UTILIZATION OF THE GUANYLYLTRANSFERASE AND METHYLTRANSFERASES OF VACCINIA VIRUS TO MODIFY AND IDENTIFY THE 5'-TERMINALS OF HETEROLOGOUS RNA SPECIES

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Received November 15, 1976

Summary

Enzymes extracted from purified vaccinia virus particles were used to catalyze the guanylylation (i.e. capping) and/or methylation of heterologous RNA species containing two or three phosphates or the structure $m^7G(5')pppN$ at their 5'-terminals. This procedure provides a novel and specific method of labeling the 5'-terminals with [α-^{32}P]GTP or S-adenosyl-[methyl-^{3}H]methionine. Analysis of the RNAs of satellite tobacco necrosis virus and tobacco mosaic virus that were modified in this manner indicated that the original 5'-terminal sequences were (p)ppApGpPy and $m^7G(5')pppGpU$, respectively, which were enzymatically converted to $m^7G(5')pppA^mpGpPy$ and $m^7G(5')pppG^mpU$.

Enzymes that form the highly modified $m^7G(5')pppN^m$ terminal structure characteristic of eukaryotic mRNA are present in purified, infectious vaccinia virus particles (1-3). Three activities, an mRNA guanylyltransferase, an mRNA (guanine-7-)-methyltransferase and an mRNA (nucleoside-2'-)-methyltransferase have been isolated (4-6). Studies of the substrate specificity of the guanylyltransferase indicated that the RNA acceptor must have a di- or triphosphate at the 5'-terminal. The further observation that homopolyribonucleotides could be modified indicated that the enzymes lack sequence specificity and might therefore be useful for modifying and labeling the 5'-terminals of heterologous RNA species. The application of this approach to determine the 5'-terminal sequences of satellite tobacco necrosis virus RNA and tobacco mosaic virus RNA is demonstrated.

Methods

Guanylylation and methylation of RNA. Guanylyl- and methyltransferases were extracted from purified vaccinia virus cores and passed through a DEAE-cellulose column to remove DNA as previously described (4). Enzyme extracts contained approximately 1.8 mg of protein per ml. Although this enzyme mixture was suitable for the investigations described here, purified enzymes (5) free of detectable endoribonuclease activity would

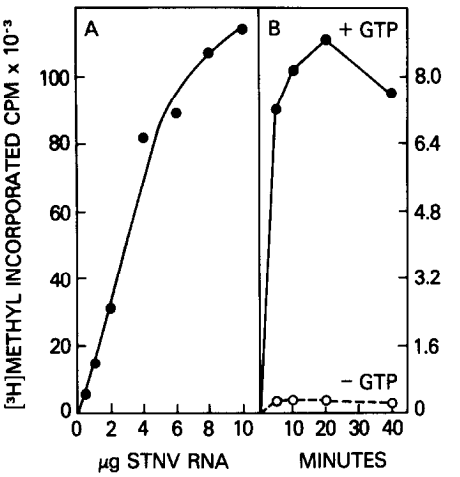

Fig. 1. Incorporation of methyl groups into satellite tobacco necrosis RNA. (A) Various amounts of satellite tobacco necrosis virus RNA were incubated with enzyme in 0.1 ml standard reaction mixtures. After 30 min at 37°, 75 µl portions were spotted on DEAE-cellulose filters. (B) Approximately 1.7 µg of satellite tobacco necrosis virus RNA was incubated with enzyme in a standard reaction mixture or one lacking GTP and Mg^{2+} and portions were removed at timed intervals as above.

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be needed for studies in which the complete integrity of the RNA must be preserved. Reaction mixtures (0.1 ml) contained 50 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 2 mM GTP, 2 mM $MgCl_2$, 0.82 µM S-adenosyl[methyl-^{3}H] methionine (12.25 Ci/mmol), 0.5 to 10 µg of RNA, and 5 µl of enzyme extract. After incubation at 37°, 75 µl portions were applied to DEAE-filter discs (Whatman DE 81) and the unincorporated precursor was removed by washing (7). The dried discs were counted in a toluene-based scintillation solvent with an efficiency of approximately 20%.

