ISOLATION AND CHARACTERIZATION OF DELETION MUTANTS OF CAULIFLOWER MOSAIC VIRUS DNA

By

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ISOLATION AND CHARACTERIZATION OF DELETION MUTANTS OF CAULIFLOWER MOSAIC VIRUS DNA

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ABBREVIATIONS

A - absorbance

amp^R - ampicillin resistant

ATP - adenosine triphosphate

bp - base pair

BSA - bovine serum albumin

CaMV - cauliflower mosaic virus

cpm - counts per minute

d - dalton

DNA - deoxyribonucleic acid

DTT - dithiothreitol

EDTA - ethylenediamine tetraacetic acid

kd - kilodalton

LSH - leaf skeleton hybridization

2-Me - 2-mercaptoethanol

NTP - nucleotide

O.D. - optical density

RNA - ribonucleic acid

rpm - revolutions per minute

SDS - sodium dodecyl sulphate

tet^S - tetracycline sensitive

U - units

CHAPTER I

INTRODUCTION

The desire to create new and better crop species that can produce higher yields, are resistant to pathogens, and are of greater nutritional value, has long been a goal of plant breeders and scientists. With the availability of new genetic engineering techniques, one possible way of accomplishing these goals is to create a plant "vector." Ideally, genes coding for desirable traits could be inserted into such a vector which could then be directly introduced into a plant where the new genes would be transcribed and expressed. Because of the ease and widespread applicability of such a system, a great deal of interest has been focused on the development of a plant genetic vector. One potential candidate that is being actively studied is the plant virus, cauliflower mosaic virus (CaMV).

Biological Properties

CaMV is classified within the taxonomic group, caulimovirus, which is the only group of plant viruses containing double-stranded deoxyribonucleic acid (DNA) as their genetic material (Harrison, et al., 1971). Other less well studied members of this group include dahlia mosaic virus (DaMV), carnation etched ring virus (CERV), mirabilis mosaic virus and strawberry vein banding virus. Like the other caulimoviruses, CaMV has a relatively limited host range. In nature,

it is transmitted only to plants of the family Cruciferae, which includes cauliflower (Brassica oleracea L.), turnip (Brassica rapa L.), mustard (Brassica campestris L.), and radish (Raphanus sativus L.) (Tompkins, 1937; Broadbent, 1957). It has been shown experimentally that some strains of CaMV can infect Nicotiana clevelandii (Hills and Campbell, 1968) or Datura stramonium (Lung and Pirone, 1972), both members of the family Solanaceae.

Disease symptoms exhibited by plants infected with CaMV usually resemble a mosaic-mottle type of disease which may be accompanied by vein clearing, vein banding, wrinkling of leaves, chlorotic local lesions or stunting (Broadbent, 1957; Shepherd, 1970).

A common insect vector, the aphid, is responsible for transmitting the virus in nature. The mechanism of aphid transmission is considered to be stylet-born and nonpersistent (see Shepherd, 1976, for review). This means the virus is picked up on the mouthparts (stylet bundle) of the aphid during feeding and carried to other plants during later feeding activity. The virus may be retained on the aphid stylet for one to two hours or, occasionally, as long as several days; but does not multiply within the insect or pass into its gut or circulatory system (Watson and Roberts, 1939; Kennedy et al., 1962).

There are at least two virus strains, CM1841 and CM4-184, which are non-aphid transmissible (Lung and Pirone, 1973; Hull, 1980). The reason for this is unknown but the absence in these strains of a virus-specified "aphid acquisition factor" that allows the virus to be transmitted, has been suggested (Lung and Pirone, 1973; 1974). CaMV may also be transmitted by mechanical inoculation of the virus or its DNA directly onto a plant (Shepherd et al., 1968) but not

through the seed of the infected plants.

Chemical and Physical Properties

Observation of CaMV under the electron microscope reveals that it is a spherical isometric particle of approximately 50 nm in diameter (Pirone et al., 1960; Day and Venables, 1960; Hull et al., 1976). It appears to reside primarily in the granular matrix of the inclusion bodies of infected cells. Inclusion bodies are elliptical masses of electron-dense material (mainly protein) that are produced within the cytoplasm of infected cells (Fujisawa, 1967). Inclusion bodies may be the site of virus assembly or synthesis.

DNA Structure

The double-stranded DNA of CaMV has a molecular weight of $4.5 - 5 \times 10^6$ daltons (d) (Shepherd et al., 1971; Russellet al., 1971). DNA isolated from virions consists of two types; a nicked circular form and a linear form. The circular form makes up 80-90% of the virion DNA and is infectious whereas the linear form is not (Russell, 1971; Shepherd and Wakeman, 1971; Hull and Shepherd, 1977). It has been proposed that the linear form arises by accidental breakage of the circular form (Hull and Shepherd, 1977). Under the electron microscope, the circular form has a twisted or tangled appearance but is not supercoiled (Shepherd et al., 1971; Shepherd, 1970). However, Menissier et al. (1982) has recently reported the presence of a supercoiled form that exists free in the cytoplasm of infected cells.

Volovitch et al. (1978) has shown that there are two or three single-stranded discontinuities or "gaps" within the circular DNA.

The gaps are at fixed sites within the DNA; two in the β strand and one in the α strand (Volovitch et al., 1978; Hull, 1980). Thus neither strand of DNA is covalently closed and this presumably explains the inability of the DNA to form a supercoiled configuration. Franck et al. (1980) and Richards et al. (1981) have shown that the gaps are actually sites of single-stranded sequence overlap. The function of the gaps is unknown but they may play some role in viral replication or transcription.

The complete nucleotide sequence of two isolates of CaMV, Cabbage B-S (Strausbourg) (Franck et al., 1980) and CM1841 (Gardner et al., 1981) have been determined. Both isolates are about 8000 base pairs (bp) long and each has at least six open reading frames (coding regions) in the α strand (Franck et al., 1980; Gardner et al., 1981). There are no open reading frames of more than 370 nucleotides (NTPs) in the corresponding β strand lending support to other evidence that only the α strand is transcribed (Howell and Hull, 1978; Guilfoyle, 1980).

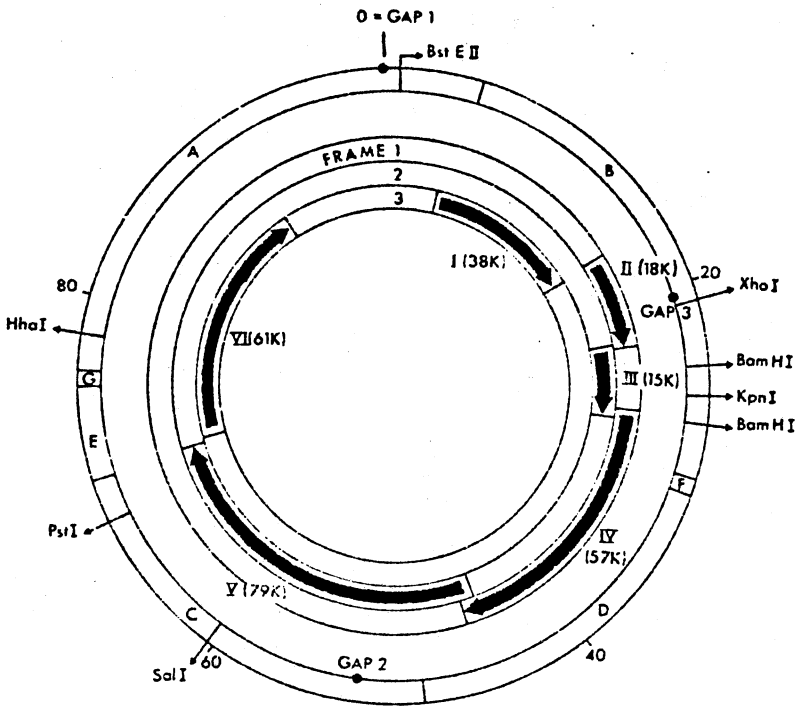
The six open reading frames are designated regions I through VI (Figure 1). The six regions are in different reading frames but there is little or no overlap between them. There are two "intergenic" regions which have no coding capacity. The largest intergenic region (∿1000 bp) is between regions VI and I and contains the only gap in the α strand (Franck et al., 1980; Gardner et al., 1981).

RNA and Protein

Only two CaMV-specific ribonucleic acid (RNA) transcripts are known to accumulate in infected cells. One is a large transcript that

Figure 1. Genetic Map of CaMV DNA.

The inner circles indicate the position of the six coding regions and the molecular weight of their translation products. The outer circle shows the location of the three gap regions and several restriction enzyme sites (from Franck et al., 1980).


0 = GAP 1
Bst E II
A
B
FRAME 1
2
3
I (38K)
II (18K)
20
Xho I
GAP 3
80
Hha I
VII (61K)
III (15K)
Bam H I
Kpn I
Bam H I
G
E
F
Pst I
IV (57K)
V (79K)
C
D
Sal I
60
GAP 2
40

covers the entire genome (Howell and Hull, 1978; Covey and Hull, 1981; Covey et al., 1981). The other transcript codes for a 61-66 kilodalton (kd) protein and corresponds to region VI on the genetic map (Odell et al., 1980; Odell et al., 1981; Covey and Hull, 1981; Covey et al., 1981).

Based on the comparison of the nucleotide sequence with the amino acid composition of the viral coat protein, it is believed that only region IV could code for the 42-43 kd coat protein (Franck et al., 1980).

No other gene products have been linked to the remaining four open reading frames. Region III must encode some essential function since its deletion results in a non-infectious virus (Lebeurier et al., 1980; 1982). A natural variant of CaMV, CM4-184, with a 421 bp deletion in region II remains infective but is not aphid transmissible, suggesting that this region may code for an aphid acquisition factor (Hull and Howell, 1978; Hull, 1978; Howarth et al., 1981). However, strain CM1841, from which CM4-184 is probably derived, is also non-aphid transmissible but has no deletion in this region (Lung and Pirone, 1973; Howarth et al., 1981).

CaMV as a Plant Genetic Vector

Several features that make CaMV an attractive genetic vector are its double-stranded DNA genome which allows direct insertion of foreign genes, its small size making it easy to manipulate, the presence of several unique restriction enzyme sites for cloning, and the capacity to spread systemically throughout the plant.

In recent years, research has focused on characterizing aspects

of CaMV that are important in order for it to be used as a plant vector. Perhaps the most important question is whether foreign DNA can be stably inserted and propagated within the genome of CaMV. To facilitate the study of CaMV and its DNA, it has been cloned into a variety of bacterial plasmids and bacteriophages via one of its unique restriction enzyme sites (Szeto et al., 1977; Hohn et al., 1980; Howell et al., 1980; Howarth et al., 1981). In the cloned state, the gaps in the DNA are sealed and the recombinant molecules become supercoiled. The cloned CaMV DNA is not infectious unless released from its vector (Howell et al., 1980). Once released, the now linear DNA is religated in vivo and the gaps regenerated in the progeny virus (Howell et al., 1980; Lebeurier et al., 1980). The inability of the cloned DNA to produce infection in the plant suggests that there may be a size limitation on the DNA that prevents its packaging into mature virions. However, the possibilities that the bacterial vectors are toxic to the virus or that the cloned DNA interrupts an essential gene function in the virus cannot be ruled out.

Howell et al. (1981) generated small insertions and deletions into several sites of cloned CaMV (isolate CM4-184) DNA in an attempt to identify regions that are nonessential for viral replication. Such regions could be eliminated in order to reduce the size of the CaMV DNA to allow for stable insertion of foreign DNA and packaging of the DNA. The only modified viral genome that retained infectivity was an 8 bp EcoRI linker insertion in the large intergenic region clockwise to the single gap in the α strand. Attempts to coinfect with two mutant genomes was sometimes successful in cases where the mutations

were not overlapping. However, analysis of the DNA from infected plants revealed that rescue was by recombination rather than complementation and resulted in expulsion of the inserted DNA.

In contrast, Lebeurier et al. (1980) showed that a bacterial plasmid insert in the large intergenic region was not infectious. In this instance, the size of the insert may have been the critical factor. Lebeurier et al. (1982) also showed that insertion of the bacterial plasmid in regions III or V abolished infectivity. In addition, it was demonstrated that the large BamHI fragment (which comprises about 7800 bp of the viral DNA) is not infectious alone but, when coinfected with the small BamHI fragment, the entire CaMV genome is regenerated and becomes infective. This finding confirms that region III, which is contained within the small BamHI fragment, encodes some essential function for viral infection.

