PROTOPLAST AND CELL FREE SYSTEMS TO STUDY THE MOLECULAR BIOLOGY OF CAULIFLOWER MOSAIC VIRUS

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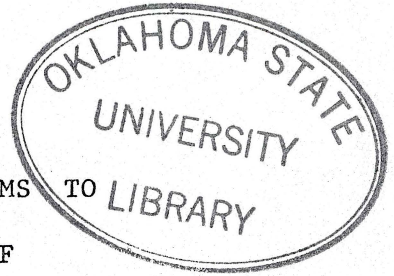
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STUDY THE MOLECULAR BIOLOGY OF

CAULIFLOWER MOSAIC VIRUS

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TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
	The Structure of the Virion	1
	CaMV Genome	2
	Transcription of CaMV	4
	Translational Products of CaMV	6
	Replication of CaMV	7
	Aim of the Present Investigation	8
II.	PROTOPLAST SYSTEM	9
	Methods	11
	Protoplast Preparation	11
	Purification of Protoplasts	12
	Evacuolation of Protoplasts	13
	Incorporation of Labelled Amino Acids into Protoplasts	13
	Purification of CaMV	14
	Purification of Plasmids Containing CaMV-DNA Sequences	15
	Packaging of CaMV and CaMV-DNA into Liposomes	17
	Infection of Protoplasts with Liposome Packaged CaMV and CaMV-DNA	18
	Detection of CaMV-DNA in the Protoplasts	19
	Detection of Coat Protein by Fluorescien-Labelled Antibodies	21
	Preparation of Antisera Against CaMV	21
	Purification of Immunoglobulin G (IgG) from the Serum	21
	Fluorescein Labelling of Goat Anti-rabbit Immunoglobulins	22
	Staining of CaMV-Infected Protoplasts with Fluorescein-Labelled Antibodies	22
	Results and Discussion	23
	Surface Sterilization of Turnip Leaves	23
	Protoplast Preparation	25
	Culture of Protoplasts	26
	Incorporation Studies	26
	Infection of Protoplasts by Liposome Packaged CaMV and CaMV-DNA	35
	Uptake of Liposomes with Protoplasts	35

Chapter | Page

Delivery of Liposomal Contents into Protoplasts.................................. 40
Fate of the Transferred Virus and DNA in the Protoplasts.................................. 42
Infection of Evacuolated Protoplasts........... 47
Transformation of Evacuolated Protoplasts from Cotton Suspension Cultures with Liposome Packaged CaMV...................... 51

III. CELL FREE SYSTEM.................................. 54

Methods.................................. 55
Preparation of Cell Free Extract........... 55

Results.................................. 56
Specificity of the Replicative Activity.... 61

Discussion.................................. 61

LITERATURE CITED.................................. 67

LIST OF TABLES

Tables | Page

I. Prehybridisation and Hybridisation Solutions.......... 20

II. Surface Sterilization of Turnip Leaves................ 24

III. Effects of Antibiotics and Polyamines on ^{3}H-Leu Incorporation into TCA Precipitable Proteins of Cultured Turnip Protoplasts.......................... 33

IV. Effect of Lipid Composition of Liposomes on Their Uptake by Protoplasts.............................. 37

V. Transfer Efficiency of ^{3}H-DNA from Positively Charged Liposomes.................................. 41

VI. Conditions Tested for Infecting Turnip Protoplasts with Liposome Packaged CaMV and CaMV-DNA........... 45

VII. Stimulation of the Replicative Activity Present in the Extract... 60

VIII. Attempts to Inhibit Replicase Activity................ 62

IX. Specificity of the Replicative Activity Present in the Extract... 63

LIST OF FIGURES

Figures | Page

1. Turnip Protoplasts 27

2. Stability of Turnip Protoplasts............................. 28

3. Time Course of ^{3}H-Leu Incorporated into TCA-Precipitable Proteins.................................... 29

4. Effects of ^{3}H-Leu Concentration on its Incorporation into TCA Precipitable Proteins............................ 31

5. Effect of Protoplast Concentration on the Incorporation of ^{3}H-Leu... 32

6. Incorporation of ^{3}H-Leu into Protoplasts when Cultured in Nagata and Takebe medium (1971) and Takebe et al. medium (1968)... 34

7. Effect of Concentration of Liposomes on Association with Protoplasts.. 39

8. Fate of CaMV-DNA in the Infected Protoplasts................ 43

9. Multiplication of CaMV in Evacuolated Protoplasts........... 48

10. Autoradiographs of the Hybridised Samples from Infected Evacuolated and Non-evacuolated Protoplasts............... 50

11. Infection of Cotton Suspension Culture Evacuolated Protoplasts with Liposome Packaged CaMV.................. 52

12. Time Course of Virus Multiplication in Cell Free System..... 57

13. Effect of EDTA on Virus Multiplication in Cell Free System.. 58

14. Effect of KCl on Virus Multiplication in Cell Free Sytem.... 59

Abbreviations Used in the Text

CaMV	Cauliflower Mosaic Virus
DNA	Deoxyribonucleic acid
HEPES	N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid
K & M buffer	Keller and Melchers (1973) buffer
Leu	Leucine
MES	2-(N-morpholino)-ethane sulfonic acid
ORF	Open reading frames
PC	Phosphatidyl choline
PEG	Polyethylene glycol
PPO	2,5-diphenyloxazole
PS	Phosphatidyl serine
PVA	Polyvinyl alcohol
SA	Stearylamine
TCA	Trichloroacetic acid

CHAPTER I

INTRODUCTION

Cauliflower Mosaic Virus (CaMV), the type virus of caulimoviruses, is an icosahedral virus whose genetic material is double stranded DNA (Shepherd et al., 1968). It infects plants belonging to the family Cruciferae. Other than crucifers, it has been shown to infect *Nicotiana clevelandii* (Hill & Campbell, 1968), and *Datura stramonium* (Lung & Pirone, 1972), both members of the family Solanaceae. It causes vein clearing, and stunting of the young leaves, and a general mosaic pattern on the infected older leaves. In nature it is transmitted by aphids, in a stylet-borne manner, but can be readily propagated in greenhouses by rubbing the leaves with a mixture of virion and abrasive. It does not get incorporated into host genomes (Guilfoyle, 1980), exists in high copy number in each cell, travels cell to cell and causes systemic infection. The suggested diagnostic features of the virus are a) restricted host range, b) high thermal inactivation point (75-80°C, 10 min), c) aphid transmissibility, and d) characteristic inclusion bodies (Shepherd, 1976).

The Structure of the Virion

The virus is a spherical particle, with a diameter of about 50 nm, and a molecular weight of 22.8 x 10^{6} Da (Hull et al., 1976). Its sedimentation coefficient ($S_{20,w}$) is 220S, and buoyant density in CsCl

is 1.37 gm/ml (Shepherd, 1970).

The virions with a diameter of 50 nm are penetrated by potassium phosphotungstate and appear empty with a hollow centre of 20 nm diameter (Shepherd, 1970). Neutron diffraction data revealed that the DNA is associated with the capsid shell, and the centre of the particles is free of DNA and protein (Chauvin et al., 1979). The DNA layer (2.0 nm) is hypothesized to be sandwiched between two protein layers: outer (6.5 nm) and inner (1.0 nm) of the capsid shell (Chauvin et al., 1979). The capsid shell of the virion consists of about 420 protein subunits, and the icosahedral triangulation number of the virion is 7 (Hohn et al., 1982; Hull, 1979).

The virus particles are usually present in specialized structures, called "inclusion bodies" or "viroplasms", in the cytoplasm of the host cell. Only the CM4-184 strain induces inclusion bodies in chloroplasts in addition to cytoplasm (Shalla et al., 1980). Viroplasms consist of many virions plus an electron dense matrix material but are devoid of any external membrane. Two types of inclusion bodies have been described a) a vacuolated structure in which virions are embedded in the matrix, and b) granular bodies which are non-vacuolated and are devoid of virions. The relative amounts of the two types of viroplasms present vary from one strain to another (Hohn et al., 1982; Shalla et al., 1980).

CaMV Genome

The genome of CaMV is double stranded DNA. The genome is AT rich and has a T_m of 87.2^{o}C. The $cot_{1/2} = 8.7 \times 10^{-3}$ M . sec^{-1}, which indicates a kinetic complexity of 4-5 x 10^6 Da. It has a buoyant density

of 1.702 g/ml in CsCl consistent with 43% GC content. CaMV-DNA sediments in neutral sucrose gradients at 19.0S and 17.1S, which correspond to relaxed circular and linear molecules (Hull, 1979). No superhelical DNA is observed in CsCl/ethidium bromide centrifugation. Denaturation by heat or alkali results in three single stranded species. One corresponds to full length linear molecules and the other two species to molecules of about 2/3 and 1/3 unit length. This shows that there is one discontinuity in one strand (called + or a) and two in the complementary (- or b) strand.

The viral DNA from three strains has been sequenced (Franck et al., 1980; Gardner et al., 1981; Balazs et al., 1982). The length ranges from 8016 to 8031 nucleotide base pairs. The three discontinuities in the viral DNA were found to contain short stretches of triple helices.

The genome contains six potential open reading frames (ORFs) for the synthesis of proteins and two intergenic regions. The noncoding regions are highly conserved, suggesting that they are involved in the initiation and termination of transcription. Although ORF II is non-essential for virus multiplication it is highly conserved. Thus the virus seems to have the ability to conserve both essential and non-essential sequences; a desirable property to become a successful vector (Gardner et al., 1981).

All the ORFs are present in the +ve strand. The -ve strand has a preponderance of stop codons, thus making it impossible to contain any potential coding region for proteins. The ORFs are present in different reading frames. Assuming ORF II and IV are present in one frame, the ORF III and V are present in the second frame, and ORF I and VI in the third frame. However, all the ORFs have the same polarity. A peculiarity of

these ORFs is that they jump abruptly from one phase to another so that in all but one case (the junction between V and VI) successive ORFs overlap slightly or are separated from one another by only a few nucleotides. Franck et al. (1980) have argued that there may not be any introns present.

Apart from the above discussed virion DNA, there exists free, supercoiled CaMV-DNA in the nuclei of the infected plants (Olszewski et al., 1982; Menissier et al., 1982). These covalently closed circular molecules possess a nucleosome structure and exist associated with histones as minichromosomes. The minichromosome has associated with it nuclear RNA polymerase II which selectively transcribes the +ve strand of DNA. Thus these covalently closed minichromosomes are transcriptionally active (Olszewski et al., 1982).

Transcription of CaMV

All the transcripts so far identified, were shown to be transcribed from the +ve strand. The major transcripts which accumulate after infection are 19S and 35S RNAs. These transcripts are polyadenylated at the 3' end, and in addition 19S RNA was shown to be capped at 5' end (Guilfoyle, 1980). 35S RNA is a full length transcript of the +ve strand. This has not yet been shown to translate into proteins. The 19S RNA is a mRNA and codes for the 62 kDa viroplasm protein (Xiong et al., 1982; Covey and Hull, 1981). Recently Condit et al. (1983) have isolated a 1800 nucleotide RNA which may have been transcribed from ORF-I. Apart from these transcripts many authors have identified a plethora of transcripts which are less well characterized and may be degradation products of the above transcripts. These post-transcriptional

modifications are probably carried out by host enzymes.

The transcription of the CaMV genome is sensitive to actinomycin D and a-amanitin. The latter shows that nuclear polymerase II is probably involved in the transcription (Guilfoyle, 1980). There is evidence that the transcription occurs in the nuclei on the viral minichromosomes (Olszewski et al., 1982).

CaMV can be transcribed in vitro from both strands by wheat germ RNA polymerases (Teissere et al., 1979), which have a binding preference for discontinuities in the genome. When cloned DNA was used, wheat germ RNA polymerases bound at 12 sites, with different affinities (Grellet et al., 1981). In contrast, turnip nuclear RNA polymerase has a preference for the +ve strand (Guilfoyle, 1980). Escherichia coli RNA polymerase can also transcribe CaMV in vitro (Volovitch et al., 1980). Meagher et al. (1977) have shown that viral RNA transcripts in E. coli direct protein synthesis, although the translation products were not related to known viral proteins. Daubert et al. (1982) have shown that antigenically related capsid protein is synthesized by E. coli transfected with plasmids containing ORF IV. The capsid protein synthesis was independent of the orientation and cloning site. Thus CaMV has an inherent ability to be transcribed by both eukaryotic and prokaryotic RNA polymerases. Sequence analysis reveals the presence of both prokaryotic and eukaryotic type transcription initiation and termination signals (Franck et al., 1981; Hohn et al., 1982). It remains to be seen, which promoters and terminators are preferentially used by the virus. Does virus optimize its coding potential by utilizing both nuclear and organelle polymerases?

