Capping of Tobacco Mosaic Virus RNA: Analysis of Viral-Coded Guanylyltransferase-Like Activity

David Dunigan  
*University of Nebraska-Lincoln*, ddunigan2@unl.edu

Milton Zaitlin  
*Cornell University*

Follow this and additional works at: [https://digitalcommons.unl.edu/plantpathpapers](https://digitalcommons.unl.edu/plantpathpapers)

Part of the [Plant Pathology Commons](https://digitalcommons.unl.edu/plantpathpapers)

Dunigan, David and Zaitlin, Milton, "Capping of Tobacco Mosaic Virus RNA: Analysis of Viral-Coded Guanylyltransferase-Like Activity" (1990). *Papers in Plant Pathology*. 124.  
[https://digitalcommons.unl.edu/plantpathpapers/124](https://digitalcommons.unl.edu/plantpathpapers/124)

This Article is brought to you for free and open access by the Plant Pathology Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Plant Pathology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Capping of tobacco mosaic virus RNA: Analysis of viral-coded guanylyltransferase-like activity

David D. Dunigan and Milton Zaitlin

Department of Plant Pathology, Cornell University, Ithaca, New York 14853, USA

Abstract

The 5’ end of tobacco mosaic virus (TMV) genomic RNA is capped with 7-methylguanosine. A virus-coded polypeptide with guanylyltransferase activity has been investigated. This enzyme is responsible for forming the 5’-5’ linkage of guanosine 5’-monophosphate to the 5’-diphosphate of an acceptor RNA, thereby forming the cap. A critical step in the mechanism for cap formation in the eukaryotic nucleus is for guanylyltransferase to bind covalently to guanosine 5’-monophosphate with the hydrolysis of pyrophosphate when guanosine 5’-triphosphate is the substrate. The TMV 126-kilodalton protein, which is most probably a component of the TMV replicase, was found to have this activity. The mechanism of this reaction has been characterized biochemically.

Abbreviations: TMV, tobacco mosaic virus; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; dd, dideoxy.

Tobacco mosaic virus (TMV) is a plus-strand RNA plant virus. The 5’ end of the genomic RNA is terminated by 7-methylguanosine. A virus-coded polypeptide with guanylyltransferase activity has been investigated. This enzyme is responsible for forming the 5’-5’ linkage of guanosine 5’-monophosphate to the 5’-diphosphate of an acceptor RNA, thereby forming the cap. A critical step in the mechanism for cap formation in the eukaryotic nucleus is for guanylyltransferase to bind covalently to guanosine 5’-monophosphate with the hydrolysis of pyrophosphate when guanosine 5’-triphosphate is the substrate. The TMV 126-kilodalton protein, which is most probably a component of the TMV replicase, was found to have this activity. The mechanism of this reaction has been characterized biochemically.

Tobacco mosaic virus (TMV) is a plus-strand RNA plant virus. The 5’ end of the genomic RNA is terminated by 7-methylguanosine. A virus-coded polypeptide with guanylyltransferase activity has been investigated. This enzyme is responsible for forming the 5’-5’ linkage of guanosine 5’-monophosphate to the 5’-diphosphate of an acceptor RNA, thereby forming the cap. A critical step in the mechanism for cap formation in the eukaryotic nucleus is for guanylyltransferase to bind covalently to guanosine 5’-monophosphate with the hydrolysis of pyrophosphate when guanosine 5’-triphosphate is the substrate. The TMV 126-kilodalton protein, which is most probably a component of the TMV replicase, was found to have this activity. The mechanism of this reaction has been characterized biochemically.
homogenate was subsequently stored at −70°C; approximately 40–70% loss of activity was noted at the end of 1 year. In some experiments, the leaf tissue homogenates were fractionated by centrifugation. Low speed centrifugation in a microcentrifuge at 13,000 × g for 10 min at 4°C yielded supernatant (S-13) and pellet (P-13) fractions.

