REGULATION OF CAULIFLOWER MOSAIC VIRUS GENE EXPRESSION

By

STEVEN D. HARTSON

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REGULATION OF CAULIFLOWER MOSAIC VIRUS

GENE EXPRESSION

Thesis Approved:

Ulrich K. Melcher
Thesis Advisor

Earl D. Mitchell Jr.

Franklin R. Leach

J. R. Sherwood

Robert J. Matts

Thomas C. Collins
Dean of the Graduate College

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LIST OF SYMBOLS

A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
ACMV	African cassava mosaic virus
appr.	approximate(ly)
bp	base pair(s)
ca.	about
CaMV	cauliflower mosaic virus
Chpt	chapter
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DHFR	dihydrofolate reductase
DTT	dithiothreitol
FDA	flourescein diacetate
FMV	figwort mosaic virus
GTPC	guanidinium thiocyanate / phenol chloroform
^3H	tritium
HBV	hepatitus B virus
Kb	kilobase(pairs)
mA	milliampres
MES	2[N-morpholino]ethanesulfonic acid
mRNA	messenger RNA
MW	molecular weight

nt	nucleotide(s)
OAc	acetate
ORF	open reading frame
^{32}P	radioisotope of phosphorus
rRNA	ribosomal RNA
RT	room temperature
PEG	polyethylene glycol
PolyA$^+$	polyadenylated
PolyA$^-$	nonpolyadenylated
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
tRNA	transfer RNA
TMV	tobacco mosaic virus
TuMV	turnip mosiac virus
TVCV	turnip vein-clearing virus
TYLCV	turnip yellow leaf curl virus
U	unit(s)
UV	ultraviolet
V	volts
v	volume
X	times or fold

CHAPTER I

INTRODUCTION

Interactions with the Host

Because viruses utilize host machinery to replicate, they are used to investigate the processes and mechanisms which occur within the cells of their hosts. Viruses provide simplified systems with which complicated processes can be studied. Additionally, the control of viral disease may be facilitated by an understanding of the biology of the offending agent. The potential benefits of pathogen control validate the pursuit of this understanding. One such pathogen which infects plants, cauliflower mosaic virus (CaMV), is introduced below.

CaMV is the type virus for the caulimovirus group. CaMV is a double-stranded DNA virus which infects members of the Crucifer family and some members of the Solanaceous family (Hills and Cambell, 1968; Lung and Pirone, 1972; Schoelz et al., 1986). Infection is transmitted by aphids in a semi-persistent manner; the virus is stylet-borne (reviewed by Hull and Covey, 1982). CaMV can also be transmitted via mechanical inoculation with virions, viral DNA, or cloned viral DNA.

In compatible hosts, cell-to-cell transfer and long distance transport lead to a systemic infection. Most or all of the cells in a systemically infected leaf contain CaMV virions (reviewed in Maule, 1985b). During infection, irregular inclusion bodies are produced in the cytoplasm of infected cells. These viroplasms may be vacuolated and contain large numbers of virions; viroplasms may also be evacuolate and contain no virions (Shepherd et al., 1980). On turnips, a commonly used host, symptoms of systemic infection include

chlorotic lesions, mosaic patterning, leaf wrinkling, stunting, and vein clearing. Systemic symptoms usually appear within 12-15 days, becoming full blown within 21-25 days (Lebeurier et al., 1980).

Physical Properties of the Virion

The CaMV virion is thought to be a 50-nm diameter icosahedron with a hollow core (Shepherd, 1970; Hull et al., 1976; Chauvin et al., 1979; Kruse et al., 1987). The primary component of the virion shell is a protein with an apparent molecular weight of 44 kD (Al Ani et al., 1979; Shockey et al., 1980); protein bands of other sizes are also observed (Brunt et al., 1975).

Appr. 16 % of the virion mass (Shepherd et al., 1968; Hull et al., 1976) consists of an 8 Kb (Franck et al., 1980) double-stranded, circular DNA molecule. Linear forms of DNA are also isolated from virions (Shepherd and Wakeman, 1971). For most strains of CaMV, three single-stranded breaks, or "gaps," interrupt the backbone of the virion DNA molecule (Hull et al., 1979; Volovitch et al., 1979; Franck et al., 1980; Richards et al., 1981). Gap 1 occurs in the minus strand (noncoding or template strand). Gaps 2 and 3 occur on the plus strand. Upon denaturation, CaMV DNA [CabbS strain (Franck et al., 1980)] dissociates into three separate strands; 8.0 Kb, 5.4 Kb, and 2.6 Kb.

Organization of the Genome

Sequence analysis (Franck et al., 1980) reveals 6 to 8 open reading frames (ORFs) located on the plus strand of CaMV DNA. ORFs one through five very closely abut or even overlap (Franck et al., 1980) and are conserved between strains, with appr. 5 % variation between isolates (Balazs et al., 1982). Two other small ORFs, VII and VIII, are less well conserved and little is known about their functions. Although the gene products for ORFs II and VII are unnecessary for infectivity, the presence and location of initiation

and termination codons in these regions are crucial for infectivity (Dixon and Hohn, 1984; Dixon et al., 1986; Melcher et al., 1986b).

CaMV as a Tool

CaMV has features which make it an especially important tool. Unlike the plant RNA viruses, CaMV has a replication cycle that is clearly divided into DNA and RNA stages. Because the double-stranded DNA of CaMV can be easily cloned, analysis of the nucleic acid sequence is much less daunting than for RNA viruses. Additionally, because cloned CaMV DNA which has been freed from the vector is infectious, it is easier to evaluate the effects of changes in the genome of CaMV than in those of other viruses. CaMV DNA also serves as an important vector for expressing exogeneous genes in plants.

The molecular biology of CaMV provides an especially appropriate model for plant molecular biology. Unlike RNA-containing plant viruses, CaMV shares the transcriptional, as well as the translational, machinery of its hosts. The CaMV 35 S promoter is already a workhorse in the area of chimeric gene constructions. Our understanding of CaMV transcription is one of the wedges that is being used to widen our understanding of plant transcription. Knowledge about other CaMV processes can only enhance our understanding of the molecular biology of plants.

Work with CaMV can contribute to the pool of knowledge for not only CaMV, but also for other viruses. CaMV is similar to several human pathogens, notably the retroviruses and the hepadnaviruses. Investigation of both the differences and similarities between CaMV and other viruses may illuminate the biology and pathology of not only CaMV, but also these other viruses.

