Translation Initiation Site of the Coat Protein Messenger Ribonucleic Acid of the Cowpea Strain of Tobacco Mosaic Virus†

Karen S. Browning‡ and John M. Clark, Jr.*

ABSTRACT: The 5′-terminus of the coat protein messenger RNA of the cowpea strain of tobacco mosaic virus contains a $m^7G(5')ppp(5')Gp\cdots$ cap group. Removal of this cap group followed by introduction of a 5′-terminal [^{32}P]phosphomonoester onto the decapped RNA and use of this ^{32}P-labeled RNA in nucleotide sequence studies establishes that the 5′-terminal nucleotide sequence of this RNA is $m^7G(5')ppp(5')$-GUAUUUGAUGAUGGCAUACUCGAUUCCGACUC-$C^U_CAG^U_C\cdots$. The codons following the two consecutive AUG sequences match the N-terminal amino acids of the coat protein of the cowpea strain of tobacco mosaic virus. Translation initiation complexes between wheat germ 80S ribosomes and this messenger RNA protect regions adjacent to the two consecutive AUG sequences of this messenger RNA from attack by ribonucleases. Therefore, one or both of these AUG sequences serve as sites for the initiation of translation of the coat protein messenger RNA of the cowpea strain of tobacco mosaic virus.

Infection of tobacco plants with the wild strain of tobacco mosaic virus (U_1-TMV)[1] results in the production of a multicomponent virion system featuring a complete genome virion, two or more encapsidated partial genome RNAs, and a small (mol wt ~280 000) U_1-TMV coat protein mRNA that lacks a site for initiation of virion assembly and therefore exists as a nonencapsidated mRNA (Hunter et al., 1976; Beachy et al., 1976; Siegel et al., 1976; Zimmern & Wilson, 1976; Beachy & Zaitlin, 1977). Infection of bean plants with the cowpea strain of tobacco mosaic virus (C_c-TMV) results in the production of a similar multicomponent virion system except that the small (mol wt ~280 000) mRNA for C_c-TMV coat protein (C_c-TMV S-RNA) is encapsidated in a small virion form because it contains a site for initiation of virion assembly (Whitfeld & Higgins, 1976; Higgins et al., 1976; Bruening et al., 1976; Beachy & Zaitlin, 1977; Fukuda et al., 1980). The amino acid sequence of the coat proteins of U_1-TMV and C_c-TMV have been determined (Anderer et al., 1960; Tsugita et al., 1960; Anderer et al., 1965; Rees & Short, 1975). These sequences demonstrate over 50% nonhomology in their amino acids, thus suggesting that the coat protein mRNAs of these two TMV strains are quite different. The nucleotide sequence of the nonencapsidated mRNA for U_1-TMV coat protein has been determined (Guilley et al., 1979) and reveals a translation initiation site for U_1-TMV coat protein beginning 9 nucleotides in from a $m^7G(5')ppp(5')Gp\cdots$ containing the 5′-terminus. No nucleotide sequence data exist for the S-RNA of C_c-TMV (i.e., the C_c-TMV coat protein mRNA).

Characterization of the nucleotides at the 5′-terminus of the C_c-TMV S-RNA allows a comparison of this sequence with the 5′-terminal sequence of the related U_1-TMV coat protein mRNA. Such a comparison can possibly interpret two important features of TMV systems. First, a comparison of the 5′-terminal untranslated regions of these related coat protein mRNAs allows detection of conserved, yet untranslated, nucleotide sequences between these mRNAs and may suggest mechanisms for the initiation of translation of these related mRNAs. Second, a comparison of the 5′-terminal regions of these related mRNAs may reveal aspects of the mechanism that generates these subgenomic RNAs. Accordingly, this paper reports the characterization of the 5′-terminus of the S-RNA of C_c-TMV.