**Standard Nucleotide-binding Assay—** The basic reaction conditions were to mix 1 part of homogenate (usually 10 μl) with 3 parts (30 μl) of Bradley buffer containing radioactive nucleotides. The nucleotides were lyophilized in an evaporating centrifuge and then solubilized with the Bradley buffer. Typically there was 5–10 μCi of nucleotide (specific activity, 3,000 Ci/mmol)/incubation. Once the incubation was quenched with electrophoresis sample buffer (final concentration, 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.715 M 2-mercaptoethanol, 0.1 mg/ml bromphenol blue) by mixing 20 μl of sample with 5 μl of 5 × concentrated sample buffer, vortexed, and the quenched samples were then heated for 3 min in a boiling water bath. Just prior to electrophoresis, the samples were clarified by centrifugation at 13,000 × g for 2 min at room temperature. The supernatant fraction was then loaded onto a 12.5% acrylamide, sodium dodecyl sulfate polyacrylamide gel for electrophoresis (SDS-PAGE) (29). 5–20 μl of sample was electrophoresed at 10 mA constant (50-100-V variable)/gel until the dye front (bromphenol blue) was completely out of the gel into the lower tank buffer. This step separated the unincorporated radionucleotides from the gel, thus greatly reducing the background radioactivity. After electrophoresis, the gel was fixed in acetic acid/methanol/water (10:10:80) for 30 min and then stained in the same solution containing 0.05% Coomassie Brilliant Blue G-250 and 0.05% Coomassie Brilliant Blue R-250 (Sigma) for 30 min at room temperature. The stained gel was then destained in the fixing solution for 15-24 h at room temperature with multiple changes of the solution. Under these conditions, noncovalently bound nucleotides would be released from the polypeptide and washed out of the gel; therefore, radioactivity associated with these reaction products was considered to be covalently bound. The destained gel was gelld onto Whatman 3MM paper and autoradiographed with preflashed (30) Kodak XAR film and intensifying screens and stored at −70°C. With longer exposures or reactions, polypeptides of molecular weight less than 120,000 were observed in both mock and infected tissue extracts. These

boiled in gel electrophoresis sample buffer and then filtered through silanized glass wool to remove the protein A-agarose. SDS-PAGE and autoradiography were performed as described above.

**Inhibitor Assays of Nucleotide Binding to TMV126kD Protein—** Possible inhibitors of [α-32P]GTP binding to TMV126kD protein were included in the reaction tube with the [α-32P]GTP prior to lyophilization in the centrifuging evaporator and then resolubilized with Bradley buffer. After mixing with S-13 fraction and incubating at 25°C, the reaction mixtures were quenched with SDS-PAGE sample buffer and analyzed by SDS-PAGE/autoradiography. In most studies, the values reported were derived from dilution curves where the possible inhibitor was diluted 1:10. In other studies, the curves’ values were derived from dilution curves where the possible inhibitor was diluted 1:2 through a concentration at which the compound had been determined previously to be an effective inhibitor.

**Nucleotide to Protein Bond Susceptibility Analyses—** TMV-infected leaf tissue homogenate was fractionated by centrifugation, and the S-13 fraction was incubated with [α-32P]GTP using the standard nucleotide-binding assay conditions. To test the susceptibility of the radioactive phosphate bond to the protein, both nuclease and phosphatase digestions were attempted. The effectiveness of the treatments was determined by a qualitative evaluation of presence (+) or absence (−) of the [32P]-TMV126kD protein. For PI nuclease digestion, an aliquot of the reaction mix was adjusted to 0.125 μM sodium acetate, pH 5.0, and 25 μg/ml of PI nuclease (Sigma); the mixture was incubated at 37°C for 90 min and then quenched with SDS-PAGE sample buffer. For RNase T1 digestion, an aliquot of the reaction mix was adjusted to 0.125 μM sodium acetate, pH 5.0, and 0.5 units of RNase T1 (Sigma); the mixture was incubated at 37°C for 90 min and then quenched with SDS-PAGE sample buffer. For RNase A digestion, an aliquot of the reaction mix was adjusted to 0.1 M NaCl, 0.01 M Tris-CI (pH 7.5), 0.005 μg/ml EDTA, and 2.5 Kunitz units of RNase A (Sigma); the mixture was incubated at 37°C for 90 min and then quenched with SDS-PAGE sample buffer. For alkaline phosphatase digestion, an aliquot of the reaction mix was adjusted to 0.2 μl Tris-Cl at pH 9.0 and 0.1 units/ml alkaline phosphatase (Boehringer Mannheim); the mixture was then incubated at 37°C for 2 h and then quenched with SDS-PAGE sample buffer. These samples were analyzed by SDS-PAGE and autoradiography along with an untreated aliquot of the reaction mix.

**Subcellular Fractionation of TMV-specific Nucleotide-binding Activity—** Crude homogenates of both mock- and TMV-infected leaf tissue in Bradley buffer were fractionated by differential centrifugation. Pelleted fractions were resuspended in equivalent cell volumes of Bradley buffer. Three fractions were obtained. A low speed pellet fraction (P-10) resulted from centrifuging at 10,000 × g for 10 min at 4°C. The resulting supernatant fraction was centrifuged at 100,000 × g for 1 h at 4°C, which yielded a high speed pellet fraction (P-100) and a high speed supernatant fraction (S-100). The S-100 was further fractionated by chromatographic methods. The buffer was exchanged by passing the S-100 through Sephacry G-25 (coarse) equilibrated with KTMDP-10 buffer (10 mM KC1, 50 mM Tris [pH 7.5], 5 mM MgCl2, 1 mM dietho- 2-thiol, 0.1 mM phenylmethylsulfonyl fluoride), and the void volume was collected. The collected void volume fraction was subsequently fractionated with DEAE-cellulose (Whatman DE52) using a linear salt gradient from 10 to 500 mM KC1 in the same buffer. Absorbance at 280 nm was monitored continuously. Column fractions were collected, and samples were tested for salt concentration by measuring the conductivity. The remaining sample of all the fractions was dialyzed against KTMDP-10 buffer, and samples of the dialysate were used in the standard nucleotide-binding assay with [α-32P]GTP as the substrate to determine which fraction contained the virus-specific activity.

