STUDIES OF THE REPLICATION ABILITY OF CAULIFLOWER MOSAIC VIRUS MUTANTS AND PARTIAL DNA SEQUENCE OF THE NY8153 STRAIN

By

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STUDIES OF THE REPLICATION ABILITY OF CAULIFLOWER

MOSAIC VIRUS MUTANTS AND PARTIAL DNA

SEQUENCE OF THE NY8153 STRAIN

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ABBREVIATIONS

ATP	-	adenosine triphosphate
bis	-	N,N'-methylenebisacrylamide
BP	-	base pair
BSA	-	bovine serum albumin
Cabb-S	-	Cabbage-S strain
CaMV	-	cauliflower mosaic virus
CPM	-	counts per minute
DNA	-	deoxyribonucleic acid
DNase I	-	deoxyribonuclease I
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetraacetic acid
ETBr	-	ethidium bromide
IG	-	intergenic
2-ME	-	2-mercaptoethanol
mRNA	-	messenger RNA
NY8153	-	New York 8153 strain
PEG	-	polyethylene glycol
PVP	-	polyvinylpyrrolidone
RNA	-	ribonucleic acid
RPM	-	revolutions per minute
SDS	-	sodium dodecyl sulfate
SSC	-	saline sodium citrate

ABBREVIATIONS (continued)

Tris - Tris(hydroxymethyl)aminomethane

tRNA - transfer RNA

UV - ultraviolet

CHAPTER I

INTRODUCTION

Biological Properties

Cauliflower mosaic virus (CaMV) is a plant virus with limited host range that usually infects members of the Cruciferae family. Some strains also infect certain members of the Solanaceae. Turnip (Brassica rapa L.) is the host used most often for studies of CaMV. CaMV is the most studied member of the caulimoviruses, a small group of plant viruses containing double-stranded DNA as their genetic material. This is somewhat unusual since most plant viruses contain RNA (Shepherd, 1979).

The symptoms of the disease produced by CaMV and their severity vary with the strain of virus and the host. In general, inoculated leaves show mild symptoms consisting of chlorotic local lesions. Symptoms on the systemic leaves are more severe and include chlorotic mottling, vein-clearing, stunting of growth, and wrinkling of young emerging leaves (Shepherd, 1970).

Cytoplasmic granules are also produced during infection. These consist of virus particles embedded in a matrix of virus-coded protein, and are called inclusion bodies or viroplasms. Most of the virions in a cell are contained within the inclusion bodies, which are thought to be the site of viral replication and/or assembly (Shepherd, 1979).

CaMV is transmitted from plant to plant in nature by aphids. When

aphids feed on infected plants they pick up virus on their mouthparts, and then transmit this to uninfected plants by further feeding. Some strains of CaMV are not aphid-transmissible. It has been shown that CaMV strains which are aphid-transmissible produce a viral protein, called the helper component for aphid transmission, which is needed for the virus to be transmitted from one plant to another (Woolston et al., 1983; Armour et al., 1983). The virus can also be mechanically transmitted by rubbing a solution containing virions or viral DNA together with an abrasive on a susceptible plant (Shepherd et al., 1968; Shepherd, 1976 and 1979).

Once inside the plant, the virus spreads to all organs of it with most cells becoming infected (Melcher et al., manuscript in preparation). The amount of virus in systemically infected leaves is much greater than in inoculated leaves (Maule et al., 1983).

Properties of CaMV DNA

The genome of CaMV consists of a circular, double-stranded DNA molecule. It contains slightly over 8,000 base pairs (BP), the exact number varying from strain to strain (Balazs et al., 1982). CaMV DNA is unusual in that it contains single-strand discontinuities at specific sites which are referred to as "gaps". These are not areas where the nucleic acid is single-stranded, but rather are "overlaps", where one DNA strand is not covalently joined and the 3' and 5' ends of the discontinuity both contain the same sequence, and thus overlap each other (Franck et al., 1980; Richards et al., 1981). One of the DNA strands of CaMV (the α strand) contains a single gap, and the other strand (β) contains one, two, or three gaps depending on the

strain. These structures are thought to result from the replication of the viral genome (Hull and Covey, 1983c). The single gap in the α strand is used as the zero point for maps of the viral genome. The physical properties of CaMV DNA have been well characterized (Hull, 1979).

Electrophoresis of DNA isolated from virus particles reveals two major CaMV DNA forms, circular and linear. Only the circular form is infectious, and the linear form may arise solely by breakage of the circular form. Various other DNA species are also present, some being products of breakage and others being twisted or knotted varieties of the circular form (Hull, 1979; Shepherd, 1979; Menissier et al., 1983). CaMV DNA can also be found free in infected cells. Circular and linear molecules are present here also, as well as forms arising from breakage at the gaps (Hull and Covey, 1983a). In addition, molecules which may be replication intermediates have been found (Marco and Howell, 1984).

Restriction endonucleases have been used to map the genome of various strains (Hull, 1980; Volovitch et al., 1981). Some strains have similar maps, while some show considerable differences from others. Several restriction endonucleases have only one site on the viral genome. These can be used to clone the entire viral genome into a bacterial plasmid such as pBR322 (Howell et al., 1980; Lebeurier et al., 1980). After cloning and passage through bacteria (which repairs the gaps of the viral DNA), the recombinant plasmid can be isolated, digested with the restriction enzyme used for cloning, and inoculated onto plants. This linear viral DNA molecule

is able to infect plants. The cloned viral genome is useful for producing large quantities of viral DNA and doing various recombinant DNA manipulations on it. Recombinant DNA techniques can be easily applied to CaMV since it contains double-stranded DNA.

The CaMV Genome and Its Products

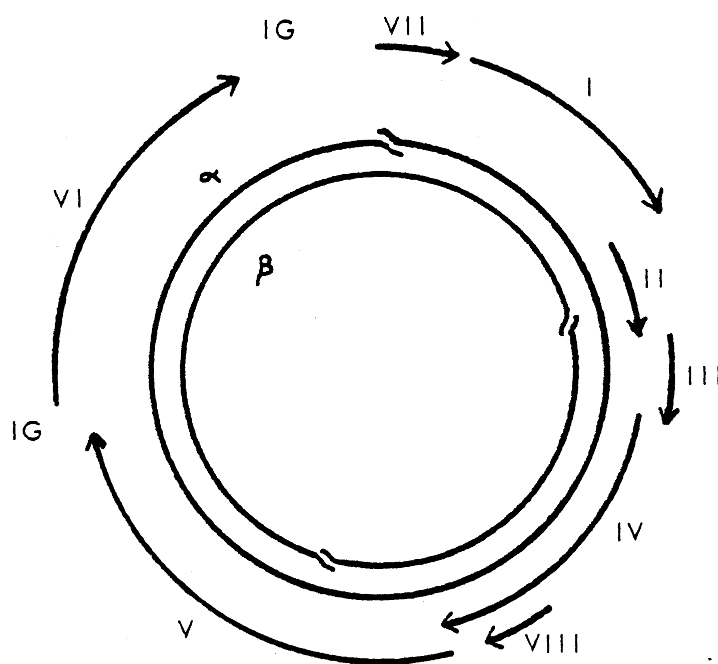
The nucleotide sequence of CaMV DNA has been determined for the Cabbage-S (Cabb-S; Franck et al., 1980), CM1841 (Gardner et al., 1981), and D/H strains (Balazs et al., 1982). These sequences show some variation from one to another, with most of the changes being base substitutions. Several insertions and deletions are also found. When each sequence is compared to the others, it differs by about five percent. The differences seem to be scattered almost randomly over the entire sequence. Base substitutions occur most often in the third base of the codons (the "wobble" base), so that changes in the amino acid sequence of the polypeptides are minimized (Balazs et al., 1982).

By looking at the distribution of start and stop codons in each of the three possible reading frames of both DNA strands, potential coding regions can be located. This has been done for the three sequenced strains, and eight open reading frames have been discovered. These are organized the same in all three strains and all are found in the β strand. These open reading frames are designated Regions I through VIII and are shown in Figure 1.

Regions I through VI are the major open reading frames and are considered most likely to produce functional viral proteins. Regions VII and VIII are minor ones and are not thought to code for any important viral proteins. Each of these coding regions has a start

Figure 1. Map of the CaMV Genome.

The two DNA strands (α and β) of a strain containing three gaps are shown. The eight potential coding regions are represented by the arcs lying outside of the DNA strands. Distance from the center of the figure to the arc specifies which reading frame it is in.


IG
VII
I
II
III
IV
VIII
V
IG
VI
α
β

codon near its beginning and ends with a stop codon. These open reading frames are packed closely together and many of them overlap (Balazs et al., 1982; Howell, 1982).

The CaMV genome also contains two intergenic regions (IG), a small one lying between Regions V and VI and a large one lying between Regions VI and VII. The nucleotide sequence in the intergenic regions is more conserved than in the coding regions. This is thought to be due to the presence of sequences important to transcription and replication (Balazs et al., 1982).

Little is known about the functions of Regions I and III and their products. Both of these coding regions are essential since mutations in them (including in-phase insertions and deletions) cause loss of infectivity (Dixon et al., 1983; Daubert et al., 1983).

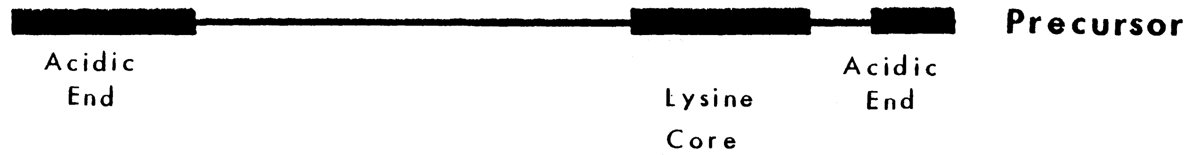
Region II is not an essential part of the viral genome since naturally occurring deletions exist (strain CM4-184) which lack almost all of this coding region (Howarth et al., 1981). Studies have shown that the product of Region II is the helper component for aphid transmissibility (Armour et al., 1983; Woolston et al., 1983). Since this area is not essential, it is an attractive location for insertion of foreign DNA into the viral genome.

The viral coat protein is produced by Region IV. This was originally determined from the amino acid composition of the coat protein and the nucleotide sequence of this coding region (Franck et al., 1980). More recent studies using an antibody against the coat protein have confirmed this assignment (Daubert et al., 1982). This coding region has an interesting structure and is shown in Figure 2.

Figure 2. Structure of Region IV.

The putative precursor of the coat protein and the functional coat protein are shown aligned with Region IV. The positively-charged lysine core is shown along with the negatively-charged, acidic ends of the precursor.

Region IV


Precursor
Acidic End
Lysine Core
Acidic End

Coat Protein

A putative precursor of the coat protein is originally synthesized. The functional coat protein is thought to arise by cleavage of the ends off of the precursor. These ends are rich in the negatively-charged, acidic amino acids glutamate and aspartate, and are thought to neutralize a lysine-rich, positively-charged area of the precursor known as the lysine core. The active coat protein then presumably packages the CaMV DNA by interaction between the lysine core and the negatively-charged viral DNA (Franck et al., 1980).

CaMV shares some similarities with hepatitis B virus and retroviruses, both of which replicate by reverse transcription (Volovitch et al., 1984). This has led to the suggestion that CaMV also replicates by reverse transcription (Guilley et al., 1983; Hull and Covey, 1983b; Pfeiffer and Hohn, 1983). Region V is thought to code for the viral reverse transcriptase since it is the only unaccounted for coding region of sufficient size to do so. It also has the most highly conserved amino acid sequence of all the coding regions, especially in the C-terminal three-fourths of the molecule (Balazs et al., 1982). Recently it has been shown that a portion of the Region V gene product is homologous to the amino acid sequence of the polymerase of Moloney murine leukemia virus, which is a retrovirus. All of this evidence points to Region V being the reverse transcriptase gene, but this has not been proven conclusively at this time.

Region VI does not overlap any other coding region, and is surrounded by the two intergenic regions. Region VI is known to code for the inclusion body protein, in which virions are embedded in the cytoplasm (Covey and Hull, 1981). This protein shows more variability in its amino acid sequence than any of the other major coding regions

(Balazs et al., 1982).

Little is known about Regions VII and VIII. Region VII is not essential since an in-phase deletion of approximately one-third of this coding region does not prevent infection (Dixon et al., 1983). Region VIII shows significantly more variability in its nucleotide and amino acid sequences than any of the other seven coding regions (Balazs et al., 1982). At present there is no evidence to suggest that either of these regions produces an important viral protein.

Transcription and Translation

A fairly large amount of information about the transcription of the CaMV genome has been obtained. Only the α strand of the viral DNA is transcribed (Howell and Hull, 1978). This gives RNA sequences equivalent to those of the β strand, which contains all of the potential coding regions.

Two major polyadenylated transcripts, termed the 19S and 35S RNA, have been found (Covey et al., 1981; Dudley et al., 1982; Odell et al., 1981). The precise locations of these on the viral genome have been mapped (Guilley et al., 1982). The 5' end of the 19S transcript is located in the small intergenic region while that of the 35S RNA is in the large intergenic region near the end of Region VI. Both of these transcripts terminate at the same position in the large intergenic region.

The 19S RNA is a transcript of Region VI and synthesizes the inclusion body protein in an in vitro system (Xiong et al., 1982; Odell and Howell, 1980; Covey and Hull, 1981). The 35S RNA is a transcript of the entire viral genome plus approximately 200 additional

nucleotides, resulting in the ends of the molecule being direct repeats. The 35S RNA has not been successfully translated in vitro. This molecule is thought to be reverse transcribed during viral replication. A covalently-closed, supercoiled CaMV DNA form has been found in infected plants (Mennissier et al., 1982), and this has been shown to be in the form of a nuclear minichromosome which is transcriptionally active (Olszewski et al., 1982). It is thought that the 35S RNA is transcribed from this minichromosome.

The 35S RNA could be cleaved apart to produce a messenger RNA for each coding region. However, there is no evidence for this. At the present time it is thought that the 35S transcript acts as a polycistronic messenger RNA and is translated according to a "relay race" rule (Dixon et al., 1983). The coding regions of CaMV are packed tightly together with the stop codon of one being very close to the start codon of the next region. A ribosome may attach to the start of the coding regions (the beginning of Region VII) and synthesize protein until it reaches a stop codon. At this point it is extremely close to the start codon of the next coding region and may immediately start to translate the next region without leaving the 35S transcript. If there is a large distance between these codons, the ribosome may fall off the RNA and not be able to initiate translation at the next coding region.

This rule does not apply to Region VI since it is separated from other coding regions by the intergenic regions and has its own separate transcript. A separate messenger RNA for this coding region exists possibly because the viral infection requires large amounts of this protein to produce the inclusion bodies.