Translational Products of CaMV

The best characterized translational products of CaMV are the capsid and viroplasm proteins. Capsid protein is synthesized as a 58 kDa precursor (Hahn and Shepherd, 1982) and is present in intact virus as a 43 kDa protein. It has been shown to be glycosylated (du Plessis and Smith, 1981) and phosphorylated (Hahn and Shepherd, 1980) too. Although it is abundant in infected plants, its mRNA has not yet been isolated.

Viroplasm protein is a 62 kDa protein which is highly hydrophobic and is translated from 19S mRNA (Xiong et al., 1982; Covey and Hull, 1981). There exists probably only one kind of protein for both kinds of inclusion bodies found in the infected plants.

Woolston et al. (1983) have shown that the inclusion bodies from aphid transmissible strains contain a 18 kDa protein which is absent in non-aphid transmissible strains. Armour et al. (1983) have shown that deletion in gene II results in the loss of the aphid transmissible property of the cloned CaMV-DNA. Thus it appears that gene II codes for the 18 kDa protein, which associates with viroplasms and helps virus transmission by aphids.

Toh et al. (1983) have shown that gene V protein sequences have considerable homology with the reverse transcriptase of Moloney murine leukaemia virus, suggesting that the gene may code for a reverse transcriptase enzyme. Nothing is known about the products of genes I and III.

It is interesting to note that all the three identified CaMV-specific products were found associated with inclusion bodies. It remains to be seen whether the other two unidentified proteins are also

associated with viroplasms. In fact close examination of the published SDS-PAGE patterns of purified viroplasms reveal more bands which are absent in uninfected plant extracts and could thus be potential candidates for the gene products I and III.

Replication of CaMV

Earlier experiments based on the accumulation of radioactive thymidine in CaMV-DNA gave conflicting results concerning the site of replication of the virus. Earlier, inclusion bodies were hypothesized to be the major sites for DNA replication (Kamei et al., 1969; Favali et al., 1973). However, later reports (Ansa et al., 1982) suggest that nuclei may be the site for DNA replication.

It has recently been hypothesized that the cytoplasm (inclusion bodies ?) of the host is the site for replication (Pfeiffer and Hohn, 1983; Hull and Covey, 1982). This mode of replication has been implicated to involve an RNA intermediate (35S RNA), which is reverse transcribed into mature viral DNA. This reverse transcription is presumably primed by the 3' end of a cytoplasmic methionine-tRNA. There exists some evidence for such a mechanism. However, more proof is required before it is accepted as a primary mode of replication.

Earlier studies with cloned CaMV-DNA sequences had shown that the cleavage at the cloning site was essential for the cloned sequences to be infectious (Howell et al., 1980). However, now partial nested dimers have been constructed which do not require restriction at the cloning site. Walden and Howell (1983) suggested that viral sequences are released from the plasmids by intergenomic recombination. Deletion and insertion mutant studies revealed that mutant DNA molecules recombine

and give rise to authentic viral DNA. No complementation has yet been observed (Howell et al., 1981; Lebeurier et al., 1982). These studies show that the viral DNA can undergo ligation, restriction, and recombination, much before viral multiplication. These events are probably host dependent. Once the viral transcription and multiplication starts, the viral products do not complement each other. This probably reflects the fact that all the products originating from the virus are restricted in one compartment. If such is the case every inclusion body may well be a clone of a single virion.

Aim of the Present Investigation

As indicated above there are many gaps concerning the knowledge of replication, transcription and translation of CaMV. This is mainly because of a lack of an in vitro system to study the molecular biology of the virus. A suitable _in vitro_ system for the study of plant viruses is transformed protoplasts (Takebe, 1975). In the present investigation, attempts were made to transform turnip protoplasts with CaMV and CaMV-DNA and to obtain a cell free extract which may support viral replication.

CHAPTER II

PROTOPLAST SYSTEM

Protoplasts, plant cells stripped of their cell walls, are naked cells, which are physiologically totipotent. They are potential objects to study the various aspects of cell physiology and the molecular biology of infectious agents surviving in these cells. Consequently, they have been extensively used for the production of subprotoplasts or organelles (Bradley, 1983), as a source of disease resistance in plants (Shepard, 1981), in somatic hybridisation (Evans and Cocking, 1977), in crop improvement (Gamborg et al., 1977), and to study the molecular biology of plant viruses (Takebe, 1975; Muhlbach, 1979).

Protoplasts were first isolated by mechanical means (for review see Cocking, 1972). In this method the tissue is preplasmolysed. During this process self-sealing of plasmodesmata occurs and the protoplast retracts from the cell wall. On cutting this plasmolysed tissue, a few protoplasts release into the media. This method is useful with tissues, whose plasmolysis results in good separation of the protoplast from the cell wall, but is particularly unsuitable for meristematic tissue.

Cocking (1960) first used enzymes to prepare protoplasts. The procedure involved prior isolation and culture of cells and their subsequent digestion with cellulase. Later, this two step procedure was found unnecessary. Nowadays a standard procedure involves the digestion with cellulase with or without pectinase and other carbohydrases, in the

presence of an osmotic stabilizer.

Takebe pioneered the use of plant protoplasts to study the molecular biology of plant viruses. His group was the first to achieve a synchronous infection of tobacco protoplasts with tobacco mosaic virus (Takebe and Otsuki, 1969). They used poly-L-ornithine (PLO) to promote the entry of virus into protoplasts. PLO probably neutralizes or reverses the surface charges of virus particles enhancing their adsorption to the protoplast surface. The uptake of virus would then be an endocytosis-like process. Burgess et al. (1973) found that PLO, in addition, causes cell membrane lesions, which are sufficient for the entry of virus particles. This method, however, results in a lower frequency of infection with naked viral genomes than compared to virions. RNAs are probably degraded by the ribonucleases present or released in the culture media, before their entry into protoplasts. Recently liposome encapsulated viruses and viral RNA have been used to infect protoplasts (Fukunaga et al., 1981; Nagata et al., 1981; Fraley et al., 1982; Rollo and Hull, 1982). Liposomes offer the unique advantage of protecting the nucleic acids against nucleases, and their use results in a higher frequency of protoplast infection. So far no DNA virus has been successfully used to infect plant protoplasts using liposomes.

CaMV has been used to infect turnip protoplasts by PLO-mediated transfer (Howell and Hull, 1978; Furusawa et al., 1980). CaMV-DNA has also been shown to infect turnip protoplasts by the same method (Yamaoka et al., 1982). However, follow-up studies were not forth-coming.

In the present investigation attempts were made to infect turnip protoplasts with liposome packaged CaMV and CaMV-DNA. During the present

investigation, liposome encapsulated CaMV has been shown not to infect turnip protoplasts, even though the same conditions result in their infection by turnip rosette virus and RNA (Rollo and Hull, 1982).

Methods

Protoplast Preparation

Protplasts were prepared in a laminar flow hood using solutions sterilized either by filtration or by autoclaving. Turnips (*Brassica rapa* cv Just right) were grown under conditions described by Gardner et al. (1980). Plants were kept in dark without watering for 24-48 h prior to harvest of leaves. Two to three younger leaves were harvested from 3-5 weeks old plants. The leaves were surface sterilized by treating successively with 70% ethanol (2-3 min), water, 1% hypochlorite (2-3 min) and water. The midrib was removed and the mesophyll tissue was cut into small pieces of about 1 mm^2, and incubated in dark at 30^oC in 100 ml of 20 mM 2(N-morpholino) ethane sulfonic acid buffer, pH 5.8, containing 0.6M mannitol (MES buffer), 1,500 units of macerase (Calbiochem., La Jolla, CA) and 5,000 units of cellulysin (Calbiochem.) in a 250 ml conical flask. After 90-120 min, the solution was decanted into another 250 ml conical flask. Fresh buffer containing enzymes was then added to the flask containing leaf tissue. Both solutions were further incubated in the dark for 90-120 min at 30^oC. The solutions were filtered through steel filters of 60 or 80, 100, and 200 mesh. The protoplasts were pelleted from the filtrate by centrifugation at 100g for 10 min at 4^oC. The protoplast pellet was suspended in 1-2 ml of MES buffer, examined with a microscope, and the number of protoplasts

counted using a hemocytometer. If the protoplast preparation was found to be contaminated with undigested tissue, the protoplasts were purified by centrifugation in a two phase system as described below.For culture they were washed once with the culture media and adjusted to 5 x 10^5 protoplasts/ml and 4-5 ml were cultured in disposable petri-dishes or culture flasks (Falcon).

Protoplasts from infected plants were prepared similarly. Two to three weeks old plants were infected either by slowly rubbing a celite and virus suspension on leaves or by dusting the leaves with carborundum, applying the virus suspension with a pipet and spreading gently with a gloved finger. After the plants developed systemic symptoms (3-4 weeks), they were treated as described for protoplast preparation. Only young systemically infected leaves were used for the preparation.

Purification of Protoplasts

For the prepartion of the two phase system (Kanai and Edwards, 1973), 3.0 ml of 20% (w/v) Dextran T-40 (Pharmacia Fine Chemicals, Uppsala, Sweden), 1.1 ml of 30% (w/v) polyethylene glycol (PEG, appr. MW 8,000, Sigma Chemicals, St.Louis, MO), 1.0 ml of 2.4M sorbitol, and 0.3 ml of 0.2M sodium phosphate buffer, pH 7.5, were pipetted in a 15 ml disposable, sterile test tube and mixed by inversion. Protoplast suspension (0.6 ml) in MES buffer was then added to the tube and mixed by inversion. The tubes were then centrifuged at 250 g for 6 min. Protoplasts were collected from the interphase. They were diluted 10-20 fold with MES buffer, centrifuged at 100 g for 10 min and resuspended in 1-2 ml of MES buffer, counted and used for further studies.

Evacuolation of Protoplasts

Evacuolation of protoplasts was performed essentially as described by Griesbach and Sink (1983). To 4.5 ml of Percoll based 100 mM $CaCl_2$, 5 mM N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES) and 9.0% mannitol, was added 1.0 ml of nonpurified protoplast suspension containing 0.5-1 x 10^6 protoplasts/ml in MES buffer. The Percoll/protoplast suspension was pipetted into a 5 ml polycarbonate centrifuge tube and centrifuged for 45 min at 18°C at 40,000 rpm (SW 50.1 rotor, Beckman).

In order to maintain axenic protoplasts following steps were taken, after the suggestion of Griesbach and Sink (1983). Percoll based HEPES buffer was filter sterilized (0.45 micron, Nalgene filters, Nalge, Rochestor, New York). The centrifuge tube, centrifuge adapter, and adapter top were sterilized by submersion in 70% ethanol for 10 min and let dry in a sterile laminar air flow hood. The sterilized tube was then loaded and balanced aseptically in a flow hood. After the centrifugation run, the centrifuge adapter was opened in a flow hood. The lower band of evacuolated protoplasts was aseptically removed with a pasteur pipette, diluted 10-fold in MES buffer and washed via centrifugation at 100 g for 10 min, counted and used for infection with liposome packaged CaMV or CaMV-DNA.

Incorporation of labelled amino acids into protoplasts

To 5.0 ml of protoplast suspension (1.25 x10^5 protoplasts/ml), ^{3}H-leu (2-10 microCi) was added and the mixture incubated at 22°C on a

gyratory shaker (40-60 rpm). The ^{3}H-leu incorporation into trichloroacetic acid (TCA) precipitable protein was determined according to Mans and Novelli (1961). The protoplast suspension (50-100 microl) was spotted onto filter paper disks (Gelman Science Inc., Ann Arbor, Michigan) and dried in hot air. The disks were kept in cold 5% TCA for 15 min - 3 days, washed twice with ether-ethanol (1:1, by vol) and twice with ether, dried and counted for radioactivity with 10 ml of scintillation cocktail (5g of phenylphenoxazole/1 of toluene).