Subsequent chapters will further explore the biology of CaMV. The introduction to Chpt. II will discuss the roles of RNA in the replication cycle of CaMV. The introduction to Chpt. III will discuss the phenomenon of cross protection as it relates to plant RNA viruses and CaMV.

CHAPTER II

REGULATION OF CAMV GENE EXPRESSION

Introduction

Transcription of CaMV DNA

Covalently closed viral DNA and gapped viral DNA are found in the nuclei (Favali et al., 1973; Menissier et al., 1982; Olszewski et al., 1983) and cytoplasm, respectively, of infected cells. In the nucleus, viral DNA is asymmetrically transcribed (Howell and Hull, 1978; Odell and Howell, 1980; Guilfoyle, 1980). Although gapped viral DNA is not considered to be transcriptionally active (Hull et al., 1979; Howell and Hull, 1978; Guilfoyle, 1980; Al Ani et al., 1980), the detection of minor transcripts with termini near or at the DNA gaps (Covey et al., 1981; Guilley et al., 1982; Condit and Meagher, 1983; Dudley et al., 1982) suggests that at least a portion of the viral transcripts may be transcribed using gapped templates.

CaMV Transcripts

Many different viral transcripts have been reported. These fall into two general classes, widely accepted transcripts and unsubstantiated ones. The predominant viewpoint is that CaMV codes for two major transcripts, the 35 S RNA and the 19 S RNA (Howell and Hull, 1978; Odell et al., 1981; Dudley et al., 1982; Covey and Hull, 1981). These RNAs are 8.2 and 1.9 Kb long, respectively (Covey et al., 1981; Dudley et al., 1982).

The 35 S RNA. The 35 S RNA is the most predominant species of CaMV RNA; visual inspection of published northern blots suggests that 35 S RNA bands yield 5 to 50

times as much signal when compared to 19 S RNA bands (Howell and Hull, 1978; Odell et al., 1981; Dudley et al., 1982; Covey and Hull, 1981; Covey et al., 1981). However, this ratio varies between, and within, laboratories. S1 nuclease analysis (Covey et al., 1981; Guilley et al., 1982) and R-looping analysis (Dudley et al., 1982) suggest that the 35 S RNA is generally not spliced. Transcription of the major 35 S RNA begins at nt 7435 (Guilley et al., 1982) on the DNA of the CabbS strain (numbered as by Franck et al., 1980), proceeds around the circular DNA template, through the origin of transcription, and terminates at nt 7615 to produce an RNA with terminally redundant ends (Guilley et al., 1982). Possible roles for this RNA are discussed elsewhere in this review. A cassette-like model for the organization of the 35 S promoter has recently been presented (Benfey and Chua, 1990).

The 19 S RNA. Transcription of the subgenomic 19 S RNA begins at nt 5765 (Covey et al., 1981) or nt # 5764 (Guilley et al., 1982) of the CabbS sequence (Franck et al., 1980) and terminates, coterminal with the 35 S RNA, at nt 7615 (Guilley et al., 1982). The 19 S RNA codes for the inclusion body protein (Al Ani et al., 1980; Odell and Howell, 1980; Covey and Hull, 1981) and is transcribed from contiguous DNA sequence (Covey et al., 1981). Transcription of the 19 S RNA is driven by the 19 S RNA promoter found in the small intergenic region between ORF V and VI. This promoter is less active than the 35 S promoter (Ow et al., 1987; Lawton et al., 1987). The 19 S RNA is capped and polyadenylated (Odell and Howell, 1980; Covey et al., 1981; Guilley et al., 1982).

Other Transcripts. A number of RNAs which appear to be transcribed from gapped DNA have been reported. An RNA sedimenting at 8 S with a length of 0.7 Kb appears to result from premature termination of transcription of the 35 S RNA at gap one (Guilley et al., 1982). Also, minor 35 S transcripts resulting from initiation and / or termination near mp 0 have been reported (Condit and Meahger, 1983; Guilley et al., 1982; Covey et al., 1981; Dudley et al., 1982). A number of unsubstantiated reports of other CaMV-specific

RNAs exist (Covey and Hull, 1981; Condit et al., 1983), but the validity of these observations remains questionable.

Replication of CaMV DNA by Reverse Transcription

The collation of a number of pieces of evidence led to a model for CaMV replication by reverse transcription (Hull and Covey, 1983; Pfeiffer and Hohn, 1983; Guilley et al., 1983). According to this model, the viral DNA is asymmetrically transcribed in the nucleus from a covalently-closed minichromosome (Guilfoyle, 1980; Olszewski et al., 1982; Hull and Covey, 1983; Menissier et al., 1982; Menissier et al., 1983; Olszewski et al., 1983). Transcripts include the greater-than-genome-length 35 S RNA (Covey et al., 1981; Guilley et al., 1982). The 35 S RNA is transported to the cytoplasm and reverse transcribed in virion-like replication bodies (Thomas et al., 1985; Marsh et al., 1985; Marsh and Guilfoyle, 1987) found associated with the viral inclusion bodies (Favali et al., 1973; Modjtahedi et al., 1984) in the cytoplasm of the host cell.

The possible linkage between reverse transcription and virion assembly has been reviewed (Hull, 1984; Fuetterer and Hohn, 1987) and a detailed model postulated (Hull et al., 1987). The key point to this model is the coregulation of the processes of reverse transcription and translation. Both processes utilize the CaMV 35 S RNA. Interference between the two processes could be prevented by temporal and / or spatial separation of the viral replication cycle into stages. [A third, pretranslation stage to the CaMV replication cycle has been proposed(Hohn et al., 1990); this subject will be addressed in a separate section of this review.]

According to the model, the first phase of the viral replication cycle is the translation of the 19 S RNA and the subsequent accumulation of the inclusion body protein. This protein facilitates translation of the 35 S RNA, resulting in the accumulation of other viral proteins. The inclusion body protein also produces a scaffold which concentrates subsequent viral molecules and processes occuring in the cytoplasm of the host cell.