Gronenborn et al. (1981) chose the XhoI site in region II as the location for insertion of foreign DNA into strain CM1841 of CaMV. Since this region shows some heterogeneity in size in some strains, it was thought to be a possible nonessential region and, thus, interruption of this gene should not destroy infectivity. For example, isolate CM4-184 has most of this region deleted (Howarth et al., 1981) and the Australian isolate has an approximately 100 bp addition in this region (Hull and Howell, 1978). A 65 bp lac operator fragment inserted at this site did not destroy replication or infectivity of the viral DNA but, after more than three successive transfers of the modified virus through turnip plants, the insert was lost. In addition, the upper limit of foreign DNA that could be propagated in the XhoI site was found to be between 250 and 500 bp (Gronenborn et al., 1981).

Although the evidence favors using region II as a cloning site for insertion of foreign DNA, its usefulness appears to be limited by the size limitation on packaging of the DNA. In vitro modification of this and other sites is one possible way of overcoming this problem. The aim of this study, then, was to create such modifications by deletion of DNA in region II and its flanking regions; and to determine their effect on infectivity of the virus in vivo.

CHAPTER II

MATERIALS AND METHODS

Viral DNA

DNA from CaMV strain New York 8153 (NY8153) which has been inserted into the Escherichia coli (E. coli) plasmid, pBR322, via their unique SalI restriction enzyme sites, was used as the starting material. The resulting plasmid, pCMS-31, transformed E. coli K-12 strain HB101. Because SalI digestion of pBR322 interrupts the tetracycline resistance gene of this plasmid but not the ampicillin resistance gene, bacteria containing pCMS-31 are tetracycline sensitive (tet^S) but remain ampicillin resistant (amp^R). The viral DNA in pCMS-31 is not infectious unless released from the plasmid vector by SalI cleavage (Howell et al., 1980).

Growth of Bacteria

HB101 (pCMS-31) or HB101 containing a deletion plasmid were streaked out on nutrient agar plates (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 2% w/v agar; all obtained from Difco) containing 0.015 mg/ml ampicillin or 0.010 mg/ml tetracycline (Sigma Chemical Co.). HB101 or HB101 (pBR322) were streaked out as controls on the same plate. Plates were incubated at 37°C in a warm air incubator overnight.

Tubes containing 5 ml of M9 minimal medium (Miller, 1972;

Table I with 50 μg/ml ampicillin were inoculated with single colonies obtained from the ampicillin nutrient agar plates and the cultures were grown overnight at 37°C. Flasks containing 250-500 ml of M9 minimal medium were inoculated with the 5 ml cultures. Flasks were placed in a 37°C gyrorotary shaker at 200-250 rpm. Growth of cells was monitored by following the increase in optical density at 550 nm ($O.D._{550}$). At $O.D._{550}$ = 0.6 to 1.0, chloramphenicol (Sigma Chemical Co. or Calbiochem) was added to a concentration of 100 μg/ml. Chloramphenicol, an inhibitor of protein synthesis, blocks replication of the E. coli genome. Since the plasmid is under relaxed control, it will continue to replicate and become amplified in the cell (Clewell, 1972). Cultures were amplified for 15-22 hr.

Plasmid Isolation

Plasmid DNA was isolated essentially by the method of Clewell and Helsinkii (1969). Volumes given are those used for a 500 ml culture and were appropriately adjusted for larger or smaller cultures. Cells were pelleted in 250 ml plastic bottles at 6160 x g for 10 min at 4°C in a Beckman J-21 B centrifuge, washed one time with 125 ml of 10 mM Tris-HCl, pH 8.5, 1 mM Na_2EDTA (ethylene diamine tetraacetic acid). The pellet was frozen at -70°C and thawed at room temperature. Pellets were combined and resuspended using 2.0 ml 0.25 M sucrose, 2 mM $MgCl_2$, and transferred to plastic centrifuge tubes. Cells were lysed by addition of 0.4 ml 20 mg/ml lysozyme (Sigma Chemical Co.), 10 mM Tris-HCl, pH 8.0 for 5 min. on ice followed by 0.8 ml 0.004 M Na_2EDTA, pH 8.0 for 5 min. and 3.2 ml 0.5% v/v Triton X-100, 0.05 M Tris-HCl, pH 8.0, 0.01 M Na_2EDTA for 20 min.

TABLE I

COMPONENTS OF M9 MINIMAL ESSENTIAL MEDIA

Ingredient	Final Concentration
Minimal Salts:	
NH_4Cl	19 mM
Na_2HPO_4	42 mM
KH_2PO_4	22 mM
NaCl	9 mM
Other Ingredients:	
$MgSO_4$	1.0 mM
$CaCl_2$	0.1 mM
Casamino Acids	0.5%
Thiamine	2.0 µg/ml
Uridine	1.0 mg/ml
Glucose	0.4%

The mixture was spun at 25,300 x g, 4°C, for 1 hr. and the clear, non-viscous supernatant containing the plasmid DNA was poured off.

Plasmid Purification

Supercoiled plasmid DNA was purified according to Radloff et al. (1967). CsCl (3.3 g) was measured into a ½ in. x 2 in. polyallomer centrifuge tube. Supernatant from the plasmid isolation and water to total 3.0 ml were added. Tubes were inverted several times to mix and dissolve the CsCl. The density of this solution was calculated to be about 1.59 g/ml. Ethidium bromide (0.3 ml 10 mg/ml, Calbiochem) was added in the dark and tubes were again inverted. Parafin oil was layered on to within 1-2 mm of the top of the tubes and was used to balance them. Tubes were placed in a Beckman SW50.1 or SW65 rotor and centrifuged for about 48 hr. at 34,000 rpm, 20°C, in a Sorvall OTD-50 ultracentrifuge or a Beckman L5-65 ultracentrifuge.

The tubes were then removed from the rotor and illuminated in the dark with a long-wave ultraviolet light (Blak-Ray UVL22, Ultra Violet Products, Inc.) to locate the band of plasmid DNA. The band was removed by piercing the tube with an 18G syringe needle and withdrawing the band into the barrel of the syringe. Ethidium bromide was removed by extracting ten times with an equal volume of NaCl-saturated (5 M NaCl, 10 mM Tris-HCl, pH 8.6, 1 mM Na_2EDTA) 1-butanol. Two volumes of water were added followed by six volumes of 95% ethanol. The plasmid DNA precipitated at -20°C overnight. The DNA was pelleted at 17,640 x g for 10-15 min. and the supernatant was discarded. The DNA was dried under vacuum and redissolved in 100 µl of DNA dissolving buffer (10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA, 10 mM NaCl). Concen-

tration of the plasmid DNA was determined by measuring the absorbance at 260 nm (A_{260}) and assuming that 50 µg/ml of DNA will have an A_{260} = 1.0.

Restriction Enzyme Digestions

Digestion of DNA with various restriction enzymes was carried out in 20-25 µl reaction mixtures at 37°C for 1-2 hrs in the appropriate buffer (Table II). All reaction mixtures were supplemented with 100 µg/ml bovine serum albumin (BSA). The amount of enzyme used varied with the quantity of DNA and the number of cleavage sites in the DNA. Enzymes were obtained from New England Biolabs or Bethesda Research Labs.

Reactions were stopped either by addition of stop reagent (4 M urea, 50% w/v sucrose, 50 mM Na_2EDTA, 1% w/v bromphenol blue, pH 7.0) prior to gel electrophoresis, or by extraction with an equal volume of phenol. Phenol was redistilled into 10 mM Tris-HCl, pH 8.5, 1 mM Na_2EDTA and mixed to saturate before use. After phenol extraction, the phenol phase was extracted again with water or 20 ml NaCl. The combined aqueous phases were then extracted twice with 1.0 ml diethyl ether. DNA was ethanol precipitated, dried, and redissolved as previously described prior to use in subsequent steps.

Exonuclease Digestions

Deletions in XhoI linearized pCMS-31 (XhoI-pCMS-31) DNA were created by digestion with either E. coli exonuclease III (New England Biolabs) or Bal-31 (Bethesda Research Labs). E. coli exonuclease III degrades duplex DNA by progressive digestion of the 3' end of each strand (Richardson

TABLE II

RESTRICTION ENZYME BUFFERS*

BglII	EcoRI
50 mM NaCl	50 mM NaCl
50 mM Tris, pH 7.5	100 mM Tris, pH 7.2
10 mM $MgCl_2$	5 mM $MgCl_2$
1 mM dithiothreitol (DTT)	2 mM 2-Me

HindIII	SalI = XhoI
50 mM NaCl	150 mM NaCl
50 mM Tris, pH 8.0	6 mM Tris, pH 7.9
10 mM $MgCl_2$	6 mM $MgCl_2$
1 mM DTT	6 mM 2-Me

*According to manufacturer's instructions.

et al., 1964). Bal-31 has an exonuclease activity that will degrade the 3' and 5' ends of duplex DNA simultaneously (Gray et al., 1975).

Exonuclease III digestion was performed according to the method of Roberts et al. (1979). Linearized pCMS-31 DNA was incubated at a concentration of 0.05 or 0.10 μg/μl in 6.6 mM Tris-HCl, pH 7.4, 6.6 mM $MgCl_2$, 6.6 mM 2-mercaptoethanol (2-Me), 60 mM NaCl with 4-8 units (U) of enzyme per μg DNA for various lengths of time. The reactions were stopped by adding an equal volume of 2X S1 buffer (100 mM NaOAC-HOAc, pH 4.0, 300 mM NaCl, 12 mM $ZnSO_4$). S1 nuclease (Bethesda Research Labs) was added to a concentration of 1.0 U/μg DNA and incubation was carried out at 37°C for 1 hr.

Bal-31 digestion of linearized pCMS-31 DNA was performed in 12 mM $CaCl_2$, 12 mM $MgCl_2$, 600 mM NaCl, 20 mM Tris-HCl, pH 8.1, 1 mM Na_2EDTA. DNA concentration varied from 0.02 to 0.09 μg/μl with 1.0 U Bal-31/μg DNA. Incubation was at 30°C for various lengths of time. Reactions using Bal-31 or exonuclease III followed by S1 nuclease were stopped as described for restriction enzyme digestions.

A portion of the exonuclease-treated DNA was digested with EcoRI as described. EcoRI digestion of XhoI-pCMS-31 produces six fragments of known size and location. By studying the EcoRI digestion pattern of exonuclease-treated molecules and comparing with that of XhoI-pCMS-31, it can be determined if the exonuclease is degrading both ends of the DNA as desired.

Ligation and Transformation

Blunt end ligation of the exonuclease digested pCMS-31 molecules was done using the optimum conditions for a sticky end (overlapping,

complementary ends) ligation of XhoI digested pCMS-31 but with a 10 fold excess of T_4 DNA ligase. DNA concentration was 0.02 to 0.04 µg/µl in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM adenosine triphosphate (ATP, Sigma Chemical Co.). Incubation was at 15°C overnight with 5.0 U T_4 DNA ligase (Bethesda Research Labs or New England Biolabs) per µg of DNA.

Transformation of HB101 with the deletion plasmids was performed according to Woods et al. (1980). The ligation reaction mixture containing 0.5 µg DNA was mixed with two volumes of competent HB101 cells. The bacteria-DNA mixture was subjected to a cold shock treatment in an isopropanol-dry ice bath, incubation at 42°C, and then at 37°C in nutrient broth (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl). The mixture (0.2 ml) was spread on an ampicillin nutrient agar plate and incubated overnight at 37°C. HB101 cells were also transformed using 0.5 µg pBR322 DNA (positive control) and with no DNA (negative control) to assure the procedure was working.

Storage of Bacteria

Selected transformants were restreaked on ampicillin or tetracycline nutrient agar plates. Those colonies that were amp^R and tet^S were incubated into 5 ml cultures of nutrient broth and grown overnight at 37°C. Cultures were retested for tet^S by streaking on tetracycline nutrient agar plates. To a 1.5 ml cryo-vial, 1.0 ml of culture and 150 µl of sterile glycerol were added and the mixture vortexed. Cryo-vials were labeled and stored at -70°C. To obtain bacteria from the cryo-vial for experimental use, the surface of the frozen culture was scratched with a heat sterilized loop and streaked on the desired

plate.