Materials and Methods

Materials. The cowpea strain of TMV was generously provided by Dr. M. Zaitlin. A mixture of 15 ^{14}C-labeled amino acids for protein synthesis assays was purchased from Amersham Corp. Carrier-free [^{32}P]P_i was from New England Nuclear Corp. [γ-^{32}P]ATP was prepared from [^{32}P]P_i by the method of Johnson & Walseth (1979) by using enzymes purchased from Boehringer Mannheim. $m^7G(5')ppp(5')G$, $m^7G(5')ppp(5')G^m$, and nuclease P_1 were obtained from P-L Biochemicals, Inc. RNase A, RNase T_1, and RNase U_2 were purchased from Calbiochem-Behring Corp. Ribonuclease Phy M was generously provided by Dr. H. Donis-Keller. Calf intestinal phosphatase was purchased from Boehringer-Mannheim. T_4 polynucleotide kinase (22 000 units/mL) was a gift from Dr. O. C. Uhlenbeck. DEAE-cellulose thin-layer chromatography plates for "wandering spot" analyses were purchased from Brinkman Co. and Analtech. Inc. Hepes buffer was purchased from Calbiochem-Behring Corp. Sparsomycin was obtained from the Upjohn Co. All other reagents were as received from Sigma Chemical Co.

Preparation of the S-RNA of C_c-TMV. An enriched preparation of the small virion of C_c-TMV was isolated from C_c-TMV infected bean leaves (*Phaseolus vulgaris*, cv. Bountiful) by the method of Bruening et al. (1976). C_c-TMV RNAs were extracted (Beachy & Zaitlin, 1977) from this preparation and then the C_c-TMV S-RNA was resolved from the other RNAs by gel electrophoresis and electroelution prior to phenol extraction and alcohol precipitation (yield = 50 μg of C_c-TMV S-RNA/200 g of infected leaves).

Preparation of the S-[5′-^{32}P]RNA of C_c-TMV. Potential 5′-terminal "cap" groups were removed from the S-RNA of C_c-TMV by the method of Richards et al. (1978), and re-

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[1] Abbreviations used: TMV, tobacco mosaic virus; U_1-TMV and C_c-TMV, the wild and cowpea strains of TMV, respectively; S-RNA, short RNA that serves as a moncistronic mRNA for viral coat protein; N, any ribonucleotide; XC, xylene cyanole FF marker dye; VSV, vesicular stomatitis virus; TYMV, turnip yellow mosaic virus; BMV, brome mosaic virus.

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sultant terminal phosphate groups were then removed with calf intestinal alkaline phosphatase prior to reisolation of the 5′-dephosphoryl S-RNA of C_c-TMV by phenol extraction and alcohol precipitation. The 5′-dephosphoryl S-RNA of C_c-TMV was converted to S-[5′-^{32}P]RNA by incubation with [γ-^{32}P]ATP and T_4 polynucleotide kinase (Leung et al., 1979), and the intact S-[5′-^{32}P]RNA of C_c-TMV (detectable by 10-min radioautographic analysis) was resolved and isolated by electrophoresis, electroelution, phenol extraction, and alcohol precipitation.

Analyses with the S-[5′-^{32}P]RNA of C_c-TMV. The homogeneity of the S-[5′-^{32}P]RNA of C_c-TMV was confirmed by a repeat of the gel electrophoretic resolution used to prepare the C_c-TMV S-[5′-^{32}P]RNA and by exhaustive digestion of the S-[5′-^{32}P]RNA with RNase T_2 (Zimmern, 1976) and subsequent chromatography of the released [^{32}P]pGp against nucleoside 3,5-bisphosphate standards (Leung et al., 1979). The 5′-terminal nucleotide sequence of the S-[5′-^{32}P]RNA of C_c-TMV was characterized by the "wandering spot" method of Silberklang et al. (1977) and by the rapid sequencing gel analysis method of Donis-Keller et al. (1977).

Preparation of S-[U-^{32}P]RNA of C_c-TMV. Uniform ^{32}P-labeled C_c-TMV S-RNA was prepared by in vivo labeling of C_c-TMV infected bean leaves with [^{32}P]P_i (Goodman et al., 1980). The ^{32}P-labeled C_c-TMV RNAs were isolated in a manner similar to that described for unlabeled C_c-TMV RNAs and then the intact S-[U-^{32}P]RNA of C_c-TMV was obtained by electrophoretic resolution, electroelution, and final phenol extraction, all as described for unlabeled C_c-TMV S-RNA.

