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Variability at the 5′ Ends of Two Plant Viruses†

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ABSTRACT: Polynucleotide phosphokinase was used to selectively phosphorylate the 5′ ends of the RNA of tobacco mosaic virus (TMV), previously shown to be naturally unphosphorylated. The 5′ ends of the RNA components of bromegrass mosaic virus (BMV) were shown by their availability toward that kinase also to be unphosphorylated. Both TMV-RNA and the various components of the quite unrelated BMV-RNA, all recently shown to be identical in their 3′-terminal sequences (-GCCCA), showed heterogeneity in the 5′-terminal region. In TMV-RNA about half of the molecules terminate in adenosine, in the BMV-RNAs about 40%, the rest being uridine > guanosine > cytidine in both types of RNA.

Polynucleotide phosphokinase has been used successfully in labeling and thus identifying the 5′- (3′-linked)[1] terminal deoxynucleotides of the DNAs of several bacteriophages (Wu and Kaiser, 1967; Weiss and Richardson, 1967; Richardson and Weiss, 1966). The isolation of a terminal oligonucleotide phosphorylated by this method has also been reported, namely of pGpGpUp from pancreatic RNase digests of monoesterase and then polynucleotide phosphokinase treated MS 2 RNA (Glitz, 1968), as well as of pAp from a strain of Qβ RNA terminating in unphosphorylated A (Young and Fraenkel-Conrat, 1970). Attempts to establish the 5′-terminal nucleotide sequence of TMV-RNA by means of this enzyme were initiated in 1965. A terminal sequence of ApUpG was indicated by the data, but 5′-terminal nucleotides other than A also occurred to lesser extents. When this study was extended to the various RNA components of bromegrass mosaic virus (BMV),[2] similar results were obtained with each, and the results resembled those obtained with TMV-RNA (terminal A > U > G > C). Many variations in techniques of enzyme isolation and treatment, purification of the modified RNAs, and degradation, as well as separation of the degradation products derived from the 5′ end, did not alter the conclusion that the RNAs of these two plant viruses are heterogeneous as far as their 5′ ends are concerned, and that all the components of BMV-RNA show very similar unphosphorylated 5′ termini.

Methods and Materials

^{14}C Labeled TMV and BMV were prepared from infected Turkish tobacco and Atlas barley held in a $^{14}CO_2$-containing atmosphere (Sugiyama and Fraenkel-Conrat, 1961). ^{3}H-labeled TMV was obtained by letting 1-day infected leaves imbibe 10–50 mCi of the ^{3}H-labeled nucleosides (the [8-^{3}H]-purine ribosides, the [5-^{3}H]pyrimidine ribosides) and harvesting them after 6–10 days (Singer, 1971). Viral RNA was prepared by standard methods. [^{32}P]ATP was prepared by the method of Glynn and Chappell (1964), frequently given to us by Dr. M. Chamberlin, and later obtained commercially. Samples of polynucleotide phosphokinase were given to us by Drs. C. C. Richardson, M. Takanami, and D. Kaiser. Later they were prepared by us according to Richardson (1965). Upon DEAE-cellulose chromatography the activity eluted largely in a later fraction than found by these authors. When the modifications of the procedure proposed by Hänggi *et al.* (1970) were used, the enzyme was obtained in better yield and with more predictable properties. Tests for nucleases were performed by incubating TMV-RNA with the enzyme in the absence of ATP. Most preparations caused little or no losses in infectivity at levels at which they caused phosphate transfer from added ATP, and were thus adequate for the desired purpose.

The kinase reaction was performed on 0.1–2 mg (0.05–1

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[1] We have in all previous publications concerned with terminal RNA sequences used the preferable terminology of 3′ linked and 5′ linked for what have since become more commonly called the 5′ and 3′ ends of nucleic acid chains, respectively. We now have regretfully decided to accept the less descriptive terms in deference to the majority of workers in the field.