**Results**

**Infection-specific Radiolabeling with [α-32P]GTP—** When crude extracts of mock- or TMV-infected tobacco leaf tissue were incubated with [α-32P]GTP, a virus infection-specific radiolabeled product of 120,000 approximate molecular weight was observed on autoradiograms (Figure 2). With longer exposures or reaction times, polypeptides of molecular weight less than 120,000 were observed in both mock and infected tissue extracts. These
lower molecular weight polypeptides were observed variably, but a guanylated polypeptide of M₅, 60,000 was often seen; this polypeptide may correspond to the cellular guanylyltransferase. Molar weight analysis of guanylyltransferase from plants has not been reported, but yeast (27) and mammalian (10) guanylyltransferases range from M₅, 52,000 to 68,000, respectively. We have not investigated this further. The radiolabeling of the virus infection-specific protein required the radiolabeled phosphorus be in the γ position. When the radiolabel was in the γ position (Figure 2; [γ-32P]GTP), no virus-specific labeling could be observed. When [α-32P]UTP was used as a substrate (Figure 2), no virus-specific radiolabeling was observed, in agreement with data shown in Figure 4.

**Immunoprecipitation Analysis of TMV126kD Protein** — The finding of the radiolabeled 120,000-dalton virus infection-specific protein suggested that this product may be the TMV126kD protein implicated in virus replication (3–5). Thus, the products of the nucleotide-binding reaction were immunoprecipitated with antisera made to the TMV126kD protein (6). Figure 3 is an autoradiogram depicting the reaction products when the incubation was allowed to continue for 150 min as well as the immunoprecipitation products. During this long incubation, several secondary reaction products were observed in both the mock- and TMV-infected samples; however, the primary product was the 120,000-dalton virus infection-specific protein. When anti-TMV126kD protein antiserum was incubated with the reaction products, there was specific precipitation of the radiolabeled protein which corresponded with the observed virus infection-specific 120,000-dalton polypeptide. No radiolabeled polypeptides were observed when preimmune serum was reacted with the reaction products. Thus, the TMV126kD protein was shown to be a nucleotide-binding protein. (The diffuse band observed in both the mock and infected samples with anti-TMV126kD protein antiserum comigrates with the immunoglobulin G heavy chain and probably represents nonspecific binding of the unincorporated [α-32P]GTP from the nucleotide-binding reaction mixture.)

**Nucleotide Specificity** — If the TMV126kD protein was to be considered a candidate as a guanylyltransferase, then the nucleotide binding should have preference for GTP as a substrate other than GTP and TMV-infected (I) leaf tissue homogenates were incubated with [α-32P]GTP for 2.5 h at 25°C using the standard nucleotide-binding assay conditions, as described under “Experimental Procedures.” One-third of the reaction mixture was quenched with SDS-PAGE sample buffer and stored at −20°C. The remaining two-thirds were split: one portion was reacted with anti-TMV126kD protein antiserum (α-TMV126kD), and the other portion was reacted with preimmune rabbit serum. The immunocomplexes were precipitated with protein A-agarose and analyzed by SDS-PAGE and autoradiography, along with the samples that were not immunoprecipitated. The samples that were not immunoprecipitated are shown here after 40 h of exposure to the film, whereas those samples that were reacted with serum (α-TMV126kD and preimmune) are shown here after 7 days of exposure. The arrow to the right of the figure indicates the position of migration of the TMV126kD protein.

**Figure 3.** Immunoprecipitation of the reaction products of the [α-32P]GTP binding with the TMV126kD protein antiserum. Mock-(M) and TMV-infected (I) leaf tissue homogenates were incubated with [α-32P]GTP for 2.5 h at 25°C using the standard nucleotide-binding assay conditions, as described under “Experimental Procedures.” One-third of the reaction mixture was quenched with SDS-PAGE sample buffer and stored at −20°C. The remaining two-thirds were split: one portion was reacted with anti-TMV126kD protein antiserum (α-TMV126kD), and the other portion was reacted with preimmune rabbit serum. The immunocomplexes were precipitated with protein A-agarose and analyzed by SDS-PAGE and autoradiography, along with the samples that were not immunoprecipitated. The samples that were not immunoprecipitated are shown here after 40 h of exposure to the film, whereas those samples that were reacted with serum (α-TMV126kD and preimmune) are shown here after 7 days of exposure. The arrow to the right of the figure indicates the position of migration of the TMV126kD protein.

