TRANSLATION OF MAIZE CHLOROTIC MOTTLE VIRUS RNA IN VITRO

By

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Dedication

For Madam Elizabeth Arhin, brother and sisters in deep appreciation of their love, admiration and encouragement.

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It was nice working with other graduate students in the department.

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LIST OF ABBREVIATIONS

ATP	-	adenosine triphosphate
CLN	-	corn lethal necrosis
DEPC	-	diethylpyrocarbonate
DMSO	-	dimethyl sulfoxide
DTT	-	dithiothreitol
EDTA	-	ethylene diamine N,N,N^1,N^1-tetraacetic acid
GTP	-	guanidine triphosphate
HEPES	-	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
MCMV	-	maize chlorotic mottle virus
NPM	-	sodium phosphate magnesium buffer
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
RNA	-	ribonucleic acid
RRB	-	ribonucleic acid resuspension buffer
SDS	-	sodium dodecylsulfate
Tris	-	tris(hydroxymethyl)-aminoethane
K	-	kilodalton
KD	-	kilodalton
KDa	-	kilodalton

CHAPTER I

INTRODUCTION

During the last decade severe yield losses in maize (Zea mays L.) crops have been reported (1) in some parts of America. The disease, corn lethal necrosis (CLN), which destroyed the maize, is known to be caused by a synergestic action between maize chlorotic mottle virus (MCMV) and any of the potyviruses which infect maize. The presence of MCMV is a requisite for the occurrence of CLN among host populations. However, by itself MCMV causes mild systemic chlorosis on maize plants (1-3).

MCMV occurs as small (30 nm) icosahedral particles with capsid protein of 29K daltons (4). It has a single-stranded RNA genome of 4360 nucleotides (1.4×10^6 daltons) as determined from agarose gel electrophoresis in 2.2 M formaldehyde (5). The virus is extremely stable. These properties put it in the southern bean mosaic virus group (sobemoviruses) (6).

MCMV is known to occur in Peru (7), in North Central Kansas and South Central Nebraska. In the United States, the virus is commonly found in affected fields located in small river valleys and irrigated districts. It was identified once in the High Plains of Texas. In Kansas, MCMV caused wide spread outbreaks of CLN and heavy crop losses in 1976, 1978 and 1980. It has also been identified in Brazil (4).

MCMV has a narrow host range. Fifteen to nineteen species of grasses native to Kansas are systemic hosts of the virus. Chrysomelid beetles have been identified as the main vector of MCMV (8), though the virus is also somehow soil transmitted in a persistant manner. It is not seed transmitted, however.

The limited geographic range of MCMV prevents a disastrous epidemic of CLN. Some control has been achieved according to Uyemoto (1983) (4). Crop rotation using non-host crops has offered effective control of outbreaks of MCMV.

Although substantial information about MCMV and other subemoviruses exists, much less is known about their genetic constitution as well as the strategy by which these viruses express their genomes in the host plants. As for MCMV, little or no information exists with regard to its genetic expression in the host plant. Considering the economic importance of MCMV and the role it plays in CLN disease, it is of great concern to seek to understand its molecular biology. Hopefully, such an understanding would prepare us for the study of the synergistic action of which results in CLN.

In monopartite viruses, the entire genetic material is contained in a single genome (as either RNA or DNA). MCMV, a monopartite virus, carries all the information required for genetic expression in one single stranded RNA molecule. However, from its electrophoretic behavior, there is indication of the presence of subgenomic materials (9).

Though monopartite viruses have simple structures, variation exists for cistron expression. In some cases, viral proteins are produced by post-translational cleavage of large polypeptides (10, 11),

or they are encoded by different genomic RNAs (12, 13). Some viral proteins, both structural and non-structural (14) can be translated only from subgenomic messages. On the other hand, related proteins can be produced from one single genomic message by a read-through of leaky termination codons (15). To identify and understand the various modes of cistron expression, both the viral RNAs and their protein products must be analyzed in vitro and in vivo.

MCMV shares properties common to the members of the southern bean mosaic group. SBMV RNA is translated into four polypeptides in vitro with molecular weights of 105 (P1), 75 (P2), 29 (P3), and 14 KDa (P4) (9). P1 and P2 originated from full-length genomic RNA and have related amino acid sequences. Read-through of a leaky termination codon accounts for the presence of P1. The SBMV coat protein (P3) is translated from a subgenomic mRNA that is encapsidated in a minor portion of the virions (16). Both the genomic RNA and the coat protein mRNA have the same 12 KDa polypeptide covalently linked to the 5' terminus of the RNA (17). P4 was translated from both the genomic RNA and the coat protein mRNA. From peptide mapping data, however, it is seen that P4 was not related to the genome-linked protein. It thus appears that full-length SBMV has two initiation sites for protein synthesis, one for P1 and P2 near the 5'-terminus and one for P4 near the 3' terminus, while the initiation site for coat protein synthesis is silent on genomic RNA. The coat protein mRNA appears to have independent initiation sites for the synthesis of both P3 and P4. The translation of turnip rosette virus (TRosV) RNA in vitro was reported by Morris-Krsinich and Hull (1981) (18). Translation products of molecular weights 105, 67, 35 and 30 kd were observed by these authors.

It appeared that genomic RNA was translated into the 105 kd polypeptide which was subsequently cleaved proteolytically to yield the 67 and 35 kd polypeptides. The TRosV coat protein (30 kd) was translated from a separate subgenomic RNA. The general translational strategy used by viruses of the SBMV group still remains unclear. Perhaps different viruses in this group use somewhat different strategies. With this in mind, we began studies of the translation of MCMV RNA. Information from this study is critical to understanding the replicative cycle of this economically important virus.

CHAPTER II

AIMS OF THE STUDY

The principal aim of this project was to study the in vitro translation of MCMV RNA in wheat germ extract. The major part of it involved the partial purification and characterization of MCMV RNA from purified MCMV, determination of optimal translation parameters of MCMV RNA in wheat germ extracts and the determination of molecular weight of the in vitro translation products.

The second aim of this work was to investigate to some extent the molecular mechanism by which MCMV RNA translates or codes for the various polypeptides. As reported by some authors (19), the separation of RNA according to sizes on sucrose gradients followed by in vitro translation of the various RNAs on wheat germ extract are helpful in assessing the role played by subgenomic messages. Also by studying the time course of (^{3}H)-leucine incorporation by MCMV RNA in polypeptides it is possible to obtain some information regarding the involvement of proteolytic processing (cleavage) in the strategy of MCMV RNA expression.

Thirdly, it was intended to determine the serological or relationship between the virion capsid protein and the major in vitro translation products and the MCMV antiserum using immunoprecipitation and serological techniques.

Presented in this report are the results, discussion and some conclusions of this project.

CHAPTER III

MATERIALS AND METHODS

Materials

Wheat germ was a gift of the Pillsbury Corp., Enid, Oklahoma and MCMV-antiserum was supplied by Dr. Jerry Uyemoto, Kansas State University. The ribonuclease inhibitor was from Boehringer Mannheim, pansorbin was from Calbiochem and (4,5-^{3}H)L-leucine was purchased from New England Nuclear.

Methods

Isolation of MCMV

The method for MCMV isolation was modified from that reported by Niblett and Paulsen (20) for panicum mosaic virus with special consideration for properties of viruses of the SBMV group. Zea mays L. cv. N 28Ht seedlings were mechanically inoculated at the four leaf stage and maintained in growth chambers operated at day temperatures of 30°C and night temperatures of 22°C on a 24 hour cycle with a 14 hour day length. After 21 days the infected leaves were harvested and stored at -70°C until required. Following thawing at 4°C, the leaves (50 gm) were cut and homogenized in a Waring blender thoroughly in a buffer (4 volumes) containing 0.1

M sodium phosphate, pH 7.0, 1% v/v 2-mercaptoethanol.

The homogenate was filtered through cheese cloth and an equal volume of cold chloroform-butanol (1:1) was added slowly. This mixture was stirred gently for 30 minutes at 4°C, then spun at 6,000 rpm for 10 minutes in a GSA rotor. The aqueous phase was recovered by aspiration and centrifuged for 4 hours at 30,000 rpm at 4°C in a Ti 45 rotor. The supernatant was discarded and the pellets were resuspended overnight in 1.5 ml/pellet of NPM buffer (0.02 M sodium phosphate, pH 7.0; 0.01 M $MgCl_2$). The resuspended pellets were pooled and centrifuged at 7,000 rpm for 10 minutes in an SS-34 rotor to separate insoluble material. The supernatant was then layered onto gradients of 10 to 40% RNase-free sucrose dissolved in NPM buffer and centrifuged at 22,500 rpm for 6 h at 4°C in an SW 25.1 rotor. The virus was located as a sharp light-scattering band in the center of the gradient and was collected by puncturing the side of the tube through vacuum grease using a syringe with an eighteen-gauge needle. The pooled virus samples were diluted approximately 1 to 1 with NPM buffer and centrifuged at 45,000 rpm for 2 hrs at 4°C in a Ti 75 rotor. The supernatant was discarded and the pellets were resuspended in 10 ml of 0.02 M sodium phosphate (pH 7.0) per pellet for 2 to 3 days at 4°C. Finally, the virus was then pelleted again as above and the pellets resuspended in 1 to 2 ml 0.02 M sodium phosphate, pH 7.0) per tube.

This method yielded approximately 0.8 to 1.0 A_{260} unit of virus per gram fresh weight of infected leaves. It was essential to treat all glassware and equipment during the latter stages of the isolation with 0.1% v/v diethylpyrocarbonate (DEPC).

Molecular Weight Determination of MCMV Coat Protein

To estimate the relative molecular weight of MCMV coat protein, 40 µl of the virus preparation was treated with equal volumes of dissolving buffer and heated at 90°C for 15 minutes to disrupt the virus. Different amounts of this mixture were analyzed on 12.5% SDS polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (21).

The gels were fixed for 30-60 minutes in 10% acetic acid/25% isopropanol, followed by a 2 hour staining in 0.1% Coomassie blue, 25% isopropanol and 10% acetic acid. Destaining in 10% acetic acid was performed over several hours, after which gels were dried. The sample dissolving buffer containing 0.0625 M Tris, 5% SDS, 5 M urea, 10% glycerol, 0.02% bromphenol blue, and 5% 2-mercaptoethanol, pH 8.9.

Serology of MCMV

The Ouchterlony agar double diffusion test was performed for MCMV in 10 cm plastic petri dishes (22, 23). About 0.2 g of Bactoagar was steamed in 24 ml of saline (85% NaCl in 0.1 M phosphate) and allowed to cool in 60°C. To this was added 1 ml of 0.5% aqueous solution of sodium azide to inhibit growth of microorganisms. The molten agar was allowed to solidify in a petri dish.

Six wells (4 mm in diameter and spaced 3-4 mm apart) surrounding a control well (of the same diameter) were punched on the agar. The punched plugs of agar were removed by aspiration. Into some of the center wells enough MCMV antiserum (1:2 or 1:4 dilution) was added with a capillary pipette to just fill the well. In other wells,

normal rabbit serum (same dilution) or saline was used as control. Pure MCMV (different dilutions) was added into the outer wells.

The petri dishes were incubated in a moist chamber for several days away from sunlight. The wells were examined for antigen-antiserum reaction.

The Microprecipitation Test

The method described by Van Slogteren (1954) (24) was used. Nine parallel lines about 7-8 mm apart were ruled on a paper card. A second set of lines were ruled at right angles to the first to give a grid of equal size squares. A grid of 7-8 mm apart lines was made on plastic petri dishes. Serial dilutions of MCMV preparation (1:2 to 1:128 in 0.02 M phosphate buffer) and MCMV antiserum (1:2 to 1:128 in saline) were made across the grid tubes.

Using a pipetteman, 15 µl of saline was placed in each square of the 7th column (vertically) and in each square of the bottom row (horizontally). Next, 15 µl of the most dilute virus solution was placed into the 6th column of each square including the square in the bottom row with saline. By proceeding from the most to least dilute virus, the same pipette was used to layer 15 µl of each virus dilution to each square of the remaining columns.