Screening for Deleted Plasmids

Attempts to screen the transformants for deleted plasmids was done by preparing single colony lysates. About one cubic mm of bacteria was scraped from a plate using a sterile loop or toothpick and resuspended in 95 µl of lysing solution. Lysing solution was composed of Tris-phosphate-EDTA buffer (Loening et al., 1967), 0.025 M Na_2EDTA, pH 7.0, 0.1 mg/ml proteinase K (Boehringer-Mannheim). 20% SDS (3 µl, sodium dodecyl sulphate) and 25 µl 0.01% bromphenol blue, 50% glycerol were added and the mixture was vortexed vigorously. The lysate solution was incubated at 70°C for 10 min. Lysates were analyzed by electrophoresis in 0.8% agarose gels (25 x 20 x 0.5 cm) at 40V, 4°C for 15-17 hours in 1X Loening buffer. Gels were stained with 0.5 µg/ml ethidium bromide and photographed through a Wratten No. 23A filter using trans-illumination with ultraviolet light.

Determination of Deletion Size

Deletion size was determined by digestion of purified plasmid DNA from selected transformants with the restriction enzymes HindIII or BglII as described. pCMS-31 DNA digested with HindIII or BglII produced fragments of known size and location and was used as a standard for comparison with restricted deletion plasmids. Restriction fragments were separated by electrophoresis in 1.0 to 1.5% agarose gels as described. The distance migrated by each fragment of digested pCMS-31 was measured. To create a standard curve, the migration distance was plotted against the log of the molecular weight of the

fragment. The migration distance of fragments of digested deletion plasmids were also measured. The molecular weight of fragments with an altered migration distance were determined by extrapolation from the standard curve.

Incubation of Turnips

Turnip plants (Brassica rapa L. cv Just Right) were maintained in a growth chamber set at 22°C for a 12 hr. day period and at 18°C for a 12 hr. night period.

Plasmid DNA used for inoculation was first digested with SalI as described to release the CaMV DNA. pBR322 vector DNA was not separated from the CaMV DNA nor was the CaMV DNA religated into a circular form prior to inoculation. The SalI reaction mixture was diluted in a solution of 1% w/v K_2HPO_4 to a DNA concentration of 10 µg/ml.

The third, fourth, and fifth leaves of 4 week old plants were inoculated by lightly dusting the leaves with carborundum or celite and rubbing a cotton-tipped swab soaked in the diluted viral DNA solution over the leaves. Inoculated leaves were identified by punching holes in the leaves. For each experiment, three control solutions, pBR322 DNA, viral DNA, and NY8153 virus, were also inoculated onto plants.

Starch Lesions

Two weeks after inoculation of turnip plants, 2-3 inoculated leaves were removed from each plant and submerged in 2-methoxyethanol at 37°C until the leaves lost their green pigment (4-8 hrs.).

Leaves were rinsed once or twice in water and KI_3 (0.06% KI and 0.006% I_2) was added to stain the leaves for detection of starch lesions. Stained leaves were photographed using transillumination.

Leaf Skeleton Hybridization

Starch stained leaves were processed for leaf skeleton hybridization (LSH) according to Melcher et al. (1981) except that ethidium bromide staining was not done and drying of leaves at 80°C in a vacuum oven was omitted. Each bag of leaves contained 20 mls of hybridization solution with at least 500,000 cpm of ^{32}P-labeled CaMV DNA per bag.

Systemic Symptoms

Development of systemic viral infection in turnip plants was determined by visual examination of the plants five weeks after inoculation. One inoculated leaf was left on each plant for five weeks for this study rather than being removed for starch staining and LSH. The presence of typical systemic symptoms such as mottling, crinkling of leaves, chlorotic lesions and stunting was the criterion for a positive result.

Viral DNA Isolation

Viral DNA was isolated according to the rapid method of Gardner et al. (1980). Systemically infected leaves (3.5 - 5.0 g of tissue) were homogenized in a Sorvall Omni-Mixer (Model 17105, E. I. Du Pont de Nemours and Co.) for 1-2 min. Pelleting of virus particles through a sucrose cushion was done in a 75Ti rotor at 31,000 rpm in a Sorvall

OTD-50 ultracentrifuge. Deoxyribonuclease I (DNase I) was obtained from Sigma Chemical Co. and was used to digest away any remaining cellular DNA. Proteinase K was obtained from Boehringer-Mannheim. Viral DNA was analyzed by digestion with HindIII as described above.

CHAPTER III

RESULTS

Construction of Deletion Mutants

The experimental scheme for construction of deletions in cloned CaMV DNA is shown in Figure 2. Purified pCMS-31 DNA was digested with the restriction enzyme, XhoI. XhoI cuts one time in the pCMS-31 plasmid producing a linear molecule. Analysis of XhoI treated pCMS-31 by agarose gel electrophoresis revealed that all detectable DNA was in the linear form and that the reaction was thus complete. The site of XhoI cleavage is in region II of the CaMV DNA portion of the plasmid. Therefore, after digesting with XhoI, deletions in region II and the coding regions flanking it can be made by synchronous digestion with an exonuclease.

Orientation of pCMS-31

EcoRI digestion of CaMV DNA produced five fragments of known size and location as determined from nucleotide sequence analysis of isolate CM1841 (Gardner et al., 1981). pBR322 is cleaved only once by EcoRI at a known site. The correct orientation of the CaMV DNA insert in pBR322 was determined by digesting with EcoRI and comparing the resulting fragments with fragment sizes expected for each of the two possible orientations of CaMV DNA relative to pBR322. An XhoI-EcoRI restriction enzyme map of pCMS-31 was constructed (Figure 3).

Figure 2. Scheme for Construction of Deletion Mutants.

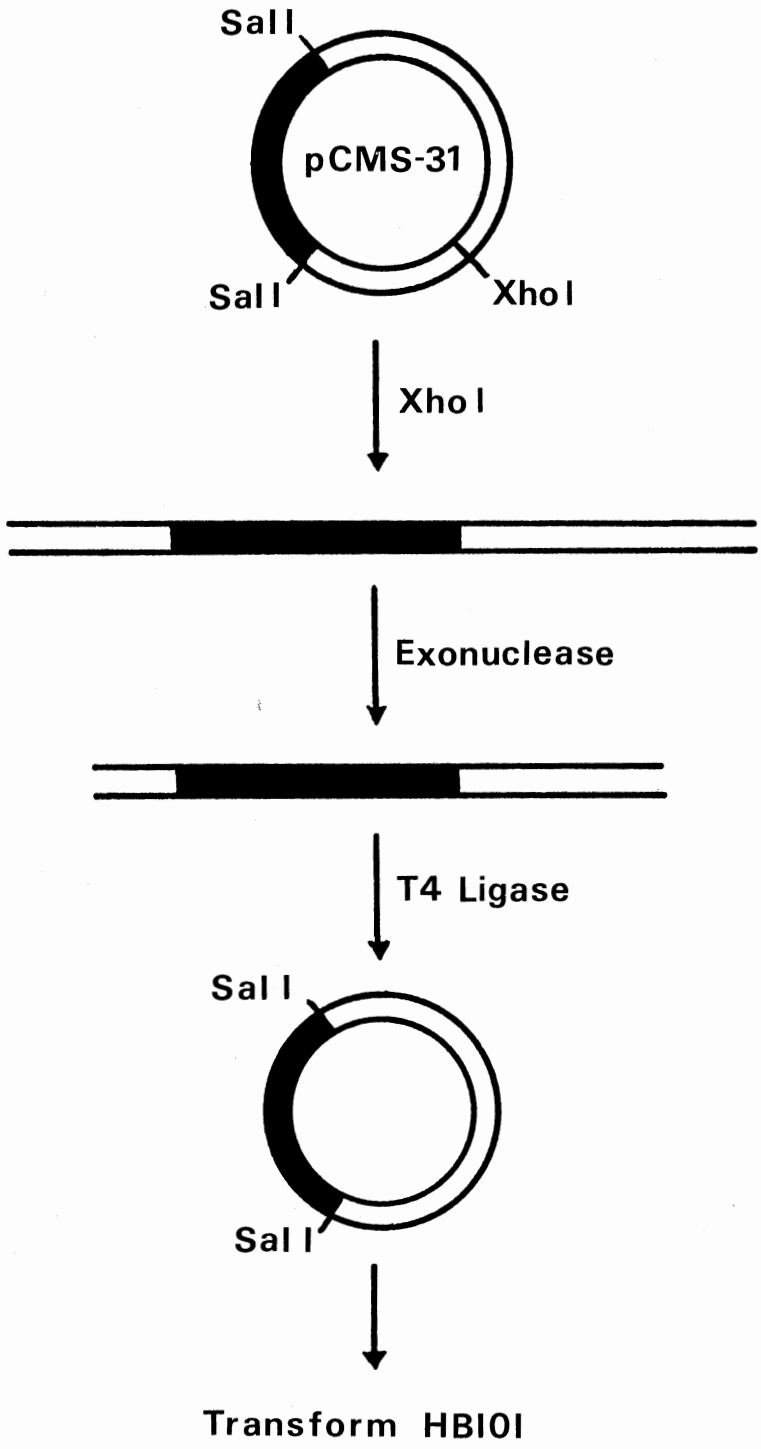
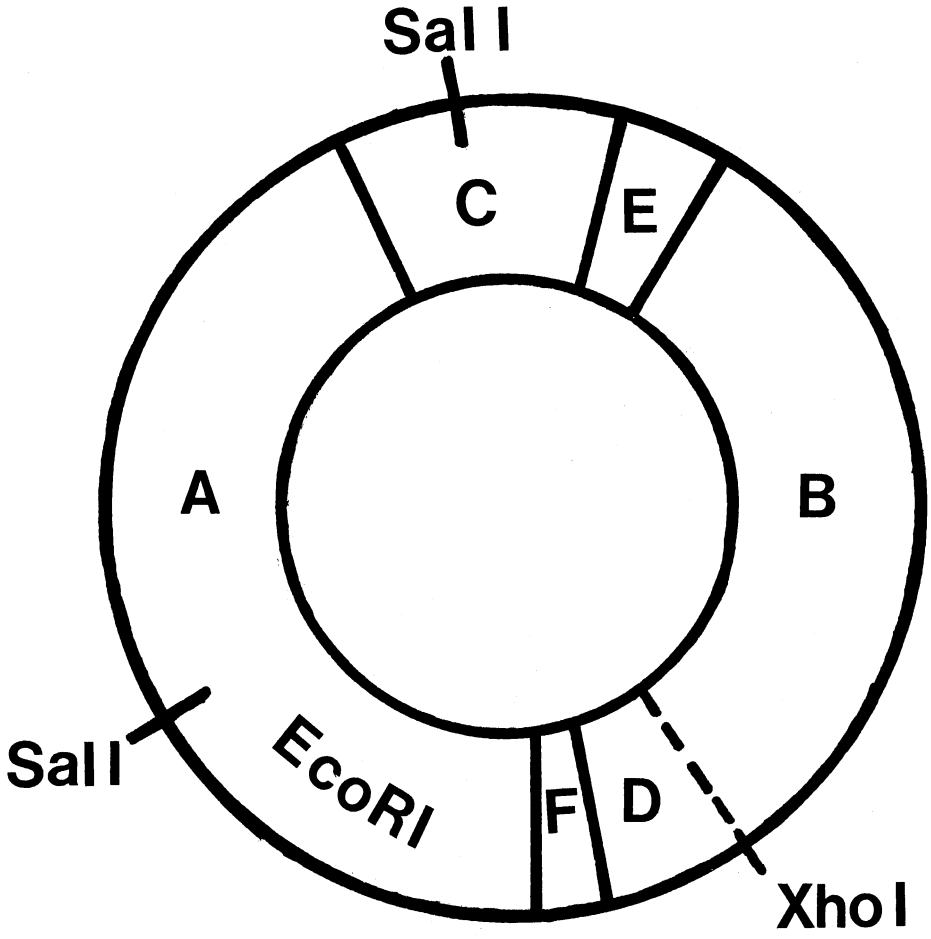

Sal I
pCMS-31
Sal I
Xho I
Xho I
Exonuclease
T4 Ligase
Sal I
Sal I
Transform HB101

Figure 3. XhoI-EcoRI Restriction Enzyme Map of pCMS-31.


Sal I
C
E
A
B
Sal I
EcoRI
F
D
Xho I

Fragments B, D, E, F and part of A and C are CaMV DNA. pBR322 DNA is located between the two SalI sites and is comprised of the remainder of fragments A and C. When XhoI-pCMS-31 (XhoI linearized pCMS-31) was treated with an exonuclease for various lengths of time and then digested with EcoRI; fragments B and D (which flank the XhoI site) can be seen to gradually decrease in size with time when analyzed on 1.0% agarose gels. The fragment sizes were estimated by extrapolation from a standard curve. The standard curve was prepared using EcoRI digested XhoI-pCMS-31 fragments as described in the Methods section. This procedure was used to assess the success of exonuclease digestion and to estimate the rate of digestion at various DNA and enzyme concentrations.