**Figure 2.** TMV infection-specific nucleotide binding. Mock (M) and TMV-infected (I) leaf tissue homogenates were incubated with either [α-32P]GTP or [α-32P]UTP, 500 μCi/ml of each, at 3,000 Ci/mmole specific activity, or [γ-32P]GTP at 30 Ci/mmole specific activity. The reaction mixtures were incubated at 25°C for 40 min and then quenched with SDS-PAGE sample buffer. The incubations were analyzed by SDS-PAGE and autoradiography, as described under “Experimental Procedures.” The numbers at the left of the figure represent the position of migration of the molecular weight standards × 10⁻³.
Figure 4. Kinetic analysis and nucleotide specificity for incorporation into TMV126kD protein. Mock- (M) and TMV-infected (I) leaf tissue homogenates were fractionated by centrifugation at 13,000 × g for 10 min at 4°C. The supernatant fractions (S-13) were reacted at 25°C with 380 μCi/ml of either [α-32P]GTP (GTP), or [α-32P]ATP (ATP), [α-32P]CTP (CTP), or [α-32P]UTP (UTP), each at 3,000 Ci/mmol specific activity. Samples were taken at 2, 4, 6, 8, 10, 15, 20, and 25 min after mixing the S-13 with Bradley buffer containing the radioactive nucleotides; both the S-13 and Bradley buffer mixtures were equilibrated to 25°C before mixing. The samples were quenched with SDS-PAGE sample buffer and then analyzed by SDS-PAGE and autoradiography, and the incorporation was quantified by scanning densitometry, as described under “Experimental Procedures.” Initial rates were determined by plotting the relative film densities versus the time of incubation (panel A). The relative rates of nucleotide incorporation into the TMV126kD protein were: GTP, 1.00; ATP, 0.04; CTP, 0.00; UTP, 0.00. Panel B shows the reaction products after 40 min of incubation. The numbers at the left of the figure represent the positions of migration of the molecular weight standards x 10^3.

The radiolabeling of the TMV126kD protein was presumed to be due to nucleotide addition, but transfer of hydrolyzed free phosphate derived from the GTP was a possibility. Figure 5 shows data that indicated that the guanine base as well as the phosphate were bound to the protein. The supernatant and pellet fractions of both mock- and TMV-infected leaf tissue extracts were incubated with [8-3H]GTP, where the radiolabel was on the base of the nucleotide. Samples were treated as described under “Experimental Procedures,” and the gel was treated with 1 M sodium salicylate for fluorographic analysis. Samples from the mock-infected materials showed no reactivity with the GTP, whereas the TMV126kD protein was labeled covalently with [8-3H]GTP in both the supernatant and pellet fractions of the TMV-infected materials.

To determine further the specificity for the nucleotide binding with TMV126kD protein, experiments with competition against [α-32P]GTP binding were performed. A variety of nucleic acids and inorganic salts was used. These data are summarized in Table I and indicate that at least two classes of molecules inhibited the [α-32P]GTP binding. The first class was the guanosine 5′-ri- or diphosphate nucleosides. Interestingly, both the 2′-deoxy-(dGTP) and 2′,3′-dideoxyguanosine (ddGTP) 5′-triphosphates inhibited as readily as did GTP, suggesting that the ribose portion of the molecule was not involved in the nucleotide-binding portion of the active site of the enzyme. GDP was a weak competitor, and GMP seemed to have no ability to compete, thus indicating that the triphosphate was the true substrate.

The second class of molecules that inhibited the GTP binding was pyrophosphate (PPi). As seen in Figure 1, step ii, the reaction products of GTP binding with guanylyltransferase are GMP bound covalently to the enzyme, and pyrophosphate. Excess pyrophosphate would drive the reaction toward the reactant side of the equation and thereby inhibit the enzyme-GMP complex formation. As seen in Figure 6 and Table I, 20 μM PPi

![Image of Figure 4 and Figure 5]

**Figure 5.** Guanine base is bound to the TMV126kD protein. Mock- and TMV-infected leaf tissue homogenates were fractionated by centrifugation at 13,000 × g for 10 min at 4°C. The resultant supernatant (S) and pellet (P) fractions (resuspended in an equal volume of Bradley buffer) were then reacted with [8-3H]GTP (Du Pont-New England Nuclear; specific activity, 15 Ci/mmol) for 60 min at 25°C. The reactions were quenched with SDS-PAGE buffer and analyzed by SDS-PAGE and fluorography. The data shown here represent a 1-month exposure. The arrow on the right of the figure represents the position of migration of the TMV126kD protein.

