TRANSLATION OF POTYVIRUS RNA IN A RABBIT RETICULOCYTE LYSATE

BY

WILLIAM GEORGE DOUGHERTY

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1979

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Ernest Hiebert, Co-chairman of my Supervisory Committee, for his friendship, willing advice, guidance, and most of all patience, during the course of this study.

I also wish to thank Dr. Henry C. Aldrich, Chairman of my Supervisory Committee, for his support and advice during my studies, and his kindness in permitting me to utilize his facilities.

I wish to extend my sincere thanks to Dr. Dan Purcifull, for supplying much of the antisera, and for his criticism and advice during this study.

I would like to thank Dr. John E. Edwardson, Dr. Harry E. Warmke, and Dr. Daryl P. Pring for their critical reading of the dissertation. The enthusiasm and interest which they, along with Richard Christie, constantly showed during these studies was deeply appreciated.

I am indebted to Drs. Francis C. Davis and James F. Preston, as members of my Supervisory Committee, for time spent in critical reading of this dissertation and their helpful comments and suggestions during the course of the study.

Sincere appreciations are accorded to June Hill, Willie Eugene Crawford, Donna Miller, Mary Conde and Steve Christie for their help, and for making the lab such an enjoyable place to work.

I am especially thankful to Dr. Ed Hoffman for bleeding my bunnies, and to him and his lab group for constant encouragement and suggestions during my studies. Sincere thanks are accorded to all the members of the

Department of Microbiology and Cell Science for their consideration, understanding, and help in many ways.

Appreciation is also expressed to Dr. Paul Smith and the Department of Microbiology and Cell Science for providing a graduate assistantship during my studies.

I would like to express my sincere appreciation to Mary Kay Sayer, who stood by me and gave me support and criticism when I needed it the most.

Most of all, I would like to thank my parents, for without their encouragement and support, this study would have been impossible.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

TRANSLATION OF POTYVIRUS RNA IN A RABBIT RETICULOCYTE SYSTEM

By

William George Dougherty

December, 1979

Chairman: Henry C. Aldrich
Co-chairman: Ernest Hiebert
Major Department: Microbiology and Cell Science

Potyviruses are filamentous plant viruses with a diameter of 12 nm and a length of 680 to 900 nm. The viral genome consists of a single-stranded, non-segmented RNA, sedimenting around 39 S on sucrose density gradients, and with an estimated molecular weight of $3.0\text{-}3.5 \times 10^6$. The capsid consists of protein monomers with an estimated size varying between $3.0\text{-}3.6 \times 10^4$ daltons (30-36 kd). The cytoplasmic cylindrical inclusion associated with all potyvirus infections consists of non-structural protein monomers with a size of 67 to 70 kd. Inclusions in the nucleus, detected only in certain potyviruses, have been shown to consist of two protein monomers with sizes of 49 kd and 54 kd. Cylindrical and nuclear inclusion characterization studies have shown the inclusions to be virus specific and host independent, but direct evidence for their genetic origin is still needed.

The primary purpose of this thesis was to determine if the genetic information coding for these non-structural proteins resides in the potyviral genome. To do this, viral RNA was isolated and translated in a rabbit reticulocyte cell-free system. Labeled products of the cell-free

translation were analyzed and compared with authentic products, using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoprecipitation, and peptide mapping. Tobacco etch virus (TEV) and pepper mottle virus (PeMV), members of the potyvirus group, were investigated. Both TEV and PeMV form cylindrical inclusions, but nuclear inclusions have not been found in PeMV infections.

The optimum conditions for cell-free translation of TEV- and PeMV-RNA are reported. The distribution of *in vitro* products was dependent on RNA quality. Full length RNA resulted in the cell-free synthesis of a 87 kd product for TEV and a 78 kd product for PeMV. RNA with limited fragmentation produced a number of discrete products in the cell-free system while highly fragmented RNA did not act as a template.

Four of the 6 discrete cell-free products identified reacted with anitsera to 4 virus-specific proteins. The cell-free 30 kd product of TEV RNA and the 33 kd product of PeMV RNA were identified as capsid protein on the basis of co-migration with authentic capsid proteins during SDS-PAGE, immunoprecipitation with the respective capsid protein antiserum, and by the comparison of the proteolytic peptide pattern with that for authentic capsid protein. Two cell-free products of TEV co-migrating with authentic nuclear inclusion proteins during SDS-PAGE, were immunoprecipitated with antisera specific to the nuclear inclusion proteins, and gave a similar proteolytic peptide pattern as authentic nuclear inclusion proteins.

A PeMV RNA cell-free product co-migrating with PeMV cylindrical inclusion protein was immunoprecipitated with antiserum to PeMV cylindrical inclusion protein. A faint product, co-migrating with TEV cylindrical

inclusion protein, was detected in the immunoprecipitation analysis of TEV cell-free products with TEV cylindrical inclusion antiserum.

A number of cell-free products, which were consistently immunoprecipitated with more than one antiserum, were detected. These products were presumed to be products of gene readthroughs on the basis of serological reactions and on the basis of size. These readthroughs were useful in linking genes on the potyviral genome.

This thesis provides direct evidence for the viral origin of the non-structural proteins (inclusions) associated with potyvirus infection. Six distinct potyviral gene products are synthesized in the cell-free system and are genetically linked on the genome. The proposed genetic map accounts for 95% of the coding capacity of the TEV RNA and 93% of the PeMV RNA.

SECTION I
REACTION CONDITIONS AND IDENTIFICATION OF
CAPSID PROTEIN AS ONE OF THE PRODUCTS OF *IN VITRO*
TRANSLATION OF TOBACCO ETCH AND PEPPER MOTTLE VIRAL RNAS

Introduction

Tobacco etch virus (TEV) (Shepherd and Purcifull, 1971) and pepper mottle virus (PeMV) (Purcifull *et al.*, 1975) are elongate plant viruses belonging to the potyvirus group (Edwardson, 1974). Potyviruses have a filamentous particle structure with a diameter of 12 nm and range in length from about 680 to 900 nm (Fenner, 1976). Infectious potyvirus RNA described for TEV (Hill and Benner, 1976; Hari *et al.*, 1979), potato virus Y (Hinostroza-Orihuela, 1975), and maize dwarf mosaic virus (Pring and Langenberg, 1972) is a single-stranded, non-segmented component, sedimenting around 39 S on sucrose density gradients. The 39 S RNA has an estimated size of 3.0-3.5 x 10^6 daltons. Potyvirus capsids consist of protein monomers with an estimated size of 3.0-3.6 x 10^4 daltons (30-36 kd) (Hiebert and McDonald, 1973; 1976). One interesting and unique aspect of the potyvirus group is the presence of non-structural proteins that form inclusions in infected host cells (Edwardson, 1974). The cylindrical (pinwheel) inclusions associated with all potyvirus infections consist of a protein monomer with a size of 67-70 kd (Hiebert and McDonald, 1973). The nuclear inclusions associated with TEV infections are composed of two distinct proteins with sizes of 49 kd and 54 kd (Knuhtsen *et al.*, 1974). These inclusions are virus-specific and

serologically unrelated to the capsid protein and to host protein (Purcifull *et al.*, 1973; Knuhtsen *et al.*, 1974).

The cell-free protein synthesizing systems, such as the wheat germ (Efron and Marcus, 1973) and the rabbit reticulocyte lysate (Gilbert and Anderson, 1970), have been used successfully to analyze the translation strategies and the genomes of a number of animal viruses and plant viruses outside the potyvirus group. Analyzing the potyviral genome in such a system is of interest because of the size and importance of this plant virus group, and because of the dearth of fundamental information on potyviral biosynthesis. Another interesting feature is the size of the potyviral RNA, which potentially carries a large amount of genetic information. Such studies will also enable one to compare the strategy of potyvirus translation with that reported for other virus groups. The unique association of large amounts of non-structural protein in potyviral infections is the most important reason for analysis of the potyviral genome in a cell-free translation system.

The conditions for efficient *in vitro* translation of TEV- and PeMV-RNA in a nuclease-treated rabbit reticulocyte lysate are reported here. Capsid protein is identified among a number of discrete products formed by the cell-free translation of a full-length potyviral RNA, indicating that the translation strategy is different from that reported for most other plant viral systems (Fraenkel-Conrat *et al.*, 1977).

Material and Methods

Virus Purification

TEV (American Type Culture Collection #69) was cultured in *Nicotiana tabacum* L. var. Havana 425. The PeMV isolate (Purcifull *et al.*,

1975) was maintained in *Nicotiana tabacum* L. var. Samsun. Tobacco leaf tissue (100 g), 3-8 weeks after inoculation, was homogenized in a 1 gal Waring blendor for 2 min at 4° in 150 ml of solution containing 20 mM Hepes, pH 7.5, 0.1% sodium sulfite (w/v), and 8% (v/v) (final concn) *n*-butanol. The homogenate was expressed through cheesecloth, and the filtrate was centrifuged at 3300 *g* (max) for 10 min. The supernatant was clarified by the addition of 1% Triton X-100 (final concn), and the virus was precipitated from the supernatant by the addition of 4% (final concn) polyethylene glycol (PEG, MW 6000) and 100 mM NaCl while stirring for 1.5 hr at 4°. The precipitated virus was collected by centrifugation (10,400 *g* max for 10 min). The resulting pellet was resuspended in 50 ml of 20 mM Hepes, pH 7.5, with the aid of a glass tissue grinder. The resuspended pellet was centrifuged at 10,400 *g* (max) for 10 min and the pellet was discarded. The supernatant was stirred for 1 hr after the addition of 8% PEG (w/v, final concn) and 100 mM NaCl. The supernatant was then centrifuged (10,400 *g* max for 15 min), and the resulting pellet was resuspended in 5-10 ml of 20 mM Hepes, pH 7.5, with the aid of a glass tissue grinder. The resuspended material was subjected to centrifugation on a CsCl gradient (30% CsCl in 20 mM Hepes, pH 7.5) generated at 140,000 *g* (max) for 16-18 hr. The virus zone was removed and diluted with an equal volume of buffer and then centrifuged at 12,000 *g* (max) for 10 min. The virus was removed from the CsCl in the supernatant by PEG precipitation.

RNA Isolation

RNA was extracted by dissociating freshly purified virus in an equal volume of 200 mM ammonium carbonate (pH 9.0), 2 mM EDTA, and 2% sodium dodecyl sulfate (SDS) (Brakke and Van Pelt, 1970a) and isolated by rate

zonal density gradient centrifugation in linear-log sucrose gradients (Brakke and Van Pelt, 1970b). The 39 S RNA fraction was collected and precipitated by the addition of sodium acetate (pH 5.0, final concn 100 mM) and two volumes of 100% ethanol. The RNA was resuspended in a small volume of H_2O and used immediately (sometimes frozen at -20° up to 2 weeks) for translation.

RNA was also isolated using the lithium chloride degradation technique (Francki and McLean, 1968), the sodium perchlorate extraction method (Wilcockson and Hull, 1974), and the pH 9.3 phenol-chloroform extraction method (Hari *et al.*, 1979).

Lysate Preparation and *In Vitro* Protein Synthesis Conditions

New Zealand white rabbits (6 weeks old) were made anemic using the phenyl hydrazine procedure of Gilbert and Anderson (1970). The rabbits were bled via cardiac puncture, and the blood was washed and lysed according to Villa-Komaroff *et al.* (1974). The lysate was made dependent on exogenous mRNA using the calcium chloride-micrococcal nuclease treatment of Pelham and Jackson (1976). The lysate (200 µl) was adjusted to a final concentration of 20 mM Tris-HCl (pH 7.6), 1 mM calcium chloride, 10 mM potassium acetate, 30 µM hemin, 50 µg/ml creatine kinase, and 10 µg/ml of micrococcal nuclease. The lysate mixture was incubated at 30° for 10 min, and EGTA was added to a final concentration of 2 mM. Protein synthesis was optimized for RNA, magnesium, and potassium concentrations in the messenger-dependent lysate. The standard protein synthesis mixture of 30 µl contained 17 mM creatine phosphate, 100 µM of each of the 19 unlabeled amino acids, 4.8 mM dithiothreitol, 3 µg of calf liver tRNA, and 2 or 4 µCi of [^{35}S] methionine (500-800 Ci/mmol).

Incubation was at 30° for 75 min, and incorporation of [^{35}S] methionine into products precipitated by trichloroacetic acid (TCA) was determined according to the method of Mans and Novelli (1961).

Reaction mixtures (30 µl) to be analyzed electrophoretically were treated with 5 µl of a solution containing 250 mM EDTA (pH 7.0) and ribonuclease A (1 mg/ml) and incubated for 15 min at 30°. Electrophoresis buffer (100 µl) containing 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol was added, and the samples were heated at 100° for 3-4 min. Samples were applied to an SDS gradient (7.5 to 15%) slab gel system, using the Laemmli (1970) discontinuous buffer system. Products were detected on dried gels by fluorography as described by Bonner and Laskey (1974), using Kodak RP Royal X-Omat X-ray film.

Antisera (kindly supplied by Dr. D. E. Purcifull) were prepared as described by Purcifull and Batchelor (1977). Immunoprecipitation was carried out according to Kessler (1975), using Cowan 1 strain of *Staphylococcus aureus* as the immune absorbent.

Partial proteolytic digestion of *in vivo* capsid proteins and the *in vitro* synthesized products, immunoprecipitable with antiserum to the capsid protein, were performed using *Staphylococcus aureus* V-8 protease as described by Cleveland *et al.* (1977).

Materials

The [^{35}S] L-methionine was obtained from New England Nuclear. Micrococcal nuclease and calf liver tRNA were obtained from Boehringer Mannheim Biochemicals. *Staphylococcus aureus* V-8 protease was obtained from Miles Laboratories. Hepes, PEG 6000, hemin, and Ribonuclease A

were obtained from Sigma Chemical Company. Proteinase K was obtained from E. M. Laboratories. DNase I was obtained from Worthington Biochemical.

Results

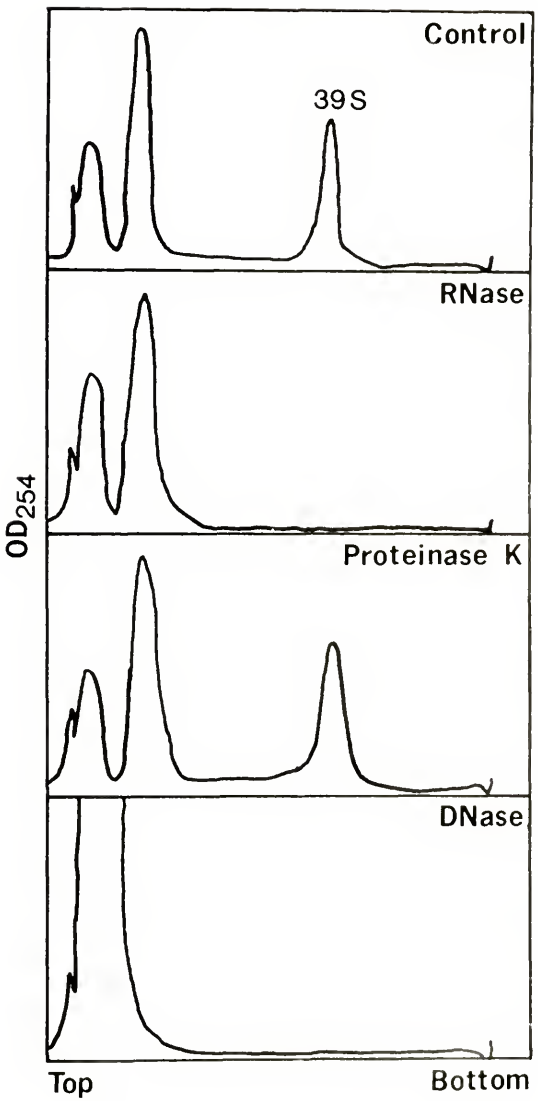
Virus Purification

Virus purification was one of the critical steps in the successful and efficent cell-free translation of TEV- and PeMV-RNA. The method of purification described for watermelon mosaic virus 2 (Purcifull and Hiebert, 1979), using phosphate buffer, invariably yielded virus preparations contaminated with host nucleic acids. Virus purified in phosphate often had 260/280 ratios greater than the expected 1.2 and yielded large amounts of nucleic acid which sedimented around 18 S (Fig. 1). This was identified as DNA by treating these preparations with Proteinase K, RNase, and DNase followed by sucrose density gradient analysis (Fig. 1). RNA preparations contaminated with this DNA did not stimulate the cell-free system. Virus purified using Hepes buffer, as outlined in Materials and Methods, was consistently free of 18 S nucleic acid and yielded RNA that was suitable for translational studies. Virus yields were in the range of 5-10 mg/100 g tissue. The use of Tris buffer during several attempts at purification produced virus yields which were unacceptably low.

RNA Isolation

Obtaining high yields of intact 39 S RNA was difficult with most of the extraction procedures. Lithium chloride precipitation and perchlorate extraction yielded 90 to 100% of the theoretical yield, but the RNA was fragmented when analyzed on 2% acrylamide-0.5% agarose

Figure 1. Analysis of TEV-RNA contaminated with a slow sedimenting nucleic acid on linear-log sucrose density gradients. Sedimentation profile of phosphate purified TEV after dissociation in sodium dodecyl sulfate-ammonium carbonate, pH 9.0, - EDTA and prior to centrifugation subjected to: no treatment (control); to RNase A (RNase); to Proteinase K (Proteinase K); to DNase I with 1 mM $MgCl_2$ (DNase). DNase I was contaminated with RNase, which explains the loss of the 39 S RNA species with this treatment.


Control
39S
RNase
Proteinase K
DNase
OD254
Top
Bottom

composite gels and on linear-log sucrose gradients. Phenol extraction alone and in various combinations with chloroform, SDS, and isopropyl alcohol consistently resulted in low yields (25%) of the 39 S RNA. The phenol-chloroform extraction at pH 9.3 described by Hari *et al.* (1979) improved the yields to about 50%. Addition of Proteinase K during extraction did not affect the quantity of the RNA. The ammonium carbonate-SDS-EDTA system (Brakke and Van Pelt, 1970a), outlined in Materials and Methods, was satisfactory for the isolation and recovery (70 to 80%) of intact RNA (Fig. 2). The RNA migrated as a single component during electrophoresis under non-denaturing conditions.