Analysis of labeled products. Reactions were terminated by immersing the tubes in a boiling water bath for 1 min and the RNA was purified by passage through a G-50 Sephadex column equilibrated with 10 mM triethylammonium bicarbonate (approximately pH 7). The labeled RNA was lyophilized and enzymatically digested as previously described (8). Alternatively, the 7-methylguanosine residue was removed by periodate oxidation and β-elimination prior to enzymatic digestion of the RNA. DEAE-cellulose column chromatography was carried out in 7 M urea at pH 7.6 (2). Solvents for descending chromatography on Whatman No. 1 paper were (A) isobutyric acid/0.5 M NH_4OH (10/6) or (B) isopropanol/H_2O/conc. NH_4OH (7/2/1). Electrophoresis on Whatman 3 mm paper was in 0.05 M ammonium formate, pH 3.5 at 3,000 volts for 4 h.

Materials. S-adenosyl[methyl-^{3}H]methionine was purchased from New England Nuclear Corp. Dinucleoside triphosphate markers were obtained from P-L Biochemical Co. and dinucleoside monophosphate markers from Sigma Chemical Co. DEAE-cellulose (DE52) and Whatman No. 1 paper were from Whatman Co. Enzymes used for digestion of RNA were supplied by Worthington Biochemical Corp. except for P_1 nuclease which came from Yamasa Shoyu Co., and nucleo-

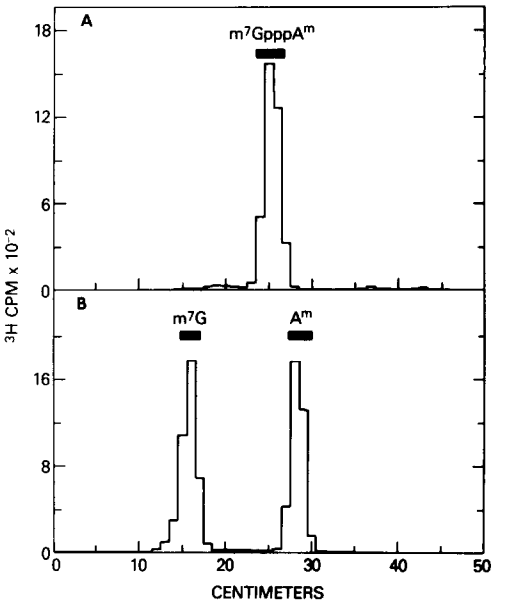

Fig. 2. Paper chromatography of digestion products of methyl-labeled satellite tobacco necrosis virus RNA. (A) RNA was digested successively with P_1 nuclease and then bacterial alkaline phosphatase. Paper chromatography was in solvent A. (B) RNA was digested successively with P_1 nuclease and then with a mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase. Paper chromatography was in solvent B.

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tide pyrophosphatase and T_2 ribonuclease which came from Sigma Chemical Co. RNA from the Rothamstead strain of satellite tobacco necrosis virus and the U1 strain of tobacco mosaic virus was a gift of Dr. Abraham Marcus of the Cancer Research Institute, Phila., Pa.

Results

Identification of the 5'-terminal sequence of satellite tobacco necrosis virus RNA. Previous studies indicated that the 5'-terminal sequence of tobacco necrosis virus RNA is (p)ppApGpU (9, 10) suggesting that it might be a substrate for the guanylyltransferase and methyltransferases of vaccinia virus. Initial experiments demonstrated that the viral enzymes catalyzed the incorporation of a GMP residue from [α-^{32}P]GTP and methyl groups from S-adenosyl[methyl-^{3}H]methionine into tobacco necrosis virus RNA. Incorporation was proportional to RNA concentration as shown for S-adenosylmethionine in Fig. 1A. Significantly, the presence of GTP and