Exonuclease III

Initial attempts to create deletions in XhoI-pCMS-31 by synchronous digestion from each end were made using E. coli exonuclease III followed by S1 nuclease to produce blunt-ended molecules. Experiments using 6U exonuclease per µg DNA for 5 or 10 min at a DNA concentration of 0.10 µg/µl followed by EcoRI digestion gave inconclusive results. In each case, all EcoRI fragments appeared to be intact but the low molecular weight bands were either faint and poorly resolved, or imperceptible. Consequently, it could not be determined if the exonuclease was working at such a slow rate that no change was detectable or whether no digestion occurred.

When a DNA concentration of 0.05 µg/µl and 8U exonuclease per µg DNA was used for 1, 5, 10 and 15 min, gel electrophoresis of EcoRI digests showed a definite decrease in the size of fragments B and D

with time (Figure 4). The rate of digestion was estimated to be approximately 40-50 bp/min.

Bal-31

Digestion of XhoI-pCMS-31 with the double-stranded exonuclease, Bal-31, was also examined. The rate of digestion with Bal-31 was much faster than with exonuclease III since after only 30 sec of treatment appreciable digestion was detectable. Several concentrations of DNA were tested (0.02 μg/μl to 0.09 μg/μl) to find the optimum conditions. After treatment with Bal-31 for various times, the samples were digested with EcoRI and analyzed on 1.0% agarose gels. For each sample, the approximate size of the deletion and the rate of Bal-31 digestion per min were determined. The average rate of digestion with Bal-31 and the standard deviation were also calculated for the different DNA concentrations in each experiment. The results are given in Table III. The data revealed two important properties of Bal-31. First, the rate of digestion with Bal-31 is independent of the DNA concentration. This is best shown by comparing the average rates of digestion at different DNA concentrations. The rate of digestion is not significantly affected by increasing or decreasing the DNA concentration. In experiments one, two, and four the rate of digestion increases slightly with increasing DNA concentration but, in experiment three, the rate decreases with increasing concentration. Furthermore, the rate of digestion does not vary linearly or in any other consistent pattern with the change in DNA concentration. The standard deviation calculated for each average rate of digestion reveals that there was a large variability between

Figure 4. Gel Electrophoresis of Linear pCMS-31 Treated with Exonuclease III for Various Times Followed by EcoRI Digestion.

S) Standard-linear pCMS-31 digested with EcoRI,
A) Exonuclease III-15 min, B) Exonuclease III-10 min,
C) Exonuclease III-5 min, D) Exonuclease III-1 min,
incomplete EcoRI digestion.

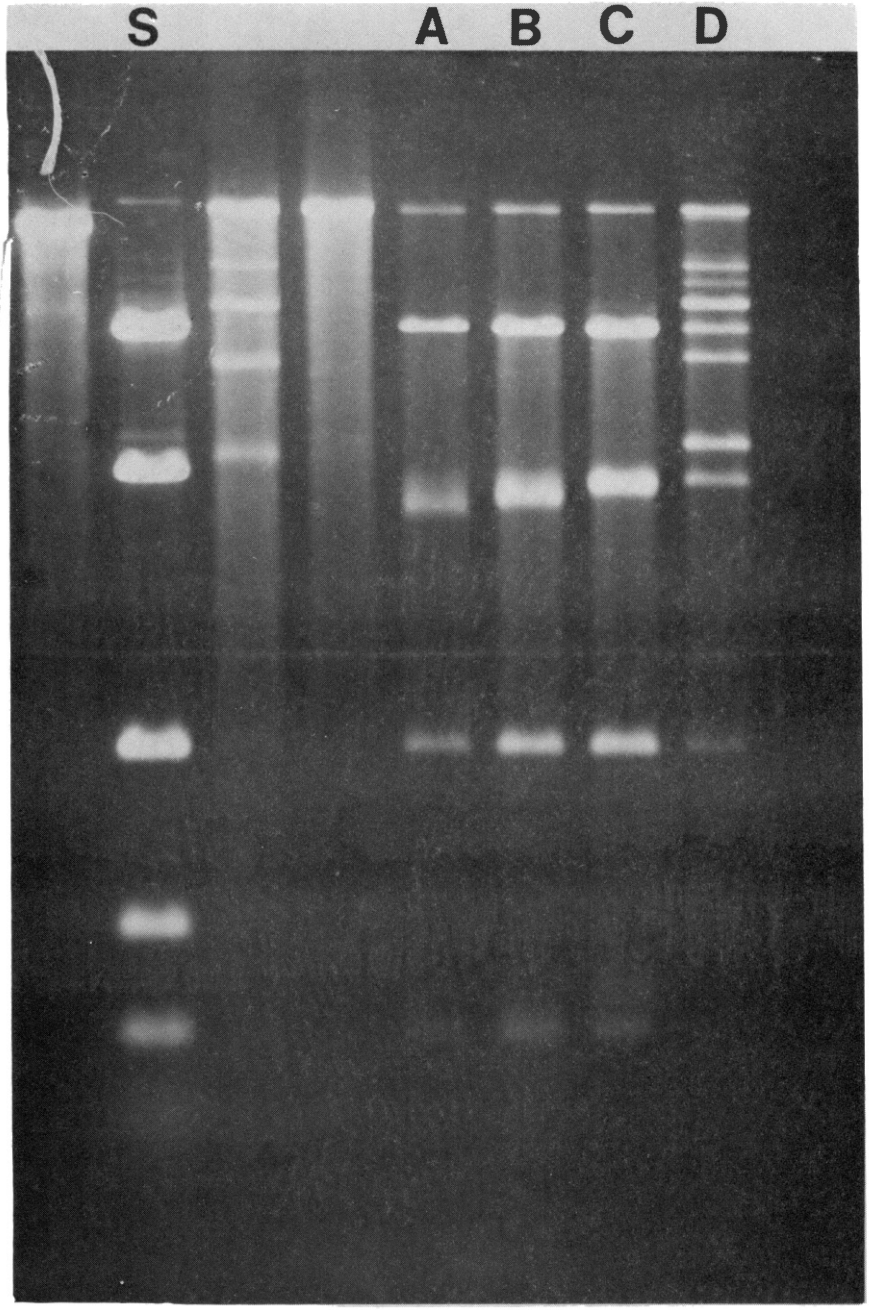

S
A
B
C
D

TABLE III

AVERAGE RATE OF BAL-31 DIGESTION AT
DIFFERENT DNA CONCENTRATIONS

DNA concentration (μg/μl)	Digestion Rate (bp/min)			
	Expt 1	Expt 2	Expt 3	Expt 4
.02 - .03	156 ± 31	—	—	88 ± 9
.05	—	301 ± 93	561 ± 170	98 ± 53
.08 - .09	—	—	394 ± 26	176 ± 19

Samples digested for different lengths of time using the same DNA and enzyme concentrations. This is probably due to inaccuracies in pipetting small amounts, or to timing and stopping reactions which were only a few seconds or minutes long.

A second conclusion drawn from the data is that the rate of Bal-31 digestion is dependent on the concentration of enzyme in the reaction mixture and also on the enzyme batch used. At the same enzyme concentration (1.0 U/μg DNA), the overall rate of digestion in experiment three is much faster than in experiments one and two. This can only be attributed to the fact that experiment three was done using a different batch of enzyme than used in experiments one and two. Experiment four was done using the same enzyme batch as experiment three. However, in experiment four, 0.5U enzyme/μg DNA was used as opposed to 1.0U/μg DNA in experiment three. The overall rate of enzyme digestion in experiment four is much less than in experiment three and, therefore, must be a result of using less enzyme.

Ligation and Transformation

To find the optimum conditions for blunt end ligation of deleted pCMS-31 molecules, sticky end ligations were done using XhoI-pCMS-31. A range of DNA concentrations (0.005 μg/μl to 0.10 μg/μl) and two T_4 DNA ligase concentrations (1.0U/μg DNA or 0.5U/μg DNA) were tested. The criterion used to determine the best ligation conditions was a decrease in the amount of linear DNA accompanied by an increase in higher molecular weight forms. The best range was found to be between 0.01 μg/μl and 0.03 μg/μl with 0.5U ligase/μg DNA. Higher DNA con-

centrations (0.07-0.10 µg/µl) gave more ligation, however the presence of several very high molecular weight bands suggested that concatamers were forming rather than open circles.

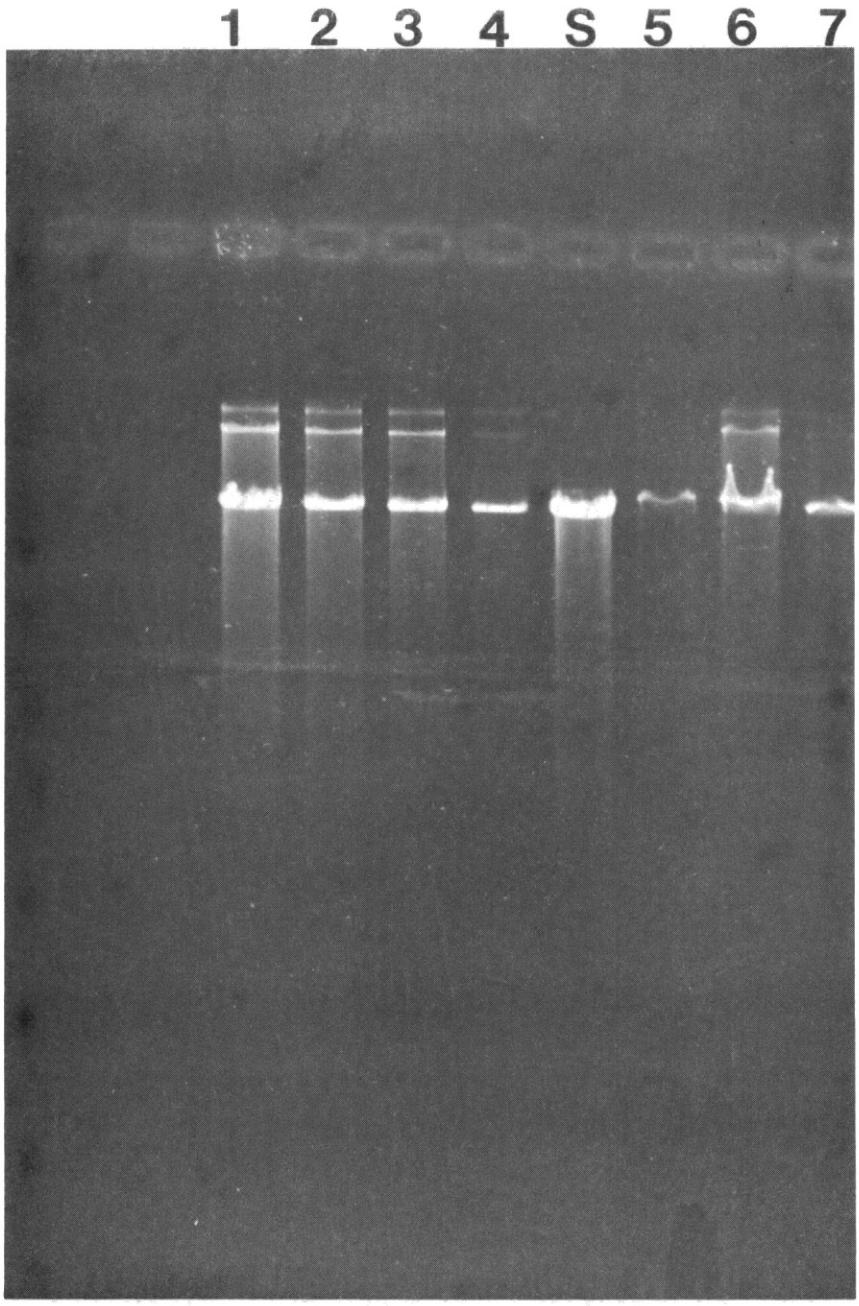
Blunt end ligations were tested using Bal-31 digested DNA at concentrations from 0.01 µg/µl to 0.05 µg/µl and either 10U or 5U of T_4 DNA ligase per µg DNA. A ten-fold excess of ligase was used for blunt end ligations because of the lower efficiency of this reaction (Sugino et al., 1977). When analyzed on an 0.8% agarose gel, each ligation reaction mixture showed three bands. The majority of the DNA was still in the linear form but there were two faint higher molecular weight bands. The migration of these two bands was identical regardless of the DNA or enzyme concentration (Figure 5). The two bands may have been concatamers but were probably not religated circular DNA since an attempt to transform competent HB101 cells with one of the reaction mixtures was unsuccessful.