The model further postulates that during the second stage of viral replication, the 35 S RNA is utilized as a template for reverse transcription rather than for translation. Immature coat protein molecules, reverse transcriptase, tRNA primer, and / or other components assemble into virion-like replication complexes. These complexes sequester the 35 S RNA prior to the onset of reverse transcription; ribosomes are excluded. Spatial separation of the processes of reverse transcription and translation prevents competition and interference between the two processes. Exclusion of ribosomes also down-regulates translation of the 35 S RNA. The binding site for the tRNA which primes reverse transcription is located just upstream of ORF VII. This strategic location suggests that tRNA-primer binding may function to coregulate the processes of translation and reverse transcription (Dixon and Hohn, 1984; Futterer et al., 1988). Proteinase processing, phosphorylation, and glycosylation might play roles in regulating the maturation of the replication complex during or after the reverse transcription of the 35 S RNA. CaMV replication and assembly may be linked in a manner similar to the interdependent replication and assembly of HBV (Hirsch et al., 1990; Yu and Summers, 1991).

Models for CaMV Gene Expression

Expression of ORF V. The CaMV reverse transcriptase may be expressed from ORF V via an ORF IV-ORF V fusion protein, as is observed for retroviruses (Covey, 1986). However, unlike retroviruses, the translation of the CaMV reverse transcriptase gene could begin at a separate AUG initiation codon (Franck et al., 1980), suggesting that translation of this ORF is not dependent on frameshifting events, but is initiated at this AUG instead. A fusion protein was not detected *in vivo* or when *in vitro* transcripts from ORF IV / V subclones were translated *in vitro* (Gordon et al., 1988). The viablility of mutants with repeated stop codons between ORF IV and V suggests that frameshifting is not the normal mode of ORF V expression (Penswick et al., 1988; Schultze et al., 1990). Furthermore,

the segment of DNA encoding the ORF IV/V overlap does not mediate frameshifting between adjacent reporter genes in a yeast system (Wurch et al., 1991).

Another model suggests that ORF V might be expressed using a subgenomic RNA. Although ORF V shows no eucaryotic promoter-like sequences immediately upstream of the transcription initiation point, a series of direct and inverted repeats occur between nt 4925 and 5070; these repeats seem to be linked to dinucleotide-primed transcription from this region *in vitro* (Cooke and Penon, 1986). Promoter activity with appr. 1 % of the strength of the CaMV 35 S promoter has also been detected when DNA sequences upstream of ORF V are used to drive expression of reporter genes in transient assays (Hohn et al., 1990). *In vitro* translation activity for a 75 kD protein can be detected in RNA populations isolated from infected plants (Plant et al., 1985). This messenger activity can be hybrid selected using subclones bearing sequences from ORF V. This activity can also be detected in fractions of sucrose gradients which sediment at 22 S (Plant et al., 1985). Unconfirmed work reported the detection of a 22 S subgenomic mRNA (Covey, unpublished; cited in Thomas et al., 1985). The existence of a subgenomic mRNA for ORF V remains uncertain.

Translation of the 35 S RNA. The 35 S RNA plays a role in replication; a possible role as a messenger RNA also exists. The 35 S RNA is polyadenylated (Odell and Howell, 1980; Covey et al., 1981) and can be isolated from polysome preparations (Odell et al., 1985; Howell and Hull, 1978). The 35 S RNA also carries the cap-scan consensus proposed by Kozak (1981) near 6 out of 8 of the CaMV ORFs (Hull, 1984). However, the 35 S RNA is translated quite poorly *in vitro* (Franck et al., 1980; Guilley et al., 1982; Gordon et al., 1988) and the long 5 ' leader of the 35 S RNA inhibits the translation of downstream genes in transient expression assays (Futterer et al., 1990). Some evidence for weak *in vitro* translation of 35 S RNA or of 35 S RNA degradation products has been advanced (Plant et al., 1985). The inclusion body protein transactivates the translation of

the second cistron of bicistronic reporter gene constructs during transient expression assays (Bonneville et al., 1989; Gowda et al., 1989), thereby demonstrating a possible resolution of the discrepancy between the implied *in vivo* translation of the 35 S RNA and the failure to achieve significant *in vitro* translation of this RNA.

The predominant model of 35 S RNA translation proposes that the closely-packed ORFs (I-III; and possibly ORFs IV & V) are read by ribosomes which translate the individual genes, but do not dissociate from the RNA before beginning translation of downstream ORFs (Sieg and Gronenborn, 1982; Dixon and Hohn, 1984). This "relay race" model is supported by the packed, polycistronic nature of the CaMV 35 S RNA, the size limits of the intergenic region (Brisson et al., 1984; Pennington and Melcher, submitted), and the observation that viability of mutants with changes in ORFs VII and II required only that these ORFs be terminated just before the initiation codon of the next downstream ORF (Seig and Gronenborn, 1982; Dixon and Hohn, 1984). Because ORFs III and IV and ORFs IV and V overlap, one must also consider the backward scanning model of ribosomal travel (Thomas and Capecchi, 1986) for these reading frames.

Accumulation of Transcriptional Template. Supercoiled CaMV DNA can be extracted from the nuclei of infected cells (Menissier et al., 1982; Olszewski et al., 1983). Supercoiled CaMV DNA appears to accumulate during incubation of purified nuclei from infected plants (Ansa et al., 1982), during replication of CaMV in PEG-infected protoplasts isolated from healthy plants (Maule, 1985a) or during prolonged culture of callus tissue from infected plants (Covey et al., 1990). The accumulation of supercoiled DNA might be a result of the cycling of a small portion of the progeny virions or viral molecules back to the nucleus (Saunders et al., 1990). Hohn et al. (1990) have suggested that this cycling might be controlled by a balance between reverse transcriptase activity, transactivation of 35 S RNA translation, and the production of coat protein. Cycling of progeny

nucleocapsids has been reported to be necessary for high levels of HBV production in infected cells (Tuttleman et al., 1986).

The Time Course of CaMV Replication in Protoplasts

Protoplasts have been infected with CaMV virions or transfected with viral DNA using various techniques (reviewed in Maule, 1985b). A large number of viral products has been characterized, including viral transcription template, transcripts, proteins, DNA, and virions. The protoplast system is in many respects a very good system for the study of virus replication. CaMV protein (Yamaoka et al., 1982) and DNA synthesis (Maule, 1983) reached levels comparable to those observed for cells in whole plants. All of the viral proteins which could be detected *in planta* could also be detected in protoplasts (Kobayashi et al., 1990). Other entities associated with viral replication and infection, such as replication complexes (Thomas et al., 1985) and virions (Yamoaka et al., 1982; Sakai and Shohara, 1982), have also been isolated from infected protoplasts.