If this rule holds true, mutations which disrupt the close association of the start and stop codons and produce a large intergenic region will prevent translation of the coding regions downstream. In-phase insertions or deletions within a region should not affect downstream translation. Also, a mutation which destroys the original stop codon but creates another one at or near the original site should not affect translation.

Other minor transcripts of the CaMV genome have been found (Condit et al., 1983; Covey and Hull, 1981; Guilley et al., 1982). No role for them has been found and they may be degradation products of other transcripts or the products of anomalous transcription by RNA polymerase.

Replication

It has recently been suggested that CaMV replicates by reverse transcription (Guilley et al., 1983; Hull and Covey, 1983b; Pfeiffer and Hohn, 1983). Evidence which supports this hypothesis includes the detection of reverse transcriptase activity in infected plants (Volovitch et al., 1984) and identification of DNA intermediates consistent with the proposed model (Marco and Howell, 1984). A model for the replication of CaMV has been described in detail (Hull and Covey, 1983c; Pfeiffer and Hohn, 1983).

This model proposes that the DNA (either viral DNA with gaps or cloned, linearized viral DNA without gaps) enters a cell and gets into the nucleus where it is repaired to form a covalently-closed supercoiled DNA molecule. This DNA becomes associated with nuclear proteins to form a minichromosome from which the 35S RNA is transcribed. This transcript is transported into the cytoplasm where it is reverse

transcribed. A transfer RNA is thought to bind near the 5' end of the 35S RNA and act as a primer for DNA synthesis. Reverse transcriptase then synthesizes a DNA strand using 35S RNA as a template, degrading the RNA as it moves. Synthesis is towards the 5' end of the RNA and stops when it reaches the end of the molecule.

Due to the direct repeats on the ends of the 35S RNA, the 3' end of the RNA can hybridize to the end of the newly synthesized DNA strand and synthesis can continue along the entire 35S RNA molecule. The RNA is degraded as this occurs and synthesis of the second DNA strand (β) can occur using the first strand (α) as a template. Synthesis is initiated at the location of the gaps and proceeds as far as possible until running into the end of the DNA molecule or another DNA strand. The first DNA strand is synthesized to the 5' end of the 35S RNA. It then hybridizes to a portion of the second DNA strand complementary to the tRNA primer and this allows DNA synthesis to be completed. The overlaps at the gaps result from a small amount of additional synthesis after the polymerase runs into another DNA strand. This model explains the presence of the gaps and other aspects of CaMV replication.

Uses of CaMV

CaMV is one of the few plant viruses which contain double-stranded DNA. This DNA is fairly small in size and can be cloned in a bacterial plasmid. These properties allow the CaMV genome to be easily manipulated by recombinant DNA techniques. Because of this, CaMV would be a good system for studying gene expression in plants.

When a plant becomes infected, CaMV spreads to most cells of

the plant. This seems to be an attractive way to get new genetic material into a plant. This combined with the other properties of CaMV makes it an attractive candidate for a plant genetic engineering vector.

One possible way to use CaMV as a vector is to insert a piece of foreign DNA into the viral DNA and then infect the plant. Essential and nonessential regions of the viral genome need to be identified to know where to make the insertion. Also, there seems to be a limit to the amount of foreign DNA the CaMV genome can carry (Gronenborn et al., 1981). Some nonessential viral DNA will need to be removed to make more room for the foreign DNA. Finally, in order to be useful, the vector must not be harmful to the plant. CaMV will need to be modified in some way so that it either does not make plants sick or else produces only very mild symptoms that do not significantly affect the growth of the plant.

The goal of the present study was to produce some deletion mutations in the CaMV genome and to determine whether any of these or other available mutants met the above criteria for a useful vector. A method for detecting CaMV replication in inoculated leaf tissue was developed to detect replication of CaMV mutants which did not produce symptoms in the inoculated plants.

CHAPTER II

MATERIALS AND METHODS

Starting Materials for Mutants

Mutants which had selected PstI restriction fragments deleted and those which had selected XbaI fragments deleted were constructed from the plasmid pCMS31. This plasmid consists of DNA from the New York 8153 (NY8153) strain of CaMV cleaved at its single SalI restriction site and ligated into the plasmid pBR322 at its unique SalI site (Armour et al., 1983). This construction interrupts Region V of the virus. Mutants with EcoRI fragments deleted were also constructed from pCMS31.

An attempt to delete the smallest EcoRI fragment utilized the plasmid pCS101 (which is identical to the plasmid Ca37 of Lebeurier et al., 1982). This plasmid consists of DNA from the Cabbage S (Cabb-S) strain of CaMV inserted into pBR322 in the same manner as pCMS31. The nucleotide sequences and restriction maps of these two strains are slightly different (Franck et al., 1980; Hohn et al., 1980; Hull, 1980; Gardner et al., 1980).

These two recombinant plasmids confer resistance to the antibiotic ampicillin. The viral DNA can be separated from pBR322 by cleaving the plasmid with SalI, and the resulting linear viral DNA is infectious (Howell et al., 1980). The two plasmids containing

the viral genomes as well as the mutants produced from them were maintained in *Escherichia coli* (*E. coli*) K-12 strain HB101.

Storage of Bacteria

Long-term storage of bacterial strains was at -70°C in 15% v/v glycerol. Isolated colonies from an antibiotic plate (1.0% w/v bacto-tryptone, 0.5% w/v yeast extract, 1.0% w/v NaCl, 2.0% w/v agar, and 50 µg/ml ampicillin) were used to inoculate a 5.0 ml tube of nutrient broth (1.0% w/v bacto-tryptone, 0.5% w/v yeast extract, and 1.0% w/v NaCl). This tube was incubated overnight at 37°C. One hundred fifty µl of sterile glycerol was placed in a sterile 1.5 ml Nunc cryovial and 1.0 ml of the bacterial culture was added to this. The cryovial was tightly capped and vortexed to mix the contents well. The vial was then stored at -70°C. Bacteria were recovered from the storage vial by scraping the frozen surface with a flame-sterilized loop and then transferring to an antibiotic plate.

Plasmid Isolation

Bacteria containing the desired plasmid were streaked from a frozen culture in a cryovial onto an antibiotic plate and grown overnight at 37°C. Isolated colonies were used to inoculate a flask containing 250 or 500 ml of nutrient broth which was incubated with shaking at 37°C for approximately 20-30 hours.

Bacterial cells were harvested from the culture by low-speed centrifugation. Cells were resuspended and lysed by either the method of Clewell and Helinski (1969), which uses lysozyme and Triton X-100 treatment, or the method of Ish-Horowicz and Burke

(1981), which utilizes NaOH and sodium dodecyl sulfate (SDS) to lyse the cells. High-speed centrifugation was then used to separate bacterial debris and precipitated material from the plasmid which remained in solution.

This crude plasmid preparation was further purified by centrifugation to equilibrium in cesium chloride with ethidium bromide present. The DNA bands were visualized by ultraviolet (UV) light, and the supercoiled plasmid band recovered by piercing the tube with a needle and syringe and drawing off the band.

The ethidium bromide was removed from the DNA by extracting several times with n-butanol saturated with 5.0 M NaCl. The DNA was then precipitated with ethanol overnight at $-20^{\circ}C$ and redissolved in 100-200 µl of DNA dissolving buffer (10 mM Tris, 10 mM NaCl, 1 mM EDTA, pH 7.5).

Partial Restriction Endonuclease Digestions

Partial digests were obtained by incubating several reaction mixtures, each containing the same amount of DNA and restriction endonuclease, for different lengths of time. The amount of enzyme used and the incubation times were determined empirically. After incubation, the digestion was stopped by extracting with phenol saturated with 10 mM Tris, 1 mM EDTA, pH 8.5 (TE buffer). The phenol was re-extracted with distilled water and the aqueous phases were combined. Residual phenol was removed by extracting twice with diethyl ether. An aliquot was removed and analyzed on an agarose gel to determine the extent of the digestion. Reactions with an intermediate amount of digestion were used for production of mutants.

The PstI partial digests used in the production of deletion mutants were obtained by incubating 2.8 μg of pCMS31 DNA in PstI buffer (Table I) with three units of PstI at 37°C for 10-60 minutes. The EcoRI partial digests were obtained by cleaving 1.5 μg of pCMS31 DNA in EcoRI buffer (Table I) with one unit of EcoRI at 37°C for 30-120 minutes. The XbaI partial digests were obtained by incubating 1.0 μg of pCMS31 in XbaI buffer (Table I) with three units of XbaI at 37°C for 20-40 minutes.

Religation of Partial Digests

Approximately 0.25-0.50 μg of the partially digested DNA was ligated to produce the deletion mutants. The DNA was placed in 100 μl of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 1 mM adenosine triphosphate (ATP), 10 mM dithiothreitol (DTT), and 1-2 units of T4 DNA ligase (New England Biolabs). The mixture was incubated at 15°C for 10-20 hours. The ligated DNA was then stored at -70°C until used for transformation of bacteria.

Transformation of Bacteria

Bacteria competent for transformation were prepared by the method of Morrison (1979). Transformation was done according to Woods et al. (1980). The DNA was mixed with competent bacteria (*E. coli* K-12 strain HB101) and kept on ice 20-30 minutes. The mixture was frozen in an isopropanol-dry ice bath, thawed at room temperature, warmed at 42°C for one minute, cooled in ice two minutes, and incubated 30 minutes at 37°C with nutrient broth added. Finally, the mixture was spread onto an antibiotic plate

TABLE I

RESTRICTION ENDONUCLEASE BUFFERS

BglI	BglII	ClaI
10 mM Tris, pH 7.4	50 mM Tris, pH 7.5	6 mM Tris, pH 7.9
60 mM NaCl	50 mM NaCl	50 mM NaCl
10 mM $MgCl_2$	10 mM $MgCl_2$	6 mM $MgCl_2$
1 mM DTT	1 mM DTT	
EcoRI	**HindIII**	**HpaI**
100 mM Tris, pH 7.2	50 mM Tris, pH 8.0	10 mM Tris, pH 7.4
50 mM NaCl	50 mM NaCl	20 mM KCl
5 mM $MgCl_2$	10 mM $MgCl_2$	10 mM $MgCl_2$
		6 mM 2-ME
PstI	**SalI**	**XbaI**
20 mM Tris, pH 7.5	6 mM Tris, pH 7.9	6 mM Tris, pH 7.9
50 mM $(NH_4)_2SO_4$	150 mM NaCl	50 mM NaCl
10 mM $MgCl_2$	6 mM $MgCl_2$	6 mM $MgCl_2$
	6 mM 2-ME	

All digests also contained 100 μg/ml bovine serum albumin (BSA).

and incubated overnight at 37°C.

With each transformation a positive and negative control were also done to ensure that the procedure was working properly. The positive control consisted of bacteria transformed with 0.1 μg of a plasmid, usually pBR322 or pCMS31, and the negative control was a transformation done with sterile water.

Small-Scale Rapid Plasmid Isolation

Colonies obtained from transformations were carefully transferred to another antibiotic plate and grown overnight at 37°C. Bacteria that grew on this second plate were used to inoculate 5.0 ml of nutrient broth in a test tube which was incubated overnight at 37°C with shaking. Plasmid DNA was isolated by the method of Ish-Horowicz and Burke (1981).

A 1.5 ml Eppendorf tube was filled with the bacterial culture using a sterile Pasteur pipet. The cells were harvested by centrifugation in a Beckman microfuge for 1-2 minutes. They were resuspended in 100 μl of 50 mM glucose, 2 mM Tris, 10 mM EDTA, pH 8.0, and were lysed with 200 μl of 0.2 M NaOH, 1.0% w/v SDS for five minutes at 0°C. One hundred fifty μl of 5.0 M K^+/H^+ acetate, pH 4.8-5.2, was added to precipitate SDS and chromosomal DNA. This was pelleted in a microfuge and the supernatant transferred to another tube. This tube was filled with absolute ethanol, incubated at room temperature two minutes, and the DNA pelleted in a microfuge. The pellet was washed with 70% v/v ethanol, dried under vacuum, and redissolved in 40 μl of DNA dissolving buffer. An aliquot of each isolated plasmid was electrophoresed on a 1.0% agarose gel to determine its size and the size of

its deletion, if any.

Screening for Desired Mutant

The partial digestion/religation procedure will produce a variety of deletion mutants with different fragments deleted. These must be screened to find the desired mutant. Screening was done by cleaving possible mutant plasmids with a restriction endonuclease and analyzing the digest by agarose gel electrophoresis. The patterns produced were compared to the pattern given by the starting material and to that predicted for the desired mutant.

PstI deletion mutants were screened by cleavage with BglI in the appropriate buffer (Table I) at $37^{o}C$. The identity of the mutant was confirmed by cleavage with BglII. These digests were electrophoresed on a 1.0% agarose gel in Tris-phosphate buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.4).

XbaI deletions were screened by cleavage with EcoRI. The digests were electrophoresed on a 1.0% agarose gel in Tris-phosphate or Tris-acetate buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.0). Digestions with SalI, XbaI, BglII, and HindIII confirmed the presence of the desired deletion. These were electrophoresed on a 1.5% agarose gel in Tris-acetate buffer.

EcoRI deletion mutants were screened by cleavage with XbaI followed by electrophoresis on a 2.0% agarose gel in Tris-acetate buffer, or a 5.0% polyacrylamide gel with a 29 to 1 weight ratio of acrylamide to N,N'-methylenebisacrylamide (bis). DNA was visualized on all gels by staining with 0.5 μg/ml ethidium bromide (ETBr) and exposing the gel to UV light.

Digestion of pCS101 with HpaI + XhoI

pCS101 DNA was cleaved in 50 µl of HpaI buffer (Table I). 2.5-3.5 units of HpaI (New England Biolabs) per microgram of DNA was used, and the reaction incubated at 37°C for 3-4 hours. Twenty-five µl of 0.4 M NaCl, 2.5 µl of 60 mM 2-mercaptoethanol (2-ME), and 5-8 units of XhoI (New England Biolabs) per microgram of DNA were added and the mixture incubated another 3-4 hours at 37°C. An aliquot was electrophoresed on an agarose gel to ensure that cleavage had occurred and had produced two fragments of approximately 1.5 and 11.0 kilobases. These were referred to as the small and large fragments, respectively.

Isolation of DNA Fragments from Agarose Gels

DNA fragments were isolated from gels using a method based on that of Weislander (1979). DNA was loaded onto a low gelling temperature agarose gel (Sigma Chemical Co.) in Tris-acetate buffer and electrophoresed as usual. The gel was stained with ETBr and the DNA bands were observed using UV light. The bands were cut out with a scalpel, and placed in a 1.5 ml Eppendorf tube. About five volumes of DNA dissolving buffer and 25 µg yeast tRNA were added and the gel was melted at 65°C for five minutes.