**Table I.** Testing of various molecules for inhibition of binding of [α-32P]GTP to TMV126kD protein.

| Compound                  | Concentration of compound needed to give 50% inhibition of [α-32P]GTP binding to TMV126kD protein |
|---------------------------|--------------------------------------------------------------------------------------------------|
| GTP1                      | 1 μM                                                                                             |
| ATP                       | >1 mM                                                                                             |
| UTP                       | >1 mM                                                                                             |
| CTP                       | >1 mM                                                                                             |
| GMP                       | >1 mM                                                                                             |
| GDP                       | 100 μM                                                                                            |
| PO4                       | >10 mM                                                                                            |
| PPi                       | 20 μM                                                                                             |
| dGTP                      | 1 μM                                                                                             |
| ddGTP                     | 1 μM                                                                                             |
| dATP                      | >1 mM                                                                                             |
| cGMP                      | >1 mM                                                                                             |
| Poly(G)                   | >1 mM                                                                                             |
| β,γ Methylene GTP         | >1 mM                                                                                             |
| Poly(A)                   | >1 mM                                                                                             |
| S-Adenosylmethionine      | >1 mM                                                                                             |
| S-Adenosylhomocysteine    | >1 mM                                                                                             |
To determine the nature of the chemical bond—To determine the nature of the bond between the GMP and the TMV126kD protein, both chemical and enzymatic methods have been utilized in an attempt to release the radioactive nucleotide from the protein. To date, only strong acid or strong base will efficiently release the bond between the GMP and the TMV126kD protein, both by 50% and thus is consistent with the predictions of the reaction mechanism.

Nature of the Chemical Bond—To determine the nature of the bond between the GMP and the TMV126kD protein, both chemical and enzymatic methods have been utilized in an attempt to release the radioactive nucleotide from the protein. To date, only strong acid or strong base will efficiently release the bond between the GMP and the TMV126kD protein, both by 50% and thus is consistent with the predictions of the reaction mechanism.

Figure 6. Pyrophosphate inhibits nucleotide binding to TMV126kD protein. TMV-infected leaf tissue homogenate was fractionated by centrifugation at 13,000 × g for 10 min at 4°C, and the resultant supernatant fraction (S-13) was incubated with [α-32P]GTP at 250 pCi/ml (specific activity, 3,000 Ci/mm) plus PPi, at various concentrations for 30 min at 25°C. The reaction products were analyzed by SDS-PAGE and autoradiography and quantified by scanning densitometry. The extent of GTP incorporation into TMV126kD protein was determined by the relative film density. The concentration of PPi required to give a 50% inhibition of [α-32P]GTP binding to TMV126kD protein was 20 μM.

Table II. Tests of various enzyme treatments on the stability of the protein-nucleotide bond

| Treatment       | Presence of 32P-TMV126kD protein after treatment |
|-----------------|-------------------------------------------------|
| Untreated       | +                                               |
| P1 nuclease     | +                                               |
| RNase T<sub>1</sub> | +                                               |
| RNase A         | +                                               |
| Alkaline phosphatase | +                                           |

was required to inhibit GTP incorporation into TMV126kD protein by 50% and thus is consistent with the predictions of the reaction mechanism.

Reaction Optimization—In order to evaluate the optimal re-action conditions for the binding of [α-32P]GTP to TMV126kD protein, temperature, protein concentration, and divalent cat-ion concentrations were investigated. 25°C was the measured temperature optimum (data not shown) and was close to the reported optimum (28°C) for TMV replicase activity (28). Figure 7 shows data in which the incorporation of GTP into either the soluble or membrane-bound form of TMV126kD protein was measured as a function of protein concentration. The incorporation was normalized to the quantity of TMV126kD protein present in the incubation, as estimated by Coomassie Blue staining. Incorporation of [α-32P]GTP into TMV126kD protein was inhibited strongly when higher concentrations of either S-13, P-13, or total homogenate fraction were used in the incubation mix. These results suggested that either there was an inhibitor of the forward reaction (that is, formation of the enzyme-GMP intermediate), or there was an activation of the guanylyltransfer step (that is, transfer of GMP to an acceptor RNA) at relatively high concentration of the fraction added to the reaction mixture. If either of these hypotheses were dependent on the presence of soluble molecules (for example, inhibition by PPi, or activation by some cofactor, or a change in the specific activity of the radionucleotide with changing amounts of nonradioactive GTP from the cell extract), then it would be predicted that the activity of the membrane-bound form in the P-13 fraction would be unresponsive to fraction concentration when resuspended in Bradley buffer. As shown in Figure 7, the accumulation of the radiolabeled TMV126kD protein from the P-13 fraction, as well as the soluble form, was strongly reduced by a high P-13 concentration. This suggested that the low yield of GMP-TMV126kD protein at relatively high concentration was not due to a soluble component of the fraction and may be because of a rapid transfer of GMP to an acceptor RNA.