Howell and Hull (1978) studied the accumulation of CaMV RNA in infected turnip protoplasts. RNA was labeled during the time course of infection by including inorganic ^{32}P in the cultures. Labeled RNA was then isolated at various times postinfection, hybridized to filter-bound DNA for various times, and the amount of bound radioactivity determined by liquid scintillation counting. Only small amounts of radioactivity were bound to the filters when RNA isolated at 2 days was hybridized, but the RNA isolated at 3 and 4 days gave a three- to four-fold increase in signal. The kinetics of hybridization were examined by determining the amount of signal bound at selected times during the hybridization. Samples shared a common plateau of signal after 12 (4-day sample) to 24 (3-day sample) hr of hybridization. However, the slopes of the hybridization curves were quite different, the 4-day sample showing a steeper hybridization slope. The authors interpreted these results to mean that the same portion of the CaMV genome was

transcribed at all times postinfection (due to the plateau) and that CaMV RNA accumulated throughout the experiment (due to the increasing slopes of the hybridization curves of samples isolated at successive time points). Other explanations can be envisioned. Characterization of this RNA suggested that the total RNA population consisted primarily of a large species of RNA complementary to the (–) strand of the viral DNA. The RNA population was appr. 30 % polyadenylated.

An unusual population of RNAs has been isolated from replication complexes that were prepared from infected turnip protoplasts (Thomas et al., 1985). Discrete RNA species of 6.7, 6.2, 4.7, 3.4, and 2.6 Kb, as well as a heterogeneous smear of RNA similar to that reported by Guilley et al. (1982), were observed.

Some differences between CaMV replication in protoplasts and in cells of whole plants may exist. It has been reported that protoplasts do not form the intracellular inclusion bodies characteristic of CaMV infection (Yamoaka et al., 1982b). Other authors have reported that the regulation of transcription in protoplasts may differ from that observed for whole plants (Ebert et al., 1987; Odell et al., 1988; Fang et al., 1989). Although the artificial nature of the system must be considered when results are interpreted, the system remains the only good way to generate synchronous replication of CaMV.

Statement of Purpose

Our understanding of the biology of CaMV has led to a model of the mechanism by which plant promoters may function. Also, studies using CaMV have illuminated alternative modes of RNA translation. Relationships between CaMV and human pathogens continue to become apparent. However, pivotal questions remain unanswered. Speculations about the existance of an ORF V RNA species have been advanced, but not addressed. This speculation is shaping hypotheses about the replication and expression of CaMV (Hohn et al, 1990) and other reverse-transcribing elements (Hohn and Futterer,

1991). Proof of this subgenomic message seems mandatory before such hypotheses become widely accepted.

Important relationships between transcription and replication remain unexplored. Although the kinetics of CaMV DNA synthesis have been well investigated (Maule, 1985a), the possibility of changes in the CaMV RNA population has not been addressed. Indeed, the only study of changes in the CaMV RNA population during replication (Howell and Hull; 1978), rather than at a given point in the infection cycle, was done over 13 years ago, before the widespread use of "northern blots"! Speculations about CaMV RNA expression are becoming annoyingly and dangerously unsupported.

An investigation of the CaMV RNA population within infected turnip protoplasts was undertaken to examine mechanisms by which CaMV gene expression might be regulated. By utilizing a synchronously-infected population of cells as a source of CaMV RNA, it was hoped that transient changes in the RNA population could be detected and analyzed. Specifically, it was hypothesized that a subgenomic ORF V RNA would be detected and that correlations between CaMV DNA accumulation and RNA expression would be apparent. Additionally, it was speculated that previously unreported RNA species might be detectable in this system. The possibility that changes in the RNA population might also be detectable *in planta* was also considered.

Materials and Methods

Overview of the System

The goal of these experiments was to examine mechanisms by which CaMV gene expression might be regulated. It was hypothesized that changes in the CaMV RNA population could be detected by harvesting the RNA from synchronously-infected protoplasts at various times postinfection.

RNA Techniques. The validity, limitations, and reproducibility of the techniques used for RNA analysis were examined using RNA isolated from infected plants rather than RNA from protoplasts due to the labor-intensive nature of protoplast work. Following the isolation of RNA samples from systemically-infected leaves of turnips, the concentration and purity of total cellular RNA was examined using UV spectroscopy. Electrophoresis, followed by staining with acridine orange and destaining, allowed analysis of the extent of degradation of the ribosomal RNAs within each sample. Gel blot assays were used to assess the amount and integrity of CaMV-specific RNA in each sample. Nuclease protection assays were used to estimate the relative amounts of CaMV RNA between samples and to determine the relative amounts of CaMV 35 S and 19 S RNA within samples.

The effects of oligo(dT)-cellulose chromatography were also characterized. RNA degradation during chromatography was assessed by electrophoresis and staining of the ribosomal RNAs present in the nonpolyadenylated fractions of RNA isolated from individual systemically-infected leaves. Gel blot assays were used to characterize the quality and quantity of CaMV-specific RNA present within polyadenylated fractions from individual samples.

Characterization of Changes in the CaMV RNA Population During Synchronous Replication. To detect changes in the CaMV RNA population during viral replication, protoplasts were isolated from leaf tissue of healthy host plants, infected with purified virions, and distributed to individual cultures. The viablility of uninfected or infected cultures was examined by a variety of techniques. CaMV DNA replication curves (Hussain et al., 1985) were used to assess the success of these PEG-mediated infections (Maule, 1983) and to examine the time course of CaMV DNA synthesis. At various times postinfection, cultures were harvested and their RNA isolated. RNA in these samples was analyzed by gel blot assays ("northern blots").

Reagents

The highest quality reagents available in the laboratory were used. Specific sources are noted where such sources are considered to be important for the success of the experimental technique.

Plasmid Contruction

The plasmid pCS101 contained the full-length CabbS genome cloned into the *Sal* I site of pBR322 (Richards, Guilley, and Jonard, unpublished). This plasmid was identical in construction to pCa37 (Lebeurier et al., 1982).

