ORGANIZATION AND *IN VITRO* TRANSLATION OF MAIZE CHLOROTIC MOTTLE VIRUS RNA

By

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PREFACE

During the course of this study, a curious infectious agent, which I reasoned to be a virus, appeared, completely displacing maize chlorotic mottle virus, the subject of this thesis. Since considerable amount of time and effort was expended in an attempt to identify and characterize this virus, and since any report of a new virus is important, the work is included in an appendix. I chose to call it "maize chamber virus" because it was discovered in the growth chamber, but as far as I can tell, it has never been reported in the field. I use quotation marks always when referring to the virus, since I have not established with absolute certainty that the particles I have identified are the infectious agent.

I would like to express my gratitude to the members of my committee, Dr. Ulrich Melcher, Dr. Richard Essenberg, Dr. Margaret Essenberg, Dr. John Sherwood, and especially my advisor, Dr. Charles O. Gardner, Jr. Thanks also to Dr. Betty Hamilton for her services and advice as regards electron microscopy, and to Dr. Robert Matts for many helpful discussions. I also wish to thank Biochemistry Department chairman Dr. Roger Koeppe for the research assistantship which supported me during my residency .

I especially want to acknowledge my mother, Doris Jourdan, my stepmother, June W. Thompson, and my late father, Mr. Arthur A. Hobbs, III. Without their moral and material support, this work would not have been possible. I wish to also extend my special thanks to Miss Aruna Prabhu, whose love and friendship helped sustain me during the latter part of this work.

My stay in the Biochemistry Department at Oklahoma State University has been a unique and rewarding experience. I will leave with many valuable lessons learned, and many good memories of the people with whom I was so fortunate to have been associated.

I wish to express my gratitude to Sue Heil and Denise Baer, for their assistance in preparing this manuscript, as well as with various clerical chores during my residency in Stillwater.

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ABBREVIATIONS

C	-	centigrade
CarMV	-	carnation mottle virus
CLN	-	corn lethal necrosis
cpm	-	counts per minute
DNase I	-	deoxyribonuclease I
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetraacetic acid
h	-	hours
Kd	-	kilodaltons
"MCV"	-	"maize chamber virus"
MCMV	-	maize chlorotic mottle virus
MDMV	-	maize dwarf mosaic virus
MDMV-B	-	maize dwarf mosaic virus, strain B
MI	-	mock inoculation
mA	-	milliampere
µCi	-	microcurie
µg	-	micrograms
min.	-	minute
µl	-	microliter
mRNA	-	messenger ribonucleic acid
Mol. Wt.	-	molecular weight
MOPS	-	(3-[N-Morpholino] propanesulfonic acid)
PAGE	-	polyacrylamide gel electrophoresis

RNase - ribonuclease

RNA - ribonucleic acid

rRNA - ribosomal ribonucleic acid

SG - subgenomic

SDS - sodium dodecylsulfate

TCA - trichloroacetic acid

Tris - Tris(hydroxymethyl)aminomethane

UF - unfractionated

VLP - virus-like particles

CHAPTER I

INTRODUCTION

Maize chlorotic mottle virus (MCMV), first described in Peru, is a relatively mild pathogen of corn (Castillo & Hebert, 1974). MCMV has been detected in the U.S. primarily in north central Kansas and south central Nebraska (Uyemoto *et al.*, 1980; Uyemoto, 1983). It is important because of its participation in corn lethal necrosis (CLN) a serious disease of corn with disastrous economic potential (Niblett & Claflin, 1978; Uyemoto & Claflin, 1981). CLN results from a synergistic interaction between MCMV and a co-infecting potyvirus, either wheat streak mosaic virus or maize dwarf mosaic virus (MDMV). To date, little has been published concerning the nature of the synergism at the molecular level, though Goldberg and Brakke (1987) found that concentrations of MCMV were up to 5.4 times higher when present in a co-infection with MDMV–B than when present alone. The concentration of MDMV did not seem to be affected by the presence of MCMV.

Understanding of the molecular basis for the synergism is hampered by limited knowledge concerning organization and expression of the RNA genomes of each of the viruses involved. Work in recent years suggests that most RNA plant viruses express their genetic material in accordance with the principle that eukaryotic ribosomes are capable of translating only the 5'-most cistron of a polycistronic mRNA, rendering internal cistrons silent (Kozak, 1978; Davies & Hull, 1982). Plant viruses adopt various strategies for the expression of these otherwise silent cistrons (Davies & Hull, 1982): (1) the RNA may be expressed as a polyprotein, which is subsequently cleaved into mature proteins; (2) the ribosomes may "read through" one or more stop codons, synthesizing more than

one protein from the same region of RNA; (3) the virus may generate subsets of its RNA (subgenomic RNA) which represent sequences not translated in the genomic RNA. Viruses may employ any one or combinations of the above strategies for the expression of their genetic material. In the case of MCMV, the only work published (Lommel, 1985) found that MCMV has a coat protein of 24.6 kilodaltons, an RNA of 1.47 X 10^6 daltons, and possible subgenomic RNAs, which may be encapsidated at low levels within the virion. Translation in rabbit reticulocyte lysates produced three major polypeptides, of 45, 28, and 25 kilodaltons.

Translational strategies of plant viruses have been studied extensively by the use of *in vitro* translation (Dougherty & Hiebert, 1985). The two most popular systems for this purpose are reticulocyte lysates and wheat germ extracts. Each system has features which make it preferrable under certain conditions to the other. Reticulocyte lysates typically have somewhat higher activity and are claimed by some to be more efficient in the translation of very long polypeptide chains (Shih & Kaesberg, 1973; Davies, 1976). Unfortunately, reticulocytes contain high levels of endogenous globin mRNA. This results in high background synthesis unless the globin mRNA is first destroyed by a nuclease. The nuclease must then by inactivated before the viral mRNA can be added (Jackson & Hunt, 1980). The wheat germ system was chosen for this study, since it contains very low levels of endogenous mRNA, making nuclease treatment unnecessary (Roberts & Paterson, 1973). It is also a plant-derived system, which was judged to be more appropriate for the analysis of a plant virus.

A large variety of biochemical techniques have evolved to detect and characterize plant viral subgenomic RNAs (Palukaitis, 1984). Sedimentation has been used to detect subgenomic RNAs from virions (Weber & Sehgal, 1982; Rutgers *et al.*, 1980). However, this technique alone can not relate the subgenomic RNAs by nucleotide sequence to the full-length, genomic RNA. Furthermore, subgenomic RNAs present at

very low concentration may not be detected in this manner. Finally, subgenomic RNAs which are not packaged into the virion are undetectable by sedimentation analysis.

Extensively used in recent years have been numerous variations on the original method of Alwine *et al.* (1977), using radiolabeled DNA probes to detect electrophoretically separated RNAs by molecular hybridization. Popularly referred to as "northern" hybridization, this technique has the twin advantages of high specificity and high sensitivity. Northern hybridizations have allowed the discovery of subgenomic RNAs which would not be possible using other, less sensitive methods (Palukaitis *et al.*, 1983; Sulzinski *et al.*, 1985). The present study employed both sedimentation and molecular hybridization in the detection and analysis of possible MCMV subgenomic RNAs.

The primary goal of this work was to identify the strategies employed by MCMV for the expression of its genetic material. A secondary goal was to determine functions for the various MCMV-specific proteins. In addition, it was of interest to deduce the location of the various cistrons from which each of the virus-specific proteins are translated, relative to each other, in the MCMV genome. With this knowledge in hand, one can further probe the molecular biology of the viral synergism underlying CLN.

CHAPTER II

MATERIALS AND METHODS

Growth and Inoculation of Plants

Maize cultivars N28ht and NB611 were obtained from Dr. Charles Gardner (Department of Agronomy, University of Nebraska, Lincoln, NE 68583). Seeds were germinated and plants maintained in vermiculite, grade two (W.R. Grace). Fertilization was by the addition of Osmocote (Sierra Chemical Company) to the growth medium. Plants were maintained, before inoculation, either on the benchtop under fluorescent lights or in the growth chamber. Benchtop conditions were room temperature, with a photoperiod of 13 1/2 h. Growth chamber conditions were a daytime temperature of 31°C, a nighttime temperature of 22°C. The photoperiod was 14 1/2 h. Plants were watered as needed with tap water. After inoculation, all plants were maintained in the growth chamber. When necessary to prevent cross-contamination, populations of plants were separated from each other by cardboard partitions and watered from separate, autoclaved flasks.

Plants were inoculated mechanically at the two to three leaf stage. Gloves were worn during inoculation to prevent cross contamination or RNase contamination when necesssary. Virus inoculations were made with crude sap from infected tissue (approximately 1 g) ground in a mortar with 10 ml distilled water. Two per cent (w/v) Celite (Johns-Manville) was used as an abrasive. Inoculations with RNA used solutions of 50 μg/ml (1.0 A_{260} = 50 μg RNA) in sterile distilled water. A small amount of Celite was added as an abrasive.

Purification of Virus

MCMV was purified from maize leaves two to three weeks after inoculation. Harvested tissue was minced with scissors and stored in 25 g portions in plastic bags at -70°C. The purification procedure used was modified from Niblett and Paulsen (1975). The tissue was blended in four volumes of cold 0.1 M NaH_2PO_4 (adjusted to pH 7.0 with NaOH), 1% ß-mercaptoethanol at 4°C for approximately one min. The blended material was filtered through cheesecloth, and mixed with an equal volume of cold chloroform:butanol (1:1, v/v). After stirring for 30 min at 4°C, the phases were separated by centrifugation at 5800 X *g* (6000 rpm in the Sorvall GSA rotor) for 10 min at 4°C. The upper aqueous phase was recovered by aspiration and filtered through miracloth into another flask. Clarified sap was then centrifuged at 70,000 X *g* (30,000 rpm in the Beckman Ti 45 rotor) for 4 h at 4°C. The pellets were drained, each covered with 1.5 ml NPM (0.02 M NaH_2PO_4, 0.01 M $MgCl_2$, adjusted to pH 7.0 with NaOH) and resuspended with the help of a rubber policeman. Resuspended pellets were combined into one 15 ml Corex tube and centrifuged at 12,000 X *g* (10,000 rpm in the Sorvall SS-34 rotor) for 10 min. The pellet was discarded and the supernatant subjected to another round of differential centrifugation as described above. The final pellet (except where noted) was layered onto 10-40% sucrose gradients made up in NPM. Gradients were centrifuged at 53,000 X *g* (22,500 rpm in the Beckman SW 25.1 rotor) for 6 h at 4°C. Fractionation was performed with the ISCO model 640 gradient fractionator. Peaks absorbing at 254 nm were pooled, diluted 1:1 with NPM, and pelleted at 130,000 X *g* (45,000 rpm in the Beckman Ti 75 rotor) at 4°C for 2 h. Pellets were covered with 1.0 ml each of NPM and resuspended overnight at 4°C. Resuspended pellets were pooled, and purified virus pelleted at 130,000 X *g* as before. These second pellets were resuspended in 1.0 ml sterile 0.02 M NaH_2PO_4 adjusted to pH 7.0 with NaOH.

Treatment of Glassware

All glassware, mortars, pestles, stir bars, spatulas and any other non-disposable vessel or tool coming in direct contact with RNA were made nuclease-free by treating with a 1% (v/v) solution of diethylpyrocarbonate (depc). Treatment was for 1 to 3 min and followed by autoclaving and baking at 65°C overnight before use. All nuclease-free materials were handled with gloves. Materials used in the purification of virus were not treated in this manner.

Extraction of Viral RNA

Virus was disrupted in the presence of sodium dodecyl sulfate (SDS) (100 mM NaCl, 10 mM Tris•HCl, 3 mM EDTA, 2% SDS, pH 7.8). After stirring at room temperature for 15 min, disrupted virions were extracted 2X with an equal volume of phenol:chloroform (1:1). The second upper, aqueous phase was precipitated with two volumes of ethanol. RNA precipitates were recovered by centrifugation at 10,000 X *g* (12,500 rpm in the Sorvall SS-34 rotor) for 30 min at 4°C. Pellets were resuspended in sterile, distilled water to a concentration of 1.0 mg/ml. RNA solutions were stored in 1.5 ml eppendorf tubes at -70°C indefinitely.

Electrophoresis

For proteins, polyacrylamide gel electrophoresis (PAGE) was performed essentially by the method of Laemmli (1970). Samples were loaded in SDS dissolving buffer (0.0625 M Tris, 5% SDS, 5M urea, 10% glycerol, 0.02% bromphenol blue, 5% mercaptoethanol pH 6.8). Molecular weight standards were purchased from Bio-Rad. They were (mol. wt. given in daltons): lysozyme (14.4 K), soybean trypsin inhibitor (21.5 K), carbonic anhydrase (31 K) , ovalbumin (45 K), bovine serum albumin (66.2 K), and phosphorylase B (92.5 K). These standards were prepared according to the manufacturer's instructions. Fluorography was performed by the method of Bonner and

Laskey (1974). Mol. Wts. were estimated form a plot of known molecular weights versus relative distance migrated.

The method for electrophoresis of RNA was modified from Seed (1982). 1% agarose gels were made in RNA gel buffer (0.02 M MOPS, 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, pH 7.0) and poured to a thickness of 2 mm onto gel bond (FMC) according to the manufacturer's instructions. RNA samples were prepared by mixing 4.44 µl RNA , 5.56 µl RNA gel buffer, and 10 µl deionized formamide in an Eppendorf tube, heating to 60°C for 10 minutes and immediately quenching on ice. After a few minutes, 5 µl of loading dye (5% v/v glycerol, 0.4% v/v bromphenol blue, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) was added and the samples loaded (15 µl/well). Electrophoresis was carried out at 25-50 mA, at room temperature. Gel buffer was recirculated by a peristaltic pump.