Purification of CaMV

CaMV was purified as described by Hull et al. (1976). All operations were performed at 4°C, and solutions were chilled before use. Systemically infected leaves (400 g) were cut into small pieces and blended in 600 ml of 0.5M potassium phosphate buffer, pH 7.2, containing 0.06M sodium sulfite, at low speed for 30 sec. The homogenate was filtered through cheese cloth and was made 1M and 2.5% with respect to urea and Triton X-100 respectively. This solubilized homogenate was centrifuged at 7,000 rpm for 10 min in a JA-14 rotor (Beckman). The virus was pelleted from this supernatant by centrifugation at 18,000 rpm for 3 h in JA-20 rotor. The pellet was dispersed in distilled water overnight, and particulate material was removed by centrifugation at 7,000 rpm for 10 min. The supernatant was layered on top of 30 ml of a sucrose density gradient (10-40% (w/v) in 0.01M potassium phosphate buffer, pH 7.2) and centrifuged at 23,000 rpm for 3 h in SW50.1 rotor (Beckman). The virus band was extracted with a syringe, diluted with an equal volume of sterile water, and centrifuged at 45,000 rpm for 1 h in Ti 75 rotor (Beckman). The pellet was resuspended in water and its

absorbance read at 260 nm. Virus concentration was calculated assuming that a 0.1% solution of CaMV has an absorbance of 7.0.

Purification of Plasmids containing CaMV-DNA Sequences

Plasmids containing CaMV-DNA sequences were purified by the alkali lysis procedure as described by Maniatis et al. (1982). Volumes given are those for 500 ml culture and were appropriately adjusted for larger cultures.

E.coli k-12 strain HB101 harboring plasmids containing CaMV-DNA sequences were streaked out on nutrient agar plates (1%, w/v, Tryptone, 0.5%, w/v, yeast extract, (from Difco laboratories, Detroit, Michigan) 1%, w/v, NaCl, 2%, w/v, agar (from Sigma Chemical Co., St. Louis, MO) containing 0.015 mg/ml ampicillin or 0.010 mg/ml tetracycline (Sigma Chemical Co.), and incubated overnight at 37°C in a warm air incubator. Single colonies from these plates were used to inoculate 5 ml of nutrient broth (1%, w/v, Tryptone, 0.5%, w/v, yeast extract, 1%, w/v, NaCl), and incubated overnight at 37°C. These 5 ml cultures were then used to inoculate 500 ml of nutrient broth. The bacteria were allowed to multiply at 37°C for 20-24 h.

The bacteria were pelleted by centrifugation at 5,000 rpm for 10 min at 4°C in a JA-14 rotor. The pellet was resuspended in 10 ml of autoclaved 25 mM Tris-HCl buffer, pH 8.0, containing 50 mM glucose and 10 mM EDTA, and let stand at room temperature for 10 min. To this was added 20 ml of freshly made 0.2N NaOH, 1.0% SDS solution (made from stock solutions of 10N NaOH and 20% SDS). The mixture was let stand for 10 min on ice. Fifteen ml of an ice-cold solution of a 5M potassium

acetate, pH 4.8, (prepared by adding 11.5 ml of glacial acetic acid and 28.5 ml of water to 60 ml of 5M potassium acetate, mixed and chilled) was added and the mixture let stand 10 min, and centrifuged at 15,000 rpm for 30 min in JA-20 rotor at 4°C. Equal quantities (approx. 18 ml) of the supernatant were transferred into each of two 30 ml Corex tubes and 0.6 volumes (approx. 12 ml) of isopropanol was added to each tube. The contents were mixed well and let stand at room temperature for 15 min. DNA was recovered by centrifugation at 12,000 g for 30 min at room temperature. The pellet was washed with 70% ethanol at room temperature, and dried in a vacuum dessicator. The pellet was dissolved in 8.0 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 10 mM NaCl, and centrifuged at 12,000 rpm for 30 min at 4°C. To the supernatant was added 0.8 ml of 3M sodium acetate and 2.4 ml of 95% ethanol and it was kept overnight at -20°C. The DNA was collected by centrifugation at 12,000 rpm, 4°C, 30 min. The pellet was washed with 70% ethanol by centrifugation at 12,000 rpm for 30 min at 4°C. The DNA was dissolved in 18 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM NaCl and 1 mM EDTA.

Closed circular plasmid DNA was purified from this total DNA by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients. CsCl (3.3 g) was weighed into nitrocellulose ultracentrifuge tubes and three ml of DNA containing solution with 0.3 ml of ethidium bromide (15 mg/ml), were added and mixed by inversion. The tubes were filled with paraffin oil, and centrifuged at 34,000 rpm at 23°C for 48 h. The lower band was collected with the help of a syringe and diluted with 2 volumes of water. To this was added 6 volumes of 95% ethanol, and the mixture was kept at -70°C for 1 h. Plasmid DNA was collected by

centrifugation at 12,000 rpm for 30 min at 4°C, washed once with 70% ethanol via centrifugation, and dried in a vacuum dessicator. The pellet was dissolved in 1.0 ml of 0.1M Tris-HCl buffer, pH 8.0, and 1 ml of phenol was mixed with the DNA and let stand at room temperature for 10 min. The mixture was centrifuged at 12,000 rpm for 15 min at 4°C, and the top aqueous phase was transferred to another 15 ml Corex tube. Twenty microliter of 5M NaCl and 2.5 ml of 95% ethanol were added. After storage at -20°C overnight, the samples were centrifuged to collect the DNA. The pellet was washed with 70% ethanol, dissolved in sterile 5 mM Tris-HCl buffer, pH 7.0, containing 50 mM NaCl, 0.4M mannitol and 0.1 mM EDTA and was used for liposome preparation. DNA concentration was calculated assuming 1 mg/ml solution of double-stranded DNA has an absorbance of 20.

Packaging of CaMV and CaMV-DNA into Liposomes

Reverse phase evaporation (REV) liposomes were prepared by the method of Szoka and Papahadjopoulos (1978) as illustrated by Fraley et al. (1982) for the encapsulation of tobacco mosaic virus RNA. All lipids were obtained from Sigma Chemical Co. The calculations were based on an average molecular weight of 775 for phospholipids. Stearylamine standards were made in ether or chloroform.

Ten micromoles of phospholipids were pipetted into screw cap vials and evaporated under nitrogen. The lipid film was dissolved in ether and evaporated under nitrogen. The process was repeated (2-3 times) until a fine transparent lipid film was obtained on the vial walls. The film was then dissolved in 1.0 ml of ether. To this was added 0.33 ml of sterile 5 mM Tris-HCl buffer, pH 7.0, containing 50 mM NaCl, 0.4 M mannitol, 0.1

mM EDTA and 100-150 micrograms of either virus or viral DNA. This mixture was sonicated in a bath type sonicator twice at room temperature for 10 sec each. The ether was then slowly removed by rotary evaporation at room temperature under reduced pressure. The contents were then dialyzed overnight at 4°C against 0.5 mM Tris-HCl buffer, pH 7.0, containing 5 mM NaCl, 0.04 M mannitol, 0.01 mM EDTA, to remove the residual ether. The liposomes were pelleted by centrifugation at 40,000 rpm for 30 min at 4°C in a SW 50.1 rotor (Beckaman). The pellet was suspended in 5.0 ml of sterile 5mM Tris-HCl buffer, pH 7.0, containing 50 mM NaCl, 0.4 M mannitol, 0.1 mM EDTA, under sterile conditions, and further used for infecting the turnip protoplasts.

Infection of Protoplasts with Liposome Packaged CaMV and CaMV-DNA

The method used followed the guidelines of Nagata et al. (1981). All steps except centrifugation were carried out in a laminar flow hood. To a protoplast pellet containing 5 x 10^5 protoplasts was added 100 microl of liposomes containing either CaMV or CaMV-DNA at room temperature, mixed gently and incubated for 5 min. One ml of 30% (w/v) PEG(6000) in 0.05 M glycine-NaOH buffer, pH 10.5, containing 0.05 $CaCl_2$ and 0.4 M mannitol (or other buffers described in results, called fusion buffers) was then added, mixed and incubated at room temperature for 10-15 min. Five ml of 0.05 M glycine-NaOH buffer, pH 10.5, containing 0.05M $CaCl_2$ and 0.4M mannitol (or other buffers described in results as washing buffers) was then added, mixed and centrifuged at 100g for 10 min. The pellet was then washed via centrifugation in Takebe et al.(1968) or Nagata and Takebe (1971) media. The protoplast pellet was

resuspended in 1.0-5.0 ml of the same medium and cultured in dark at 23°C, on a gyratory shaker at about 50 rpm for about 5 days.

Detection of CaMV-DNA in the Protoplasts

The cultured protoplasts (0.1-1.0 ml) were centrifuged at 100g for 10 min and the pellet was resuspended in 10-20 microl of 20 mM MES buffer, pH 5.8, containing 0.6M mannitol, and 5-10 microl of the suspension was spotted in duplicates or triplicates on nitrocellulose sheet (Schleicher and Schuell, Keene, N.H.). The CaMV-DNA was detected following the method of Brandsma and Miller (1980) described for the detection of Epstein-Barr viral DNA. The sheet was laid for 5 min on a piece of 3MM Whatman filter soaked in 0.5 M NaOH. The sheet was neutralized by laying it on two different 3MM Whatman filters soaked in 1.0M Tris-HCl buffer,pH 7.0, containing 0.6M NaCl for about 1 min. The sheet was then neutralized for 5 min by laying it on Whatman paper soaked in 0.5M Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl. The sheet was washed twice with chloroform, dried in a vacuum oven at 80°C for 2 h. The sheet was wetted in 4 x SSC (4 x 0.015 M sodium citrate, 0.15 M NaCl) and then treated with prehybridization solution (Table 1) for 2 h at 65°C. The paper was then hybridized with hybridisation solution (Table 1) containing nick translated CaMV-DNA, prepared according to Rigby et al.(1977). The hybridisation was performed at 65°C for 18-24 h. After hybridisation the nitrocellulose sheet was washed twice with 20 ml of 2xSSC containing 0.1% SDS at 65°C for 10 min. The sheet was exposed to X-ray film at -70°C with an intensifying screen (Dupont) and autoradiographs were developed using Kodak solutions as described by the manufacturers. For counting, the areas corresponding to spots on the

TABLE I

PREHYBRIDISATION AND HYBRIDISATION SOLUTIONS

Materials	Prehybridisation solution	Hybridisation solution
Water	5.0 ml	4.3 ml
Sonicated Calf Thymus DNA (2 mg/ml)	1.0 ml	0.25 ml
^{32}P-CaMV-DNA	-----	0.5 million cpm (about 25 microl)
Heat in bioling water bath for 5 min. Cool immediately. Then add		
20 x SSC	2.5 ml	0.25 ml
50 x Denhardt*	1.0 ml	0.1 ml
1M sod. phosphate+	0.5 ml	0.1 ml

* 50 x Denhardt solution was prepared by dissolving 1.0 g of polyvinyl pyrrolidine, 1.0 g of ficoll and 1.0 g of bovine serum albumin in 100 ml of water.

+ prepared by dissolving 81.0 g of Na_2HPO_4 and 89.3 g of NaH_2PO_4,H_2O or 51.5 g of NaH_2PO_4 in 1.0 l of water.

radiograph were cut out and counted in 5 ml of toluene based cocktail (5 g of PPO/1 of toluene).

Detection of CaMV Coat Protein by Fluorescein-labelled Antibodies

Preparation of antisera against CaMV. Antibodies against CaMV were raised in rabbits. Preimmune blood was collected before the first immunization. Immunization was started by injecting 1.0 ml of 0.01M phosphate buffer, pH 7.0, containing 0.85% NaCl (PBS) and 0.2 mg of CaMV into the ear vein. After one week 1.0 ml of Freund's adjuvant containing CaMV, prepared by adding 5.0 ml of PBS containing 1.0 mg of CaMV to 5.0 ml of Freund's complete adjuvant, was administered subcutaneously. The subcutaneous injections were repeated twice at fortnight intervals. A week after the last subcutaneous injection, approximately 20 ml of blood was collected from an ear vein. Booster subcutaneous injections were given every month thereafter and blood was collected a week later.

The blood was left to clot at room temperature for 2-3 h. The clot was gently scraped from the tube walls and refrigerated overnight. The sera were decanted into two 15 ml Corex tubes and centrifuged at 2,500 rpm for 30 min at $4^{o}C$ (JA-20 rotor). The sera were collected, made 0.1% with respect to sodium azide, and stored in cryovials at $-70^{o}C$.