The plasmid pSRL51 contained a 1.2 Kb fragment of *Arabidopsis thaliana* genomic DNA inserted into a bacterial vector, pUC119. The inserted fragment included half of the α-tubulin gene including two introns and the 3' terminus. pSRL51 was constructed by Steven Ludwig (Ludwig et al., 1987).

The plasmid pSH115 was created by inserting the 438 *Pst*I / *Sac*I fragment of pCS101 between the corresponding sites of Bluescript SK (+) [Stratagene]. pSH115 therefore contained 438 nt of pCS101-derived DNA corresponding to nt 5390 to nt 5828 of the CabbS viral DNA (numbering of Franck et al., 1980). The orientation of the insert was such that synthesis of RNA / DNA primed from the T-3 promoter / primer would result in a a fragment of RNA / DNA complementary to the viral coding (+) strand or viral RNA.

All plasmids were constructed using the general guidelines recommended by Maniatis et al. (1982). Recombinant plasmids were identified by restriction analysis and / or limited DNA sequencing.

Protoplast Isolation

Turnip plants were grown as previously described (Gardner et al., 1980). The plants were moved to total darkness and held at 25° C for 20 hr prior to protoplast isolation.

Protoplasts were isolated following the guidelines of Ulrich et al. (1980). Peeled tissue (2-3 g from the 4th-7th leaves of 4-5 week-old plants) was digested in 25 ml of filter-sterilized enzyme solution [1% Cellulase Onozuka RS (Yakult Pharmaceutical Co.), 0.1% Macerozyme R10 (Yakult Pharmaceutical Co.), 5 mM MES, pH 5.6, 4 mM $CaCl_2$, 0.4 M mannitol] for 4 hr at room temperature. The digestion mixture was then filtered through cheesecloth and the protoplasts were washed thoroughly in 5 mM MES, pH 5.6, 4 mM $CaCl_2$, 0.4 M mannitol.

Turnip plants were infected with CabbS virions as previously described (Gardner et al., 1980). At time of harvest, the first primary leaf was designated as leaf number one and subsequent leaves numbered proceding from oldest to youngest.

Infection of Turnip Protoplasts with CaMV Virions

CaMV virions were isolated and purified as described by Hull et al. (1976), filter-sterilized, and mixed with freshly-isolated protoplasts. PEG (MW 6000, Sigma) was used to promote infection following the procedure of Maule (1983). For small scale work, the original volumes specified were used; for large-scale work, this procedure was scaled up four-fold.

Culture of Protoplasts

After infection, protoplasts (6×10^6) were cultured at room temperature under ambient light in 3 ml of a modified A medium (AΔ medium) in 60 x 15 mm sterile, disposable petri dishes whose edges had been sealed with parafilm. AΔ medium consists of A medium (Kao and Michayluk, 1981) in which the hormone and $CaCl_2$ concentrations were changed to those used in 8p medium (Kao and Michayluk, 1975). Alternatively, protoplasts (0.8×10^6) were cultured in Costar Mark II 24-well tissue culture plates in 0.4 ml of AΔ medium. Carbenecillin was routinely included in the culture medium at a concentration of 0.50 mg/ml. Protoplasts were cultured for up to four days before harvest at the time of harvest,

aliquots from each culture were spotted to nitrocellulose as described below and the rest of the culture was collected in a 10 ml centrifuge tube and centrifuged for 5 min. at 100 x g. Appr. 9 ml of solution were removed by aspiration. The cells were resuspended in appr. one ml of culture media and transfered to a microcentrifuge tube. These tubes were centifuged for 5 min. at appr. 100 x g, the packed cell volume was estimated, the supernate was removed by aspiration, and the cells were lysed for RNA isolation as described below.

Dot Blot Assay of Infection

Replication of viral DNA in transfected protoplasts was followed via dot blot hybridization (Hussain et al., 1985). At various times postinfection, triplicate aliquots (5 µl, appr. 10^4 cells / aliquot) of the cultures were spotted directly on an untreated nitrocellulose membrane (Schleicher & Schuell). After all samples were collected, the dry filter was laid for 5 min on two layers of 3 MM Whatman paper saturated with 0.5 N NaOH. The nitrocellulose filter was then neutralized by two successive 1 min incubations on 3MM paper saturated with 1.0 M Tris-HCl, pH 7.5, 0.6 M NaCl followed by a 5 min incubation on 3MM paper saturated with 0.5M Tris-HCl, pH 7.5, 1.5 M NaCl. The blot was then washed twice with chloroform, dried at room temperature, and baked for 2 hr in a vacuum oven at appr. 80 °C. The baked blot was hybridized with radiolabeled (Feinberg and Vogelstein, 1983), cloned CaMV DNA (pCS101) using conditions recommended by the membrane manufacturer. After hybridization, the membrane was washed 4 times with 2X SSC/0.1% SDS for at 25 ° C for 10 min followed by a final wash under the same conditions for 30 min. The washed membrane was exposed to X-ray film. After autoradiography, the spots were excised and total radioactivity for each spot was determined by liquid scintillation counting. The hybridization conditions used for DNA dot blot assays were the same as those used for RNA gel blot assays; these conditions are described more fully below.

Isolation of RNA from Leaves and Protoplasts

All solutions, plasticware, and glassware for RNA work were treated and handled as recommended by Maniatis et al. (1982). A modification the RNA isolation procedure of Chomczynski et al. (1987) was done in 1.5 ml microcentrifuge ("Eppendorf") tubes or in 15 ml Corex centrifuge tubes. Individual leaves were frozen and ground in liquid nitrogen. One volume of Solution D (4.0 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5 % Sarkosyl, 0.1 M 2-mercaptoethanol) was added to 0.1 to 1.0 volume of the frozen tissue and the mixture was vigorously vortexed. Immediately thereafter, 1 volume of water-saturated phenol, 0.2 volume of chloroform:isoamyl alcohol (24:1), and 0.1 volume of 2 M sodium acetate (pH 4.0) were added to each tube and the mixture was again vortexed vigorously . After a 20 min incubation on ice, the phases were separated by centrifugation and the aqueous phase was collected. One volume of isopropanol was added to the aqueous phase and the samples were then incubated at -20 ° C for one hour. Samples were routinely stored at this point or, alternatively, the RNA was pelleted at 10,000 x g, rinsed with 70:30 (v / v) ethanol / TE-SDS (10 mM Tris pH 7.0, 1 mM EDTA, 0.1% SDS) and stored at -70 ° C. For protoplasts, RNA was extracted from 3 x 10^6 to 6 x 10^6 cells as described above; however, the cells were not frozen before disruption.