The mixture was cooled to 25-35°C and extracted with one volume of phenol saturated with TE buffer. The aqueous phase was removed and the phenol re-extracted with distilled water. The aqueous phases were combined and the phenol extraction repeated. The resulting aqueous phase was extracted with one volume of phenol:chloroform (1:1). The aqueous phase was removed, and the organic phase

re-extracted with distilled water. The combined aqueous phases were extracted with chloroform, and the chloroform re-extracted with distilled water. The aqueous phases were combined.

The DNA was precipitated by adding two volumes of absolute ethanol and placing at $-20^{o}C$ overnight. The DNA was pelleted and redissolved in a small volume (20-50 µl) of DNA dissolving buffer.

Cleavage of Small Fragment with EcoRI

Approximately 0.1 µg of the small fragment isolated from an agarose gel was cleaved in EcoRI buffer (Table I) plus 6 mM 2-ME. Twenty units of EcoRI was added and the reaction incubated at $37^{o}C$ for four hours. After incubation the mixture was phenol extracted and ethanol precipitated.

Blunt-End Ligation

0.05-0.5 µg of DNA fragments were placed in 100 µl of 50 mM Tris, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 100 µg/ml BSA. Usually 800 units of T4 DNA ligase and 8 units of T4 RNA ligase (both from New England Biolabs) were added and the reaction incubated at $15-20^{o}C$ for 18-70 hours. Some ligations used different amounts of these enzymes. Some reactions also included 15% w/v polyethylene glycol (PEG; Zimmerman and Pheiffer, 1983). After incubation, the ligated DNA was stored at $-70^{o}C$ until used for transformation or electrophoresis.

Mutants Constructed by Others

Several mutants constructed by other people were used in these

studies. pCMS34 was constructed from pCMS31 and lacks most of Region III. pCS101-4Δ30, pCS101-4Δ33, and pCS101-4Δ50 are all deletions of approximately 100 base pairs in Region IV. pUM37 is a larger deletion of about 1,000 base pairs. pUM13 has an 8 base pair insertion in Region IV. All of these Region IV mutants were constructed from pCS101. pCS101-4Δ30, pCS101-4Δ33, and pCS101-4Δ50 were constructed by Ken Richards, and the remainder by Ulrich Melcher.

Preparation of DNA for Inoculation

Plasmid DNA was prepared for inoculation by cleavage with SalI to separate the viral sequences from the pBR322 sequences of the recombinant plasmid. The desired amount of plasmid DNA was cleaved in 50 μl of SalI buffer (Table I). The amount of enzyme added depended on the amount of DNA present, with at least one unit of SalI per microgram of DNA used. Reaction mixtures were incubated at 37°C for approximately three hours. After the incubation was completed a small portion was electrophoresed on an agarose gel to ensure that the digestion had worked. The cleaved DNA was stored at -70°C until used for inoculation.

Immediately before use the desired amount of DNA was diluted with water and 0.6 M potassium phosphate, pH 8.5, to final concentrations of 20 μg DNA/ml and 60 mM potassium phosphate. The linear pBR322 DNA was not separated from the linear viral DNA.

Inoculation of Plants

Turnips (*Brassica rapa L.* cv. Just Right) were used as the host

on which wild-type CaMV was grown and mutants were tested. Plants were grown in a growth chamber with 12 hour day and night periods. The temperature for these two periods was approximately 21°C (68°F) and 16°C (61°F), respectively. Three to four week old plants were used for inoculation. Plants at this stage had 4-6 leaves greater than 1.0 cm in length. Two or three of the youngest of these 4-6 leaves per plant were inoculated and the remainder removed.

Leaves to be inoculated were sprinkled with a small amount of celite (diatomaceous earth) and the inoculum pipetted onto the leaf surface. 0.5 μg of SalI-cut plasmid in 25 μl of 60 mM potassium phosphate was used for the inoculation of each leaf. Once on the leaf, the inoculum was rubbed gently over the entire surface with the tip of a gloved finger. Finally, the inoculated plants were watered and placed back in the growth chamber so that they were not in contact with each other.

After inoculation, plants were observed periodically for inoculation damage, condition of plant, and appearance of symptoms of viral infection. These items were noted and recorded.

Rapid Viral DNA Preparation

Viral DNA was prepared according to the method of Gardner and Shepherd (1980) with some modifications. Leaves were harvested from plants 12-14 days after inoculation. 2.5 grams of leaf tissue (excluding the midrib) were placed in a mortar, liquid nitrogen added, and the leaf tissue ground to a fine powder. Ten ml of 1.5 M urea, 200 mM Tris, 20 mM EDTA, pH 7.5, was added to the mortar and mixed well with the ground leaf tissue. The homogenate was poured

into a 30 ml Corex centrifuge tube. The mortar was rinsed with 2.0 ml of 10% v/v Triton X-100 and this was added to the homogenate. This was mixed well and stored on ice for 2-4 hours with occasional mixing.

The homogenate was spun at 12,000 X g for ten minutes to remove large debris. The supernatant was filtered through one layer of miracloth, and then layered on top of 3.0 ml of 15% w/v sucrose (in the same buffer added to the ground leaf tissue) in Oak Ridge centrifuge tubes and spun at 34,000 revolutions per minute (RPM) for 2½ hours in a Beckman Ti75 rotor. The supernatant was aspirated off of the viral pellet.

The pellet was resuspended in 200 µl of 100 mM Tris, 2.5 mM $MgCl_2$, pH 7.5. 2.0 µl of 1.0 mg/ml deoxyribonuclease I (DNase I) was added and incubated at 37°C for ten minutes. 4.0 µl of 0.5 M EDTA, pH 7.5, 12.5 µl of 20% w/v SDS, and 50 µl of 2.5 mg/ml Proteinase K were added and the solution incubated at 65°C for ten minutes. Ten µl of 5.0 M NaCl was added and the solution was extracted with an equal volume of phenol saturated with TE buffer. The aqueous phase was recovered and extracted with diethyl ether. The viral DNA was precipitated with 2-3 volumes of ethanol and redissolved in 100 µl of DNA dissolving buffer.

Total Plant DNA Preparation

Leaves were harvested from plants 10-12 days after inoculation. Ten to twenty grams of leaf tissue were placed in a mortar, liquid nitrogen was added, and the material ground to a fine powder and kept frozen. For each gram of leaf tissue the following was added:

50 μl of 2.0 M Tris, pH 8.0, 50 μl of 20% w/v SDS, 100 μl of 0.5 M EDTA, pH 8.0, and 1.0 ml of phenol saturated with 3 X saline sodium citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate). The ground tissue was allowed to thaw and was mixed well with the added liquid. The homogenate was poured into a centrifuge tube and spun at 2,500 RPM in a Sorvall GLC-2 centrifuge for five minutes. The upper aqueous phase was transferred to another tube and extracted with an equal volume of phenol:chloroform (1:1). Centrifugation was performed as above to separate the phases. The aqueous phase was removed and extracted several times with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was recovered and the DNA precipitated by adding 1-2 volumes of ethanol until a fluffy precipitate appeared. The DNA was placed at $-20^{o}C$ overnight, pelleted, and redissolved in 1.0 ml of DNA dissolving buffer.

Electroblotting

Agarose gels to be electroblotted were stained with ETBr and photographed. Excess agarose was cut away from the areas of interest, and the gel exposed to UV light an additional 5-10 minutes longer to facilitate transfer by damaging the DNA. The DNA in the gel was denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for approximately one hour with gentle shaking. The gel was then neutralized by shaking it in 3.0 M NaCl, 0.5 M Tris, pH 7.0 for another hour.

A sheet of nitrocellulose (Schleicher and Schuell) was cut to the size of the gel and immersed in the electroblot buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.5). The nitrocellulose was placed in contact with the bottom of the gel and put in a Bio-Rad

electroblot apparatus. The transfer was done at 5-10 volts, 200-250 milliamperes overnight at $4^{o}C$.

After the electroblotting was finished, the nitrocellulose was taken out of the apparatus and air-dried. The gel was restained with ETBr to determine how well the transfer had worked.

Nick Translation

0.1 μg of viral or plasmid DNA was labelled with ^{32}P by nick translation (Rigby et al., 1977). The DNA was placed in 10 μl of a solution containing 6.6 mM Tris, pH 7.4, 6.6 mM $MgCl_2$, 1.0 mM DTT, 50 mM KCl, 50 μM each of dATP, dGTP, and dTTP, 5 μM dCTP, and 5-10 μCi (7.5-15 pmol) of α-^{32}P-dCTP (New England Nuclear). The DNA was nicked by adding 10 pg of DNase I (Sigma Chemical Co.) and incubated at $15^{o}C$ for three hours.

After incubation, 40 μg of sonicated calf thymus DNA was added as a carrier, and sodium acetate was added to a concentration of 0.3 M. The solution was extracted with phenol and ethanol precipitated overnight at $-20^{o}C$. The nick translated DNA was redissolved in 100 μl of DNA dissolving buffer and an aliquot was counted before use.

Hybridization of ^{32}P-Labelled DNA to Electroblots

The dried nitrocellulose filter was baked at approximately $80^{o}C$ for two hours. It was then immersed in 6 X SSC and placed in a heat-sealable bag. Twenty or thirty ml of prehybridization solution was added, depending on the size of the bag. This solution consisted of

6 X SSC, 0.5% w/v SDS, 0.1% w/v polyvinylpyrrolidone (PVP), 0.1% w/v BSA, 0.1% w/v Ficoll, and 100 μg/ml sonicated calf thymus DNA which was denatured by heating the solution at $100^{o}C$ for ten minutes and then cooling in ice. The nitrocellulose was incubated for 2-4 hours at $65^{o}C$.

The prehybridization solution was removed and 20 or 30 ml of hybridization solution added. The composition of this solution was the same as the prehybridization solution but also contained 10 mM EDTA and 0.5 to 1.0 X 10^{6} counts per minute (CPM) of nick translated DNA. This solution was also heat denatured and cooled in ice before addition to the bag.

The hybridization was carried out at $65^{o}C$ for 24-72 hours. When completed, the nitrocellulose was washed three or four times with 2 X SSC at $65^{o}C$ for ten minutes each. It was dried briefly in air, covered with Handi-wrap, and autoradiographed using Kodak X-Omat AR film (XAR-5).

Restriction Enzyme Digestions for Sequencing

Twenty μg of pDLS19 DNA in 100 μl of HindIII buffer (Table I) was digested with 65 units of HindIII for 4-5 hours. The reaction mixture was phenol extracted and ethanol precipitated before labelling the ends as described in the next section. The labelled fragments were cleaved with 15 units of ClaI in the appropriate buffer (Table I) for 3-4 hours. The mixture was once again phenol extracted and ethanol precipitated.

3'-End-Labelling of Restriction Fragments

The DNA pellet was dissolved in 20 μl of 40 mM Tris, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM of each deoxynucleotide triphosphate needed, and 10-50 μCi (15-75 pmol) of α-^{32}P-dCTP (New England Nuclear). 2.5 units of the Klenow fragment of DNA polymerase I (Bethesda Research Labs) was added and incubated 30-45 minutes at 37°C.

After incubation, 80 μl of distilled water was added and the solution extracted with one volume of phenol saturated with TE buffer. The phenol was re-extracted with distilled water. Sodium acetate was added to a concentration of 0.3 M, and the labelled DNA precipitated with two volumes of ethanol. The DNA was pelleted, washed with 70% v/v ethanol, and dried under vacuum.

Polyacrylamide Gel Electrophoresis and Isolation of Labelled Fragments

After labelling, the fragments were cleaved with a second restriction endonuclease to generate DNA fragments which were labelled at only one end. These labelled fragments were separated on a 5.0% polyacrylamide gel (with a 29 to 1 weight ratio of acrylamide to bis) in Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3).

After electrophoresis, one glass plate was removed and the remaining plate and gel wrapped in parafilm and marked with radioactive ink to allow the film to be aligned with the gel after autoradiography. The gel was autoradiographed for 15-30 minutes.

After the film was developed it was positioned on top of the gel and cuts were made through the film, parafilm, and gel above and below the labelled DNA fragments of interest. These gel slices were placed in a 1.0 ml disposable plastic syringe and crushed through the syringe into a capped plastic scintillation vial. 1.5 ml of 20 mM Tris, 200 mM NaCl, 2 mM EDTA, pH 8.0 (gel elution buffer) and 10 μg of yeast tRNA were added and the vial shaken overnight at 37°C to elute the DNA from the gel slice.

The eluted DNA was filtered through Whatman 3 MM paper to remove gel fragments, and then bound onto a DE-52 ion exchange column. The column was washed twice with gel elution buffer, and the labelled DNA eluted with 200 mM Tris, 1.5 M NaCl, 2 mM EDTA, pH 8.0, which was at a temperature of 90-100°C. The DNA was precipitated with 2-3 volumes of ethanol, pelleted, washed with 70% v/v ethanol, redissolved in distilled water, and the precipitation procedure repeated. The DNA pellet was finally dissolved in 23 μl of dissolved water in preparation for the sequencing reactions.

Sequencing Reactions

Sequencing reactions were done according to Maxam and Gilbert (1980) with a few modifications. The G+A reaction was done at 37°C instead of 20°C and the reaction time cut down to 30 minutes. The other three reactions were carried out as described. The reaction times of all reactions were varied somewhat to produce good cleavage in the area of the DNA which was being sequenced.

Sequencing Gels

Sequencing gels were done according to Maxam and Gilbert (1980) with a few modifications. Eight or 20% w/v acrylamide gels (with a 19 to 1 w/w ratio of acrylamide to bis) containing Tris-borate buffer and 6.0 M urea were used. Thirty-eight cm long gels were used with 20% acrylamide, and either 38 or 84 cm long gels with 8%. Gels were run at 800-2000 volts until the tracking dyes had migrated the desired distance.

After electrophoresis the 20% gels were wrapped in Handi-wrap and autoradiographed at -70°C with an intensifying screen without any further processing. Eight per cent gels were soaked in 10% acetic acid, 25% ethanol and then dried on a Hoefer gel dryer. The dried gel was then autoradiographed using Kodak X-Omat AR film (XAR-5). After the desired length of exposure the autoradiograms were developed.

CHAPTER III

RESULTS

Construction of Deletion Mutants

Mutants with specific restriction fragments deleted were constructed using a partial digestion/religation procedure. Time course digestions with a restriction endonuclease were carried out to obtain partial digests in which a significant portion of the plasmid molecules were cleaved at more than one restriction site. Upon analysis by gel electrophoresis, such digests showed only a small amount of original supercoiled and circular plasmid remaining. Linear plasmid molecules and completely-cut fragments were present, as well as several bands corresponding to intermediates of digestion. It was desirable to generate digests containing as many of these intermediates as possible since these are the molecules giving rise to deletion mutants upon religation. Since several different intermediates can be produced, several possible deletion mutants can result. Also, since supercoiled and open circular molecules remain in the digest and linear molecules can be religated to produce the original plasmid, some of the final transformants will contain the original starting material.