By the same technique, 15 µl of different dilutions of MCMV antiserum was added to each square starting from the most to the least dilute. Thus, the petri dish contained a grid of squares in which every dilution of antiserum is treated against every dilution of virus. The 7th square in each row and each column is a saline

control to detect any spontaneous precipitates occurring in the antiserum or virus preparations.

The entire plate was flooded with heavy mineral oil to cover and prevent drops of virus and antiserum from dehydration. Observations for precipitation in each square was made three hours after preparation and again periodically for 2 to 3 days. The relative amounts of precipitates were recorded as +++, ++, + or -.

Isolation of MCMV RNA

The starting material was usually 30 to 40 A_{260} units of MCMV in 10 ml of 0.1 M NaCl, 3 mM EDTA, 2% SDS and 20 mM Tris base adjusted to pH 7.8. The mixture was stirred for 15 minutes at room temperature to disrupt the virus. RNA was extracted three times with chloroform-phenol (1:1) at room temperature and resuspended in RRB (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH 8.0) and the RNA was recovered from the final aqueous phase by ethanol precipitation. Again, it was essential to use only DEPC treated glassware and autoclaved solutions. The recovery of viral RNA was 70 to 85 percent based on the absorbance of the purified virus at 260 nm.

Isolation of RNA from Membrane-Bound Polysome

Whenever it was necessary to isolate polysomal RNA from uninfected maize seedlings, the method used was a modification of that of Jackson and Larkins (25). Fresh uninfected young maize leaves were surface sterilized with 4% clorox for 5 minutes, rinsed in autoclaved water and excised into a Waring blender. Homogenization was performed for two 30 sec periods in 100 ml polysome isolation

buffer (200 mM Tris, 400 mM KCl, 200 mM sucrose, 35 mM $MgCl_2$, 25 mM EDTA, pH 9.0) and 0.1% DEPC. The homogenate was sieved through cheese cloth and then through miracloth. The pellets were collected by centrifugation at 16,000 rpm for 15 minutes in an SS-34 rotor.

Working at room temperature, the pellets were carefully resuspended in autoclaved RNA extraction buffer (2.5 ml per pellet) containing 1% SDS, 0.1 M NaCl, 0.05 M Tris and 0.01 M Na_2EDTA, pH 9.0. Ten ml of chloroform-phenol (1:1) was added and the mixture was stirred gently for 30 minutes. Subsequently, the aqueous phase containing the RNA was collected after centrifugation at 12.5 K rpm for 10 minutes and the extraction was repeated 3 times. The rest of the procedure used was similar to that described earlier on for MCMV RNA. The purified polysomal RNA (0.250 mg/ml) was stored at -70°C in sterile water and used as a marker for RNA electrophoresis.

RNA Molecular Weight Determinations by Agarose Gel Electrophoresis Under Denaturing Conditions

The method of choice was that described by Schwinghamer and Shepard (1980) (26). MCMV RNA (10 to 20 µg) or maize polysomal RNA was heated in 2.2 M formaldehyde (pH 7.0) in the presence of 25 mM phosphate, 2.5 mM Na_2EDTA, pH 7.2 and 50% formamide at 60°C for 10 minutes. The samples were cooled to room temperature after which 13 µl of SUEB (50% sucrose, 4 M urea, 50 mM EDTA, 0.1% bromphenol blue) was added. Agarose gels were prepared by heating a 1.5% agarose in 50 mM Na-phosphate, 5 mM EDTA (pH 7.2) and 2.2 M

formaldehyde (5). The gel was poured into slab and allowed to polymerize at room temperature.

The denatured RNA samples were applied to the gels and electrophoresed at 20 to 30 volts for 12 to 16 hours at room temperature. Staining of gels was achieved in autoclaved water containing 1 µg ethidium bromide for 2 to 3 hours, and was destained in autoclaved water (changed periodically). After destaining, the gels were photographed under short wave UV radiation. The molecular weight of MCMV RNA was estimated by the extrapolation of the log mobility vs. the square root of base pairs plot. The number of bases in MCMV RNA was estimated from the plot.

The Use of Wheat Germ Cell-Free Extracts for In Vitro Translation of MCMV RNA

The wheat germ cell-free translation system has been described in several publications for several plant viral RNAs (27-29). The wheat germ provides tRNA, ribosomes, and many enzyme systems necessary for protein synthesis.

Its efficiency is comparable to animal cell free systems except that many incompletely synthesized polypeptides may accumulate. However, it is generally preferred because of its availability and ease of preparation. The entire operation for preparing the extract from commercial wheat germ normally takes 75 minutes. It may contain low levels of ribonuclease; thus, a concentrated extract can result in degradation of the messenger RNA.

Preparation of Wheat Germ Extract. All glassware were treated with 0.1% v/v DEPC. A crude wheat germ extract was prepared according to Marcu and Dudock (28). All steps were performed at 4°C. Two grams of commercial wheat germ were ground dry for approximately 60 seconds in a cooled mortar with an equal weight of powdered glass. Four mls of extraction buffer containing 20 mM HEPES (pH 7.6, adjusted with KOH), 100 mM potassium acetate, 1 mM magnesium acetate, 2 mM calcium acetate and 1 mM DTT (added after autoclaving the buffer) was then followed by gentle swirling for about 30 seconds. The resultant thick slurry was scraped out of the mortar with a sterile, nuclease-free spatula and spun at 16K rpm for 12 minutes in a Sorvall SS-34 rotor. After centrifugation, the supernatant was carefully removed with a pipette. The thick lipid layer was avoided. Two mls of supernatant was layered on a Sephadex G-25 column (medium) which had previously been washed with several volumes of 0.1% DEPC to render it nuclease free. The column was subsequently equilibrated with 20 mM HEPES (pH 7.6), 120 mM potassium acetate, 5 mM magnesium acetate and 1 mM DTT. Ten drops (about 0.5 ml) per fraction were collected starting before the appearance of cloudy material. All fractions of about 90 OD/ml A_{260} were pooled and the supernatant was saved after centrifugation for 20 minutes at 16K rpm in an SS-34 rotor.

The extract obtained by this procedure was stable for long periods of time at -70°C without considerable loss of activity. For most translations, 30 µl of extract was used to obtain the optimum level of amino acid incorporation in a 100 µl assay.

In Vitro Translation of MCMV RNA in Wheat Germ Extracts (S-30)

McMV RNA was translated in wheat germ extract according to the procedure reported (29) using two assay mixtures.

Mix One. The energy generating source consisted of:

Na_2 creatine phosphate	128 mM
Tris acetate (pH 7.4)	400 mM
Na_2 ATP	16 mM
Creatine phosphokinase	0.64 mg/ml

When stored at -70°C, this mixture remains stable for several months.

Mix Two. This mix provided the necessary ionic environment and the 18 cold amino acids except lysine and leucine. The composition of this mixture was:

Potassium acetate	500 mM
Magnesium acetate	5 mM
Spermidine	1 mM
GTP	0.2 mM
DTT	30 mM
18 cold (unlabeled) amino acids	0.30 mM

The mixture is stable at -70°C.

The protein synthesis was carried out in a total volume of 100 µl containing 30 µl of wheat germ extract, 33 mM Tris acetate (pH 7.4), 3 mM DTT, 1 mM ATP (neutralized with KOH), 20 µM GTP, 10 mM sodium creatine phosphate, 50 µg/ml creatine phosphokinase, 2 mM magnesium

acetate, 90 to 100 mM potassium acetate, 0.1 mM spermidine, 25-30 μM of all 19 unlabeled amino acids, 20 to 50 μCi of (^{3}H)-leucine and 75 to 100 μg/ml MCMV RNA. In the control experiments, RNA was replaced with water.

To achieve the conditions listed above, the translation mixtures were set up as follows:

Mix 1	10 μl
Mix 2	10 μl
1.25 mM lysine	5 μl
(^{3}H)-leucine (1 mCi/ml)	5 μl
H_2O (sterile)	30 μl
RNA	10 μl
Wheat germ extract	30 μl
Total Volume	100 μl

Usually, the translation mixture was incubated for 90 minutes at 20 to 26°C after which the synthesis was arrested by the addition of equal volume (100 μl) of PAGE dissolving buffer.

TCA precipitation and assay of translation products was performed as follows: 20 μl of translation products and 1 ml of 10% TCA w/v containing 1 mg/ml cold leucine was incubated on ice for 30 to 60 minutes. The mixtures were heated to 90°C for 15 minutes (to hydrolyze all aminoacyl-tRNAs) and finally cooled on ice for 30 to 60 minutes. The resultant protein precipitates were collected on glass fiber filter (Type A/E, Gelman Sciences, Michigan) (previously moistened with cold 10% TCA/leucine mixture). Assay tubes were rinsed thoroughly with 10% TCA and all filters were washed an additional three times with 10%

TCA. Finally, the filters were washed one time with absolute ethanol, dried for 20 to 30 minutes on a hot plate and counted in a 10 ml portion of 5 g diphenyloxazole/liter toluene in a Beckman LS-3150 T scintillation counter.

Identification of Translation Products by SDS-PAGE

To identify the various polypeptides made in vitro according to molecular weight SDS-PAGE (21) was used. About 20 to 40 µl of each assay was electrophoresed on either 10% or 12.5% polyacrylamide gels at 6 mA for 6 to 9 hours at room temperature. The protein standards used were the same as mentioned earlier on under the materials and methods section.

To detect the (^{3}H)-labeled protein, the gels were fluorographed as described by Bonner and Laskey (1974) (30). After destaining, the wet gels were dehydrated for 3 hours (three changes) in DMSO, soaked in PPO/DMSO (22.4% w/v) for an additional 2 hours, rinsed several times in cold water (1 to 2 hours) before being dried. The dried gels were exposed to preflashed RP Royal "X"-Omat film at -70^{o}C for several hours or days.

Optimization of Translation Conditions for MCMV RNA in Wheat Germ Extracts

To determine the optimal ionic strengths (Mg^{2+} or K^{+}) for efficient translation of MCMV RNA in wheat germ extract, (^{3}H)-leucine incorporation into TCA precipitable materials under varying K^{+} and Mg^{2+} concentrations in the translation system was performed (29) taking

into account the levels of K^+ and Mg^{2+} already present in the wheat germ extract.

Similarly, the optimal RNA level for efficient translation was studied under optimal ionic strengths of Mg^{2+} and K^+ but with varying amounts of MCMV RNA. All other conditions were the same as mentioned elsewhere in this chapter.

Fractionation of MCMV RNA According to Sizes

Sucrose gradient fractionation of MCMV RNA was as described by Rutgers et al. (1980) (19). After 15 minutes incubation at 60°C in 10 mM Tris-HCl (pH 8.0), 1.5 mM Na_2EDTA, 20 mM NaCl, the RNA (132 µg) was chilled rapidly on ice and was immediately layered on 0.2 to 0.8 M sucrose gradient containing 25 mM Tris-HCl, pH 7.8, 1.0 mM Na_2EDTA, and 100 mM potassium acetate. Maize polysomal RNA (132 µg) was used as a marker. The gradient was centrifuged for 14 hours at 25K rpm at 4°C in an SW 28 rotor. Various fractions (1 ml/tube) were obtained using an ISCO Model 640 gradient fractionator. The A_{260} measurement was performed with Beckman spectrophotometer. The absorbance pattern is shown in Chapter IV.

Translation of the Various Size Classes of MCMV RNA

The gradient obtained as described above was divided into several regions (A-F) based on A_{260}. The RNAs were concentrated by ethanol precipitation in the presence of 100 mM potassium acetate using 50 µg yeast tRNA as a carrier at -20°C for 3 days. The RNAs were pelleted at 17.5K rpm for 20 minutes and the dried pellet resuspended in 100 µl

sterile autoclaved water.

To determine the translation products and efficiency of the various fractions, 50 µl of each region (A-E) was translated on wheat germ extracts as described previously (28). The analysis of (^{3}H)-leucine labeled products synthesized by the RNAs was performed on 12.5% gels.

