IN VITRO ENZYMATIC METHYLATION OF TMV RNA

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1. Introduction

Structures of the type $m^7G^{5'}ppp^{5'}N^m$- (referred to as caps) are present at the 5'-terminus of many eukaryotic [1], animal viral [2–5], and plant viral RNAs [6–8]. Formation of these termini has been most readily studied with those DNA and RNA viruses that contain transcriptase activity which makes mRNA in vitro. The nature of the enzyme(s) which methylate RNAs of single stranded RNA viruses which do not contain transcriptase activity, such as many plant viruses and the animal togaviruses (alphaviruses), have not been investigated. Here, I report the in vitro methylation of tobacco mosaic virus (TMV) specific RNAs and characterization of the enzymatic activity involved in methylation of RNA.

2. Materials and methods

Tobacco plants (*Nicotiana tabacum* L. cv. Havana 38) 10–15 cm high were inoculated with TMV. Four days after inoculation, de-ribbed leaves were ground in a mortar and the membrane fraction (31 000 × *g* pellet) and cytosol (31 000 × *g* supernatant) were prepared by differential centrifugation [9]. The standard RNA polymerase assay contained 4 mM $MgCl_2$, 7.5 mM dithioerythritol, 25 mM $(NH_4)_2SO_4$, 10 μg/ml actinomycin D, 100 mM Tris–HCl (pH 8.2 at 35°C), 0.1 μmol/ml each of ATP, CTP, and GTP and 1 nmol [^{3}H]UTP (16 Ci/mmol) or [^{32}P]UTP (300 Ci/mmol). The assay of the methyl transferase was similiar to RNA polymerase assay except that UTP (0.1 μmol/ml) was added unlabeled and 0.75 nmol *S*-adenosyl [*methyl*-^{3}H]methionine (65 Ci/mmol) was added. The reactions were terminated by transferring two 40 μl samples into two 10 ml portions of cold 5% trichloroacetic acid containing 1% sodium pyrophosphate and collecting the precipitates on Whatman GF/C glass fiber filter discs and further processing as in [10]. RNA was purified by phenol extraction of scaled up reaction mixtures for further characterization, by analysis on 3% polyacrylamide gels [10], and by RNA hybridization as in [11]. Enzymatic hydrolysis of labeled RNA product for analysis of nucleosides was performed as in [12]. The hydrolysate was applied to Eastman thin-layer cellulose sheets (20 × 20 cm) and ascending chromatography was performed in ethyl acetate/7.5 M NH_4OH/2-propanol/*t*-butanol (3/2/2/1). After drying the plate, the position of authentic standards was determined by examination under ultraviolet light. The labeled products were detected by counting 0.75 cm strips in 0.4% Omniflour and 2% NCS solubilizer in toluene.

3. Results

When *S*-adenosyl [*methyl*-^{3}H]methionine was added to cold RNA polymerase reaction mixtures containing TMV replicase (31 000 × *g* pellet), label was incorporated into trichloroacetic acid-insoluble material or into ethanol precipitates of phenol-extracted assay mixtures. To further characterize the nature of the labeled material, phenol-extracted [^{3}H]methyl product was incubated for 30 min in protease (10 μg/ml), DNase (5 μg/ml in 5 mM Mg^{2+}), RNase a (10 μg/ml), sterile water at 37°C, or 18 h in 0.3 M KOH and the amount of acid-precipitable material was again determined. The % of acid-precipitable material after treatment compared to water control was 85%, 77%, 29% and 2.6% for protease, DNase, RNase, and KOH, respectively. These results suggest that the major labeled product was RNA.