Analyses of the S-[U-^{32}P]RNA of C_c-TMV. The 5′-terminal [^{32}P]m^{7}G(5′)ppp(5′)Gp was obtained from the S-[U-^{32}P]RNA of C_c-TMV by exhaustive digestion with RNase T_1 or T_2 and subsequent electrophoresis on DEAE-cellulose paper (Zimmern, 1976). The character of this 5′-terminal capping group was confirmed by exhaustive digestion of this compound with phosphomonoesterase and subsequent two-dimensional chromatography (Gross et al., 1978). Ribosome protection analyses of the S-[U-^{32}P]RNA of C_c-TMV were performed as described by Browning et al. (1980) with the exception that the assays featured 0.1-mL reactions containing (5×10^5)–(1.5×10^6) dpm of S-[U-^{32}P]RNA (i.e., 1–2 μg of RNA). Specific ^{32}P-labeled initiation fragments were resolved by gel electrophoresis and extraction from the gel (Browning et al., 1980) prior to assay for the presence of a 5′-terminal capping group by exhaustive digestion with RNase T_2 and subsequent electrophoresis on DEAE-cellulose paper (Zimmern, 1976).

Results

Characterization of a Capping Group at the 5′-Terminus of the S-RNA of C_c-TMV. All previously characterized TMV RNAs, and most other eucaryotic mRNAs, contain a 5′-terminal m^{7}G(5′)ppp(5′)Np··· capping group (Shatkin, 1976; Zimmern, 1976; Guilley et al., 1979). One preliminary report (Fukuda et al., 1980) also suggests that the S-RNA of C_c-TMV contains a 5′-terminal capping group. We have developed three lines of evidence that establish that the S-RNA of C_c-TMV contains a 5′-terminal m^{7}G(5′)ppp(5′)Gp··· group. First, we have determined that treatment of the C_c-TMV S-RNA with chemical steps known to remove 5′-terminal capping groups (Richards et al., 1978) results in a 75% drop in the in vitro translation potential of this mRNA. This loss in in vitro translation potential characteristic of 5′-terminally capped eucaryotic mRNAs does not reflect extensive cleavage of the C_c-TMV S-RNA for identical treatment of an uncapped control mRNA, STNV RNA, does not significantly change its in vitro translation potential. Second, we observe that

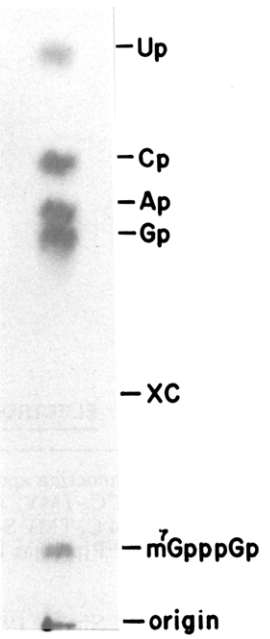

FIGURE 1: Radioautogram of the DEAE-cellulose paper electrophoretic resolution of the ^{32}P-labeled products released from the S-[U-^{32}P]RNA of C_c-TMV by exhaustive digestion with RNase T_2.

treatment of the S-RNA of C_c-TMV with chemical treatments known to remove 5′-terminal capping groups and a subsequent phosphatase treatment known to remove the 5′-terminal phosphates remaining after chemical decapping results in a 27-fold increase in the quantity of ^{32}P incorporated into intact C_c-TMV S-[5′-^{32}P]RNA by 5′OH kinase (T_4 polynucleotide kinase) in the presence of [γ-^{32}P]ATP. RNase T_2 dependent exhaustive digestion of this C_c-TMV S-[5′-^{32}P]RNA yields [^{32}P]pGp as the only detectable product and suggests some form of a 5′-terminal m^{7}G(5′)ppp(5′)Gp structure for the original RNA. Third, we have determined that RNase T_1 and T_2 dependent exhaustive digestion of in vivo labeled S-[U-^{32}P]RNA of C_c-TMV followed by paper electrophoretic resolution (Zimmern, 1976) yields a readily detectable ^{32}P-labeled m^{7}G(5′)ppp(5′)Gp group (Figure 1). The release of this material by RNase T_1, an enzyme that cannot cleave adjacent to 2′-*O*-methylguanosine residues in RNAs (RajBhandary et al., 1967; Samuelson & Keller, 1972; Reid et al., 1972), and two-dimensional thin-layer chromatographic analysis (Gross et al., 1978) both establish that the original capping group lacks additional methylation on the 2′-hydroxyl group of the G residue.