Religation of the partial digests was carried out at a low DNA concentration. This promoted intramolecular ligation and the formation of circular plasmid molecules rather than linear molecules. Religated

circular molecules lacking the ampicillin resistance gene and linear molecules, which do not transform bacteria, will not produce colonies after transformation and plating on ampicillin-containing agar plates.

Several restriction fragments were determined to be good candidates for deletion. These fragments were all fairly small (less than 1,000 base pairs) so that their deletion would affect only a limited portion of the viral genome. They were located in interesting areas of the viral genome, and a screening method existed to identify the presence of a deletion and distinguish it from other deletion mutants produced.

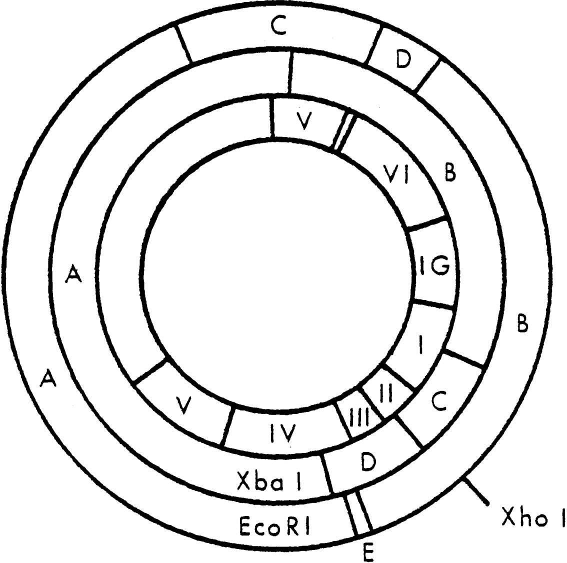
XbaI Deletions

The plasmid pCMS31 contains four XbaI restriction sites as shown in Figure 3. Fragment C contains portions of Regions I and II, and its deletion removes 50% of Region I and 75% of Region II. Since the function of Region II is known (Armour et al., 1983), this deletion mutant may shed light on the function of Region I.

Two different partial digests were ligated and used to transform bacteria. Plasmid was isolated from the transformants and analyzed on an agarose gel. Plasmids with sizes close to that of pCMS31 were screened by digestion with EcoRI followed by gel electrophoresis.

Thirty-six colonies obtained from using the first partial digest were analyzed. Only two of them were noticeably smaller than pCMS31, and these two appeared to be identical in size. One of the smaller plasmids and seventeen of the larger ones were screened. All of the larger plasmids gave the same restriction pattern as pCMS31 and probably resulted from intact pCMS31 remaining in the partial digest

Figure 3. XbaI, EcoRI, and XhoI Restriction Map of pCMS31.



C
D
V
VI
B
IG
A
B
I
A
II
C
V
IV
III
D
Xba I
EcoRI
E
Xho I

or from linear pCMS31 DNA which had been religated. Upon EcoRI digestion, the smaller plasmid produced one band which migrated slightly slower than the EcoRI A fragment (6071 BP). This pattern was expected for a plasmid consisting of the XbaI A fragment (6792 BP) which had recircularized. This plasmid was called pCMXD59. Presumably the other small plasmid which was not screened, pCMXD80, was identical to this one.

A second XbaI partial digest was made in order to obtain a more extensive digestion. Seven out of 36 plasmids obtained using this digest were smaller than pCMS31. There were three sizes of these smaller plasmids: one which migrated only slightly faster than pCMS31 and two which migrated moderately faster. One plasmid from each size class and the plasmids co-migrating with pCMS31 were screened.

The smallest (fastest-moving) plasmid, pCMXD120, gave a restriction pattern consistent with a recircularized XbaI A fragment. The next larger plasmid, pCMXD122, gave a pattern indicating it contained A and D fragments. The plasmid migrating only slightly faster than pCMS31, pCMXD130, gave a restriction pattern consistent with pCMS31 which had lost the XbaI D fragment. The presence or absence of the C fragment could not be determined using this screening method. All of the plasmids which were the same size as pCMS31 gave EcoRI restriction patterns identical to that of pCMS31.

Up to this point the desired mutant lacking only the XbaI C fragment had not been found. In order to reduce the number of plasmids which would possibly need to be screened, the ligated partial digest was phenol extracted and digested with XhoI. pCMS31 contained a single XhoI site which is located within the XbaI C fragment. Plasmids

lacking this fragment will not be cleaved and will remain circular. Plasmids containing this fragment will be cleaved to produce linear molecules that do not transform bacteria. After XhoI cleavage, the DNA was used to transform bacteria and plasmid was isolated from the resulting transformants.

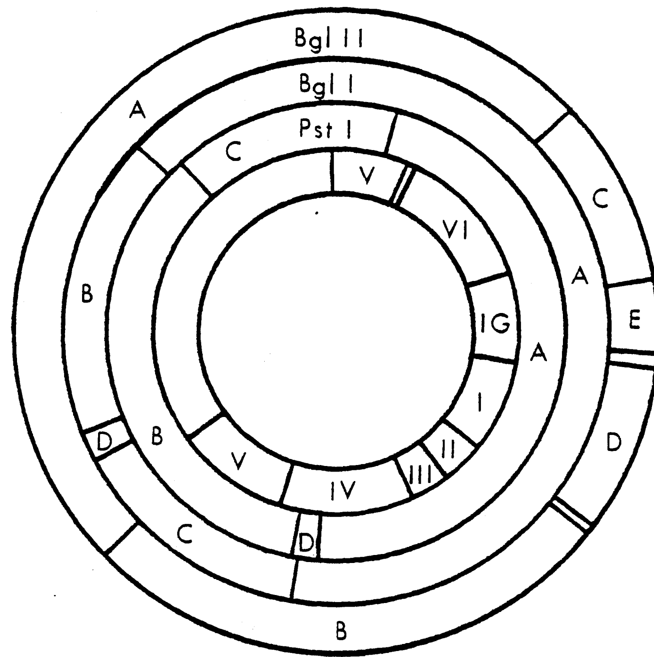
A wide variety of plasmid sizes was obtained, ranging from almost the size of pCMS31 to much smaller. Several plasmids close to the size of pCMS31 were analyzed further. Restriction digests with XbaI, EcoRI, SalI, and HindIII followed by gel electrophoresis revealed that several of the plasmids (pCMXD215, pCMXD226, and pCMXD232) lacked the XbaI C and D fragments. Others (pCMXD230, pCMXD233, and pCMXD234) gave restriction patterns which indicated they lacked fragments C and D plus various amounts of DNA flanking these fragments. This was possibly the result of contaminating exonuclease action in the XbaI partial digestion and/or religation reaction, followed by a blunt-end ligation that occurred during religation. This would also explain the greater than expected variety of plasmid sizes obtained with this procedure.

One plasmid gave an EcoRI restriction pattern consistent with a plasmid lacking only the XbaI C fragment. This deletion mutant was designated pCMXD319. It was digested with SalI, XbaI, BglII, and HindIII and compared to pCMS31 digested with the same enzymes. pCMXD319 gave restriction patterns different from those of pCMS31 and consistent with desired deletion.

PstI Deletions

pCMS31 contains four PstI sites as shown in Figure 4. The PstI

Figure 4. PstI, BglI, and BglII Restriction Map of pCMS31.


Bgl II
Bgl I
Pst I
A
B
D
C
B
C
E
D
A
A
B
D
C
V
VI
IG
I
II
III
IV
V

D fragment is 195 base pairs in length and lies within Region IV, the coat protein gene. This fragment spans almost the entire length of an area in the coat protein known as the lysine core (Franck et al., 1980), which is thought to interact with the viral DNA in the virus particle. A mutant with this fragment deleted may yield information on the function of the coat protein and the importance of the lysine core.

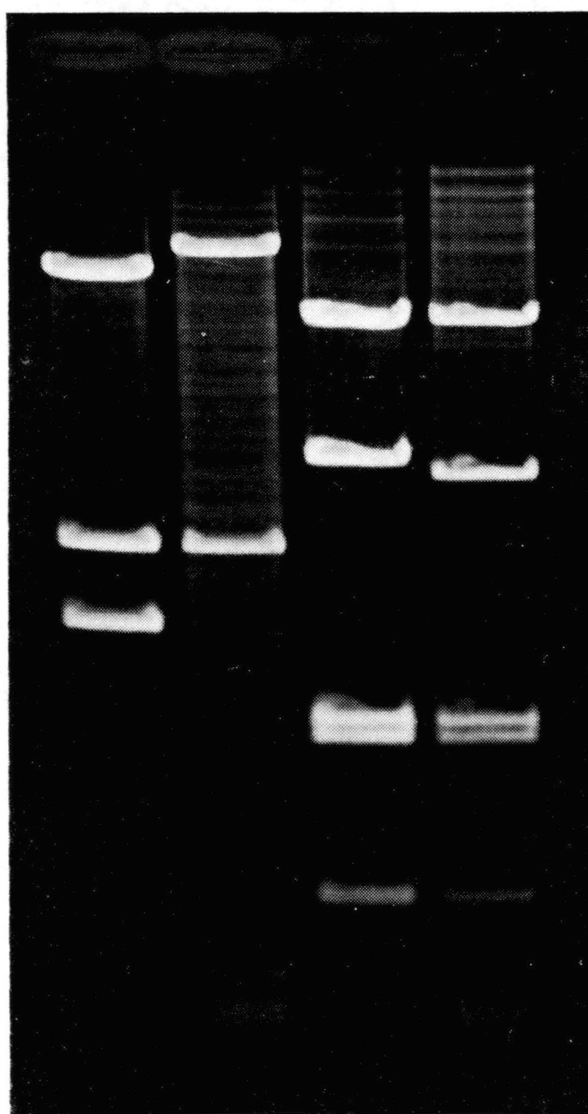
The partial digestion/religation procedure in this case produced two sizes of plasmids: large and small. The large plasmids result from intact pCMS31 and pCMS31 lacking only the PstI D fragment. The small plasmids consist of pCMS31 which has lost the A fragment or both A and D fragments. Fragments B and C must always be joined together as in the starting material since deletion of one of them will destroy the ampicillin resistance gene which is needed for the transformants to grow on ampicillin agar plates.

The large plasmids were screened by cleavage with BglI followed by gel electrophoresis. Plasmids lacking the PstI D fragment also lack the BglI site which separates the BglI A and C fragments. Screening produced several plasmids in which the A and C fragments were absent and a larger fragment was present, indicating the correct deletion. One of these was called pDLS19 and used for further studies. The deletion of the PstI D fragment was confirmed by cleavage with BglII (see Figure 5). PstI digestion showed that the ligation had regenerated a PstI site between the PstI A and B fragments as expected.

This mutant has lost 195 base pairs which is equivalent to 65 amino acids of the coat protein. The coding sequence located after

Figure 5. Gel Electrophoresis of pCMS31 and pDLS19 Digested with BglI and BglII.

1) pCMS31 digested with BglI; 2) pDLS19 digested with BglI; 3) pCMS31 digested with BglII; 4) pDLS19 digested with BglII.



1 2 3 4

the deletion remains in phase with the beginning of the region.

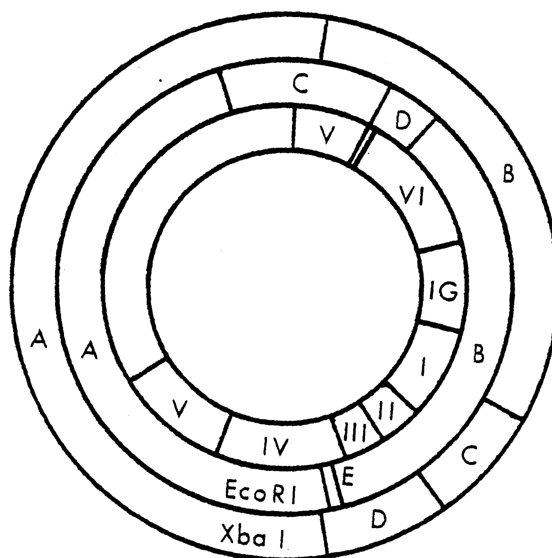
EcoRI Deletions

The smallest EcoRI fragment of pCMS31 contains only 60 base pairs (Figure 6). It lies in the N-terminal portion of Region IV in one of the areas thought to be cleaved off of the precursor to give an active coat protein. A mutant lacking this fragment should produce information about possible functions of the coat protein precursor and the processing needed to give the functional viral coat protein.

Several different ligations were done using several different partial digests. Resulting plasmids were screened by cleavage with XbaI followed by gel electrophoresis. In one of these procedures a fairly extensive partial digest was used. This resulted in all 17 plasmids which were analyzed being smaller than the original starting material. Most of them were significantly smaller than pCMS31. When one of these, pCMED105, was characterized by XbaI digestion, it gave a restriction pattern consistent with a recircularized EcoRI A fragment. The E fragment may also have been present, since this screening method is not able to determine its presence or absence. The remainder of the plasmids were only slightly smaller than pCMS31. Analysis of one of these, pCMED114, showed that it consisted of pCMS31 with the EcoRI C fragment deleted. The D fragment may or may not have been present since this also could not be determined using this method.

From the other partial digests and ligations a total of 104 plasmids were isolated and analyzed. Sixteen of them were noticeably

Figure 6. EcoRI and XbaI Restriction Map of pCMS31.


C
V
D
B
VI
IG
A
A
I
B
II
V
III
IV
C
E
EcoRI
D
Xba I

smaller than pCMS31. Some of these were further characterized and found to be the same deletion mutants as produced by the more extensive partial digest. In addition, one of these plasmids, pCMED246, had a size consistent with a deletion of the EcoRI B fragment. Eighty-six (out of the 104) plasmids that were the same size as pCMS31 were cleaved with XbaI and analyzed on either agarose or polyacrylamide gels. All of them gave a restriction pattern identical to that of pCMS31 and thus were not the desired deletion mutant. It seems that this method is not capable of efficiently producing the desired mutant for some reason, and because of this, a different procedure was tried.