The mobility of the methylated product was compared in gel electrophoresis with that of the products

of membrane-bound TMV replicase which usually contains TMV RI RNA and TMV RF RNA [13]. RNA polymerase reaction mixtures were prepared with [^{32}P]UTP or methyl transferase reaction mixtures with *S*-adenosyl [*methyl*-^{3}H]methionine, reactions were initiated by adding replicase preparations. RNA was purified and analyzed on polyacrylamide gels (fig.1A). [^{32}P]UMP was incorporated into 2 major peaks, RI (gel slice n. 4–7) and RF (gel slice n. 8–11). No free single-stranded TMV RNA was synthesized (expected gel slice n. 16). RNA prepared in the presence of *S*-adenosyl [*methyl*-^{3}H]methionine revealed incorporation into products that comigrated with [^{32}P]UMP labeled RI and RF, though the highest specific activity of RI occurred in a different fraction than for RI. In most instances, RI was labeled to a greater extent than RF.

RNA hybridization was performed to further confirm that the product synthesized in the presence of *S*-adenosyl [*methyl*-^{3}H]methionine was TMV specific. [^{3}H]methyl RNA was tested for the degree of self annealing and for the effect of either TMV RNA or denatured double-stranded (ds) TMV RNA on the reannealing process. Reannealing in absence of exogenous RNA (table 1,D) was relatively high. This may be due to the large amounts of TMV RNA, RI and RF extracted from the membrane preparation. Hybridization of labeled product from membrane-bound replicases from other sources also revealed high self-annealing characteristics (20–72% depending on experiments [11,14]). Addition of TMV RNA (F) slightly increased the reannealing reaction when compared to the self-annealing reaction. Addition of melted ds TMV-RNA stimulated the annealing to a greater degree than did TMV RNA alone (F). These results suggest that the [^{3}H]methyl RNA was TMV specific and that the plus strand was methylated. It is not possible from the results to determine accurately whether the complementary strand was methylated.

To determine which nucleoside was labeled, the [^{3}H]methyl RNA was analyzed on thin-layer chromatography after the RNA was digested with RNase followed by phosphodiesterase and alkaline phosphatase treatment. Two major radioactive peaks were detected (fig.2). The first peak (2.5–3.5 cm) was not associated with a visible spot under ultraviolet light and was detected in reaction mixtures containing preparations from the cytosol of either healthy (fig.2) or TMV-infected tissues (not shown). The nature of this radioactive material is unknown but it is not