A number of divalent cations were tested to determine their dependence or inhibition of the [α-32P]GTP incorporation into TMV126kD protein. Under conditions tested, neither the rate nor the extent of the reaction could be increased by the addition of Mn<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, or Cu<sup>2+</sup> chloride salts when tested in a range from 10 nM to 10 mM (data not shown). However, relatively high levels of Zn<sup>2+</sup> (≥10 mM) or Co<sup>2+</sup> (≥100 μM) inhibited the reaction. The presence of 10 mM EDTA completely abolished the reaction, which indicated that there was a requirement for divalent cation. This requirement must have been sat-
isfied by endogenous ions in the homogenate, as the reaction would not respond to additional exogenous divalent cations.

Subcellular Fractionation — In order to characterize further the TMV126kD protein guanylyltransferase-like activity, subcellular fractionation was employed. As indicated in Figures 5 and 7, the TMV126kD protein as well as the virus-specific guanylyltransferase-like activity partitioned in both the membrane-bound phase and the soluble phase. Figure 8 shows data of a differential centrifugal fractionation of mock- and TMV-infected tobacco leaf tissue homogenates followed by incubation with [α-32P]GTP and analysis by SDS-PAGE/autoradiography. The low speed pellet fraction (P-10) of the mock-infected sample showed four major bands. The TMV-infected sample showed the same four bands and the infection-specific product, the TMV126kD protein. The high speed pellet fraction (P-100) contained only the TMV126kD protein in the infection-derived material, whereas the mock-infected-derived material had no detectable products. The high speed supernatant fraction (S-100) contained several nucleotide-binding products in both the mock- and virus-infected-derived materials, but only the virus-infected-derived samples contained the TMV126kD protein. Thus, although host-derived nucleotide-binding products partitioned to either the low speed pellet or the high speed supernatant fractions with centrifugation, the TMV126kD protein (with guanylyltransferase-like activity) distributed rather uniformly throughout the fractions.

To fractionate the TMV126kD protein guanylyltransferase-like activity from the host-derived nucleotide binding products further, the S-100 fraction of both the mock- and TMV-infected-derived extracts was desalted and then chromatographed on DEAE-cellulose using a linear KCl gradient (10–500 mM) to elute bound material. The protein content was monitored by absorbance (280 nm), and the concentration of KCl was monitored by measuring the conductivity. The DEAE fractions were then equilibrated with the KTMDP-10 buffer by dialysis and then sampled for their ability to bind [α-32P]GTP, as determined by SDS-PAGE/autoradiographic analysis.

**Figure 8.** Subcellular fractionation of TMV126kD protein nucleotide-binding activity. Mock- (M) and TMV-infected (I) leaf tissue homogenate was fractionated by differential centrifugation, 10,000 × g at 4°C for 10 min, which resulted in a pellet fraction (P-10); the resultant supernatant fraction was subsequently centrifuged at 100,000 × g at 4°C for 1 h and resulted in a pellet fraction (P-100) and a supernatant fraction (S-100). The pellet fractions were resuspended in an equal volume of Bradley buffer. The fractions were tested for their ability to bind [α-32P]GTP using the standard nucleotide-binding assay, as described under “Experimental Procedures.” Equal cell volumes were loaded onto the gel. The numbers to the left of the figure indicate the position of migration of the molecular weight standards × 10^3.