Optimal Reaction Conditions

The optimum conditions for translation of both potyviral RNAs in the messenger dependent reticulocyte system was determined. Rates of incorporation of [^{35}S] methionine into TCA precipitable products with PeMV- and TEV-RNA showed maximal stimulation at 0.8-1.0 mM magnesium ion concentration (Fig. 3a). The potassium ion requirement for maximal stimulation of [^{35}S] methionine incorporation ranged from 50 mM to 175 mM for TEV-RNA and 75 mM to 125 mM for PeMV-RNA (Fig. 3b). TEV-RNA concentrations of 1-3 µg/30 µl assay volume provided the greatest stimulation of the cell-free system, but higher concentrations were inhibitory (Fig. 3c). A RNA concentration profile for PeMV showed a similar pattern, and 2-3 µg RNA/30 µl assay was subsequently used. Addition of degraded RNA, as indicated by analyses on linear-log sucrose gradients and composite acrylamide-agarose gels, greatly reduced stimulation. A time course study for both TEV- and PeMV-RNA stimulation of the reticulocyte system showed an increase in the incorporation of

Figure 2. Sedimentation profile of PeMV- and TEV-RNA in a linear-log sucrose gradient. UV absorbing material at the top of the gradient is dissociated capsid protein. Only the stippled area of the 39 S RNA peak was collected for use in translation.

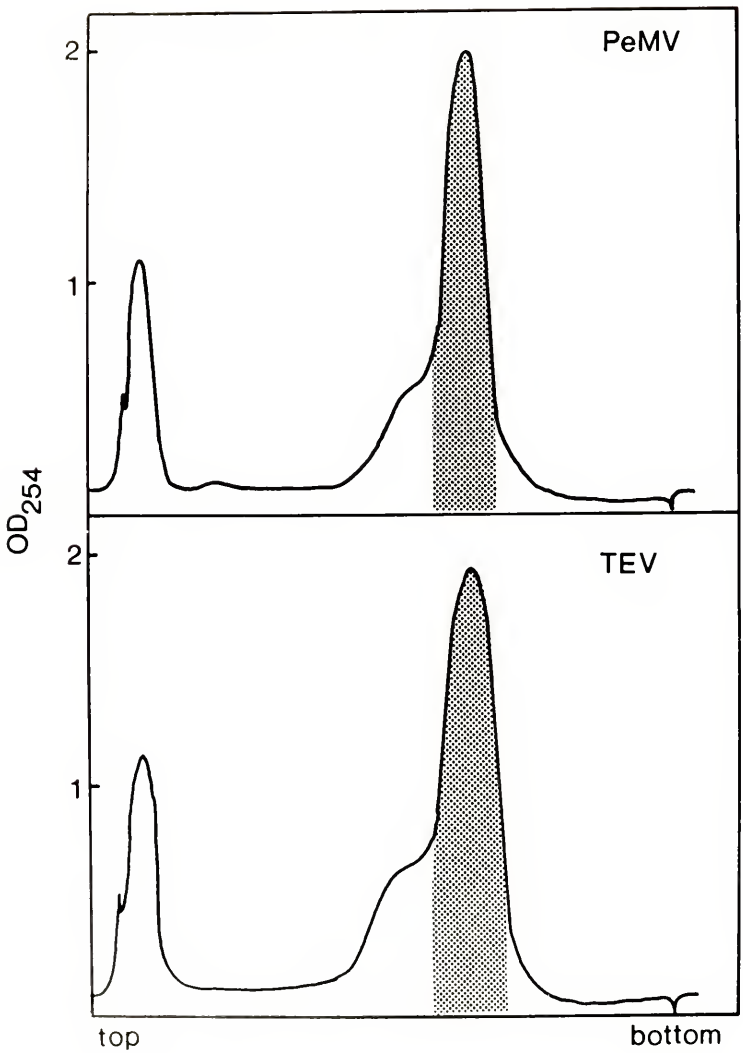

PeMV
TEV
2
1
2
1
OD_{254}
top
bottom

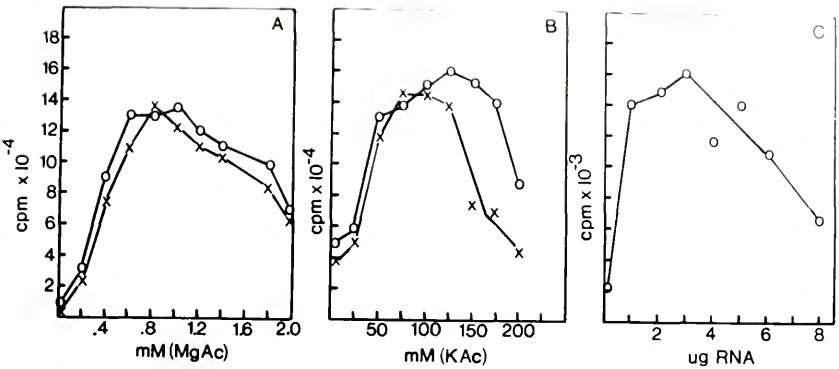
Figure 3. Characteristics of [^{35}S] methionine incorporation directed by PeMV RNA (-x-x-x-) and TEV RNA (-0-0-0-) in the reticulocyte lysate system. All values have been corrected for low levels of endogenous activity. Aliquots of 10 µl were counted.

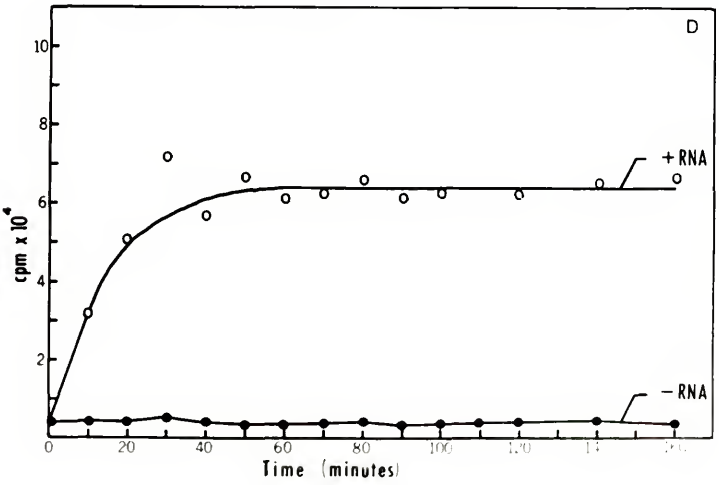
A. Magnesium dependence of TEV- and PeMV-RNA in the cell-free translational system. Potassium ion concentration was 100 mM and each 30 µl reaction sample contained 4 µCi of [^{35}S] methionine and 2 µg of RNA

B. Potassium dependence of TEV- and PeMV-RNA in a cell-free translation system. Cell-free protein synthesis was carried out at 1 mM magnesium and each 30 µl reaction sample contained 2 µg of RNA and 4 µCi of [^{35}S] methionine.

C. RNA dependence of the cell-free translation of TEV-RNA. Cell-free synthesis was carried out with a magnesium ion concentration at 1 mM and potassium ion concentration at 100 mM. Each 30 µl sample contained 2 µCi of [^{35}S] methionine.

D. Time course study of the translation of TEV-RNA. Conditions were the same as described in Figure 3C except 4 µCi of [^{35}S] methionine were used.


A
B
C
cpm x 10^-4
cpm x 10^-4
cpm x 10^-3
mM (MgAc)
mM (KAc)
ug RNA


D
+RNA
−RNA
cpm x 10^-4
Time (minutes)

[^{35}S] methionine into TCA precipitable products for up to 50 to 60 min (Fig. 3d). No further stimulation of the lysate was observed with time, and therefore an incubation time of 60 to 75 min was used routinely. Under the above conditions a 15 to 20-fold stimulation of [^{35}S] methionine incorporation into TCA precipitable products over endogenous levels was observed.

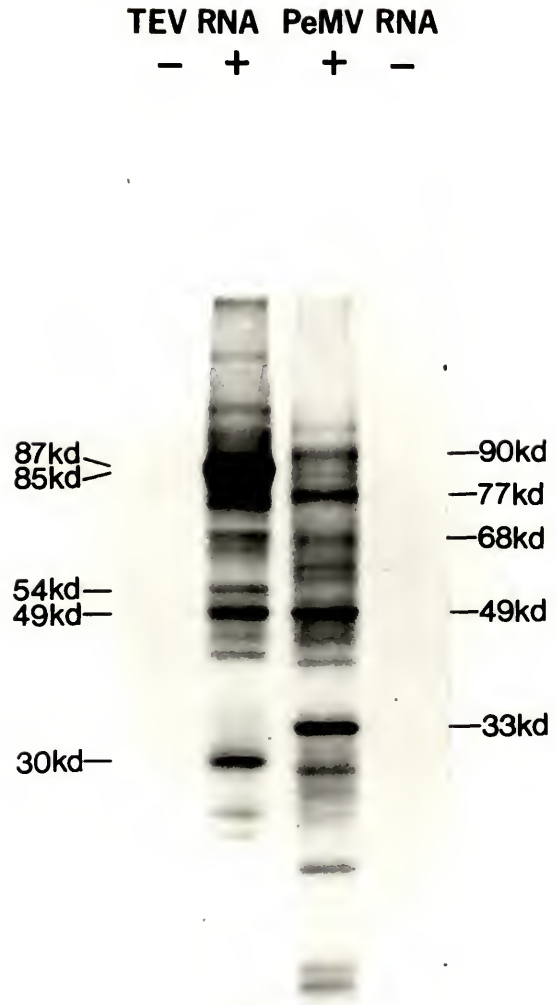
Electrophoretic Analysis of Synthetic Products

The *in vitro* translation products of both TEV- and PeMV-RNA were analyzed by electrophoresis in SDS discontinuous polyacrylamide gradient gels (PAGE). Translation of each viral genome resulted in unique products under identical conditions (Fig. 4). The major products of TEV translation had estimated sizes of 87 kd, 85 kd, 54 kd, 49 kd, and 30 kd; whereas the products of PeMV translation had estimated sizes of 90 kd, 77 kd, 68 kd, 49 kd, and 33 kd. The 30 kd product for TEV and 33 kd product for PeMV co-migrated with TEV and PeMV capsid protein, respectively. The non-capsid products mentioned above and a number of other products evident in the fluorograms of both TEV and PeMV translations have been identified by selective immunoprecipitations with antisera to virus-specific proteins, and these data will be presented in Sections 2 and 3.

Fidelity of Translation

To test the antigenicity of the 30 kd product for TEV and the 33 kd product for PeMV, cell-free products made in reticulocyte lysates were immunoprecipitated with antiserum prepared against the respective viral capsid protein. Analysis of the immunoprecipitations by PAGE shows that

Figure 4. Electrophoretic fractionation of the *in vitro* translation products of TEV- and PeMV-RNA. The products were fractionated on a sodium dodecyl sulfate polyacrylamide slab gel (7.5 to 15%) gradient (PAGE). Translation conditions were identical for both viral RNAs. Negative lanes represent endogenous translation, and positive lanes represent the translation of exogenous viral RNA. Molecular weight markers used in all electrophoretic studies were as follows: β-galactosidase 125,000 daltons (125 kd); phosphorylase B (94 kd); TEV cytoplasmic inclusion protein (70 kd); TEV nuclear inclusion proteins (54 kd and 49 kd); carbonic anhydrase (29 kd); tobacco mosaic virus capsid monomer (17.5 kd); ribonuclease (13 kd); and myoglobin (10 kd). The significant products of the translations, based on serological specificity, are identified by their estimated molecular weights in the figure.


TEV RNA
PeMV RNA
– + + –
87kd
85kd
54kd
49kd
30kd
90kd
77kd
68kd
49kd
33kd

the 30 and 33 kd products were immunoprecipitated by the TEV- and PeMV-capsid antisera, respectively (Fig. 5). No polypeptides were detected by immunoprecipitation when the cell-free products were tested with normal serum, with potato virus x capsid antiserum, with antiserum prepared to healthy tobacco proteins, and with antiserum prepared to watermelon mosaic virus 1, a potyvirus unrelated to either TEV or PeMV (Fig. 5, lanes 3-5). Minor products, smaller than 30 or 33 kd immunoprecipitated by their respective antiserum, are considered to be premature termination products, because these products were not immunoprecipitated by antisera prepared to three other virus-specific products. The immunoprecipitation products larger than 30 and 33 kd (largely in the size range of 70 to 90 kd) appear to be readthroughs of the adjacent gene or genes on the viral RNAs. Evidence for these readthroughs is based on the molecular weight estimates on PAGE and on the co-immunoprecipitation of these products by antisera prepared to capsid protein, to 54 kd protein, and to other virus-specific proteins (Section 3).

The co-migration and serological specificity of the 30 kd and the 33 kd products, compared to their respective *in vivo* products, suggest that the capsid protein gene for both TEV and PeMV is faithfully translated by the reticulocyte lysate. To test this possibility further the peptide maps of capsid protein were compared with the *in vitro* 30 and 33 kd products. The [^{35}S]methionine labeled 30 and 33 kd products were immunoprecipitated from the lysate and digested with V-8 protease. The peptides resulting from the partial protease digestion of the cell-free products, and the capsid proteins were resolved by PAGE. The peptide digest patterns shown in Fig. 6 compare the labeled peptides detected by fluorography with the unlabeled capsid peptides detected by Coomassie

Figure 5. Analysis of the *in vitro* translation products of TEV- and PeMV-RNAs by immunoprecipitation. The figure illustrates [^{35}S] methionine labeled products separated by PAGE and detected by fluorography.

A. (lane 1) Total products of TEV-RNA translation; (lane 2) products immunoprecipitated with antiserum to TEV capsid protein; (lane 3) immunoprecipitation with antiserum to potato virus X capsid protein; (lane 4) immunoprecipitation with antiserum to healthy tobacco proteins; and (lane 5) immunoprecipitation with antiserum to watermelon mosaic virus 1 capsid protein.

B. (lane 1) Total products of PeMV-RNA translation; (lane 2) products immunoprecipitated with antiserum to PeMV capsid protein; and (lanes 3-5) as in A. Immunoprecipitation of TEV and PeMV cell-free products with normal serum was negative. Arrows indicate the migration distance of the respective authentic capsid protein.

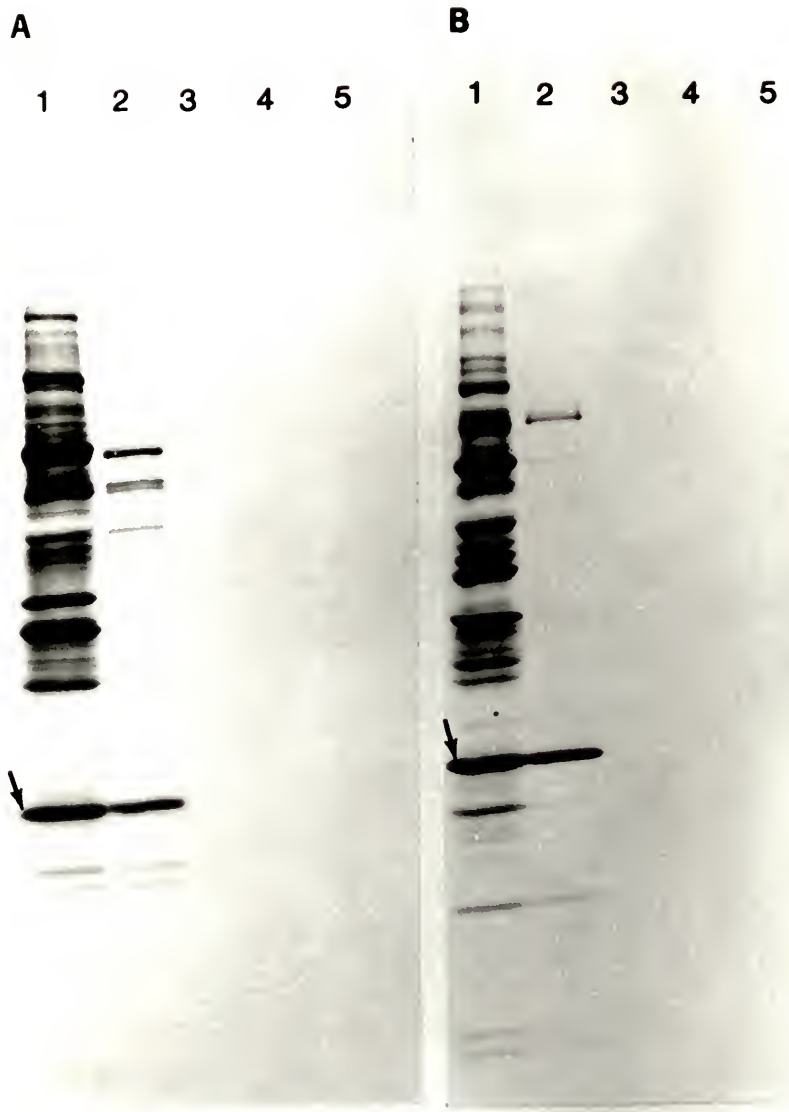
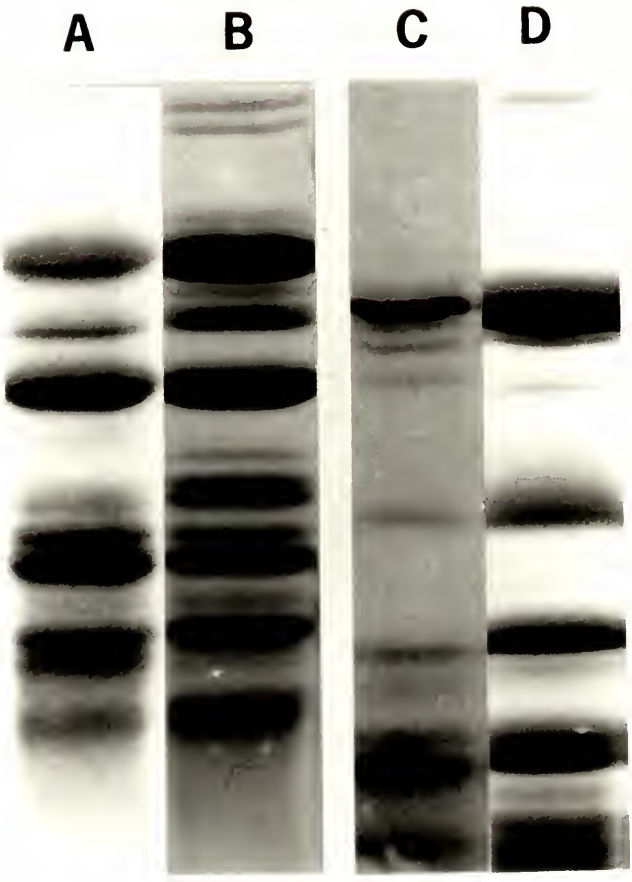

A
1 2 3 4 5
B
1 2 3 4 5

Figure 6. *Staphylococcus aureus* V-8 protease digest pattern comparison of the *in vivo* capsid protein with the *in vitro* product immunoprecipitated with antiserum to the capsid protein. The peptides were separated by SDS-PAGE. Lane A is a fluorogram of the V-8 protease digest pattern obtained from labeled, PeMV cell-free product immunoprecipitated with antiserum to PeMV to capsid protein. Lane B represents a Coomassie brilliant blue stained digest pattern of the *in vivo* PeMV capsid protein. A similar comparison of labeled, TEV cell-free product and stained TEV capsid protein is present in lanes C and D, respectively. The two bands at the top of columns B and D represent components of the V-8 protease preparation consistently observed in all Coomassie brilliant blue stained digest patterns.