To determine whether pre-translational RNA cleavage by nucleases played a role during translation, the various RNAs (sucrose gradient fractions, A-E) were translated as described above in the presence of ribonuclease inhibitor (2.5 units/100 µl) and the products were analyzed by SDS-PAGE and fluorography.

Analyses of In Vitro Translation Products by Immunoprecipitation

Immunoprecipitation of in vitro translation products was carried out similarly to Morris-Krsinich et al. (31). Samples from the translation of MCMV RNA were diluted with one volume of phosphate-buffered saline (PBS, 3 m$\underline{M}$ KH_2PO_4, 7 m$\underline{M}$ Na_2HPO_4, 0.15 M NaCl, pH 7.0), and 10 to 40 µl antiserum to MCMV was added. After an overnight incubation of the mixture at 4°C, 0.2 volumes of 3 m$\underline{M}$ KCl was added to the complex.

Pansorbin (100 µl) was washed with 1 ml PBS and pelleted at 2600 rpm in GLC at 4°C for 15 minutes. The immunocomplex mixture was added to the pansorbin pellet and allowed to bind during a 30 to 60 minutes incubation (on ice) period. Centrifugation was performed at 2600 rpm for 15 minutes (4°C) in GLC to obtain the antigen-antibody/pansorbin complex. This complex was washed three times (each

time with 1 ml PBS) before elution of the immunocomplex with 300 µl Laemmli (21) sample buffer after heating to 90°C for 3 minutes. Samples were analyzed by 12.5% SDS-PAGE and fluorography (30).

In control experiments, normal rabbit serum was substituted for the MCMV antiserum. In some cases, goat anti-rabbit serum was used to capture the immunocomplexes instead of pansorbin. Such reactions were performed at 30°C for 20 minutes. The rest of the procedure was similar to that described for the pansorbin except that no centrifugation was done after addition of the dissolving buffer during the final step. At every step, aliquots of supernatant were saved for radioactive analyses.

CHAPTER IV

RESULTS

Molecular Weight Determination of MCMV Coat Protein

Molecular weight determination of MCMV coat protein by 12.5% PAGE is shown in Figures 1 and 2. As seen, the major protein band from pure MCMV corresponds to 29 KDa.

At a position corresponding to 50 KDa, a minor band was observed. It may be dimers of coat protein. The other minor band (<1% of the viral structural protein) of 30 KDa could have emerged from a modified form of the MCMV coat protein.

Ouchterlony Test

The immunological relationship between MCMV coat protein and the MCMV antiserum is presented in Figure 3. As seen, the MCMV coat protein was recognized by the antiserum raised against MCMV.

At viral dilutions up to 1/128, cross reactivity with MCMV antiserum (1:4; central well) was observed. The extent of MCMV-antibody reaction is inhibited as the coat protein became more and more diluted. This observation suggests that MCMV capsid protein reacts with the MCMV antiserum. Results also show that the capsid protein is the major MCMV protein.

Figure 1. SDS-PAGE Showing the Molecular Weight of MCMV Coat Protein.

Thirty to forty microliters of MCMV was incubated with equal volumes of SDS-dissolving buffer at 90°C for 15 minutes. Samples were electrophoresed on 10 or 12.5% gels for 6-8 hours at 6 mA and stained in Coomassie Blue. Lanes 1-3, different amounts of the virus. Lane 4, low molecular weight standards: A: phosphorylase B (91.5K); B: bovine serum albumin (BSA) (66.2K); C: Ovalbumin (45K); D: carbonic anhydrase (31K); E: soybean trypsin inhibitor (21.5K); F: lysozyme (14.4K).

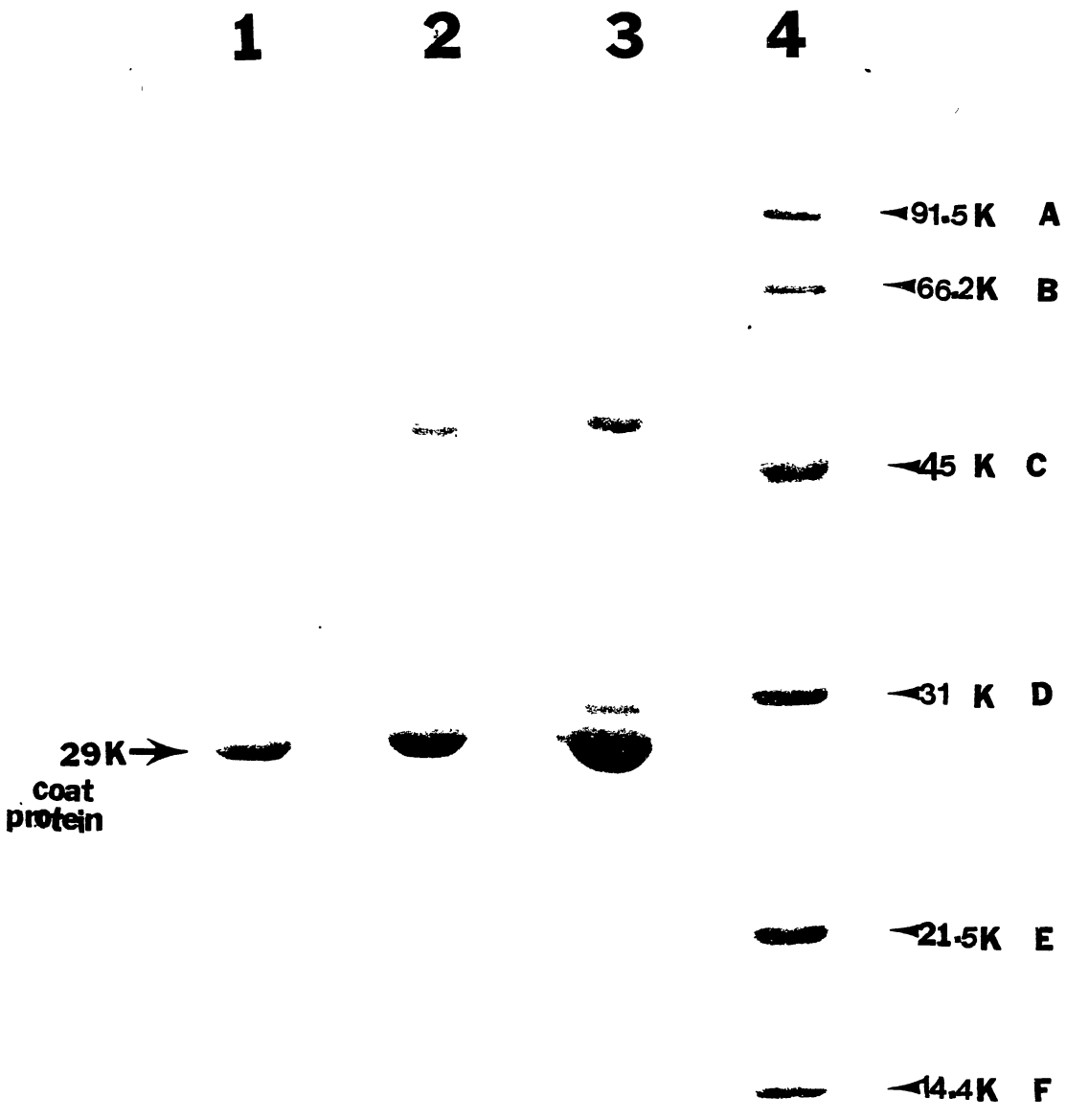

1
2
3
4
91.5K A
66.2K B
45 K C
31 K D
29K
coat protein
21.5K E
14.4K F

Figure 2. Estimation of the Molecular Weight of MCMV-Coat Protein.

A-F are low molecular weight standards described earlier and X is the MCMV-coat protein.

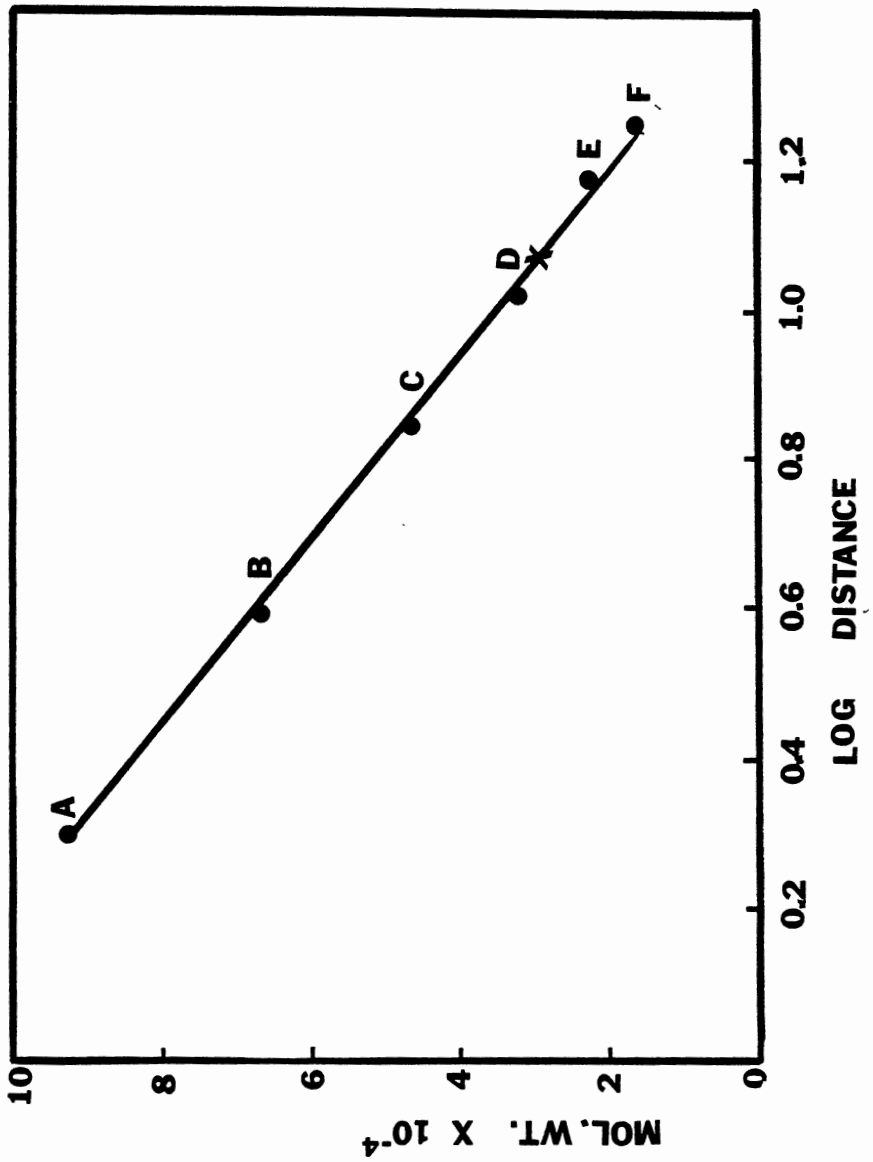
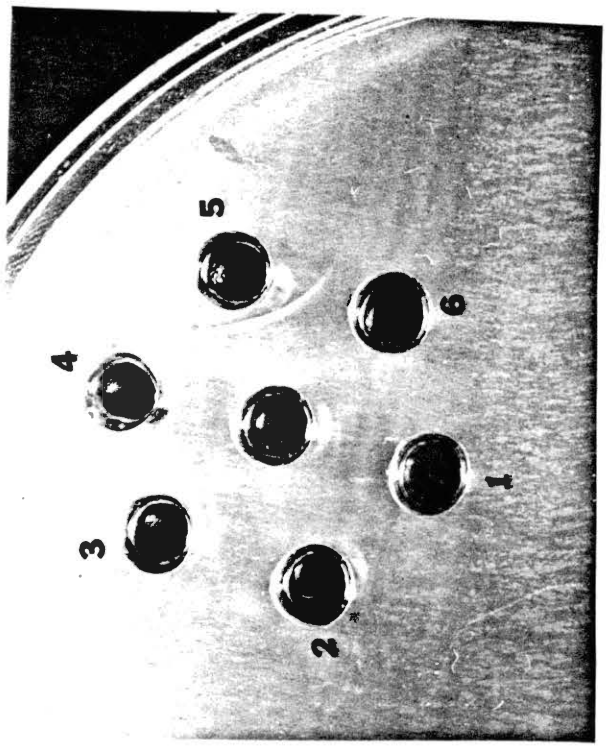

MOL. WT. X 10⁻⁴
10
8
6
4
2
0
0.2
0.4
0.6
0.8
1.0
1.2
LOG DISTANCE
A
B
C
D
E
F

Figure 3. Ouchterlony Test Showing Cross Reactivity Between MCMV-Antiserum and Pure Preparation of MCMV.