**Figure 9.** DEAE chromatographic isolation of TMV126kD protein nucleotide-binding activity. Mock- (panel A) and TMV-infected (panel B) leaf tissue homogenates were fractionated by centrifugation, as described in Figure 8. The buffers of the S-100 fractions were subsequently exchanged to KTMDP-10 buffer by passing over Sephadex G-25 (coarse). The resulting void volumes were then loaded onto Whatman DE52 cellulose; the unbound fraction was collected, the column was washed with the same buffer, and the bound material was eluted with a linear KCl gradient from 10 to 500 mM in KTMDP buffer (upper half of panels A and B). The collected fractions were assayed for salt concentration by conductivity measurement and then dialyzed against KTMDP-10 buffer overnight. The dialyzed fractions were assayed for nucleotide-binding activity, as described under “Experimental Procedures” (lower half of panels A and B).
The TMV126kD protein was shown previously to be a nucleoside 5′-triphosphate-binding protein by radiolabeling the protein with UV light activation of either 8-N3-μ[γ-32P] ATP or 8-N3-μ[γ-32P] GTP or 8-N3-μ[γ-32P] ATP (5). These data are supported by reports from Young et al. (3) that the TMV126kD protein is a component of the viral replicase complex. This type of γ-labeled NTP affinity for the protein is significantly different from that measured in the experiments presented here. First, in our experiments, the nucleotide binding of the guanylyltransferase-like activity observed required no exogenous energy (e.g. UV light) to form a covalent bond between the polypeptide and the nucleotide. Second, the product of the guanylyltransferase-like activity was GMP bound in such a fashion that the phosphate moiety was not susceptible to nuclease or phosphatase digestion (Table II), nor was it susceptible to mild acid or mild base, which is consistent with the suggestion that GMP binds to guanylyltransferase through a phosphoamide bond, probably at a lysine residue (17, 26). Third, the specificity of the guanylyltransferase-like activity for GTP, dGTP, or ddGTP was at least 25-fold greater than for any other nucleoside 5′-triphosphate tested (Table I), whereas specificity for labeling with the UV light-activated azido derivatives was not limited to either purine or pyrimidine ribonucleoside 5′-triphosphates (5). These two different results for nucleotide binding to TMV126kD protein may represent two unique binding sites for the protein and thereby represent two unique activities, RNA-dependent RNA polymerase and guanylyltransferase.

The conditions of incubation altered significantly the rate and extent of reaction to form a stable GMP-TMV126kD protein complex, as well as the type of host-specific products formed. A variety of host-specific proteins was found which distributed to various fractions after fractionation of the total homogenate (Figure 8). Yet, the TMV-specific guanylyltransferase-like activity was found distributed nearly uniformly among the membranes fraction (P-10), the ribosomal fraction (P-100), and the cytoplasmic fraction (S-100). Curiously, there was a distinct concentration effect for the TMV-specific guanylyltransferase-like activity, especially with the P-13 fraction (Figure 7). The activity was enhanced by dilution of the fractions. This “dilution activation” may be due to the release of an inhibitor from the protein or may result when the intermediate of the guanylyltransferase reaction (enzyme-GMP) was diluted to such an extent that the probability of interacting with the acceptor RNA was low, and so the intermediate accumulated.

No significant dependence for exogenously added divalent cations could be observed for the virus-specific guanylyltransferase-like activity, yet the reaction was sensitive to EDTA, which indicated a divalent cation requirement. The requirement may have been fulfilled in vivo. This apparent lack of sensitivity to exogenously added divalent cations differs from guanylyltransferases from other sources. The HeLa cell enzyme has sensitivity to both Mn2+ and Mg2+ for enzyme-GMP complex formation (15) and guanylyltransferase activity (10). At their optima (2 mM for Mn2+ and 2–10 mM for Mg2+), these activities are approximately 2–4-fold greater in incubations with Mn2+ than with Mg2+. Optimal capping activity is found at 0.5 mM MnCl2 and 5 mM MgCl2 in wheat germ extracts with nearly equal activity at these optima (18). The vaccinia virus guanylyltransferase activity is also sensitive to divalent cations, but the optimal activity is found with Mg2+. At 2.5 mM, guanylyltransfer is approximately a 10-fold greater rate in the presence of Mg2+ than in the presence of Mn2+ (33). Thus, at
least three classes of guanylyltransferase may be distinguished by their sensitivity to divalent cations; the TMV-specific guanylyltransferase-like activity may represent yet another class.

The TMV-specific guanylyltransferase-like activity in the S-100 fraction was isolated from the host nucleotide-binding activity by anion exchange chromatography (Figure 9). Three major host-specific products were eluted in the DEAE-bound low salt-eluting fractions (mock fractions 19–21, TMV fractions 21–33), whereas the TMV-specific activity was not bound to the DEAE-cellulose and eluted in fractions 3 and 4 (Figure 9B). In both the mock and TMV samples, the unbound material eluted in two peaks, but the TMV material had an enhanced first peak (Figure 9B, fractions 1–6) that corresponded to the TMV-specific guanylyltransferase-like activity. The TMV material also had an enhanced second peak in the salt-eluting fractions (fractions 23–25). It is not clear why this second peak of the $A_{280}$ absorbing material was so much stronger in the TMV samples than in the mock samples.

We believe the primary significance of these data is that for the first time, a specific enzymatic activity may be assigned to the major nonstructural protein implicated in TMV replication. The coding region of the RNA which specifies the TMV126kD protein has been shown to have regions of sequence similarity to those of several other members of the Sindbis-like viruses (34, 35) as well as to other proteins involved in nucleic acid replication (36, 37). One important structural feature common to all the members of the Sindbis-like viruses is the 5’ RNA capped genomes, and, to the best of our knowledge, all members of this group are replicated in the cytoplasm of the infected cell. Thus, we postulate that other members of this group of viruses have virus-coded guanylyltransferases, possibly associated with their nonstructural proteins.