Another experiment was done using a DNA concentration of 0.03 µg/µl and 5U ligase/µg DNA but only DNA in the linear form was seen. Transformation of HB101 with these reaction mixtures was successful. The number of transformants obtained for these reaction mixtures (30-40 colonies/0.5 µg DNA) was small compared to the standard (>300 colonies/0.5 µg pBR322 DNA). It is possible that the ligation reaction was so inefficient that only a small proportion of DNA was religated and thus, could not be detected on the gel. The success of all subsequent ligation reactions was tested by transformation of HB101 rather than by looking for evidence of ligation on a gel.

Single colony lysates were prepared from transformants and analyzed on 0.8% agarose gels. Because of the large size of the

Figure 5. Gel Electrophoresis of Blunt-end Ligation Products of Bal-31 Treated DNA Using Different DNA and T4 Ligase Concentrations.

S) Non-ligated Bal-31 treated DNA. DNA concentrations were: 1,5) 0.05 µg/µl, 2,6) 0.03 µg/µl, 3,7) 0.02 µg/µl, 4) 0.01 µg/µl. Ligase concentrations were: 1-4) 5U/µg DNA, 5-7) 10U/µg DNA.


1 2 3 4 S 5 6 7

pCMS-31 plasmid (about 12,000 bp), it is difficult to detect a deletion of less than 10% (1200 bp) by observing a change in plasmid size on an agarose gel. Therefore, single colony lysates were useful as a screening method only for eliminating from consideration plasmids with large deletions.

Deletion Plasmids

The six deletion plasmids isolated and characterized in this study were obtained from two separate experiments. The first group of deletion plasmids was created by digesting XhoI-pCMS-31 with Bal-31 (1U/μg DNA) for 45 sec or 90 sec. Analysis of EcoRI digests of the Bal-31 treated DNA indicated that the rate of exonuclease digestion was approximately 250-300 bp/min. Religation followed by transformation of HB101 with the ligation mixtures produced 30-40 colonies/0.5 μg DNA. Seventy single colony lysates were prepared and analyzed by agarose gel electrophoresis. The majority of the plasmids migrated much faster than the pCMS-31 plasmid and thus, probably had large deletions. Four plasmids that were fairly close in size to pCMS-31 were selected for further characterization. The four plasmids were designated pSA125, pSA126, pSA216 and pSA226. Although the estimated rate of Bal-31 digestion suggested that most of the DNA should have deletions less than 800 bp, the size of the plasmids obtained from colony lysates indicated that most of the deletions were greater than 2000 bp.

A second group of deleted plasmids was made to try and obtain plasmids with smaller deletions. In this case, Bal-31 digestion was carried out for 10 sec and 25 sec using 1U/μg DNA. EcoRI digests were

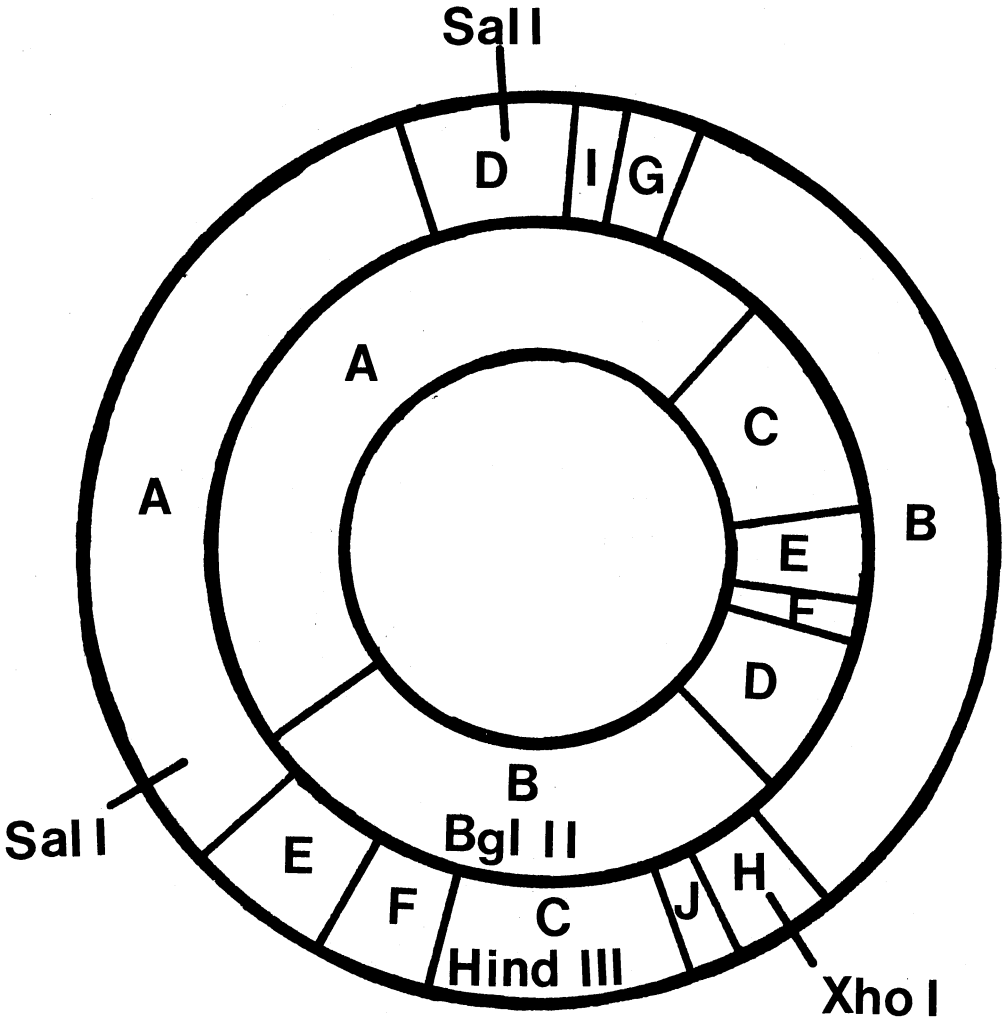
not done. Transformation of HB101 produced 4 colonies/0.5 µg DNA on the 10 sec digest plate and about 100 colonies/0.5 µg DNA on the 25 sec digest plate. Colony lysates were prepared from 30 colonies. All of the plasmids except one migrated the same as the standard, pCMS-31. Two plasmids were chosen at random for further characterization and were designated pSA102 and pSA103.

Mapping of Deletion Size

The restriction enzymes, Hind III and BglII, were chosen to map the size of deletion in each of the selected plasmids. Both enzymes cleave at several sites in pCMS-31 and give fragments in a size range that can easily be separated on agarose gels. A restriction enzyme map of HindIII-pCMS-31 and BglII-pCMS-31 was constructed (Figure 6). HindIII cleaves pCMS-31 into ten fragments of which B, C, E, F, G, H, I, J and part of A and D are CaMV DNA. pBR322 DNA lies between the two SalI sites and includes the remainder of fragments A and D. Electrophoresis of HindIII-pCMS-31 fragments on 1.0-1.5% agarose gels gives clear resolution of fragments A, B, C and D. Fragments E, F, G, and H do not give clearly separated bands, and fragments I and J migrate as a single band. BglII cleaves pCMS-31 into six fragments of which B, C, D, E, F and part of A are CaMV DNA. The remainder of fragment A which lies between the two SalI sites is pBR322 DNA. Electrophoresis of BglII fragments produces five well-resolved bands. Fragment F is too small to be detected on the agarose gels.

Deletions created by Bal-31 digestion would be expected to produce an altered restriction enzyme pattern for those fragments flanking

Figure 6. HindIII and BglII Restriction Enzyme Map of pCMS-31.


Sal I
D
I
G
A
C
A
B
E
F
D
B
Bgl II
Sal I
E
F
C
Hind III
J
H
Xho I

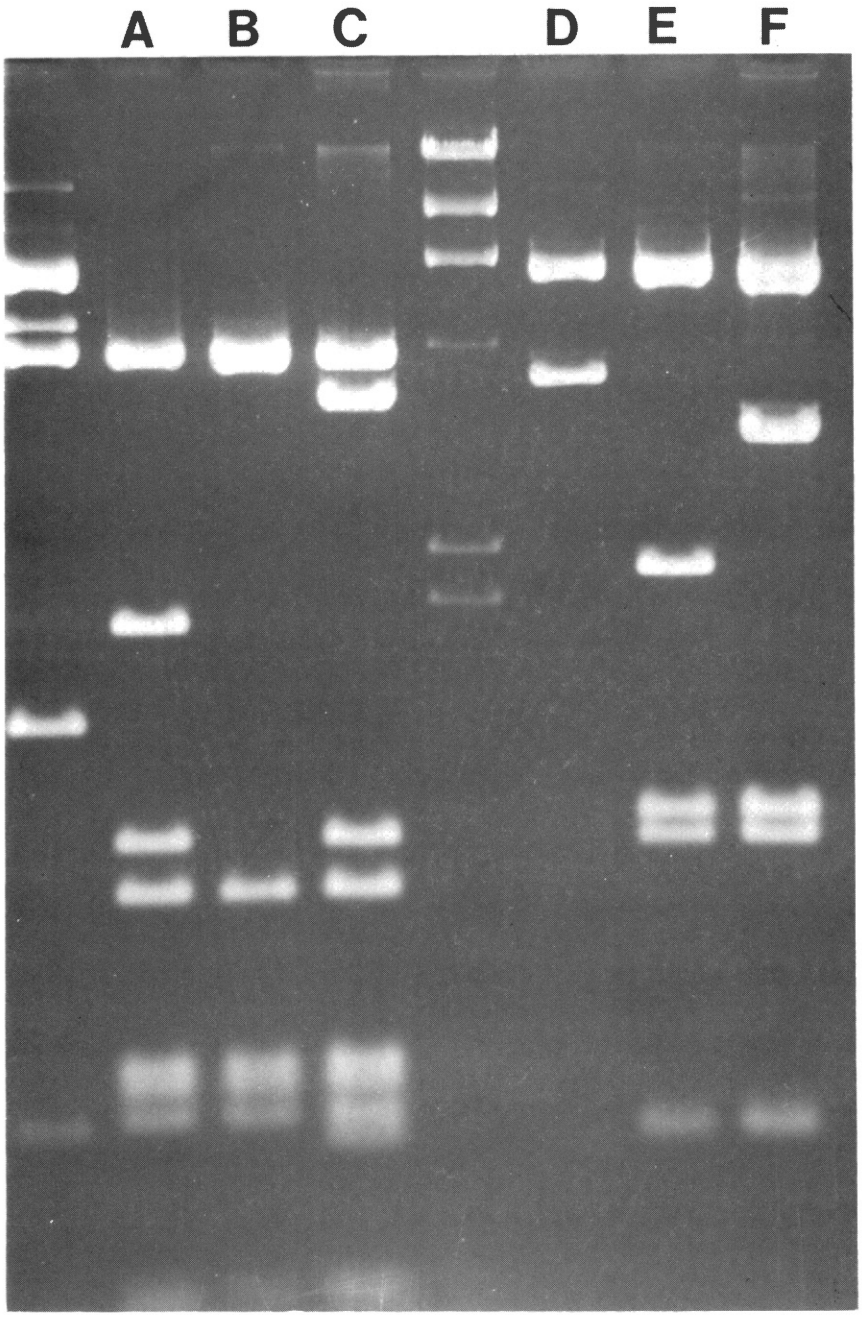
the XhoI site. The number of fragments affected depends on the extent of Bal-31 digestion. For HindIII digestion, fragments H, J, C and B are most likely to be deleted or reduced in size enough to alter the restriction enzyme pattern. For BglII, fragments B and D are most likely to be reduced in size and migrate faster on agarose gels. Thus, each deletion plasmid was digested with HindIII and BglII and the fragment pattern analyzed on agarose gels. The results obtained for each plasmid are discussed below.

pSA125 and pSA126

Deletion plasmids pSA125 and pSA126 were each digested with HindIII and BglII (Figure 7). BglII digested pSA125 gave a fragment pattern similar to the control (BglII digested pCMS-31) except that fragment B was smaller and had migrated to a lower position. A corresponding change in fragment D (as expected if synchronous digestion with Bal-31 had occurred) was not seen. HindIII digested pSA125 showed that fragments B, C, H and J (shown by the reduced staining intensity of the I-J band) were absent. The apparent loss of HindIII fragment B but not the corresponding BglII fragment D was explained after the amount of DNA deleted was determined. The deletion in pSA125 is primarily in the clockwise direction but enough DNA was deleted in the counterclockwise direction to eliminate the HindIII site bounded by fragments B and H. After religation, fragment B was fused to the remainder of C producing a new fragment nearly identical in size to A and thus, migrating with it on the gel. The deletion size in pSA125 was estimated to be approximately 1000-1100 bp. A deletion of this size primarily in the clockwise direction

Figure 7. Gel Electrophoresis of HindIII and BglII Digestion Products of pSA125 and pSA126.