The 5′-Terminal Nucleotide Sequence of the S-RNA of C_c-TMV. The ability to produce a homogeneous [5′-^{32}P]RNA from the S-RNA of C_c-TMV facilitates the characterization of 5′-terminal nucleotides of this RNA. "Wandering spot" nucleotide sequence analyses (Silberklang et al., 1977) define the sequence GUAUUUGAUGAUG for the first 13 nucleotides following the 5′-terminal capping group of C_c-TMV S-RNA (Figure 2). Polyacrylamide gel electrophoretic analysis of the ^{32}P-labeled oligonucleotides produced by partial digestions of the S-[5′-^{32}P]RNA of C_c-TMV with specific nucleases (Donis-Keller et al., 1977; Simoncsits et al., 1977; Leung et al., 1979) characterizes 32 nucleotides near the 5′-terminus of the S-RNA of C_c-TMV (Figure 3). The overlapping homology of these different characterizations and the codon assignments for the N-terminal amino acids of

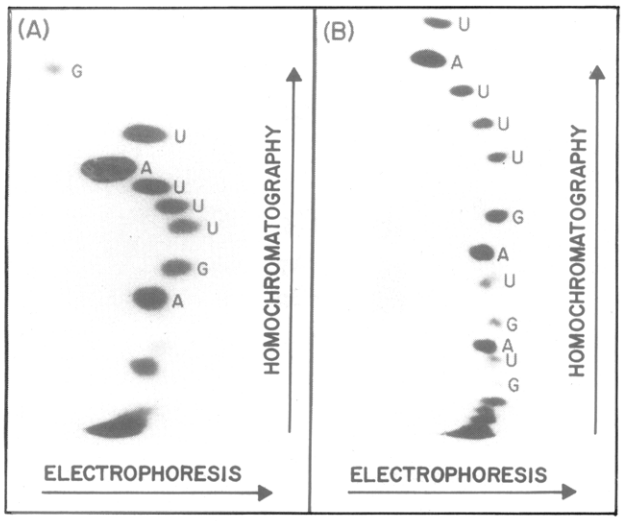

FIGURE 2: Radioautograms of "wandering spot" analyses of the 5′-terminus of the S-[5′-^{32}P]RNA of C_c-TMV. (A) Analysis of the first 8 nucleotides at the 5′-terminus of C_c-TMV S-RNA; (B) Similar analysis in which the 5′-terminal [^{32}P]pG has been eluted off the chromatogram.

C_c-TMV coat protein (Rees & Short, 1975) establish the 5′-terminal nucleotide sequence and translation reading frame:

Met Ala Tyr

$m^7G(5')ppp(5')GUAUUUGAUGAUGGCAUAC-$

Ser Ile Pro Thr Pro Ser

$UCGAUUCCGACUCC\begin{matrix}U\\C\end{matrix}AG\begin{matrix}U\\C\end{matrix}\cdots$

The Protection of the S-RNA of C_c-TMV by Wheat Germ 80S Ribosomes. Ribosome protection assays detect specific regions of a mRNA that associate with ribosomes and suggest portions of mRNAs that function in the correct recognition of initiator AUGs (Steitz, 1969; Browning et al., 1980). Accordingly, we examined the protection of the S-[U-^{32}P]-RNA of C_c-TMV by wheat germ 80S translation initiation complexes. Gel electrophoretic resolution of the ^{32}P-labeled materials protected by these translation initiation complexes (Figure 4) reveals that these 80S ribosomes protect approximately 37, 35, and 30 nucleotide long initiation fragments from RNase T_1 and approximately 31, 29, and 28 nucleotide long initiation fragments from RNase A. We have used two approaches to characterize these specific initiation fragments. First, we used the method of Zimmern (1976) to establish that the approximately 37, 31, 30, and 28 nucleotide long initiation fragments of Figure 4 each contain a ^{32}P-labeled $m^7G(5')$-ppp(5′)Gp group while the approximately 35 and 29 nucleotide long initiation fragments of Figure 4 lack this group (Zimmern, 1976). Second, we combined these characterizations of the presence or absence of a 5′-terminal capping group with the sizes of the specific initiation fragments, the specificities of the ribonucleases used to generate the fragments, and the 5′-terminal nucleotide sequence of the S-RNA of C_c-TMV to obtain the characterizations of these initiation fragments shown in Figure 5.