References
1. Zimmern, D. (1975) Nucleic Acids Res. 2, 1189-1201.
2. Palukaitis, P., and Zaitlin, M. (1986) in The Plant Viruses (van Regenmortel, M. H. V., and Fraenkel-Conrat, H., eds) Vol. 2, pp. 105-131, Plenum Publishing Corp., New York.
3. Young, N., Forney, J., and Zaitlin, M. (1987) J. Cell Sci. 7, (suppl.) 277-285.
4. Wilson, T. M. A. (1988) Oxford Surv. Plant Mol. Cell. Biol. 5, 89-144.
5. Evans, R. K., Haley, B. E., and Roth, D. A. (1985) J. Biol. Chem. 260, 7800-7804.
6. Hills, G. J., Plaskitt, K. A., Young, N. D., Dunigan, D. D., Watts, J. W., Wilson, T. M. A., and Zaitlin, M. (1987) Virology 161, 488-496.
7. Flotch, S. J., Bouloy, M., Ulmanen, I., and Klug, R. M. (1981) Cell 23, 847-858.
8. Abraham, G., Rhodes, D. P., and Banerjee, A. K. (1975) Cell 5, 51-58.
9. Mizumoto, K., and Lioman, F. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4961-4965.
10. Venkatesan, S., Gershowitz, A., and Moss, B. (1980) J. Biol. Chem. 255, 2829-2834.
11. Venkatesan, S., and Moss, B. (1980) J. Biol. Chem. 255, 2835-2842.
12. Venkatesan, S., and Moss, B. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 340-344.
13. Mizumoto, K., Kaziro, Y., and Lipman, F. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 1693-1697.
14. Wang, D., Furuichi, Y., and Shatkin, A. J. (1982) Mol. Cell. Biol. 2, 993-1001.
15. Shuman, S. (1982) J. Biol. Chem. 257, 7237-7245.
16. Yagi, Y., Mizumoto, K., and Kaziro, Y. (1983) EMBO J. 2, 611-616.
17. Tovara, R., Mizumoto, K., Nakahara, Y., Tatsuno, T., and Kazird, Y. (1983) EMBO J. 2, 2195-2202.
18. Keith, J. M., Venkatesan, S., Gershowitz, A., and Moss, B. (1982) Biochemistry 21, 327-333.
19. Furuichi, Y., Muthukrishnan, S., Tomasz, J., and Shatkin, A. J. (1976) J. Biol. Chem. 251, 5043-5053.
20. Yamakawa, M., Furuichi, Y., and Shatkin, A. J. (1982) Virology 118, 157-168.
21. Monroy, G., Spencer, E., and Hurwitz, J. (1978) J. Biol. Chem. 253, 4490-4498.
22. Spencer, E., Loring, D., Hurwitz, J., and Monroy, G. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4793-4797.
23. Venkatesan, S., Gershowitz, A., and Moss, B. (1980) J. Biol. Chem. 255, 903-908.
24. Shuman, S., Surks, M., Fremeaux, H., and Hurwitz, J. (1980) J. Biol. Chem. 255, 11588-11598.
25. Shuman, S., and Hurwitz, J. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 187-191.
26. Roth, M. J., and Hurwitz, J. (1984) J. Biol. Chem. 259, 13488-13494.
27. Itoh, N., Yamada, H., Kaziro, Y., and Mizumoto, K. (1987) J. Biol. Chem. 262, 1989-1995.
28. Bradley, D. W., and Zaitlin, M. (1971) Virology 45, 192-199.
29. Dreyfus, G., Adam, S. A., and Choi, Y. D. (1984) Mol. Cell. Biol. 4, 415-423.
30. Laskey, R. A., and Mills, A. D. (1977) FEBS Lett. 82, 314-316.
31. Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83-88.
32. Sefton, B. M., Beemon, K., and Hunter, T. (1978) J. Biol. Chem. 253, 957-971.
33. Martin, S. A., and Moss, B. (1975) J. Biol. Chem. 250, 9330-9335.
34. Ahlquist, P., Strauss, E. G., Rice, C. M., Strauss, J. H., Haseloff, J., and Zimmern, D. (1985) J. Virol. 53, 536-542.
35. Goldbach, R., and Wellink, J. (1988) Intervirology 29, 260-267.
36. Gorbaleyna, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, M. (1988) Nature 333, 22.
37. Hedgman, T. C. (1988) Nature 333, 22-23.