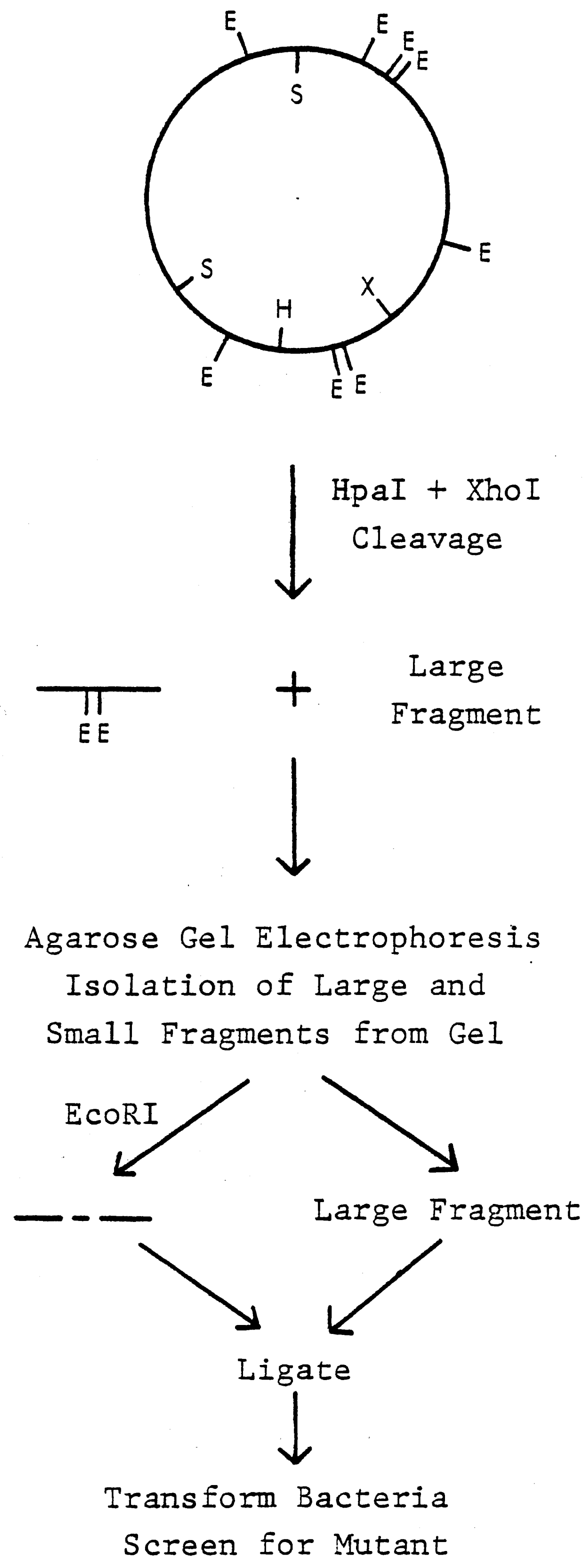
EcoRI Deletion Attempted Using pCS101

pCS101 has a slightly different restriction map than pCMS31. Because of this, a different method (Heusterspreute and Davison, 1983) could be used in an attempt to delete the EcoRI E fragment. The scheme for this is shown in Figure 7. The plasmid was first cleaved with HpaI and then with XhoI, resulting in production of a large and a small fragment. The two fragments were separated on a low gelling temperature agarose gel, and both of them isolated from the gel.

The isolated small fragment was digested with EcoRI to cleave the 60 base pair fragment out of the small fragment. This digestion proved to be more difficult than would be expected, possibly due to inhibitors released from the agarose gel during the isolation procedure. Doubling the reaction volume to dilute inhibitors, using a large amount of enzyme, and including 6 mM 2-ME in the reaction

Figure 7. Construction of an EcoRI Deletion Mutant from pCS101.

Restriction sites are labelled as follows: E = EcoRI, S = SalI, H = HpaI, X = XhoI.


E
E
E
E
S
E
S
H
X
E
E E
HpaI + XhoI
Cleavage
E E
+
Large
Fragment
Agarose Gel Electrophoresis
Isolation of Large and
Small Fragments from Gel
EcoRI
Large Fragment
Ligate
Transform Bacteria
Screen for Mutant

mixture was found to be necessary for a good digestion. However, electrophoresis of the digest showed that traces of the intact small fragment usually remained, and that some of the two smaller fragments produced still retained the 60 base pair fragment. Because of the low yield from the gel isolation procedure, the 60 base pair fragment was left in the mixture.

The EcoRI-digested small fragment was mixed together with the isolated large fragment and the mixture blunt-end ligated (since HpaI generates blunt ends). Success of the ligation was determined by agarose gel electrophoresis and transformation of bacteria.

Several blunt-end ligations were done using different amounts of T4 DNA and RNA ligase, different incubation times, and different amounts of DNA. Some of these reactions were analyzed by gel electrophoresis. In all cases the large and small fragments disappeared and material migrating slower than the large fragment was produced. A band or bands migrating at approximately the position of open circular pCS101 were produced, and in one case, very large material which did not leave the origin was produced. Hence, it seems that some type of ligation had occurred.

However, when bacteria were transformed using this DNA, virtually no colonies appeared. Six ligations produced only three colonies. These grew about twice as fast as normal plasmid-containing E. coli HB101, and were suspected to be contaminants rather than transformants.

One of these colonies, pCMED404, gave an insoluble plasmid preparation which was probably not DNA. The other two, pCMED401 and pCMED402, gave plasmid DNA preparations which migrated as two faint, diffuse bands on an agarose gel. These bands moved significantly

slower than pCS101. A diffuse band migrating somewhat slower than the tRNA in the preparations was also present. These were digested with several restriction enzymes and electrophoresed, but the resulting patterns gave no clue as to their identity.

Hybridization experiments were also done in which the ability of these two plasmids to bind various radioactively-labelled probes was determined. The plasmids were unable to bind viral DNA, which indicates that they probably did not originate from the original starting material. They were able to hybridize to pBR322 DNA. This was expected since the unknown plasmids probably carry an ampicillin resistance gene which is likely to have some homology with the same gene of pBR322. However, the band which hybridized comigrated with supercoiled pBR322 control DNA, and did not migrate like any of the bands seen in the plasmid preparation. The hybridization may thus be entirely due to some type of contamination. All of this information indicates that the colonies are contaminants rather than transformants.

Since large DNA molecules were being formed by the ligation, it was possible that long linear polymers were being formed instead of circular plasmids. These will not transform bacteria and not give rise to colonies. To overcome this possible problem, a portion of the ligated DNA was phenol extracted and then cut with XhoI, which cleaves pCS101 only once. This mixture was phenol extracted again and religated at low DNA and T4 DNA ligase concentrations which will favor production of circular molecules by sticky-end ligation. Unfortunately, this procedure also did not yield any transformants.

Since the ligation seemed to be working, it was possible that the lack of transformants was due to a problem with the transformation.

One possibility was that something in the fairly complex blunt-end ligation mixture was inhibiting transformation. To test this, 5.0 ng of pCMS31 DNA was placed in sterile water and in the blunt-end ligation mixture. These two mixtures were used to transform the same amount of competent bacteria. The pCMS31 DNA in sterile water gave several hundred transformants, while pCMS31 DNA in the ligation mixture did not give any. This showed conclusively that the blunt-end ligation mixture was inhibiting transformation to a large extent.

It was originally thought that this inhibition was due to the polyethylene glycol which was added to improve ligation efficiency. pCS101 was cut with HpaI and XhoI then phenol extracted and ethanol precipitated. This mixture of large and small fragment (not digested with EcoRI) was blunt-end ligated without PEG and analyzed by electrophoresis and transformation. Several bands larger than the large fragment were present on the gel, indicating that ligation had occurred. However, no transformants were obtained, suggesting that something was still interfering with transformation. When 5.0 ng of pCMS31 DNA was added to this ligated DNA solution and compared to the same amount in sterile water, the former gave about 20-25 colonies while the latter gave several hundred. Something else in the ligation mixture other than PEG was inhibiting transformation. However, since this experiment produced a few transformants whereas the previous one (which included PEG) did not, it seems that PEG was responsible for some of the inhibition.

An attempt was made to remove these inhibitors. This same ligated DNA mixture was phenol extracted and precipitated out of 2.5 M ammonium acetate with three volumes of ethanol. The pellet was

washed with 70% v/v ethanol and redissolved in sterile water. 5.0 ng of pCMS31 DNA was added and the mixture used to transform bacteria. 5.0 ng of pCMS31 DNA in sterile water was used as a control. Several hundred transformants were produced by each of these preparations, with the control definitely producing more. Hence, this purification procedure seems to remove a large part of the inhibition, although some still seemed to remain.

When the remainder of this ligated DNA mixture (the same one as in the previous two paragraphs) was purified in this way and used for transformation, this purified DNA gave about 200 transformants. This indicates that the ligation had worked and that the purification of the ligated DNA relieved the inhibition.

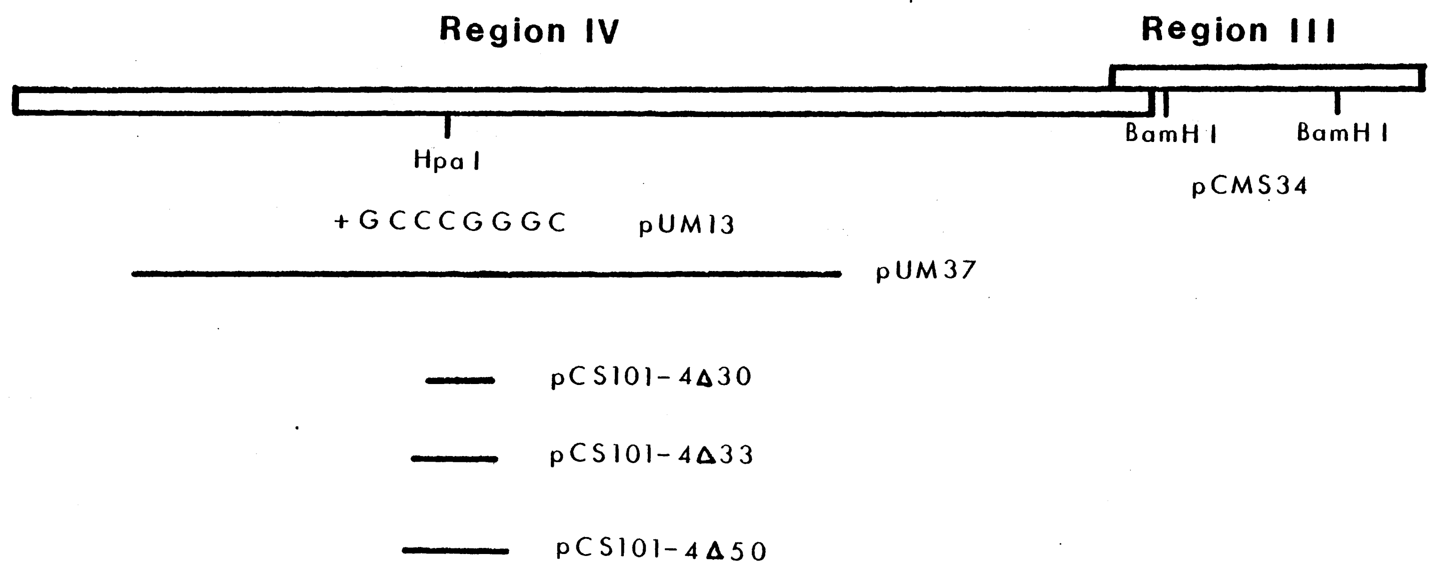
Next, large and uncut small fragments isolated from a gel were blunt-end ligated. Electrophoresis showed that larger material had formed, and one band co-migrated with the open circular form of pCS101. The ligated DNA was purified according to the above procedure and used to transform bacteria. Only three transformants resulted. This suggests that there are inhibitors remaining in the DNA isolated from the agarose gel, since all other components were the same as in the previous experiment. Unfortunately, these do not seem to be easily removed from the ligated DNA, and they effectively prevent the production of the desired mutant by this method.

Other Mutants

Several mutants constructed by other workers were also used in the replication studies. These mutations affect Regions III and IV and are diagrammed in Figure 8. pCMS34 was constructed from pCMS31

Figure 8. Other Mutants Used for the Replication Ability Studies.

The solid lines under Region IV indicate the areas which have been deleted.


Region IV
Region III
Hpa I
BamH I
BamH I
pCMS34
+GCCCGGGC
pUM13
pUM37
pCS101-4Δ30
pCS101-4Δ33
pCS101-4Δ50

by deleting the BamHI C fragment which lies entirely within Region III. This removes 222 base pairs which is slightly over half of the region. The end of the coding sequence remains in phase with the beginning.

The remaining mutants all affect Region IV and were constructed from pCS101 using its single HpaI site. This site was cleaved and a SmaI linker ligated into it to give the insertion mutant pUM13. The other mutants were constructed by treating HpaI-linearized pCS101 with exonuclease Bal-31 to shorten the DNA molecule. This was then recircularized by ligating in a SmaI linker. pCS101-4Δ30, pCS101-4Δ33, and pCS101-4Δ50 have about 100 base pairs deleted, while pUM37 has over 900 deleted. The only one of these Region IV mutants to remain in phase is pCS101-4Δ50.

Inoculation Studies

During the course of these experiments, questions arose concerning the effects of the plant's age and the inoculation lag time (the length of time the inoculum stays on the leaf before it is rubbed over the leaf surface) on the ability of the plant to become infected. It was speculated that the chances of infecting a plant decreased as the plant got older or the lag time increased. Some simple experiments were done to test these possibilities.

Plants of different ages were inoculated with SalI-cut pCMS31 DNA. These plants were 4, 5, and 6 weeks old. Plants are normally inoculated when they are 3½ weeks old. The 4 and 5 week old plants started showing faint symptoms at about 12-14 days post-inoculation, as usually occurs in a normal inoculation. At three weeks post-

inoculation, the 4 week old plant showed systemic symptoms as normally infected plants do. The 5 week old plant required another 3-7 days before it developed extensive symptoms. The 6 week old plant remained healthy for 4 weeks. However, between 4 and 5 weeks post-inoculation it also became sick. This experiment showed that older plants are definitely able to be infected, although as a plant becomes older symptoms take longer to appear.

Plants were also inoculated using different lag times. The inoculum was pipetted onto the leaf and rubbed over the leaf surface immediately or else left there for various lengths of time before rubbing. Lag times of 1, 2, 5, 10, 15, and 30 minutes were tested. All of the plants became sick at about the same time. There were minor differences between some plants but these did not fit into a pattern and were not considered to be significant. Inoculation lag times of as much as 30 minutes do not seem to have an effect on the ability to infect a plant. In practice, lag times of more than a few minutes are not encountered, and these will have no effect on the inoculation.

Determination of Symptom Production and Replication Ability of Mutants

Symptom Production

Mutants were inoculated onto turnips to determine if they still retained the ability to make the plant sick. For each inoculation experiment a positive control, SalI-cut pCMS31 DNA, and a negative control, inoculation buffer alone, were done. In all cases the

positive control produced symptoms of viral infection, while the negative control plant remained healthy. The mutants tested were pCMS34, pDLS19, pCMXD319, pUM13, pUM37, pCS101-4Δ30, pCS101-4Δ33, and pCS101-4Δ50. These mutations affected Regions I+II, III, and IV. None of these mutants produced symptoms in the turnip plants. The inoculated plants were observed for about five weeks after inoculation, well beyond the usual time required to produce symptoms (2-3 weeks).