[2] Abbreviations used are: BMV, bromegrass mosaic virus; TMV, tobacco mosaic virus.

nmole) of RNA in 1–2 ml of 0.05 M pH 7.8 Tris buffer, containing 0.01 M $MgCl_2$ and 0.01 M mercaptoethanol, for 30–90 min at 37°. ~-[^{32}P]ATP, containing about 2 mCi/μmole, was added to 20–100 μM concentrations. At the end of the reaction 0.1 M EDTA (pH 7), in excess over the Mg^{2+} present, and 3 volumes of ethanol at 0° were added. The precipitated RNA was redissolved in water and reprecipitated in the same manner, as well as by addition of an equal vo'ume of 4 M NaCl, and again with alcohol. The salt precipitation was of great importance for the removal of most of the ATP. The RNA was then generally placed on a 5–20% sucrose gradient (SW 25.1) and centrifuged for 17 hr at 24,500 rpm. The presence of RNA and its extent of ^{32}P labeling were determined across the gradient by spectrophotometry and counting. The specific ^{32}P activity was usually constant, in terms of counts per minute per absorbancy for the leading part of the RNA peak (usually two-thirds down the tube), and increased toward the top of the tube. If ^{14}C- or ^{3}H-labeled RNAs were used the ^{32}P activity could also be related to the respective label in the bases using a two-channel scintillation counter. The intact RNA of constant ^{32}P specific activity was isolated by alcohol precipitation. Frequently, polyacrylamide gel electrophoresis (2.5%) was used to further purify phosphorylated TMV-RNA or in the case of BMV-RNA to separate the A, B, C and components (Fowlks and Young, 1970).

Degradation of the RNA for the purpose of obtaining terminal fragments was achieved by (1) snake venom phosphodiesterase to yield 5′ nucleotides, including the labeled (p*-) terminals[3] (5 μg/20 μg of RNA, 0.02 M pH 8.8 borate, or preferably 0.1 M pH 8.8 ammonium bicarbonate, 4 hr at 37°); (2) pancreatic ribonuclease A to yield p*-(Pu-p)$_n$Py (usually 1 unit/20 μg of RNA = about 1–2% of the RNA in 0.01 M pH 7.5 Tris-HCl, 4 hr at 37°); (3) T-1 ribonuclease (under the same conditions as 2 to yield p*-(N†p)$_n$G (N† = A, U, or C); (4) spleen diesterase, to degrade 5′-unphosphorylated oligonucleotides to nucleotides (1 U/33 μg of RNA, in 0.15 M ammonium succinate (pH 6.5), 2 × 10^3 M EDTA, 0.05% Tween 80, 3 hr, 37°); (5) 0.5–1 N KOH, 37°, 18 hr, then neutralized with IRC 50 or, if further procedures were not sensitive to salt, with an equivalent amount of HCl (the alkali serves to degrade the 5′-phosphorylated and labeled oligonucleotides from 2, 3, and 4 to ascertain their composition and in some cases their sequence); (6) *Escherichia coli* phosphatase (1 μg/20 μg of RNA, pH 8) to remove 2 charges (at pH 7) from 3′-nucleotides and oligonucleotides and 4 charges from 5′-phosphorylated oligonucleotides, p-(Np)$_n$.

The methods used to separate the four nucleotides or the nucleoside 3′,5′-diphosphates from one another were (1) Whatman No. 3MM or DE-81 paper electrophoresis at pH 3.5 in 0.05 N formate or pyridine acetate. On DE-81 the diphosphates were well separated from the nucleotides, but the separation of Ap from Gp and of pCp from pGp was imperfect. On Whatman No. 3MM paper the furthest moving nucleotide, Up, usually overlapped with one of the least moving diphosphates pCp or pAp. (2) Chromatography on Whatman 1 paper impregnated with 10% saturated ammonium sulfate, with 76% ethanol as solvent.

Oligonucleotides were separated according to number of charges on DEAE-cellulose columns in the presence of 7 M urea (Tomlinson and Tener, 1962). Fractions obtained in this manner were desalted, after dilution, on DEAE-carbonate-cellulose, eluting with triethylammonium carbonate. Short oligonucleotides were desalted by means of charcoal ad- and desorption (at pH 3 and by ethanolic ammonia, respectively). Oligonucleotides were also separated by a two-dimensional method (Sanger and Bronwlee, 1967).

Results

5′-End Groups of TMV-RNA. TMV-RNA accepted in different experiments and with different kinase preparations from 0.2 to 1.0 mole of phosphate per mole of RNA (2 × 10^6 daltons). This finding thus confirmed earlier conclusions that the 5′ end of that RNA is predominantly if not entirely unphosphorylated (Fraenkel-Conrat and Singer, 1962; Sugiyama and Fraenkel-Conrat, 1963).