A
B
C
D

brilliant blue staining. The peptide pattern for the 33 kd protein (Fig. 6, lane A) resembles that generated for PeMV capsid protein (Fig. 6, lane B). Likewise the 30 kd peptide pattern (Fig. 6, lane C) appears similar to the TEV capsid protein peptide pattern (Fig. 6, lane D). However, the peptide pattern for PeMV capsid protein is distinct from the pattern for TEV capsid protein.

Discussion

The reaction conditions necessary for translation of TEV- and PeMV-RNA in a rabbit reticulocyte lysate system have been demonstrated. *In vitro* translation of other potyvirus RNAs under similar conditions has also been successful. Thus, the rabbit reticulocyte lysate appears to be an efficient probe of the genetic information carried by potyviral RNAs. On the other hand, preliminary attempts to translate PeMV-RNA by the wheat germ system were unsuccessful.

Capsid protein appears as one of the products of the cell-free translation of TEV- and PeMV-RNA. This is based on the analysis of the cell-free products by PAGE, which shows a product co-migrating with authentic capsid protein. Furthermore, this co-migrating product is immunoprecipitated by antiserum specific to the respective capsid protein. A comparison of the proteolytic peptide fragments of the co-migrating product with the proteolytic peptide fragments of authentic capsid protein provides additional evidence for the faithful translation of the capsid protein gene in our cell-free system. A number of other products are formed during the *in vitro* translation of potyviral RNAs, and these will be shown in Chatpers 2 and 3 to be unique genomic proteins.

A number of distinct products, larger than 30-33 kd and not immunoprecipitated by capsid protein antisera, are formed in addition to capsid protein during *in vitro* translation of TEV- and PeMV-RNA. This suggests that potyviral RNA may act as a polycistronic messenger *in vitro* and contains interior initiation sites. This is supported by the fact that the 39 S potyviral RNA was most efficient in stimulating the cell-free translation system. All attempts to date to fractionate the potyviral RNA and isolate a small RNA, monocistronic for capsid protein, have failed. For example, heating TEV- and PeMV-RNA to 85° for 3 min followed by quick cooling, did not yield RNA monocistronic for capsid protein or for any other virus-specific protein (Section 3). Further evidence for a polycistronic message *in vitro* appears in the immunoprecipitation analysis of the translation products. The immunoprecipitation of TEV-RNA cell-free translation products with TEV capsid protein antiserum shows, in addition to the expected 30 kd protein, products with estimated sizes ranges from 70 to 87 kd (Fig. 5A, lane 2). These 70 to 87 kd products are also immunoprecipitated by antiserum produced to TEV nuclear inclusion 54 kd protein (Section 3). The co-immunoprecipitation of 87 kd protein can be explained by a 54 kd gene-30 kd gene readthrough; the others such as the 70 kd protein, by a 54 kd gene-30 kd gene readthrough with a premature termination in the 30 kd gene. The latter observation is consistent with the number of proteins smaller than 30 kd immunoprecipitated only by TEV capsid protein antiserum (Fig. 5A, lane 2). Evidence for the *in vitro* production of capsid protein as well as other proteins from an apparently non-segmented viral genome has also been shown for carnation mottle virus (Salomon *et al.*, 1978) and for tobacco necrosis virus (Salvato and Fraenkel-Conrat, 1977). In contrast,

the strategy for *in vitro* translation of plant viruses such as tobacco mosaic (Bruening *et al.*, 1976), brome mosaic (Shih and Kaesberg, 1973, 1976), and turnip yellow mosaic (Pleij *et al.*, 1977) requires a small monocistronic RNA for efficient capsid protein synthesis. Recently Pelham (1979a) has presented evidence for proteolytic processing of cowpea mosaic virus proteins formed in the cell-free translation as a possible strategy for plant viral translation. Several analyses of the time course of product appearance with both TEV- and PeMV-RNA cell-free translations have shown no signs of proteolytic processing of the translation products.

The cell-free translation of potyviral RNAs has several interesting and unique features compared to previously studied plant viral translation systems. The size of the non-segmented viral genome (estimated to be $3.0\text{-}3.5 \times 10^6$ d) represents a large amount of genetic information. The availability of antisera to non-structural, virus-specific proteins (inclusion proteins) as well as to the capsid protein is proving useful in analyzing translation products. The *in vitro* expression of many genes in the RNA, the presence of gene readthroughs, and the availability of antisera to most of the viral-specific proteins should allow the development of an accurate genetic map of the potyviral RNA.

SECTION 2
IDENTIFICATION OF NUCLEAR INCLUSION PROTEINS AS PRODUCTS OF TOBACCO ETCH VIRUS RNA CELL-FREE TRANSLATION AND CYLINDRICAL INCLUSION PROTEIN AS A PRODUCT OF THE POTYVIRUS GENOME

Introduction

The conditions for efficient *in vitro* translation of tobacco etch virus (TEV) and pepper mottle virus (PeMV), both members of the potyvirus group have been described in Section 1. Capsid protein was identified as one of the products of the cell-free translation of TEV RNA and PeMV RNA. A number of other distinct translational products were observed, and here an attempt is made to identify some of these products and to relate them to *in vivo* proteins associated with these viruses.

Potyvirus Inclusions

The association of large amounts of non-structural proteins with potyviruses is unique among plant viruses. These non-structural proteins are aggregated in host cells to form distinct morphological structures known as inclusions. Cylindrical inclusions (also known as pinwheels, and cytoplasmic inclusions) are observed in the cytoplasm of potyvirus-infected cells (Edwardson, 1974). Inclusions in the nucleus have been observed for only a limited number of potyviruses (Edwardson, 1974; Christie and Edwardson, 1977). Both TEV and PeMV form cylindrical inclusions, but nuclear inclusions have not been observed in PeMV infections.

The nuclear inclusions associated with TEV infections have a three dimensional morphology described as that of a truncate, four-sided pyramid (Knuhtsen *et al.*, 1974; Matsui and Yamaguchi, 1964). TEV nuclear inclusions have been shown to be proteinaceous by proteolytic digestion in thin sections of infected tissue (Shepard, 1968) and by characterization studies of partially purified preparations (Knuhtsen *et al.*, 1974). The nuclear inclusions consist of two distinct protein monomers with molecular sizes of 49,000 daltons (49 kd) and 54 kd. Serological analysis of the nuclear inclusion proteins shows them to be unrelated to healthy host proteins, to TEV cylindrical inclusion, and to TEV capsid protein (Knuhtsen *et al.*, 1974).

The cylindrical inclusions induced by TEV and PeMV differ in morphology. In cross section, TEV cylindrical inclusions appear as pinwheels with triangular shaped laminated aggregates attached to the central core. The PeMV cylindrical inclusions appear as pinwheels with scrolls and short curved laminated aggregates attached to the central core. Cylindrical inclusions associated with TEV and PeMV have been purified and partially characterized (Hiebert and McDonald, 1973). The inclusions consist of proteinaceous subunits with a size of 68 kd for PeMV and 70 kd for TEV. The cylindrical inclusion morphology and serology have been demonstrated to be virus specific and host independent (Edwardson, 1974; Purcifull *et al.*, 1973).

The evidence for these non-structural proteins being of potyviral origin is based on the constant association of inclusions with potyviral infection, but direct evidence is still needed. In this section, the non-structural components associated with TEV and PeMV infections are demonstrated to be virus-coded proteins. Products which co-migrate

with authentic TEV nuclear inclusion proteins, which react serologically with antisera to nuclear inclusion proteins, and which generate a proteolytic peptide pattern similar to authentic nuclear inclusion proteins, are detected in TEV RNA stimulated cell-free translation. Cell-free translation of both TEV RNA and PeMV RNA also results in products which co-migrate with cylindrical inclusion protein and which react with antisera to the respective cylindrical inclusion protein.

Material and Methods

Virus Propagation

TEV (American Type Culture Collection #-69) was cultured in *Nicotiana tabacum* L. var. Havana 425 and *Datura stramonium* L. PeMV (Purcifull *et al.*, 1975) was maintained in *Nicotiana tabacum* L. var. Samsum.

Virus Purification

TEV and PeMV were purified from infected tobacco leaf tissue using Hepes buffer. Clarification and concentration of the virus have been described in Section 1.

RNA Isolation

RNA was isolated from purified virus using the ammonium carbonate (pH 9.0)-SDS-EDTA method of Brakke and Van Pelt (1970a). The dissociated virus was centrifuged (260,000 *g* max) for 4 hr at 14°. The 39 S RNA zone was collected using an ISCO Model UA-4 UV analyzer and an ISCO density gradient fractionator. The RNA was precipitated from the sucrose by addition of two volumes of 100% ethanol, and sodium acetate (pH 5.0)

to a final concentration of 100 mM. The precipitated RNA was resuspended in water and stored at -20°.

Cell-free Reticulocyte Translation System

Blood was obtained from anemic New Zealand white rabbits via cardiac puncture, and washed and lysed according to Villa-Komaroff *et al.* (1974). The lysate was made messenger dependent as follows. To 183 µl of lysate were added: 4 µl of 1.0 M Tris-HCl (pH 7.6), 2 µl of creatine kinase (5 mg/ml) in 50% ethylene glycol, 1.5 µl of 4 mM hemin, 2 µl of 100 mM calcium chloride, 1 µl of 2.0 M potassium acetate, and 2 µl of micrococcal nuclease (1 mg/ml). The mixture was incubated for 10 min at 30°, and the digest was stopped by the addition of 4 µl of 100 mM EGTA.

The messenger dependent cell-free system (360 µl) contained 200 µl of predigested lysate, 100 mM potassium acetate, 1.0 mM magnesium acetate, 4 µCi of [^{35}S] methionine (specific activity 500-800 Ci/mmol) or [^{3}H] leucine (specific activity 137 Ci/mmol), 100 µm of each of the 19 unlabeled amino acids, 17 mM creatine phosphate, 36 µg of calf liver tRNA, 4.8 mM DDT, and 24 µg of viral RNA. Translation of the viral genome was carried out at 30° for 75 min, and the reaction was stopped by the addition of 60 µl of RNase A (5 mg/ml) in 125 mM EDTA. Total incorporation was determined by the trichloroacetic acid (TCA) precipitation of 10 µl aliquots on filter discs according to Mans and Novelli (1961).

Samples to be analyzed by polyacrylamide gel electrophoresis (PAGE) or by immunoprecipitation were dissociated by the addition of two volumes of a solution containing 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol in 62.5 mM Tris-HCl (pH 6.8). Samples were heated at 90° for 3-5 min.

Analysis of Labeled Products

Analysis of *in vitro* products was carried out using a SDS polyacrylamide slab gel (1 mm x 140 mm x 150 mm). Electrophoresis in a 7.5% to 15% linear gradient gel, containing 0.1% SDS, was performed using the discontinuous buffer system of Laemmli (1970). Products were detected on dried gels by fluorography according to Bonner and Laskey (1974).

Immunoprecipitation was carried out with modifications to the procedure described by Kessler (1975). The dissociated lysate (40 µl) was incubated with 10 µl of antiserum and 80 µl of NET buffer (150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.4) containing 0.05% NP-40, ovalbumin (1 mg/ml), and 2 mM methionine. After incubating the sample for 1 hr at room temperature, 20 µl of (10% w/v) *Staphylococcus aureus* (*S. aureus*) strain Cowan I was added and incubated for 30 min at room temperature. The solution was centrifuged (2000 *g* for 5 min) and the pellet was resuspended in 250 µl of 0.05% NP-40 NET buffer. The material was precipitated (2000 *g* for 5 min) and resuspended three more times in this manner. After the third washing, the pellet was resuspended with 50 µl of a solution containing 62.5 mM Tris-HCl (pH 6.8), 2.0% SDS, 5.0% 2-mercaptoethanol, and 10% glycerol, and heated at 90° for 5 min. The material was centrifuged at 2000 *g* for 5 min, and the supernatant was layered on a gel for PAGE.

Partial proteolytic digestion of the *in vivo* and *in vitro* inclusion proteins was performed using *S. aureus* V-8 protease as described by Cleveland *et al.* (1977).

Purification of TEV and PeMV Cylindrical Inclusions

Systemically infected tissue (5 to 8 wk after inoculation) was homogenized in a Waring blender for 2 min with a solution containing 2 ml

of 500 mM potassium phosphate buffer (PB), pH 8.2, 5 mg sodium sulfite, 0.5 ml chloroform, and 0.5 ml carbon tetrachloride per g of tissue. The homogenized material was centrifuged in a Sorvall GSA rotor at 1020 *g* (max) for 5 min. The pellet, containing organic solvents, was re-extracted with 0.5 ml of PB per g of tissue and recentrifuged at 1020 *g* (max) for 5 min. The two supernatants were combined and centrifuged at 13,200 *g* for 15 min. The pellets were resuspended with the aid of a Sorvall Omnimixer in 0.5 volume (per g of starting material) 50 mM PB containing 0.1% 2-mercaptoethanl (2-ME). Triton X-100 was added to a final concn of 5%. After stirring for 1 hr at 4°, this mixture was centrifuged at 27,000 *g* for 15 min. The pellet was resuspended in 20 mM PB containing 0.1% 2-ME (5 ml PB per 100 g starting material). The resuspended material was homogenized for 30 seconds and then layered on a sucrose step gradient made up of 10 ml of 80%, 7 ml of 60%, and 7 ml of 50% (w/v) sucrose in 20 mM PB. The gradient was centrifuged for 1 hr at 21,000 rpm in a Beckman SW 25.1 rotor. The inclusions, which layered on top of the 80% sucrose zone, were collected dropwise from the bottom of the centrifuge tube. To remove the sucrose, the inclusions were diluted four times with 20 mM PB and pelleted by centrifugation at 27,000 *g* for 15 min. The pellet was resuspended in a small volume of 20 mM Tris-HCl (pH 8.2). Yields up to 25 A_{280} units (1 A_{280} = 1 mg protein) per 100 g tissue were obtained.

TEV Nuclear Inclusion Isolation

Datura leaf tissue (300 g) was homogenized in a Waring blender for 1.5 min at 4° in 900 ml of 100 mM PB (pH 7.5) containing 0.2% sodium sulfite. The homogenate was expressed through cheesecloth and Miracloth. Triton X-100 was added to the filtrate to a final concn of 5%. After

clarification by stirring for 1 hr at 4°, the material was centrifuged (1000 *g* for 10 min). The pellets were resuspended in 400 ml of 20 mM PB (pH 8.2) containing 0.5% sodium sulfite. The solution was centrifuged (1000 *g* for 10 min), and the pellets were resuspended in 30 ml of 20 mM PB containing 40% (w/v) sucrose and 0.5% sodium sulfite. This material was homogenized in a Sorvall Omnimixer (setting 3 for 2 min) and layered onto a 50%, 60%, 80% (w/v) sucrose discontinuous gradient. The gradients were centrifuged in a SW 25.1 rotor for 20 min (4°) at 15,000 rpm. The entire 80% sucrose zone was collected and diluted with three volumes of 20 mM PB (pH 8.2) containing 0.5% sodium sulfite. The inclusions were pelleted (1000 *g* for 10 min) and resuspended in 100 ml of 20 mM PB containing 0.5% sodium sulfite and 5% Triton X-100. The solution was stirred for 1 hr at 4° and the inclusions were pelleted by centrifugation (1000 *g* for 10 min). The material was resuspended in 40% sucrose in PB (pH 8.2) and centrifuged on sucrose gradients as before. The material was diluted with three volumes of PB, and the inclusions were pelleted (1000 *g* for 10 min). The inclusion pellet was resuspended in 20 mM Tris-HCl (pH 8.2) containing 0.1% sodium sulfite and stored in the freeze-dried state at -20°. Yields up to 27 A_{280} units of protein per 100 g tissue were obtained.

Separation of 49 kd and 54 kd Nuclear Inclusion Proteins

Separation of the 49 kd and 54 kd nuclear inclusion proteins was obtained with SDS-PAGE, using the Laemmli (1970) discontinuous buffer system. A linear 7.5% to 15% gel gradient was used in a slab gel

apparatus (3 mm x 140 mm x 150 mm) to separate the inclusion proteins. Six mg of purified TEV nuclear inclusion proteins were dissociated in 0.8 ml of a solution containing 100 mM Tris-HCl (pH 6.8), 2.5% SDS, 5% 2-ME, and 5% sucrose. The dissociated sample was layered on top of the gel and electrophoresed for 14 hr at a constant voltage of 80. The 49 kd and 54 kd protein bands were detected in the gel either by scanning gel strips at 280 nm wavelength in a spectrophotometer, or by fluorescence of dansyl chloride labeled nuclear inclusion protein (Talbot and Yphantis, 1971) when exposed to short wavelength ultraviolet light. The gel bands were excised and crushed with a mortar and pestle. Ten volumes of water were added to the crushed gel. This material was frozen at -20° for a few hr and then incubated at room temperature for 24 hr. The material was centrifuged at 1000 *g* for 5 min. The pellet was re-extracted with an equal volume of water and centrifuged at 1000 *g* for 5 min. The supernatants were combined and the water was removed by freeze-drying. The residue was resuspended in 2 ml of water and dialyzed for 8 hr against 20 mM Tris-HCl (pH 6.8) containing 0.05% SDS. The dialyzed inclusion protein was stored at -20° after freeze-drying.

Antiserum Production

Antisera to the 49 kd and 54 kd nuclear inclusion proteins were prepared in New Zealand white rabbits. Freeze-dried antigen (about 1 mg) in 0.75 ml of water was homogenized with 0.75 ml of complete Freund's adjuvant. Part of this emulsion (0.2 ml) was injected into the rabbit's footpad, while the rest of the emulsion was administered intramuscularly into the hind leg. This injection procedure was repeated three times at

weekly intervals, substituting incomplete Freund's adjuvant for complete adjuvant during emulsification. The rabbits were bled at weekly intervals after the final injection.

The other antisera (kindly supplied by Dr. Dan E. Purcifull) were prepared as described by Purcifull and Batchelor (1977).