Discussion

This paper characterizes the 5′-terminal nucleotides and the translation-initiation site of the S-RNA of C_c-TMV. Three features of this overall characterization deserve further comment. First, the nucleotide sequence provides a unique pair of consecutive AUG codons either or both of which can serve as an initiator AUG. We have not investigated which of these

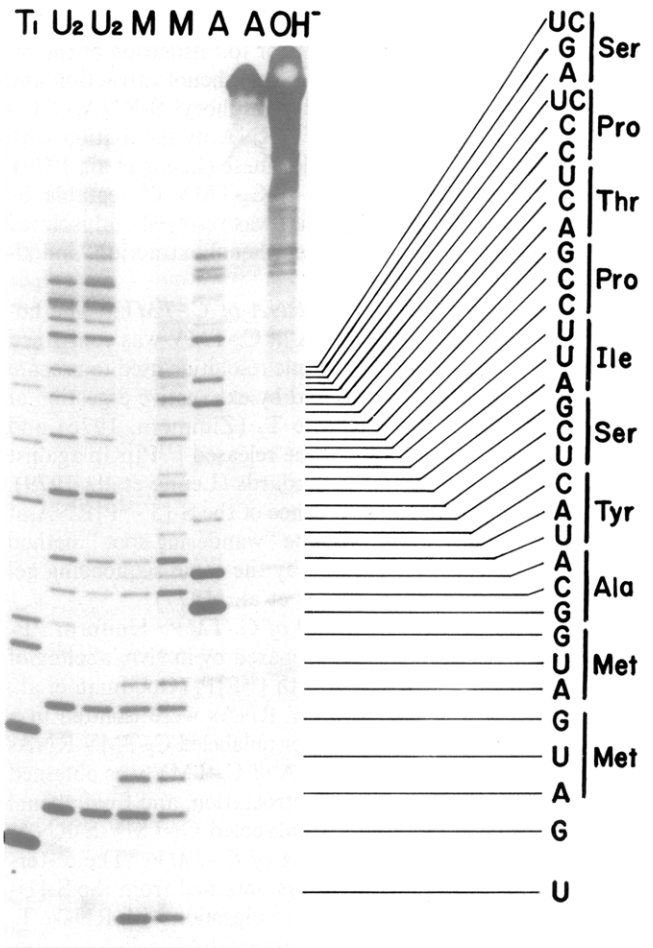

FIGURE 3: Radioautogram of the gel electrophoretic resolution of the ^{32}P-labeled digestion products derived from the S-[5′-^{32}P]RNA of C_c-TMV by partial enzymatic digestions with specific nucleases. The oligonucleotides derived from the first 5 nucleotides at the 5′-terminus of the S-[5′-^{32}P]RNA of C_c-TMV have been run off the gel. The column headings T_1, U_2, etc. refer to the specific RNases used to treat the S-[5′-^{32}P]RNA before electrophoretic resolution. OH^- represents partial hydrolysis of the S-[5′-^{32}P]RNA with alkali before electrophoretic resolution.

AUG codons serve as initiator codons. Preproparathyroid hormone mRNA (Kronenberg et al., 1979) is currently the only other mRNA known to have two consecutive potential initiator AUGs. As a matter of precedence, an in vitro system from the wheat germ recognizes the first of the consecutive AUGs on preproparathyroid mRNA as the initiator AUG (Kemper et al. 1976a,b). C_c-TMV coat protein has the N-terminal sequence Ala-Tyr-Ser··· (Rees & Short, 1975). Therefore, all methionine residues preceeding the Ala-Tyr-Ser··· sequence must be removed during or after the in vivo synthesis of C_c-TMV coat protein. Second, the sequence includes two consecutive out-of-phase UGA terminator codons at, and just preceding, the initiation codon. Thus, this mRNA is like many other mRNAs in that premature out-of-phase translations are stopped before the correct initiation of translation begins. Third, the sequence does not suggest any degree of secondary structure at or near the 5′-terminus of the mRNA. Instead, C_c-TMV 5-RNA is similar to the VSV mRNAs (Rose, 1978), the TYMV coat protein mRNA (Briand et al., 1978), and the small virion RNA of BMV (Dasgupta et al., 1975) in that a short 5′-terminally capped and unstructured region precedes the initiator AUG.

