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Identity of the 5′-Terminal RNA Nucleotide Sequence of the Satellite Tobacco Necrosis Virus and Its Helper Virus: Possible Role of the 5′-Terminus in the Recognition by Virus-Specific RNA Replicase*

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Abstract. A pancreatic ribonuclease digest of ^{14}C-labeled tobacco necrosis virus RNA was fractionated according to charge by column chromatography. Individual fractions were dephosphorylated with alkaline phosphatase and rechromatographed. The fraction, originally containing oligonucleotides with seven negative charges, separated into two components corresponding to five (−5) and two negative charges (−2). The −5 fraction was derived from the internal oligonucleotides while the −2 fraction must have originated from a 5′-pyrophosphorylated terminal trinucleotide. The sequence of this terminal trinucleotide was determined by column chromatography on DEAE-cellulose in a triethyl ammonium carbonate gradient, using the appropriate markers. The radioactivity chromatographed with a (ApGp)U marker. The order of the Ap and Gp was determined after ribonuclease T_1 and alkaline phosphatase digestion. The radioactivity in the product chromatographed with an ApG marker. The 5′-terminus of tobacco necrosis virus RNA was therefore determined as ppAp-GpUp . . ., which is identical to the terminus of the RNA of its satellite virus as previously determined (*J. Mol. Biol.*, **38,** 59 (1968); *Science*, **160,** 1452 (1968)). The 5′ pyrophosphate in both viruses was probably formed by an *in vivo* enzymatic removal of a γ-phosphate from a triphosphate, and its presence in both viruses suggested a common site of synthesis. The identity of the 5′-terminal sequences is considered not to be fortuitous and is discussed from the standpoint of their role as a recognition site for the virus-specific RNA replicase.

Sequence studies of the mRNA's of viral origin[1–3] surprisingly failed to reveal the presence of a protein-chain initiating codon near the 5′-terminus. It must therefore be concluded that these nucleotide sequences are not part of a cistron and their biological role remains obscure. De Wachter and Fiers[3] showed that the 5′-terminal sequence of Q_β-RNA was identical to the sequence of the "little variant"[4] reported by Bishop.[5] In view of the template activity of the little variant, the conservation of the 5′-terminus would suggest its possible role as a recognition site for Q_β-RNA replicase.

Experiments with partly purified extracts of plants infected with tobacco necrosis virus (TNV) in our laboratory, showed that labeled nucleotides were incorporated into high molecular weight RNA under the direction of both TNV-

RNA and its satellite (STNV) RNA, whereas other plant viral RNA's failed to serve as a template.[6] In view of these results and the limited information contained in the satellite tobacco necrosis virus genome[7, 8] it is likely that the STNV-RNA utilizes at least a portion of the TNV-RNA replicase, and has an identical recognition sequence. Since the coat proteins of the two viruses are unrelated,[9, 10] any homologies in the two RNA's are likely to reflect RNA replicase recognition sites.

The 5′ nucleotide sequence of the STNV-RNA was previously determined in this laboratory.[11] For the sake of comparison, similar studies of the TNV-RNA were made and are the subject of this communication.

Materials and Methods. Preparation of ^{14}C-labeled TNV-RNA: ^{14}C-labeled tobacco necrosis virus was grown in a STNV-free chamber as described previously.[9, 11] Purification of the virus and isolation of its RNA were also described.[9]

Enzymatic hydrolyses: Pancreatic ribonuclease (RAP, 7 da, phosphate free, Worthington, Freehold, N. J.) digestions were made in 0.1 *M* Tris-HCl buffer, pH 7.5, at 37°C for 24 hr with an enzyme to substrate ratio of 1:15 (W:W).

Ribonuclease T_1 (RT_1, Worthington) digestions were made in 0.1 *M* Tris-HCl, 0.002 *M* EDTA, pH 7.5, at 37°C for 30 min using 500 units RNase T_1 per mg RNA.