Results

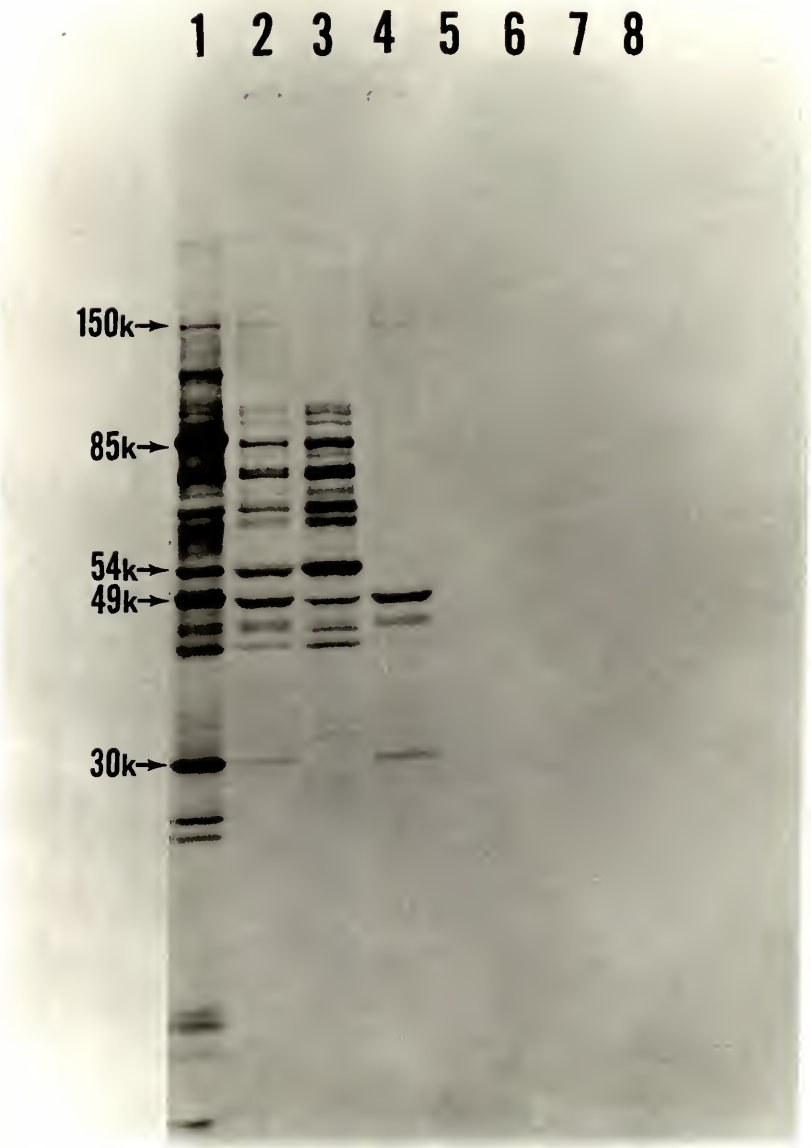
The rabbit reticulocyte lysate cell-free translation of TEV RNA and PeMV RNA produced a number of products which co-migrated with purified *in vivo* inclusion proteins on SDS-PAGE. These cell-free products were analyzed further in order to determine their possible relationship with *in vivo* proteins.

Nuclear Inclusion Proteins

The cell-free translation of TEV RNA produced two products which co-migrated with the 49 kd and 54 kd *in vivo* nuclear inclusion proteins (Fig. 7, lane 1). When [^{35}S] methionine was used as the labeled amino acid, the 49 kd cell-free product appeared as the more labeled band compared to the 54 kd product. When [^{3}H] leucine was used as the labeled amino acid, the 54 kd product appeared as the more labeled band, thus reflecting differences in the amino acid composition of the two proteins.

The antigenicity of the TEV RNA stimulated cell-free products was analyzed using antiserum prepared against sucrose gradient purified nuclear inclusion proteins. Both the co-migrating 49 kd and 54 kd cell-free products were precipitated with this antiserum (Fig. 7, lane 2). Products larger than 54 kd were also immunoprecipitated, and these were

Figure 7. Analysis of the *in vitro* translation products of TEV RNA by immunoprecipitation with antisera prepared to TEV nuclear inclusion proteins. The figure illustrates [^{35}S] methionine labeled products separated in a sodium dodecyl sulfate (SDS) polyacrylamide slab gel (7.5% to 15%) gradient (PAGE) and detected by fluorography. Molecular weight markers used for PAGE were as follows: Myosin, 200,000 daltons (200 kd); β-galactosidase, 125 kd; phosphorylase B, 94 kd, TEV nuclear inclusion proteins, 54 kd and 49 kd; carbonic anhydrase, 29 kd; tobacco mosaic virus capsid protein, 17.5 kd; ribonuclease, 13 kd; and myoglobin, 10 kd. Lane 1, total products of TEV RNA translation; lane 2, products immunoprecipitated with antiserum to total nuclear inclusion protein; lane 3, products immunoprecipitated with antiserum to PAGE purified 54 kd nuclear inclusion protein; lane 4, products immunoprecipitated with antiserum to PAGE purified 49 kd nuclear inclusion protein; lane 5, immunoprecipitation with antiserum to potato virus x capsid protein; lane 6, immunoprecipitation with antiserum to watermelon mosaic virus 1 capsid protein; lane 7, immunoprecipitation with antiserum to healthy tobacco proteins; and lane 8, immunoprecipitation with normal serum.


1 2 3 4 5 6 7 8
150k
85k
54k
49k
30k

presumed to be gene readthroughs on the basis of their serological reactivity with antisera to other virus-specific proteins. Products with molecular weights less than 49 kd were also precipitated, and these were considered premature terminations of the 49 kd and 54 kd cell-free products.

Antisera prepared against the PAGE purified 49 kd or 54 kd nuclear inclusion protein enabled the distinction of the products immunoprecipitated by antiserum to total nuclear inclusion proteins. Antiserum to the 49 kd nuclear inclusion protein selectively precipitated a major 49 kd product as well as three minor products of 30 kd, 44 kd, and 45 kd (Fig. 7, lane 4). The three minor products were believed to be premature terminations of the 49 kd gene. A large product around 150 kd was also immunoprecipitated and will be discussed later. The 54 kd cell-free product was not immunoprecipitated by the 49 kd antiserum, indicating that the 49 kd and 54 kd proteins are different.

Immunoprecipitation of TEV cell-free products was also carried out using antiserum directed against PAGE purified 54 kd nuclear inclusion protein. This antiserum was contaminated with antibodies to the 49 kd protein as determined by SDS-immunodiffusion tests. The 54 kd cell-free product was selectively precipitated over the 49 kd product (Fig. 7, lane 3). Products larger than 54 kd were also immunoprecipitated and appear to be readthrough products of the 54 kd nuclear inclusion gene and the 30 kd capsid protein gene. These products, around 65 kd to 90 kd in size, were serologically reactive to the 54 kd protein antiserum and were not immunoprecipitated by anitserum to the 49 kd protein. Immunoprecipitation products with molecular weights less than 49 kd are

considered to be premature terminations of the 54 kd gene. All the products immunoprecipitated by either the 49 kd or 54 kd antisera were also immunoprecipitated by antiserum against total nuclear inclusion proteins. Immunoprecipitation analyses of the products of TEV RNA cell-free translation with antisera to potato virus x capsid protein, watermelon mosaic virus 1 capsid protein, healthy tobacco proteins, and with normal serum were all negative (Fig. 7, lanes 5 through 8).

The *S. aureus* V-8 protease digest patterns of total authentic nuclear inclusion proteins were compared with the digest patterns of the cell-free products immunoprecipitated with antiserum against total nuclear inclusion proteins. In order to minimize the complications of the presence of large gene readthroughs, cell-free products were absorbed first with antiserum to TEV capsid protein. Products were then immunoprecipitated with antiserum to total nuclear inclusion protein, and digested with the V-8 protease. The PAGE digest pattern of the [^{35}S] methionine labeled cell-free proteins was similar to the pattern generated by authentic nuclear inclusion proteins stained with Coomassie brillant blue (Fig. 8).

Cylindrical Inclusion Proteins

The cell-free translation of PeMV RNA produced a 68 kd product which co-migrated with PeMV cylindrical inclusion protein (Fig. 9, lane 1). Immunoprecipitation of the cell-free products with antiserum to PeMV cylindrical inclusion protein selectively precipitated two major polypeptides with sizes of 68 kd and 110 kd, as well as a few minor products

Figure 8. *Staphylococcus aureus* V-8 protease digest pattern comparison of the *in vivo* nuclear proteins with the *in vitro* proteins immunoprecipitated with antiserum to total nuclear inclusion proteins. The peptide cleavage products were separated by SDS-PAGE. Total *in vitro* lysate products were treated with TEV capsid protein antiserum first to absorb out the 54 kd nuclear inclusion-capsid protein readthrough products. The lysate was then immunoprecipitated with antiserum to total nuclear inclusion protein and this material was digested with *S. aureus* V-8 protease. Lane A represents the Coomassie brilliant blue stained digest pattern of purified TEV nuclear inclusion proteins. Lane B represents the digest pattern of [^{35}S] methionine labeled cell-free products detected by fluorography. Two products observed at the top of lane A are components of the V-8 protease preparation and are not digest products of the nuclear inclusion protein.

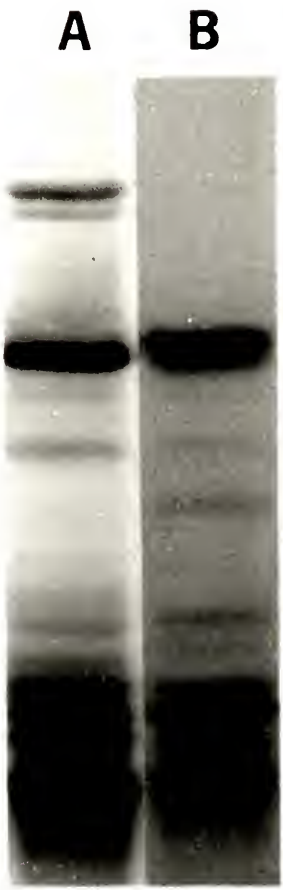
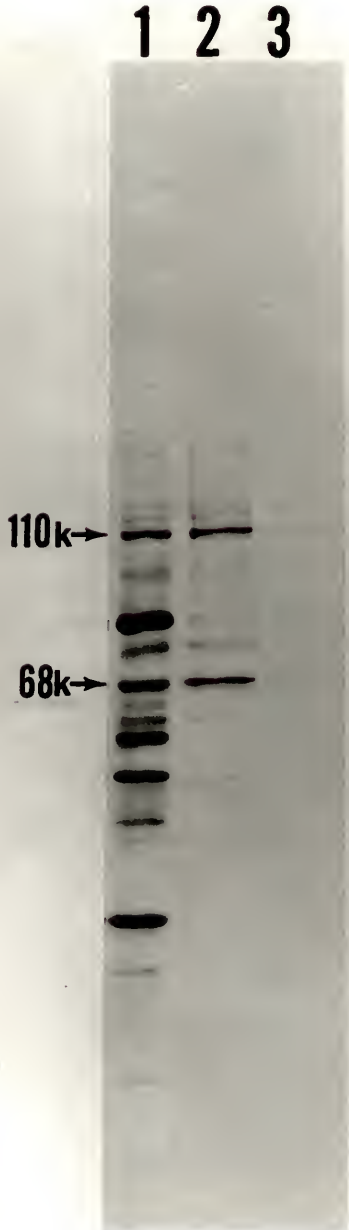

A
B

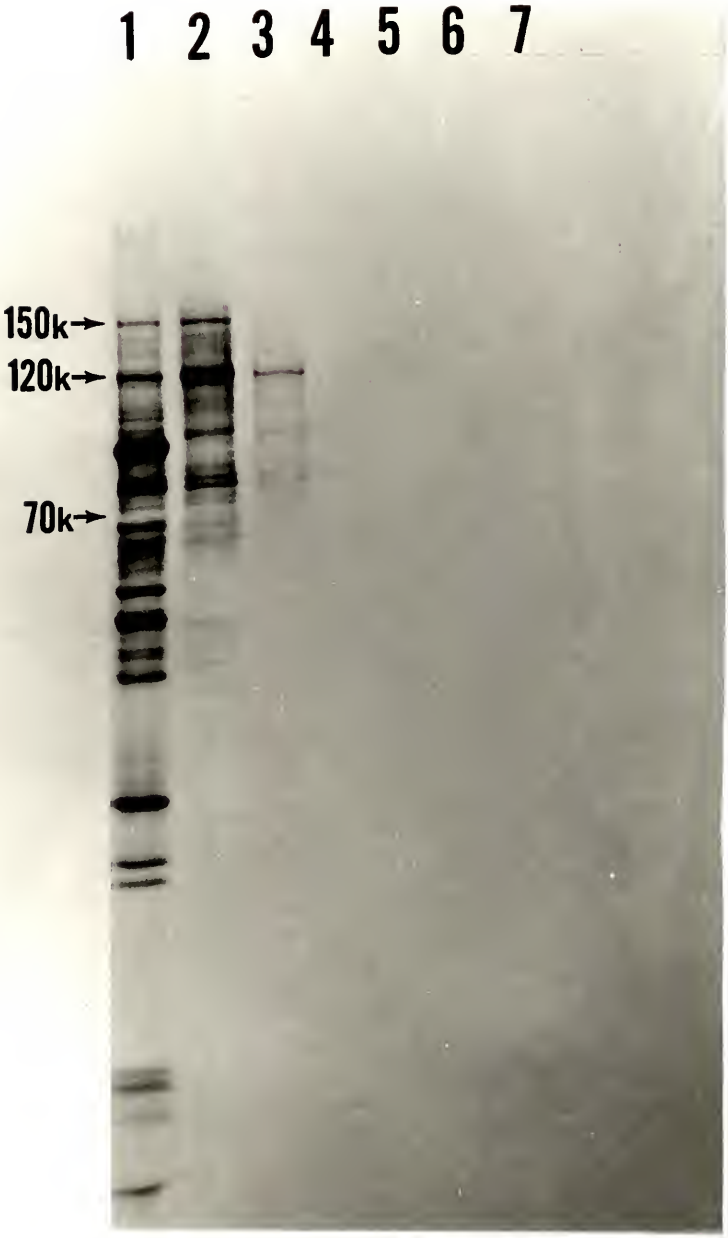
Figure 9. Analysis of the cell-free translation products of PeMV RNA by immunoprecipitation with cylindrical inclusion antisera. The figure represents [^{35}S] methionine labeled products which have been separated by PAGE and detected by fluorography. Estimated molecular weights of products reacting with cylindrical inclusion antisera are given in the figure. Lane 1, total products of PeMV RNA *in vitro* translation; lane 2, products immunoprecipitated with antiserum prepared against PeMV cylindrical inclusion protein; and lane 3, immunoprecipitation with antiserum to TEV cylindrical inclusion protein. Control antisera, as in lanes 4 through 7 of Fig. 10, used in this analysis were negative.


1 2 3
110k
68k

(Fig. 9, lane 2). The 110 kd product was presumed to contain the PeMV cylindrical inclusion protein amino acid sequence (or part of it) and adjacent gene product(s). Antiserum to TEV cylindrical inclusion proteins (distantly related to PeMV cylindrical inclusions) inefficiently precipitated the same products as the PeMV cylindrical inclusion antiserum (Fig. 9, lane 3). Immunoprecipitation tests with antisera to cylindrical inclusions of an unrelated potyvirus (watermelon mosaic virus 1) were negative.

Cell-free products, coded for by TEV RNA, have a gel profile distinct from PeMV RNA directed cell-free translation. Only a minor band, co-migrating with TEV cylindrical inclusion protein (70 kd), was observed among the products of TEV cell-free protein synthesis (Fig. 10, lane 1). This 70 kd protein, as well as products with molecular weights of 75 kd, 77 kd, 95 kd, 120 kd, and 150 kd, were immunoprecipitated with antiserum to TEV cylindrical inclusion protein (Fig. 10, lane 2). The products larger than 70 kd are presumed to contain the TEV cylindrical inclusion protein amino acid sequence (or part of it) and an adjacent gene product(s). Antiserum to the distantly related PeMV cylindrical inclusion protein (Purcifull *et al.*, 1975) precipitated the same products as the TEV cylindrical inclusion antiserum, but not as efficiently as the homologous serum, as indicated by the intensity of the products bands (Fig. 10, lane 3). Antiserum to cylindrical inclusion protein of an unrelated potyvirus (watermelon mosaic virus 1) did not immunoprecipitate any of the TEV cell-free products (Fig. 10, lane 4). Immunoprecipitation tests with antisera to healthy tobacco proteins, potato virus x capsid protein, and with normal serum were also negative (Fig. 10, lanes 5 through 7).

Figure 10. Analysis of TEV RNA *in vitro* translation products by immunoprecipitation with cylindrical inclusion antisera. The figure illustrates [^{35}S] methionine labeled products separated by SDS-PAGE and detected by fluorography. Estimated molecular weights of products reacting with cylindrical inclusion antisera are given in the figure. Lane 1, total products of TEV RNA cell-free translation; lane 2, immunoprecipitation with antiserum prepared against TEV cylindrical inclusion protein; lane 3, immunoprecipitation with antiserum prepared against PeMV cylindrical inclusion protein; lane 4, immunoprecipitation with antiserum to watermelon mosaic virus 1 cylindrical inclusion protein; lane 5, immunoprecipitation with antiserum to healthy tobacco proteins; lane 6, immunoprecipitation with antiserum to potato virus x capsid protein; and lane 7, immunoprecipitation with normal serum.


1 2 3 4 5 6 7
150k
120k
70k

Discussion

The 49 kd and 54 kd nuclear inclusion proteins associated *in vivo* with TEV infection are also produced during *in vitro* translation of TEV RNA. This conclusion is based on the analysis of the TEV RNA stimulated cell-free protein synthesis by PAGE, which shows products co-migrating with authentic nuclear inclusion proteins. These co-migrating products are immunoprecipitated by antiserum specific to the nuclear inclusion proteins. A comparison of the proteolytic peptide maps of the co-migrating *in vitro* products with authentic nuclear inclusion proteins also provides evidence that the nuclear inclusion proteins are virus-coded proteins.

The availability of antisera to four TEV specific proteins and the technique of immunoprecipitation has enabled the characterization of many of the polypeptides generated during the *in vitro* translation of TEV RNA. The consistent serological specificity of the antisera, and the consistent negative serological reactions with appropriate control antisera in all the immunoprecipitation analyses of the cell-free products, provided evidence that the immunoreactive products smaller or larger than the homologous antigen represented premature gene terminations or gene readthroughs, respectively. Confirmation of the smaller products as premature termination and the larger products as gene readthroughs needs to be determined by peptide mapping and N-terminal analyses.