Total Plant DNA Isolation and Hybridization

Total plant DNA was isolated from turnip leaves which had been inoculated with mutant DNA to see whether any replication of the mutant DNA could be detected. These DNA preparations were electrophoresed on an agarose gel and transferred to nitrocellulose by electroblotting. The electroblots were hybridized to either CaMV DNA or pBR322 DNA which had been radioactively labelled by nick translation. Bands on the electroblot which hybridized to the labelled DNA were detected by autoradiography.

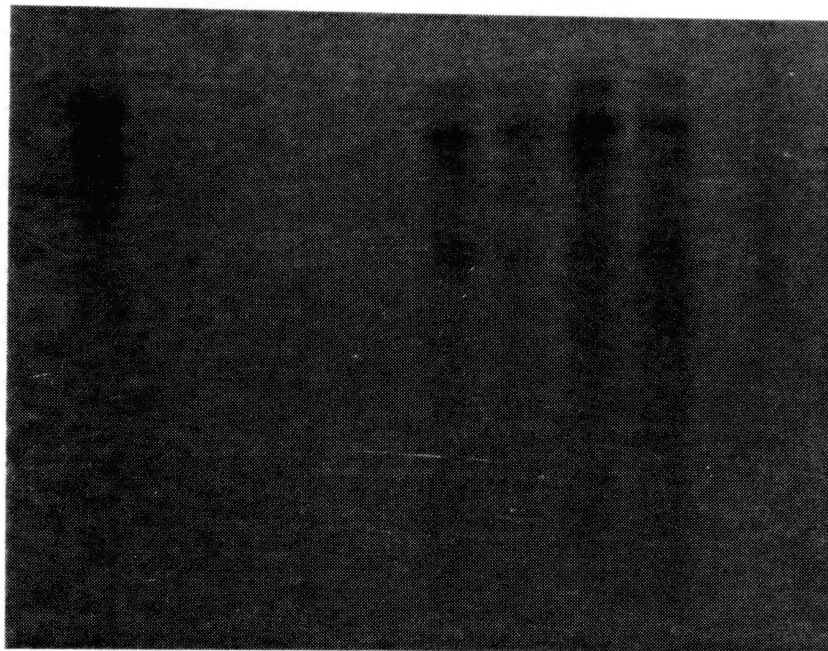
Preliminary experiments were done to ensure that this method was able to detect viral replication in inoculated leaves. Total DNA was isolated from four plants which had been inoculated with SalI-cut pCMS31 DNA. Negative controls, which consisted of total plant DNA from two plants inoculated with SalI-cut pBR322 DNA and two plants inoculated with only inoculation buffer (called "blank" plants), were also done. These eight DNA preparations were electrophoresed with standards of pBR322 and CM4-184 viral DNA, electroblotted, and hybridized with nick translated CaMV DNA or pBR322 DNA. The results of this experiment can be seen in Figures 9 and 10.

Figure 9. Autoradiogram of an Electroblot Hybridized with ^{32}P-labelled CaMV DNA.

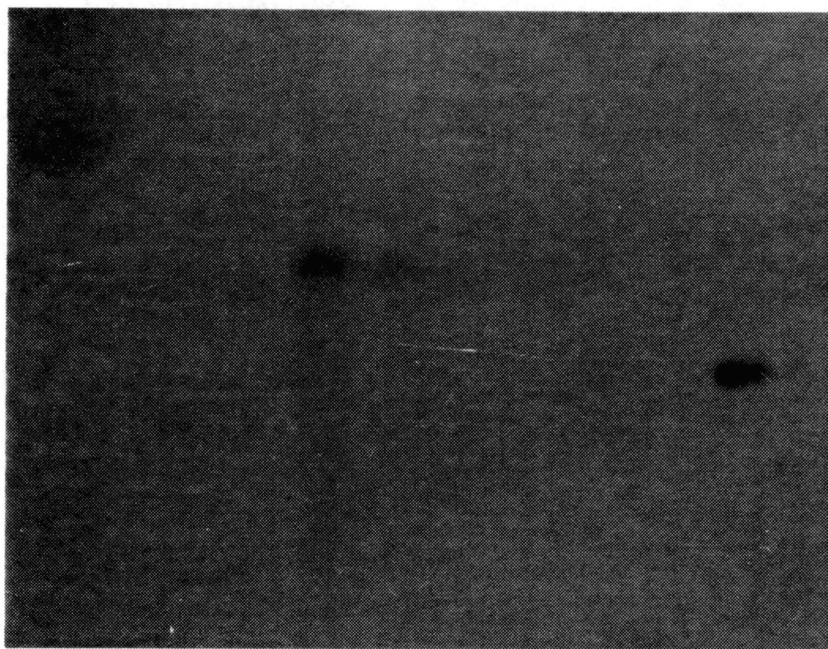
1) CM4-184 DNA standard. Total plant DNA from plants inoculated with: 2-3) Blank inoculation; 4-5) pBR322 DNA; 6-9) pCMS31 DNA. 10) pBR322 standard.

Figure 10. Autoradiogram of an Electroblot Hybridized with ^{32}P-labelled pBR322 DNA.

The samples are the same as in Figure 9.



1 2 3 4 5 6 7 8 9 10



The electroblot in Figure 9 was hybridized with labelled CaMV DNA. A standard of CM4-184 DNA can be seen in the first lane. The next four lanes are the negative controls -- total DNA isolated from the blank plants (lanes 2 and 3), and from plants inoculated with pBR322 (lanes 4 and 5). As expected, nothing in these four lanes hybridizes with CaMV DNA. The positive controls (lanes 6 through 9) are total plant DNA preparations from plants inoculated with pCMS31 DNA, and all of these lanes hybridize labelled CaMV DNA.

The most slowly migrating band of the CM4-184 DNA standard is the open circular form of the viral DNA and the band below this is linear viral DNA (Hull, 1979; Shepherd, 1979). The total plant DNA preparations give similar patterns which presumably correspond to the same types of molecules as in the standard. The bands in the CM4-184 DNA standard migrate slightly faster than the bands of the total plant DNA preparation since the NY8153 strain contains a slightly larger DNA molecule. The material migrating faster than the linear viral DNA probably consists of supercoiled and knotted molecules, and possibly damaged molecules which have been broken at the gaps.

This method seems to be able to detect replicating virus in inoculated leaves quite well. The viral DNA is readily visible in all four lanes, and all of the plants inoculated with viral DNA gave total plant DNA preparations which hybridized to the probe. Also, there are several bands present in these preparations, not just the original linear viral DNA which was used for inoculation. This suggests that the material being detected is replicating DNA, and not merely the original inoculum.

Figure 10 shows the same samples probed with nick translated

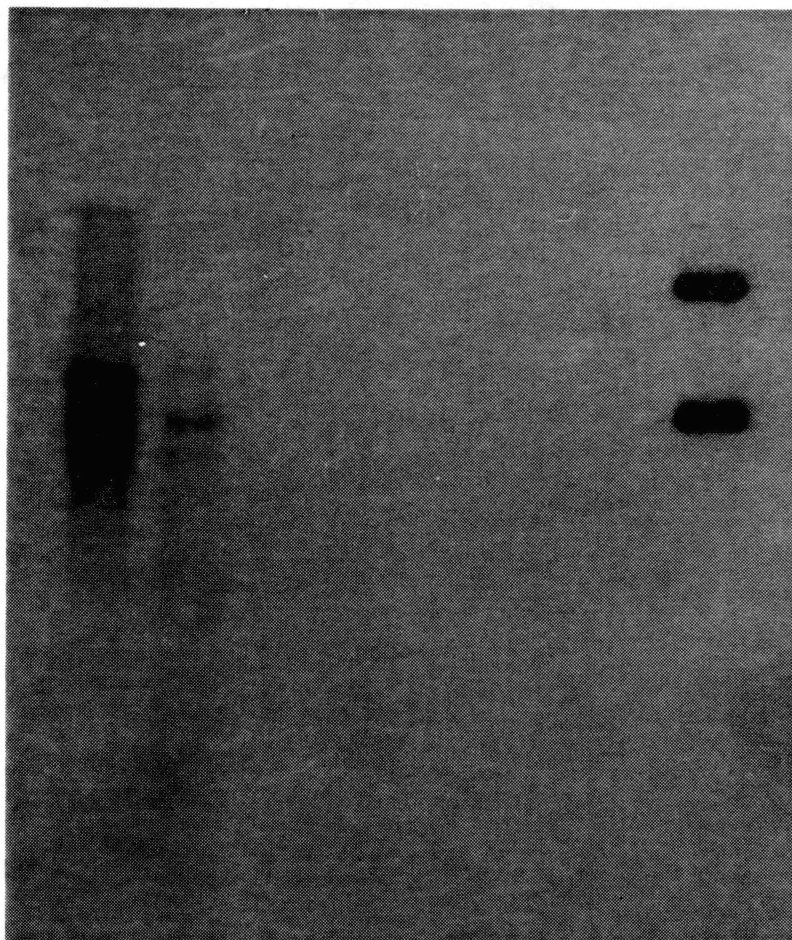
pBR322. A standard of pBR322 plasmid DNA can be seen in the last lane. This probe did not hybridize to the total DNA from the blank plants (lanes 2 and 3) or from plants inoculated with pCMS31 (lanes 6 through 9). However, it did hybridize to total plant DNA from the two plants inoculated with SalI-cut pBR322 (lanes 4 and 5). The band seen on the autoradiogram migrated between the supercoiled and open circular forms of the plasmid pBR322, just slightly faster than the open circle. It was thought that this material was linear pBR322 DNA from the inoculum which remained on the plants.

Further experiments were done to better characterize this material. When SalI-cut pBR322 DNA was used as a standard, these bands co-migrated with it. When these total DNA preparations were digested with SalI they showed no change in their migration. However, when digested with EcoRI (which will cleave 651 base pairs off of SalI-cut pBR322) the bands migrated somewhat faster than before. All of this information indicates that the material from the pBR322-inoculated plants was SalI-cut pBR322 remaining from the inoculum.

Figure 11 shows total plant DNA preparations from plants which had been inoculated with some Region IV mutants. The first lane contains a standard of CM4-184 DNA. The next lane (lane 2) is total plant DNA from a pCMS31-inoculated plant, and the last lane is a pCMS31 DNA standard. The remaining lanes (4-8) contain total plant DNA isolated from plants inoculated with pDLS19, pUM13, pCS101-4Δ30, or pCS101-4Δ33. They do not bind the probe to any detectable extent. The same study was also done with pCMS34, pUM37, and pCS101-4Δ50 with the same results. None of the mutants tested gave total plant DNA preparations able to hybridize nick translated CaMV DNA. Hence, no

Figure 11. Autoradiogram of an Electroblot Containing Total Plant DNA from Plants Inoculated with Mutant DNA.

1) CM4-184 DNA standard. Total plant DNA from plants inoculated with: 2) pCMS31; 3-4) pDLS19; 5) pUM13; 6) pCS101-4Δ30; 7) pCS101-4Δ33. 8) Empty lane. 9) pCMS31 DNA standard.



1 2 3 4 5 6 7 8 9

replication of the mutant DNA appears to be occurring.

Viral DNA Isolation and Hybridization

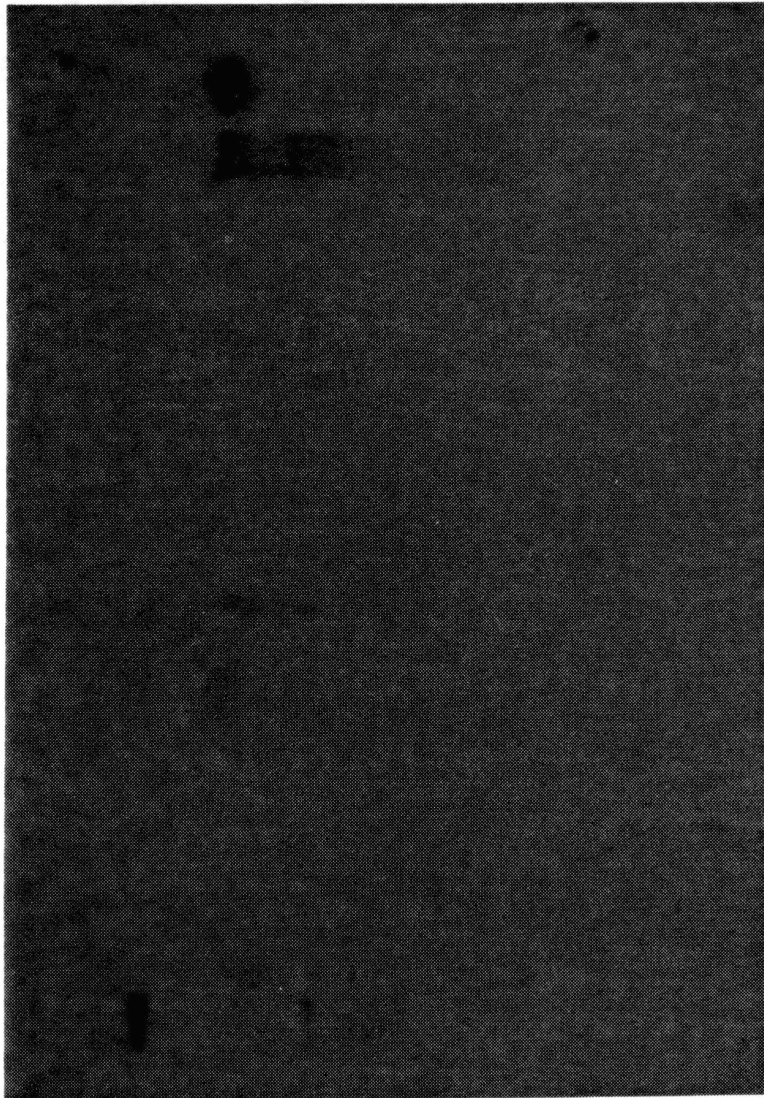
Other studies have shown that encapsidated viral DNA is not recovered by total plant DNA isolation procedures (Menissier et al., 1982). It was possible that for some reason these mutants had replicated and all of the DNA was packaged into virus particles at the time total DNA was isolated. If this were the case no viral DNA would be detected. This possibility was considered unlikely, especially since most of the mutants tested had mutations which seriously affected the coat protein gene.

In order to test this possibility, several mutants were used to inoculate turnip plants. The inoculated leaves were put through a viral DNA isolation procedure. These viral DNA preparations were electrophoresed, electroblotted, and hybridized with labelled CaMV DNA as before. The mutants pDLS19 and pCS101-4Δ50 were tested since their deletions are in-phase and are more likely to produce a functional coat protein and do not violate the relay race rule of CaMV translation. pCS101-4Δ33, an out of phase deletion, was also tested. Controls consisted of plants inoculated with only inoculation buffer and with SalI-cut pCMS31 DNA. The results are shown in Figure 12.