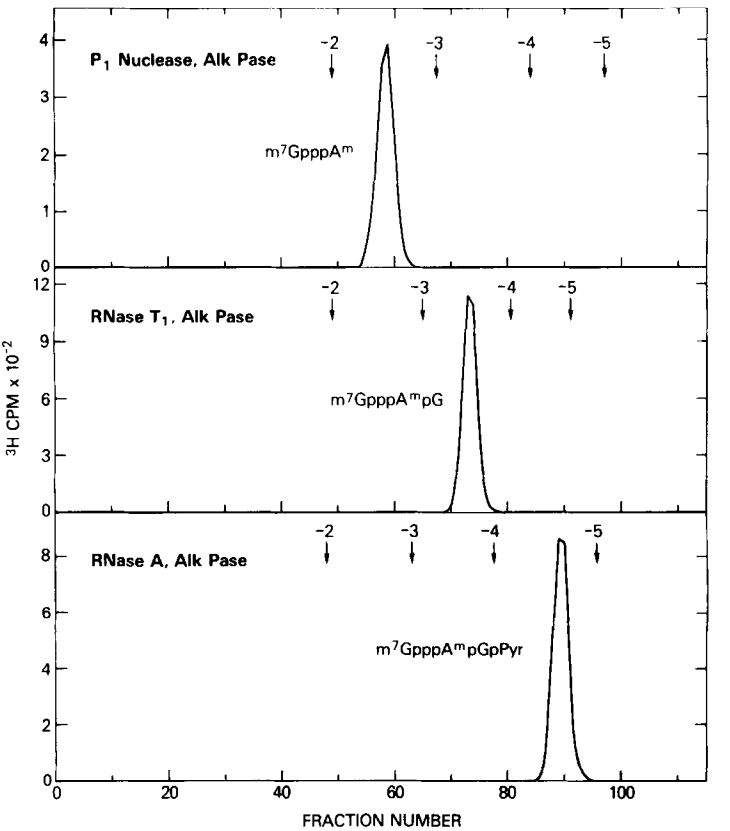

Fig. 3. DEAE-cellulose column chromatography of digestion products of methyl-labeled satellite tobacco necrosis virus RNA. RNA was digested with either P_1 nuclease followed by bacterial alkaline phosphatase, a mixture of RNase T_1 and bacterial alkaline phosphatase, or a mixture of RNase A and bacterial alkaline phosphatase. The numbered arrows indicate the negative charges and elution positions of a series of oligoadenylate markers. The proposed structures are written next to each peak.

Mg^{2+} was required for methylation (Fig. 1B) and a plateau occured when approximately 1.6 pmol of methyl were added per pmol of RNA. After P_1 nuclease (11) and alkaline phosphatase digestion of the methylated RNA, virtually all of the labeled material co-chromatographed with the expected product $m^7G(5')pppA^m$ (Fig. 2A). Equal amounts of labeled m^7G and A^m were recovered after further digestion with snake venom phosphodiesterase and alkaline phosphatase (Fig. 2B). Had the reaction not gone to completion, partially methylated $m^7G(5')pppA$ products would have formed. These

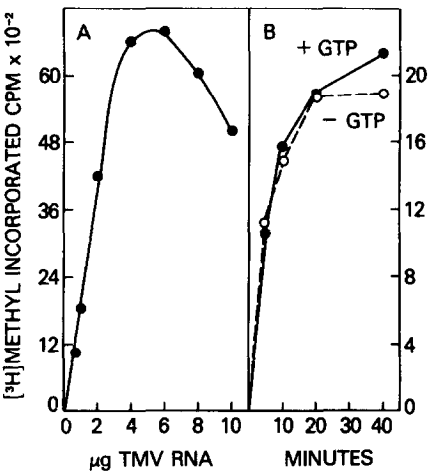

Fig. 4. Incorporation of methyl groups into tobacco mosaic virus RNA. (A) various amounts of tobacco mosaic virus RNA were incubated with enzyme in 0.1 ml standard reaction mixtures lacking GTP and Mg^{2+}. After 30 min at 37°, 75 µl portions were spotted on DEAE-cellulose filters. (B) approximately 8 µg of tobacco mosaic virus RNA was incubated with enzyme in a standard reaction mixture or in one lacking GTP and Mg^{2+}. Portions were spotted on to DEAE-cellulose filters at timed intervals.

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experiments confirmed that the 5'-terminal of satellite tobacco necrosis virus RNA is (p)ppA. To determine the adjacent nucleotides, methylated tobacco necrosis virus RNA was digested with (1) P_1 nuclease and alkaline phosphatase, (2) RNase T_1 and alkaline phosphatase, and (3) RNase A and alkaline phosphatase and then chromatographed on DEAE cellulose columns in the presence of 7 M urea at pH 7.6. In each case only a single peak of methyl-labeled material was detected (Fig. 3). Based on the specificity of P_1 nuclease for all phosphodiester bonds, of RNase T_1 for those containing guanosine and of RNase A for those containing pyrimidines and the net negative charges of the digestion products, the oligonucleotides were identified as $m^7G(5')pppA^m$, $m^7G(5')pppA^mpG$, and $m^7G(5')pppA^mpGpPy$ (Fig. 3). Accordingly, the original 5'-terminal sequence of tobacco necrosis virus RNA was inferred to be (p)ppApGpPy.