Samples of sucrose gradient purified 5′-terminally labeled TMV-RNA were completely degraded with alkali, or with snake venom phosphodiesterase. The alkaline digests were subjected to paper electrophoresis to isolate the four nucleoside 3′,5′-diphosphates and their radioactivity was determined. From the snake venom digests the 5′-nucleotides were electrophoretically separated and their content in radioactive nucleotides was established. Both methods indicated the 5′-(terminally) labeled residues to be heterogeneous, with A:U:G:C = 54:17:18:11, all ±2. Later experiments were performed with terminally labeled TMV-RNA samples that had been purified by polyacrylamide gel electrophoresis. TMV-RNA tended to form varying amounts of an aggregate of lesser polyacrylamide gel mobility upon kinase treatment, probably attributable to the presence of enzyme, since this slow-moving material was diminished by phenol extraction of the modified RNA and/or by using less RNA per gel tube.[4] Various parts of the RNA from such gels, derived from the trailing and leading half of both the retarded and the typical RNA peaks, were subjected to end group analysis. To facilitate quantitative extraction of the RNA from gel slices, these were first treated with T-1 ribonuclease (0.5 ml of 0.01 M pH 7.4 Tris, 0.01 ml of 0.1% T-1, 4 hr, 37°), and the extract was then brought to 0.5 N with KOH and held for 17 hr at 37°. The digests were then electrophoresed on DE-81 paper, and the radioactivity in the area of the 4 diphosphates was counted. They all showed the same labeling pattern, pAp:pUp:pGp:pCp = 46:27:16:11 (±2) (Table I). In an earlier experiment with the same procedure but without subfractionation of the polyacrylamide gel bands, these ratios were 49:29:14:8. The slight discrepancy in regard to the distribution of the minor components between these and the earlier experiments in which different techniques had been employed was not further investigated.

Since the total amount of phosphate accepted was usually at or below the stoichiometric amount in RNA fractions of 2 × 10^6 daltons, as judged by their movement in sucrose gradients and upon polyacrylamide gel electrophoresis, it appears improbable that the observed 5′ terminals are due to hidden breaks or contaminating oligonucleotides in the RNA. To further diminish this possibility, the kinase-treated RNA was fractionated on 4–7 M urea gradients instead of

[3] p* and p stand for ^{32}P-labeled and unlabeled phosphate, respectively; Pu, Py for purine and pyrimidine nucleosides; N for any nucleoside.

[4] Many RNA samples (both TMV and BMV) that had been treated with a particular batch of polynucleotide phosphokinase lost all ^{32}P upon gel electrophoresis. Upon pretreatment of the enzyme-treated RNA with 0.1 M pH 9.2 ammonium bicarbonate at 37° for 30 min the phosphate became fixed and then coelectrophoresed with the RNA. It is suggested that a complex between RNA (5′ end) and activated, possibly phosphorylated, enzyme might be an intermediate that was unusually stable in the case of this particular enzyme preparation.

TABLE I: Distribution of 5′-Terminal Phosphate Groups in TMV-RNA and Components of BMV-RNA.[a]

	TMV-RNA	BMV-RNA Components			
	(%)	A1[b] (%)	A2 (%)	B (%)	C (%)
pAp	46	38	39	38	41
pUp	27	26	29	25	25
pGp	16	19	18	21	18
pCp	11	17	14	16	16

[a] Average of three experiments. Molecular weights of A1, A2, B, and C: 1.09×10^6, 0.99×10^6, 0.7×10^6, and 0.3×10^6. [b] Contains a little A2.

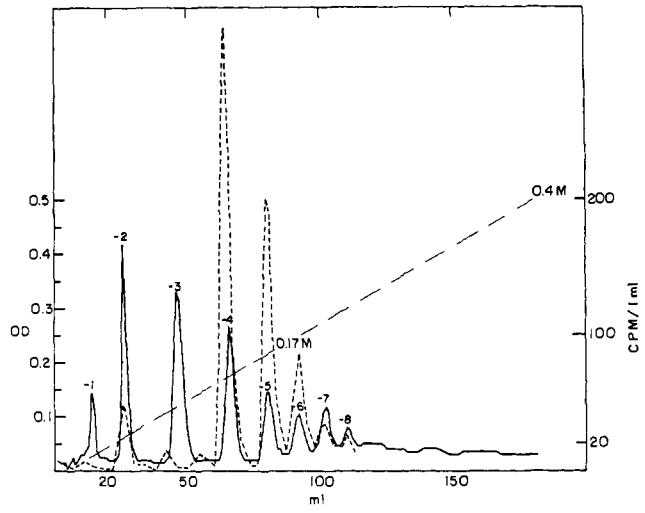

FIGURE 1: Fractionation of a pancreatic RNase digest of terminally ^{32}P-phosphorylated TMV-RNA according to Tomlinson and Tener (1962). The solid line represents absorbancy, the short-dash line ^{32}P radioactivity, the diagonal the NaCl concentration. The numbers above peaks represent the number of charges at pH 7 characterizing each fraction, −2 representing free nucleotides, *e.g.*, Cp and Up.