RNA was detected in agarose gels by modification of previous protocols for silver staining (Willoughby & Lambert, 1983; Peats, 1983; L. Lane, personal communication). Immediately after electrophoresis, the gel was covered in Fix 1 (50% methanol, 12% acetic acid, 2.2% (w/v) glycerol) for 30 min. The gel was then removed, dried partially by blotting for 10 min, then to completion by blow drying for 8-10 min. Dried gels were covered in Fix 2 (0.15 M sulfosalicylic acid, 0.3 M trichloroacetic acid (TCA), 0.3 M zinc sulfate) for 10 min, followed by washing with two changes of distilled water for 30 min. Gels were then covered in 95% ethanol for 15 min, washed with water again for 5 min, and dried briefly by patting gently with a kimwipe. Staining was performed by mixing two volumes solution B (1.0 g NH_4NO_3, 1.0 g $AgNO_3$, 5.0 g tungstosilicic acid, 7.0 ml 37% formaldehyde per 500 ml solution) with one volume solution A (25 g anhydrous Na_2CO_3 per 500 ml solution) and the mixture added to the dried gels with gentle agitation. Staining was stopped with 1% acetic acid. Mol. wt. standards were tobacco mosaic virus RNA (mol. wt. 2 X 10^6 daltons) and brome mosaic virus RNAs (mol. wt. 1.1 X 10^6, 1.0 X 10^6, 0.7 X 10^6, and 0.3 X 10^6 daltons). Molecular weights were determined by

plotting the square root of molecular weight versus the log of mobility (Lehrach *et al.*, 1977).

Sucrose Density Gradients of RNA

RNA was sedimented through sucrose gradients by the method of Morris-Krsinich and Forster (1983). Samples were prepared by pelleting ethanol precipitates of RNA as described, and resuspending the pellets in RNA SDG buffer (0.1 M LiCl, 5 mM EDTA, 10 mM Tris, 0.2 % SDS, 50% deionized formamide, pH 8.0), heating at 60°C and quenching on ice.

In Vitro Translations

Cell-free translations were carried out in wheat germ extracts prepared by the method of Marcu and Dudock (1974). Raw wheat germ (grade one) was the kind gift of Pillsbury, Inc., Enid, OK. Translations were performed in sterile, disposable tubes at 20 to 25°C for 90 min under conditions previously optimized for MCMV (Gardner, unpublished). Added constituents of the translation and their final concentrations are as follows: 0.01 M disodium creatine phosphate, 0.03 M Tris-acetate (pH 8.0), 1.34 mM ATP, 1.66 U creatine kinase, 0.05 M potassium acetate, 0.45 mM each L-amino acid (except leucine and lysine), 2 mM dithiothreitol, 0.1 mM spermidine, 0.002 mM GTP, 0.6 mM magnesium acetate, 0.06 mM L-lysine, 5 µCi (4.2 X 10^{-6} µM) L-[4,5-^{3}H(N)] - leucine, and 10 µg RNA. Total volume was typically 100 µl, of which 30 µl was wheat germ extract. Reactions were assayed by scintillation counting of ^{3}H-leucine incorporation into TCA-insoluble material.

Isolation of RNA From Tissue

Total RNA was extracted from maize tissue by the method of Otal and Hari (1983). Polysomal RNA from infected or uninfected tissue was isolated as described by Palukaitis

(1984). Maize ribosomal RNA was isolated essentially by the procedure of Jackson and Larkins (1976), except that the sucrose density gradient step was omitted. Ribosomal RNA was recovered from low-speed polysomal pellets by the extraction and ethanol precipitation methods described above for the extraction of RNA from MCMV.

Immunoprecipitation

The immunoprecipitation protocol was the method of Dougherty and Hiebert (1980), except that the precipitation buffer was NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4), containing 0.05% NP-40, 1 mg/ml bovine serum albumin, and 2 mM leucine. MCMV antiserum was originally raised in rabbits by J. Uyemoto to the Kansas isolate of MCMV and was the kind gift of Dr. Steve Lommel.

Complementary DNA Probes

Single-stranded probes of complementary DNA were synthesized *in vitro* by randomly-primed reverse transcription. The template was gradient-purified MCMV genomic RNA. Primers were prepared by digesting sonicated calf thymus DNA with DNase I (Taylor *et al.*, 1976). The resulting oligonucleotides were incubated with genomic RNA (10 µg primers/10 µg RNA/ reverse transcription) at 90°C for 10 min, shifted to 42°C for 15 to 20 min, then allowed to cool on the benchtop until reaching room temperature. Reactions were assembled and incubated at 42°C for 3 h exactly as described by Maniatis *et al.* (1982). Reactions contained 25 to 50 µCi [α-^{32}P] - dCTP each and were assayed by scintillation counts of ^{32}P incorporated into TCA-insoluble material as compared to identical background tubes which contained no reverse transcriptase. RNA templates were destroyed by base hydrolysis, after which the reaction was neutralized (Maniatis *et al.*, 1982). Whole reaction volumes were added to a minimal volume of hybridization buffer (5.0 ml for bags, 10.0 ml for the chamber, see below for clarification) for denaturation and hybridization. 100,000 to 500,000 cpm were used per gel or blot.

Northern Hybridizations

Two methods were used to perform northern hybridizations. One was by blotting electrophoretically separated RNA onto nitrocellulose, which then served as a medium for hybridization carried out in sealed plastic bags at 42°C. The other was to hybridize to RNA directly in dried-down agarose gels. For the blotting method, the protocol of Thomas (1980) was followed exactly. The direct in-gel hybridization was derived from Tsao *et al.* (1981). In this procedure, agarose gel electrophoresis of RNA was performed as described above. After the gel was dried to completion, the lane containing RNA molecular weight markers was cut out with a paper cutter and stained by the silver stain procedure. The rest of the gel was pre-hybridized and hybridized exactly as if it were nitrocellulose (Thomas, 1980) with two exceptions. First, the 80°C baking step was omitted. Second, the pre-hybridization and hybridization steps were carried out in a small lexan chamber instead of in sealed plastic bags. To maximize contact between the buffer and the gel, the gel bond, with dried gel side facing up, was adhered to the bottom of the chamber with a thin coat of vaseline. The gel was then covered in buffer and the chamber was placed at 42°C. After hybridization, the gel was washed just as with nitrocellulose. After washing, it was necessary to redry the gel with a blow dryer. Either the gel or the nitrocellulose were exposed to X-Omat film (Kodak) at -70°C with an intensifying screen for periods ranging from 15 min to 5 h. The probe in either case was complementary DNA made as described above.

Electron Microscopy

Samples were prepared by adhering solutions of virus (1 to 10 µg/ ml) to formvar-coated grids. Viruses were stained by floating grids on a 2% solution of uranyl acetate. Microscopy was performed on the JEOL 100 cx electron microscope.

Phosphodiesterase Digestions

When genomic RNA was digested with snake venom phosphodiesterase, the RNA was first treated with calf intestinal phosphatase (0.1 unit /10 μg RNA in the reaction) as described by Maniatis *et al.* (1982) for bacterial alkaline phosphatase. This was done to remove any potential 3' phosphates, since the venom phosphodiesterase requires a free 3' hydroxyl as a substrate. Phosphatase digests were extracted twice with phenol-chloroform (1:1). The second aqueous phase was ethanol precipitated and stored at -20°C. RNA precipitates were recovered by microfuging for 10 minutes. Dried pellets were resuspended in water to a concentration of 1 mg/ml. For this purpose recovery of RNA was assumed to be 100% of the original amount in the reaction.

Phosphatase-treated RNA was digested with snake venom phosphodiesterase by a method derived from Ho and Gilham (1973). RNA was mixed with an equal volume of venom phosphdiesterase buffer (0.2M Tris•Cl, 0.04 M magnesium acetate, pH 8.0) on ice. Snake venom phosphodiesterase (0.05 units / 100 μg RNA) was then added. Reactions were incubated on ice. Aliquots were removed at desired times, and the reactions stopped by phenol-chloroform extraction. The RNA was recovered by ethanol precipitation.

When genomic RNA was digested with calf spleen phosphodiesterase, it was, except where noted, first digested with proteinase K to remove a proposed genome-linked protein, covalently attached to the 5' end (Lommel, personal communication). RNA (1 mg/ml) was made to 100 mM NaCl, 10 mM Tris•Cl, 3 mM EDTA, 2% SDS, pH 7.8. Proteinase K (1 μg/μg RNA)was added, the reaction incubated at 65°C for 10 minutes, and chilled on ice. RNA was recovered by phenol-chloroform extraction and ethanol precipitation.

Proteinase K-treated RNA was digested with calf spleen phosphodiesterase by essentially the same method used for the venom enzyme, except that the buffer was 0.3 M ammonium acetate, 0.05 M EDTA, pH 6.5 (Ho and Gilham, 1973). 0.066 units

enzyme/100 μg RNA were used. Incubation, reaction stoppage, and RNA recovery were as described for the snake venom enzyme.

CHAPTER III

RESULTS AND DISCUSSION

Organization of the MCMV Genome

MCMV was purified from symptomatic corn leaves as described in Chapter II. MCMV is reported to sediment as a single species (Niblett & Claflin, 1978; Niblett *et al.*, 1977). That is also the case in this study (Fig. 1). However, RNA extracted from MCMV sediments as two species on sucrose density gradients [Fig. 2(a)]. These species were separated and further purified on subsequent gradients [Fig. 2 (b) and (c)]. The RNAs were analyzed by agarose gel electrophoresis under denaturing conditions as shown in Figure 3. The molecular weight of the major RNA band is estimated to be 1.5 X 10^6 daltons. The material from the upper peak in sucrose gradients was not resolved by this method, but consists of a smear ranging in molecular weight from about 1.0 X 10^6 to 0.3 X 10^6 daltons. Somewhat better resolution was seen by northern hybridization using the blotting method described in Chapter II. Figure 4 shows such a blot. Lane 2 contains the upper peak RNA from sucrose gradients. Dark areas in the smear corresponding to molecular weights of approximately 1.0, 0.7, and 0.5 X 10^6 are visible, but smearing (presumably degraded MCMV RNA) obscured them. Discrete RNAs were best detected by direct in-gel northern hybridization to polysomal RNAs from infected plants (see Fig. 5, lane 4). This approach yielded bands at 1.5, 1.07, 0.73. and 0.52 X 10^6 daltons. Since these RNAs are actually on ribosomes (so presumably are true messenger RNAs), since they are detected with an MCMV-specific probe, and appear only in MCMV-infected tissue (compare lanes 4 and 5), it is concluded that the three smaller species found in the

Figure 1. Sedimentation Profile of MCMV in 10 to 40% Sucrose Density Gradients.

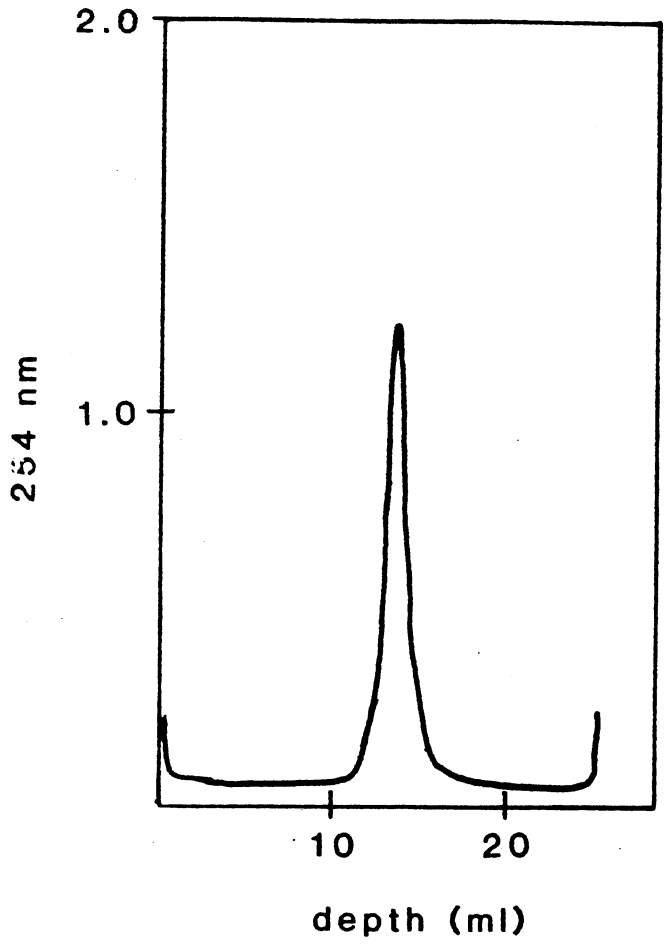
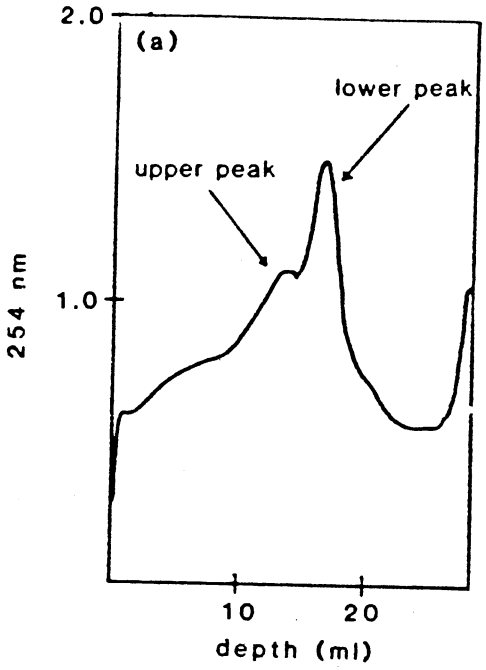
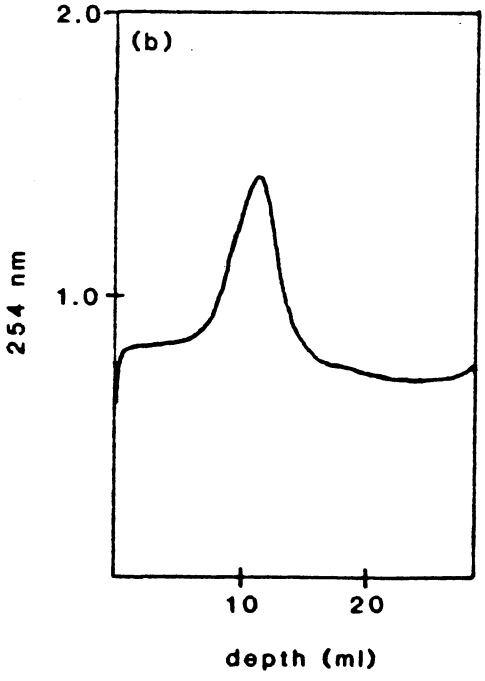

2.0
1.0
254 nm
10
20
depth (ml)

Figure 2. Sedimentation Profile of MCMV RNA in 0.2 to 0.8 M Sucrose Density Gradients.