Central well: 1:2 or 1:4 dilution of MCMV-antiserum; outer wells: 1:- 1:4, 2:- 1:8, 3:- 1:16, 4:- 1:64, 5:- 1:128, 6:- saline.


5
6
4
1
3
2

Microprecipitation Assay

The results of the microprecipitation assay of MCMV using MCMV-antiserum raised against purified MCMV is displayed in Figure 4. At MCMV dilutions down to 1/128 cross reactivity with several dilutions of MCMV antiserum occurred. However, the titer for MCMV antiserum was estimated to be 1/64 as shown by the microprecipitates formed between dilutions of MCMV and MCMV-antiserum. The reasons for this low liter are unknown.

The Molecular Weight of MCMV RNA

The molecular weight of MCMV RNA was estimated from a plot of log mobility versus square root of base pairs (Figure 6) of the data obtained from 1.5% agarose gel in the presence of 2.2 mM formaldehyde. By extrapolation, MCMV RNA occurred at a position corresponding to 4360 bases. From this, its molecular weight was estimated to be 1.4 x 10^6 daltons.

Gel Filtration of Wheat Germ Extracts

Figure 7 is an elution pattern of wheat germ extract on Sephadex G-25 (medium) column. Fractions with A_{260} between 0.4 and 0.8 (enclosed by arrows) and under peak 1, were pooled and the supernatant was collected as potential in vitro translation system. Peak 2 fractions which probably contains some nucleotides and other materials that might inhibit translation were avoided.

Figure 4. Cross Reactivity Between MCMV-Antiserum and Pure MCMV in a Microprecipitation Assay.

Both the MCMV-antiserum and MCMV were diluted down to 1/128. Extent of reactivity is represented by dots.

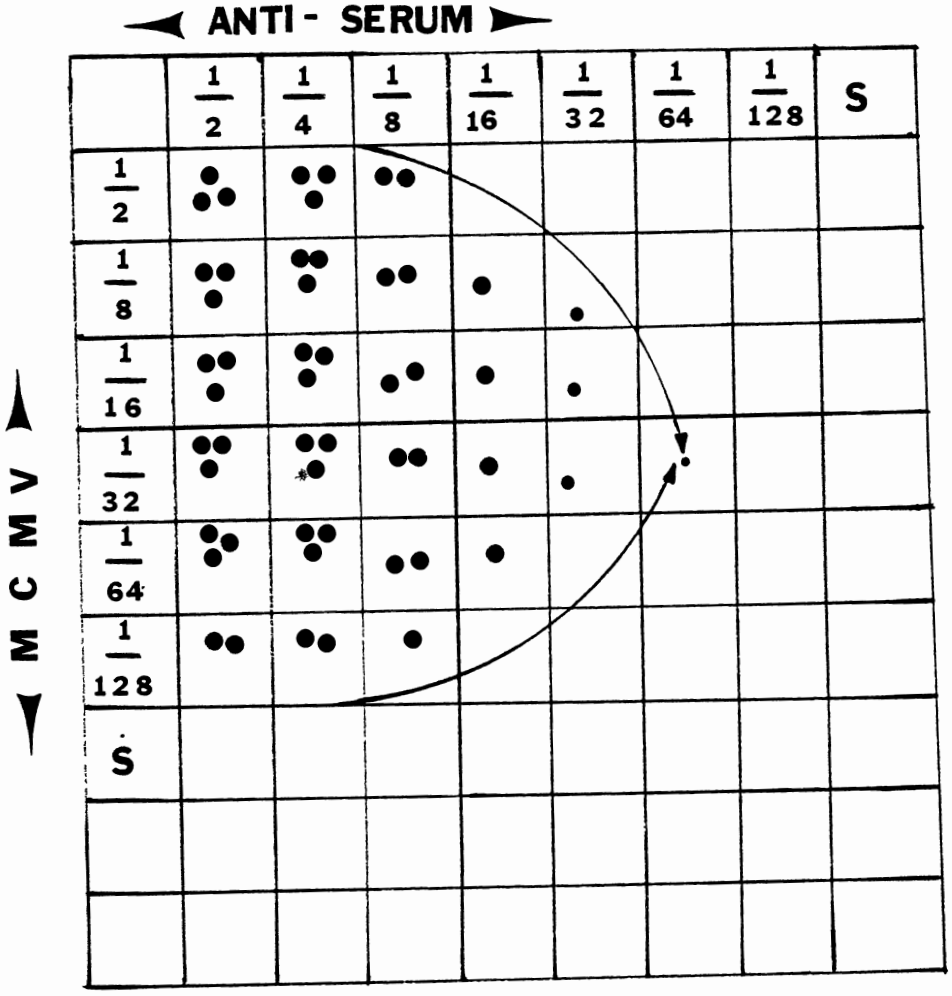

ANTI - SERUM
MCMV
1/2
1/4
1/8
1/16
1/32
1/64
1/128
S

Figure 5. Agarose Gel Electrophoresis of MCMV RNA in the Presence of 2.2 m*M* Formaldehyde.

Lane 1, 3 and 4 are maize polysomal RNAs used as standard, lane 2 is MCMV RNA. Details are in materials and methods section.

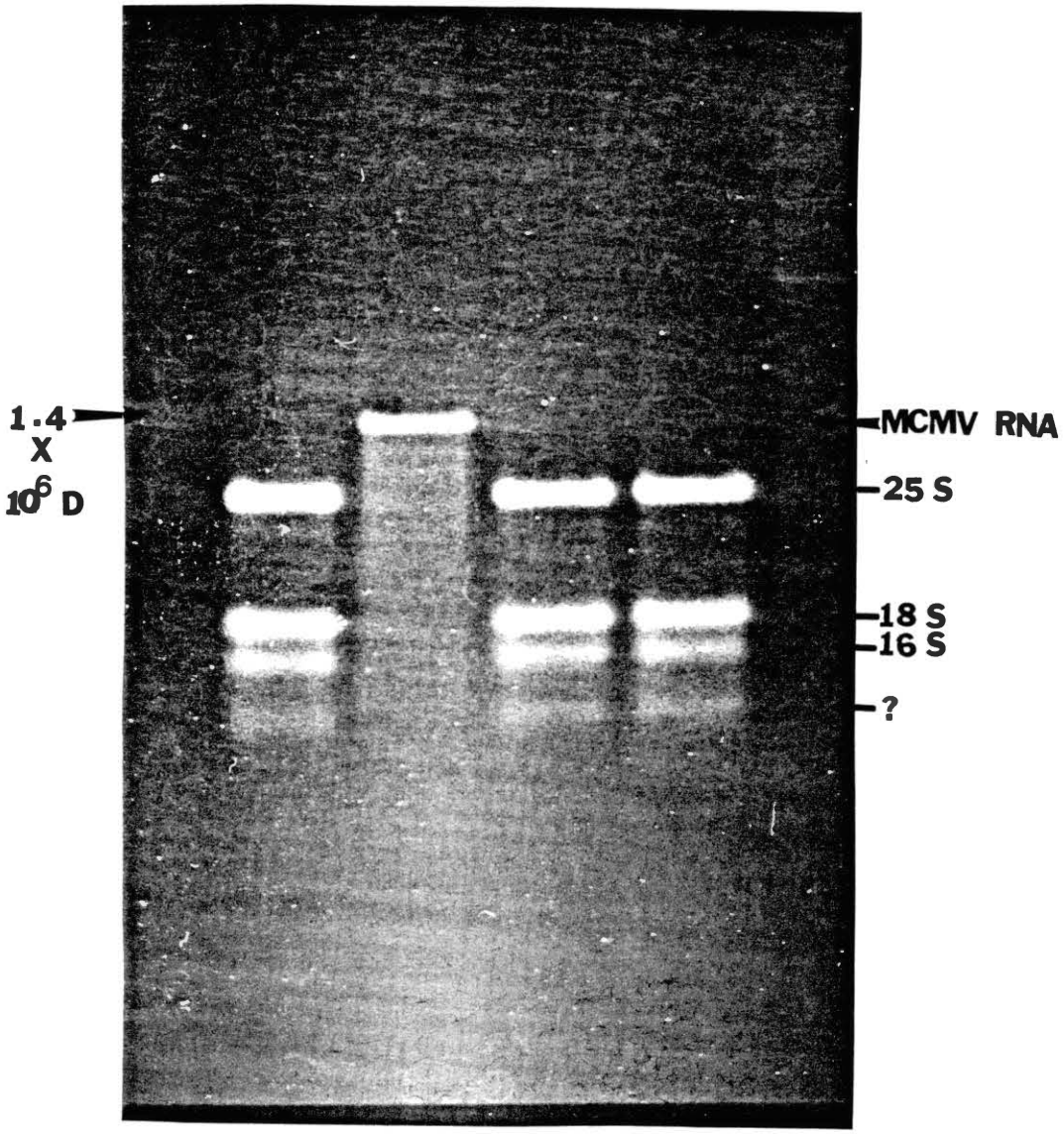

1.4
X
10^6 D
MCMV RNA
25 S
18 S
16 S
?

Figure 6. A Plot of Log Mobility Versus Square Root of Base Pairs Showing the Molecular Weight of MCMV RNA.

Mobilities were estimated from data in Figure 5. Standards were maize polysomal RNAs (A:- 25S, B:- 18S, C:- 16S, and E:- MCMV RNA.