Purification of Immunoglobin G (IgG) Fraction from the Serum. To 1.0 ml of antiserum was added 9.0 ml of distilled water, and 10.0 ml of saturated ammonium sulfate solution, and the mixture was left at room temperature for 1 h. The solution was centrifuged at 2,500 rpm for 30 min at $23^{o}C$. The pellet was dispersed in 2.0 ml of 0.5 x PBS, and

dialysed against 500 ml of 0.5 x PBS with two changes of buffer every 24 h at 4°C. The dialysed IgG fraction was applied on a 3-5 ml DE-52 column preequilibrated with 0.5 x PBS. The column was washed at room temperature with 0.5 x PBS and fractions of about 2.0 ml were collected. The absorbance of the effluent was measured at 280 nm. The first protein fraction eluted from the column was pooled and adjusted to read approximately 1.4 absorbance, which corresponds to a concentration of 1 mg of IgG per ml.

Fluorescein Labelling of Goat Anti-rabbit Immunoglobulins. Antibodies against rabbit IgG were raised in goat and were labelled with fluorescein isothiocyanate (FITC) by the dialysis technique described by Clark and Shepard (1963).

The purified IgG fraction was dialysed against 0.02M bicarbonate buffer, pH 9.8, overnight at 4°C. Fifteen ml of 1% IgG in 0.02M bicarbonate buffer,pH 9.8, was dialysed overnight at 4°C against 200 ml of 0.02M bicarbonate buffer, pH 9.8, containing 0.01% FITC. The dialysis was continued for another 24 h against PBS. The residual unreacted FITC was removed by gel chromatography on a Sephadex G-25 column. Three grams of Sephadex G-25 were prewetted in PBS and packed into a column with 1 cm diameter. The column was equilibrated with PBS, and the dialysis mixture was applied at 4°C. The column was washed with PBS at 2-3 ml x h^{-1} flow rate and 5 ml fractions were collected. Absorbance at 280 and 490 nm was registered. The labelled protein fractions were collected.

Staining of CaMV-infected Protoplasts with Fluorescein-Labelled Antibodies. Staining of protoplasts with fluorescein-labelled antibodies was performed as described by Otsuki and Takebe (1969). A few drops of

Meyer's albumin (Harleco, N.J.) were smeared on to a glass slide with the help of another slide. One drop of thick protoplast suspension was added and quickly dried under hot air (slow drying may result in the crystallization of mannitol). The slide was immersed in acetone for 30 min for fixation. The slide was then washed by immersing in PBS for 2 h with four changes, with constant stirring. The protoplasts were covered with anti-CaMV serum (or purified IgG) and incubated at 37°C for 30-60 min, washed once with PBS for 15 min. The slide was then covered with fluorescein labelled anti-IgG and incubated at 37°C for 30-60 min, washed with PBS FOR 1 h with four changes. The slide was mounted with PBS containing 10% glycerin and observed under epifluorescence using Zeiss fluorescent microscope.

Results and Discussion

Surface Sterilization of Turnip Leaves

To optimize conditions for surface sterilization, turnip leaves were treated with 70% ethanol and 1% hypochlorite for different time intervals. After the treatment the leaves were placed on sterile nutrient agar plates and incubated at 37°C. Growth of bacteria was visually examined after 1 and 4 days (Table 2). Since bacterial colonies were not observed when the leaves were treated with 70% ethanol for 3 min followed by 3 min of treatment with 1% hypochlorite, turnip leaves were routinely surface sterilized by treating with 70% ethanol for 3 min followed by 3 min treatment with 1% hypochlorite.

No bacterial contamination was observed in protoplast cultures, when leaves used to prepare protoplasts were surface sterilized as

Table II

SURFACE STERILIZATION OF TURNIP LEAVES

Time of treatment with		Bacterial colony growth after	
70% ethanol and	1% hypochlorite	1 day	4 days
1 min	1 min	+	+
3 min	1 min	+	+
5 min	1 min	+	+
1 min	3 min	-	+
3 min	3 min	-	-
5 min	3 min	-	-

described above and in methods. Howell and Hull(1978) have reported occasional contamination of their protoplast cultures even in the presence of antibiotics. The contamination might have been due to inadequate surface sterilization.

Protoplast Preparation

The preparation of turnip protoplasts was adversely affected by the starch grains present in leaf mesophyll tissue. Incubation of the plants in darkness prior to protoplast isolation decreased the number of starch grains and increased the protoplast yield. The increased yield may be due to less interference of starch grains with cellulose hydrolysis or increased susceptibility of cell walls to digesting enzymes.

Protoplasts from infected and uninfected turnip mesophyll tissue were released by a mixture of cellulase and pectinase. Experiments were performed to optimize the amounts of enzymes required for protoplast preparation. The amounts of enzymes described in methods were the smallest amounts of enzymes which gave maximum yield of protoplasts. No attempts were made to compare the enzyme preparations from different commercial sources.

Two difficulties, variable yields and impurity, were encountered. The biggest problem in protoplast preparation was unpredictable yields ($1-200 \times 10^4$ protoplasts/g of tissue). The reasons for the variation in yield were hard to assess. It was not due to a) variation in enzyme activities, b) age of plants, and c) age of leaf. It is possible that environmental conditions may have been responsible. The other problem was the contamination of protoplasts with undigested tissue. Since the attempts to digest all the tissue with increased enzyme concentrations

and longer incubation periods failed, attempts were made to purify the protoplasts. Protoplasts could be purified by centrifugation in the PEG-Dextran two-phase system (Fig.1).

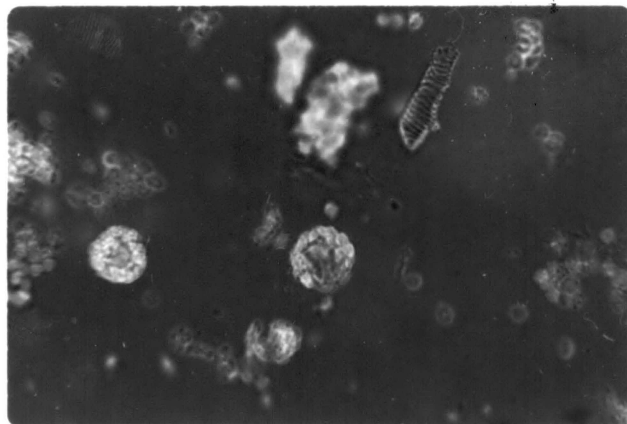
Culture of protoplasts

Howell and Hull (1978) encountered difficulties in culturing turnip protoplasts due to protoplast lysis. The same problem was encountered here during the culture of protoplasts and thus attempts were made to stabilize turnip protoplasts. Since cations including polyamines have been shown to stabilize oat protoplasts (Altman et al., 1977), they were used in an attempt to stabilize turnip protoplasts in the present investigation (Fig.2).

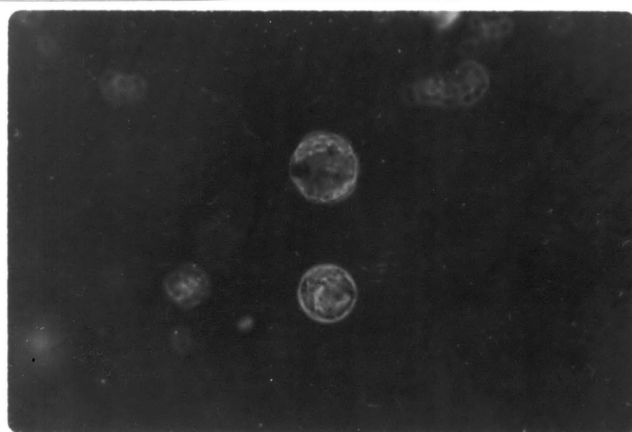
The stability of the protoplasts was determined by counting the number of protoplasts remaining intact after various times of incubation. The stability of turnip protoplasts was greater when divalent cations and polyamines were added to the buffer (Fig.2). In all cases the disintegration ranged between 20-35% within 30-40 h. Divalent cations were generally better than the polyamines in stabilizing protoplasts. Thus two media were selected which had concentrations equal to or greater than the concentration of Ca^{2+} and Mg^{2+} used in these studies. The media are those described by Takebe et al. (1968) and Nagata and Takebe (1971).

Incorporation Studies

The incorporation of ^{3}H-leu into TCA-precipitable protein was linear up to 6-7 h and reached a plateau at about 15 h (Fig.3). The amount of ^{3}H-leu incorporated into protoplasts (1.25×10^{5} / ml) at 18 h



(A) NON-PURIFIED PROTOPLASTS



(B) PURIFIED PROTOPLASTS

Figure 1. Turnip Protoplasts

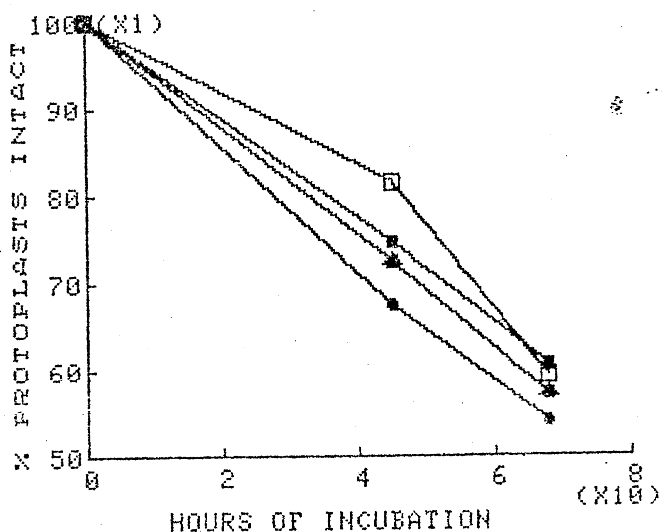


Figure 2. Stability of Turnip Protoplasts.
2.5 x 10^5 protoplasts were incubated at 22°C in 1.0 ml of MES buffer with or without compounds. Aliquots (appr. 50 microlitres) were withdrawn asceptically at the indicated times and protoplasts were counted in a hemocytometer. a) MES buffer alone (●—●—●—●—●—●), b) 20 mM Ca^{2+} (▲——▲——▲——▲——▲), c) 20 mM Ca^{2+} and 2 mM Mg^{2+} (■—■—■—■—■—■), d) 1 mM spermine (□—□—□—□—□—□—□—□—□—□).

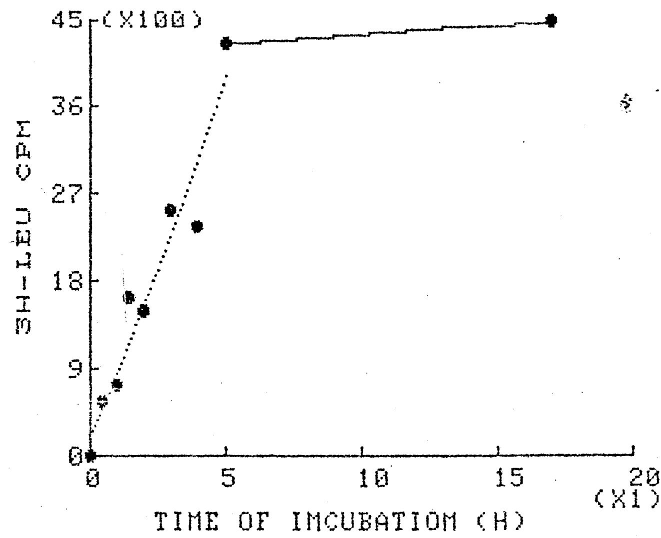


Figure 3. Time Course of ^{3}H-Leu Incorporation into TCA-Precipitable Proteins.
To 2.2 x 10^{5} protoplasts in MES buffer was added 5 microCi of ^{3}H-leu. At different time intervals 75 microl of the protoplasts were withdrawn and the incorporation was determined as described in methods.

was linear with the concentration of labelled leu (Fig.4). Incorporation of labelled leu (10 microCi) was also linear with the number of protoplasts (1-20 x 10^5 protoplasts / ml) under similar conditions (Fig.5). The incorporation was sensitive to kanamycin, chloramphenicol, and cycloheximide (Table 3). Since cycloheximide inhibits cytosol protein systhesis and kanamycin and chloramphenicol inhibit organelle protein synthesis, these studies showed that protein synthesis was occuring both in cytosol and organelles. At lower concentrations of labelled leu (less than 1 microCi) no appreciable incorporation was observed perhaps due to either dilution or competition of the label with endogeneous leu. In short, the incorporation of leu into TCA precipitable proteins was leu concentration dependent, antibiotic sensitive and was linear with the number of protoplasts.