A) HindIII digested pSA126, B) HindIII digested pSA125,
C) HindIII digested pCMS-31, D) BglII digested pSA126,
E) BglII digested pSA125, F) BglII digested pCMS-31.


A
B
C
D
E
F

would result in elimination of all of coding region III, at least 60% of region II and 30-40% of region IV.

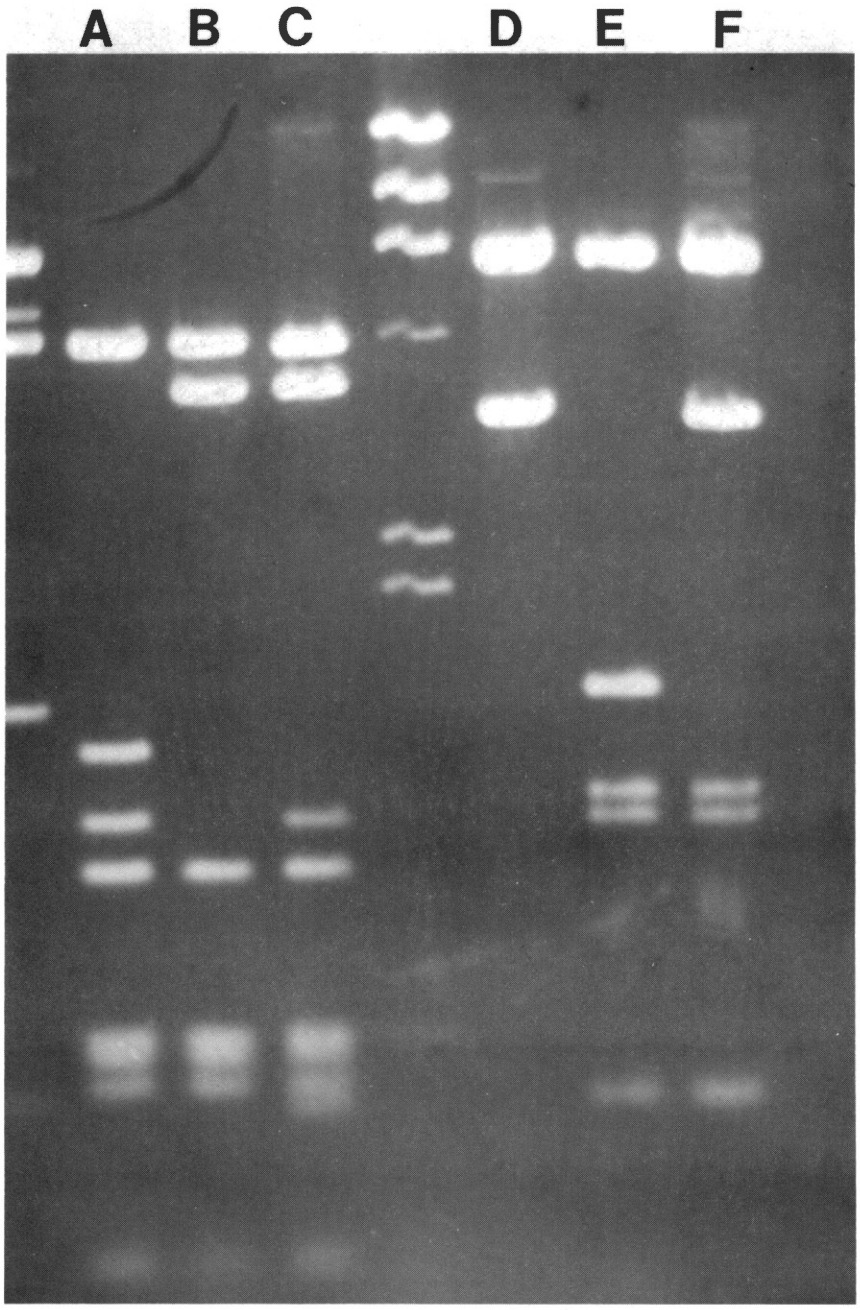
BglII digested pSA126 gave a fragment pattern quite different from the control. Fragments C, D and E were all absent and fragment B was now larger and migrated slower. Analysis of HindIII digested pSA126 showed that fragment H was absent and fragment B was much smaller as indicated by its faster migration. Taken together, these results indicate that, in pSA126, the deletion was primarily in the counterclockwise direction. The increased size of BglII fragment B results from the fusion of the remainder of fragments B and C. XhoI cleaves at a site in BglII fragment B that is about 340 bp from the BglII site bounded by fragments B and D. At least this much of B is eliminated by Bal-31. Digestion with Bal-31 may have occurred to some degree in the clockwise direction but not enough to eliminate the HindIII site bounded by fragments H and J. J appears to be intact since the staining intensity of the I-J band is not noticeably reduced as in HindIII digested pSA125. The deletion size in pSA126 was estimated to be about 1900-2100 bp. A deletion of this size in the counterclockwise direction would eliminate coding region I, 60-80% of the intergenic region, and at least half of region II. Digestion in the clockwise direction by Bal-31 was probably not more than 100-150 bp and therefore, should not affect region III.

pSA216 and pSA226

pSA216 and pSA226 were each digested with Hind III and BglII and the results are shown in Figure 8. BglII digested pSA216 gave a

Figure 8. Gel Electrophoresis of HindIII and BglII Digestion Products of pSA216 and pSA226.

A) Hind III digested pSA226, B) HindIII digested pSA216,
C) HindII digested pCMS-31, D) BglII digested pSA226,
E) BglII digested pSA216, F) BglII digested pCMS-31.


A
B
C
D
E
F

fragment pattern similar to BglII digested pSA125 except that fragment B in pSA216 was even smaller than in pSA125. This indicated that pSA216 had a deletion larger than pSA125. Analysis of HindIII digested pSA216 showed that fragments C and H were absent. Fragment J must also be absent, although the intensity of the I-J band did not seem reduced, since it is located between C and H. The estimated size of the deletion in pSA216 was 1700-1850 bp. In this case, the deletion was entirely in the clockwise direction. If there was any Bal-31 digestion in the counterclockwise direction, it must have been less than 130 bp since the HindIII site bounded by fragments H and B was still intact. This is somewhat different from what was observed with pSA125 where a small amount of Bal-31 digestion in the counterclockwise direction was detected. A deletion of 1700-1850 bp in the clockwise direction would eliminate coding region III and most of coding regions II and IV.

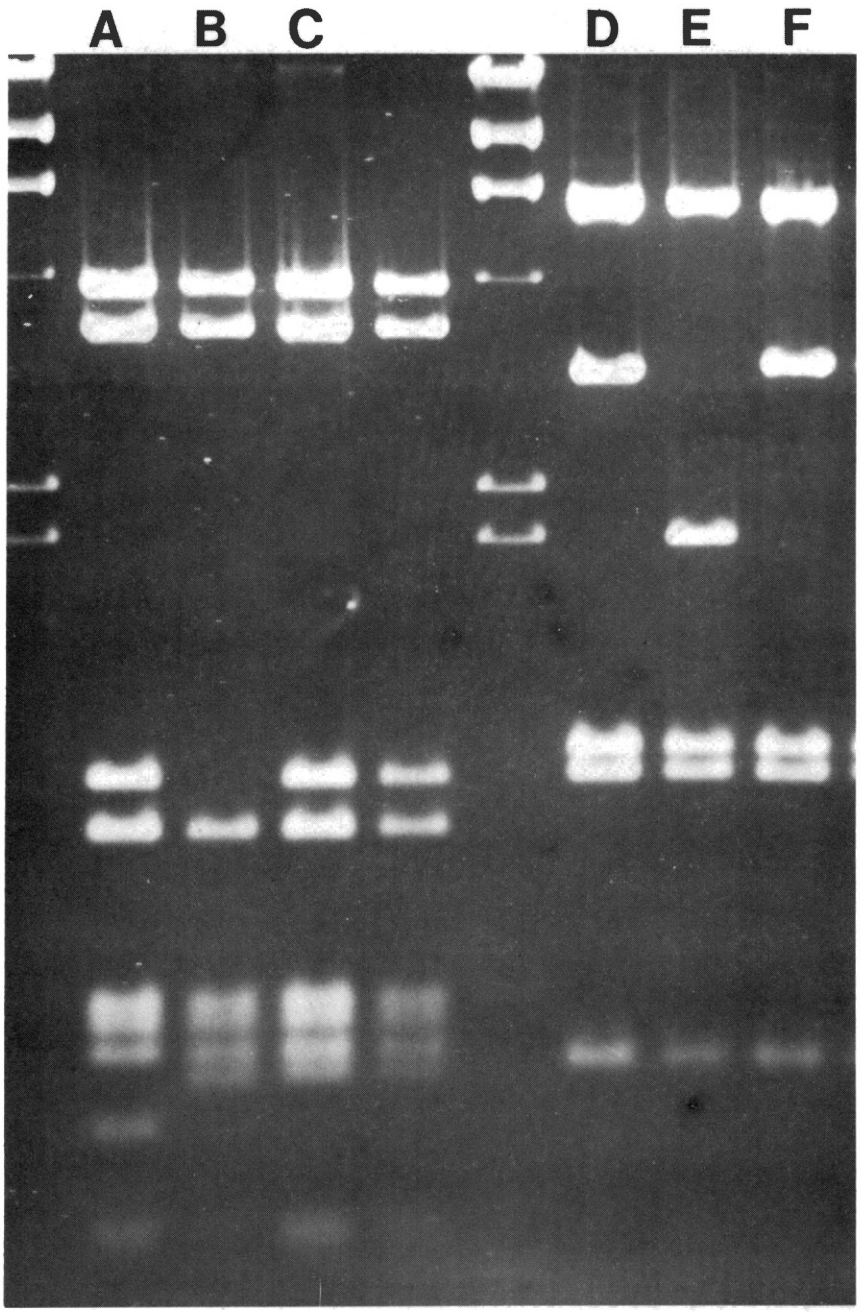
Analysis of BglII digested pSA226 showed that fragments C, D, and E were absent indicating there was a large deletion in the counterclockwise direction. HindIII digested pSA226 showed that fragment H was absent and fragment B was much smaller. The estimated size of the deletion was 2400-2500 bp. Again, no Bal-31 digestion was detectable in the clockwise direction. A deletion of this size would eliminate region I, the intergenic region, and a portion of regions II and VI.

pSA102 and pSA103

The results of the restriction enzyme digestion of pSA102 and pSA103 using BglII and HindIII are shown in Figure 9. The BglII

Figure 9. Gel Electrophoresis of HindIII and BglII Digestion Products of pSA102 and pSA103.

A) HindIII digested pSA103, B) HindIII digested pSA102,
C) HindIII digested pCMS-31, D) BglII digested pSA103,
E) BglII digested pSA102, F) BglII digested pCMS-31.


A
B
C
D
E
F

fragment pattern of pSA102 was similar to pSA125 and pSA216. Fragment B was smaller whereas the remaining fragments are unchanged. HindIII digestion shows only two changes in the fragment pattern: the loss of fragments C and J (shown by the reduced intensity of the I-J band). Fragment H appeared to be intact. Upon calculation of the deletion size, it could be shown that the remainder of fragment C fused to the remainder of fragment H would create a new fragment nearly identical in size to the intact fragment H. This new fragment was the one seen on the gel in the position of the H fragment. The deletion size was estimated to be 1100-1200 bp, all in the clockwise direction. The deletion is similar to that in pSA125 except there is no evidence for any Bal-31 digestion in the opposite direction as occurred with pSA125. Again, this type of deletion eliminated all of region III and part of regions II and IV.