sucrose gradients. The urea gradient runs required 50% more time to bring the midpoint of the broad RNA peak (mound) below the middle of the gradient tube. The amount of ^{32}P that remained associated with the bottom half of the RNA in such gradient tubes was the same as that in the 30S component in sucrose gradients. If one assumes that H-bonded poly- or oligonucleotide would be separated by this treatment, the detected multiple end groups must be attributed to the macromolecules.

Urea (8 M) was also used in polyacrylamide gel electrophoresis of RNA. This led to extremely sharp bands, but the movement down the gels was greatly diminished for all RNAs. TMV-RNA did not enter the 2.5% gels at all so that this procedure did not seem suitable for its further purification.

5′-End Groups of BMV-RNA. BMV-RNA shows three components on sucrose gradient centrifugation, and three bands, the least moving, *i.e.*, largest, of which is a doublet, upon polyacrylamide gel electrophoresis (Fowlks and Young, 1970). The former belief that the 2 smaller pieces (molecular weight about 0.7×10^6 and 0.3×10^6) represent fragments of the large one (molecular weight about 1.0×10^6) is now regarded as erroneous, since evidence has been reported that the doublet actually represents two different components (molecular weight 1.09×10^6 and 0.99×10^6) and that these two (A1, A2) plus the medium-sized one (B) are required for infectivity (Lane and Kaesberg, 1971).

The first question, whether the components of BMV-RNA were unphosphorylated at the 5′ end, was tested by performing the kinase treatment with or without prior treatment with *E. coli* phosphatase. The amount of ^{32}P that became bound by all components of BMV-RNA, while usually less than stoichiometric (0.2–0.8 mole/mole of RNA with different kinase preparations), was about the same with and without phosphatase treatment, suggesting that all 5′ ends were unphosphorylated.

We then performed experiments of the type described above with TMV-RNA, in determining the nature of the 5′-labeled nucleotides of BMV-RNA that had been kinase treated and then fractionated on sucrose gradients or gels. When the dual nature of the large (A) component came to our attention, we prepared by RbCl density gradient fractionation (Lane and Kaesberg, 1971) virus samples containing B and C that were enriched in A1 and A2, respectively. RNA was prepared from these, treated with kinase, fractionated by polyacrylamide gel electrophoresis, and analyzed for end groups. The results of all these experiments were similar. All four components of BMV-RNA appear to terminate predominantly with A but also contain chains with terminal U and G, and much less with C (see Table I).

5′-Terminal Sequences in TMV-RNA. Fractionation of pancreatic RNase or T-1 RNase digests of a homogeneous terminally labeled RNA on DEAE-cellulose or Sephadex in the presence of urea should yield only one ^{32}P-labeled oligonucleotide. When such studies were performed with kinase-treated TMV-RNA repurified by sucrose gradient centrifugation, ^{32}P counts of decreasing magnitude appeared in several oligonucleotide fractions starting with the trinucleotide fraction, carrying 4 negative charges at pH 7 (Figure 1). This −4 fraction contained ATP, and no other identifiable ^{32}P-labeled products. The next one, in the −5 position of pancreatic RNase digests, contained material that behaved like pApUp and its ^{14}C counts, when base-labeled RNA was used, cochromatographed with authentic ApU after phosphatase treatment and rechromatography in the same system (−1 peak). None of the other ^{32}P-labeled oligonucleotides arising from the terminally labeled TMV-RNA digests could be unambiguously identified by this methodology. The largest peak from a T-1 digest, also of −5 charge, did not yield detectable amounts of UpG, CpG, or ApG after enzymatic dephosphorylation.