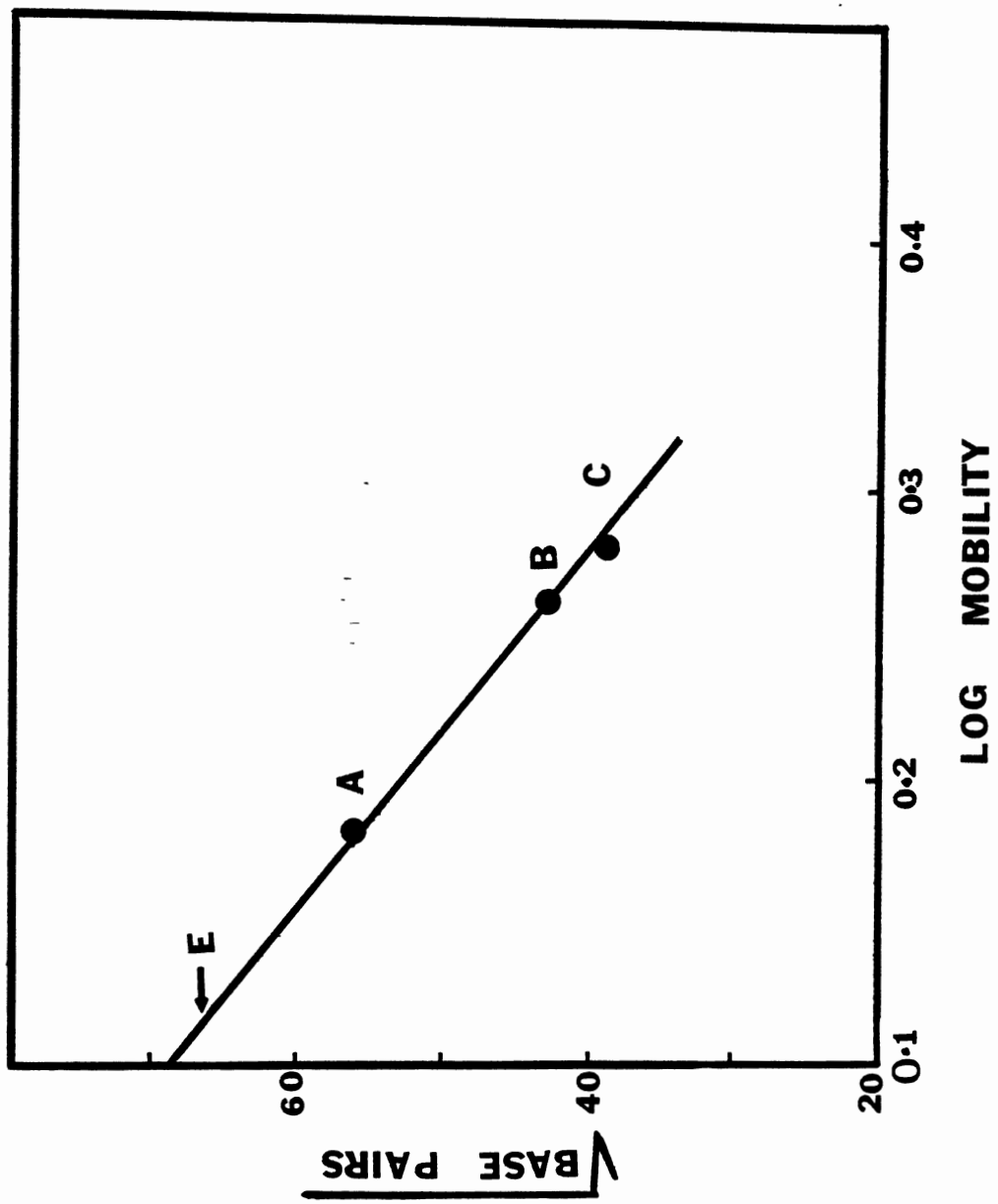
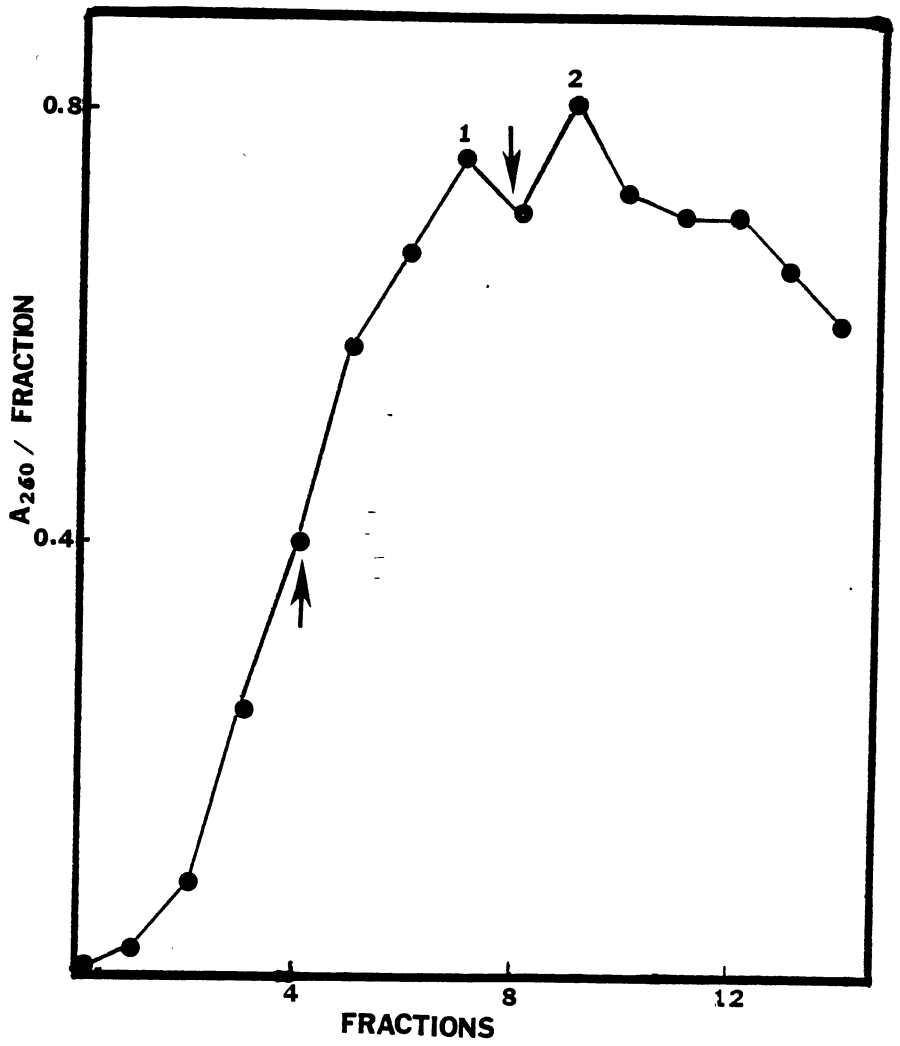

√BASE PAIRS
60
40
20
0·1
0·2
0·3
0·4
LOG MOBILITY
A
B
C
E

Figure 7. Gel Filtration Pattern of Wheat Germ Extraction on Sephadex G-25 (Medium).

Column dimensions were 2.5 x 45 cm and 0.5 ml fractions were collected in the elution buffer described in materials and methods section. Peak 1 fraction enclosed (arrows) were pooled and used for transition.


0.8
0.4
A_{260} / FRACTION
1
2
4
8
12
FRACTIONS

Effect of K^+ on (^3H)-Leucine Incorporation and Protein Synthesis In Vitro by MCMV RNA

The amount of K^+ in the translation system influenced the rate of incorporation of (^3H)-leucine into proteins in the presence of 125 µg/ml RNA and 1.5 m$\underline{M}$ Mg^{2+} (Figure 8).

An initial stimulation of (^3H)-leucine incorporation was observed up to 80 m$\underline{M}$ total K^+ in the translation system. At K^+ concentrations higher than 80 m$\underline{M}$ total, strong inhibitory effect on translation is seen. In fact, translation is completely halted at 160 m$\underline{M}$ K^+ total in the translation medium. However, the pattern of translation products remained unchanged over a wide range of K^+ concentrations.

Mg^{2+} Dependence of (^3H)-Leucine Incorporation In Vitro by MCMV RNA

Figure 9 shows that in vitro translation of MCMV RNA in wheat germ extracts is Mg^{2+} dependent. Optimum translation is achieved at 2.1 m$\underline{M}$ total Mg^{2+} (corresponding to 0.6 m$\underline{M}$ added) in the translation system since the wheat germ extract contributes 1.5 m$\underline{M}$ Mg^{2+}.

At Mg^{2+} concentrations higher than 2.1 m$\underline{M}$ total, there is a substantial inhibition of (^3H)-leucine incorporation.

It was observed from SDS-PAGE analysis of in vitro translation products that the pattern of polypeptides synthesized is not influenced by Mg^{2+} levels though the absolute amounts of the various polypeptides changed.

Figure 8. Effect of K^+ on (^3H)-Leucine Incorporation into Polypeptides by MCMV RNA In Vitro.

Translation was performed in the presence of 1.5 mM $Mg(OAc)_2$, 125 µg/ml RNA at 25°C for 90 minutes. KoAc concentration (total) was varied from 40-160 mM in the translation medium.

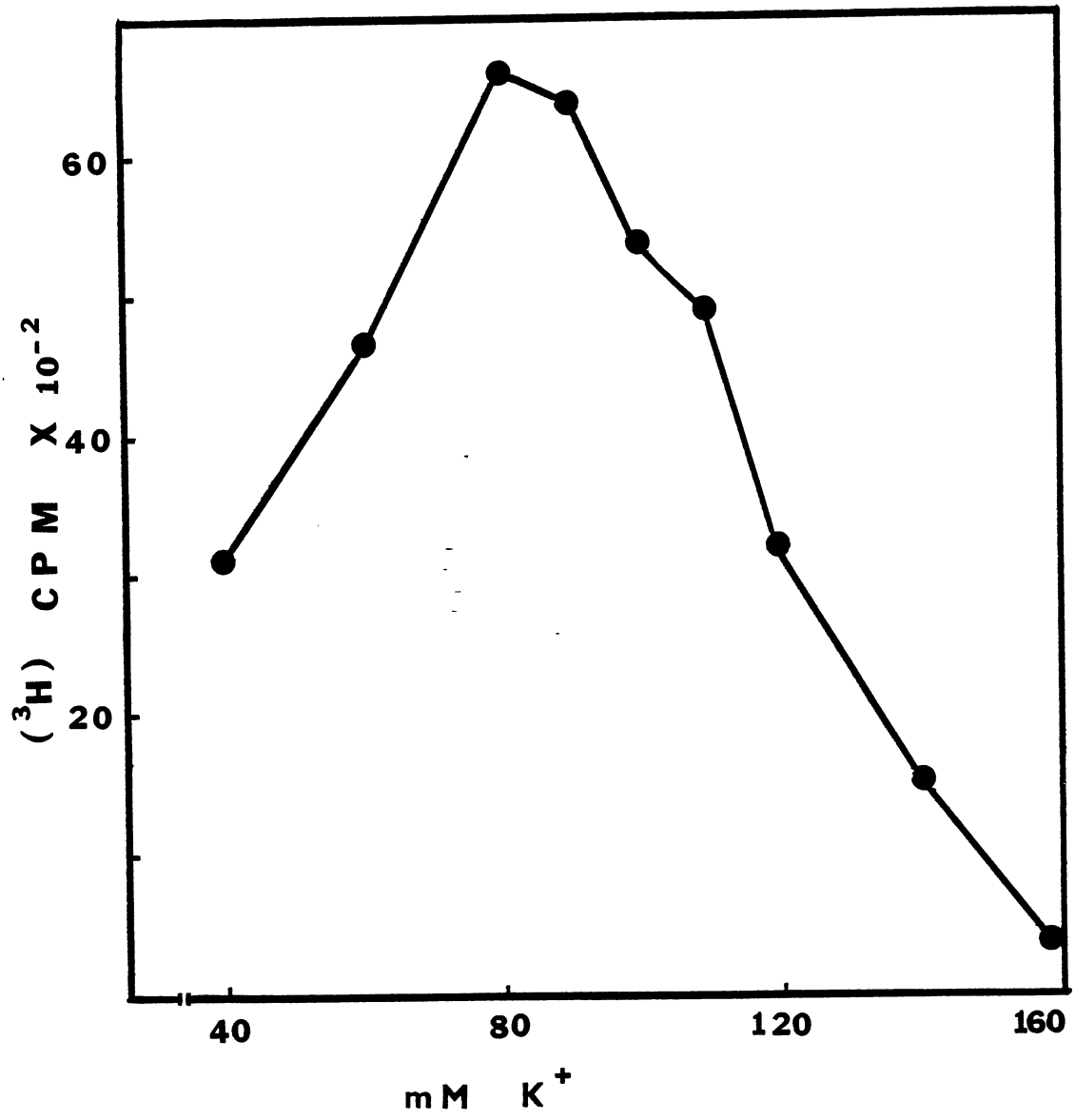
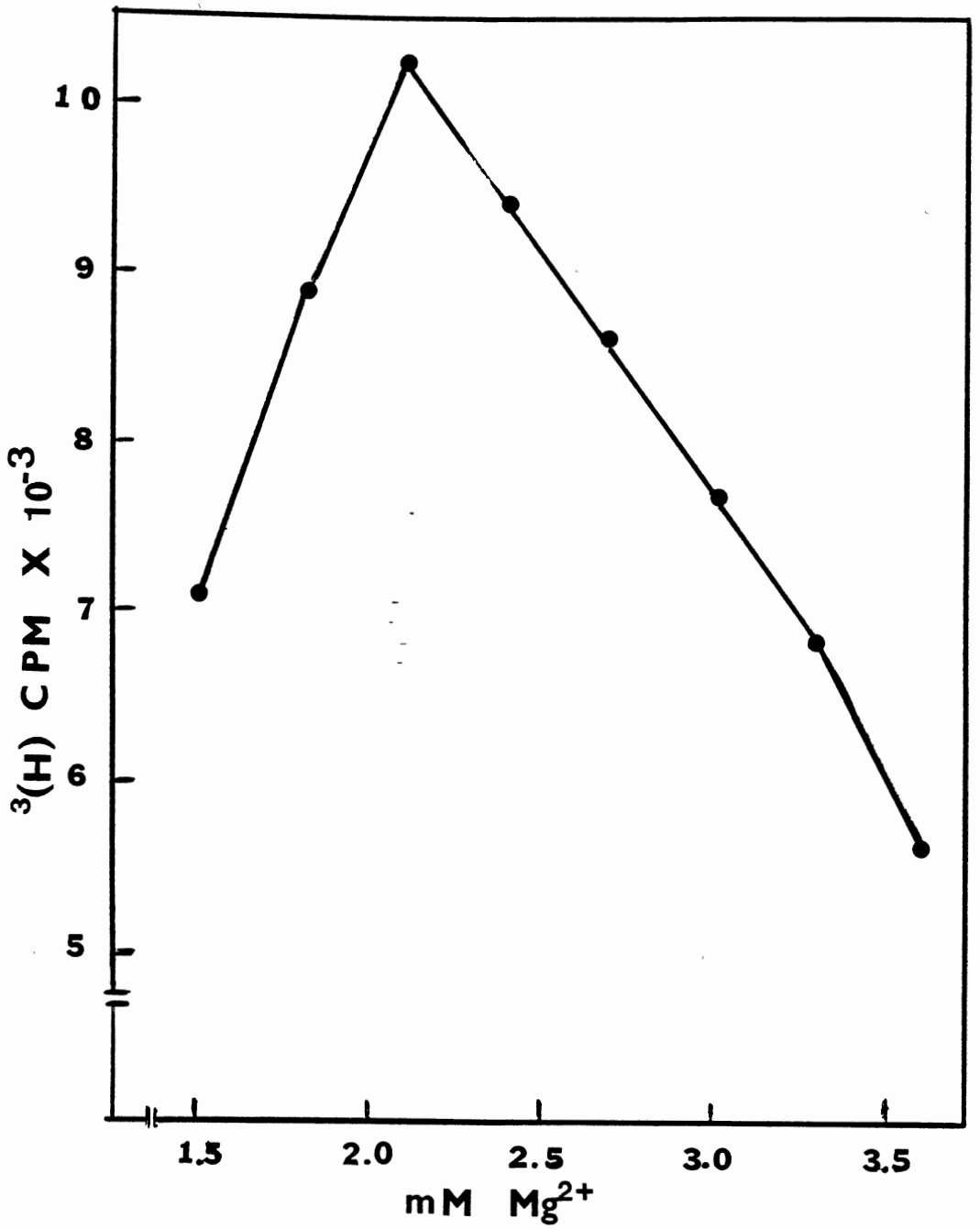