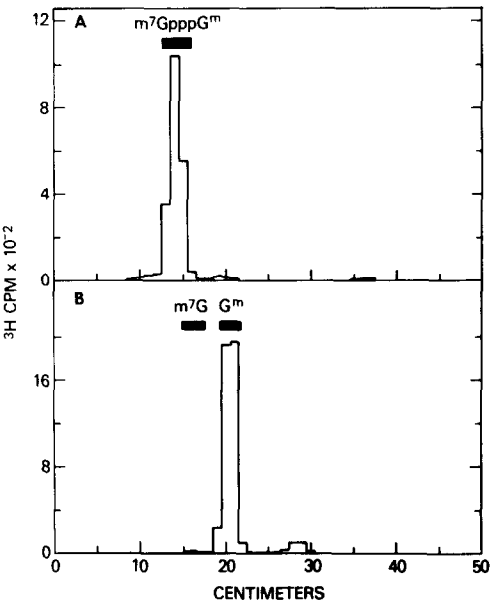

Fig. 5. Paper chromatography of digestion products of methyl-labeled tobacco mosaic virus RNA. (A) RNA was digested with P_1 nuclease and then bacterial alkaline phosphatase. Paper chromatography was in solvent A. (B) RNA was digested with P_1 nuclease and then with a mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase. Paper chromatography was in solvent B.

Identification of the 5'-terminal sequence of tobacco mosaic virus RNA

Tobacco mosaic virus RNA was chosen as a second model since its 5'-terminal sequence $m^7G(5')pppG$ (12) should also serve as a substrate for the vaccinia virus nucleoside-2'-methyltransferase but not for the guanylyltransferase or guanine-7-methyltransferase. Incorporation of methyl groups was proportional to the concentration of RNA up to values of 60 µg per ml (Fig. 4A). As anticipated, GTP and Mg^{2+} were not required for methylation of tobacco mosaic virus RNA (Fig. 4B). Incorporation plateaued when less than one pmol of methyl was added per pmol of RNA. After P_1 nuclease and alkaline phosphatase digestion nearly all of the labeled material co-chromatographed with $m^7G(5')pppG^m$ (Fig 5A). Further digestion with snake venom phosphodiesterase indicated that only G^m was labeled (Fig. 5B). This result confirmed that the original 5'-terminal of tobacco mosaic virus RNA is $m^7G(5')pppG$.

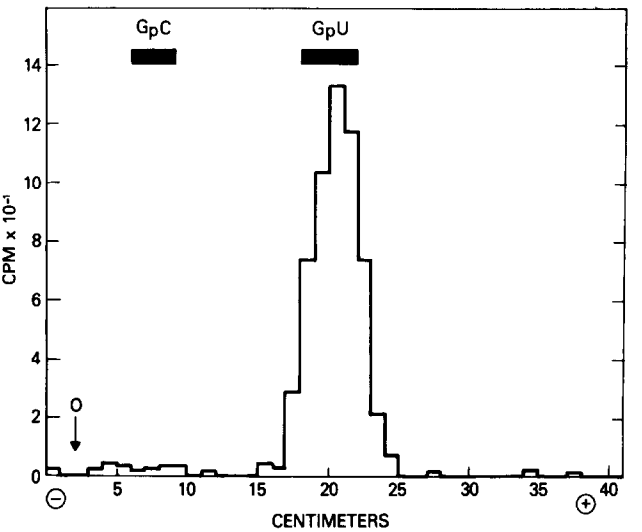

Fig. 6. Paper electrophoresis of dinucleoside monophosphate derived from the methyl-labeled 5'-terminal of tobacco mosaic virus RNA. The terminal 7-methylguanosine residue of methyl-labeled tobacco mosaic virus RNA was removed by periodate oxidation and β-elimination. Following this treatment the RNA was digested with RNase A and bacterial alkaline phosphatase and the resistant dinucleoside monophophosphate was isolated by DEAE-cellulose chromatography in 7 M urea. The desalted dinucleoside was then subjected to paper electrophoresis at pH 3.5 with appropriate markers.