A standard of pCMS31 plasmid DNA can be seen in the first lane. CM4-184 DNA is also present (lane 7), but did not turn out well due to a wrinkle in the nitrocellulose during the transfer. All of the viral DNA preparations from plants inoculated with pCMS31 (lanes 6, 14, and 15) hybridized to the probe. The negative controls (lanes 2 and 3)

Figure 12. Autoradiogram of an Electroblot Containing Viral DNA from Plants Inoculated with Mutant DNA.

1) pCMS31 DNA standard. Viral DNA from plants inoculated with: 2-3) Blank inoculation; 4-5) pDLS19; 6) pCMS31. 7) CM4-184 DNA standard. Viral DNA from plants inoculated with: 8-10) pCS101-4Δ33; 11-13) pCS101-4Δ50; 14-15) pCMS31.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

did not bind the probe. All of the remaining lanes contain viral DNA isolated from plants inoculated with mutants. None of the viral DNA preparations from these mutants hybridized labelled CaMV DNA.

Partial DNA Sequence of the NY8153 Strain of CaMV

A portion of the DNA sequence of the NY8153 strain of CaMV was determined using the base specific chemical cleavage method of Maxam and Gilbert (1980). pDLS19 DNA was used for this since the original goal was to sequence across its deletion.

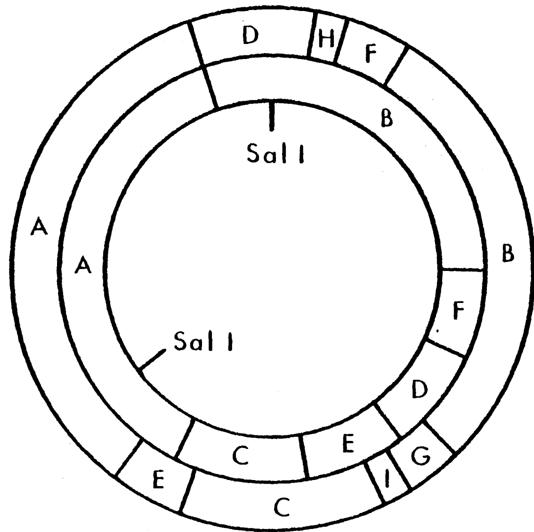
The DNA was first cleaved with HindIII. The ends were labelled with ^{32}P by filling in the overhangs with the Klenow fragment of DNA polymerase I. The labelled DNA was then digested with ClaI to generate fragments with only one end labelled. This process is shown in Figure 13.

The labelled fragments were separated on a 5.0% polyacrylamide gel and visualized by autoradiography. Bands which were well-separated from others and contained a fragment labelled at only one end were excised from the gel. The labelled DNA was extracted from the gel, purified, and used for the sequencing reactions.

The 166, 184, 270, 376, and 693 base pair (BP) fragments were all excised and extracted. In addition, a band containing the 897 and 936 BP fragments was excised. This DNA was electrophoresed again on a denaturing acrylamide gel in hopes of obtaining the labelled strand of the 897 BP fragment which contains the mutated site of pDLS19. However, after electrophoresis the DNA appeared as a fast moving smear with a few very faint slow moving bands. Presumably contaminating

Figure 13. Generation of Labelled Fragments for Sequencing.

The number of asterisks on each labelled fragment indicates the number of labelled ends the fragment has.



Hind III →

		Label; Cla I →
A	4241	4235*, 6*
B	3696	2139*, 693*, 864
C	1411	897*, 514*
D	936	936**
E	560	376*, 184*
F	476	476**
G	436	270*, 166*
H	224	224**
I	218	218**

nucleases present somewhere in this procedure degraded the fragments.

The other five fragments were isolated and sequenced. The results are shown in Figures 14 through 17. In cases where the identity of the nucleotide could not be determined precisely, the various possibilities are placed in parentheses.

The sequence obtained for NY8153 was compared with the sequence of other strains which have been sequenced (Balazs et al., 1982). The differences between these strains are listed in the figures. The sequences listed are from the β strand of the viral DNA. The sequence obtained for the 184 BP fragment was that of the α strand, but this has been converted to the complementary sequence of the β strand in Figure 15. The sequences run from the 5' end to the 3' end, and the positions of the terminal nucleotides of these sequences relative to the three sequenced strains have been listed.

When the 270 BP fragment was sequenced it gave strange results. In some cases there were bands in all four lanes, sometimes there were bands in three lanes, and sometimes bands in both the G+A and C+T lanes. The same pattern was seen in two different sequencing attempts using fragments that were isolated at different times. The two fragments (166 and 693) which are directly adjacent to the 270 BP fragment in pDLS19 did not show this problem when they were sequenced. The reason for these results is not known, although a nick in the DNA will cause a band in all four lanes, and heterogeneity in the DNA can cause inconsistent bands. Two different strands of labelled DNA present will also cause this problem, but this is considered unlikely since the 270 BP fragment was well-separated from other bands.

Figure 14. Partial Nucleotide Sequence of the 166 BP Fragment of pDLS19.

The differences between the NY8153 strain and the other sequenced strains are listed above and below the sequence. A dash indicates a deletion. Bases or areas which could not be determined with certainty are enclosed in parentheses. The positions of the terminal nucleotides in the three sequenced strains are listed before and after the NY8153 sequence.

Figure 15. Partial Nucleotide Sequence of the 184 BP Fragment of pDLS19.

```
 D/H  (1790)              A
CM1841(1794)              A
NY8153       CGATCAAAGA (A/G)GGATTAAAG AATATTATTG GCTGAAATGG
Cabb-S(1794)              A                          A

                   G
CTAATCTTAA TCAAATCCAA AAAGAAGTCT CTGAAATCCT CAGTGACCAA AAATCCATGA
              G

                            T                                  (1947)
  T                         T GC                    C          (1951)
AAGCGGATAT AAAAGCTATC TTAGAAATAT TAGGATCCCA AAATCCTATT AAAGAAAG
                            T                                  (1951)
```

```
 D/H  (3778) T                            A                 A
CM1841(3776)                              A                   T
NY8153       CTTATGCATA GCATCCAAGT TCGTCATTCC AGAAGAACAT TGGGTCAATG
Cabb-S(3779)  C                           A

              C            T            T   G  C        A   A       C
                                        T               A           C
CAGAAAGACC AATAATGGTC AAAATAGCAG ATGGAAGCTC AATTACCATC (A/G)GCAAAGT(C/T)T
              T                                         A           C

   G       T   C          C   A  AA  A         CATA    C  (3934)
                          C      A   A         AAAA    C  (3932)
GCAAAGACAT AGACTTGATC ATAGTCGGCG TG(A/T)TATTC (A+G)4 TTTCC
                          C      A   A         AGAA    C  (3935)
```

Figure 16. Partial Nucleotide Sequence of the 376 BP Fragment of pDLS19.

The differences between the NY8153 strain and the other sequenced strains are listed above and below the sequence. A dash indicates a deletion. Bases or areas which could not be determined with certainty are enclosed in parentheses. The positions of the terminal nucleotides in the three sequenced strains are listed before and after the NY8153 sequence.

Figure 17. Partial Nucleotide Sequence of the 693 BP Fragment of pDLS19.

```
 D/H  (4148)   T            A       A--- -  -  --- ----------
CM1841(4146)                        AAGA    A           A
NY8153       CCGGTGAACA TTTCGACAAA (A+G)4T(A/G)GAA AATCCGCTAG
Cabb-S(4149)   A            T       AAGA    A           A

---                                                  T
                                                     T
AAGAAATTGC TATTCTTTCA GAGGGGAGGA GGTTATCAGA AGAAAAACTC

      T       T     G                 T                      A
      T       T                       T              A       A
TTCA(T/C)CAC(T/C)C AACAAAGAAT GCAAAAAACC GAAGAACTAC TTGAGAA(A/G)GT
      T       T                       T                      A

                                         G     A        G      . (4316)
                                         G     A        G        (4335)
ATGTTCAGAA AATCCATTAG ATCCTAACAA GACTAA(G/A)C(A/G)A TG(G/A)ATGAAG
                                         G     A        G        (4338)


 D/H  (1336)             ---  -  -       A
CM1841(1335)                  A
NY8153       CTGTGGAGAA TAAA(T/A)TGAGC ATTACGGGTC AACCGCATGT
Cabb-S(1335)                T  A                A

         A

TTATAAAAAG GATACTATTA TTAGACTAAA ACCATTGTCT CTTAATAGTA ATAATAGAAAG
         A

             A               A      G
             A               A      G
TTATGTTTTT (G/A)GTTCCTCAA A(A/G)GG(G/A)AACAT TCAAAATATA ATTAATCATC
             A               A      G

               A      T      T           (1511)
                      T      T           (1515)
TTAACAACCT CAATGAGAT(T/A) G(T/A)AGGAAGAA G
                      T      T            (1515)
```

CHAPTER IV

DISCUSSION

Production of Mutants by the Partial Digestion/Religation Procedure

The partial digestion/religation procedure worked quite well in the production of the PstI deletion mutant, pDLS19. It also worked fairly well in the production of the XbaI deletion, pCMXD319, although an additional selective step (XhoI cleavage) was added and more mutants needed to be screened. Unfortunately, extensive use of this procedure failed to produce the EcoRI deletion mutant lacking only the smallest EcoRI fragment.

At one point it was thought that the NY8153 strain lacked one of the EcoRI sites at the ends of the EcoRI E fragment which are present in other strains (Cabb-S and CM1841). If one of these sites is absent, this deletion mutant cannot be produced. However, other studies have shown that both of these sites are present in NY8153 and that a 60 base pair fragment is produced upon EcoRI digestion (Gardner et al., 1980).

Another potential problem would be a slow rate of cleavage of the EcoRI sites flanking the E fragment. Studies have shown that restriction endonucleases do not cleave all of their sites at the same rate (Nath and Azzolina, 1981). If one or both of these EcoRI sites

were not cleaved as rapidly as the other sites, then the probability of producing a molecule with only these two sites cleaved would be significantly decreased. This would make it more difficult to produce the desired mutant. Several observations suggest that this is occurring.

First of all, the small fragment produced by HpaI and XhoI cleavage of pCS101 contains the two EcoRI sites flanking the E fragment. When this small fragment was isolated from a gel, it was found to be more difficult to digest than expected. This could have been a result of a slower rate of cleavage of these sites, although isolation from the gel may also have been a factor.

Secondly, eleven of the thirty-three EcoRI deletion mutants produced seem to be lacking the EcoRI C fragment (plus possibly also the D fragment; see Figure 6). However, only one of the thirty-three mutants seemed to lack the B fragment (plus possibly also the D and/or E fragments). This suggests that it is easier to cleave out the C fragment than the B fragment. Since both of these require cleavage at the same location (either of the EcoRI sites flanking the D fragment), it seems that it is easier to cleave the EcoRI site separating the A and C fragments than it is to cleave the B fragment away from the A fragment by cleaving either of the two EcoRI sites flanking the E fragment. Hence, these sites all cleaved slower than at least one of the other EcoRI sites. The slower rate of cleavage hinders theproduction of the mutant.

The restriction site preference of EcoRI has been studied using adenovirus DNA (Forsblom et al., 1976) and bacteriophage lambda DNA (Thomas and Davis, 1975). Thomas and Davis (1975) suggest that this differential preference is due to the nucleotide sequence surrounding the EcoRI

sites. These flanking sequences for lambda DNA (Sanger et al., 1982) and cloned CaMV DNA (strains Cabb-S and CM1841 since the sequences for NY8153 are not known; Balazs et al., 1982) are listed in Table II. Site number 1 of lambda DNA is the left-most EcoRI site on the left arm of the bacteriophage DNA. The remaining sites are numbered consecutively moving to the right. Site 5 is cleaved 5-10 times more frequently than sites 1 and 2. The preferences for sites 3 and 4 are intermediate between these two extremes. Site number 1 for the cloned CaMV is the EcoRI site of pBR322 (see Figure 6) and the site number increases as one moves clockwise around the plasmid. Sites 4 and 5 flank the 60 base pair EcoRI E fragment.

The flanking sequences of the fast-cleaving and slow-cleaving sites were compared and contrasted to determine if any patterns existed. A readily apparent similarity exists between sites 4 and 5 in the cloned viral DNA. All of these sites contain GAAGAA in the 5' flanking sequence and three out of four contain an additional GAA or GGA. Perhaps this sequence somehow slows the rate of cleavage.

Also, in the slow-cleaving sites (1 and 2) of lambda DNA the nucleotides immediately before and after the EcoRI recognition sequence are not complementary to each other. In the remaining sites (3 through 5) these adjacent nucleotides are complementary. These nucleotides may play a role in the rate of cleavage, with sites having complementary adjacent nucleotides being cleaved faster. This may result from a more stable cruciform structure which can be formed at the recognition site. If this is true, the EcoRI sites flanking the E fragment (4 and 5) should be slow-cleaving since their adjacent nucleotides are not complementary.

TABLE II

EcoRI SITE FLANKING SEQUENCES OF LAMBDA AND CaMV DNA

Site No.	Flanking Sequences of Lambda
1	TGGGGCCGGTGA-GGCCTTTCCGGC
2	GATCTCCGCTTA-ATTTCAGCATTT
3	AGAACCCTGTTT-ACTTCCGGCGTG
4	TTATTCGTCAGA-TGGCGAATCCTC
5	ACTGTCTGTCCT-ATTAGTAATAGT
Site No.	**Flanking Sequences of Cabb-S**
1	TTTCGTCTTCAA-TCATGTTTGACA
2	TTCCTTTCAAGA-AATAAGGTTAAT
3	GTCCACATCAGG-CAATCCCACCAA
4	GAAGAAGAATCA-CTTCTAGCAATA
5	GGAGAAGAACCT-GAGCAAGTTCGA
Site No.	**Flanking Sequences of CM1841**
1	TTTCGTCTTCAA-TCATGTTTGACA
2	TTCCTTTCAAGA-AATAAGGTTAAT
3	GTCCTCATCAGG-CAATCCCACAAA
4	TCGGAAGAATCA-CTTCTAGCAATA
5	GGAGAAGAACCT-GAACAAGTTCGA

A dash represents the EcoRI recognition sequence, GAATTC. The site numbering is explained in the text.

No other patterns could be easily discerned when the nucleotide sequence or base compositions were studied. It is possible that the site preference is affected by sequences located at greater distances away from the cleavage site, or by long-range secondary structure of the DNA.