There are a number of products smaller than 49 kd immunoprecipitated by antiserum to the 49 kd nuclear inclusion protein. The 30 kd product, which co-migrates with TEV capsid protein, is believed to be a specific

premature termination of the 49 kd gene and not the non-specific binding of capsid protein. The evidence is based on two observations. Firstly, the 49 kd antiserum showed no immunoreactivity with TEV capsid protein when analyzed in SDS immunodiffusion assays. Secondly, an immunoprecipitation analysis of the time course of product appearance of TEV RNA *in vitro* translation revealed that the 49 kd product, along with its presumed premature termination 30 kd product, appeared as early as 5 min, while capsid protein could not be detected before an incubation period of 15 min.

The 150 kd TEV cell-free product immunoprecipitated by antiserum to 49 kd nuclear inclusion protein also reacted with antiserum to TEV cylindrical inclusion protein. This product is probably a readthrough of these two genes and a third gene (discussed in Section 3) which codes for a product (40 kd to 50 kd), unreactive with antisera to four TEV specific proteins. Products of 40 kd and 50 kd were detected by Siegel and Hari (1977) in their *in vivo* labeling of TEV and potato virus Y infected tissue.

A number of products, in the size range of 65 kd to 90 kd, were immunoprecipitated by the 54 kd nuclear inclusion protein antiserum. These large products were also immunoprecipitated with antiserum to TEV capsid protein and/or TEV cylindrical inclusion protein and, thus, were considered to be gene readthrough products. This subject will be discussed in Section 3. The 54 kd antiserum also precipitated a 49 kd product. This immunoprecipitation could represent a premature termination of the 54 kd gene, but more likely was due to the presence of contaminating antibodies to 49 kd protein. The 54 kd antiserum

formed a faint precipitin band when tested against PAGE purified 49 kd protein in a double immunodiffusion test. Antiserum prepared against PAGE purified 49 kd protein did not react with 54 kd protein.

The analysis of the cell-free translation of PeMV RNA provides direct evidence that the cylindrical inclusions associated with potyviruses are virus-specific proteins. This is based on co-migration of a cell-free product with authentic cylindrical inclusion protein and on the immunoprecipitation studies with antiserum prepared to cylindrical inclusion protein. *Staphylococcus aureus* V-8 proteolytic peptide digest pattern analysis has not been carried out on the [^{35}S] methionine PeMV labeled cell-free product, immunoprecipitated by antiserum to PeMV cylindrical inclusion protein, due to the low molar ratio of methionine in the inclusion protein (one methionine per inclusion monomer) (unpublished data). Attempts to detect peptide patterns with [^{3}H] leucine labeled product or to label the 68 kd cell-free protein, excised from preparatory PAGE, with ^{125}Iodine have been unsuccessful to date.

The TEV RNA stimulated cell-free protein synthesizing system produced much less product (based on fluorogram band intensity) co-migrating with TEV cylindrical inclusion protein, compared to the PeMV cell-free system. This suggests that the initiator codon for the TEV cylindrical inclusion gene is not as available as the one for PeMV inclusion gene in this cell-free system. However, the immunoprecipitation studies indicate that the TEV cylindrical inclusion gene is translated efficiently as part of the 120 kd readthrough product. A 110 kd readthrough product is also formed in the PeMV RNA stimulated cell-free system, indicating a translation strategy similar to TEV.

In vitro translation studies with these two potyviral RNAs are the culmination of evidence implicating the potyviral associated inclusions as specific products of the viral genome. Previous evidence was based on the constant association of cylindrical inclusion with potyvirus infections (Edwardson, 1966), and on the observations that cylindrical inclusion morphology and serological properties are virus specific and host independent (Edwardson, 1974; Purcifull *et al.*, 1973).

The direct demonstration in this report that inclusions associated with the potyvirus group are virus-coded proteins confirms the proposals of Edwardson (1966) and Edwardson and Christie (1978) that inclusions may be used in diagnosing potyvirus infections and in taxonomically grouping potyviruses into distinct cytological groups (Edwardson, 1974). Furthermore, inclusions must be considered in potyvirus characterization studies because, as in the case of TEV, they represent approximately 50% of the viral genetic information.

SECTION 3
STRATEGY OF POTYVIRUS *IN VITRO* TRANSLATION

Introduction

In the first two sections, the reaction conditions necessary for efficient translation of pepper mottle virus (PeMV) RNA and tobacco etch virus (TEV) RNA were described. The capsid protein of these two viruses was found to be synthesized *in vitro* as determined by co-migration in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with their respective *in vivo* capsid protein, by immunoprecipitation data, and by the comparison of the partial proteolytic digest pattern of the products immunoprecipitated with antiserum to capsid protein with *in vivo* capsid protein pattern. TEV nuclear inclusion proteins also have been demonstrated to be synthesized using the same criteria. Cylindrical inclusion protein was translated *in vitro* with PeMV RNA, as determined by co-migration with the *in vivo* protein and immunoprecipitation. TEV RNA did form the cylindrical inclusion monomer but at a very low level. Larger proteins, presumed to be readthrough products, also were identified by co-immunoprecipitation with multiple antisera.

Characterization of Potyvirus RNA

There is a paucity of data available concerning potyvirus nucleic acid. The infectious nucleic acid has been shown to be a 39 S sedimenting RNA species with an extrapolated molecular weight of 3.2×10^6 (Hill and Benner, 1976; Hinostroza-Orihuela, 1975). A population of TEV RNA has

been shown to be polyadenylated at the 3' end as determined by binding assays on polyuridylic acid agarose columns (Hari *et al.*, 1979).

Strategies of *In Vitro* Translation

The infectious RNAs of multicomponent viruses are translated as monocistronic messengers in the cell-free system. Brome mosaic virus (BMV) (Shih and Kaesberg, 1973; 1976) and alfalfa mosaic virus (AMV) (Rutgers, 1977) are the two viral systems studied most extensively to date. Capsid protein was formed *in vitro* only when the small monocistronic RNA was present. Translation of the other RNA species (RNAs 1, 2, and 3) resulted in the formation of one product per RNA species. Multiple products observed with a single RNA species have been shown to contain identical N-termini, and the smaller products are thought to be formed via premature termination during translation (Van Tol and Van Vloten-Doting, 1979).

The infectious RNA of unicomponent spherical plant viruses can be translated in a monocistronic or polycistronic fashion. Cell-free studies with turnip yellow mosaic virus (TYMV) indicate that the RNA-protein product relationship is analogous to the BMV or AMV system. Capsid protein was formed only if a small monocistronic RNA species was present. Only one large molecular weight polypeptide was translated from the infectious RNA. Multiple products, when observed, were thought to be premature terminations (Mellena *et al.*, 1979).

The infectious RNA species of two plant viruses, carnation mottle virus (CarMV) (Salomon *et al.*, 1978) and tobacco necrosis virus (TNV) (Salvato and Fraenkel-Conrat, 1977), appear to be translated as polycistronic messengers in the cell-free system. Multiple products were

detected and were shown to be discrete proteins. Capsid protein was formed by both of these viral RNAs *in vitro*.

Pelham (1979a) has recently implicated proteolytic cleavage as a possible mode of cell-free product formation in the cowpea mosaic virus (CoMV) system. Although post-translational proteolytic cleavage has been well documented in a number of animal virus systems (Shih *et al.*, 1978; Pelham, 1978b), confirmation of his hypothesis, as the viable process for CoMV *in vivo*, will have to await correlation of the processed products *in vitro* with those observed *in vivo*.

The RNA of only two elongated viruses has been translated in a cell-free system. The infectious RNA of tobacco mosaic virus (TMV) has been shown to form two products from the infectious 2.0×10^6 dalton RNA; a 130,000 dalton (130 kd) product and a 160 kd readthrough product (Pelham, 1978a). Capsid protein was formed only if the low molecular weight component (LMC) was present (Bruening *et al.*, 1976). This particle contains the monocistronic messenger for the capsid protein. A 30 kd gene product is also formed if the subgenomic RNA I_2 species is present (Beachy and Zaitlin, 1977). Pelham (1979b) has recently shown that the 30 kd gene of the intact RNA may be translated *in vitro* if the infectious RNA is enzymatically fragmented prior to translation.

Potato virus x (PVX) has been translated in a wheat germ and rabbit reticulocyte system. Multiple products were synthesized, but none has been related to possible *in vivo* products (Ricciardi *et al.*, 1978).

Immunoprecipitation Analysis

Immunoprecipitation with homologous antiserum has been used as a specific probe to analyze the products of cell-free translation. Coupled

with an immunoabsorbent (*i.e., Staphylococcus aureus* Cowan strain 1), this type of product analysis is rapid and definitive. The assay is limited, however, by the availability of antisera to viral products. Because of the various inclusions formed *in vivo* with potyvirus infections, and the availability of inclusion and capsid protein antisera, the genetic analysis of the potyvirus genome using immunoprecipitation has been undertaken.

The synthesis of potyvirus cell-free products was affected by the RNA quality. Potyvirus RNA isolated in the presence of Proteinase K yielded a higher percentage of intact 39 S RNA, as determined on linear-log sucrose gradients. This RNA stimulated the incorporation of [^{35}S] methionine into trichloroacetic acid (TCA) precipitated products 50 to 60 times over endogenous levels. Potyvirus RNA isolated without Proteinase K typically stimulated the cell-free system 15 to 20 times over endogenous levels. *In vitro* products analyzed by SDS-PAGE, varied depending on the RNA used. RNA purified in the presence of Proteinase K was translated into one major product and a number of minor products larger than 150 kd. The major product had a molecular weight of 87,000 (87 k) for TEV and 78 k for PeMV RNA. RNA purified without Proteinase K also synthesized the major protein in the cell-free system in addition to a number of major products which have been shown to be similar to *in vivo* proteins. The RNA appears to be translated as a monocistronic message with only the gene at the 5' end of the intact RNA translated efficiently. Fragmentation of the RNA, during isolation, may open internal genes to *in vitro* translation that were closed in the intact 39 S RNA species. Discrete low molecular weight RNA species or LMCs

were not detected. Using immunoprecipitational analyses of products and translation of intact and fragmented RNAs, a genetic map of the potyvirus genome is proposed.

Material and Methods

Virus Source and Propagation

PeMV (Purcifull *et al.*, 1975) was maintained in *Nicotiana tabacum* L. var. Samsum. TEV (American Type Culture Collection #69 and the Simon's [1976] isolate) was cultured in *Nicotiana tabacum* L. var. Havana 425. Watermelon mosaic virus 1 (WMV-1) and WMV-2 were propagated in *Cucurbita pepo* L. "Small Sugar" (Purcifull and Hiebert, 1979).

Virus Isolation

PeMV and TEV were purified as previously described (Section 1). WMV-1 and WMV-2 were purified as described by Purcifull and Hiebert (1979).

RNA Isolation and Fractionation

Freshly purified virus was dissociated by the addition of an equal volume of 200 mM ammonium carbonate (pH 9.0), 2.0% SDS, and 2 mM EDTA (Brakke and Van Pelt, 1970a). Proteinase K, when used, was added at a concentration of 10 µg per mg of virus. Dissociation took place at room temperature for either 15 min or 12 hr. The RNA was isolated by rate zonal density-gradient centrifugation in linear-log sucrose gradients (Brakke and Van Pelt, 1970b). The 39 S RNA fraction was collected and precipitated by the addition of sodium acetate (pH 5.0) to a final

concentration of 100 mM and 2 volumes of 100% ethanol. The RNA was resuspended in a small volume of water and used for translation studies, or the RNA was brought up in 10 mM sodium phosphate buffer (pH 7.0) for further analysis.

Further analysis of the 39 S RNA fraction was made by denaturing with 50% deionized formamide (final concentration) buffered with 10 mM sodium phosphate buffer (pH 7.0) and heating the sample at 85° for 4 min. The RNA sample was then quick-cooled in an ice-water bath and diluted with 7 to 10 volumes of 10 mM sodium phosphate buffer (pH 7.0). The RNA sample was layered on a linear-log sucrose gradient and centrifuged (260,000 *g* max) in a SW 41 rotor for 5 hr at 14°. One ml fractions were collected, and the RNA was precipitated as previously described and resuspended in a small volume of water.

Lysate Preparation and *In Vitro* Reaction Conditions

Lysate preparation and the *in vitro* reaction conditions were carried out as described in Section 2.

Analysis of the Cell-free Products

Immunoprecipitation was carried out as previously described in Section 2. All cell-free products were separated by SDS-PAGE on a slab gel apparatus (1 mm x 140 mm x 150 mm). The Laemmli (1970) discontinuous buffer system was used in a 7.5% to 15% linear gradient gel. Products were detected either by staining with Coomassie brilliant blue or by fluorography (Bonner and Laskey, 1974) using Kodak Royal X-Omat X-ray film.

Serologically Specific Electron Microscopy

Serologically specific electron microscopy (SSEM) was carried out as described by Derrick and Brlansky (1976). Material was examined in a Philips EM-200 electron microscope.

Results

Analysis of PeMV Particles

Virus particles were anlayzed for the possible existence of individually encapsidated subgenomic RNAs. Leaf dips of PeMV infected tissue, examined using serologically specific electron microscopy, did not reveal the existence of shorter virus particles. A high percentage (92%) of the particles examined (1400) were 700 to 800 nm in length (Fig. 11).

Analysis of TEV RNA After Various Dissociation Procedures

Proteinase K, added to the dissociation solution, affected the quality of the RNA recovered. RNA, dissociated with the ammonium carbonate-SDS-EDTA system, had comparable rates of recovery (70 to 80%) with Proteinase K present or absent in the dissociation solution. This value was consistent whether the virus was dissociated for 15 min or 12 hr at room temperature. RNA analyzed on linear-log sucrose gradients, however, possessed distinct sedimentation profiles (Fig. 12). Virus dissociated for 15 min resulted in a major RNA species sedimenting at 39 S. Exposure to Proteinase K during dissociation did not affect the non-denatured RNA profile. Virus which was dissociated for 12 hr at room temperature, without Proteinase K, resulted in a sedimentation profile

Figure 11. Histogram representation of TEV particle length. Examination of leaf dips of TEV infected tobacco tissue was made using serologically specific electron microscopy. Formvar coated grids were sensitized with antiserum to TEV capsid protein. Leaf sap was allowed to incubate with the grid, and after a period of 1 hr, the grids were washed and then stained with 0.2% uranyl acetate. Grids were examined and photographed in a Philips EM 200. Calibration of particle length was made from a diffraction grating replica (E. F. Fullam Co., N.Y.) contained 21,600 lines per centimeter.

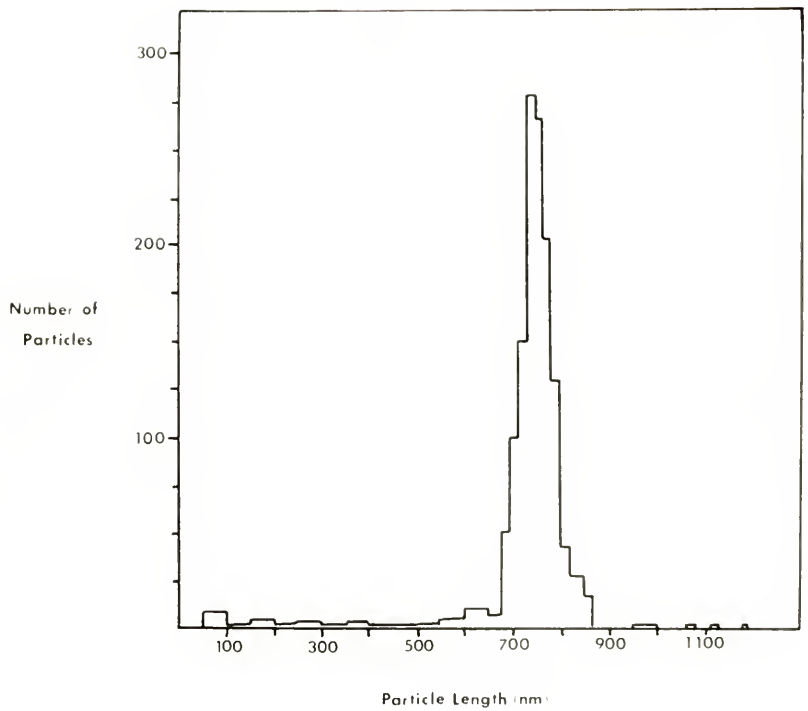
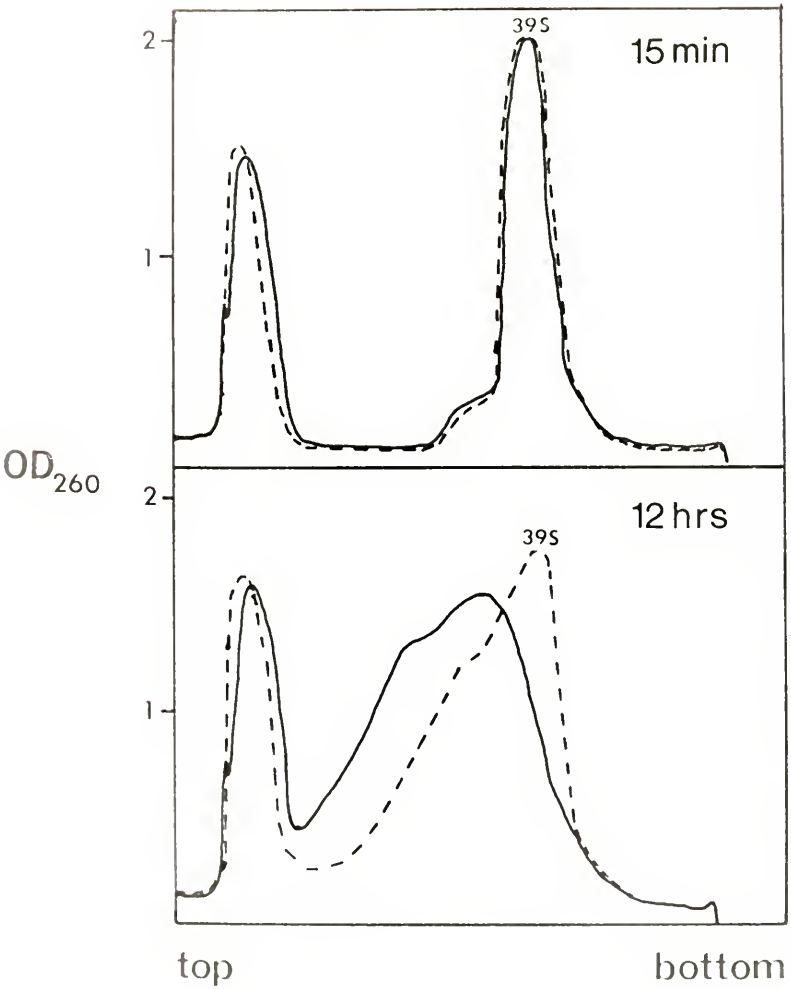

300
200
100
Number of
Particles
100
300
500
700
900
1100
Particle Length (nm)

Figure 12. Analysis of TEV RNA after various dissociation procedures on linear-log sucrose density gradients. Sedimentation profile of TEV RNA after purified virus was dissociated in the ammonium carbonate-SDS-EDTA system in the presence (-------) or absence (————) of Proteinase K (10 µg/1 mg of virus), is shown for incubation periods of 15 min and 12 hr. Centrifugation was in a SW 41 rotor at 260,000 *g* for 4 hr at 14°.