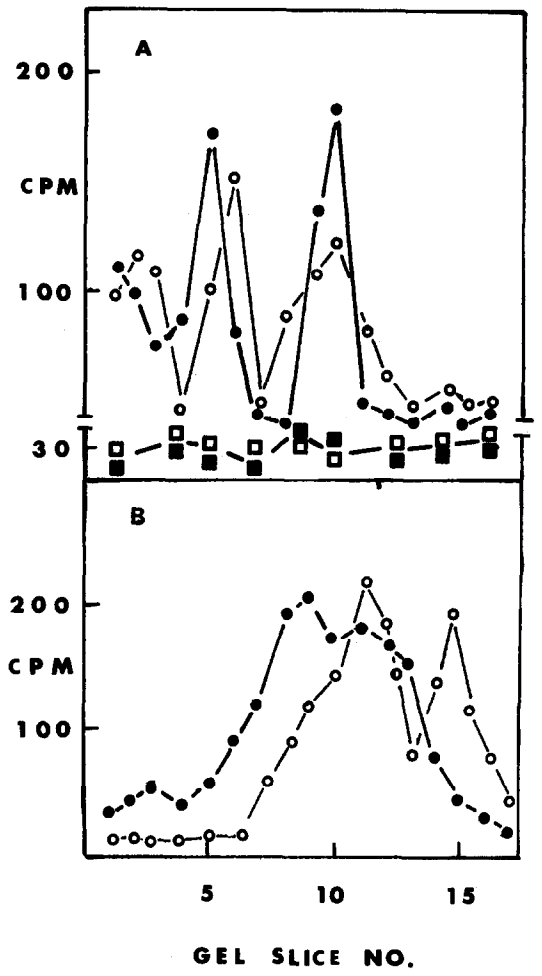

Fig.1. Polyacrylamide gel electrophoresis of in vitro labeled products. RNA polymerase assays were performed with [^{32}P]UTP; methyl transferase assays were performed with *S*-adenosyl [*methyl*-^{3}H]methionine. (A) RNA was analyzed on 3% acrylamide gels. [^{32}P]UMP RNA (•——•) and [^{3}H]-methyl RNA (○——○) was isolated from scaled-up reaction mixtures containing 31 000 × *g* pellets from TMV-infected tissue. [^{32}P]UMP RNA (▪——▪) and [^{3}H]methyl RNA (□——□) from reaction mixtures containing 31 000 × *g* pellets from uninfected tobacco. TMV replicative intermediate and TMV replicative form are located in gel slice n. 4–7 and 9–11, respectively. (B) RNA isolated from reaction mixtures containing 31 000 × *g* supernatant from uninfected tobacco as the enzyme source and analyzed on 10% acrylamide gels. [^{32}P]UMP (•——•) and [^{3}H]methyl RNA (○——○). Similar results were obtained when the enzyme source was 31 000 × *g* supernatant from TMV-infected tobacco.

Table 1
Hybridization experiments to determine the nature of the product synthesized by TMV methyl transferase

Condition	Acid-precipitable cpm	
	Expt. 1	Expt. 2
a. Before melting; no RNase	1145	599
b. Melting, no reannealing; RNase	66	49
c. Reannealed; no RNase	1213	628
d. Reannealed; RNase (self)	491	214
e. Reannealed with melted ds-TMV RNA; RNase	654[a]	307[b]
f. Reannealed with TMV RNA; RNase	566[c]	233[d]
g. Reannealed with tobacco rRNA	–	208[d]

[a] Reannealed with 0.4 μg melted ds-TMV RNA; [b] Reannealed with 1.6 μg melted ds-TMV RNA; [c] Reannealed with 0.2 μg RNA; [d] Reannealed with 0.8 μg RNA

virus-specific. The second labeled peak was detected at 6.0–7.0 cm and comigrated with authentic 7-methyl guanosine and was not detected in reaction mixtures with cytosol or membrane preparations from healthy tobacco.

The incorporation of [^{3}H]methyl-label into acid-insoluble products depended on the presence of 4 nucleotides and Mg^{2+} for activity (table 2). *S*-adenosyl L-homocysteine, a known inhibitor of RNA and DNA methylases [1], inhibited enzyme activity ~80%. At the same concentration, the inhibitor reduced [^{3}H]UMP incorporation into RI and RF by ~40% (not shown). Inhibitors of RNA polymerization, pyrophosphate and cordycepin triphosphate, also reduced methylase activity (table 3). Omission of actinomycin D from reaction mixtures increased [^{3}H]methyl incorporation suggesting that there may have been contaminating DNA-dependent methylase(s) present.

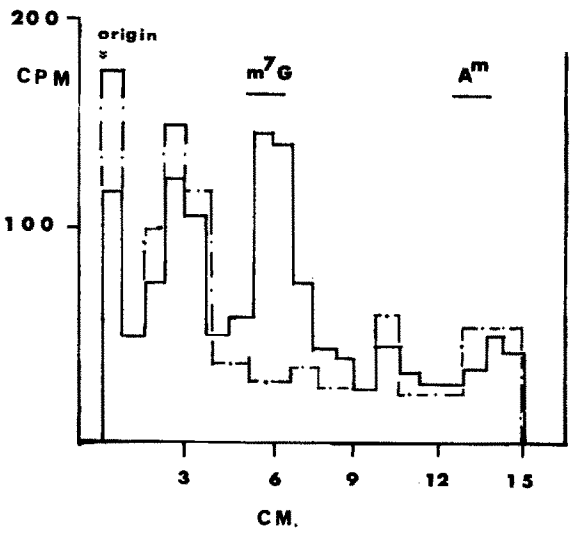

Fig.2. Thin-layer chromatography of methylated nucleoside products. RNA synthesized in vitro with 31 000 × *g* pellet (——) from TMV-infected tobacco or 31 000 × *g* supernatant (–·–) from uninfected tobacco in standard methyl transferase assay. RNA was isolated and digested and nucleosides were analyzed on thin-layer chromatography as described in section 2. The origin and location of marker 7-methyl guanosine (m^{7}G) and 2′-*O*-methyladenosine (Am) are indicated.