RNA from virions is shown in (a). "Upper" and "lower" peaks are indicated. Fractions containing each peak were pooled seperately, and RNA recovered was subjected to a second, identical gradient. Second gradient profiles for upper peak RNA (b) and lower peak RNA (c) are shown.


2.0
(a)
lower peak
upper peak
254 nm
1.0
10
20
depth (ml)


2.0
(b)
254 nm
1.0
10
20
depth (ml)

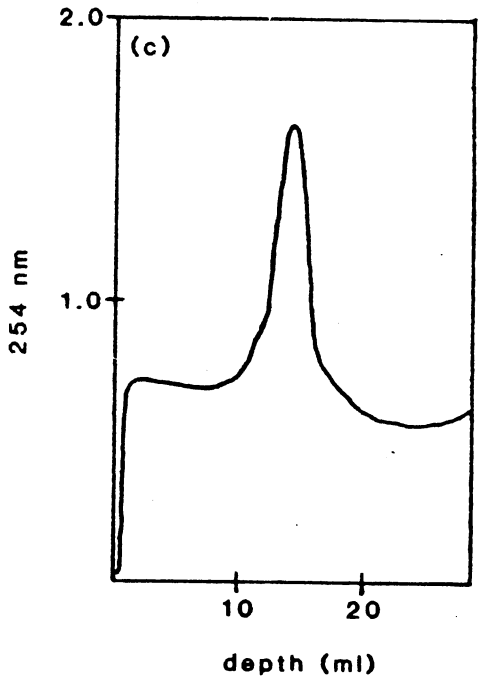

2.0
(c)
254 nm
1.0
10
20
depth (ml)

Figure 3. Agarose Gel Electrophoresis of MCMV RNA.

Lane 1 contains molecular weight standards as described in Chapter II. Lane 2, unfractionated MCMV RNA. Lane 3, upper peak MCMV RNA from a second sucrose gradient [see Fig. 2(b)]. Lane 4, Lower peak RNA from a second sucrose gradient [see Fig. 2 (c)].

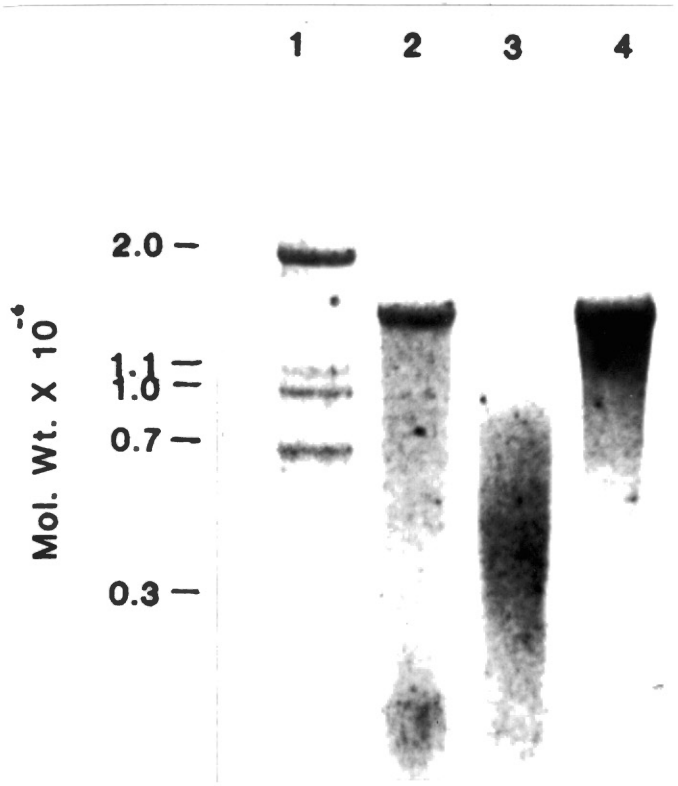

1
2
3
4
2.0
1.1
1.0
0.7
0.3
Mol. Wt. X 10⁻⁶

Figure 4. Northern Hybridization of MCMV RNA.

Lane 1, unfractionated RNA. Lane 2, upper peak (subgenomic) RNA. Lane 3, lower peak (genomic) RNA. Obscure bands are seen in the subgenomic smear at approximately 1.0, 0.7, and 0.5 x 10^6 daltons. The probe was cDNA made using twice gradient-purified MCMV genomic RNA as the template.

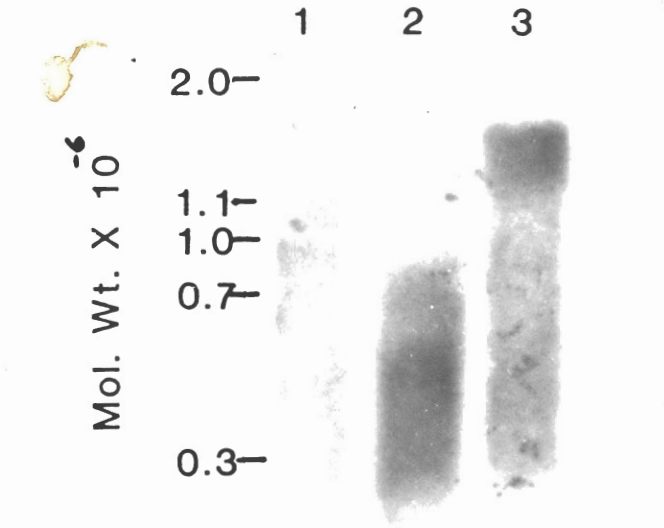
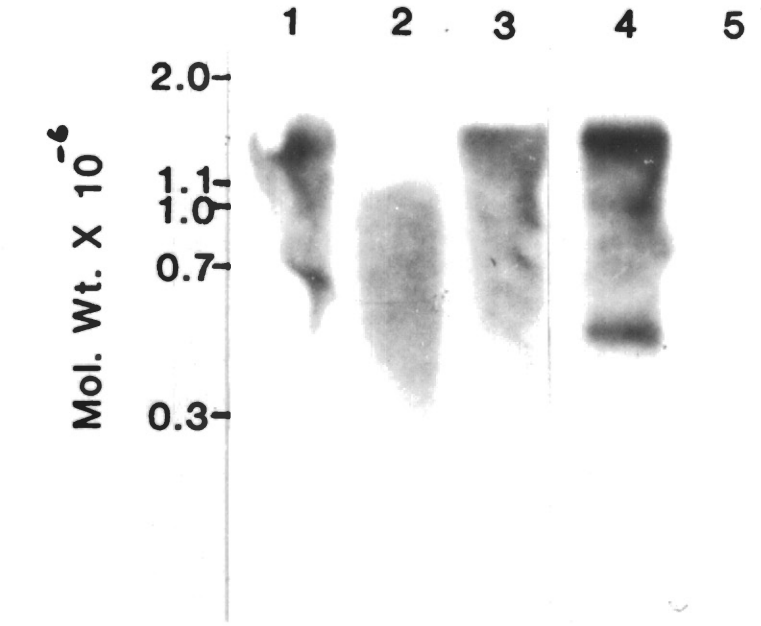

1
2
3
2.0
1.1
1.0
0.7
0.3
Mol. Wt. X 10⁻⁶

Figure 5. In-Gel Hybridization of MCMV RNA and Polysomal RNA.

Lane 1, unfractionated virion RNA. Lane 2, subgenomic RNA fraction. Lane 3, genomic RNA fraction. Lane 4, polysomal RNA from plants infected with MCMV. Lane 5, polysomal RNA from uninfected plants.


1
2
3
4
5
2.0
1.1
1.0
0.7
0.3
Mol. Wt. X 10⁻⁶

upper peak are true subgenomic RNAs and that the 1.5 X 10^6 dalton species found in the lower peak is the genomic RNA.

That subgenomic RNAs purified from virions seem to have somewhat discrete bands in northern hybridization experiments corresponding to molecular weights of RNAs detected on polysomes suggests that all the species of subgenomic RNA can be encapsidated. However, they probably do not all exist in every particle, since these bands could not be reliably detected in hybridizations to unfractionated RNA. This implies a low concentration of these species. RNA sedimentation data seems to argue that the subgenomic RNAs should be detectable in silver-stained agarose gels, since the relative area under the subgenomic peak is roughly half the area under the genomic peak (Fig. 2). However, when one considers that each lane in an agarose gel contains approximately 2.66 μg RNA, then there would be only 0.293 μg of each subgenomic RNA per lane, assuming each of the three subgenomics identified by other means are present in equimolar concentrations. It is reasonable to assume that these levels would not be detectable above the background of degraded MCMV RNA in agarose gels. If all subgenomic RNAs were present in every particle, one would expect that each would be in equal molar concentration with the genomic RNA, and hence be easily detectable.

Infectivity of MCMV RNA

Table I summarizes experiments on infectivity of MCMV RNA. MCMV symptoms were seen only when unfractionated RNA (UF) was used as inoculum. Since virus infection can sometimes be symptomless, two other assays for infectivity were used. Tissue inoculated with either unfractionated RNA, gradient-purified subgenomic RNA (UP), or water (MI for "mock inoculated") was subjected to an MCMV purification. This material was either observed in the electron microscope in an attempt to detect virus-like particles (VLPs) or extracted in an attempt to recover RNA co-migrating with MCMV RNA. Figure 6 shows electron micrographs comparing VLPs from RNA-inoculated

TABLE I

RNA INFECTIVITY

Inoculum	Symptoms	VLPs?	RNA?
UP	0/92	no	no
UF	90/233	yes	yes
MI	0/108	no	no

Numbers represent symptomatic/inoculated plants.
Abbreviations used: UF, unfractionated; MI, mock-inoculated; UP, upper peak.

tissue and virus-inoculated tissue. The particles appear to be of identical size and morphology, and closely resemble published reports of electron micrographs of MCMV (Gordon *et al.*, 1984). VLPs from RNA-inoculated tissue were seen only when the inoculum was unfractionated RNA. Likewise, RNA co-migrating with MCMV RNA was recovered only from tissue inoculated with unfractionated RNA (Fig. 7). Subgenomic RNA was never infectious by any assay. Since the unfractionated RNA contains the genomic RNA, but the subgenomic fraction lacks it (Fig. 3, lanes 1 and 2), the RNA infectivity results suggest that the genomic RNA is required for viral replication or possibly for cell-to-cell movement of the virus. To further establish this, gradient-purified genomic RNA was inoculated onto maize plants. Though only 2 of 20 plants so inoculated developed symptoms, this suggests that the genomic RNA alone is infectious, and the inability of subgenomic RNA to infect the plant when it is used as inoculum is not an artifact of RNA fractionation.

In Vitro Translation of MCMV RNA

Figure 8 shows PAGE of proteins produced by wheat germ extracts programmed with MCMV RNA. The major species produced are of the following molecular weights (in daltons): 45,000, 36,000, 29,000, 18,000, and 16,000. The band apparent at approximately 8 Kd in Figure 8 was not detected consistently, so was not considered a major product. Likewise, double bands in the 45 Kd and 29 Kd regions were evident in some gels, but not all, and were thus not considered as separate polypeptides among the major products. It is interesting to note that gradient-purified subgenomic RNA directs the synthesis of all but the 36Kd and 45Kd proteins (Fig. 8, compare lanes 1 and 2). Since this is the only difference in translational activity between infectious and uninfectious RNA, the data imply that these proteins may have the above-mentioned role in replication or cell-to-cell movement of the virus. It is also interesting that gradient-purified genomic RNA seems to have the capacity to direct the synthesis of all the proteins made by MCMV

Figure 6. Electron Micrographs of Virus Recovered from MCMV-Inoculated Plants (a) or from Plants Inoculated with Unfractionated RNA (b).

In each case, the particles measure approximately 31 nm in diameter. Magnification is 131,200 x.

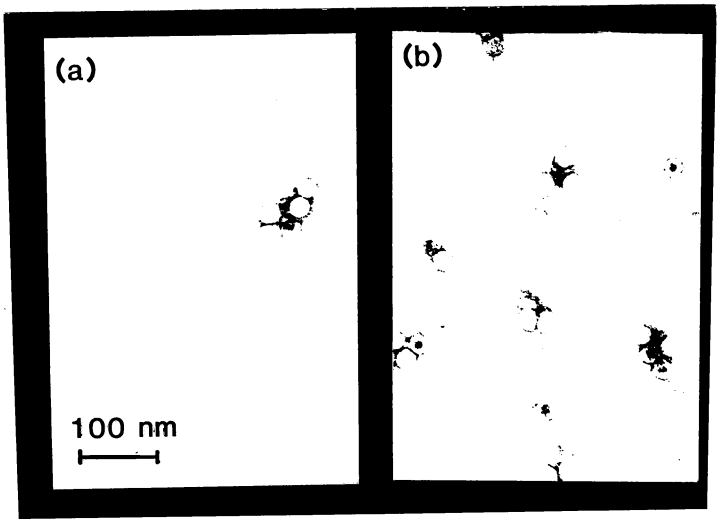

(a)
(b)
100 nm

Figure 7. Agarose Gel Electrophoresis of RNA Recovered from Tissue Inoculated with RNA Either Including or Excluding the Lower Peak RNA.

Lane 1, molecular weight standards. Lane 2, MCMV RNA recovered from virus-inoculated plants. Lane 3, RNA recovered from plants inoculated with unfractionated RNA. Lane 4, RNA recovered from plants inoculated with only upper peak RNA. Lane 5, RNA recovered from mock-inoculated plants.