BglII digested pSA103 showed no obvious changes in fragment pattern from BglII digested pCMS-31. However, after careful measurement of band migration distances, fragment B was determined to be slightly smaller. Digestion of pSA103 with HindIII showed one small change. Fragment H was noticeably smaller. The estimated size of the deletion was 70-75 bp and affected only coding region II.

Analysis of the Ability of Deletion Mutants to Replicate and Induce Infection in Turnip Plants

Each of the six deletion mutants of CaMV DNA were tested for their ability to replicate and produce infection in turnip plants. Controls used in each experiment were NY8153 virus, CaMV (isolate

NY8153) DNA, and pBR322 DNA. After recombinant plasmids were digested with SalI to release the CaMV DNA portion, the reaction mixtures were diluted to a final DNA concentration of 10 μg/ml. Each DNA or virus sample was inoculated onto two four-week old turnip plants. Leaves were rinsed with water from a spray bottle after inoculation to prevent DNA sticking to the leaves. Disposable gloves were worn and changed between each sample inoculation to prevent cross-contamination. After two weeks, 2 or 3 inoculated leaves were removed from each plant and processed for starch staining and LSH.

Starch staining allows for detection of starch lesions which are analogous to the chlorotic local lesions produced by infection with CaMV. Starch lesions are identified as small unstained circles in the dark purple background of the stained leaf.

LSH is a method whereby viral nucleic acid can be detected in leaves by molecular hybridization with radioactive homologous DNA. The viral nucleic acid in infected leaves appears as dark circles on autoradiographs of leaf skeletons. The autoradiographic spots correspond closely to starch lesions. The presence of one or two small isolated spots or large irregular areas of darkening on leaf autoradiographs can not always be taken as positive evidence of viral nucleic acid replication. The criterion for a positive result in this study was the presence of more than two dark circular areas or a high background of radioactivity throughout the leaf. Leaves that show no hybridization to the probe DNA have only enough low level background radioactivity to produce a faint leaf outline.

Deleted CaMV DNA from pSA125, pSA126, pSA216 and pSA226

Deleted CaMV DNA excised from the plasmids pSA125, pSA126, pSA216, and pSA226 was inoculated onto turnip plants. The plants used in this experiment suffered some leaf damage possibly from roaches in the growth chamber prior to and after inoculation. Two weeks after inoculation, 2 or 3 leaves were removed from each plant and stained with KI_3. Staining, in general, was poor. Many leaves stained only partially or not at all thus, hampering detection of starch lesions. Four NY8153 inoculated leaves stained well enough to detect many lesions but CaMV DNA inoculated leaves stained so poorly that no lesions could be seen. No lesions were seen on pBR322 DNA inoculated leaves. Staining of leaves inoculated with deleted CaMV DNA from pSA125, pSA126, pSA216 and pSA226 varied from poor to moderate. No lesions were detected on any of these leaves.

Starch staining was followed by LSH. Based on the criterion stated above, the results of LSH in this experiment are summarized in Table IV. The absence of starch lesions and any evidence for hybridization on leaf skeletons of leaves inoculated with CaMV DNA from pSA125, pSA126, pSA216 and pSA226 indicated that replication of these mutant viral DNAs was not occurring in the plants. Furthermore, since the overall condition of the plants inoculated with these deleted DNAs was good, no systemic infection study was done.

Deleted CaMV DNA from pSA102, pSA103, and pSA125-pSA126

A second experiment was done using deleted CaMV DNA from plasmids

TABLE IV

RESULTS OF LSH ON TURNIP LEAVES

Inoculum	leaves +/total	
	Expt 1	Expt 2
NY8153	5/6	1/3
CaMV DNA	2/5	2/4
pBR322	0/4	0/3
pSA125	0/6	-
pSA126	0/6	-
pSA216	0/5	-
pSA226	0/6	-
pSA102	-	0/4
pSA103	-	2/4
pSA125 + pSA126	-	0/4

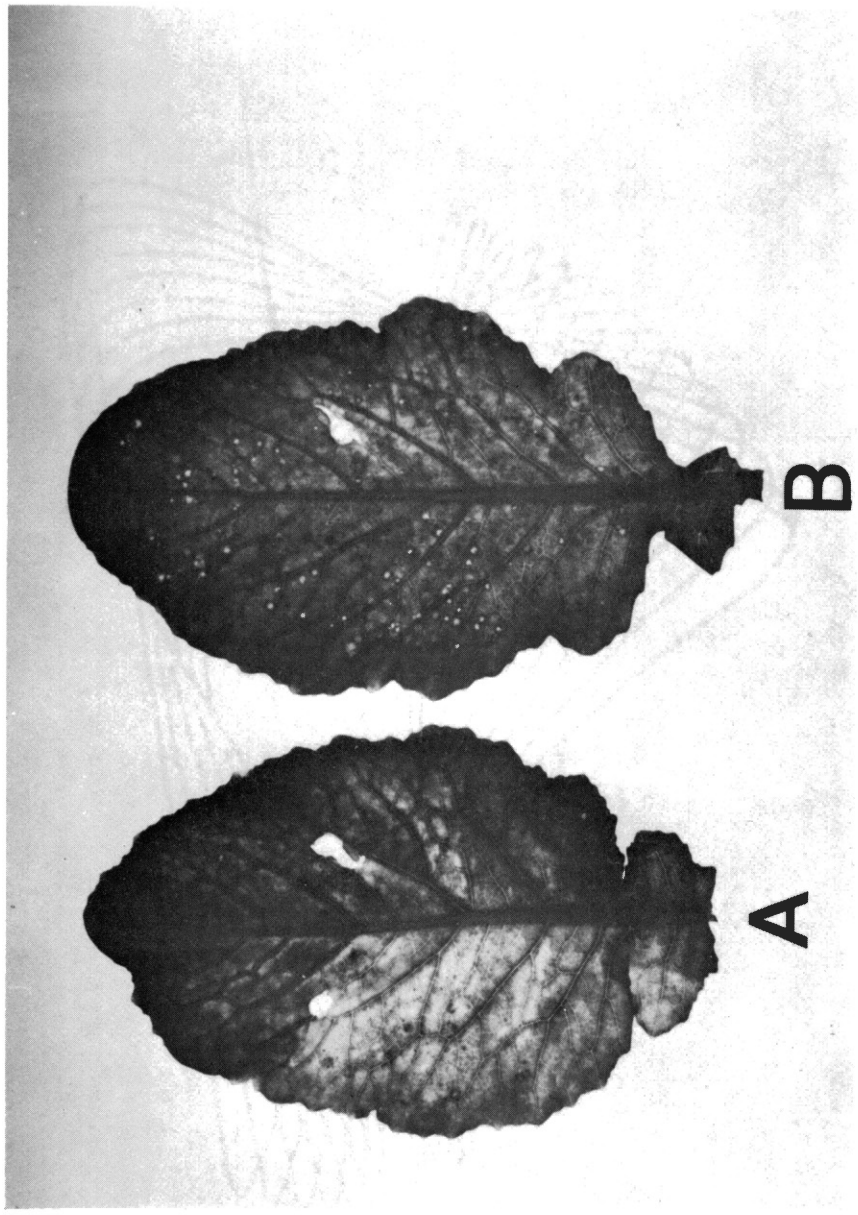
pSA102, pSA103, pSA125 and pSA126. DNA from pSA125 and pSA126 was mixed in equal amounts to a final DNA concentration of 10 μg/ml and inoculated onto turnip plants. Since these two DNA molecules had deletions in different regions, it was of interest to determine if they could rescue each other by complementation or recombination. Plants used in this experiment also had some damage possibly from roaches prior to inoculation. At the end of ten days, two leaves were removed from each plant and subjected to starch staining and LSH. Again, starch staining of leaves varied from good to poor. NY8153 inoculated leaves had many clear lesions on all leaves. CaMV DNA inoculated leaves had good lesions on one leaf but there were no obvious lesions on the others. Leaves inoculated with either pBR322 DNA or deleted CaMV DNA from pSA102 were all negative. Out of four leaves inoculated with deleted DNA from pSA103, one had 7 to 12 good lesions (Figure 10). The other three leaves had no definite lesions. Leaves inoculated with the mixture of deleted DNA from pSA125 and pSA126 had no apparent lesions.

The results of LSH in this experiment are summarized in Table II. Leaves inoculated with DNA from pSA102 or the mixture of DNA from pSA125 and pSA126 were all negative. However, two leaves inoculated with DNA from pSA103 did show positive hybridization indicating that replication of this deleted viral DNA was occurring.

After three additional weeks, plants were observed for evidence of systemic infection. Both NY8153 and CaMV DNA inoculated plants were systemically infected as shown by the mottled, wrinkled appearance of the leaves and the stunted condition of the plants. Plants inoculated with deleted DNA from pSA103 were also systemically

Figure 10. Turnip Leaves Stained with KI_3 for Detection of Starch Lesions.

A) Leaf inoculated with deleted CaMV DNA from pSA103,
B) Leaf inoculated with NY8153 virus.


A
B

infected. However these plants did not appear to be as advanced in infection as those of NY8153 and CaMV DNA since they still had some large older leaves and were less stunted. Of two plants inoculated with DNA from pSA102, one was healthy and the other was obviously infected. The reason for the systemic infection in this plant was unknown since the leaves had no apparent starch lesions and were negative for LSH. Plants inoculated with DNA from pSA125 and pSA126 were healthy.

Because of the unexplained infection by deleted DNA from pSA102, an experiment to retest this DNA and deleted DNA from pSA103 and pSA125 was done. After two weeks, plants were inoculated with NY8153 or CaMV DNA were already showing signs of infection. However, plants used in this experiment were not very healthy. Many leaves had died or had pink or yellow edges after only two weeks. This can probably be attributed to lack of fertilizer. Staining of leaves was excellent but only those leaves inoculated with NY8153 or CaMV DNA showed lesions. LSH was unsuccessful due to a bad batch of ^{32}P-labeled CaMV DNA used as the probe. After five weeks, plants were observed for systemic infection. Plants inoculated with DNA from pSA102 and pSA125 were healthy. Plants inoculated with DNA from pSA103 were systemically infected but again the infection was less advanced than in the positive controls.

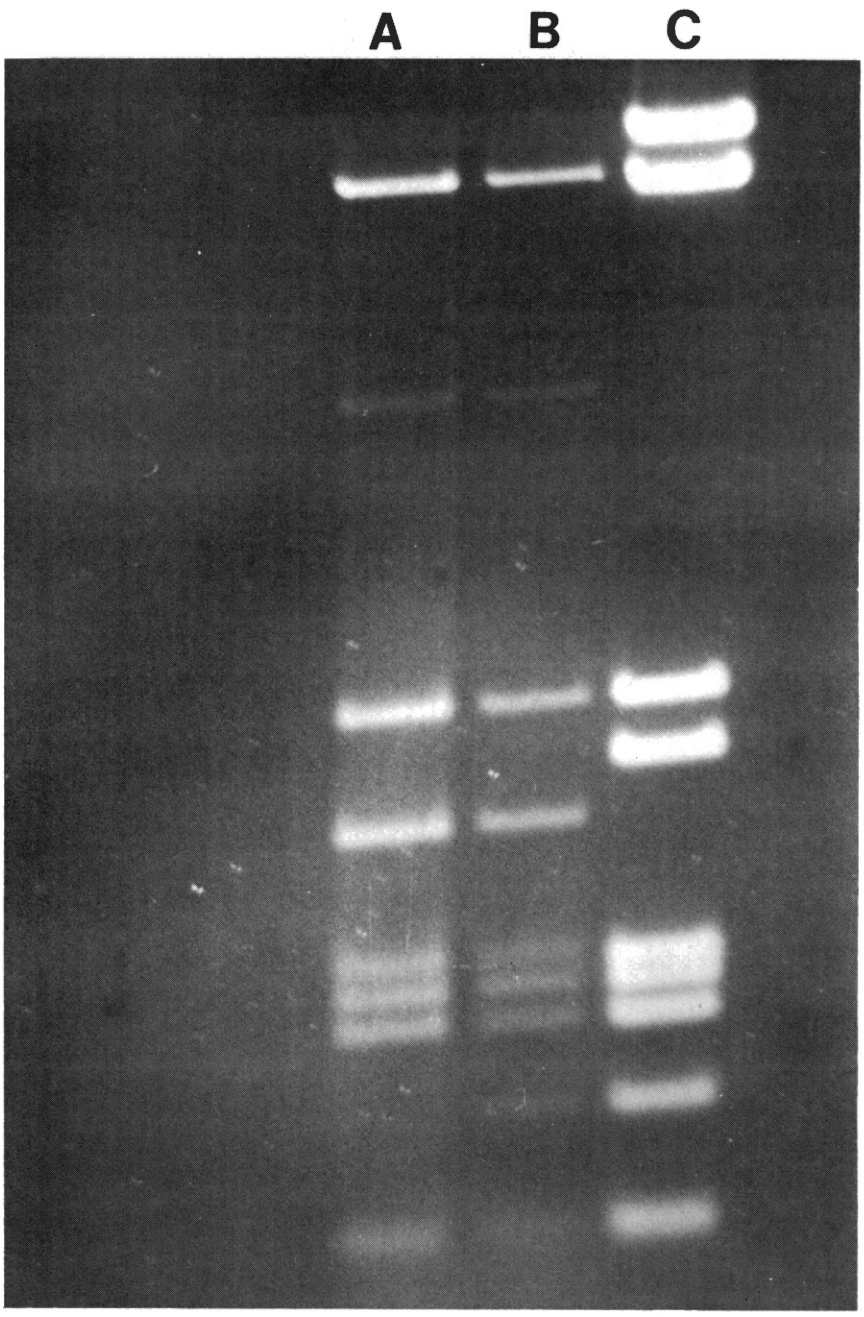
Analysis of Viral DNA from Plants Inoculated with Infectious Mutant CaMV DNA