The polyamines inhibited the incorporation of the ^{3}H-leu in the protoplasts (Table 3). Altman et al. (1977) have observed that incorporation of ^{3}H-uridine into RNA of protoplasts was inhibited to various extents by spermine and spermidine. Thus it appears that spermine and spermidine stabilize the protoplasts, and cause decreased uptake of nutrients. It is possible that the increased integrity of protoplasts may be due to the interaction of negatively charged lipids present in the plasma membrane of protoplasts and positively charged polyamines. Such interactions may hinder the uptake of nutrients, thus resulting in less incorporation of labelled compounds from the media into the protoplasts.

The Nagata and Takebe (1971) medium, which contains vitamins, hormones, major and minor elements and an energy source supported less ^{3}H-leu incorporation than did Takebe et al. (1968) medium (Fig.6). The

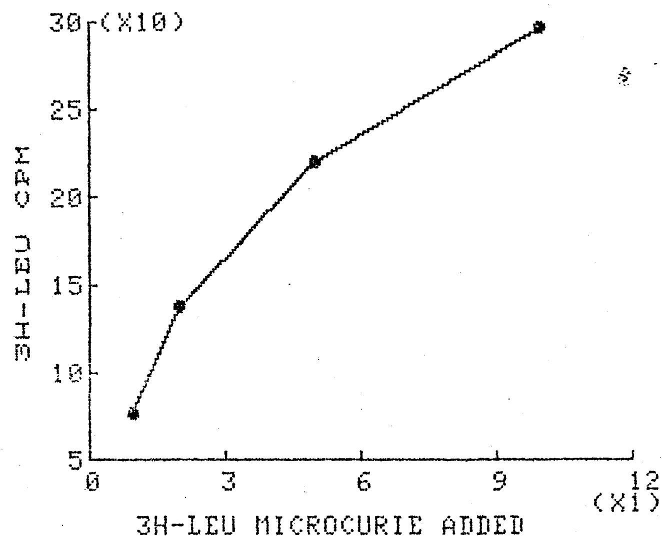


Figure 4. Effect of ^{3}H-Leu Concentration on its Incorporation into TCA Precipitable Proteins.

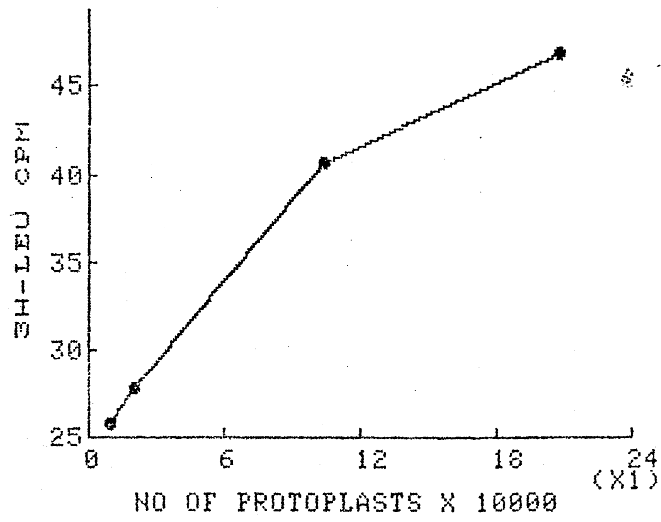


Figure 5. Effect of Protoplasts Concentration on the Incorporation of ^{3}H-Leucine.

TABLE III

EFFECTS OF ANTIBIOTICS AND POLYAMINES ON ^{3}H-LEU INCORPORATION INTO TCA PRECIPITABLE PROTEINS OF CULTURED TURNIP PROTOPLASTS

Compounds (concentration)		% inhibition
Chloramphenicol	(10 microg/ml)	42
Kanamycin	(10 microg/ml)	49
Cycloheximide	(13 microg/ml)	67
Spermidine	(1 mM)	56
Spermine	(1 mM)	32

To 0.5 ml of protoplast suspension (1.25 x 10^{5}) in MES buffer was added 2.5 microCi of ^{3}H-leu. Samples were cultured with and without the compounds listed for 18 h.

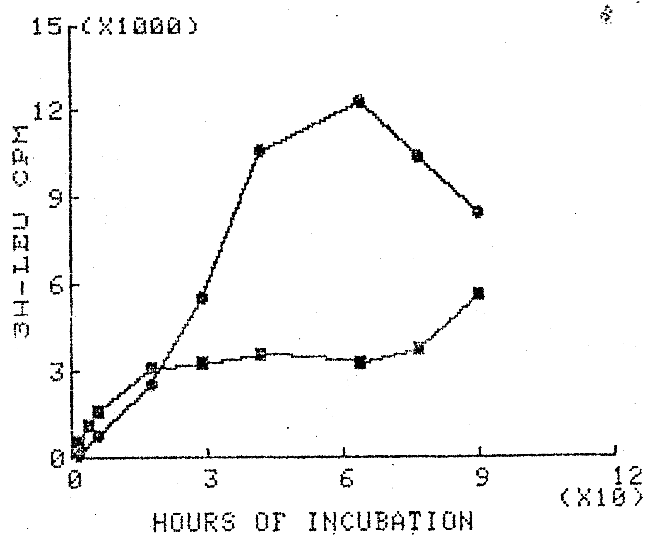


Figure 6. Incorporation of ^{3}H-Leu into Protoplasts when Cultured in Nagata and Takebe Medium (1971) (■—■—■) Takebe et al. Medium (1968) (●—●—●—●—●—●—●—●).

Nagata and Takebe (1971) medium supported less incorporation of ^{3}H-leu compared to MES buffer (data not shown). Thus it appears that simplified Takebe et al. (1968) medium is better suited to stabilize protoplasts as well as to study the incorporation of labelled compounds into newly synthesized products. Due to the same reasons it should be a better medium to study the molecular biology of viruses multiplying in the protoplasts. Some lysis of protoplasts occurs in this medium. There was no decrease in incorporation parallel to the decrease in protoplast number. This may either be due to the preferential degradation of metabolically less active protoplasts or more vigorous uptake into surviving cells due to lack of competition.

Infection of Protoplasts with Liposome Packaged CaMV and CaMV-DNA

CaMV or pLW111D-C was successfully encapsulated into liposomes by the method of Szoka and Papahadjopoulos (1978). The presence of CaMV and pLW111D-C in the liposomes was confirmed by hybridisation with ^{32}P-CaMV-DNA.

Uptake of liposomes by protoplasts. Liposomes were allowed to interact with protoplasts in the presence and absence of fusogens (compounds e.g. polyalcohols, which can appose membranes by excluding water, which subsequently results in the fusion of artificial membranes). Protoplasts were incubated with liposomes for 5 min, fusogens were then added and the mixture was incubated for a further 10 min. After this treatment protoplasts were collected by centrifugation at 100 g for 10 min. This low speed centrifugation does not pellet

liposomes. Thus the radioactive lipid found associated with protoplasts should be due to specific uptake or non-specific association of liposomes with protoplasts. To minimize non-specific association protoplasts were washed twice via centrifugation. The radioactivity remaining with the protoplast pellet was used to calculate % uptake relative to the same amounts of liposomes added to untreated, washed protoplasts.

Only about 5% of the added phosphatidylcholine (PC, neutral) liposomes were found associated with the protoplasts in the absence of any fusogen. When glycerol or polyvinyl alcohol (PVA) was used there was a less than one fold increase in the % uptake of liposomes (Table 4). With polyethylene glycol (PEG) the % uptake was about three-fold that with glycerol or PVA, and about 5-fold that with no fusogen (Table 4). When PC-phosphatidylserine (PC-PS, negative) liposomes were used similar results were obtained. Thus the % uptake in the presence of PEG was about 2.5-fold that in the presence of glycerol or PVA. Thus PEG appears to be a better fusogen for the uptake of liposomes by turnip protoplasts. These results are in accordance with those of Fraley (1983) and Fukunaga et al. (1981) who found that PEG is a better fusogen for infecting petunia and Vinca rosea protoplasts with tobacco mosaic virus (TMV) RNA encapsulated in PS-liposomes. However, Fraley et al. (1982) and Nagata et al. (1981) found PVA to be a better fusogen for the delivery of TMV-RNA encapsulated in PS-liposomes into tobacco protoplasts. Uptake of PC-stearylamine (PC-SA, positive) liposomes was not increased by fusogens (Table 4). These positively charged liposomes are probably apposed to protoplast membranes by ionic interactions.

These studies also showed that positively charged liposomes

TABLE IV

EFFECT OF LIPID COMPOSITION OF LIPOSOMES ON THEIR UPTAKE BY PROTOPLASTS

Fusogens in K & M buffer	% Liposomes Uptaken		
	PC	PC:PS(7:3, molar)	PC:SA(7:3, molar)
No fusogen	5.23	N.D.	38.47(2.45)
10% PVA (w/v)	7.6(2.55)	9.85(1.63)	22.66(0.95)
20% Glycerol(v/v)	9.7(4.53)	9.67	24.42(2.52)
30% PEG (w/v)	25.5(6.58)	21.95(10.00)	36.73(3.06)

Protoplasts ($5x10^5$) were incubated with 0.3 micromole phospholipids as described in methods. They were washed with MES buffer via centrifugation and suspended in 1.0 ml of MES buffer. ^{3}H-phosphatidylcholine radioactivity was determined in 50-100 microl aliquots. Radioactivity was counted in 5 ml of Instagel (Packard).

N.D.-- not determined

Numbers in parentheses represent standard deviation amongst different experiments.

associated with protoplasts more than neutral or negatively charged liposomes did(Table 4). Uchimiya (1981) reported that tobacco protoplasts take up neutral vesicles much more readily than charged vesicles during 1 h of incubation. He did not find any significant difference between neutral and positively charged vesicles with respect to efficiency of liposome uptake by carrot protoplasts. In both cases negatively charged vesicles resulted in the lowest uptake. Thus it appears that the effect of the surface charge of liposomes, on their association with protoplasts from different species is different.

The % uptake of all liposomes was generally greater when they were incubated with protoplasts and 30% PEG in 0.05M glycine/NaOH buffer, pH 10.5, containing 0.05M $CaCl_2$ and 0.4 M mannitol (K & M buffer; described by Keller and Melchers (1973) for protoplast fusion), than when MES buffer, pH 5.8 (used in the protoplast preparation), and 5mM Tris/HCl buffer, pH 7.0, containing 50 mM NaCl, 0.4 M mannitol and 0.1 mM EDTA (described by Fraley et al (1982)) was used (data not shown).

To study the amount of liposomes required for their maximum association with protoplasts, positively charged liposomes were used (Fig.7). Assuming 100% recovery of phospholipids during liposome formation, it was calculated that maximum association occurred when 0.3 micromoles of phospholipids were incubated with 5.5×10^5 protoplasts. Ohgawara et al. (1983) reported that the efficiency of liposome encapsulated DNA uptake was saturable in the neighborhood of 1.2 micromoles of lecithin / 10^6-10^7 protoplasts prepared from suspension cultures of Daucus carota. The amount of phospholipids required in the present experiment was higher, perhaps due to the assumption that 100% lipids were recovered during liposome formation.

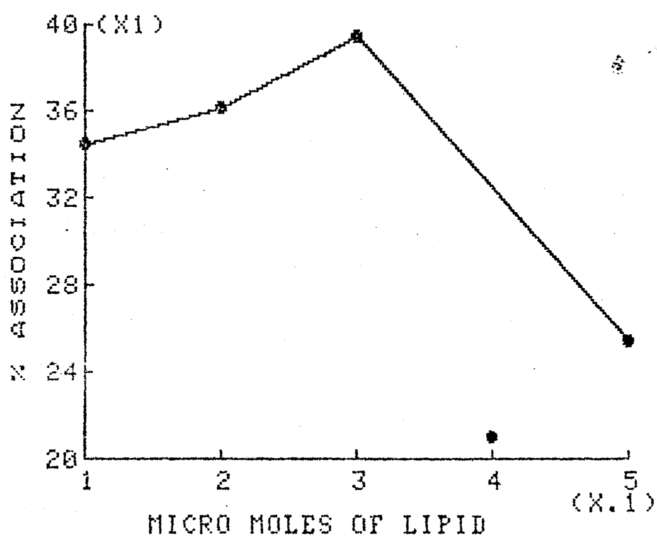


Figure 7. Effect of Concentration of Liposomes on Association with Protoplasts.
To pellets containing 5.5 x 10^5 protoplasts were added different amounts of positively charged liposomes and the mixture incubated as described in methods. The fusogen used was 30% PEG in K and M buffer. One hundred microl was used for determining the radioactivity. Radioactivity was counted as described in Table 4.