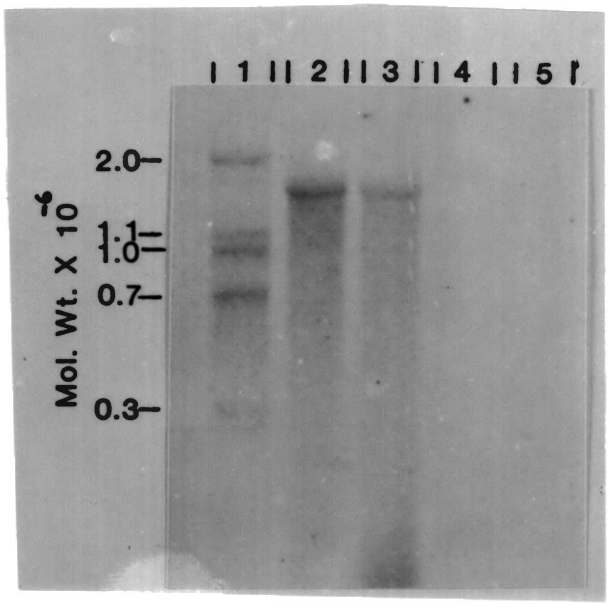
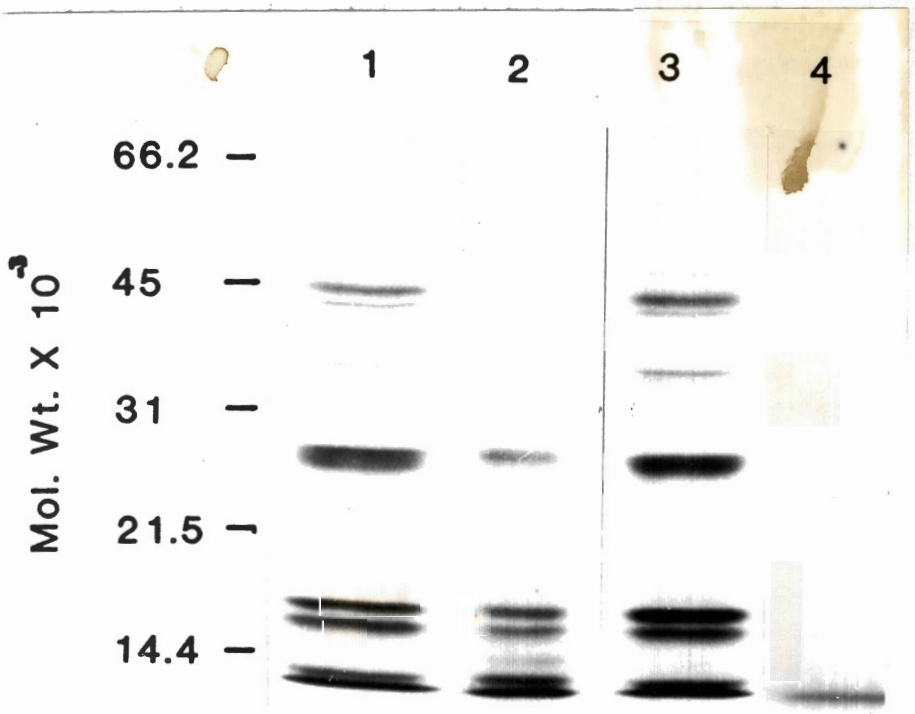

1 2 3 4 5
Mol. Wt. X 10⁻⁶
2.0
1.1
1.0
0.7
0.3

Figure 8. Fluorograph of PAGE of Proteins Produced by Wheat Germ Extract Programmed with MCMV RNA.

Lane 1, translation programmed with unfractionated RNA. Lane 2, translation programmed with subgenomic RNA. Lane 3, translation programmed with genomic RNA. Lane 4, unprogrammed wheat germ. Loading volumes were adjusted when necessary to give approximately equal amounts of radioactivity (scintillation counts) in each lane.


1
2
3
4
66.2
45
31
21.5
14.4
Mol. Wt. X 10³

in vitro (Fig. 8, compare lanes 1 and 3). Whether this is due to the generation of subgenomic RNAs during the course of *in vitro* translation or the ability of ribosomes to read cistrons throughout the length of the genomic RNA is unknown.

One virally encoded protein whose function could be clearly assigned was the viral coat protein. Figure 9 shows PAGE of coomassie blue-stained, disrupted virus particles vs. *in vitro* translation products which were immunoprecipitated with MCMV-specific antiserum (lanes 2 and 3). Disrupted virus particles produce only one protein, at 29,000 daltons. This was unaltered by increasing boiling times up to fifteen minutes. The major species which reacts with MCMV-specific antiserum is also at 29 kilodaltons. A minor, 25 Kd protein also is precipitated by MCMV antiserum. Since no protein of this size was ever recovered from purified, disrupted virions, it is concluded that the MCMV coat protein has a molecular weight of approximately 29,000 daltons. It is baffling that the value reported here for the coat protein (29 Kd) differs from the previously reported value of 24.6 Kd (Lommel, 1985). It is possible that the 25 Kd protein is either a proteolytic breakdown product of the 29 Kd coat protein, or is a truncated translation product of the same cistron.

Translation products directed by MCMV RNA in wheat germ extracts agree roughly with those reported to be synthesized in the rabbit reticulocyte lysate (Lommel, 1985). However, experiments with the wheat germ system yield a 36 Kd protein, as well as proteins of 18 Kd and 16 Kd, which were not reported in the reticulocyte system. An experiment directed at resolving the nature of the 36 Kd protein suggests that it may be an artifact, since polysomal RNA from infected tissue seems to be incapable of directing its synthesis by wheat germ extracts (Fig. 10, lane 3). This situation is complicated, however, by the apparent ability of total RNA from infected plants to direct synthesis of the 36 Kd protein. (Fig. 10, lane 5). The 18 Kd and 16 Kd proteins are always major products. They are listed as such but what function, if any, they may have *in vivo* is unknown. It is possible that they are products of the same cistron. They are also made

Figure 9. Identification of MCMV Coat Protein.

Lane 1, PAGE fluorograph of the *in vitro* translation products seen in MCMV- programmed wheat germ. Lane 2, coomassie blue-stained disrupted virus particles. Lane 3, products of MCMV-programmed *in vitro* translation which were precipitated by MCMV antiserum. Lane 4, same as lane 3, except the tube contained no MCMV antiserum.

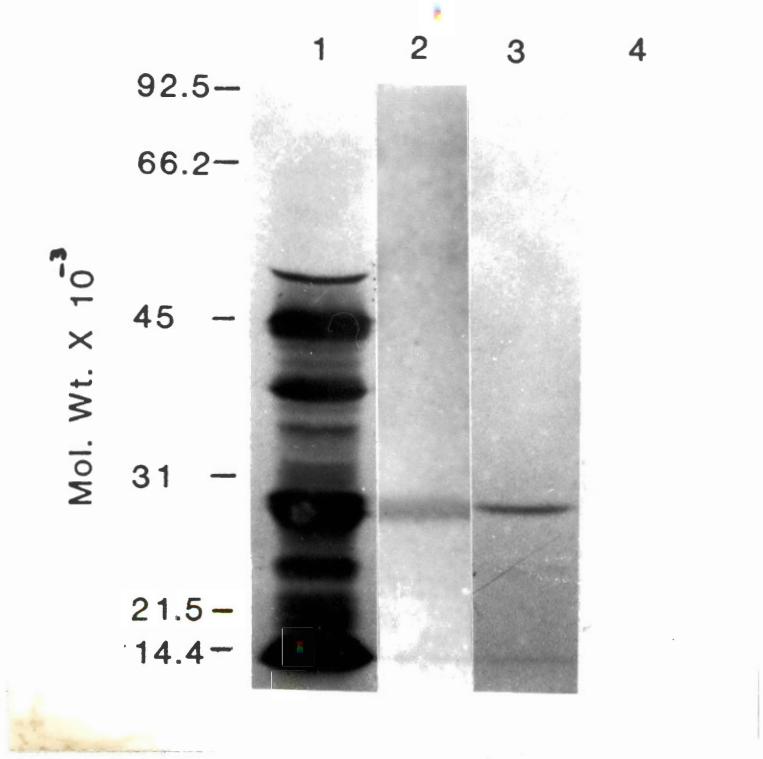
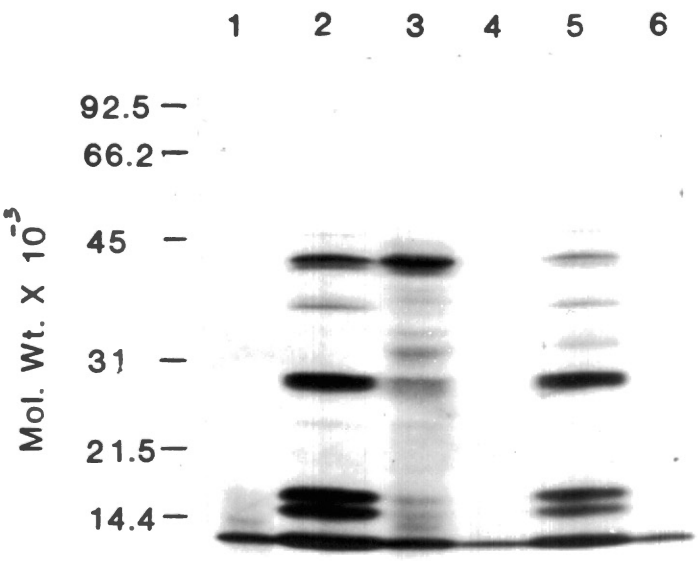

1
2
3
4
92.5
66.2
45
31
21.5
14.4
Mol. Wt. X 10⁻³

Figure 10. PAGE Fluorograph of *In Vitro* Translation Products.

Wheat germ was programmed with: lane 1, water; lane 2, unfractionated MCMV RNA; lane 3, polysomal RNA from MCMV-infected plants; lane 4, polysomal RNA from uninfected plants; lane 5, total RNA from MCMV-infected plants; lane 6, total RNA from uninfected plants.


1
2
3
4
5
6
92.5
66.2
45
31
21.5
14.4
Mol. Wt. X 10^{-3}

by wheat germ programmed with polysomal RNA from infected tissue (Fig. 10, lane 3). Presumably they are made *in vivo,* unless wheat germ ribosomes behave differently from maize ribosomes, which would be surprising.

One could argue that the 16 Kd and 18 Kd are unfinished polypeptides or proteolytic breakdown products of other MCMV proteins. However, they are synthesized by the gradient-purified subgenomic RNA, which apparently lacks the 45 Kd and 36 Kd cistrons. They also are not precipitated by MCMV-specific antiserum. This argues that the 16 Kd and 18 Kd proteins are not subsets of larger proteins synthesized *in vitro,* since they are made in the absence of two of the three larger proteins which could serve as precursors and have no detectable serological relationship with the other.

One explanation for the differences in MCMV translation products between those presented here and the earlier report is the difference in translation systems. Lütcke *et al.* (1987) have demonstrated that the rabbit reticulocyte system shows a hierarchy of translational efficiencies, depending on the nucleotide at the -3 position relative to the AUG initiation codon. They did not find such a hierarchy in the wheat germ system.Since wheat is a host for MCMV (Gordon *et al.,* 1984), translation of MCMV RNA in wheat germ extracts constitutes a homologus system. Thus, it is possible that the 16 Kd and/or 18 Kd would be made *in vivo* but not in an animal-derived *in vitro* system due to low efficiency of the reticulocyte ribosomes to initiate protein synthesis properly.

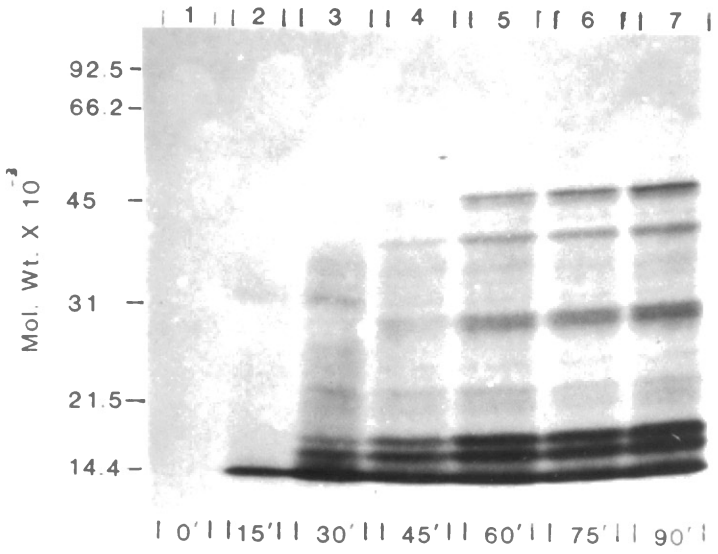
The fidelity of the wheat germ system is also supported by the previous observation that virus-specific proteins of 47, 45, 37, 30, 24, and 20 Kd were synthesized in maize protoplasts infected with MCMV. In addition, a 29 Kd protein was isolated from infected maize leaves and was precipitated with MCMV antiserum, suggesting that it is the MCMV coat protein (Subramanian, 1984).