As mentioned earlier, a comparison of the 5′-terminus of the S-RNA of C_c-TMV with the 5′-terminus of the related

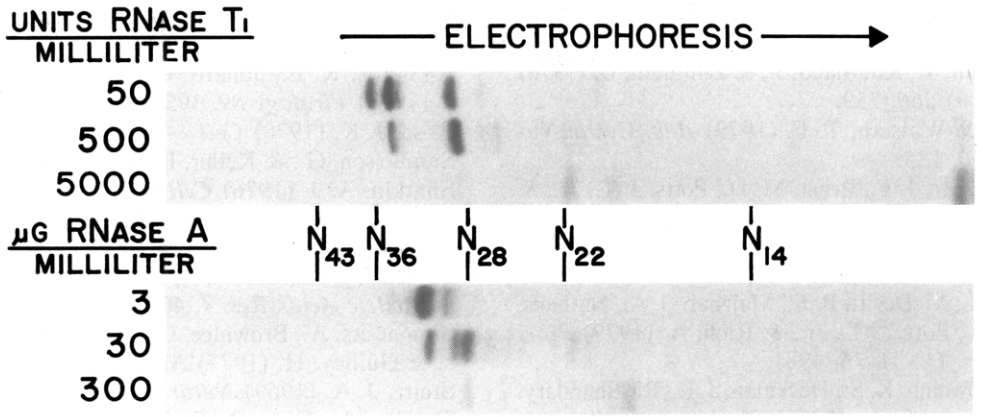

FIGURE 4: Radioautogram of the gel electrophoretic resolution of the ^{32}P-labeled initiation fragments of C_c-TMV S-[U-^{32}P]RNA that are protected from increasing concentrations of RNase T_1 and RNase A by wheat germ 80S translation initiation complexes. N_{43}, N_{36}, N_{22}, and N_{14} indicate the location of specific size standards generated by partial digestion of S-[5′-^{32}P]RNA of C_c-TMV with RNase T_1 and subsequent resolution of the ^{32}P-labeled materials in parallel tracks on the gel.

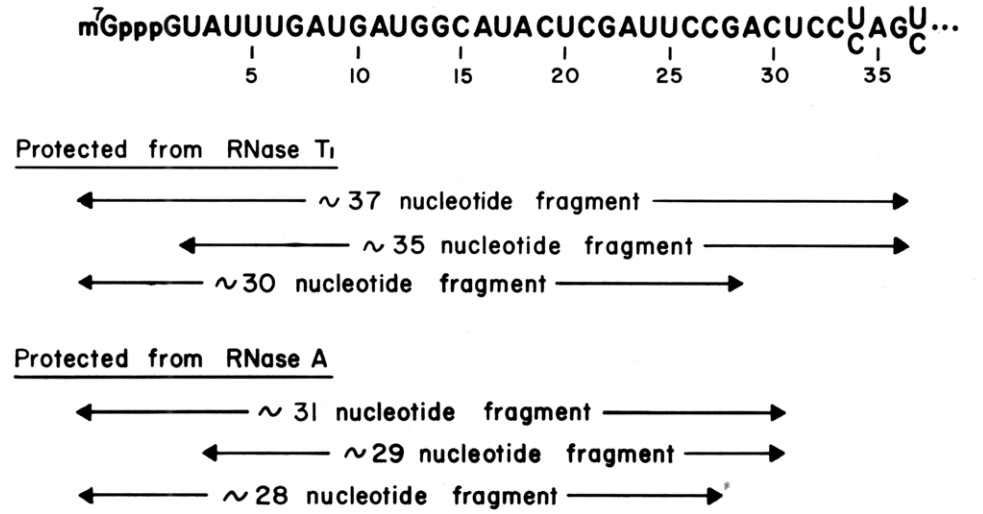

FIGURE 5: Proposed origins for the 80S initiation fragments of the S-[U-^{32}P]RNA of C_c-TMV.