Replication Ability of Mutants

One possible way to use CaMV as a plant genetic engineering vector is to produce a mutant in the viral genome which would prevent disease production yet still allow the virus to replicate and spread throughout the plant. It is desirable that such a mutation be a deletion of a portion of the viral DNA since there seems to be a limit to the amount of foreigh DNA the CaMV genome can carry (Gronenborn et al., 1981). This may be due to a limitation in the amount of DNA that can be packaged into a CaMV virus particle. Deletion of part of the viral DNA would allow more foreign DNA to be inserted.

Several insertion and deletion mutants (both in-phase and out of phase) have been constructed and have been inoculated onto plants (Gronenborn et al., 1981; Howell et al., 1981; Dixon et al., 1983; Daubert et al., 1983). Symptom production was the only criterion used to decide whether the mutant was active in the plant or not. Viral DNA was isolated from plants showing symptoms and further characterized to show that the original mutation was still present in the isolated DNA. Neither viral DNA nor total plant DNA was isolated from plants that did not show symptoms. Hence, these studies could not have detected a replicating mutant which did not

produce disease symptoms.

In an attempt to find such a mutant, total plant DNA and viral DNA were isolated from inoculated leaves of plants which did not show symptoms when inoculated with mutants. The viral DNA preparation should contain DNA which is encapsidated in virus particles, while the total plant DNA preparations would contain viral DNA free in the plant cells (Menissier et al., 1982). No CaMV sequence could be detected by hybridization in either of these preparations with any of the mutants studied. Wild-type viral DNA cloned in a bacterial plasmid gave a positive response with both DNA preparations. The mutants definitely behave differently in the plants than wild-type virus does.

These results can be accounted for by either of two general explanations. The first is that the mutant is unable to replicate. All of the mutants tested have significant portions of one coding region deleted. The loss of the gene product of Region III or IV could quite possibly stop replication of the virus. Also, most of the mutations in Region IV are out of phase. If the relay race rule of CaMV translation holds true, these mutants will also lack the gene product of Region V. Since this coding region is thought to produce the viral reverse transcriptase, it seems unlikely that these mutants could replicate. The in-phase mutants (pCMS34, pDLS19, and pCS101-4Δ50) should produce the Region V gene product. Their lack of activity should result solely from lack of the Region III or IV gene product. It is possible these play a role in replication.

Secondly, a mutation preventing transport of the virus within the plant would also produce these results. Infection of a plant may result from introduction of viral DNA into very few cells during the

inoculation. If the virus replicated in these cells of the plant, there probably would not be enough DNA to detect. A mutant defective in transport could be detected by using a protoplast system (Yamaoka et al., 1982) or a cell-free system (Hussain et al., manuscript in preparation). These systems do not require transport of the virus in order to detect replication. Further study of these and other mutants needs to be done using these systems.

pDLS19 is an in-phase deletion in Region IV which lacks the lysine core. It was originally thought that this mutant was not active because it lacked an area of the coat protein thought to be needed for interaction with the viral DNA. However, another in-phase deletion, pCS101-4Δ50, is also inactive yet it still contains the lysine core. Perhaps the inactivity of both of these mutants is due to a shorter coat protein. This altered coat protein may not be able to package the viral DNA, and this may prevent transport of the virus out of the originally infected cells. However, very little is known about the transport process, and the Region IV gene product may have other functions such as serving as an enzyme.

Melcher et al. (manuscript in preparation) have looked for replication of mutants in uninoculated leaves using the method of squish hybridization. They were unable to detect viral DNA in the plants which did not show symptoms. The present study used DNA preparations from inoculated leaves in order to detect possible mutants defective in long-range transport (leaf to leaf), but capable of short-range transport (cell to cell). No such mutants were found in this study. Since both of these studies use some of the same mutants (pCMS34, pUM13, and pUM37), no evidence was found here to suggest that two

separate transport mechanisms exist.

Daubert et al. (1983) constructed several mutants which produced milder symptoms than wild-type virus. When the amount of virus in these plants was measured it was found to be less than that found in plants infected with wild-type virus. Hence, the severity of symptoms seems to be proportional to the extent of replication of the virus (at least in the limited number of studies done). This suggests that the disease is a direct result of the replication of the virus (in contrast to the suggestions of Zaitlin, 1979). This is consistent with studies of Melcher et al. (manuscript in preparation) who have shown that none of the six major coding regions has symptom production as its only function. If disease symptoms result from viral replication, then a mutant which still replicates but is completely symptomless cannot exist.

Tomlinson and Shepherd (1978) have reported the production of a "symptomless" mutant. Actually, this mutant produces pale lesions on inoculated leaves. They also briefly mention one strain of CaMV that is symptomless on Brussels sprouts. The presence of virus in the plants was determined by grinding up tissue of the inoculated Brussels sprouts and showing that it was able to infect turnips. The plants may have had very mild symptoms difficult to detect. Perhaps further physical, cytological, or biochemical studies would have revealed abnormalities in the plant. Neither one of these cases has been shown to be a true symptomless infection.

Nucleotide Sequence of the NY8153 Strain of CaMV

The areas of the NY8153 viral genome which were sequenced are

are shown in Figure 18. Approximately the first third of Regions II and III and portions of the first half of Region V were sequenced. Altogether, 686 bases of the NY8153 strain have been determined. This is approximately 8.6% of the total genome.

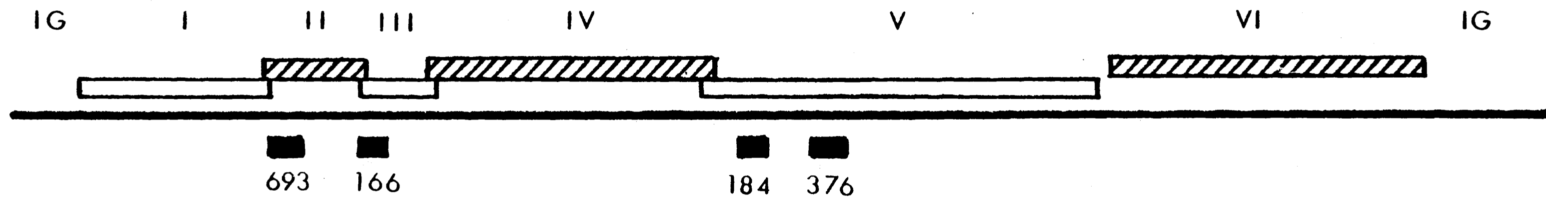
The differences between the NY8153 strain and the other sequenced strains are quantitated in Table III. Two to five percent of the sequenced nucleotides of NY8153 are different as compared to the other sequenced strains (Balazs et al., 1982). This is approximately the same amount of difference found among the three previously sequenced strains.

The NY8153 strain seems to be more closely related to the CM1841 and Cabb-S strains than to the D/H strain. In addition to a larger number of base substitutions, the D/H strain also has 26 bases deleted from the NY8153 (and CM1841 and Cabb-S) sequence. From the limited amount of sequence available here, it is not possible to determine which of the other two strains is more closely related to NY8153.

When the amount of base substitution between the different sequenced fragments is studied, several observations can be made. First of all, the 693 BP fragment clearly has the lowest amount of variation. This sequence comes almost entirely from Region II, which is the most highly conserved coding region of the virus (Balazs et al., 1972). In addition, the 5' half of this coding region is conserved more than the 3' half. The sequence of the 693 BP fragment comes from the 5' half of Region II and seems to follow this high degree of conservation. The second-most conserved coding region is Region III, from which most of the 166 BP fragment comes. This sequence shows more variability than the previous one, but not as much as the remaining two.

Figure 18. Sequenced Areas of the NY8153 Genome.

Regions of CaMV



Areas Sequenced

TABLE III

COMPARISON OF NY8153 DNA SEQUENCE TO OTHER STRAINS

Fragment	No. of Bases Sequenced	Bases Substituted, Percentage of Total			Average Percentage	Bases Deleted from the NY8153 Sequence		
		D/H	CM1841	Cabb-S		D/H	CM1841	Cabb-S
166	158	1,0.6	6,3.8	3,1.9	2.1	0	0	0
184	157	19,12.1	6,3.8	7,4.5	6.8	0	0	0
376	190	5,2.6	4,2.1	5,2.6	2.5	21	0	0
693	181	3,1.7	0,0.0	3,1.7	1.1	5	0	0
Total	686	28,4.1	16,2.3	18,2.6	3.0	26	0	0

The sequences from the 184 and 376 BP fragments lie within Region V. This region shows a considerable amount of variation, primarily located in the 5' one-fourth of the coding region (Balazs et al., 1982). The 184 BP fragment lies within this area, and it shows the most sequence variation of all the sequenced fragments. The sequence of the 376 BP fragment shows more variability than the first two fragments, but not nearly as much as the 184 BP fragment. It comes from Region V, but does not seem to lie completely within the 5' one-fourth of the region. This seems to decrease the amount of variability between it and the other strains.

Although there is considerable variability in the base sequence of Region V, the amino acid sequence shows the least amount of change among all the major coding regions. The 3' three-fourths of the coding region shows very few amino acid changes among the three strains. This may indicate the area of the putative protein which is essential for its activity. The portion of Region V which shows homology to the polymerase gene of Moloney murine leukemia virus is located within this highly conserved area (Toh et al., 1983; Volovitch et al., 1984). The 5' one-fourth of the region which shows tremendous variability may not be essential to the protein's activity and may be a peptide which is cleaved off of the precursor to give the active product of Region V.

A base that is changed in one strain tends to also be changed in the other strains, although there are many cases where a base of NY8153 is different in only one of the other three strains. These base substitutions seem to occur at random. All of this information about the sequenced portion of the NY8153 genome indicates that it

is very similar to that of the other strains.

The amino acid changes in the putative proteins produced by the base changes were determined. These are quantitated in Table IV. As expected, there are more amino acid differences between NY8153 and the D/H strain than between NY8153 and the other two strains. However, CM1841 shows more amino acid differences (9) from NY8153 than Cabb-S does (5). This may indicate that Cabb-S is more closely related to NY8153 than CM1841, even though both of them have approximately the same percentage of base substitutions (Table III).

The amino acid sequence of the 693 BP fragment shows the smallest variability of the sequenced fragments. This sequence comes from the 5' end of Region II which has a highly conserved amino acid sequence in the other three strains (Balazs et al., 1982).

The greatest amount of sequence variability is found in the 184 BP fragment. This comes from the 5' one-fourth of Region V which shows a tremendous amount of variation among the other strains. The 376 BP fragment also comes from Region V, but seems to lie mostly outside of this variable area and does not show nearly the amount of amino acid variability found in the previous fragment.

The amino acid sequence from the 166 BP fragment is primarily from region III but includes a small portion of the end of Region II. It shows an intermediate amount of amino acid differences when compared to the other strains. Curiously, this area shows a larger amount of amino acid differences between the NY8153 and CM1841 strains than between NY8153 and the other strains. The significance of this is not known.

Most of the amino acid changes are conservative, with many of them

TABLE IV

COMPARISON OF NY8153 AMINO ACID SEQUENCE TO OTHER STRAINS

Fragment	No. of Amino Acids in Sequenced Area	Amino Acids Changed			Percent Changed			Average Percentage
		D/H	CM1841	Cabb-S	D/H	CM1841	Cabb-S	
166	49	1	5	1	2.0	10.2	2.0	4.7
184	52	8	3	3	15.4	5.8	5.8	9.0
376	63	1	1	1	1.6	1.6	1.6	1.6
693	58	1	0	0	1.7	0.0	0.0	0.6
Total	222	11	9	5	5.0	4.1	2.3	3.8

them being one hydrophobic side chain replaced by another. Some of them involve addition or deletion of a hydroxyl group, and a few occur in which a charge is created by the change of a neutral amino acid to lysine or arginine. These types of changes are also seen among the other three sequences (Balazs et al., 1982). The information on the amino acid sequence and its variability also suggests that the NY8153 strain is generally very similar to the other three strains.

CHAPTER V

SUMMARY

A partial digestion/religation procedure was used to construct mutants of CaMV which had specific restriction fragments deleted. A recombinant plasmid (pCMS31) containing NY8153 viral DNA cloned into the bacterial plasmid pBR322 was partially digested with a restriction endonuclease and then ligated with T4 DNA ligase. The religated DNA was used to transform bacteria and the resulting plasmids screened by restriction endonuclease digestion and gel electrophoresis to find the desired mutant.

The PstI D fragment was deleted from pCMS31 to give pDLS19, which has 195 BP deleted from Region IV, the viral coat protein gene. The XbaI C fragment was deleted to produce pCMXD319. This mutant lacks 864 BP from Regions I and II. This method was extensively used in an attempt to delete the EcoRI E fragment of pCMS31. This was not successful, possibly because one or both of the EcoRI sites flanking the E fragment were more resistant to cleavage than the other EcoRI sites.

These mutants plus some others affecting Region III or IV (constructed by other people) were inoculated onto plants to determine what abilities they retained. None of the mutants were able to produce disease symptoms.

Further studies were done in an attempt to detect mutants able to

replicate but unable to induce symptoms. Total plant DNA was isolated from leaves inoculated with mutant DNA. In addition, leaves inoculated with some of the mutants were put through a viral DNA isolation procedure. Both of these DNA preparations were electrophoresed, transferred to nitrocellulose, and hybridized with ^{32}P-labelled CaMV DNA. Both DNA preparations from plants infected with wild-type viral DNA hybridized to the probe while preparations from healthy plants did not, indicating that this is a reliable way to detect replicating virus in inoculated leaves. None of the mutants tested gave total plant DNA or viral DNA that would hybridize to the probe.

These results suggest that the mutants are doing very little, if anything, in the host plants, and certainly do not behave as wild-type virus does. This could be caused by obstruction of either viral replication or transport out of initially infected cells. Further studies need to be done to determine which is actually occurring.

A portion of the nucleotide sequence of the NY8153 strain of CaMV was determined using the chemical degradation method of Maxam and Gilbert. Fragments were generated by digesting pDLS19 with HindIII, labelling the 3' ends, and cleaving with ClaI. Approximately 8.6% (686 BP) of the viral genome was sequenced.

The NY8153 sequence was compared to the other three sequenced strains. Most of the differences were base substitutions amounting to 2-5% of the total number of bases and seemed to be randomly scattered. The CM1841 and Cabb-S strains were more similar to NY8153 than the D/H strain, which had more base substitutions and some nucleotides deleted from the NY8153 sequence.

The three previously sequenced strains have areas of their

genomes which are conserved to various degrees. When the sequenced areas of NY8153 were compared to the other three strains, they seemed to follow the degree of conservation very closely. The structure of the NY8153 genome seems to be very similar to that of the other sequenced strains.

The amino acid sequences were also compared. Once again, NY8153 seems to follow the degree of conservation fairly closely. The amino acid changes between NY8153 and the other strains are minor, most often being one hydrophobic side chain replaced by another. However, some are the addition or deletion of hydroxyl groups or charged groups.

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