OD260
2
1
39S
15 min
2
1
12 hrs
39S
top
bottom

characteristic of fragmented RNA, with only a minor amount of intact 39 S RNA remaining. Virus dissociated for 12 hr in the presence of Proteinase K had a greater proportion of intact 39 S RNA, although a considerable amount of degradation was evident (Fig. 12).

Potyvirus 39 S RNA fractionated from the previously described dissociation preparations was tested for translational activity in the rabbit reticulocyte lysate system. TEV RNA isolated from virus dissociated for 12 hr without Proteinase K, did not stimulate the cell-free system and was slightly inhibitory to endogenous levels (Table 3-1). This inhibition of endogenous activity by highly fragmented RNA has been observed on numerous occasions. TEV RNA acquired from virus dissociated for 12 hr with Proteinase K present in the dissociation solution stimulated the cell-free translation 15 to 20 times over endogenous levels. Comparabel levels of [^{35}S] methionine incorporation was obtained with TEV and PeMV RNA isolated from virions dissociated for 15 min without Proteinase K (Table 3-1). However, RNA prepared by dissociating PeMV or TEV for 15 min in the presence of Proteinase K stimulated the cell-free system 50 to 60 times over endogenous levels (Table 3-1).

Product Analyses

Analysis of cell-free products by SDS-PAGE resulted in product distribution patterns distinctive for the respective RNA preparations. TEV RNA isolated after dissociation with Proteinase K for 12 hr did not synthesize products larger than 100 kd, as analyzed by SDS-PAGE. The major cell-free product had a molecular weight of 87 k. Products with molecular weights less than 87 k were formed but not as major translational products (Fig. 13, lane 2).

Table 3-1. Effect of RNA extraction methods on the translational activity of TEV- and PeMV-RNA.

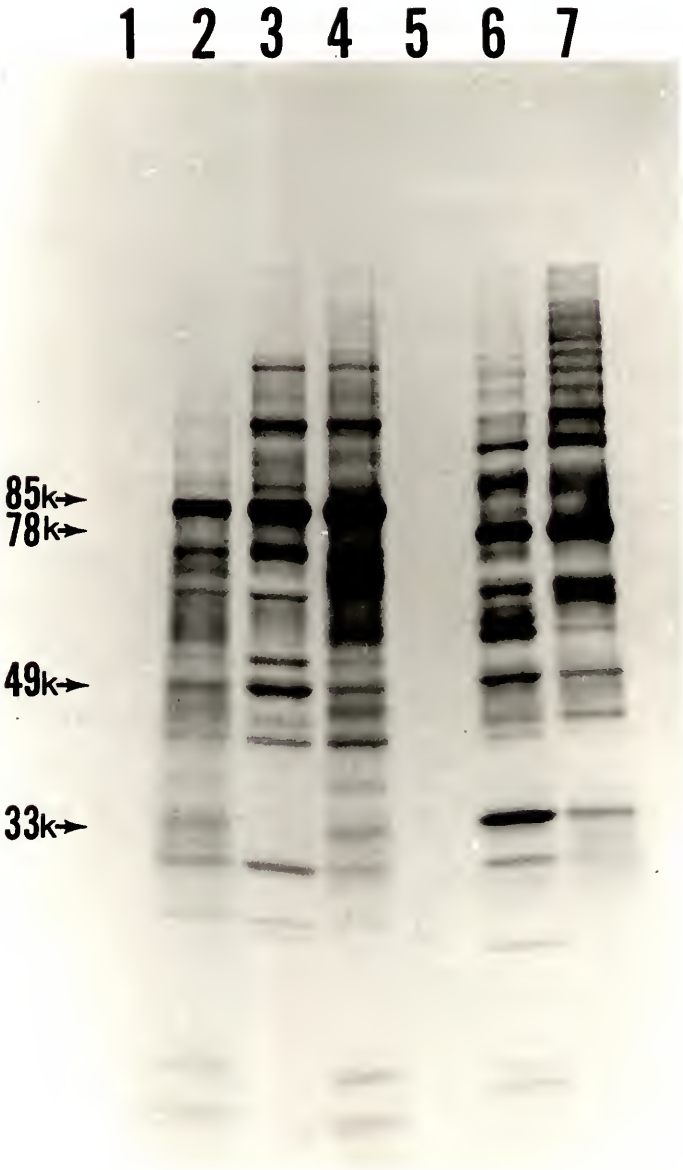
RNA PREPARATION[a]	TEV RNA	PeMV RNA
- Proteinase K (12 hr)	11,167[b]	nt
+ Proteinase K (12 hr)	235,291	nt
- Proteinase K (15 min)	245,656	241,389
+ Proteinase K (15 min)	701,657	691,854
Minus RNA	12,635	11,942

[a]Purified virus was dissociated in the ammonium carbonate-SDS-EDTA system in the presence (+) or absence (-) of Proteinase K (10 µg/mg of virus) for the specified time. Minus RNA represents the endogenous activity.

[b]Assay values are cpm per 10 µl aliquot of TCA precipitable products. Values have not been corrected for endogenous activity.

nt, not tested.

Figure 13. Polyacrylamide gel electrophoresis fractionation of the *in vitro* translation products of TEV- and PeMV-RNA isolated by various dissociation procedures. The products were fractionated on a SDS polyacrylamide slab gel (7.5% to 15%) gradient. Translation conditions were identical for all the RNA preparations and have been described in Section 2. The figure illustrates [^{35}S] methionine labeled products detected by fluorography. The RNA preparations, used to stimulate the cell-free synthesis of the observed products are as follows: (lane 1) TEV RNA isolated after incubating purified virus for 12 hr in the dissociation solution lacking Proteinase K; (lane 2) TEV RNA isolated after incubating purified virus for 12 hr in the dissociation solution with Proteinase K; (lane 3) TEV RNA isolated after incubating purified virus for 15 min in the dissociation solution after Proteinase K; (lane 4) TEV RNA isolated after incubating purified virus for 15 min in the dissociation solution with Proteinase K; (lane 5) empty lane; (lane 6) PeMV RNA isolated after incubating purified virus for 15 min in the dissociating solution without Proteinase K; and (lane 7) PeMV RNA isolated after incubating purified virus in the dissociating solution containing Proteinase K for 15 min.


1 2 3 4 5 6 7
85k
78k
49k
33k

In vitro translation products of TEV- and PeMV-RNA were distinct regardless of the RNA isolation procedure employed. PeMV- and TEV-RNA extracted with Proteinase K absent coded for discrete products generally ranging in molecular weights from 30 k to 150 k as analyzed on SDS-PAGE (Fig. 13, lanes 3 and 6). RNAs prepared with Proteinase K were able to synthesize these products but at a lower level. Instead, there was the tendency to form proteins greater than 100 kd during translation, in addition to synthesizing the major protein (87 kd for TEV and 78 kd for PeMV) at an increased level (Fig. 13, lanes 4 and 7).

PeMV RNA Separation and Cell-free Translation of RNA Fractions

PeMV dissociated with or without Proteinase K generated the same RNA sedimentation profile after initial separation on linear-log sucrose gradients (Fig. 14A). These two PeMV 39 S RNA samples were further characterized by formamide denaturation followed by separation on linear-log sucrose gradients. PeMV RNA extracted with Proteinase K sedimented predominately as a 39 S RNA species. A minor amount of trailing RNA was detected (Fig. 14B). This was in contrast to PeMV RNA isolated without Proteinase K during dissociation, which generated a profile indicative of fragmented RNA (Fig. 14B). RNA from this gradient was fractionated into 12 one ml fractions, and the analysis of the translation products on SDS-PAGE is shown in Fig. 15. The RNA from each of the individual fractions was responsible for the synthesis of discrete polypeptides, most of which have been observed when unfractionated RNA of this quality was translated. Translation of RNA from fraction 4 and 5 formed 5 major products with molecular weights less than 33 k. Of these products, 4 are thought to contain part of the capsid protein amino acid sequence as

Figure 14. Analysis of PeMV RNA isolated from dissociated virus with Proteinase K present or absent.

A. Sedimentation profile of PeMV RNA isolated from purified PeMV which was dissociated for 15 min with the ammonium carbonate-SDS-EDTA system containing Proteinase K (------) or lacking Proteinase K (———). The dissociated virus was layered on linear-log sucrose gradients and centrifuged in a SW 41 rotor at 260,000 *g* for 4 hr at 14°. UV absorbing material at the top of the gradient is dissociated capsid protein.

B. Sedimentation profile of the 39 S PeMV RNA, isolated from the gradients in A, after denaturation. The 39 S RNA was denatured in 50% formamide as described in Material and Methods section. The denatured RNA was layered on the top of a linear-log sucrose gradient and centrifuged in a SW 41 rotor at 260,000 *g* for 5 hr at 14°. Numbers at the bottom of the figure represent 1 ml fractions that were collected and precipitated for further translational analyses.

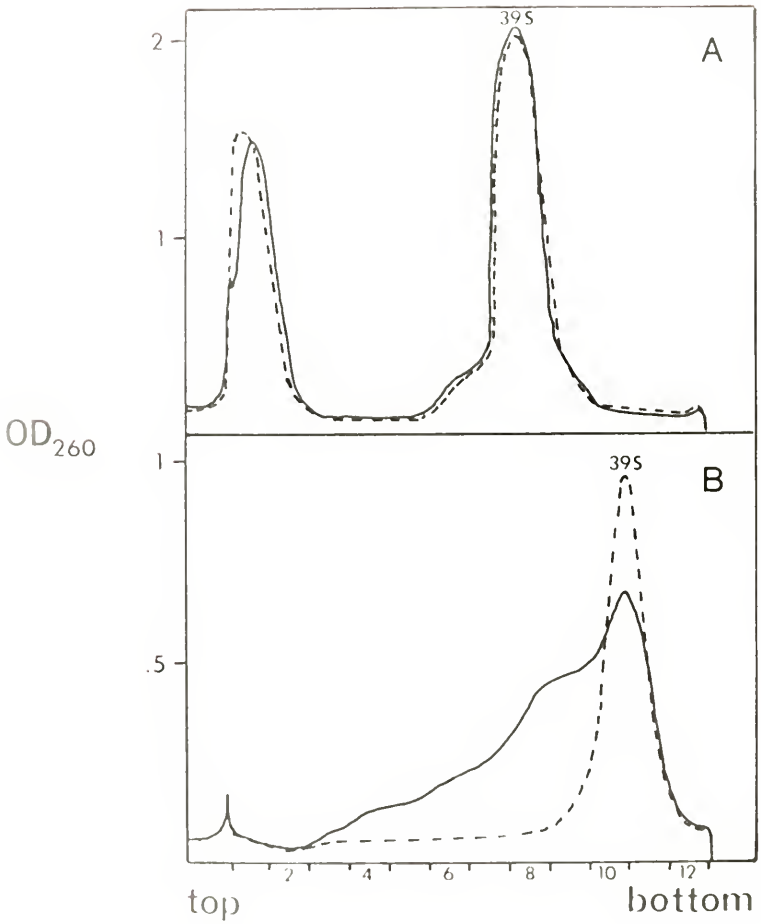
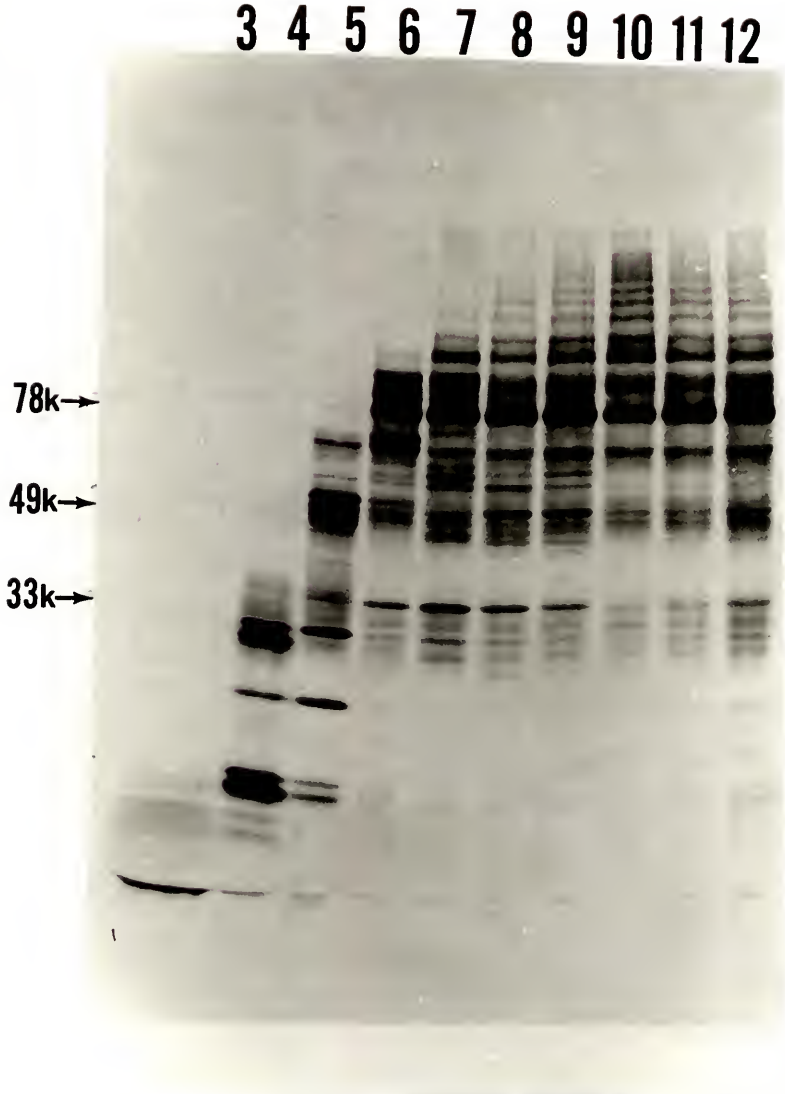

OD260
2
1
1
.5
39S
A
39S
B
2
4
6
8
10
12
top
bottom

Figure 15. Electrophoretic separation of the cell-free translation products of fractionated, denatured PeMV RNA. The products were separated on a 7.5% to 15% linear gradient gel using SDS-PAGE and detected using fluorography. Lane numbers refer to the fraction number of the denatured PeMV RNA (Fig. 14B) extracted without Proteinase K and separated on linear-log sucrose gradients.


3 4 5 6 7 8 9 10 11 12
78k
49k
33k

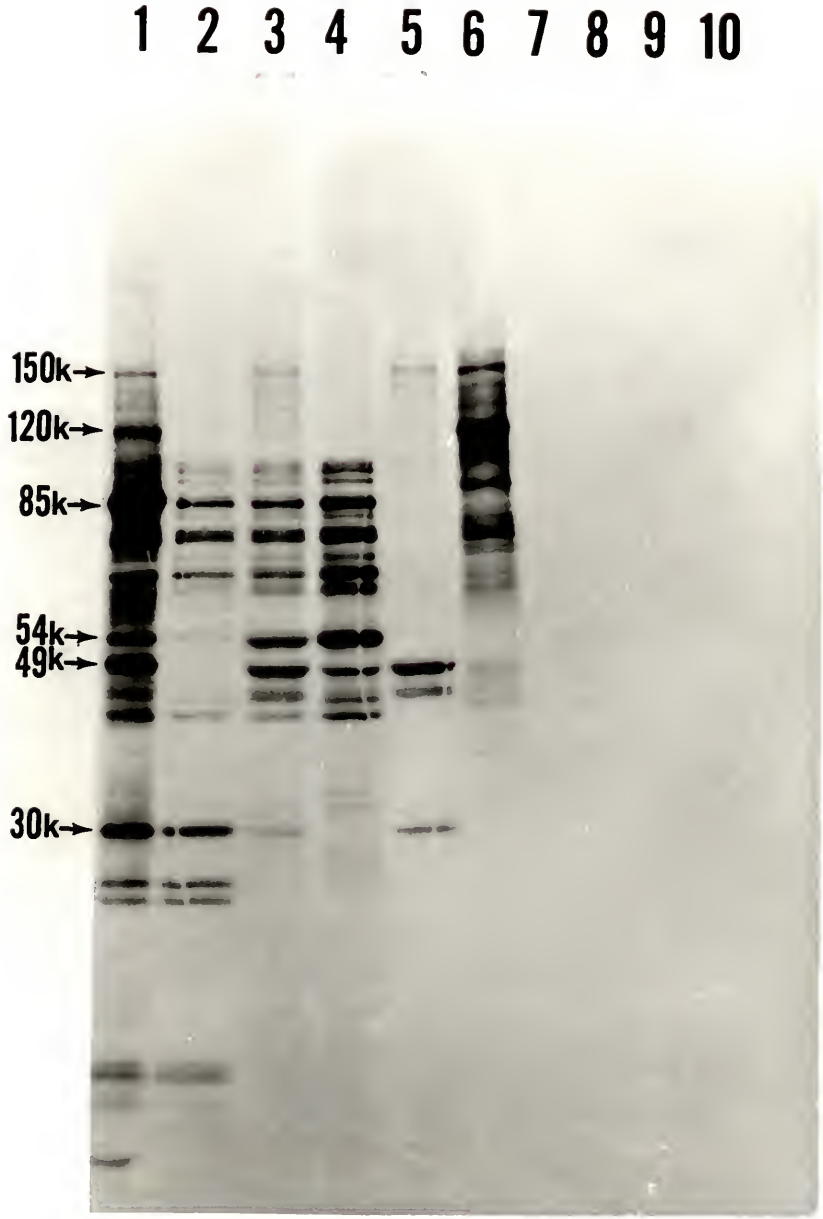
determined by immunological studies. RNA from fractions 5 through 9 was involved in the synthesis of discrete products with molecular weights ranging from 100 k to 33 k. The 33 kd protein product synthesized with fractions 6 through 9 co-migrated with PeMV capsid protein. RNA from fractions 5 through 9 also appeared to be involved in the synthesis of the 49 kd protein in addition to other major polypeptides around 70 kd. A 78 kd protein was detected as the major translation product of RNA from fractions 6 through 12. RNA fractions 10 and 11, which co-migrated with the 39 S RNA, synthesized this 78 kd protein almost exclusively. Large molecular weight products also predominated in these fractions and were thought to be readthrough products of adjacent genes. Few low molecular weight products were oberved as major cell-free products.