U_1-TMV coat protein mRNA may reveal conserved features of these two mRNAs that are involved in their replication or translation. However, as seen in the sequence comparison below, the 5′-terminal untranslated regions of these two related

C_c-TMV coat protein mRNA

$$m^7G(5')ppp(5')GUAUUUGAUGAUG\cdots \quad (\overline{\text{Met}})$$

U_1-TMV coat protein mRNA

$$m^7G(5')ppp(5')GUUUUAAAUAUG\cdots \quad (\overline{\text{Met}})$$

mRNAs are rather similar in both size and nucleotide composition. Thus, if the second AUG of C_c-TMV coat protein mRNA is defined as the initiator AUG and this codon is matched with the initiator AUG of the U_1-TMV coat protein mRNA, one detects homology (or conservation) of four nucleotides in the untranslated 5′-terminal regions of these mRNAs. If one matches the 5′-terminal capping groups as a point of reference for evaluation of nucleotide homology, one detects homology of six untranslated nucleotides in these mRNAs. Yet, the rather similar character of the untranslated regions of these mRNAs dictates that data on the character of the 5′-terminal untranslated regions of other TMV coat protein mRNAs must be obtained before one can utilize nucleotide homologies as an indication of function in the initiation of translation or in the replication process that yields the multicomponent RNAs of TMV systems.

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[Tetrakis(3-nitrotyrosine)]insulin†

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ABSTRACT: The reaction of Zn-free bovine insulin with a 2.5-fold excess (over the tyrosines) of tetranitromethane at pH 8 results in the modification of all four tyrosine residues in the insulin molecule. The maximum amount of 3-nitrotyrosine produced in the reaction occurs at 35 min and amounts to ~2.7 residues/molecule of insulin. The remainder of the modified tyrosines are unaccounted for by amino acid analysis and are probably involved in the formation of intermolecularly cross-linked derivatives consisting of dimers and higher polymers of insulin which compose 70% of the reaction mixture. After removal of polymeric material from the reaction mixture by gel chromatography on Sephadex G-50, the monomeric material was separated according to charge by DEAE-cellulose chromatography in 7 M urea at pH 8.0 to give a 10% yield of [tetrakis(3-nitrotyrosine)]insulin—a derivative in which each of the four tyrosines of insulin has been nitrated at the 3 position of the aromatic ring. Spectrophotometric titration indicated that each of the nitrotyrosine phenolic groups had the same pK_a value of 7.3. Sedimentation equilibrium experiments indicated that the [tetrakis(3-nitrotyrosine)]insulin did not aggregate at pH 7.4 even in the presence of Zn^{2+}. At pH 2, however, the material aggregated to a much larger extent than insulin under similar conditions. The circular dichroism spectrum of [tetrakis(3-nitrotyrosine)]insulin was similar to that of desoctapeptide-(B23–30)-insulin, an inactive derivative which also does not aggregate at pH 8. The spectrum, in contrast to that of insulin, was not perturbed by pH, protein concentration, or the presence of Zn^{2+}. [Tetrakis(3-nitrotyrosine)]insulin exhibited a hormonal response in the fat cell assay of 55% and in the mouse convulsion assay of 50–70% of that of the native hormone. Thus, [tetrakis(3-nitrotyrosine)]insulin represents a unique derivative of insulin in which the physical properties have been altered without undue effect on the hormonal activity. The inability of [tetrakis(3-nitrotyrosine)]insulin to aggregate at physiological pH values indicates that the monomeric form is the active species in the hormonal response. The fact that the introduction of the nitro group had the expected effect of lowering the pK_a values of the phenolic groups from 10.4 in insulin to 7.3 in [tetrakis(3-nitrotyrosine)]insulin but with retention of 50% of the hormonal activity implies a minor role for some if not all of the tyrosines in eliciting the hormonal response.

The study of the structural aspects of the insulin molecule that are responsible for its hormonal activity has been approached in this laboratory via the preparation of homogeneous insulin derivatives obtained through chemical or enzymatic modifications of the native hormone. Investigations of the hormonal and physical properties of the derivatives yield an insight into the significance of the changes for the function of the insulin molecule (Blundell et al., 1972). Since three of the four tyrosines in insulin are conserved in all of the species whose sequences have been determined (Dayhoff, 1976), an important functional role can be inferred for these groups. That this role may involve the hormonal action of insulin is implied by the fact that extensive iodination of the molecule leads to a loss of hormonal activity (Izzo et al., 1964; Rosa et al., 1967). The introduction of tetranitromethane as a relatively specific agent for modifying tyrosines (Riordan et al., 1966; Sokolovsky et al., 1966) stimulated our interest in

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