Membrane preparations from uninfected tobacco did not synthesize high molecular weight RNAs (fig.1A). All enzyme fractions tested, from either healthy or infected plants, synthesized a small, non-viral related, ds-RNA, 4–6 S (fig.1B) that was resolved on 10% polyacrylamide gels [15]. The RNA-dependent RNA polymerase that synthesized the 4–6 S RNA was predominantly located in the cytosol but was also present in 31 000 × *g* pellets. To determine if methyl transferase was present in extracts from uninfected plants, RNA was isolated from scaled-up methyl transferase assays that contained a membrane preparation from uninfected tobacco. The radioactivity recovered in ethanol precipated RNA was from 10–20-times less than that recovered from comparable preparations from TMV-infected tissues. Since this amount was insufficient for further analysis, I characterized the methyl transferase in cytosol preparations where larger amounts of RNA-dependent

Table 2
Characteristics of the methyl transferase in the membrane preparation from TMV-infected tobacco

Condition	Acid-precipitable cpm (% of complete)
Complete[a]	100[b]
$-Mg^{2+}$	12
–(ATP, CTP, UTP, GTP)	10
$+PP_i$ (1 mM)	68
+3′-deoxyadenosine triphosphate (0.1 μmol/ml)	64
+*S*-adenosyl L-homocysteine (0.5 mM)	21
–Actinomycin D	128

[a] The complete reaction mixture was as described in section 2
[b] Depending on experiment, 100% ranged from 1742–3127 cpm. Preparations from healthy plants were generally 10–20-fold less than the comparable preparation from infected tissue

RNA polymerase are located. The cytosol preparations from healthy plants incorporated [^{3}H]methyl labeled into RNase-sensitive products (not shown) that migrated slightly faster than [^{3}H]UMP-labeled products (fig.1B). Enzymatic hydrolysis of the RNA and analysis of nucleosides on thin layer chromatography failed to reveal label in 7-methylguanosine (fig.2). An unidentified peak at 2.5–3.5 cm was present but was not visible under ultraviolet light.

4. Discussion

I have shown that crude membrane preparations that contain TMV replicase activity also possess guanine-7-methyl transferase. Since [^{3}H]methyl-label was detected predominantly in RI, that methylation of virion RNA may occur before the newly synthesized plus strand is released from RI. The fraction of RI showing the greatest methylation migrated faster than the fraction showing greatest incorporation of [^{32}P]UMP, which would be expected if methylation is not random but occurs more frequently on one class of product, such as full length RNA. RNA isolated from TMV virions is capped [7] but capping of subgenomic TMV mRNAs has not yet been reported [16].

Whether the enzyme(s) required for capping is coded for by the virus is unknown. The viral replicase may also possess the ability to cap newly synthesized RNA. Healthy plants are known to contain an RNA-dependent RNA polymerase predominantly located in the cytosol that synthesizes a small, non-viral related RNA [15,17]. It has been strongly suggested that plant viruses utilize this enzyme as their RNA replicating enzyme and that they do not code for their own replicase [17]. The methyl transferase activity may be a useful aid in purifying viral RNA polymerases since the RNA-dependent RNA polymerase from healthy plants apparently does not incorporate label from *S*-adenosyl [*methyl*-[^{3}H]-methionine into 7-methylguanosine.

The presence of non-virus specific methylation should not interfere with purification of TMV methyl transferase. When TMV replicase was solubilized from membranes, TMV methyl transferase activity was also released. No methyl incorporation could be detected in similiar preparations from uninfected tissues (unpublished).

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