Marker oligonucleotides were dephosphorylated with 0.3 units/A260 of *Escherichia coli* alkaline phosphatase (BAPF, ribonuclease-free, Worthington, Freehold, N. J.) in 0.1 *M* Tris-HCl buffer, pH 8.0, for 2 hr at 37°C. In the case of the ^{14}C-labeled oligonucleotides the digestion time was extended to 4 hr or overnight in order to digest any possible pyrophosphate[11] or triphosphate containing oligonucleotides.[12]

Column chromatography and desalting: These were as previously described.[11] Specific details are presented in the legends.

Oligonucleotide markers: The $(Ap)_nA$ series of markers were obtained from Miles Laboratories, Inc. Larger oligonucleotides were prepared by dephosphorylating the appropriate fractions of a pancreatic ribonuclease digest of 50 mg bulk yeast RNA (RNA 8KA Worthington, Freehold, N. J.) separated by the method of Tomlinson and Tener.[13]

Polyacrylamide gel electrophoresis: The ^{14}C tobacco necrosis virus coat protein obtained during the isolation of the ^{14}C TNV-RNA was solubilized and electrophoresed on SDS-polyacrylamide gels.[9] Unlabeled coat protein markers of the two viruses were included. After they were stained and the marker positions recorded, the gels were sliced into 1-mm slices with stacked razor blades and counted for radioactivity.

Radioactive counting: All samples were counted in a Beckman LS 250 liquid scintillation counter in toluene, PPO, POPOP, and BBS-3 solubilizer.

Results. The preparations of ^{14}C-labeled tobacco necrosis virus were checked for possible contaminations with satellite tobacco necrosis virus by SDS-polyacrylamide gel electrophoresis of the coat protein. The two coat proteins are sufficiently different in molecular weights to be well separated by this method.[9] To increase the sensitivity, the gels were intentionally overloaded. Under these conditions the dimer of tobacco necrosis virus coat protein was detectable in small quantities. As shown in Figure 1, no radioactive counts were detectable in the region corresponding to the STNV marker.

The elution profile of a pancreatic ribonuclease digest of ^{14}C-labeled TNV-RNA (1.2×10^6 cpm) from DEAE-cellulose in 7 *M* urea is shown in Figure 2. The fractions higher than −4 were desalted, dephosphorylated, and rechromatographed with appropriate nonradioactive markers. To avoid dephosphorylation of markers by residual alkaline phosphatase activity,[11] only markers

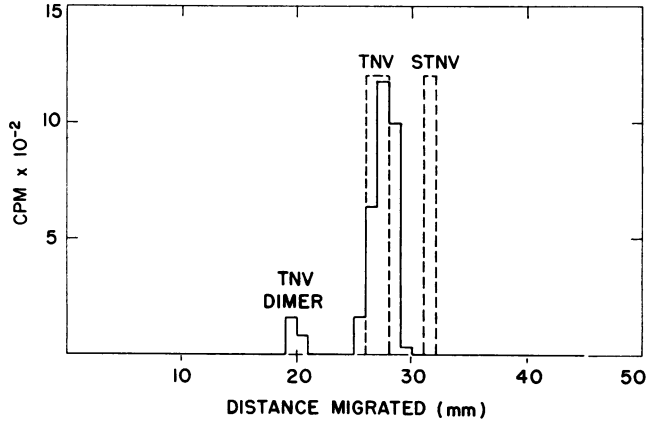

FIG. 1.—SDS-polyacrylamide gel electrophoresis of ^{14}C-labeled tobacco necrosis virus coat protein. The sample was electrophoresed with tobacco necrosis virus and satellite tobacco necrosis virus coat protein markers on a 5% gel and stained as described previously.[9] After the positions of the markers were recorded (*broken line*), the gel was sliced into 1-mm sections with stacked razor blades and assayed for radioactivity (*solid line*). The marker in the dimer position is now shown.

devoid of terminal phosphates were used. In all rechromatographed fractions, with the exception of −7, the radioactivity was found in a single peak whose position corresponded to two negative charges less than the original fraction. The absence of radioactivity in any other position indicated that these fractions contained only internal oligonucleotides and that the dephosphorylation was quantitative. Different results were obtained with fraction −7 as shown in Figure 3. In addition to the major radioactive peak −5, significant counts were detected in the −2 and −7 position.