Similar pancreatic or T-1 ribonuclease digests of terminally ^{32}P-labeled [^{3}H]TMV-RNA were subjected to two-dimensional electrophoresis (Sanger and Brownlee, 1967) and radioautographed. Again not one but several fragments containing different amounts of ^{32}P were detected. The strongest of these were eluted, treated with spleen diesterase to digest away any contaminating not 5′-terminally phosphorylated oligonucleotides, and reelectrophoresed in the same dimension (in 7% formic acid). The 5′-terminally phosphorylated oligonucleotides were largely resistant to this treatment and moved in unchanged manner, although free pNp was apparent in some instances. The 5′-phosphorylated oligonucleotides were then digested with alkali which unfortunately caused considerable loss of ^{3}H label from the 8-labeled purines. To remedy this, digestion was at times performed at pH 8.8 with

a mixture of 3 ribonucleases (*e.g.*, pancreatic, T-1, and T-2, 12, 17, and 1 unit, respectively, per 0.2 mg of nucleotide material). The composition of such digests determined after electrophoresis on Whatman 3 MM paper indicated the presence of pApUp, pCpUp, and pUpCpGp among other less clearly identified oligonucleotides.

Discussion

Studies of the end groups of viral RNAs represented the opening wedge into the structural analysis of genomes in general and RNAs in particular. These studies started with the finding that TMV-RNA carried 3′-terminal adenosine (Sugiyama and Fraenkel-Conrat, 1961), and that the terminal sequence was -GCCCA (Steinschneider and Fraenkel-Conrat, 1966; Mandeles, 1968). Recently the same sequence was found in all BMV-RNA components (Glitz and Eichler, 1971), and the existence of at least 3 cytidine residues, with or without a terminal A, has been found in all viral RNAs that have been investigated. One variant of this structural feature was the finding that in the RNA of the satellite of tobacco necrosis virus (STNV-RNA, molecular weight about 400,000) the terminal C was partly 3′-phosphorylated (Horst *et al.*, 1972), an indication that homogeneity in terminal sequences of viral RNAs need not always pertain.

Early studies of the 5′ ends of TMV-RNA and TYMV-RNA indicated the prevalence of unphosphorylated A (Sugiyama and Fraenkel-Conrat, 1963; Suzuki and Haselkorn, 1968), but no further analyses of that end of plant viral RNA were published, possibly because of the problems created by the marked heterogeneity now reported for TMV-RNA and the BMV-RNAs in this part of the molecule. Again STNV is exceptional in that it appears to have a unique 5′-terminal base sequence, variably 5′-phosphorylated (pppAGU as well as ppAGU-) (Horst *et al.*, 1972). The triphosylated state seems to be the rule for bacteriophage RNAs (Glitz, 1968; Young and Fraenkel-Conrat, 1970; de Wachter and Fiers, 1969) while the RNA of some animal viruses, like STNV-RNA, shows a tendency to lose one phosphate (Levin *et al.*, 1970).

The present finding of a variety of 5′ ends in TMV-RNA and BMV-RNA may *a priori* be regarded as indicative of the presence of contaminating oligonucleotides. The fact that the total number of such ends was never in excess of the expected for the viral RNA termini makes this interpretation unlikely. Further, the use of sucrose or urea gradients as well as gel electrophoresis in the purification of the treated RNA would be expected to diminish or abolish such contamination with oligonucleotides. Since the heterogeneity of ends was observed to be similar, regardless of the method of purification of the labeled RNA, it appears improbable that it can be attributed to such causes. The absence of significant numbers of hidden breaks in TMV-RNA is indicated by many recent experiments in which the bulk of this RNA retained its 30S character after treatment under conditions which dissociate the RNA of tumor viruses (*e.g.*, Rous sarcoma virus). Thus dimethyl sulfoxide as well as heat, in the presence or absence of formaldehyde or 5 M urea, does not noticeably degrade TMV-RNA.

The varible state of phosphorylation of the 5′ ends of viral RNAs and the fact that sequence heterogeneity is observed when that end is unphosphorylated would suggest the possibility that the heterogeneity might result from 5′-exonuclease attack on the unphosphorylated end. In that case a unique sequence should be detectable in the various labeled terminal oligonucleotides, such as p*ApUpGp, p*UpGp, p*Gp, after T-1 RNase digestion. The data do not support this hypothesis, the number and nature of terminally labeled oligonucleotides being greater and more complex than one would expect on this basis. Regretfully one must conclude that the chemical structure of a single species of biopolymer can be variable in what one must presume to be an unessential part of the molecule.

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