Oligo(dT)-Cellulose Chromatography of RNA

Immediately before oligo(dT)-cellulose (obtained from Sigma Chemical Company) chromatography, RNA samples ("total RNA" or "total cellular RNA") stored in isopropanol or ethanol were centrifuged at 10,000 x g for 10 min. The pellet containing the RNA was incubated at 70 ° C in 200 µl of TE-SDS, chilled on ice, the insoluble materials sedimented by centrifugation at 10,000 x g for 5 min., and the supernatant was collected. To each sample, 1/9 volume of 3 M sodium acetate, pH 6.0 and 2.5 volumes of ethanol

were added and the samples were incubated at -20 to -70 ° C for 1 or more hr. Samples were then centrifuged at 10,000 x g and the RNA pellets were redissolved in 200 µl of TE-SDS. The concentration of the RNA in each sample was estimated spectrophotometrically.

Oligo(dT)-cellulose chromatography was carried out for each individual sample as described by Kingston (1989) using batch chromatography at room temperature in microfuge tubes. Briefly, each sample (RNA in TE-SDS) was brought to 0.5 M LiCl and mixed for 10-45 min with oligo(dT)-cellulose that had been preequilibrated with TE-SDS, 0.5 M LiCl. Equal amounts (40-400 µg) of each RNA sample were applied to each "column" (40-200 µl packed oligo(dT)-cellulose, appr. 20-100 mg). The "columns" were then washed four times with mid-wash buffer (10 mM Tris, pH 7.0, 1 mM EDTA, 0.1% SDS, 0.15 M LiCl) and polyadenylated RNA was eluted from the washed oligo(dT)-cellulose "pellet" by two successive 10-30 min incubations with 150 µl of TE-SDS. Pairs of eluted fractions were pooled, mixed, and extracted with buffered phenol / chloroform / isoamyl alcohol (25:24:1, v/v). The RNA in each sample was precipitated with 25 µg of tRNA carrier, 1/9 volume of 3 M NaOAc, pH 6.0, and 2.5 volumes of 95% (or absolute) ethanol. Precipitated samples were stored at -70 ° C. Nonpolyadenylated RNA was also collected by precipitating aliquots of the unbound fraction (appr. 15 µg) with 1/9 vol of 3 M sodium acetate, pH 6.0 and 2.5 volumes of ethanol. The integrity of nonpolyadenylated RNA was assessed by agarose gel electrophoresis (McMaster and Carmichael, 1977).

Gel Blot Assays ("Northern Blots")

For gel blot analysis of RNA samples, RNA was redissolved, denatured with glyoxal, and separated on 1% agarose gels following the recommendations of Thomas (1980). After electrophoresis at a field strength of 0.6 V / cm on 14 cm gels for 4 - 5 hr, RNA was capillary transferred to charge-modified nylon membranes (S + S Nytran) using 10X SSC. Alternatively, RNA was electrotransferred to Nytran membranes following the membrane manufacturer's recommendations. Membranes were baked for 2 hr at appr. 80 ° C under

vacuum, washed with hot TE-SDS, and prehybridized for 4-8 hr at 42 ° C. Prehybridization and hybridization buffers contained 50% formamide, 5X SSPE, 5X Denhardt's solution, 0.5% SDS, and 200 µg/ ml sheared, denatured salmon sperm DNA. CaMV-specific RNA was detected using 2 - 5 x 10^6 cpm of radiolabeled (Feinberg and Vogelstein, 1983) cloned CaMV DNA (pCS101) per ml of hybridization solution. In hybridizations of blots designed to detect CaMV-specific RNA from transfected protoplasts, hybridization buffer was mixed 4:1 (v / v) with 50 % dextran sulfate. Hybridizations were incubated for appr. 20 hr at 42 ° C.

After hybridization, the membranes were washed four times for 10 min each in 2X SSC / 0.1% SDS at room temperature. A final stringency wash with 0.1X SSC / 0.1% SDS was done at 65 ° C for 30 min. Membranes were exposed to X-ray film at -70 ° C. The time of exposure and use of intensifying screens depended on the amount of radioactivity bound to each blot.

For blots of RNA isolated from protoplasts, previously bound probe was removed by boiling the membrane in TE-SDS for 30 min followed by washing in 2X SSC / 0.1% SDS for 5 min. Efficiency of stripping was assessed by exposing the stripped membrane to X-ray film or, alternatively, by Geiger-Mueller monitoring. Membranes were rehybridized with a radiolabeled plasmid containing 1.7 Kb of coding sequence for the *Arabidopsis thaliana* tubulin gene (pSRL51; Ludwig et al., 1987). Hydridizations with the tubulin-gene clone were carried out as described above, but the final stringency wash was at 25 ° C in 2X SSC / 0.1% SDS.

In Vitro Transcription Probe for Nuclease Protection Assays of CaMV 35 S and 19 S RNA

Nuclease protection assays were performed using a small radiolabeled probe that would detect and differentiate between the CaMV 19 S RNA and CaMV RNAs which do