60
40
20
(^{3}H) CPM X 10^{-2}
40
80
120
160
mM K^+

Figure 9. Mg^{2+} Dependence of (^3H)-Leucine Incorporation In Vitro by MCMV RNA in Wheat Germ Extracts.

In vitro translation of MCMV RNA was performed under varying Mg^{2+} concentration as outlined in the materials and methods section.


10
9
8
7
6
5
^{3}(H) CPM X 10^{-3}
1.5
2.0
2.5
3.0
3.5
mM Mg^{2+}

The Effect of mRNA Concentration on (^{3}H)-Leucine Incorporation

In vitro translation of MCMV RNA in wheat germ extract is mRNA dependent. As seen in Figure 10, optimum incorporation of (^{3}H)-leucine into TCA precipitable proteins was obtained at 75-125 µg/ml RNA in the translation system. Inhibition of translation occurred at higher RNA levels as can be seen by the 80% decrease in the rate of (^{3}H)-leucine incorporation at 200 µg/ml RNA.

It is, however, not known whether the composition of the translation products synthesized was affected by RNA levels during translation.

Time Course of (^{3}H)-Leucine Incorporation into Polypeptides by MCMV RNA In Vitro

The time course of MCMV RNA translation in wheat germ extracts under optimized conditions is shown in Figure 11. As seen, there is an initial lag phase during the first 5 minutes of (^{3}H)-leucine incorporation. This is followed by a rapid linear phase of incorporation over a 30 minute period, and there is a gradual decline. The synthesis reaches a plateau after an hour of incubation.

The sigmoidal nature of the curve may be a reflection of the nature and amounts of polypeptides made in vitro over the 90 minutes period.

Figure 10. Effect of MCMV RNA on (^{3}H)-Leucine Incorporation into Polypeptides In Vitro Translation.

Translation was performed in the presence of 2 mM $Mg(OAc)_2$, 100 mM KOAc at 25°C for 90 minutes as described in materials and methods. MCMV RNA was varied from 0-200 µg/ml in the translation medium.

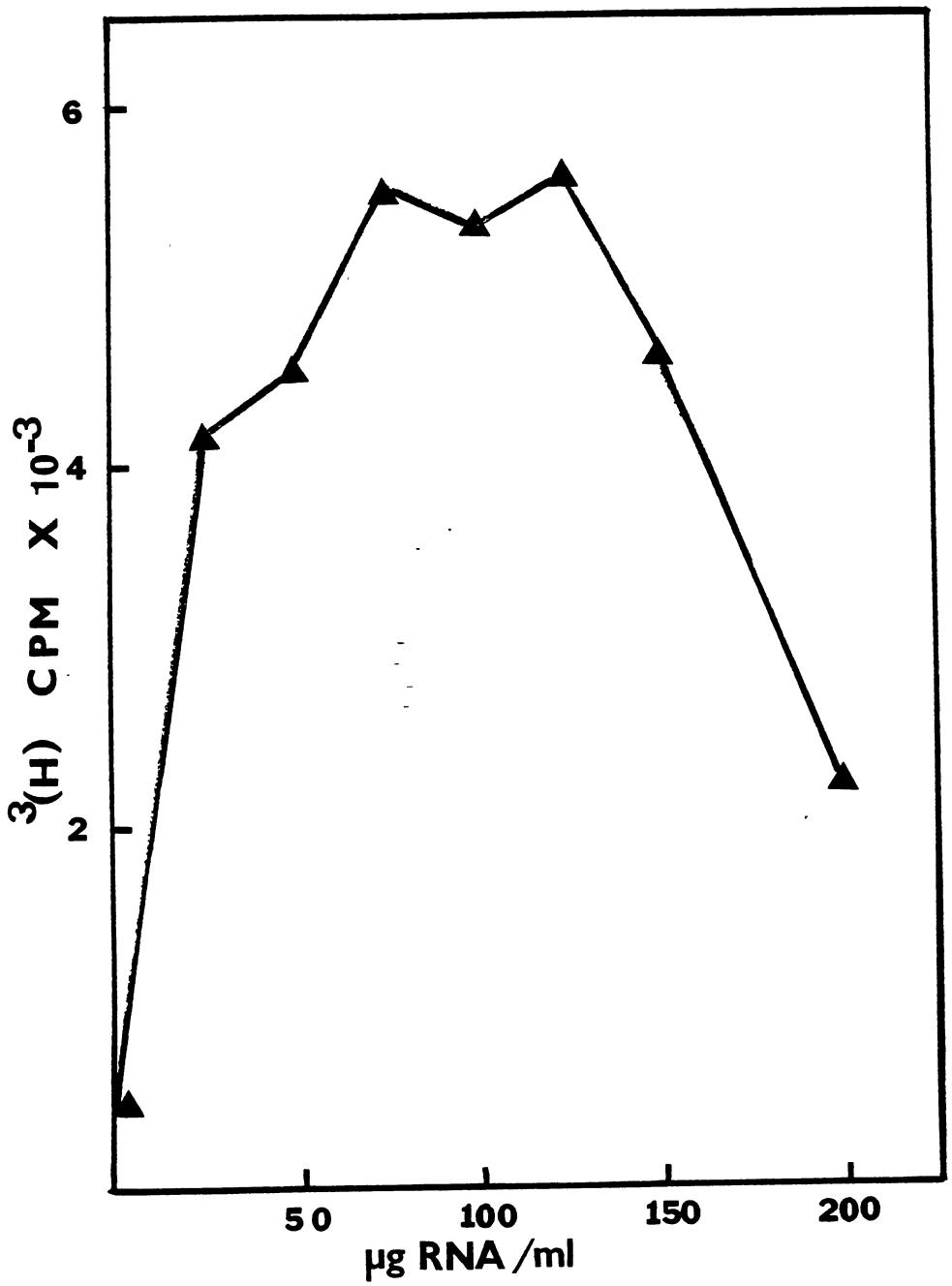
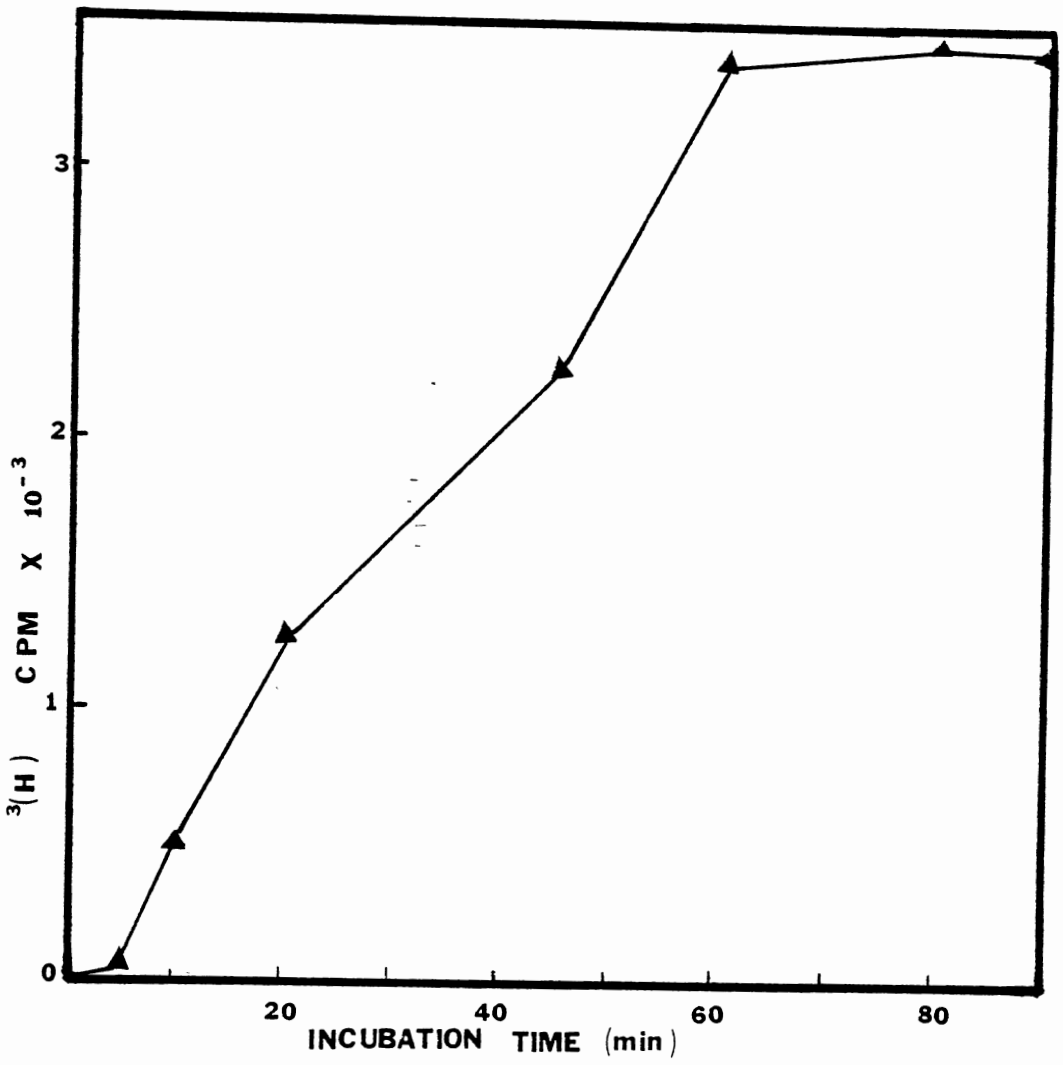

6
4
2
^{3}(H) CPM X 10^{-3}
50
100
150
200
µg RNA /ml

Figure 11. Time Course of (^{3}H)-Leucine Incorporation into Polypeptides by MCMV RNA In Vitro.