To assure that the mutant CaMV DNA from pSA103 was producing the infection in turnip plants and not a contaminant or reverted form,

viral DNA was isolated from systemically infected leaves. Leaf tissue (3.5 g) infected with mutant CaMV from pSA103 was weighed out and processed as described in the methods section. Approximately 7.0-8.5 μg DNA/μg of tissue was recovered according to A_{260} measurements. However, the actual amount was probably somewhat less. When visualized on an agarose gel, the faintness of the bands indicated that there was less DNA present than calculated. The isolated mutant viral DNA was digested with HindIII and analyzed on an agarose gel. Controls used on the gel were HindIII digested pSA103 DNA and HindIII digested CM4-184 viral DNA. Figure 11 shows that the viral DNA isolated from the infected plants had essentially the same fragment pattern as DNA from pSA103, the only differences being those fragments in pSA103 that are changed due to the pBR322 portion of the plasmid. It is clear that the altered size of HindIII fragment H is preserved in the viral DNA of plants infected with CaMV DNA from pSA103. This was positive proof that the small deletion in region II of the CaMV DNA did not interfere with the ability of the DNA to replicate and produce infection in turnip plants.

Figure 11. Gel Electrophoretic Analysis of HindIII Digestion Products of Viral DNA Isolated from Plant Infected with pSA103.

A) HindIII digested CM4-184 viral DNA, B) HindIII digested viral DNA from plant infected with pSA103, C) HindIII digested pSA103 plasmid DNA.


A
B
C

CHAPTER IV

DISCUSSION

Six deletion mutants of CaMV DNA were isolated and characterized. The results are summarized in Table III. Of the six mutants isolated, only one, pSA103, was still able to replicate and produce infection in turnip plants. pSA103 had a small deletion of approximately 70-75 bp which affected only coding region II in the CaMV DNA. Several turnip leaves inoculated with the mutant DNA from pSA103 gave positive results when examined for starch lesions and hybridization spots. Inoculated plants also became systemically infected although the appearance of symptoms was slightly delayed compared to positive control plants. The reason for this is unknown. These results show that region II is not essential for the viral DNA to replicate and produce infection in plants. This conclusion is supported by other evidence. Gronenborn et al. (1981) showed that small amounts of foreign DNA could be inserted into and propagated at the XhoI site. In addition, a natural variant of the virus, CM4-184, has nearly all of region II deleted and is still infective (Howarth et al., 1981). Alkaline gel electrophoresis indicates that the single-stranded gap region just counterclockwise to the XhoI site has also been lost in the mutant viral DNA isolated from infected leaves (U. Melcher, unpublished observations).

Three mutants, pSA125, pSA216, and pSA102, had large deletions

TABLE V

CHARACTERISTICS OF DELETION MUTANTS OF CaMV

Deletion Plasmid	Deletion Size (bp)	Coding Regions Affected	Starch Lesions	LSH	Systemic Symptoms
pSA125	1000-1100	II,III,IV	-	-	-
pSA126	1900-2100	I,II,Intergenic	-	-	-
pSA216	1700-1850	II,III,IV	-	-	NT*
pSA226	2400-2500	I,II,Intergenic,VI	-	-	NT
pSA102	1100-1200	II,III,IV	-	-	-,(+)
pSA103	70-75	II	+	+	+

*Not tested.

primarily in the clockwise direction. The CaMV DNA coding regions affected by these deletions were II, III and IV. The role of the coat protein (region IV) in replication and infection is not known. However, if packaging of viral DNA is a prerequisite for cell to cell transport, a deletion in region IV may block systemic infection. Since there was no conclusive evidence using LSH that these mutant DNAs were replicating in turnip leaves, it can be assumed that an essential function necessary for DNA replication was destroyed. During the course of this investigation, evidence that showed region III to be an essential region for viral infection was published (Lebeurier et al., 1982).

Two other mutants, pSA126 and pSA226, had large deletions in the counterclockwise direction. The coding regions affected by these deletions were I, II, the intergenic region and, in pSA226, region VI. Although the functions of region I and the intergenic region are unknown, at least one of these must play some role in DNA replication since leaves inoculated with these mutants were negative for starch lesions and LSH. Howell et al. (1981) attempted to insert small pieces of foreign DNA into both region I and the intergenic region. The insert in region I destroyed the infectivity of the viral DNA, but the insert in the intergenic region did not. The insert in the intergenic region was just clockwise to the single-strand interruption in the α strand. Due to the extent of the deletions in pSA126 and pSA226 the single-stranded interruption must have been deleted. This gap (the only one in the α strand) may be important in initiation of transcription or replication.

Coinfection with the two mutant viral DNAs from pSA125 and

pSA126 was unsuccessful. Howell et al. (1981) and Lebeurier et al. (1982) have shown that when two viral genomes with deletions or foreign DNA insertions at different sites are used to coinfect plants, recombination may occur between the two mutant DNAs producing a normal CaMV DNA molecule. The inability of deleted DNA from pSA125 and pSA126 to coinfect plants may be due to the fact that the deletions overlap at one end making recombination difficult.

The aim of this study was to determine how much of region II and its flanking regions could be deleted and retain infective viral DNA. It has been shown that region II itself is nonessential but whether regions I or III are essential can not be determined from the results of this study. However, it seems possible that at least two functions are required for DNA replication. In each mutant (except pSA103 and pSA226) two regions clockwise or counterclockwise from region II are deleted. Regardless of the direction of the deletions, the mutant CaMV DNA is not infective. Thus, at least one of these regions on each side of region II must play some role in viral DNA replication.

The original experimental plan called for creating deletions in XhoI linearized pCMS-31 using an exonuclease that synchronously degrades the DNA from each end. Bal-31 was chosen for use over exonuclease III and S1 nuclease because it eliminated the extra S1 nuclease step, did not require altering the pH extensively, and seemed to work faster. EcoRI digests of Bal-31 treated molecules indicated that digestion was occurring down each end of the linear molecule. However, the only religated transformants obtained had either no deletion or had deletions almost entirely in one direction

or the other. In addition, the efficiency of the ligation was very poor and religated circular molecules could not be seen on gels. The primary reason for these problems now appears to be that the Bal-31 enzyme concentration was much too high. In each experiment, an enzyme to DNA concentration ratio was used to determine the amount of Bal-31 used. The volume of these reaction mixtures was small enough that the total enzyme concentration in the sample was high. The amount of Bal-31 used should have been based on the concentration of enzyme in the reaction mixture regardless of the DNA concentration. It is probable that Bal-31 digestion did occur synchronously down each end of the linear molecule as the EcoRI fragment pattern suggests and that only a small population of DNA molecules undetected on gels was degraded primarily from one end. Landick (1982) mentions that a Bal-31 concentration of greater than 1 U/ml seems to leave nonligatable ends. Since the enzyme concentrations used in these experiments were greater than 1 U/ml, this may explain the low ligation efficiency. Why the unidirectionally deleted molecules were able to religate but not the bidirectionally deleted molecules is unknown. Although the Bal-31 conditions were incorrect for optimal ligation, mutants with unidirectional deletions were able to provide some answers to the problem addressed in this study.

Three techniques were utilized to determine if the six deletion mutants of CaMV were able to replicate and infect turnip plants. Care was taken to minimize the variability between plants and experiments by using plants of the same age, inoculated and grown under the same conditions. Staining of starch in inoculated leaves was

not always successful, thus preventing accurate detection of lesions on some leaves. The reason for poor staining of starch is the lack of starch reserves in the leaves. The absence of starch may be due to any one of a variety of factors such as leaf age, poor soil fertility, or high temperatures in the growth chamber.

LSH was used as a further test of the ability of the mutant CaMV DNAs to replicate in leaves. Results, in some cases, were difficult to interpret objectively due to the presence of occasional isolated spots and dark patches on autoradiographs of control and mutant DNA inoculated leaves. Therefore, criteria were adopted for deciding which leaves could be considered positive. It is possible that some positive results may not have been counted. However, leaves that had starch lesions and/or later became systemically infected were definitely positive with LSH. The only exception to this was the one systemically infected plant inoculated with DNA from pSA102 which had no starch lesions or hybridization spots and was considered to be a false positive. In the remaining cases, plants with no apparent starch lesions had only occasional or no hybridization spots and did not become systemically infected. Although there were some problems with each of the tests used, the results of all three taken together gave fairly conclusive answers concerning the infectivity of the mutant viral DNAs.

The retention of infectivity by the mutant CaMV DNA from pSA103 poses an interesting question. What, if any, gene function has been disrupted by the deletion? As previously mentioned, it has been speculated that region II may encode an aphid acquisition factor that allows the virus to be transmitted from plant to plant by aphids

in a stylet-born manner (Howarth et al., 1981). It has been shown that non-aphid-transmissible isolates of the virus may be transmitted if the aphids are first allowed to probe plants infected with a transmissible isolate. If the aphids probe leaves infected with a non-transmissible isolate first, and then plants infected with a transmissible virus, only the latter is transmitted by the aphids. These findings suggest that an aphid acquisition factor produced by the virus or by the infected plant is the agent which allows the aphids to acquire the virus (Lung and Pirone, 1974). Preliminary results of aphid acquisition studies using the mutant DNA from pSA103 indicate that the virus is no longer transmissible by aphids (T. P. Pirone, unpublished results). Whether this is due to the loss of the gene product from region II, destruction of the gap region, or a low concentration of the mutant virus in infected leaves has not yet been determined. The second explanation does not seem plausible since one non-transmissible isolate, CM1841, has an intact gap region. The third explanation remains to be tested.

Although it would have been desirable to obtain additional mutants with smaller deletions affecting regions I and/or III, some information has been gained from the mutants isolated. Additional work will need to be done to determine exactly what the roles of region II and the other coding regions are.

CHAPTER V

SUMMARY

Mutants of CaMV DNA were constructed that had deletions in coding region II and its flanking regions. The mutants were created in an attempt to determine the amount of DNA that could be eliminated and still retain infective viral DNA that would replicate and induce infection in turnip plants.

pCMS-31 is a recombinant plasmid containing a full length copy of the viral DNA (isolate NY8153) cloned into the bacterial plasmid, pBR322, via their unique SalI sites. Mutants of the CaMV DNA were made by first digesting with XhoI which cuts once in region II of the CaMV DNA portion of the plasmid producing a linear molecule. The linear molecule was then treated with the double-stranded exonuclease, Bal-31, and the deleted plasmids were religated and used to transform HB101 cells. Six transformants were selected for further study. Three deletion plasmids had large deletions in the clockwise direction and affected coding regions II, III, and IV. Two mutants had large deletions in the counterclockwise direction and affected coding regions II, I, the intergenic region, and, in one of these, region VI also. None of these deletion mutants showed evidence of replication or infection when inoculated onto turnip plants. The sixth deletion plasmid, pSA103, had only a small deletion of 70-75 bp in region II. This mutant gave positive results when examined for starch lesions

and hybridization spots using LSH; and inoculated plants became systemically infected. These results show that region II is a nonessential region of the viral DNA. A possible function for this region is discussed in Chapter IV. The role or importance of other deleted coding regions in viral DNA replication and infection could not be ascertained from this study.

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