Mapping the MCMV Genome

Several experiments were conducted in an attempt to discern the order of cistrons on the genomic RNA. One approach to this problem was to take advantage of the natural 5' to 3' progression of ribosomes along an mRNA molecule during translation. If translation of the genomic RNA is stopped at various times, the proteins coded for by cistrons closest to the 5'-end cistrons should accumulate first, while those derived from 3'-end cistrons should accumulate last. Figure 11 shows a PAGE fluorograph of such a time-course translation. Interestingly, the first labeled product appeared at 15 minutes, and had a molecular weight of approximately 33 Kd, which was not a major polypeptide when translation was allowed to proceed for the standard period (90 minutes). By 30 minutes, the 16 Kd, 18 Kd, and 25 Kd proteins were present, and the 33 Kd species was more prominent. The 36 and 45 Kd proteins were still missing, however. At 45 minutes, the 36 and 45 Kd proteins finally appeared. Also interesting is that the 33 Kd protein was abruptly replaced by the 29 Kd coat protein. Since no other, smaller bands increased in intensity with the apparent loss of the 33 Kd protein, this suggests a relationship between the 33 Kd protein and the 29 Kd coat protein. The most obvious explanation of this relationship is that the 33 Kd protein is a precursor of the coat protein, which is proteolytically processed to the mature coat protein. By 60 minutes the standard MCMV translation profile was present. The early accumulation of an apparent coat protein precursor suggests that the coat protein cistron maps closest to the 5' end of the genomic RNA. Accordingly, the late appearance of the 36 and 45 Kd proteins suggests that they are coded for by a cistron(s) at the 3' end of the genomic RNA. The 18 and 16 Kd protein cistrons map between the 45 Kd and coat protein cistrons. It seems unlikely that the largest proteins accumulate last simply because it takes ribosomes longer to pass through larger cistrons. If this were true, the 33 Kd protein would appear after the 18 and 16 Kd, since it is translated from a larger cistron. This was evidently not the case.

Figure 11. PAGE Fluorograph of Time-Course *In Vitro* Translation Programmed with Gradient-Purified Genomic RNA.

Aliquots were removed at the times indicated below and translation stopped by mixing with an equal volume of SDS dissolving buffer.


1
2
3
4
5
6
7
92.5
66.2
45
31
21.5
14.4
Mol. Wt. X 10⁻³
0'
15'
30'
45'
60'
75'
90'

A second approach to mapping the MCMV genomic RNA was to digest gradient-purified genomic RNA with snake venom phosphodiesterase (E.C. 3.1.9.1). This enzyme is a 3' exonuclease. In other words, it sequentially removes nucleotides from the 3' end of a polynucleotide. The strategy for this approach to mapping was to digest genomic RNA for various periods of time, thereby eliminating larger and larger segments of RNA from the 3' end. Then each aliquot was used to program translation in wheat germ extracts. The earlier in digestion that the RNA lost the capacity to direct the synthesis of a given protein, the closer the cistron for that protein was assumed to be to the 3' end of the genomic RNA. Figure 12 shows agarose gel electrophoresis of RNA digested with snake venom phosphodiesterase for various times as indicated. Digestion resulted in smears with the slowest-migrating (highest molecular weight) portion of the smear migrating further as digestion progressed, indicating a loss in molecular weight, as one would expect if the enzyme is successfully degrading the RNA. When these time aliquots were used to program wheat germ extracts, the results showed an early loss of the 45 Kd and 36 Kd proteins (Fig. 13). This agrees well with time-course translations, which also suggest that the 45 and 36 Kd cistrons map at the 3' end.

It should be noted that it is possible that the degradation of genomic RNA is due to contaminating nuclease activities present in the commercial preparation of the snake venom phosphodiesterase. It could thus be argued that the 45 Kd protein cistron is lost first simply because it is the largest, and would therefore be attacked more often as a matter of random statistical probability. The presence of such contamination is acknowledged by the manufacturer (Boehringer-Mannheim). However, the most prevalent of these activities, a 5' nucleotidase, is listed at only 0.1% of the phosphodiesterase activity. Given that the assay (*in vitro* translation) involves millions of molecules of RNA, it seems doubtful that the inclusion of a small percentage of aberrantly-degraded molecules would cause the marked reduction in 45Kd intensity evident in Figure 13. In addition, the reaction rate was controlled by incubation on ice. This would presumably have a generally

Figure 12. Agarose Gel Electrophoresis of Phophatase-Treated Genomic RNA Digested with Snake Venom Phosphodiesterase.

Aliquots were removed at the times indicated below, and the reaction stopped with an equal volume of SDS dissolving buffer. Lanes 1 and 9, molecular weight markers; lane 10, MCMV RNA incubated on ice for 50 minutes, with no enzyme.

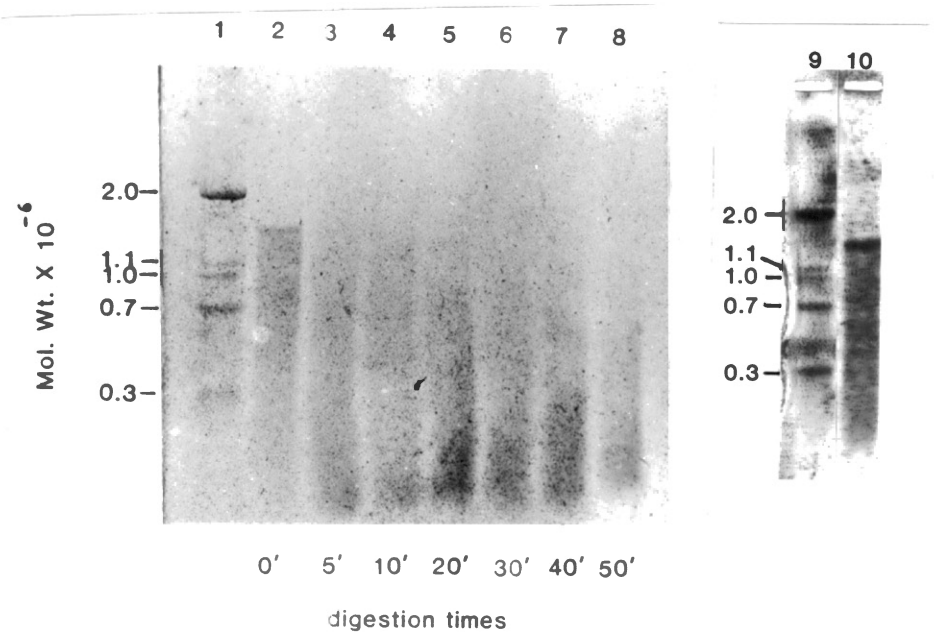
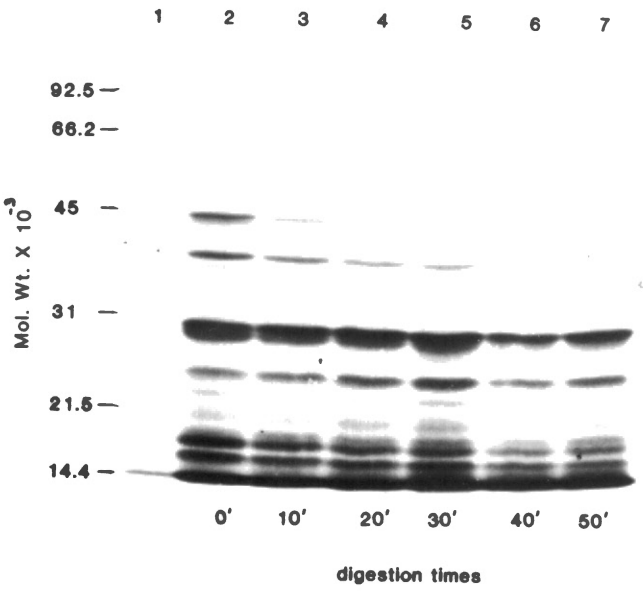

1 2 3 4 5 6 7 8
9 10
Mol. Wt. X 10⁻⁶
2.0
1.1
1.0
0.7
0.3
0' 5' 10' 20' 30' 40' 50'
digestion times

Figure 13. PAGE Fluorograph of *In Vitro* Translation Products of Wheat Germ Extracts Programmed with Venom Phosphodiesterase-Digested Genomic RNA.

Lane 1, no RNA control. Lanes 1 through 7, translations programmed with RNA that had been digested for the times indicated below.


1
2
3
4
5
6
7
92.5
66.2
45
31
21.5
14.4
Mol. Wt. X 10⁻³
0'
10'
20'
30'
40'
50'
digestion times

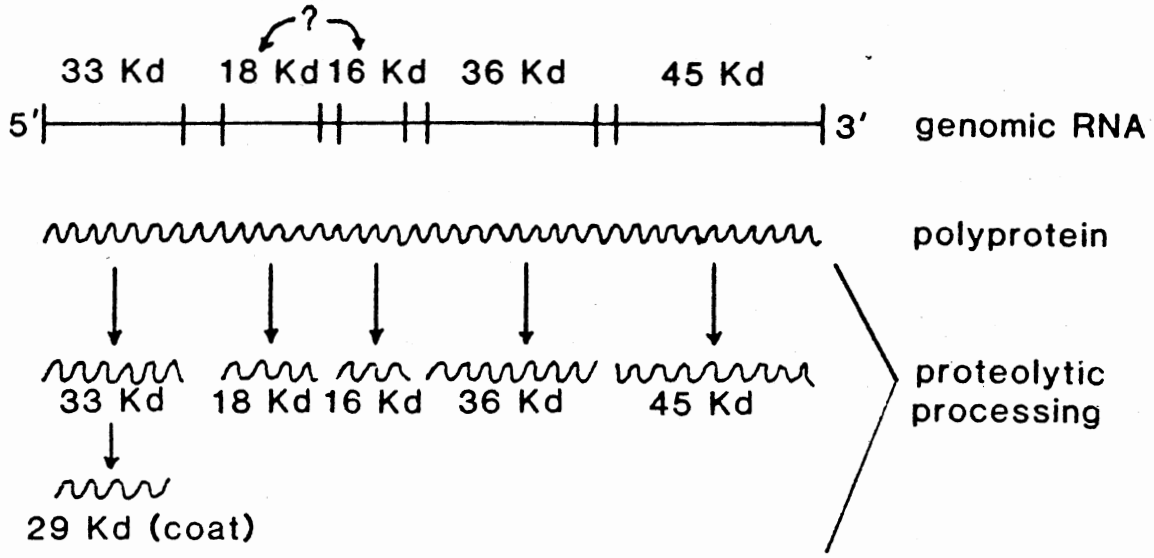
depressing effect on enzyme activity. Low reaction temperature has been used before with the venom phosphodiesterase to control the splitting of internal phosphodiester bonds (Singer & Fraenkel-Conrat, 1963). Finally, given that the 29 Kd polypeptide is at least 50% as large as the 45 Kd cistron, one would expect random internal degradation to cause a substantial loss in intensity of the 29 Kd band. However, when wheat germ was programmed with RNA which had completely lost the capacity to direct the synthesis of the 45 Kd protein, little if any loss in intensity of the 29 Kd band was apparent.

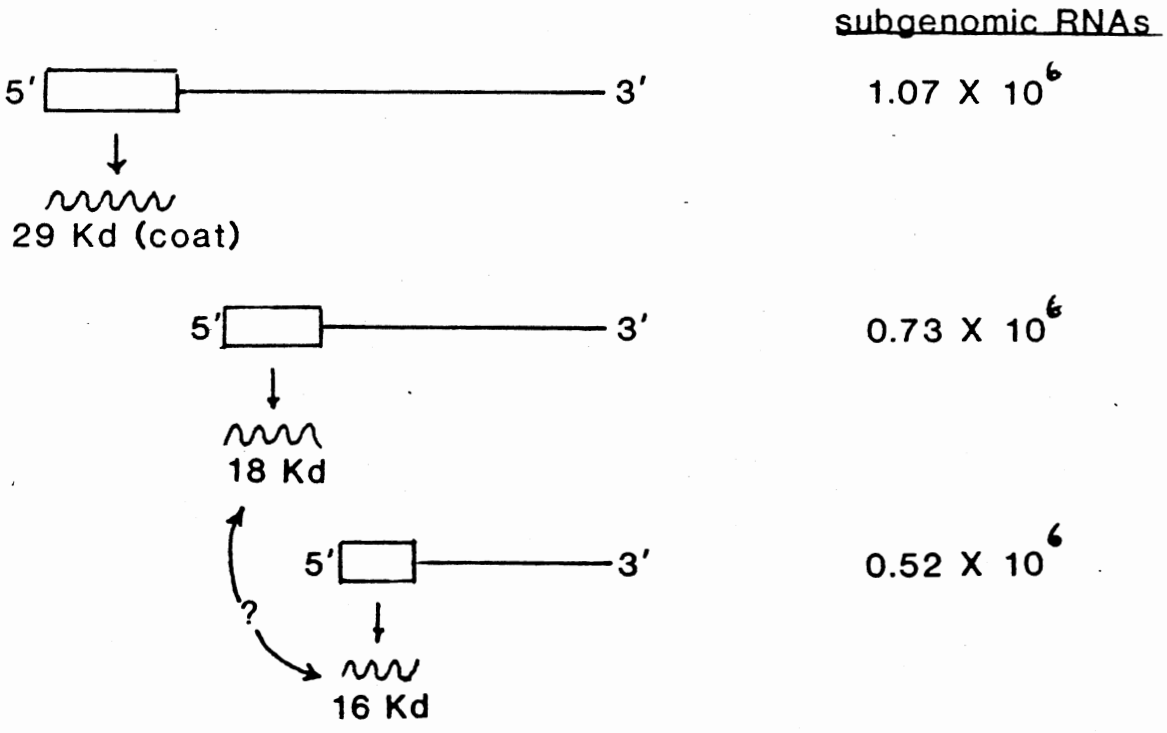
From these data, a genome map can be inferred for MCMV, and is shown in Figure 14. If this map is accurate, it would represent a significant departure from most other RNA plant viruses. Typically, the coat protein cistron is at the 3' end of the genomic RNA (Davies & Hull, 1982; Dougherty and Hiebert, 1985). The map also seems to be inverted from the standpoint of the current thinking on why plant viruses generate subgenomic RNAs - to express otherwise silent cistrons. Subgenomic RNAs almost invariably map to the 3' end of the genomic RNA. In the case of the model presented here, however, the genomic-specific proteins (the 45 Kd and 36 Kd proteins, see Fig. 8) map to the 3' end, while the subgenomic-specific proteins (16 Kd, 18 Kd, and 29 Kd, see Fig. 8) map at the 5' end.