Translation in wheat germ extracts was performed under optimized concentrations of Mg^{2+}, K^+ and MCMV RNA as described in materials and methods. At different incubation times, (^{3}H)-leucine incorporation was determined by scintillation analysis.


^{3}(H) CPM X 10^{-3}
0
1
2
3
20
40
60
80
INCUBATION TIME (min)

Pattern of Protein Synthesized During the Time Course Studies

Figure 12 is an autoradiogram showing the pattern of proteins made in vitro by MCMV RNA during a time course study.

Clearly, no polypeptide is made during the first five minutes (lag period) (lane 1). Low molecular weight polypeptides appeared first (lanes 2-3), followed by some high molecular weight products during the late periods of translation (lanes 4-7).

Ten minutes after the commencement of translation, the synthesis of 16 KDa and 18 KDa polypeptides is completed. The coat protein (29 KDa) does not appear until after 10 minutes. However, its synthesis appears to be completed after 20 minutes. It then begins to be processed or modified partially into a 28 KDa product just 20 minutes after its appearance. The synthesis of 25 KDa polypeptides begins just 10 minutes during translation and it is probably completely made after 20 minutes.

The higher molecular weight products 36, 38, 45, and 46 KDa began to appear 40 minutes into the translation period.

Generally, more and more of all the translation products (lanes 1-7) are made as translation advanced.

Immunoprecipitation of MCMV RNA In Vitro Translation Products

The immunoprecipitation of some in vitro translation products is shown in Figure 13. There was specific cross reactivity between the MCMV antiserum and the 29 KDa and 25 KDa translation products. Thus,

Figure 12. Fluorogram Showing Pattern of Polypeptides Synthesized by MCMV RNA During Time Course Studies.

As described in the footnote of Figure 11, translation products were analyzed by SDS-PAGE (10% gels) after different incubation times. Fluorograms were exposed to Kodak O-mat film at 70°C for 14 days. Lane 1: -5 minutes, 2:- 10 minutes, 3:- 20 minutes, 4:- 40 minutes, 5:- 60 minutes, 6:- 80 minutes, 7:- 90 minutes.

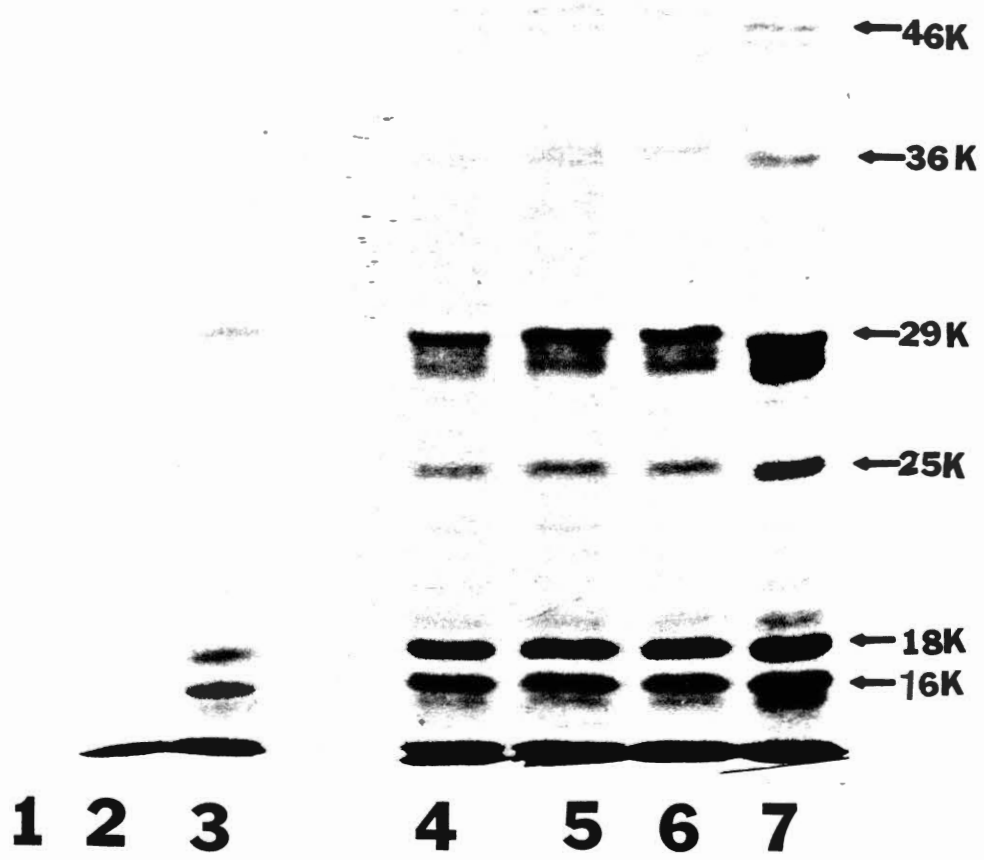
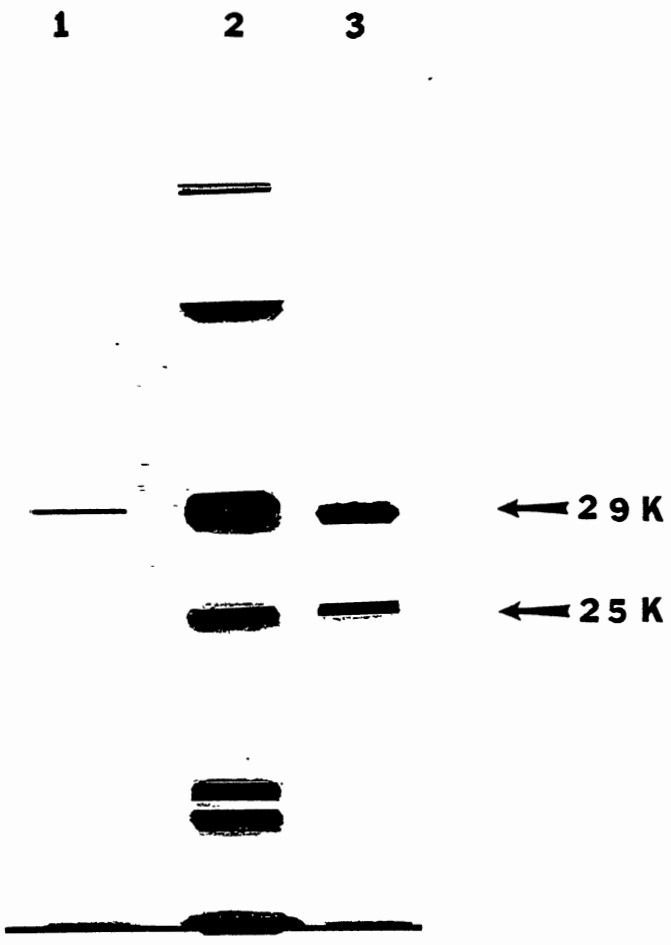

46K
36K
29K
25K
18K
16K
1 2 3 4 5 6 7

Figure 13. Fluorogram Showing Immunoprecipitation of Some In Vitro Translation Products by Anti-MCMV Serum.

Translation products were reacted with MCMV antiserum as outlined under materials and methods section. Immunocomplexes were captured on pansorbin bed, analyzed on 12.5% gels and fluorographed. Lanes 1: normal serum, 2: complete in vitro translation products, 3: MCMV-antiserum.


1
2
3
29K
25K

compared to the control, enrichment in radioactivity is seen. A rather unexpected observation is the fact that the 25 KDa polypeptide showed higher affinity for the MCMV-antiserum than the 29 KDa material.

Sucrose Gradient-Fractionation of MCMV RNA

MCMV RNA was fractionated according to size on a sucrose gradient. Results in Figure 14 show that A_{260} was highest in region E and indicates the presence of full length RNA in this region. The A_{260} material in regions B-D may indicate the presence of subgenomic RNAs.

(^{3}H)-Leucine Labeled Products Synthesized by Sucrose Gradient MCMV RNA Fractions

The results presented in Figure 15 show the (^{3}H)-leucine labeled products synthesized by various sucrose gradient MDMV RNA regions. Region E (containing full length MCMV RNA) induced a protein synthesis similar to that of unfractionated RNA (lane F). The sub-genomic region (B-D) induced the synthesis of mostly low molecular weight major polypeptides. Message from region D seemed to induce the synthesis of more materials than regions B and C.

The synthesis of high molecular weight polypeptides (36, 45 and 46 KDa) is induced by full length RNA (region E). It must be stated, however, that the full length RNA also induced the synthesis of the low molecular weight polypeptides similar to that of regions B-D and the unfractionated RNA.

The cost protein (29 KDa) and the 25 KDa polypeptide were synthesized by messages from all regions (B-E) except A. The sub-genomic

Figure 14. Sucrose Gradient of MCMV RNA.

Sedimentation was from right to left. Each of the regions A-F were pooled as indicated in the figure.