RNA of sufficient concentration was not collected from fraction 1 through 8 of PeMV RNA extracted with Proteinase K. RNA from fractions 9 through 11 was collected, and this RNA synthesized translation products which were identical to those observed in fraction 10 and 11 in Fig. 15.

Immunoprecipitation of TEV RNA Translation Products

The products of TEV RNA cell-free translation were analyzed using antisera to TEV proteins and antisera unreactive with TEV antigens in SDS double diffusion agar assays. TEV RNA, isolated without Proteinase K present during dissociation, was used in the translation system. A major product with a molecular weight of 87 k was the only distinct protein not immunoprecipitated with any of the antisera used. Antiserum to TEV capsid protein (Fig. 16, lane 2) immunoprecipitated the 30 kd product which has been shown to be similiar to native capsid protein. Proteins with molecular weight less than 30 k were presumed discrete capsid protein

Figure 16. Analysis of the *in vitro* translation products of TEV RNA by immunoprecipitation. The figure illustrates [^{35}S] methionine labeled products separated by SDS-PAGE and detected by fluorography. (lane 1) Total products of TEV RNA cell-free translation; (lane 2) products immunoprecipitated with antiserum to TEV capsid protein; (lane 3) products immunoprecipitated with antiserum to total nuclear inclusion protein; (lane 4) products immunoprecipitated with antiserum to SDS-PAGE purified 54 kd nuclear inclusion protein; (lane 5) products immunoprecipitated with SDS-PAGE 49 kd nuclear inclusion protein; (lane 6) products immunoprecipitated with antiserum to TEV cylindrical inclusion protein; (lane 7) products immunoprecipitated with antiserum to watermelon mosaic virus 1 cylindrical inclusion protein; (lane 8) products immunoprecipitated with antiserum to potato virus x capsid protein; (lane 9) products immunoprecipitated with antiserum to healthy tobacco proteins; and (lane 10) products immunoprecipitated with normal serum.


1 2 3 4 5 6 7 8 9 10
150k
120k
85k
54k
49k
30k

premature terminations. Products larger than 30 kd are also precipitated with capsid protein antiserum, with 75 kd, 77 kd, and 85 kd proteins being the major products immunoprecipitated.

Antiserum to total nuclear inclusion proteins precipitated the 49 kd and the 54 kd products, which co-migrated with *in vivo* nuclear inclusion proteins (Fig. 16, lane 3). A large number of other products were also selectively precipitated. Products related to the nuclear inclusion proteins were characterized further by utilizing antisera prepared against SDS-PAGE purified 54 kd and 49 kd nuclear inclusion proteins.

Antiserum to the partially purified 54 kd inclusion protein selectively precipitated the 54 kd cell-free product (Fig. 16, lane 4). The larger products precipitated with the 54 kd antiserum co-migrated with products that were also immunoprecipitated with antiserum to capsid protein (Fig. 16, lane 2). A 49 kd protein and smaller proteins were also precipitated and have been discussed previously (Section 2). Antiserum to the SDS-PAGE purified 49 kd nuclear inclusion precipitated the 49 kd cell-free product (Fig. 16, lane 5). A large 150 kd product was also precipitated exclusively by the 49 kd antiserum and not by the 54 kd antiserum. The large number of cell-free products, ranging in molecular weight from 65 k to 100 k and immunoprecipitated by antiserum to the 54 kd nuclear inclusion protein and the capsid protein, are not immunoprecipitated with antiserum to the 49 kd *in vivo* nuclear inclusion protein.

Antiserum to TEV cylindrical inclusion protein was also used to analyze the TEV RNA cell-free translation products (Fig. 16, lane 6). A major 120 kd product was precipitated by TEV cylindrical inclusion antiserum. Antisera to other TEV specific proteins or control antiserum did not react with this protein. The 150 kd protein, immunoprecipitated with antisera

to total nuclear inclusion proteins and to purified 49 kd nuclear inclusion protein, was precipitated with antiserum to TEV cylindrical inclusion. Two proteins with molecular weights of 77 k and 75 k were also precipitated. Immunoprecipitation with antisera to WMV-1 cylindrical inclusion protein (an unrelated potyvirus), potato virus x capsid protein, healthy tobacco proteins, and with normal serum were all negative.

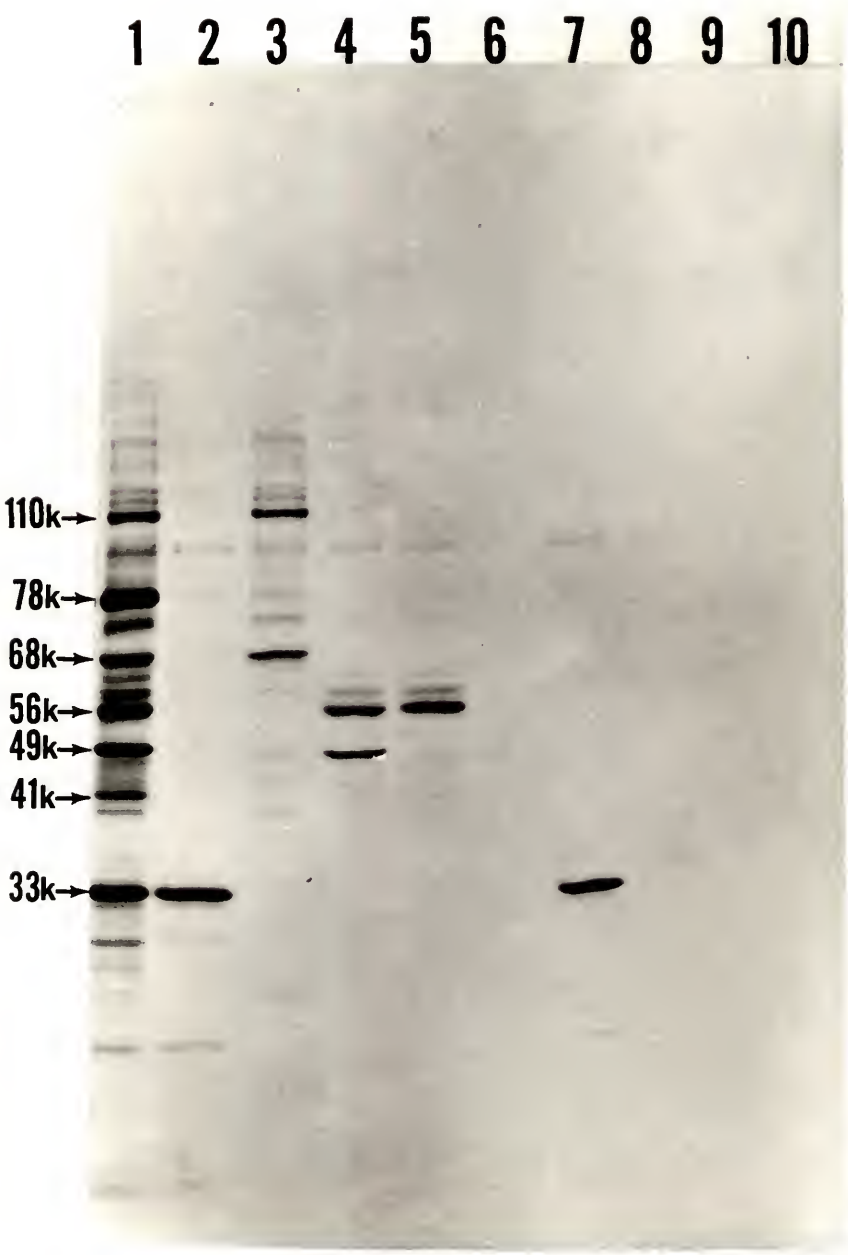
Immunoprecipitation of PeMV RNA Cell-free Translation Products

PeMV RNA cell-free translation products were analyzed with antisera to PeMV associated proteins and unrelated proteins. Antisera to TEV associated proteins, which were serologically related to PeMV protein, were also used.

Antiserum to PeMV capsid protein selectively precipitated the 33 kd protein, smaller presumed premature termination products, and a protein with a molecular weight of 90 k (Fig. 17, lane 2). TEV capsid protein antiserum, whcih shows a reaction of partial identity with PeMV capsid protein in SDS double diffusion immunoassays, immunoprecipitated proteins with the same molecular weights (Fig. 17, lane 7). Antiserum to PeMV cylindrical inclusion protein was also used to analyze the cell-free products (Fig. 17, lane 3). A 68 kd protein was a major immunoprecipitated product along with a 110 kd protein. The 68 kd protein, co-migrated with the *in vivo* cylindrical inclusion protein.

Antiserum to TEV total nuclear inclusion protein immunoprecipitated two major products of the PeMV RNA cell-free translation, with estimated molecular weights of 49 kd and 56 kd. A 90 kd protein and other minor products were also immunoprecipitated (Fig. 17, lane 4). Further characterization using antisera to SDS-PAGE purified 49 kd and 54 kd TEV nuclear

Figure 17. Analysis of the *in vitro* translation products of PeMV RNA by immunoprecipitation. The figure illustrates [^{35}S] methionine labeled products separated by SDS-PAGE and detected by fluorography. (lane 1) Total products of PeMV RNA cell-free translation; (lane 2) products immunoprecipitated with antiserum to PeMV capsid protein; (lane 3) products immunoprecipitated with antiserum to PeMV cylindrical inclusion protein; (lane 4) products immunoprecipitated with antiserum to TEV total nuclear inclusion proteins; (lane 5) products immunoprecipitated with antiserum to SDS-PAGE purified TEV 54 kd nuclear inclusion protein; (lane 6) products immunoprecipitated with antiserum to SDS-PAGE purified TEV 49 kd nuclear inclusion protein; (lane 7) products immunoprecipitated with antiserum to TEV capsid protein; (lane 8) products immunoprecipitated with antiserum to potato virus x capsid protein; (lane 9) products immunoprecipitated with antiserum to healthy tobacco proteins; and (lane 10) products immunoprecipitated with normal serum.


1 2 3 4 5 6 7 8 9 10
110k
78k
68k
56k
49k
41k
33k

inclusion proteins, resulted in only the 54 kd antiserum reacting. The 54 kd antiserum selectively precipitated the 56 kd cell-free product, in addition to a minor band at 90 kd and minor bands at 60 kd. Antiserum to the 49 kd nuclear inclusion protein did not react with any of the products of PeMV cell-free translation. Immunoprecipitation of PeMV RNA translation products with normal serum and antisera to potato virus x capsid protein and healthy tobacco proteins were negative. Major cell-free products with estimated molecular weights of 78 k and 41 k were not immunoprecipitated by any of the antisera used (Fig. 17, lanes 8 through 10).

Effect of m^7GTP on TEV RNA *In Vitro* Translation

The cap analogue m^7GTP was added to the rabbit reticulocyte system with TEV RNA as the exogenous message. Increasing the concentration of the cap analogue resulted in a decrease in the incorporation of [^{35}S] methionine into TCA precipitable counts. Cap analogue at 1 mM concentration had the same effect on intact 39 S TEV RNA or fragmented TEV RNA, with a 72% reduction in TCA precipitable counts observed (Table 3-2).

Discussion

The genetic material coding for the virus capsid protein and inclusion proteins, observed with viral infection, is contained in the potyvirus RNA. Cell-free translation of the intact 39 S RNA and subgenomic fragments, and immunological analyses of the products have enabled the identification of gene products and the mapping of their relationship to each other on the potyvirus genome.

Table 3-2. The effect of the cap analogue m^7GTP on TEV RNA *in vitro* translation.

mM m^7GTP	TEV RNA isolated[a] with Proteinase K (15 min)	TEV RNA isolated without Proteinase K (15 min)
0.0	702,658[b]	254,657
0.05	689,793	247,383
0.10	669,411	235,215
0.25	675,908	220,570
0.50	565,795	152,052
0.75	354,795	119,664
1.00	193,403	69,789

[a]TEV was dissociated for 15 min in the presence or absence of Proteinase K, and the 39 S RNA fraction was collected and precipitated. Cell-free reaction mixtures (30 µl) were assayed with varying m^7GTP concentrations for the two RNA samples. The *in vitro* reaction conditions for both RNA preparations were identical and have been reported in Section 1.

[b]Assay values are cpm per 10 µl aliquot of TCA precipitable product. Values have been corrected for endogenous activity.

Whether the potyvirus genome is translated as a monocistronic or polycistronic messenger RNA remains open to speculation. The data presented in this section, utilizing PeMV- and TEV-RNA, would tend to support the concept that the intact 39 S RNA is translated as a monocistronic message. Translation of TEV-and PeMV-RNA, isolated after two cycles of rate zonal sucrose density gradient centrifugation and presumed to be homologous for the 39 S RNA species, resulted in the synthesis of one predominant polypeptide and a number of larger products. The large products were presumed to be readthroughs of adjacent genes. Synthesis of these large molecular weight products was in contrast to the multiple smaller products formed when fragmented RNA was translated.

There are three hypotheses which would account for the multiplicity of products observed *in vitro* if the 39 S RNA is monocistronic as suggested. First, the formation of products may be due to intermediate size RNAs being translated, similiar to the situation found with TYMV. All the intermediate RNAs are derived from the infectious TYMV RNA species containing the same 5' end and only varying in length (Mellena *et al.*, 1979). Translational products formed would contain a similiar N-terminus and would be serologically related. This serological relationship between products, using antisera to four virus-specific proteins, was not observed in the potyvirus system. The second possible explanation is that the multiple products may be the result of subgenomic pieces of RNA, individually encapsidated, analogous to certain strains of TMV. The S-RNA, which is the monocistronic message for the capsid protein, is encapsidated in the cowpea strain of TMV (Bruening *et al.*, 1976). When this particle is isolated, and the RNA extracted and translated in a cell-free system, capsid protein is synthesized efficiently (Bruening *et al.*, 1976; Siegel

et al., 1976). The *in vivo* encapsidation of a monocistronic message has also been reported for a number of spherical plant viruses such as BMV (Shih and Kaesberg, 1973), TYMV (Pleij *et al.*, 1977), CMV (Schwinghamer and Symons, 1977), AMV (Mohier *et al.*, 1975), and cowpea chlorotic mottle virus (Verduin, 1978; Davies and Verduin, 1979). Another distinct subgenomic RNA species, I_2, is encapsidated in three strains of TMV. This TMV RNA species has been translated *in vitro* and forms a 30 kd protein (Beachy and Zaitlin, 1977). Analysis of potyviruses from infected leaf tissue using SSEM did not reveal many less than full-length particles, which could contain encapsidated monocistronic messengers. Analysis of purified potyviruses on sucrose gradients was not possible due to the aggregation problems encountered with purified virus preparations. The third possible explanation of the multiple products observed may be due to the limited fragmentation of the intact 39 S RNA during isolation and handling, which may open internal initiation sites previously closed to translation. Isolation of RNA with Proteinase K, and subsequent RNA and translational analyses would support this theory. Pelham (1979b) has been able to open internal initiation sites by limited enzymatic fragmentation of intact CoMV-and TMV-RNA. Most of the products synthesized from the fragmented RNA, however, were not normally found *in vivo* or *in vitro*. Addition of the cap analogue m^7GTP did not inhibit their synthesis *in vitro*, indicating that they are uncapped fragments or uncapped *in vivo* messengers. Limited fragmentation of the 39 S potyviral RNA may account for the multiple products observed. However, if it occurs, some high degree of specificity must be involved, due to the reproducibility of the cell-free products formed. Also, cap analogue inhibition studies

did not selectively inhibit the synthesis of any one product, but a general inhibitory effect was observed with the *in vitro* translation of intact 39 S RNA or fragmented RNA preparation. Further analyses of potyvirus RNA, using RNA purified under denaturing conditions, hybridization studies with complementary intact 39 S RNA and subgenomic fragments, and ribosome binding studies are needed to confirm the manner in which the 39 S RNA species acts as a message during *in vitro* translation.

Immunological analyses of the products formed *in vitro* have been used successfully in a number of animal virus systems. Products of adenovirus messenger RNA have been studied using immunoprecipitation (Esche *et al.*, 1979). Numerous members of the retrovirus group have been translated in a cell-free system and their products analyzed by immunoprecipitation (Kamine and Buchanan, 1977; Palmiter *et al.*, 1978; Gallis *et al.*, 1979). Analyses by immunological methods have been minimal in most of the plant viruses translated in a cell-free system. Siegel *et al.* (1976) used antiserum to TMV capsid protein in a double diffusion assay to partially identify TMV capsid protein as a product of cell-free translation. Formation of immune complexes, utilizing the addition of unlabeled AMV capsid protein, has been used in the analysis of AMV cell-free products (Mohier *et al.*, 1975). CarMV capsid protein, synthesized *in vitro*, was detected using antibodies to CarMV capsid protein linked to a solid phase followed by elution and analysis of the products bound to them by SDS-PAGE (Salomon *et al.*, 1978). The immunoprecipitation method described by Kessler (1975), employing *Staphylococcus aureus* Cowen strain I as an immunoabsorbent, has not previously been utilized in the analysis of any cell-free plant virus translation study.

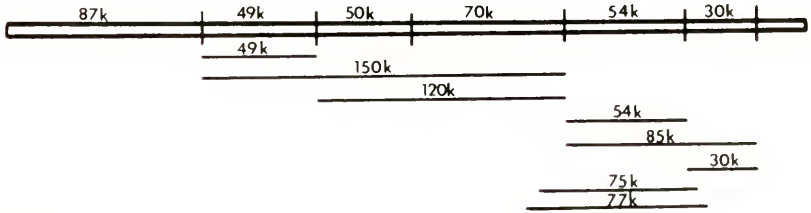
Immunoprecipitation is an extremely sensitive and specific probe. Not only were reactions between homologous antibodies and antigens observed, but heterologous reactions were also observed. Co-migration of *in vitro* products with *in vivo* identifiable proteins, availability of antisera to four virus-specific proteins, apparent gene readthrough products, and the technique of immunoprecipitation has enabled the construction of a map of the PeMV-and TEV-genome (Fig. 18).

The 87 kd protein of TEV and the 78 kd protein of PeMV have been located at the 5' end of the 39 S RNA. These two proteins were always observed to be synthesized, and in the case of intact or fragmented RNAs, were the predominant products. In a number of plant virus systems (Shih and Kaesberg, 1973; Mohier *et al.*, 1975; Beachy and Zaitlin, 1977) the gene located at the 5' end of a multigenomic piece of RNA is read most efficiently. Immunological analyses of the 78 kd and 87 kd products were not possible. Antisera to these two proteins were not available, nor did any of the antisera used in this study react with these two proteins.