Interestingly, Salomon *et al.* (1978) used methods analogous to those used here to study carnation mottle virus (CarMV) RNA. They found that limited phosphorolysis of the 3' terminus caused selective loss of a 77 Kd protein product in wheat germ extracts, which paralleled a loss of RNA infectivity. For MCMV, as has been discussed above, the genomic RNA is infectious, but subgenomic RNA is not. The only difference in translational activity between infectious and uninfectious MCMV RNA is the synthesis of the 45 Kd and 36 Kd proteins, which are shown by 3'-specific digestion of the genomic RNA to map close to the 3' end. However, more recent work involving the analysis of the nucleotide sequence of CarMV RNA has returned that virus to the realm of convention, placing the coat protein cistron at the 3' end of the viral RNA, but expressed through a 1.5

Figure 14. Tentative, Proposed Map and Expression Strategy for MCMV RNA.

Wavy lines represent proteins, straight lines represent RNA. Open boxes are coding regions on subgenomic RNAs. Suggested cistron order is shown by hash marks on the genomic RNA. Uncertainty about the position of the 18 Kd and 16 Kd cistrons is indicated by arrows and a question mark. Subgenomic RNAs are identified by their approximate molecular weights, in daltons.


?
33 Kd
18 Kd
16 Kd
36 Kd
45 Kd
5'
3'
genomic RNA
polyprotein
33 Kd
18 Kd
16 Kd
36 Kd
45 Kd
proteolytic processing
29 Kd (coat)


subgenomic RNAs
5'
3'
1.07 X 10^6
29 Kd (coat)
5'
3'
0.73 X 10^6
18 Kd
5'
3'
0.52 X 10^6
?
16 Kd

kilobase subgenomic RNA (Carrington & Morris, 1986). MCMV RNA sequencing work currently underway in another laboratory may show that MCMV fits the same pattern as other, single-stranded plant viruses. Indeed, this work has mapped the coat protein cistron at the 3' end of the genomic RNA, opposite of its position as proposed in this study (Nutter & Lommel, 1987).

Expression of the Genomic RNA

It was noted above that gradient-purified genomic RNA is capable of directing the synthesis of the full complement of MCMV proteins *in vitro*. There are a number of possibilities as to how this could occur. One is that otherwise "closed" 3' cistrons could be "opened" by nuclease activity in the wheat germ extract. This would, in effect, be the equivalent of generating subgenomic RNAs *in vitro*. Another possibility is that the genomic RNA is translated as a polyprotein, which is proteolytically cleaved into mature MCMV proteins. A third possibility is the presence of internal initiation sites for translation on the genomic RNA.

The possibility of nuclease cleavage of genomic RNA seems unlikely, since the inclusion of a human placental ribonuclease inhibitor had no effect on the translation profile of wheat germ progammed with genomic RNA over an inhibitor concentration of 0 to 20 units per 100 µl translation assay.

A number of methods have been used to demonstrate viral polyproteins by *in vitro* translation. The inclusion on zinc ion (Butterworth & Korant, 1974), use of amino acid analogs (Jacobsen *et al.*, 1970) and the alteration of dithiothreitol concentration (Pelham, 1979) all aim in different ways to inhibit the activity of proteolytic enzymes. When MCMV genomic RNA was used to program wheat germ extract in the presence of varying concentrations of Zn^{2+} (0 to 1.0 mM) there was no variation in the electrophoretic profile of MCMV-specific proteins. Likewise, amino acid analogs canavanine and fluorophenylalanine (0.45 mM each) had no effect on *in vitro* translation products directed

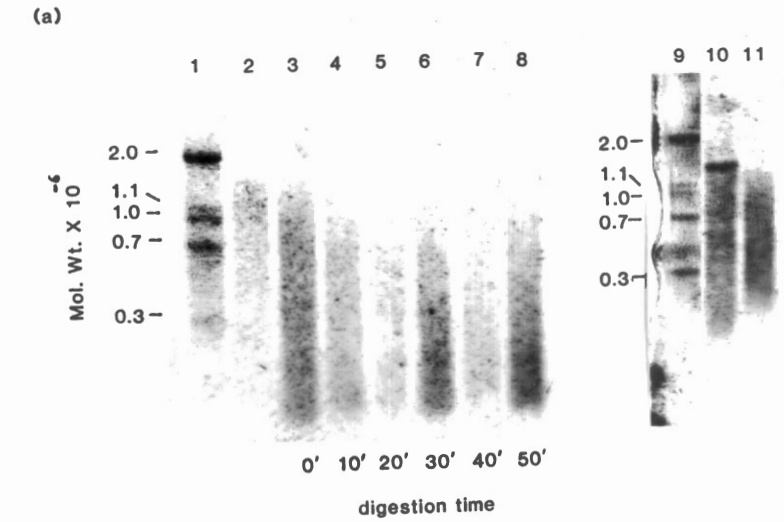
by genomic RNA. Finally, variation of the dithiothreitol concentration (from 0 to 4.0 mM) similarly produced no change in the proteins directed by genomic RNA in wheat germ extracts.

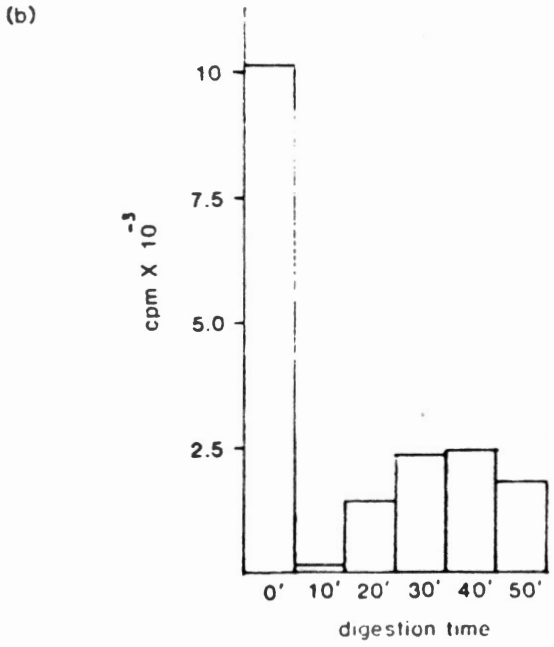
The inability to detect a polyprotein under these conditions, however, does not rule out this strategy for genomic RNA expression. Some indirect evidence supporting the formation of a polyprotein was found. Genomic RNA was digested with proteinase K (to remove a putative protein covalently attached to the 5' end; Lommel, personal communication) followed by digestion for various periods with calf spleen phosphodiesterase (E.C. 3.1.16.1). This enzyme is a 5' exonuclease. In other words, it degrades polynucleotides from the 5' end, opposite from the activity of the venom phosphodiesterase. Figure 15 shows that very early in digestion, translational activity is depressed, but recovers slightly as degradation from the 5' end proceeds. This observation was reproducible, suggesting that it is not merely an artifact. This is contrary to the situation with the snake venom enzyme, in which extensive degradation from the 3' end reduces the amount of translational activity little or not at all. Figure 15 can be interpreted as suggesting that a single site for initiation of translation exists near the 5' end of the genomic RNA. When it is eliminated enzymatically, translational activity drops off sharply. One could speculate the the mild recovery of activity indicates that internal sites of initiation, normally silent, were exposed for ribosome binding.

As regards the possibility of multiple sites for translation initiation, all of the known exceptions to the general pattern of eucaryotic mRNA translation are viral RNAs (Kozak, 1986). However, multiple initiation events have not been demonstrated rigorously for any plant virus, though such a mechanism has been suggested for southern bean mosaic virus (Ghosh *et al.*, 1981), carnation mottle virus (Salomon *et al.*, 1978) and tobacco necrosis virus (Salvato and Fraekel-Conrat, 1977). No data is offered in this work which would eliminate this mode of expression for MCMV. Therefore, the possibility of multiple initiation sites cannot be ruled out.

Figure 15. Agarose Gel (a) and Graph (b) Showing Digestion of Genomic RNA with Calf Spleen Phosphodiesterase.

(a)-Lanes1 and 9, molecular weight markers. Lane 2, MCMV RNA. Lanes 3 through 8, proteinase K-treated genomic RNA, digested with phosphodiesterase for the times indicated below. Lane 10, MCMV RNA. Lane 11, genomic RNA, not pre-treated with proteinase K, digested with calf spleen phosphodiesterase for 50 minutes.(b)- Graph showing translational activity, judged by incorporation of ^{3}H-leucine into TCA-precipitable products, of RNA digested with calf spleen phosphodiesterase. ^{3}H was measured by liquid scintillation counting (cpm).


(a)
1 2 3 4 5 6 7 8
9 10 11
Mol. Wt. X 10^-6
2.0
1.1
1.0
0.7
0.3
0' 10' 20' 30' 40' 50'
digestion time

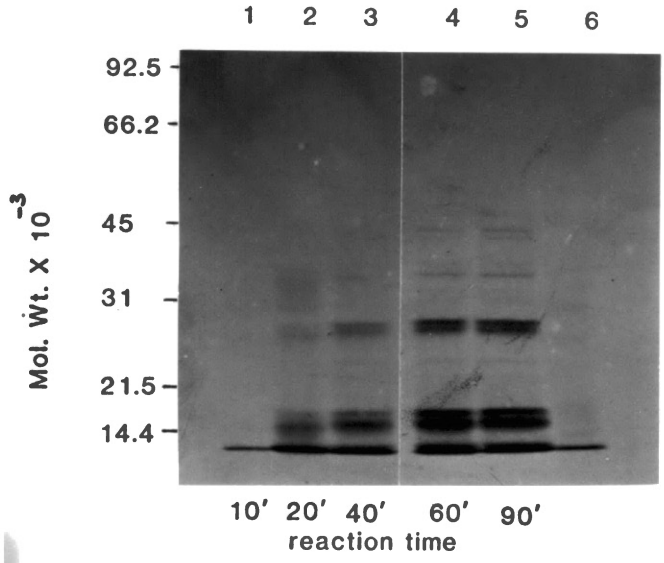

(b)
cpm X 10^-3
10
7.5
5.0
2.5
0' 10' 20' 30' 40' 50'
digestion time

A somewhat more exotic possibility for the expression of MCMV genomic RNA is that of RNA self-splicing or self-cleaving, which has become well-established in recent years (Cech, 1987). Such a model for the virusoid from lucerne transient streak virus has been proposed (Forster & Symons, 1987). Self-splicing or self-cleaving has never been demonstrated for a plant viral RNA, however.

The data point, albeit tentatively, to the formation of a polyprotein, which is subsequently cleaved into mature MCMV proteins, as the mechanism for the expression of the genomic RNA. This raises the question of why MCMV should generate subgenomic RNAs in the first place, if the genomic RNA is capable of expressing all of its cistrons in the absence of subgenomic RNA. Possibly, subgenomic RNAs are more efficient in translation than the genomic RNA. When time-course translations were performed with unfractionated MCMV RNA, protein products appeared strictly according to molecular weight (Fig. 16). It will be remembered that this was not the case in time course translations programmed with gradient-purified genomic RNA. This observation is most easily explained by assuming that the subgenomics are monocistronic, and that their protein products would accumulate at a rate proportional to their size. The 33 Kd putative coat protein precursor detected in time-course translations of genomic RNA appears before the smaller 16 Kd and 18 Kd proteins, presumably because ribosomes must first pass through the coat protein cistron before reaching the 18 and 16 Kd cistrons. If the proteins appear strictly according to size in the presence of subgenomic RNAs (as is the case when translating unfractionated RNA) it can be interpreted as meaning that the smaller proteins are accumulating first because they are being translated from monocistronic (subgenomic) RNAs. The higher translational efficiency of subgenomic RNAs becomes more evident when earlier results suggesting that subgenomic RNAs are present at low concentration (Figs. 2 and 4) are considered. Though the subgenomic RNAs are assumed to be monocistronic, and are portrayed that way in the proposed model, that is not necessarily so. However, even if the subgenomics are polycistronic RNAs, they could still be

Figure 16. PAGE Fluorograph of a Time-Course Translation Programmed with Unfractionated RNA.

Aliquots were removed at the indicated times and translation stopped by mixing with an equal volume of SDS dissolving buffer. Lane 6 is a no RNA, 90 minute control.


1 2 3 4 5 6
92.5
66.2
45
31
21.5
14.4
Mol. Wt. X 10 -3
10' 20' 40' 60' 90'
reaction time

translated more rapidly than the genomic RNA simply because ribosomes could travel their length in a shorter period of time. One could also speculate that aspects of secondary structure which would reduce translational efficiency are present in the genomic RNA but not in the subgenomic RNAs.

A tentative strategy for expression of the MCMV genome, then, combines the generation of subgenomic mRNAs with synthesis of a polyprotein, which is subsequently cleaved into mature MCMV proteins (Fig. 14). The advantage to the virus in such a strategy could be in the temporal control of the appearance of given viral proteins. Early in infection, viral proteins other than the coat protein must be synthesized - most notably an RNA polymerase. Later, an abundance of coat protein is required for purposes of packaging viral RNA. The proposed model would allow for a relatively high ratio of polymerase to coat protein during the early, polyprotein phase of the viral replication cycle. Later, after the polymerase had synthesized more genomic and subgenomic RNA, the high translational efficiency of the subgenomic RNAs would provide the virus with the needed excess of coat protein. Such a model has been postulated for brome mosaic virus (Shih & Kaesberg, 1973), which expresses its coat protein cistron through a subgenomic RNA.

Future work in this field should first deal with the precise mapping of cistrons on the MCMV RNA by nucleotide sequencing. Once this is accomplished, the role of MCMV in viral synergism can be explored in detail by synthesizing infectious and translationally active transcripts from MCMV cDNA clones. Using DNA as a chemical intermediate will allow for the use of recombinant DNA techniques to remove or alter specific cistrons or controlling regions in the resultant transcripts. This approach has been used extensively in recent years to study other viruses (Carrington & Morris, 1986; Ahlquist *et al.*, 1984; French *et al.*, 1986; Dawson *et al.*, 1986; Meshi *et al.*, 1986). Apparently, no one has used infectious, recombinant transcripts to study co-infection. This strategy could yield

precise information about which virus-specific products are responsible for the synergism underlying CLN.