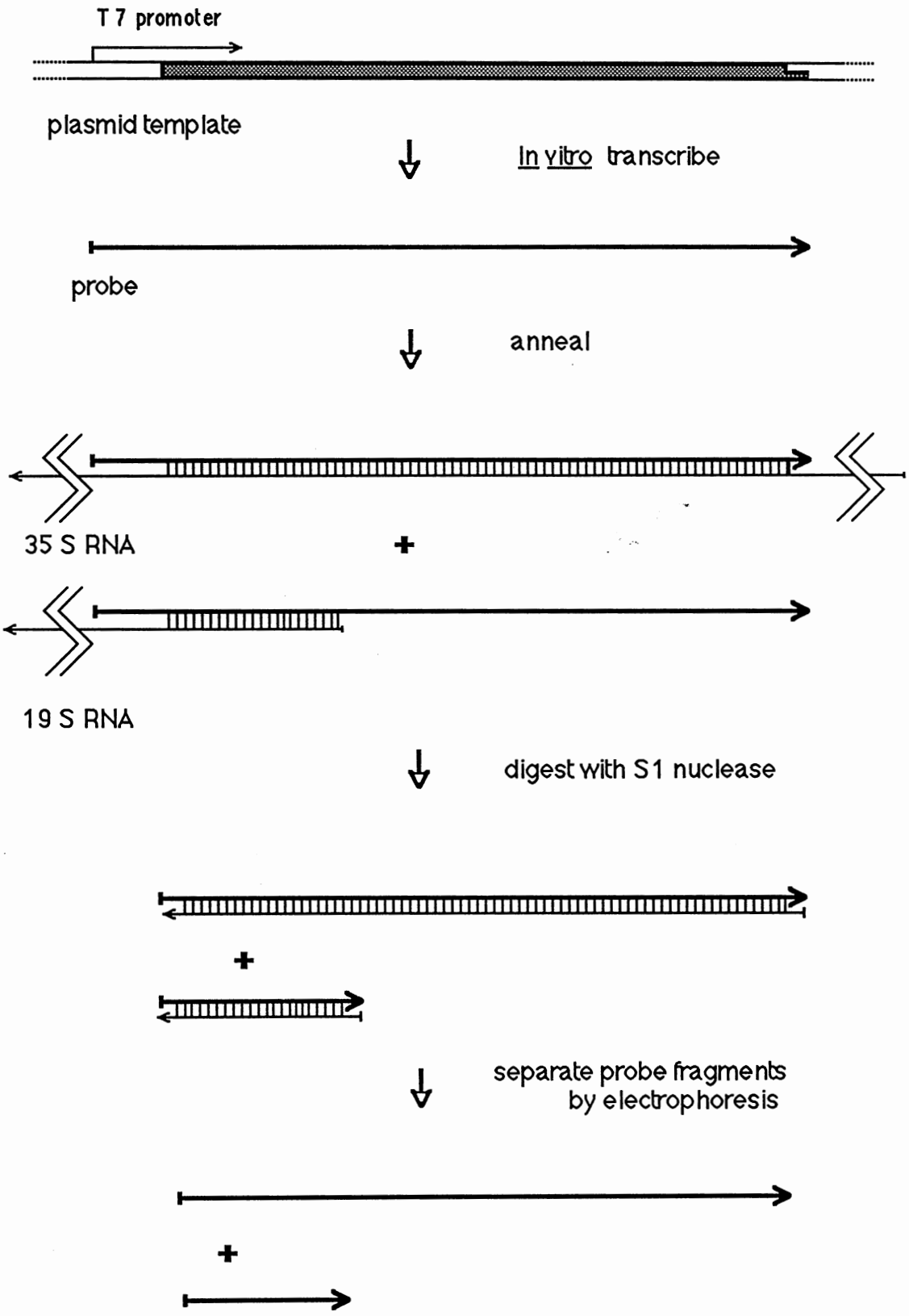
not have termini within the DNA sequences encompassed by the probe. To generate this probe, the plasmid pSH115 was digested with *Eco*RI before being used as an *in vitro* transcription template. This digestion resulted in two fragments, the larger of which contained 180 nt of DNA complementary to CaMV CabbS DNA. Specifically, CabbS DNA sequence from nt 5650 to nt 5829 [numbered as by Franck et al. (1980)] was present downstream of the T3 promoter of the vector on the larger fragment. *In vitro* transcription reactions using T3 RNA polymerase were assembled following the recommendations of the enzyme manufacturer. These reactions generated a 194-nt radiolabeled RNA probe consisting of 180 nts of RNA complementary to CaMV CabbS DNA and a 5 ' leader of 14 nts complementary to vector sequence. The CaMV 35 S RNA contains sequence complementary to 180 nt of the probe; the CaMV 19 S RNA contains sequence complementary to 66 nt at the 5 ' end of the probe (Fig. 1).

Nuclease Protection Assays

S1 nuclease protection assays were performed following the guidelines of Kedzierski and Porter (1990) using the probe described above. The *in vitro* transcription template was removed by acidic phenol extraction (Kedzierski and Porter, 1991). RNA samples (1 / 4 of the polyadenylated RNA purified from 50 µg of total RNA or, alternatively, 10 µg of total RNA) were annealed with 1.4 X 10^6 cpm (appr. 1.4 ng) of gel-purified probe in 30 µl of hybridization buffer containing 60 mM Tris·HCl, pH 7.5, 0.9 M NaCl, 6 mM EDTA, 4 mM DTT, and 40% formamide. Carrier tRNA dissolved in annealing buffer was added to bring the final RNA concentration to 25 µg/30 µl. After annealing overnight at 50 ^{o} C, 25 µl of each annealed sample were brought to 200 µl with 1X S1 nuclease buffer containing 1 U/µl of S1 nuclease(BRL) and digested for 1 hr at 37 ^{o} C. Digested samples were then extracted with buffered phenol/chloroform/ isoamyl alcohol (25:24:1) and the undigested RNA was precipitated with 10 µg of tRNA and 2.5 volumes of ethanol in the presence of 2 M ammonium acetate. The RNA was then redissolved in 80% formamide,

Figure 1. Diagram of Strategy for Nuclease Protection Assays.

Reactions were carried out as described in Materials and Methods. Stippled area on the plasmid template indicates CaMV sequence. DNA sequences are represented by thick lines. RNA sequences are represented by thin lines.


T 7 promoter
plasmid template
In vitro transcribe
probe
anneal
35 S RNA
+
19 S RNA
digest with S1 nuclease
+
separate probe fragments
by electrophoresis
+

10 mM EDTA, 0.1% bromophenol blue, 0.1 % xylene cyanol, heated at 85 ° C for 5 min, and electrophoresed on 8% sequencing gels at 50 V/cm for 1 hr. Gels were exposed wet overnight. Alternatively, exposures were also done for 5 days using intensifying screens.

Results

Integrity of CaMV Virions During RNA Isolation.

In the RNA isolation procedure (Chomczynski and Sacchi, 1987) used to extract RNA from plant tissues, DNA is removed from RNA samples by a phenol extraction done at low pH. To determine if this procedure disrupts CaMV virions, purified CaMV virions were subjected to a slightly modified version of this procedure. When done at neutral pH, the RNA isolation protocol yielded only trace amounts of ethidium bromide-fluorescing material from virions (Fig. 2). This material did not comigrate with authentic virion DNA. Little or no CaMV DNA was recovered from CaMV virions. Thus, virions were resistant to disruption by the RNA isolation procedure.