Similar results were obtained previously with STNV-RNA[11] except that the radioactivity in peak −7 in Figure 3 is significantly higher. To eliminate the possibility of incomplete dephosphorylation, this peak was redigested overnight with alkaline phosphatase. However, the counts remained in the −7 position. Incomplete pancreatic ribonuclease digestion could have resulted in an alkaline

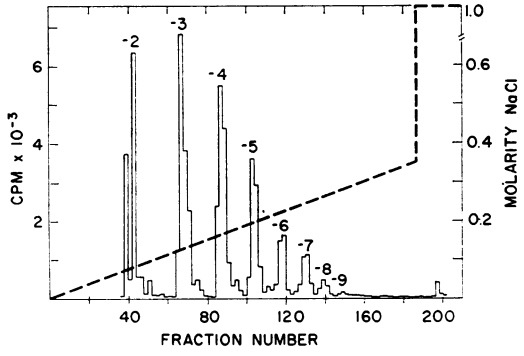

FIG. 2.—Chromatography of a pancreatic ribonuclease digest of ^{14}C-labeled TNV-RNA (1.2×10^6 cpm) on a 0.9×50 cm DEAE-cellulose column. The sample was eluted with a sodium chloride gradient (*broken line*) in 7 *M* urea and 0.005 *M* Tris-HCl buffer, pH 7.5. Total vol 500 ml.

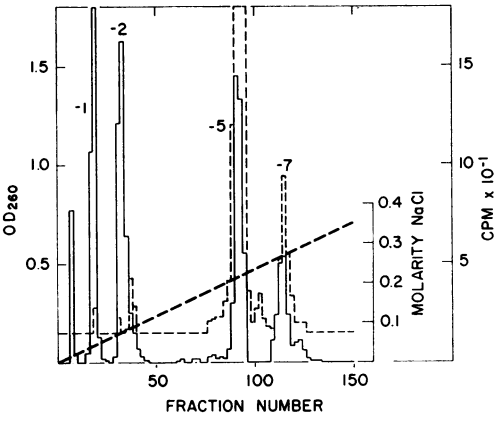

FIG. 3.—Chromatography of the dephosphorylated −7 fraction shown in Fig. 2 with the appropriate markers on a 0.6 × 40 cm DEAE-cellulose column. The sample was eluted with a sodium chloride gradient (*heavy broken line*) in 7 *M* urea and 0.005 *M* Tris-HCl buffer, pH 7.5. Total vol 300 ml. The fractions were assayed for absorbance (*solid line*) and radioactivity (*broken line*).

phosphatase resistant cyclic $(Np)_6Np$! Alternately a protein contaminant in the RNA preparation could have chromatographed in this position.

Peak −2, obtained from fraction −7 (Fig. 3), was desalted and chromatographed together with (ApGp)U and (ApGp)C nonradioactive markers on DEAE-cellulose with a triethyl ammonium carbonate gradient as shown in Figure 4. Under these conditions the nonisomeric trinucleotide markers were well separated. It should be noted that the ApApA peak originated from the −2 marker shown in Figure 3. The fractions corresponding to each peak were pooled and aliquots were examined for radioactivity. Only the (ApGp)U peak contained radioactivity.

From the data discussed above it can be concluded that as in STNV-RNA the 5′-terminal fragment is a pyrophosphorylated trinucleotide (ApGp)U. Since the isomeric trinucleoside diphosphates do not separate on DEAE-cellulose with a triethyl ammonium carbonate gradient (Fig. 4) the order of the Gp and Ap had to be determined. This was accomplished by the examination of an alkaline phosphatase treated T_1 digest of the radioactive trinucleoside diphosphate. A distinction in the two isomeric triplets could be made since in one case the expected product would be ApG + U whereas its isomer would have