Unfortunately, this work sheds no new light on the taxonomic relationships of MCMV. Recent nucleotide sequencing work in another laboratory has led some to classify MCMV as a relative of CarMV, based on a high degree of deduced amino acid sequence homology between the replicase of CarMV and a 45 Kd open reading frame from MCMV (Lommel, personal communication). However, the conclusion is questionable, since relationships can be drawn on the same basis for such highly diverse viruses as tobacco mosaic virus, brome mosaic virus, alfalfa mosaic virus, and even poliovirus (Guilley *et al.*, 1985). MCMV shares a number of characteristics with the sobemoviruses, including particle size, coat protein molecular weight, and molecular weight of RNA (Shepherd, 1971; Gordon *et al.*, 1984). Until more information is available, MCMV is perhaps best classified as a possible member of the sobemovirus group.

CHAPTER IV

CONCLUSIONS

The major conclusions of this study can be summarized as follows:

1. The MCMV genome is organized into one genomic RNA of approximately 1.5 X 10^6 daltons, and three subgenomic RNAs of approximately 1.07, 0.73, and 0.52 X 10^6 daltons.
2. The genomic RNA is required for infectivity, but the subgenomic RNAs are not.
3. *In vitro* tranlations in wheat germ extracts programmed with MCMV RNA suggest that MCMV RNA codes for major poteins of 45, 36, 29, 18, and 16 Kd. Gradient-purified subgenomic RNAs are able to direct the synthesis of all but the 45 and 36 Kd proteins. Taken together with the RNA infectivity data, this argues that the 45 and 36 Kd proteins are involved with viral replication and/or cell-to-cell movement of the virus. The genomic RNA was capable of directing the synthesis of the full complement of MCMV-specific proteins. Immunoprecipitation of *in vitro* translation products and disruption of purified virions indicate that the 29 Kd protein is the MCMV coat protein. No *in vivo* functions for the 18 and 16 Kd proteins were demonstrated.
4. Translations with genomic RNA, terminated at various times during the course of translation, and translations programmed with genomic RNA digested specifically from the 3' end, suggest a tentative order of cistrons on the genomic RNA as follows: 5'- coat-18 Kd-16 Kd-36 Kd-45 Kd-3'. The order of the 18 and 16 Kd cistrons given is arbitrary. No data was gathered to pinpoint the locations of these two cistrons.

It must be cautioned that in drawing conclusions on the *in vivo* behavior of a virus based on *in vitro* translations, one runs the risk of interpreting the various possible artifacts of the system used (Wilson & Glover, 1983; Efron & Marcus, 1973; Roberts *et al.*, 1973; Neeleman *et al.*, 1985) as reflective of the situation in nature. This is not necessarily the case. Still, *in vitro* translation is valuable in that it is inexpensive, relatively easy to prepare and has yielded useful information on a number of plant viruses (Dougherty & Hiebert, 1985).

The MCMV genomic RNA has a coding capacity of approximately 166,000 daltons, which exceeds the sum of the major proteins synthesized in wheat germ programmed with MCMV RNA (about 144,000 daltons). This indicates that MCMV contains enough genetic information to code for the number and molecular weight of proteins *in vivo* that it does *in vitro*. While this does not support the fidelity of the wheat germ system, it certainly maintains the principal findings of this study within the bounds of possibility.

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APPENDIX

"MAIZE CHAMBER VIRUS" – A POSSIBLE NEW VIRUS OF MAIZE

Discovery and Partial Characterization

During the course of this work, MCMV was maintained in continuous culture in the growth chamber. That is, MCMV-infected tissue from one batch of infected plants was used as inoculum to infect the next batch of plants. At one point, however, when symptomatic leaves were harvested and subjected to a standard MCMV purification procedure, an unusual sedimentation profile appeared in sucrose gradients (Fig.17). Instead of the standard single sedimenting species, there were several peaks. Electron microscopy of this material showed that 3 of the 4 major peaks contained spherical particles ranging from 18-23 nm in diameter, with most measuring approximately 20 nm in diameter (Fig.18). This discovery resulted in an effort to determine if this particle was MCMV, a heretofore undiscovered part of MCMV, or a contaminating entity. The purified material was subjected to spectrophotometric analysis. The unknown material shows a UV absorbance profile typical of those for viruses (Fig.19). The A_{260}/A_{280} ratio is consistently 2.0 or greater. This suggests the presence of nucleic acid, since nucleic acids absorb more strongly at 260 nm than do proteins, and conversely, proteins absorb more strongly at 280 nm than do nucleic acids. This virus-like material was subjected to an RNA extraction by the usual MCMV protocol. The ethanol precipitate was assayed by absorbance at 260 nm as with MCMV RNA, and analyzed by agarose gel elecrophoresis (Fig.20). The electrophoretic pattern of this extract was clearly different from that of MCMV RNA. Four obvious bands were detectable with approximate molecular weights of 0.450, 0.326, 0.192, and 0.097 X 10^6 daltons. Interestingly, these bands migrated quite

Figure 17. Composite of Sedimentation Profile of "Maize Chamber Virus" Through 10 to 40% Sucrose Density Gradients.

Top, first gradient with peaks identified by number. Below, sedimentation profile of each peak on second, identical gradients.

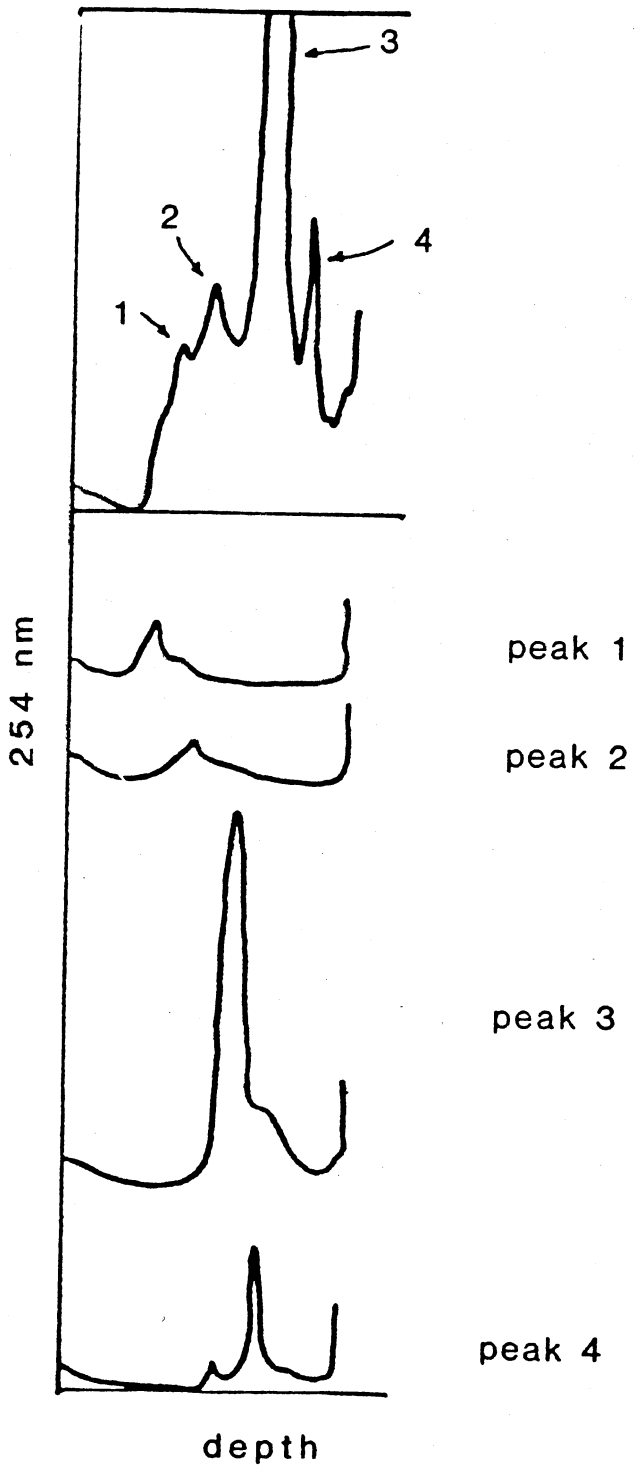

3
2
4
1
254 nm
peak 1
peak 2
peak 3
peak 4
depth

Figure 18. Electron Micrograph of "MCV" Particles from Peak 3.

Peaks 2 and 4 contained similar particles. Magnification is 48,000X.

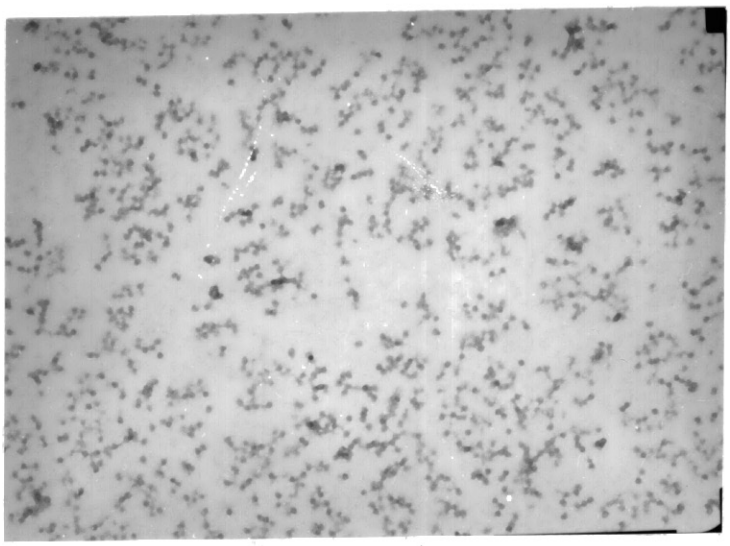


Figure 19. Ultraviolet Absorbance Spectrum for "MCV", Peak 3.

Virus was diluted 100-fold in water. The spectrum was not corrected for light scattering.

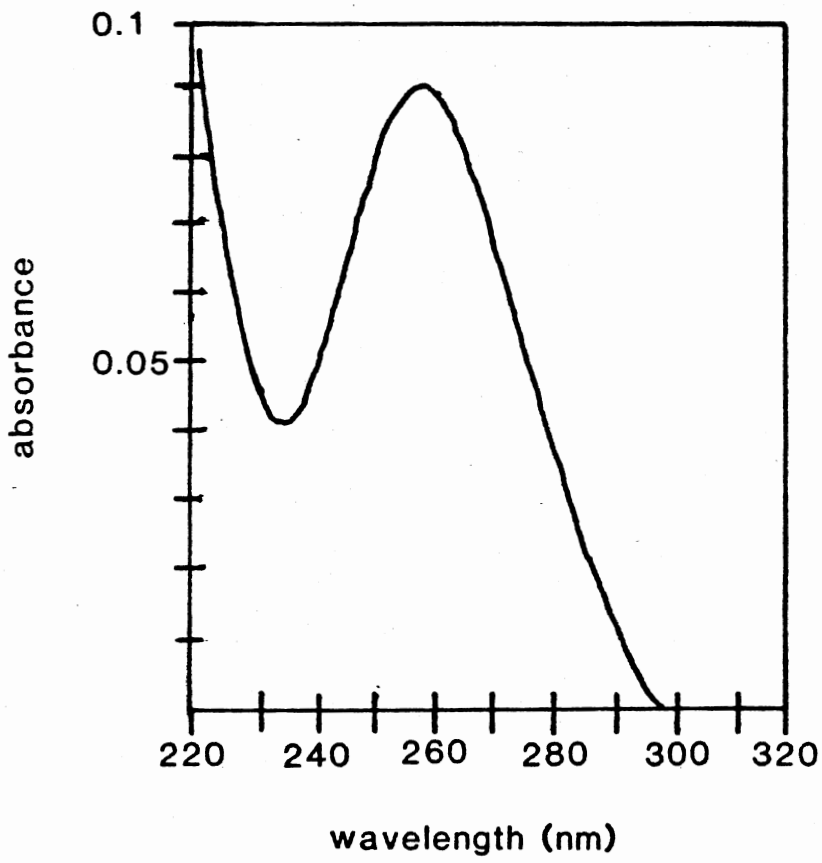
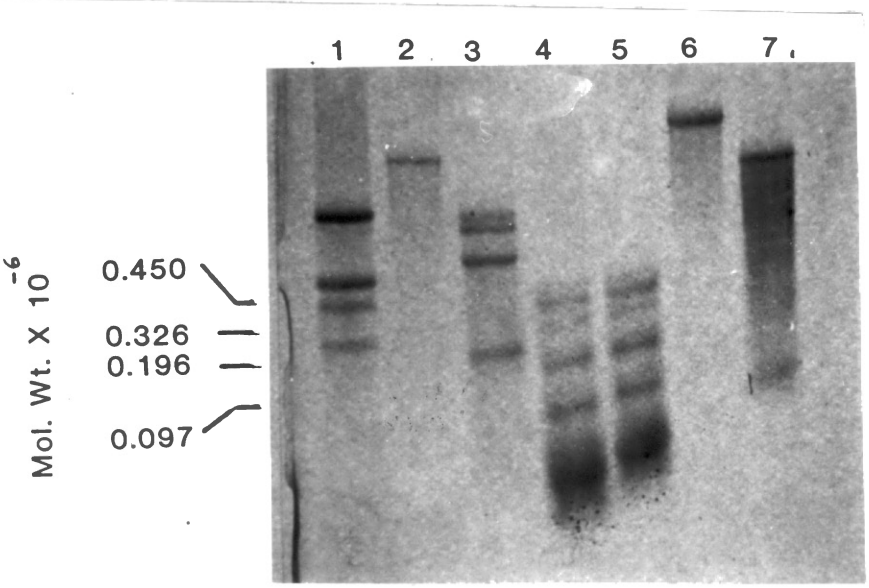

0.1
0.05
absorbance
220
240
260
280
300
320
wavelength (nm)

Figure 20. Agarose Gel Electophoresis of "MCV" Extract, Compared with Common RNAs.