Delivery of Liposomal Contents into Protoplasts

The uptake measured earlier does not distinguish between the association of liposomes with protoplast membranes or actual endocytosis of whole liposomes. Theoretically it can be argued that if liposomes associate with protoplast membranes, their subsequent fusion with the membrane would result in loss of most of their contents into the medium. If the liposomes were endocytosed then the % contents delivered should be close to % liposomes uptaken by protoplasts.

To study the delivery of liposomal contents, PC-SA liposomes were prepared encapsulating ^{3}H-pLW111D-C. Since the non-specific association with free (DNA is negatively charged) ^{3}H-pLW111D-C is minimal, the radioactivity found associated with protoplasts should represent the amount of DNA transferred inside protoplasts. When such studies were performed about 30% of the liposomal contents were transferred to protoplasts (Table 5). This percentage delivery parrallels the percentage uptake of liposomes by protoplasts (compare with Table 4). It appears that most of the liposomal contents were transferred to protoplasts, suggesting uptake of liposomes by endocytosis.

There are conflicting reports concerning the uptake of liposome-encapsulated or free foreign nucleic acids. Because Fraley et al. (1982) and Nagata et al. (1981) found negatively charged liposomes encapsulating TMV-RNA resulted in much greater infection of protoplasts than other liposomes, they suggested that the PS-liposomes were selectively endocytocized by an active process. Lurquin and Rollo (1983), however, suggested that liposomal-DNA was delivered via fusion and there was no evidence for endocytosis. Uchimiya and Murashige (1977)

TABLE V

TRANSFER EFFICIENCY OF ^{3}H-DNA FROM POSITIVELY CHARGED LIPOSOMES

Fusogens	% ^{3}H-radioactivity delivered
No fusogen	30.96(8.22)
30%PEG	29.73(6.22)
10%PVA	29.74(0.11)

Numbers in parentheses represent standard deviation amongst different experiments

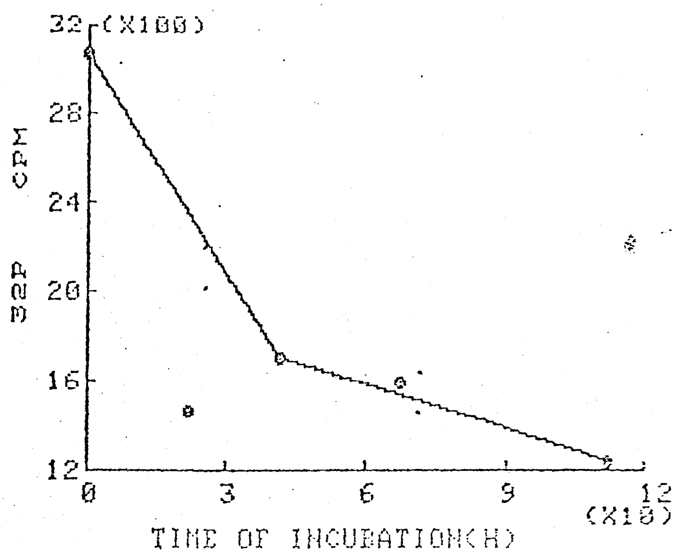
found that uptake of free DNA was by energy-dependent pinocytosis. Detailed studies concerning the endocytosis of liposomes by kidney cell lines have been performed by Straubinger et al. (1983). They found that liposomes are actively uptaken by endocytosis.

Fate of the Transferred Virus and DNA

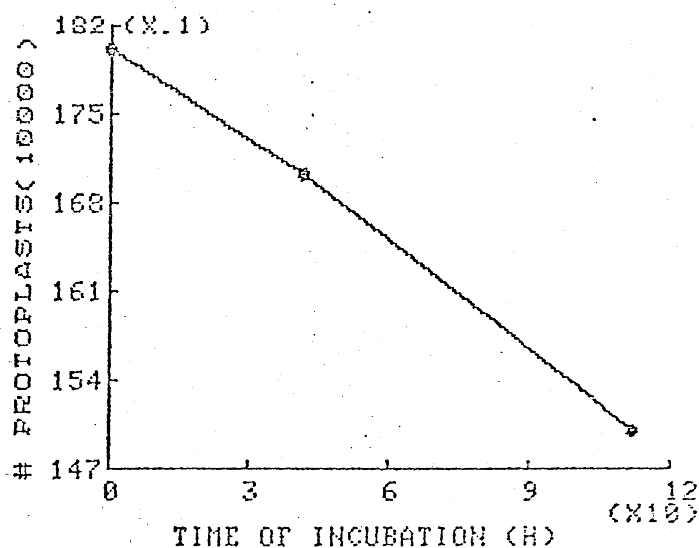
Since the assay used in the present study only detects viral DNA associated with protoplasts, there exist only two possibilities concerning the fate of the DNA. First the DNA will not be detected if the protoplasts become destabilized due to liposome fusion and eventual lysis occurred. In this case loss of DNA should parallel with lysis of protoplasts. Secondly, the liposomes are endocytosed and the DNA can be degraded intracellularly by protoplasts. In such an instance the rate of breakdown or loss of DNA will be higher than the rate of protoplast lysis. Eventually, since CaMV infects turnips, there should be an increase in the viral DNA due to virus multiplication.

The fate of viral DNA was studied by hybridization. After the introduction of CaMV or DNA, the amount of hybridizable CaMV-DNA decreased with time. This decrease was much faster than the decrease in intact protoplasts (Fig.8a & 8b). This indicates that the CaMV or CaMV-DNA was preferentially degraded by the host enzymes or excreted. No evidence for any increase could be obtained. It should be pointed out that foreign endocytosed DNA is known to be degraded in protoplasts, and the products are reutilized by the protoplasts (Uchimiya and Murashige, 1977). No evidence for excretion of foreign DNA is available in the literature.

Virus multiplication can also be determined by assaying for an



(A) DEGRADTION OF CAMV-DNA



(B) DISINTEGRATION OF PROTOPLASTS

Figure 8. Fate of CaMV-DNA in the Infected Protoplasts.
CaMV-DNA (A) in the protoplasts, which were infected with positively charged liposomes containing CaMV, was detected by hybridisation as described in methods. Protoplasts (B) were counted in a haemocytometer.

increase in coat protein. Viral coat protein was detected in infected protoplasts by fluorescein labelled antibodies as described in methods. When protoplasts were infected with liposome packaged virus no appreciable increase in coat protein with time occurred. Infection of protoplasts with liposome packaged DNA did not result in detection of coat protein at any time.

These two independent methods, revealed that the virus was being degraded with time and that the virus had no chance to multiply in the protoplasts. These results are in agreement with those of Rollo and Hull (1982). They showed that under identical conditions turnip protoplasts supported turnip rosette virus and RNA multiplication, but did not support CaMV and CaMV-DNA replication. The infection could not be achieved by changing lipid composition of liposomes, fusogens, time of incubation of liposomes with protoplasts, pH of the fusogen containing buffer, or culturing media (Table 6).

The inability to detect CaMV replication in these experiments was not due to an inherent inability of turnip protoplasts to support CaMV replication. Turnip protoplasts have been shown to support CaMV and CaMV-DNA replication if infected with the help of PLO (Howell and Hull, 1978; Furusawa et al. (1980); Yamaoka et al. (1982) and PEG (maule, 1983). Furusawa et al. (1980) and Yamaoka et al. (1982) have detected virus multiplication by labelling with fluorescein labelled antibodies. Thus failure in the present investigation was not due to insensitivity of the assay.

The results suggest that the virus and DNA were being delivered to a compartment which degrades the DNA, rather than to the compartment which supports its replication. It is known that CaMV transcription

TABLE VI

CONDITIONS TESTED FOR INFECTING TURNIP PROTOPLASTS
WITH LIPOSOME PACKAGED CAMV AND CAMV-DNA

Lipid composition of liposomes:- PS, PC, SA, PS:Cholesterol (5:5, molar), PC:Cholesterol (5:5, molar), SA:Cholesterol (5:5, molar), PC:PS:SA:Cholesterol (2:2:2:4, molar), PC:PS (7:3, molar), PC:SA (7:3, molar)

Fusogens:- PEG (5-45%, w/v), PVA (5-20%, w/v), Glycerol (5-50%, v/v).

Time of incubation:- 5-30 min

Buffer:- MES buffer, pH 5.8, K and M buffer, pH 10.2, 5mM Tris/ 50mM NcCl/ 0.4M mannitol/ 0.1 mM EDTA

Media for culture:- Takebe et al. (1968), Nagata and Takebe (1971), Howell and Hull (1978)

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occurs in nuclei (Guilfoyle, 1980) and that the transcription is essential for viral multiplication (Pfeiffer and Hohn, 1983; Hull and Covey, 1983). It can be argued that the virus had not been delivered to nuclei, and was thus not replicated. Uchimiya and Murashige (1977) reported that 60% of the uptaken DNA was found associated with the nuclear fraction. Lurquin and Rollo (1983) found that the DNA associated with nuclei was more accessible to deoxyribonuclease I than the host DNA, suggesting that most of the radioactivity found associated with the nuclear fraction was bound outside the nuclei. Uchimiya and Murashige (1977) also presented evidence that most of the uptaken E. coli DNA was degraded at longer incubation times.

Straubinger et al. (1983) showed that in a kidney cell line the endocytosed liposomal contents are processed intracellularly by the coated vesicle pathway to the lysosomes. They found that acidification of the endocytic vesicles, rather than liposomal fusion, permits escape of certain molecules to the cytoplasm. If such a process is occurring in the plant cells, liposomes will be delivered to the cytoplasm and then transported to vacuoles, after their endocytosis. Some contents may leak out in the cytoplasm due to acidification of liposomes. These may get associated or taken up by other organelles in the cytoplasm. It is probable that the molecules which leak out of liposomes into the cytoplasm are more important in the process of infection of virus than those delivered to vacuoles, because virus delivered to vacuoles may be preferentially degraded. In such a case, removal of the vacuole would result in the delivery of all the liposomal contents into the cytoplasm. They would subsequently be transferred to different organelles by cytoplasmic streaming. To test such a possibility evacuolated protolasts

were prepared and infected with liposome packaged CaMV.

Infection of Evacuolated Protoplasts

Evacuolated protoplasts could be prepared by centrifugation in Percoll following the method of Griesbach and Sink (1983). After Percoll gradient centrifugation two different layers were observed. The top layer had undigested tissue and some protoplasts. The lower layer contained pure evacuolated protoplasts with little undigested tissue. Attempts to remove undigested tissue from evacuolated protoplasts by PEG-Dextran two phase were unsuccesful. When protoplasts were purified by the PEG-Dextran two phase system, before centrifugation in Percoll, evacuolation did not occur.

During the course of incubation of protoplasts fused with CaMV-containing liposomes (positively and negatively charged), a decrease in the amount of CaMV-DNA associated with protoplasts was observed during the first 22h. During further incubation, a significant increase in the amount of CaMV-DNA detected by hybridisation ocurred (Fig. 9), so that at the end of the incubation 11-13 fold more DNA was detected than was found at 22h. At the end of the incubation period virus multiplication had not yet reached a plateau. An eclipse period for the multiplication of CaMV in protoplasts has been observed previously by others. Howell and Hull (1978) could only detect an increase in CaMV 96h after infection of turnip protoplasts with CaMV in the presence of PLO. Furusawa et al. (1982) could not detect any increase in the number of viral particles by electron microscopy 48h after infection of turnip protoplast with CaMV, and Maule (1983) could detect an increase in CaMV only at 72h post inoculation.