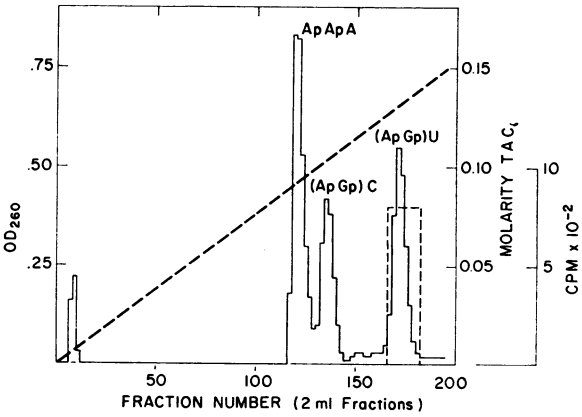

FIG. 4.—Chromatography of the −2 peak shown in Fig. 3 with (ApGp) U and (ApGp) C markers on a 0.6 × 40 cm DEAE-cellulose column. Elution was carried out with a 400 ml linear triethyl ammonium carbonate (TAC) gradient (*heavy broken line*). Fractions were assayed for absorbance (*solid line*). Each peak was pooled, flash evaporated, and assayed for radioactivity (*broken line*).

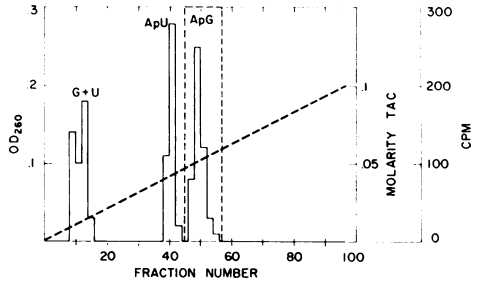

FIG. 5.—Chromatography of a dephosphorylated ribonuclease T_1 digest of the (ApGp)U peak shown in Fig. 4 on a 0.6 × 40 cm DEAE-cellulose column. Elution was performed with a 200 ml linear triethyl ammonium carbonate (TAC) gradient (*heavy broken line*). Fractions of 2 ml were collected and assayed for absorbance (*solid line*). The ApU and ApG peaks were flash evaporated and assayed for radioactivity (*broken line*).

yielded G + ApU. Figure 5 shows the analysis of the product with the appropriate markers. Radioactivity was found only in the nucleoside (not shown) and in the ApG fractions. The sequence ppApGpUp . . . was therefore assigned to the 5′-terminus of TNV-RNA.

Discussion. The RNA's of all bacterial viruses so far examined contained a triphosphate in the 5′ position.[2, 12, 14] This is consistent with the current theory of the mechanism of RNA synthesis. The 5′-RNA terminus of some plant viruses, however, was reported to be nonphosphorylated,[15, 16] which suggests an *in vivo* enzymatic removal of the triphosphate.

In recent reports from our laboratory,[11, 17] the possible enzymatic removal of a γ-phosphate resulting in a 5′-pyrophosphorylated end has been discussed in connection with the structure of STNV-RNA. The identical degree of phosphorylation of TNV-RNA is not surprising since the sites of synthesis of a dependent virus and its helper virus would be expected to be identical or at least adjacent. Some unique structural features of the coat proteins of the two viruses have already suggested the probability of a common site distinct from other plant viruses.[10]

The identity of the 5′-terminal triplet of TNV-and STNV-RNA is unlikely to be accidental and suggests a functional importance. In view of our data[6] and the report on the invariance of the 5′ end in Q_β-RNA and its monster[3, 5] it would seem that the function of the 5′ end pertains to replicase recognition.

Because of the failure of partly fragmented Q_β-RNA to exhibit template activity, it was suggested that both ends of the Q_β-RNA molecule are required in replicase recognition.[18] Although the circular structure postulated in this mechanism did not materialize, a recent investigation[19] suggests an alternate means of bringing the two ends together. A high degree of secondary structure was found within a cistron of the R17 RNA, and it was proposed that the sequence of a viral RNA is determined not only by the need to specify a certain amino acid sequence but also by its need to assume a particular secondary structure. It may very well be that one of these structural requirements results in the spatial juxtaposition of the two RNA termini. This model is particularly attractive since it would also explain the template activity of the complementary strand[20] and also possibly put a restriction on the lower limit of the size of the little variant.[4] A comparison of the 3′-terminal sequences of the RNA's of TNV and STNV should provide information pertinent to these problems. These studies and the investigation of the extent of the homologies are currently in progress.

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