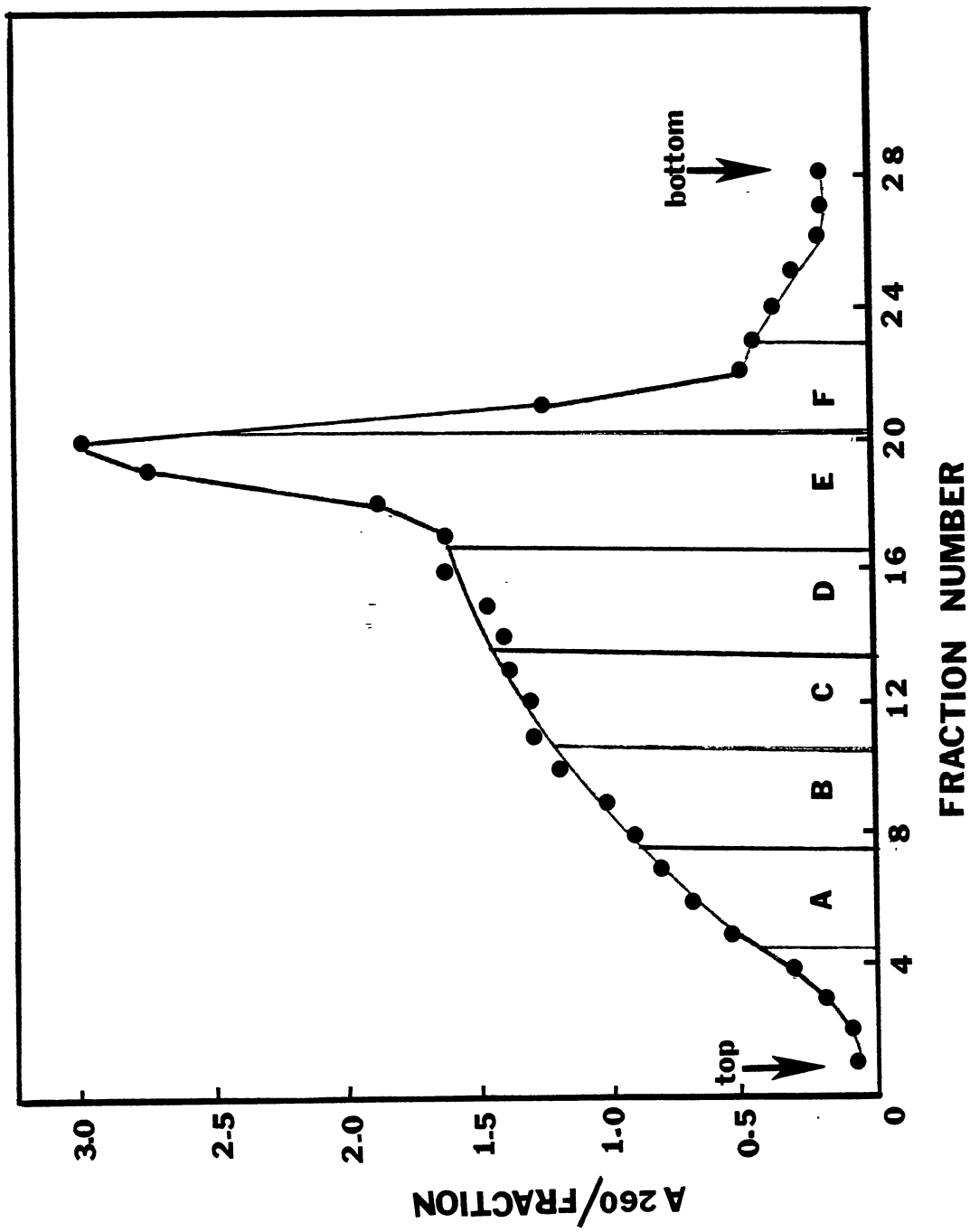
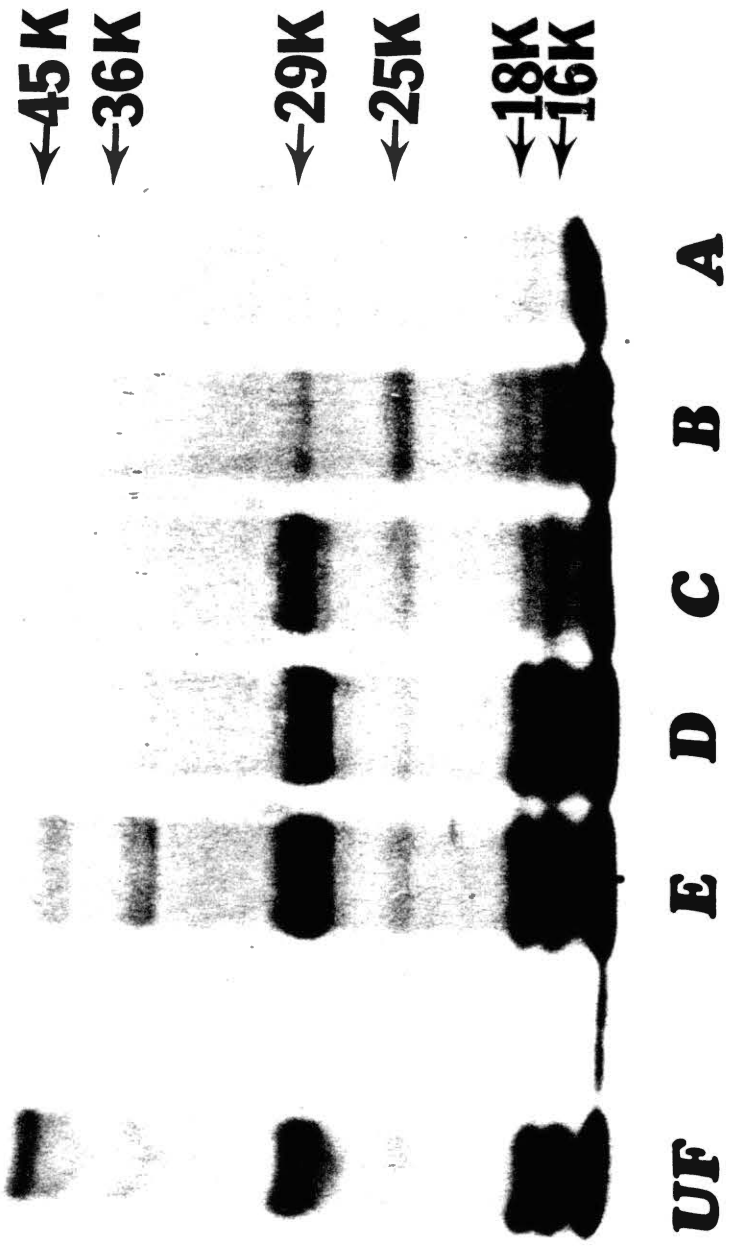

A 260/FRACTION
3.0
2.5
2.0
1.5
1.0
0.5
0
top
bottom
A
B
C
D
E
F
4
8
12
16
20
24
28
FRACTION NUMBER

Figure 15. A Fluorogram Showing Polypeptides Synthesized by Sucrose Gradient Fractions of MCMV RNA.

Equal volumes of sucrose gradient MCMV RNA fractions (A-E) were translated separately. Products were analyzed on 12.5% gels. Gels were exposed to Kodak O-mat films for 14 days at -70°C.


45K
36K
29K
25K
18K
16K
UF
E
D
C
B
A

region B appeared a more efficient messenger for the synthesis of 25K than all regions (C-E). No specific region induced the synthesis of either the coat protein (29 KDa) or the 25 KDa alone.

The pattern of protein synthesis seen here may have been influenced by some factors including nucleases action and/or the aggregation of messages during translation. To investigate this, translation of various RNA regions was done in the presence of ribonuclease inhibitor in the translation medium.

Translation of Sucrose Gradient Fractions of MCMV RNA in the Presence of Ribonuclease Inhibitor

To investigate whether nucleases contributed towards the in vitro translation of message to polypeptides, 2.5 units ribonuclease inhibitor per 100 µl was used in the translation set up.

Essentially, there was no difference in the nature of translation products encoded for by the RNAs compared to the situation when there was no ribonuclease inhibitor in the translation medium. The only obvious difference was the synthesis of more high molecular weight materials when the inhibitor was present in the translation medium.

CHAPTER V

DISCUSSION AND CONCLUSION

The molecular weights of the MCMV coat protein (29 KDa) and the MCMV RNA (1.4×10^6 Da) as presented in Figures 1 and 5, respectively, appear to be similar to other members of the sobemoviruses (6). Another interesting observation was the fact that the single sharp MCMV RNA band is followed by a streak of lower molecular weight material. This characteristic is found among some members of the sobemovirus group in which such a streak includes sub-genomic RNAs. This speculation was later confirmed from the RNA fractionation experiment described in Figure 14.

The serological cross reaction between MCMV antiserum and its major capsid protein is demonstrated by the data obtained from the serological assays. It should, however, be mentioned that MCMV-antiserum showed a low titer value (1:64) in the microprecipitation assay. In the experiments described in Figure 13, MCMV-antiserum cross reacted with the 29 KDa and 25 KDa products made in vitro, thus suggesting a relationship between these two products. The normal serum showed slight reaction with these products. Perhaps, a mono-specific antiserum raised against the viral capsid protein when used could lead to a better understanding of the serological relationship between MCMV and the in vitro translation products made by the viral RNA.

As seen in Figures 8 and 9, respectively, translational efficiency of MCMV RNA is influenced by the levels of K^+ and Mg^{2+} in the environment. Both Mg^{2+} and K^+ caused a substantial stimulation of (^{3}H)-leucine incorporation into proteins by MCMV RNA. The optimal levels of Mg^{2+} (2.1 m$\underline{M}$) and K^+ (80-100 m$\underline{M}$) were noted to cause maximum translational efficiency. The inhibitory effect of high concentrations of Mg^{2+} and K^+ on in vitro translation may be due to the effect of these ions on the secondary structure of some of the enzymes involved in trnaslation or on the structure of the mRNA. It is interesting, however, that the types of polypeptides coded by MCMV RNA remained unchanged at all the Mg^{2+} and K^+ concentrations tested. Thus, Mg^{2+} and K^+ levels do not seem to alter the sites of initiation of protein synthesis, although the rate at which the message is translated may be affected.

The level of RNA in the translation system also influenced the translational efficiency to a great extent as seen in Figure 10. However, it is not known whether this effect affects the types of products synthesized in vitro. Such an information could be obtained if the in vitro translation products made under varying amounts of MCMV RNA were analyzed by SDS-PAGE.

When 75 to 100 µg/ml MCMV RNA was introduced into the wheat germ translation system several high and low molecular weight polypeptides appeared. The major ones were 46, 45, 36, 29, 25, 18 and 16 KDa.

From theoretical considerations, a single stranded RNA of 4360 bases is capable of coding for an average of 1453.3 amino acids based on the genetic code of 3 bases per amino acid. Then, on the assumption that each amino acid contains 110 daltons, MCMV RNA is capable of

directing the synthesis of a total of 160 KDa of protein. However, when put together, it is estimated that over 340 KDa of protein is encoded for by MCMV RNA. Thus, MCMV RNA seems to operate at a level which exceeds twice its coding capacity. Obviously, this virus resorts to some unknown mechanism for complete expression of its entire genome.

The synthesis of some polypeptides by the sub-genomic region of MCMV RNA as shown in Figure 16 suggests that this virus employs sub-genomic RNAs in addition to the full length RNA for the expression of its genome. The sub-genomic RNAs contain the message necessary to code for the synthesis of complete polypeptides. By this mechanism, MCMV seems to behave like SBMV (16). Considering the products made by MCMV RNA relative to SBMV gene products, one could speculate that the 25 KDa is similar to 14 KDa, 29 KDa protein is similar to 29 KDa and the higher molecular weight proteins are similar. In contrast, however, the capsid protein of MCMV does not appear to be made exclusively by the MCMV sub-genomic RNA. Also, the full length RNA does not make coat protein in SBMV but does for lucerne transient streak virus (31). This point is supported by the observation that both the full length and the sub-genomic RNAs contained messages for the synthesis of the coat protein. A similar result was obtained even in the presence of ribonuclease inhibitor.

It is known that in some plant viruses proteolytic processing of large polypeptide precursors into smaller products form an active part of the strategy of gene expression. To investigate whether proteolytic cleavage is part of the mechanism by which MCMV translate its entire message into various polypeptides, in vitro translation of the viral

RNA in wheat germ extracts under optimum conditions of Mg^{2+}, K^{+} and RNA was performed in a time course study. The data presented in Figure 12 show that low molecular weight products appeared first followed by the heavier ones as translation advanced. This observation does not support an active involvement of proteolytic processing as part of the MCMV RNA translational strategy. Another interesting observation was the fact that the 29K product seems to be processed into a 28K product by an unknown mechanism (perhaps proteolysis) as translation advanced. It was also noticed that more of each product built up with time until translation plateaus at 60-90 minutes of incubation.

In conclusion, MCMV, a virus with a capsid protein of 29 KDa and possessing a single stranded RNA genome of 1.4×10^{6} daltons codes for several polypeptides in vitro in wheat germ extract. Under optimal conditions of 2.1 mM Mg^{2+}, 80 to 100 mM K^{+}, and 75 to 125 µg/ml RNA, the proteins encoded for by the viral RNA include the 46, 45, 36, 29, 25, 18 and 16 kdaltons. Among these products, the 29 KDa and 25 KDa materials were recognized by an antiserum raised against the virus.

The mechanism by which these polypeptides are made still remains unclear. From our findings, however, one could propose that processing of sub-genomic messages in addition to the full length RNA plays an important role in the strategy of gene expression of MCMV. The involvement of proteolytic cleavage as part of the mechanism is not supported by our findings.

CHAPTER VI

SUMMARY

Maize chlorotic mottle virus (MCMV), a single stranded RNA virus possesses a major coat protein of molecular weight 29 KDa. Its single stranded genome of 4360 bases (1.4×10^6 Da) coded for several polypeptides when translated in vitro in wheat germ extracts. Operating at a capacity of about twice that of its coding capacity, MCMV RNA encoded for major polypeptides including the 46, 45, 36, 29, 25, 18 and 16 KDa. The major products are made in abundance when translation conditions are optimal (2.1 mM Mg^{2+}, 80 to 100 mM K^{+}, and 75 to 125 µg/ml RNA). These 29 KDa and 25 KDa products cross reacted with an antiserum prepared against MCMV. These products also slightly cross reacted with the normal serum which was used as control.

While proteolytic processing did not seem to be important in the formation of these products, the virus appeared to employ sub-genomic RNAs as part of its translational strategy. This observation offers partial explanation to the mechanism of gene expression by MCMV.

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