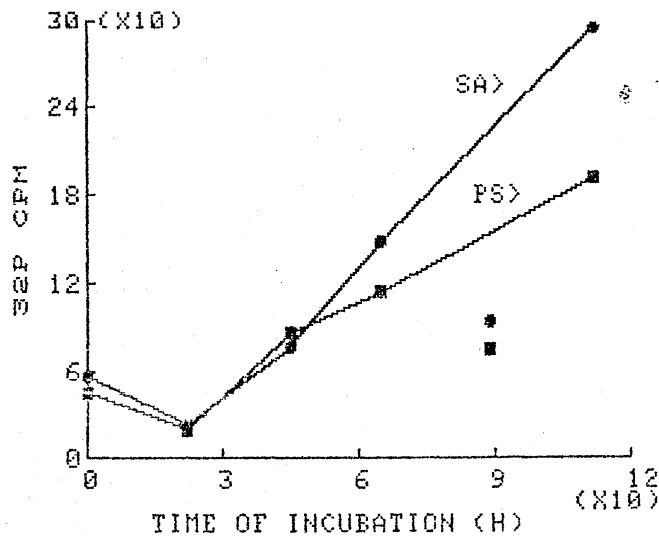

Figure 9. Multiplication of CaMV in Evacuolated Protoplasts.
To about 4.6 x 10^4 evacuolated protoplasts in 0.1 ml of MES buffer was added 25 microl of PC:PS (7:3, molar) or PC:SA (7:3, molar) liposomes encapsulating CaMV (appr. 0.0125 micromole of phospholipid in 5 mM Tris/HCl, 50 mM NaCl, 0.6M mannitol, 0.1 mM EDTA, pH 7.0) and incubated at room temperature for 5 min. One ml of 20 mM potassium phosphate buffer, pH 6.8, containing 0.6 M mannitol and 30% PEG was added to the protoplast-liposome mixture, mixed gently and incubated at room temperature for further 10 min. Then 5 ml of K and M buffer was added and centrifuged at 100g for 10 min. The evacuolated protoplasts were washed by centrifugation with Nagata and Takebe medium (1971), and suspended in 1 ml of the same medium and cultured at 23°C. At each time interval 150 microl of the cultured evacuolated protoplasts was taken out in sterile conditions, centrifuged at 100g for 10 min, suspended in 10 microl of MES buffer and two 5 microl were spotted on nitrocellulose sheets and hybridized with ^{32}P-CaMV-DNA as described in methods.

To ascertain whether the infection of evacuolated protoplasts was indeed due to evacuolation, the following controlled experiment was peformed. After the protoplast preparation, they were divided into two aliquots. One was used to purify protoplasts by two phase system and then the purified protoplasts were fused with liposomes directly, while the other aliquot was evacuolated by Percoll gradient centrifugation prior to fusion with liposomes. Fig. 10 shows the autoradiographic spots from dot hybridisation of aliquots of these two preparations. CaMV-DNA was synthesized in the evacuolated protoplasts after an initial degradative phase and only degradation was seen in protoplasts that retained their vacuoles at the time of infection. Attempts to infect non-purified protoplasts gave the same results, suggesting that the inability to infect protoplasts was not due to their purification by the two phase system.

The successful infection of evacuolated protoplasts as opposed to non-evacuolated protoplasts, substantiates the assumption that liposomes were delivered to vacuoles. But there is no _a priori_ reason to believe that liposomes encapsulating RNA viruses are not routed to vacuoles. Then why do RNA viruses multiply in protoplasts? The reason probably is that RNAs delivered to cytoplasm are less degraded compared to DNA, and that the RNA viruses exploit the machinery present in the cytoplasm for their multiplication. In case of CaMV, it needs to be transported to nuclei for its multiplication.

The above argument still does not explain why protoplasts can be infected with CaMV and CaMV-DNA with the assisstance of PLO. The mode of action of PLO is little understood. It has been hypothesized that it can help appose virus particles with protoplasts. Burgess et al. (1973) have

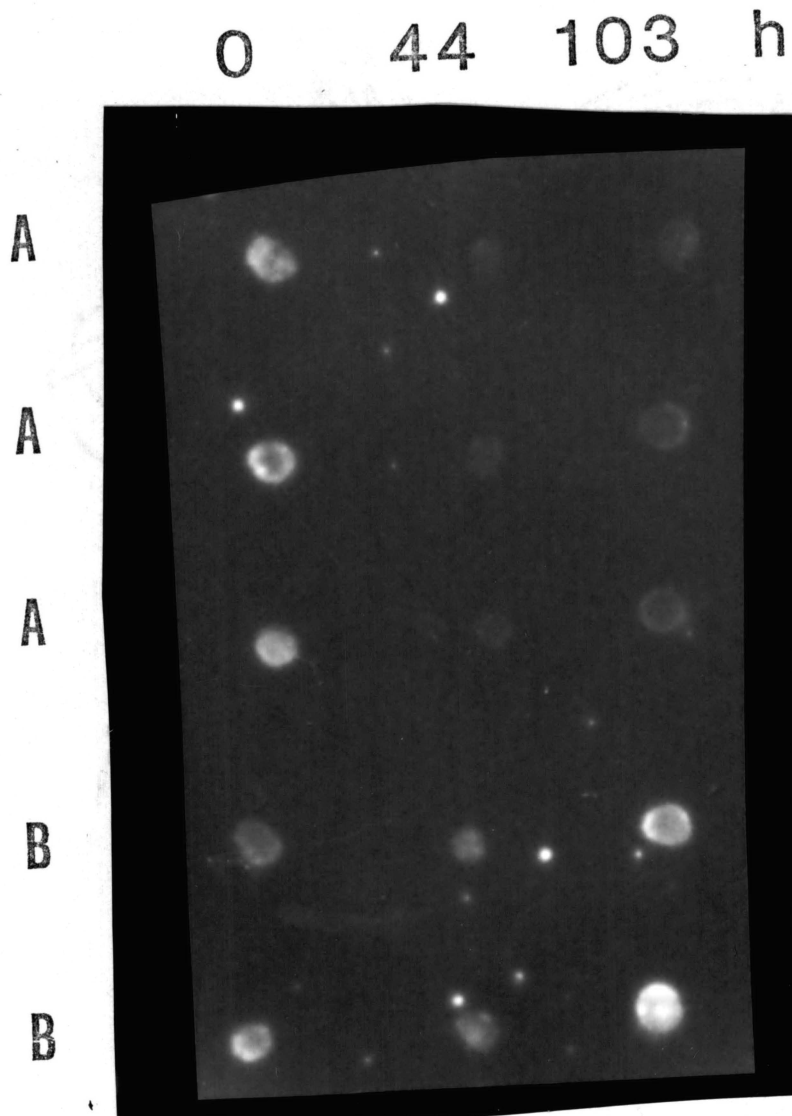

Figure 10. Autoradiographs of the Hybridised Samples from Infected Evacuolated and Non-evacuolated Protoplasts.
Non-evacuolated(A) and Evacuolated(B) protolasts were infected with PC:SA (7:3, molar) liposomes encapsulating CaMV as described in Fig. 9.

shown that it causes cell damage sufficient to help virus entry. It is possible that such damage might hurt the vacuoles, and their contents are oozed out, or the damaged cell wall synthesis is more important than degradation of foreign materials. Apart from these reasons, it should be remembered that in the case of PLO, virus and DNA are inroduced as virus and DNA. Virus and its DNA might have evolved some features to avoid their delivery to vacuoles. Similar arguments may explain the successful infection of plants by viruses with the help of aphids in nature and with abrasives in laboratories.

Transformation of Evacuolated Protoplasts from Cotton Suspension Cultures with Liposome Packaged CaMV

Cotton suspension cultures were those described by Ruyack et al. (1979). Protoplasts were prepared as described by Ruyack (1975). Cotton suspension culture protoplasts were evacuolated as described earlier for turnip protoplasts. In this case, however, protoplasts were centrifuged in Percoll gradient for 1h at 23^{0}C. After centrifugation in Percoll only one layer was observed which contained predominantly evacuolated protoplasts.

When cotton suspension culture protoplasts were fused with negatively charged liposomes containing CaMV, there was an initial decrease in hybridisable DNA up to 4h. However, longer periods of incubation resulted in about a ten fold increase in hybridisable DNA at 114h, at which time the virus multiplication had not yet reached a plateau (Fig.11).

It should be noted that cotton is not a host for CaMV (Melcher,

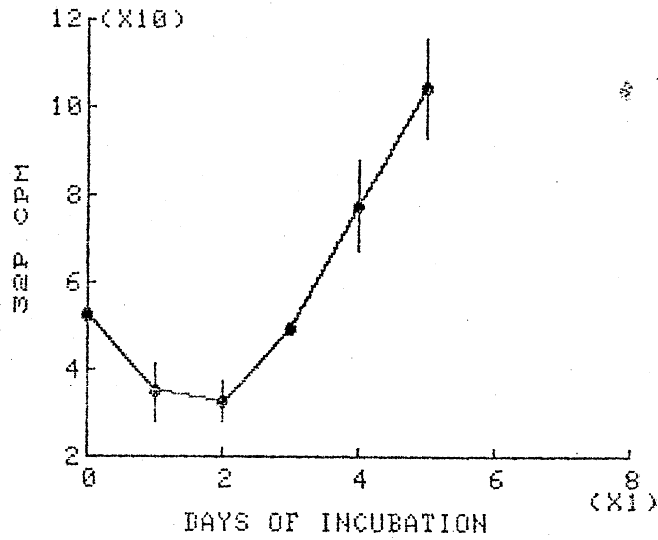

Figure 11. Infection of Cotton Suspension Culture Evacuolated Protoplasts with Liposome Packaged CaMV.
Cotton suspension culture evacuolated protoplasts (1.36×10^5) in 0.1 ml of MES buffer were infected with 100 microl of PC:PS (7:3, molar) liposomes as described in Fig. 9 (Results of one experiment).

unpublished). Thus it is possible that the constraint imposed by cotton plants for CaMV multiplication were absent in suspension culture protoplasts. This is a first report describing transformation of any suspension culture cells by CaMV. These results should encourage one to test diferent non-host plant protoplasts for their ability to support viral multiplication.

CHAPTER III

CELL FREE SYSTEM

No virus has been shown to complete its life cycle in cell free extracts obtained from host tissue. However, partial stages of their life cycle have been studied outside the cellular environment. Study of translational products of RNA viruses has been accomplished with a number of viruses, in related and unrelated cell free systems. In vitro systems to study the replication of viruses and plasmids are available e.g. adenovirus, a DNA virus, (Challberg and Kelly, 1979) and yeast 2 micron plasmid (Jazwinski and Edelman, 1979; Kojo et al., 1982). These systems have provided invaluable information to understand eukaryotic DNA replication (Challberg and Kelly, 1982).

Guilfoyle (1980) and Ansa et al. (1982) have presented evidence of transcription and replication of CaMV in nuclei isolated from infected turnip leaves. Pfeiffer and Hohn (1983) have described an extract from a mixture of nuclei and inclusion bodies, purified from CaMV infected turnip leaves, which supported transcription and replication of endogeneous CaMV. In the present study a cell free extract from healthy turnip leaves is described which may have supported CaMV-DNA synthesis. The first observations concerning the increase in hybridizable CaMV-DNA in healthy turnip extracts were made by Mr.Q.C.Mei in this laboratory.

Methods

Preparation of Cell Free Extract

All operations except centrifugation were carried out in a laminar flow hood. Solutions were sterilized either by filtration or autoclaving. Young leaves from 3-5 weeks old healthy turnip plants were collected and their midribs were removed. The midrib-less leaves (3 g) were surface sterilized by incubating in 0.2% sodium hypochlorite for 5 min at room temperature. The leaves were washed four times with sterile distilled water, and cut into small pieces with scalpel blades. The cut tissue was ground in the presence of glass powder in a mortar with 0.5 ml of 0.05M Tris-Cl buffer, pH 8.0, containing 0.35 M NaCl (Tris buffer). To this ground tissue was added 4.0-4.5 ml of the same buffer and the mixture was ground further. The contents were filtered through four layers of cheese cloth. The mortar and pestle were rinsed with 5.0-5.5 ml of the buffer and filtered through the cheese cloth. The filtrate was centrifuged at $4^{o}C$ for 5 min in a GLC-2 centrifuge (Beckman) at 800 rpm (about 100 g). The supernatant was recovered. In a 17 mm tube was pipetted 5.0 ml of Tris buffer containing 30% sucrose, and 2.0 ml of buffer containing 5% sucrose was layered on top. To this discontinuous sucrose gradient was added 5.0 ml of the supernatant and the tube was centrifuged for 30 min at $4^{o}C$ in a GLC-2 centrifuge at 2,200 rpm. From each tube about 6-7 ml of the top layer was removed and diluted 1:1 with the Tris buffer. The virus or DNA and different compounds as described in results were added to 1.0 ml of this extract, incubated at $23^{o}C$ for about 65-72 h in most cases.

For virus detection, 10-50 microl of incubation mixture was diluted with equal volumes of 1N NaOH and incubated at room temperature for a minimum of 10 min. Five to ten microl were pipetted on to a nitrocellulose sheet and neutralized, baked in vacuum oven, prehybridized and hybridized as detailed for the protoplast system, and described by Maule et al. (1983).