Lane 1, maize ribosomal RNA. Lane 2, tobacco mosaic virus RNA. Lane 3, brome mosaic virus RNA. Lanes 4 and 5, "MCV" extract. Lane 6, wheat streak mosaic virus RNA. Lane 7, MCMV RNA. Approximate molecular weights for the "MCV" extract are indicated at left.


1
2
3
4
5
6
7
Mol. Wt. X 10^-6
0.450
0.326
0.196
0.097

differently from RNAs of viruses which might conceivably contaminate maize (brome mosaic virus, wheat streak mosaic virus) or from tobacco mosaic virus RNA. The bands from the unknown, virus-like agent also migrated differently from maize rRNA, which is a possible mulitple-RNA contaminant. These extracts are both RNase-sensitive (data not shown) and capable of directing the synthesis of proteins *in vitro* (Fig.21). It is concluded form these data that the extract from the unknown virus-like agent in indeed RNA.

Given the particle size, morphology, and that it is readily mechanically transmissable, it seems very likely that the unknown agent is a virus. That it is not MCMV, or a part of MCMV, is indicated clearly by its sedimentation profile, RNA content, and particle size. The possibility that the RNAs of the unknown virus are actually MCMV subgenomics is doubtful, since the two viruses appeared to have no common *in vitro* translation products (Fig.21). Further evidence differentiating the two viruses was uncovered quite by accident. The unknown virus seems incapable of causing symptoms when mechanically inoculated onto maize cultivar NB611. However, NB611 is susceptible to MCMV (Fig. 22). For purposes of brevity and clarity, the unknown virus will hereafter be referred to as "maize chamber virus" ("MCV") because it has been found only in the growth chamber.

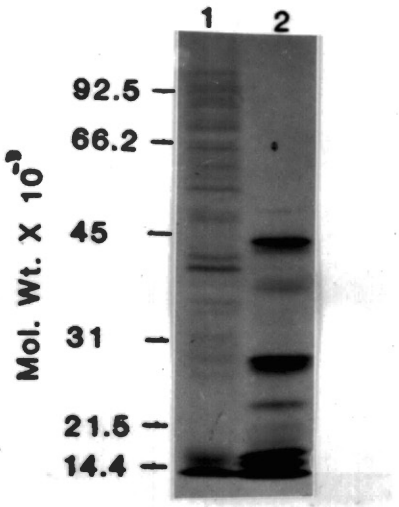
Various sources of plant viral taxonomy (Francki *et al.* 1985; Mathews, 1981; Fraenkel-Conrat, 1985; Gibbs & Harrison, 1976) were consulted in an attempt to identify "MCV" as a previously-described virus. Apparently, no virus with the combination of particle size, morphology, and RNA content of "MCV" has been described before.

Possible Origin of "MCV"

The two most obvious possibilities for the origin of "MCV" are that it is a contaminant introduced from outside the growth chamber (probably via an insect vector) or that it has always existed at low levels in MCMV-infected plants. In this latter scenario, the act of continually transmitting MCMV many times might somehow cause "MCV" to replicate at a very high rate, evidently at the expense of MCMV.

Figure 21. PAGE Fluorograph of Translation Products from Wheat Germ Extract Programmed with Either MCMV RNA or "MCV" RNA.

Lane 1, "MCV" RNA translation products. Lane 2, MCMV RNA translation products.


1
2
92.5
66.2
45
31
21.5
14.4
Mol. Wt. X 10⁻³

MCMV was eventually reestablished by inoculating plants with dried-down, MCMV-infected tisssue stored at 4°C since 1981. Upon electron microscopy of virus pruified from these plants, a few small, 20 nm particles were detected in the same field as 30 nm MCMV particles (Fig.23). These were possibly left over from glassware used in previous "MCV" purifications, though all glassware was washed thoroughly and baked at 60°C overnight after use. The other possibility is that "MCV" was present at low levels as far back as 1981, and may have always been present, but never detected.

It is unlikely that "MCV" is a satellite virus of MCMV, since it is capable of replication independent of MCMV. Other workers have found recently that "MCV" has little or no serological relationship to MCMV (Fulton & Gordon, unpublished). This suggests that "MCV" is in fact a separate virus, and not an artifact of MCMV replication; for example, encapsidation of satellite RNAs by MCMV coat protein (for a review of plant virus satellites, see Francki, 1985).

Another possibility is that "MCV" is a cryptic virus. Cryptic viruses have been discovered in a number of plant hosts. They are characterized by lack of symptoms produced in carrying hosts, lack of transmissability by mechanical inoculation or grafting, high rates of seed transmission, and low concentration in plants (Boccardo *et al.*, 1983). However, given that "maize chamber virus" is apparently transmissable mechanically and is easily purified, the possibility that "MCV" is a cryptic virus seems remote.

Attempts to establish the 20 nm particles as the infectious agent by the demonstration of Koch's postulates unfortunately resulted in a reestablishment of MCMV. "MCV"-infected tissue that had been frozen for many months at -70°C was used to inoculate maize N28Ht. Virus purified from these plants was in turn used as inoculum on a second batch of N28Ht. Electron micrographs of the pure virus inoculum show a mixed population of particles, with many 30 nm (dia.) spheres. Purified virus from the second batch of N28Ht contained only 30 nm (dia.) particles (Fig 24). RNA from this material co-migrated with MCMV RNA (data not shown).

Figure 22. "MCV" Symptoms on Maize.

Above, chlorotic symptoms of "MCV" infection on cultivar N28 ht. Below left, cultivar NB 611 inoculated with "MCV", showing no symptoms. Below right, cultivar N28 ht inoculated with "MCV", showing typical "MCV" symptoms.

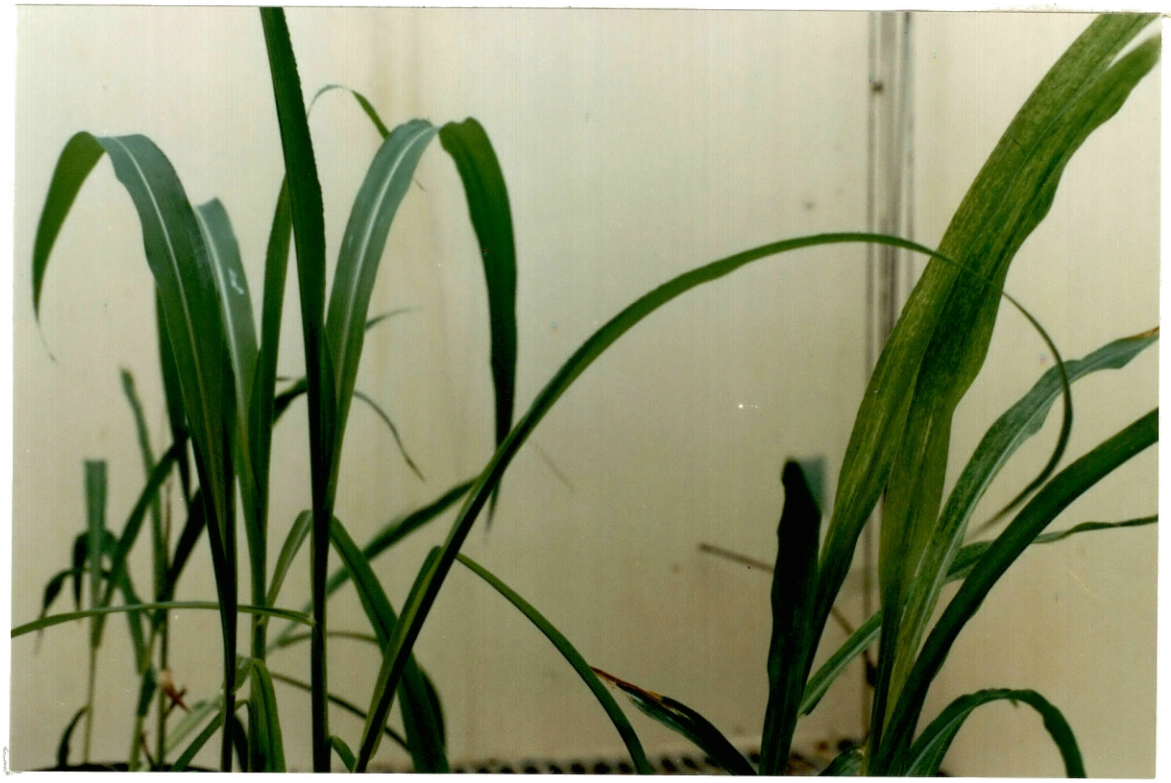
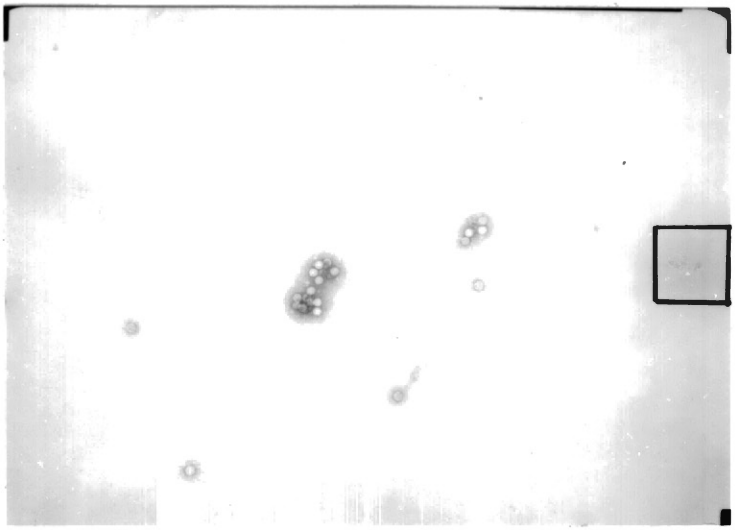


Figure 23. Electron Micrograph of Virus Purified from Maize Which had Been Inoculated with Dried-Down MCMV Infected Tissue from 1981.

Larger particles measure approximately 31 nm in diameter. Enclosed in the box at right is a cluster of smaller particles, approximately 20 nm in diameter. Magnification is 48,000X. The box is shown below left, enlarged photographically to a total magnification of 131,200X. At below right is a section of figure 18, showing "MCV" particles, for comparison. This photo is enlarged to the same magnification as at below left.



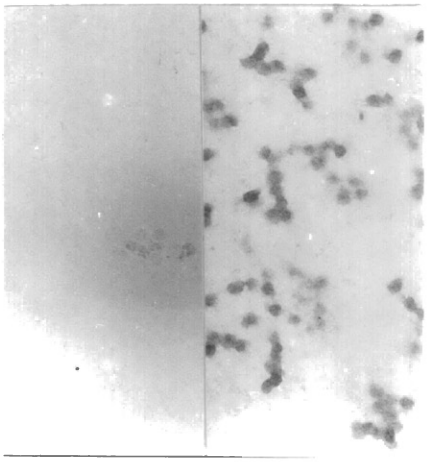
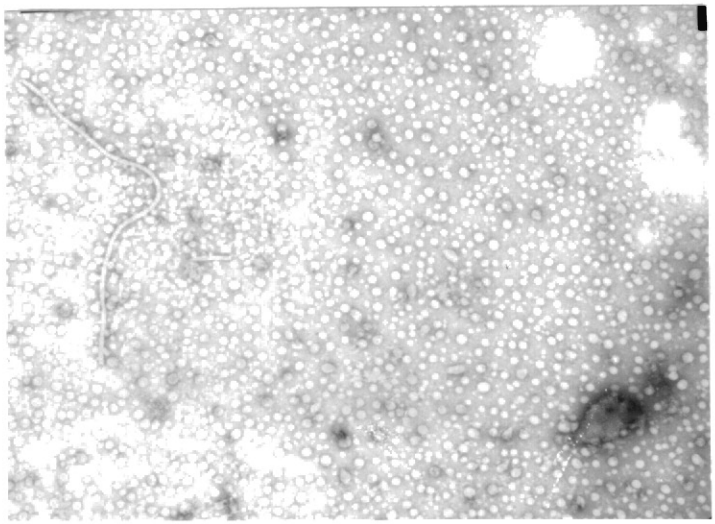
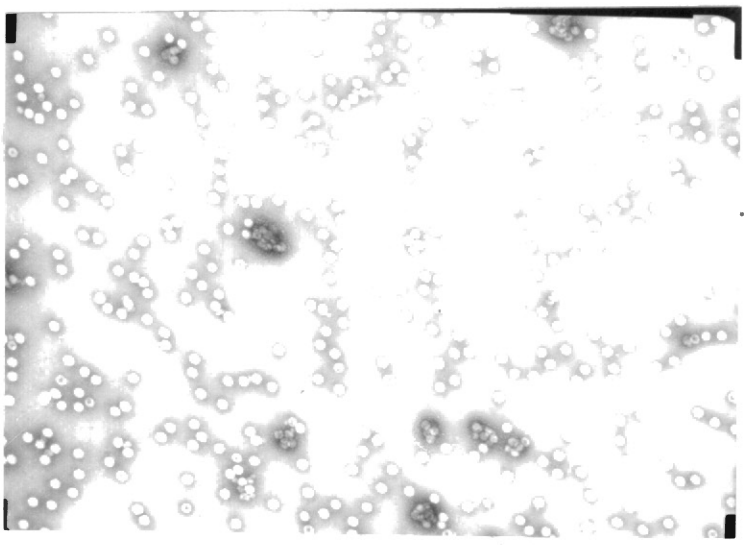


Figure 24. Electron Micrographs of Virus Purified from Maize.

"MCV"-infected tissue was used to inoculate a batch of maize. Virus was purified from symptomatic tissue (a) and used to inoculate a second batch of maize plants. Virus purified from the second batch (b) was compared to that of the first batch. Note the mixture of particle sizes in (a), which is less apparent in (b).





The nature of the appearance and subsequent disappearance of "MCV" is a mystery. It is evidently a virus, not a part of, or dependent on, MCMV for replication, and has apparently never before been described. "Maize chamber virus" has possibly been present in co-infection at low levels with MCMV for some time. If this is the case, then the role of this virus in MCMV infection, to say nothing of CLN, makes it worthy of further study.

VITA

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