The next nucleotide of tobacco mosaic virus RNA was shown to be a pyrimidine since a single product with a net change of -3.5 was obtained by DEAE-cellulose column chromatography after treatment with RNase A and alkaline phosphatase. The following steps were taken to identify the pyrimidine. First, methyl-labeled tobacco mosaic virus RNA was treated with periodate and aniline to remove the m^7G residue. The β-eliminated RNA was then digested with RNase A and alkaline phosphatase following which G^mpPy was isolated as a -1 charge peak by DEAE-cellulose column chromatography. Upon paper electrophoresis at pH 3.5, the labeled dinucleoside monophosphate co-migrated with GpU and well ahead of GpC indicating that the pyrimidine was uridine (Fig 6). Consequently the 5'terminal sequence of the original tobacco mosaic virus RNA was m^7G(5')pppGpU. Digestion of methylated

tobacco mosaic virus RNA with RNase T_1 did not yield an oligonucleotide that could be eluted from the DEAE-cellulose column with 0.5 M NaCl suggesting that the next guanosine residue is far from the terminal or inaccessible to RNase T_1 because of secondary structure.

Discussion

A novel and highly specific method of enzymatically modifying and labeling the 5'-terminal of RNA has been described. A mixture of guanylyltransferase, guanine-7-methyltransferase, and nucleoside-2'-methyltransferase extracted from vaccinia virus particles was used. The procedure is applicable to at least two types of RNA species. The first group, exemplified by satellite tobacco necrosis virus RNA, consists of molecules retaining two or three 5'-terminal phosphates of the primary transcript and includes some prokaryotic, eukaryotic, viral and in-vitro synthesized RNA species. Such RNA molecules may be modified with [α-^{32}P]GTP to form G(5')pppN terminals or with GTP and S-adenosyl[methyl-^{3}H]methionine to form m^7G(5')pppN^m terminals.

A second class of RNA exemplified by tobacco mosaic virus RNA, contains m^7G(5')pppN type terminals. Such terminals, which are found in plant viral RNAs (12-15), some animal viral RNAs (16, 17) and yeast mRNA (18) may be labeled only with S-adenosyl[methyl-^{3}H]methionine unless the terminal m^7G residue is removed by periodate oxidation and β-elimination.

Following labeling, digestion of the RNA with specific ribonucleases and chromatographic procedures may be used to identify the terminal nucleotide as well as some adjacent ones. For example, we have confirmed the 5-terminal sequence (p)ppApGpPy of satellite tobacco necrosis virus RNA and extended by one nucleotide the 5'-terminal sequence of tobacco mosaic virus RNA which is m^7G(5')pppGpU. Using this procedure it should be possible to rapidly identify the 5'-terminals of many other RNAs. An initial screening by measuring S-adenosyl[methyl-^{3}H]methionine incorporation in the presence and absence of GTP will serve to distinguish between (p)ppN and m^7G(5')pppN terminals. The sensitivity is extremely high

since in the case of satellite tobacco necrosis virus RNA, more than 100,000 cpm were incorporated in a 0.1 ml reaction mixture containing several μg of RNA and 1 μCi of S-adenosyl[methyl-^{3}H]methionine. Moreover, labeling is highly specific and false results are not obtained even if the RNA contains internal nicks. Some investigators may also find it useful to modify the 5'-terminals of ^{32}P-labeled RNA species in order to isolate the 5'-terminal fragments. The added 7-methylguanosine residue, which contain free 2', 3'-hydroxyls, will allow the 5'-terminal oligonucleotides to bind to supports containing covalently bound dihydroxyboryl groups (19, 20). Since the 7-methyl-guanosine residue is necessary for efficient translation of some mRNAs (21) still another potential use of the guanylyl- and methyltransferases of vaccinia virus will be to modify synthetic polyribonucleotides or unmethylated natural RNA species to study protein synthesis.

Acknowledgements

Satellite tobacco necrosis virus RNA and tobacco mosaic virus RNA were kindly supplied by Dr. A. Marcus of the Institute for Cancer Research, Phila., Pa.

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