Results

When CaMV was incubated with the extract, there was a significant increase in the amount of hybridizable DNA after 40 h (Fig.12). After 68 h there was 1.5-2.0 times as much hybridizable DNA as was originally added to the extract. The increase was linearly dependent on the amount of virus (0.12-0.3 microg) added (data not shown). Attempts were made to enhance this activity.

When increasing concentrations of EDTA were added to the reaction mixture, a stimulation of the replicative activity was seen. An optimal stimulation occurred at 5-10 mM (Fig.13). The stimulation due to 5 mM EDTA was about 1.5-2.0 fold. Inhibition due to higher concentrations of EDTA (up to 50 mM) was not greater than 10-20% relative to the activity present at 5 mM (data not ahown). Similarly KCl (up to a concentration of 80 mM) activated the replicative activity (Fig.14). The stimulation of activity at 80 mM KCl was 4-fold. Higher concentrations significantly inhibited the activity. The increase in the hybridizable DNA after 72 h of incubation due to EDTA (5 mM) and KCl (80 mM) was 9-16 fold (Table 7).

The activity was not inhibited by antibodies raised against CaMV in one rabbit. It was insensitive to aphidicolin and actinomycin D (Table

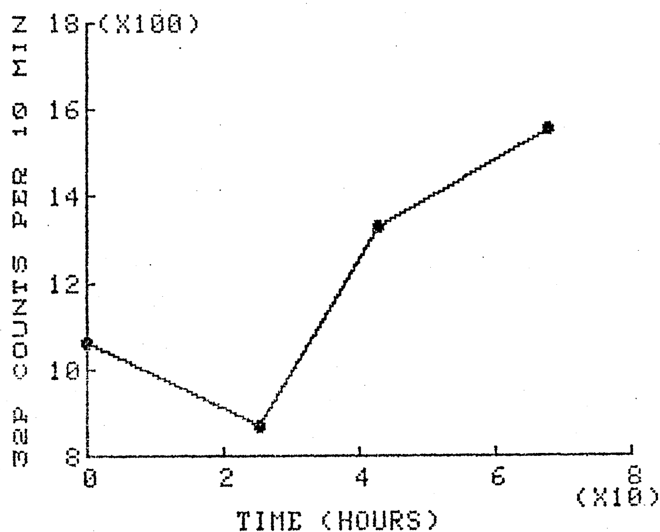

Figure 12. Time Course of Virus Multiplication in Cell Free System.
To 1.0 ml of extract was added 2.4 microg of virus and incubated at 23°C. At different times the incubation mixture was gently vortexed and 20 microl were mixed with equal volumes of 1.0 N NaOH. Ten microl were spotted on nitrocellulose sheet in triplicate. The samples were hybridized with ^{32}P-CaMV-DNA. Averaged counts were plotted against time.

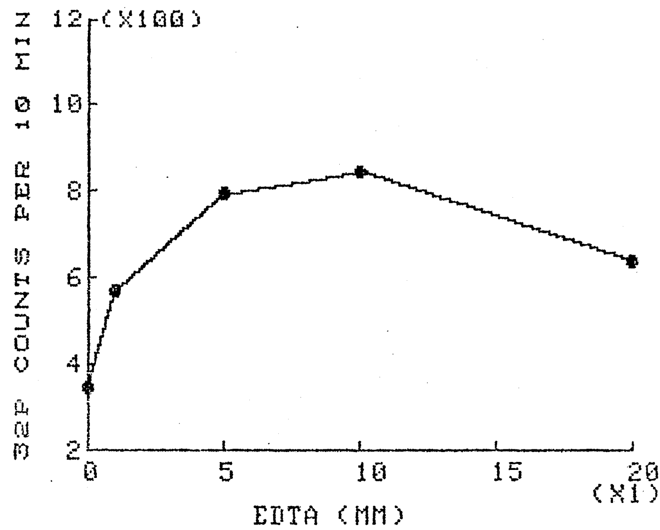

Figure 13. Effect of EDTA on Virus Multiplication in Cell Free System.
To 1.0 ml of extract (described in preparation of cell free extract), in duplicate, was added EDTA (0.5M stock) to the final concentrations, incubated at 23°C for 74 h. Aliquots were spotted in triplicate. Zero hour cpm were subtracted from 74 h cpm, averaged, and plotted against the concentration of EDTA.

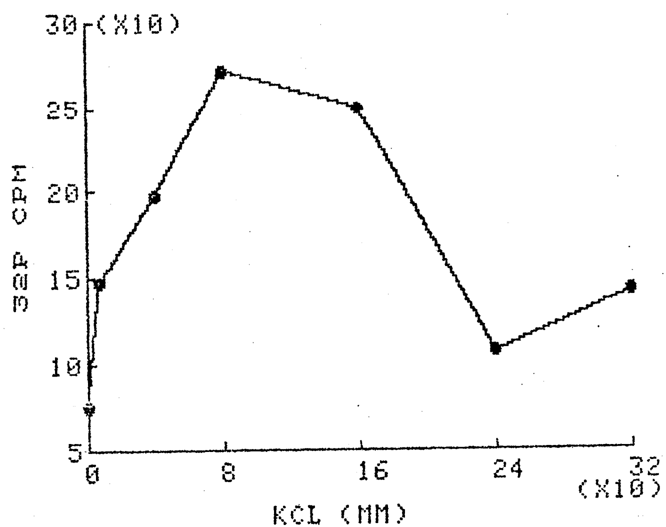

Figure 14. Effect of KCl on Virus Multiplication in Cell Free System.
The experiments were performed as described in Fig.10. The KCl stock was 4.0 M. (Results of one experiment).

TABLE VII

STIMULATION OF THE REPLICATIVE ACTIVITY PRESENT IN THE EXTRACT

Compounds	Times increase in hybridizable DNA
Extract	1.5-2.0
Extract + 5 mM EDTA	2.3-4.0
Extract + 5 mM EDTA + 80 mM KCl	9.0-14.0

To 1.0 ml of extract was added the indicated compounds and the mixture incubated at 23°C. Blank values (spots of virus free extract hybridised) were subtracted were from zero and final (66-74) hour counts. Times increase in final counts with respect to zero hour are reported.

8).

Specificity of the Replicative Activity

To determine which DNA templates can be replicated by the activity, CaMV-DNA and bacterial plasmids containing CaMV sequences undigested and digested with different restriction endonucleases, and bacterial plasmids containing no CaMV sequences were tested (Table 9). The extract supported an increase in hybridizable DNA at 66 h when CaMV strain CM4-184 DNA was added to the extract. There was an increase in the DNA sequences hybridizable to ^{32}P-CaMV-DNA and ^{32}P-pBR322, when pLW111D-C was added to the extract. However, there was no increase in the CaMV-DNA sequences when CM4-184 or pCMS31 was added after their cleavage with restriction endonucleases. Undigested pCMS31 did not replicate. There was no increase in the hybridisable ^{32}P-pBR325 when undigested and digested pBR325 and pCMS31 templates were incubated with the extract.

Discussion

The extract, prepared from healthy turnip leaves, was able to carry out replication of added CaMV resulting in increased amounts of hybridizable CaMV-DNA sequences. The activity was stimulated by EDTA and KCl. EDTA was tested with a view to inhibit nuclease activity. Maximum activity was observed when the EDTA concentration was 5-10 mM. Higher concentration of EDTA (upto 50 mM) inhibited the activity to about 10-20% compared to the activity at 5 mM. Since in the absence of EDTA, CaMV-DNA added to the extract was observed to be degraded rather than being replicated (Q.C.Mei, unpublished), EDTA probably was effective in inhibiting nuclease activity. It may also have complexed with some

TABLE VIII

ATTEMPTS TO INHIBIT REPLICASE ACTIVITY

Compounds (concentration)	Inhibition
Preimmune sera (50 microl/ml)	No
Rabbit anti-CaMV sera (50 microl/ml)	No
Aphidicolin (10 microM)*	No
Actinomycin D (36 microg/ml)	No

To 1.0 ml of extract was added different compounds to the indicated final concentrations, incubated at 23°C for about 66-74 h. Inhibition was compared to the control which had no compounds. In all cases no inhibition was observed. (* represents results of one experiment)

TABLE IX

SPECIFICITY OF THE REPLICATIVE ACTIVITY PRESENT IN THE EXTRACT

DNA Templates tested	^{32}P-CaMV-DNA and	^{32}P-pBR325 probes
CM4-184 DNA	+	
CM4-184-DNA digested with SalI	-	
CM4-184-DNA digested with EcoRI	-	
pLW111D-C	+	+
pCMS31	-	-
pCMS31 digested with SalI	-	-
pBR325		-
pBR325 digested with SalI		-

Hybridizable DNA sequences were probed with ^{32}P-CaMV-DNA and ^{32}P-pBR322-DNA. If cpm at the end of incubation (ca. 70 h) were more than at zero h, the results were reported positive. If there was no significant increase or there was a decrease in the cpm the results were reported as negative. (Results of one experiment).

inhibitory divalent cations.

KCl was tested because it was shown to be required by turnip DNA polymerase beta (b) and gamma(g) (Thomas et al., 1983). These authors have shown that the major DNA polymerase activities present in the turnips are alpha(a), b, and g type. DNA polymerase a and b require Mg^{2+}. "A" activity did not require KCl and maximum activity was at 125 mM. DNA polymerase g requires KCl with an optimum of 150 mM. In the present investigation optimum activity was observed at 80 mM. Higher concentrations of KCl significantly inhibited the activity. The replicative activity profile obtained in the presence of different concentrations of KCl, very much resembled that of DNA polymerase g from spinach chloroplasts in the presence of different concentration of KCl (Sala et al., 1980).

Aphidicolin is a specific inhibitor of "a" like DNA polymerase activity of plant cells (Sala et al., 1980). Thomas et al. (1983) have shown that turnip DNA polymerase b and g are not inhibited by aphidicolin, but "a" activity was sensitive to aphidicolin. The activity present in the extract was not inhibited by aphidicolin. Insensitivity towards aphidicolin and stimulation by KCl suggest that the activity responsible for increase in hybridizable CaMV-DNA sequences is not due to DNA polymerase a.

The activity was insensitive to actinomycin D, suggesting that the increase in hybridisable CaMV sequences was not due to transcription. This was confirmed by the fact that treatment of the incubated extract with ribonuclease did not result in any decrease in hybridizable sequences (U.Melcher, unpublished). It showed that the product was probably DNA.

Attempts to inhibit the increase in hybridisable DNA after the addition of virus into extract by antibodies against CaMV were negative. This indicates that either the encapsidated DNA was accessible to the replicative activity or the uncoating of the virus was occurring in the extract which was insensitive to antibody binding.

To study the specificity of the replicative activity, different DNA templates were added to the extract. When CaMV strain CM4-184 DNA was added to the extract, there was an increase in hybridisable sequences. However, when CM4-184 DNA was digested with SalI or EcoRI, the increase was abolished. The extract caused increase in hybridisable sequences when pLW111D-C was added. pLW111D-C is a partial nested dimer of CaMV-DNA, which does not need restriction for its infectivity. It was believed that intragenomic recombination results in native DNA formation, which subsequently infects the plants, whereas the vector sequences are lost (Walden and Howell, 1983). In the extract there was an increase in vector sequences, probed with ^{32}P-pBR322, as well as CaMV sequences, probed with ^{32}P-CaMV-DNA. It appears that the molecule was being replicated prior to release of CaMV sequences, or no release of CaMV sequences from vector sequences occurred in the extract.

In short it appears that the extract from healthy leaves contains enzyme(s) which replicate(s) CaMV-DNA. It remains to be seen whether this activity is important in the life cycle of CaMV. We hypothesize that this activity may represent an early event in the life cycle of CaMV. It is known that virus DNA is transported to nuclei, soon after infection, and forms minichromosomes by associating with host histones. Newly replicated DNA, and subgenomic DNA (due to partial replication or degradation) may also form minichromosomes. These minichromosomes are

known to be transcribed in nuclei (Guilfoyle, 1980). 35S RNA and other RNAs are transported to cytoplasm. All RNAs except 35S RNA probably get translated in the cytoplasm. The 35S RNA has been hypothesized to get transcribed into DNA and the DNA is packaged into virion with coat protein (Pfeiffer and Hohn, 1983; Hull and Covey, 1983). The activity identified in the present investigation may replicate the DNA in host nuclei. Alternatively the replication observed may be due to DNA polymerase g activity present in the organelles.

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VITA 2

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