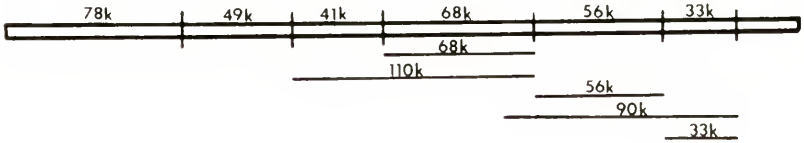
The 49 kd, 50 kd (or 41 kd), and 70 kd (or 68 kd) cell-free products have been mapped to their respective locations on the genome, using immunoprecipitation data of the discrete products and co-immunoprecipitation of the readthrough products with different antisera. Their interrelationship is shown in Fig. 18. Molecular weights of the distinct products, and of the readthrough products precipitated with the antisera, are given. TEV 49 kd *in vitro* protein was immunoprecipitated with antisera to total nuclear inclusion protein and with SDS-PAGE purified 49 kd *in vivo* inclusion protein. The 49 kd protein formed during cell-free translation of PeMV RNA was not immunoprecipitated with the TEV 49 kd antiserum.

Figure 18. A tentative genetic map. The figure illustrates the interrelationship observed between the *in vitro* products as determined by immunoprecipitation data. The elongated figure represents the viral RNA with an estimated molecular size of 3.2×10^6 daltons (9,697 nucleotides). Final product molecular weight, determined by *in vivo* and *in vitro* observation, are presented above the map. Major cell-free products and readthrough products (————) and their molecular weights, immunoprecipitated in each viral system, are presented below the map. Distance from the 5' end to the first initiator codon, spacers between genes, and distance from the termination codon of the coat protein gene to the 3' end, are unknown. The figure includes data for (A) TEV, (B) PeMV, and (C) a proposed general map for the potyvirus group considering other pertinent data.

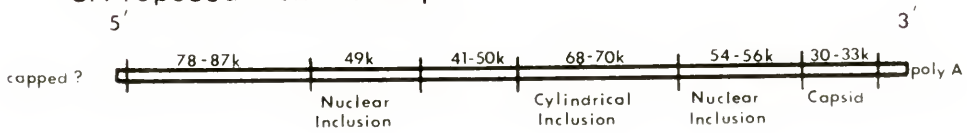
A. TEV



B. PeMV



C. Proposed Genetic Map



The 49 kd antiserum consistently reacted with a 150 kd TEV cell-free protein. This 150 kd protein was co-precipitated with antiserum to TEV cylindrical inclusion protein.

The evidence for the existence of a 41 kd protein in PeMV RNA cell-free translation and a 50 kd product in TEV RNA cell-free translation is speculative. A 41 kd cell-free product was observed during PeMV RNA *in vitro* translation but was not immunoprecipitated with any of the antisera used. The formation of a 50 kd product was observed during TEV RNA cell-free translation that was not immunoprecipitatable. Siegel and Hari (1977), however, have shown a 41 kd and a 50 kd protein to be synthesized in TEV and potato virus Y infected leaf tissue. Whether the 41 kd and the 50 kd cell-free products are distinct gene products or possible premature termination of the 78 kd and 87 kd cell-free products, respectively, is unknown. Indirect evidence in the form of the 150 kd and 120 kd readthrough products of TEV RNA translation, and the presence of a 110 kd readthrough in the PeMV RNA stimulated cell-free system, supports the existence of the 41 kd and 50 kd gene products.

A co-migrating product immunoprecipitated with antiserum to cylindrical inclusion protein was observed in PeMV RNA translation studies. A significant product (70 kd) co-migrating with *in vivo* TEV cylindrical inclusion protein was not observed in TEV cell-free translation. A small amount of 70 kd co-migrating product was observed after immunoprecipitation of the TEV cell-free products with antiserum to TEV cylindrical inclusion protein. A 120 kd and a 150 kd product were immunoprecipitated in the TEV RNA translation system with antiserum to TEV cylindrical inclusion protein. A 110 kd protein in the PeMV RNA

translation system was immunoprecipitated with antiserum to PeMV cylindrical inclusion protein, indicating that the translation strategy for this portion of the potyvirus genome may be comparable for both viruses.

The 54 kd nuclear inclusion protein gene is linked to the capsid protein gene. This was based on numerous readthrough proteins, 70 kd to 90 kd, immunoprecipitated by antisera to these two products. Although the antiserum to TEV 49 kd nuclear inclusion protein did not react with the PeMV RNA 49 kd *in vitro* protein, cross reactivity was exhibited with other TEV antisera and PeMV RNA cell-free products. Antiserum to SDS-PAGE purified 54 kd nuclear inclusion protein did react with a 56 kd protein formed during the *in vitro* translation of PeMV RNA. A 56 kd-33 kd readthrough product (90 kd) was also detected, linking these two genes in the PeMV RNA translation system.

The respective capsid protein genes have been located at the 3' end, based on immunological precipitation data. Previous reports dealing with BMV (Shih *et al.*, 1972), AMV (Gould and Symons, 1978), CMV (Schwinghamer and Symons, 1977), TYMV (Mellena *et al.*, 1979), and TMV (Beachy *et al.*, 1976) have also located the capsid protein cistron at the 3' end.

The maps presented account for 95% of the estimated coding capacity of TEV and 93% of PeMV RNA. The potyvirus RNA appears to be capped at the 5' end based on competition studies with m^7GTP. Confirmation of this must await 5' terminal analysis of the 39 S RNA. Hari *et al.* (1979) have shown TEV RNA to be polyadenylated at the 3' end. The gene linkage map described in this section relies principally on

immunoprecipitation data. WMV-1, WMV-2, and the Simons (1976) isolate of TEV have also been translated and their products analyzed. These *in vitro* translation studies also support the proposed genetic map. The map presented in this study may not be applicable to all potyviruses and will require further refinement. The functions of five of the six gene products remain to be elucidated. The characterization of these viral-coded proteins may in the future provide a gene product or gene products through which effective potyvirus control may be achieved.

LITERATURE CITED

Beachy, R. N., Zaitlin, M., Bruening, G., and Israel, H. W. (1976). A genetic map for the cowpea strain of TMV. Virology 73, 498-507.

Beachy, R. N., and Zaitlin, M. (1977). Characterization and *in vitro* translation of the RNAs from less-than-full-length, virus related, nucleoprotein rods present in tobacco mosaic virus preparations. Virology 81, 160-169.

Bonner, W. M., and Laskey, R. A. (1974). A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46, 83-88.

Brakke, M. K., and Van Pelt, N. (1970a). Properties of infectious ribonucleic acid from wheat streak mosaic virus. Virology 42, 699-706.

Brakke, M. K., and Van Pelt, N. (1970b). Linear-log gradient for estimating sedimentation coefficients of plant viruses and nucleic acids. Anal. Biochem. 38, 56-64.

Bruening, G., Beachy, R. N., Scalla, R., and Zaitlin, M. (1976). *In vivo* and *in vitro* translation of the ribonucleic acids of a cowpea strain of tobacco mosaic virus. Virology 71, 498-517.

Christie, R. G., and Edwardson, J. R. (1977). Light and electron microscopy of plant virus inclusions. Fla. Agric. Expt. Stn. Monogr. Ser. No. 9. 150 p.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252, 1102-1106.

Davies, J. W., and Verduin, B. J. M. (1979). *In vitro* synthesis of cowpea chlorotic mottle virus polypeptides. J. Gen. Virol. 44, 545-549.

Derrick, K. S., and Brlansky, R. H. (1976). Assay for viruses and mycoplasmas using serologically specific electron microscopy. Phytopathology 66, 815-820.

Edwardson, J. R. (1966). Electron microscopy of cytoplasmic inclusions in cells infected with rod-shaped viruses. Am. J. Bot. 53, 359-364.

Edwardson, J. R. (1974). Some properties of the potato virus Y-group. Fla. Agric. Expt. Stn. Monogr. Ser. No. 4. 398 p.

Edwardson, J. R., and Christie, R. G. (1978). Use of virus-induced inclusions in classification and diagnosis. Ann. Rev. Phytopathol. 16, 31-55.

Efron, D., and Marcus, A. (1973). Translation of TMV-RNA in a cell-free wheat embryo system. Virology 53, 343-348.

Esche, H., Schilling, R., and Doerfler, W. (1979). *In vitro* translation of adenovirus type 12-specific mRNA isolated from infected and transformed cells. J. Virol. 30, 21-31.

Fenner, F. (1976). Classification and nomenclature of viruses. Intervirology 7, 4-115.

Fraenkel-Conrat, H., Salvato, M., and Hirth, L. (1977). The translation of large plant viral RNAs. In "Comprehensive Virology" (H. Fraenkel-Conrat and Robert R. Wagner, eds.) Vol. 11, pp. 201-235. Plenum Press, New York.

Francki, R. I. B., and McLean, G. D. (1968). Purification of potato virus X and preparation of infectious nucleic acid by degradation with lithium chloride. Austral. J. Biol. Sci. 21, 1311-1318.

Gallis, B., Linial, M., and Eisenman, R. (1979). An avian oncovirus mutant deficient in genomic RNA: Characteristic of the packaged RNA as cellular messenger RNA. Virology 94, 146-161.

Gilbert, J. M., and Anderson, W. F. (1970). Cell-free hemoglobin synthesis. II Characteristics of the transfer ribonucleic acid-dependent assay system. J. Biol. Chem. 245, 2342-2349.

Gould, A. R., and Symons, R. H. (1978). Alfalfa mosaic virus RNA. Determination of the sequence homology between the four RNA species and a comparison with the four RNA species of cucumber mosaic virus. Eur. J. Biochem. 91, 269-278.

Hari, V., Siegel, A., Rozek, C., and Timberlake, W. E. (1979). The RNA of tobacco etch virus contains poly (A). Virology 92, 568-571.

Hiebert, E., and McDonald, J. G. (1973). Characterization of some proteins associated with viruses in the potato Y-group. Virology 56, 349-361.

Hiebert, E., and McDonald, J. G. (1976). Capsid protein heterogeneity in turnip mosaic virus. Virology 70, 144-150.

Hill, J. H., and Benner, H. I. (1976). Properties of potyvirus RNAs: Turnip mosaic, tobacco etch, and maize dwarf mosaic viruses. Virology 75, 419-432.

Hinostroza-Orihuela, A. M. (1975). Some characteristics of infectious RNA from potato virus Y. Virology 67, 276-278.

Kamine, J., and Buchanan, J. M. (1977). Cell-free synthesis of two proteins unique to RNA of transforming virions of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA 74, 2011-215.

Kessler, S. (1975). Rapid isolation of antigens from cells with a staphylococcal protein A - antibody absorbent: Parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. 115, 1617-1624.

Knuhtsen, H., Hiebert, E., and Purcifull, D. E. (1974). Partial purification and some properties of tobacco etch virus induced intranuclear inclusions. Virology 61, 200-209.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. Nature (London) 227, 680-685.

Mans, R. J., and Novelli, G. D. (1961). Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch. Biochem. Biophys. 94, 48-53.

Matsui, C., and Yamaguchi, A. (1964). Electron microscopy of host cells infected with tobacco etch virus. I. Fine structures of leaf cells at later stages of infection. Virology 22, 40-47.

Mellena, J.-R., Benicourt, C., Haenni, A.-L., Noort, A., Pleij, C. W. A., and Bosch, L. (1979). Translational studies with turnip yellow mosaic virus RNAs isolated from major and minor virus particles. Virology 96, 38-46.

Mohier, E., Hirth, L., LeMeur, M.-A., and Gerlinger, P. (1975). Translation of alfalfa mosaic virus RNA's in mammalian cell-free systems. Virology 68, 349-359.

Palmiter, R. D., Gagon, J., Vogt, V. M., Ripley, S., and Eisenman, R. N. (1978). The NH_2-terminal sequence of the avian oncovirus *gag* precursor polyprotein ($Pr^{2}76$ gag). Virology 91, 423-433.

Pelham, H. R. B. (1978a). Leaky UAG termination codon in tobacco mosaic virus RNA. Nature 272, 469-471.

Pelham, H. R. B. (1978b). Translation of encephalomyocarditis virus RNA *in vitro* yields an active proteolytic processing enzyme. Eur. J. Biochem. 85, 457-462.

Pelham, H. R. B. (1979a). Synthesis and proteolytic processing of cowpea mosaic virus proteins in reticulocyte lysates. Virology 96, 463-477.

Pelham, H. R. B. (1979b). Translation of fragmented viral RNA *in vitro*. Initiation at multiple sites. FEBS Lett. 100, 195-198.

Pelham, H. R. B., and Jackson, R. J. (1976). An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. 67, 247-256.

Pleij, C. W. A., Mellema, J. R., Noort, A., and Bosch, L. (1977). The occurrence of the coat protein messenger RNA in the minor components of turnip yellow mosaic virus. FEBS Lett. 80, 19-22.

Pring, D. R., and Langenberg, W. G. (1972). Preparation and properties of maize dwarf mosaic virus ribonucleic acid. Phytopathology 62, 253-255.

Purcifull, D. E., Hiebert, E., and McDonald, J. G. (1973). Immunochemical specificity of cytoplasmic inclusions induced by viruses in the potato Y group. Virology 55, 275-279.

Purcifull, D. E., Zitter, T. A., and Hiebert, E. (1975). Morphology, host range, and serological relationships of pepper mottle virus. Phytopathology 65, 559-562.

Purcifull, D. E., and Batchelor, D. L. (1977). Immunodiffusion tests with sodium dodecyl sulfate (SDS) - treated plant viruses and plant viral inclusions. Fla. Agric. Expt. Stn. Tech. Bull. 788. 39 p.

Purcifull, D. E., and Hiebert, E. (1979). Serological distinction of watermelon mosaic virus isolates. Phytopathology 69, 112-116.

Ricciardi, R. P., Goodman, R. M., and Gottlieb, D. (1978). Translation of PVX RNA *in vitro* by wheat germ. I. Characterization of the reaction and product size. Virology 85, 310-314.

Rutgers, A. S. (1977). *In vitro* and *in vivo* translation of the RNAs of alfalfa mosaic virus. Ph.D. Thesis, University of Leiden. p. 147.

Salomon, R., Bar-Joseph, M., Soreq, H., Gozes, I., and Littauer, U. Z. (1978). Translation *in vitro* of carnation mottle virus RNA: Regulatory function of the 3'-region. Virology 90, 288-298.

Salvato, M. S., and Fraenkel-Conrat, H. (1977). Translation of tobacco necrosis virus and its satellite in a cell-free wheat germ system. Proc. Nat. Acad. Sci. USA 74, 2288-2292.

Schwinghamer, M. W., and Symons, R. H. (1977). Translation of the four major RNA species of cucumber mosaic virus in plant and animal cell-free systems and in toad oocytes. Virology 79, 88-108.

Shephard, J. F. (1968). Electron microscopy of subtilisin treated tobacco etch virus nuclear and cytoplasmic inclusions. Virology 36, 20-29.

Shepherd, R. J., and Purcifull, D. E. (1971). Tobacco Etch Virus C.M.I./ A.A.B. Description of Plant Viruses No. 55.

Shih, D., Lane, L. C., and Kaesberg, P. (1972). Origin of the small component of brome mosaic virus RNA. J. Mol. Biol. 64, 353-362.

Shih, D. S., and Kaesberg, P. (1973). Translation of brome mosaic viral ribonucleic acid in a cell-free system derived from wheat embryo. Proc. Nat. Acad. Sci. USA 70, 1799-1803.

Shih, D. S., and Kaesberg, P. (1976). Translation of RNAs of brome mosaic virus: The monocistronic nature of RNA_1 and RNA_2. J. Molec. Biol. 103, 77-88.

Shih, D. S., Shih, C. T., Kew, O., Pallansch, M., Rueckert, R., and Kaesberg, P. (1978). Cell-free synthesis and processing of the proteins of poliovirus. Proc. Nat. Acad. Sci. USA 75, 5807-5811.

Siegel, A., Hari, V., Montgomery, I., and Kolacz, K. (1977). A messenger RNA for capsid protein isolated from tobacco mosaic virus-infected tissue. Virology 73, 363-371.

Siegel, A., and Hari, V.. (1976). Comparison of the replication and translation of two non-divided genome plant viruses. Colloques Internationaux C. N. R. S. No. 261 - Acides Nucleiques et Synthese des Proteines chez les Vegetaux. p. 669-672.

Simons, J. N. (1976). Aphid transmission of a nonaphid-transmissible strain of tobacco etch virus. Phytopathology 66, 652-654.

Talbot, D. N., and Yphantis, D. A. (1971). Fluorescent monitoring of SDS gel electrophoresis. Anal. Biochem. 44, 246-253.

Van Tol, R. G. L., and Van Vloten-Doting, L. (1979). Translation of alfalfa-mosaic-virus RNA 1 in the mRNA-dependent translation system from rabbit reticulocyte lysates. Eur. J. Biochem. 93, 461-468.

Verduin, B. J. M. (1978). Characterization of cowpea chlorotic mottle virus and its assembly. Ph.D. Thesis, Agriculture University of Wageningen. p. 134.

Villa-Komaroff, L., McDowell, M., Baltimore, D., and Lodish, H. F. (1974). Translation of reovirus mRNA, poliovirus RNA, and bacteriophage QB RNA in cell-free extracts of mammalian cells. Methods in Enzymology 30, 709-723.

Wilcockson, J., and Hull, R. (1974). The rapid isolation of plant virus RNAs using sodium perchlorate. J. Gen. Virol. 23, 107-111.

BIOGRAPHICAL SKETCH

William George Dougherty was born in Washington, D.C., on March 10, 1952. In June, 1970, he graduated from Saint Joseph's Preparatory School located in Philadelphia, Pennsylvania. From September, 1970, to June, 1974, he attended Rutgers University in New Brunswick, New Jersey, from which he received his Bachelor of Arts degree with a major in biology. He attended the University of Florida from September, 1974, to June, 1976, as a member of the Department of Botany from which he earned his Master of Science. He has been a member of the Department of Microbiology and Cell Science since September, 1976.

He is a member of the American Society for Microbiology, The American Society for Cell Biology, the American Phytopathological Society, and the Mycological Society of America. In 1977, he was the recipient of an American Society for Microbiology Presidential Fellowship.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Henry C. Aldrich

Henry C. Aldrich, Chairman
Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ernest Hiebert

Ernest Hiebert, Co-Chairman
Associate Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dan Purcifull

Dan Purcifull
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John E. Edwardson

John E. Edwardson
Professor of Agronomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James F. Preston
Associate Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Francis Davis
Associate Professor of Microbiology and Cell Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1979

Jack T. Fry
Dean, College of Agriculture

Dean, Graduate School