Analysis of RNA Techniques - RNA from Whole Plants

The applicability of the techniques used for RNA analysis were examined using RNA isolated from systemically-infected leaves of turnips. Presented in Fig. 3 to Fig. 10 are results from analysis of RNA samples isolated from individual leaves of four different plants. Plant number one was harvested 28 days postinfection. Plants number two, three, and four were harvested at 14 to 15 days postinfection.

Analysis of RNA Purity and Quantity. The concentration and purity of RNA samples was examined by measuring the UV absorbance at 260 and 280 nm. Old leaves yielded less RNA per gram of stemless tissue than young leaves (not shown). RNA samples extracted from older leaves (longer than 15 cm) typically had A_{260} / A_{280} ratios of appr. 1.6 while samples from younger tissues typically had correct (Sambrook et al., 1989)

Figure 2. Effect of GTPC Treatment on CaMV Virions.

CaMV virions (50 µg of the CabbS strain) were treated by the method of Gardner and Shepherd (1980) using Proteinase K and SDS or by a modification of the method of Chomczynski and Sacchi (1987) in which the extraction was done at neutral pH with guanidine thiocyanate. Nucleic acids were then purified by extraction with buffered phenol / chloroform (5:1), precipitated with isopropanol in the presence of 50 µg of tRNA carrier, and redissolved in TE. One-fifth of the nucleic acid isolated using Proteinase K / SDS (Pro/SDS) and all of the nucleic acid isolated using guanidine thiocyanate (GTPC) were applied to a 1 % agarose gel. After electrophoresis at 7.7 V / cm for 45 min, the gel was stained with 1 µg / ml ethidium bromide. The sizes (Kb) of selected bands of the DNA size standards (stds; BRL 1 Kb ladder) are shown.

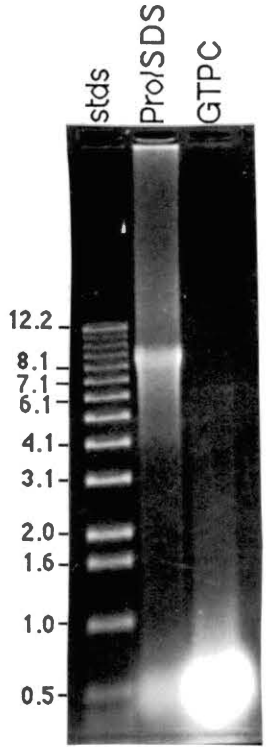

stds
Pro/SDS
GTPC
12.2
8.1
7.1
6.1
4.1
3.1
2.0
1.6
1.0
0.5

Figure 3. Analysis of Integrity of RNA Isolated from Turnip Leaves at 28 Days Postinoculation-Plant #1.

RNA was isolated from the indicated leaves (numbered as described in Materials and Methods) of an infected plant. The leaflets and shoot apex were designated leaf #24. RNA from an uninoculated plant was also analyzed (healthy). The positions of the 25 S and 18 S cytoplasmic rRNAs have been indicated.

(A) Seven μg of total RNA were glyoxalated, separated by electrophoresis, and stained with acridine orange.

(B) RNA samples were subjected to oligo(dT)-cellulose chromatography and an aliquot (5 μg) of nonpolyadenylated fractions were glyoxalated, separated by electrophoresis, and stained with acridine orange.

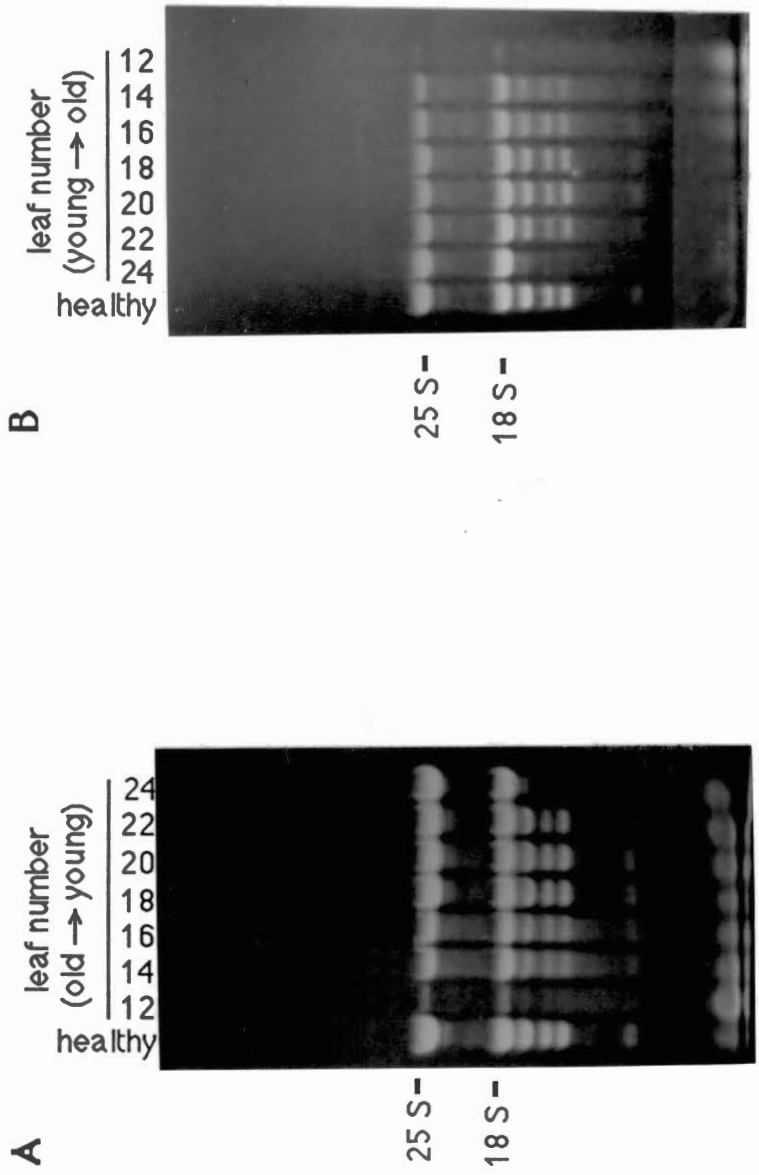

B
leaf number
(young → old)
12
14
16
18
20
22
24
healthy
25 S –
18 S –
A
leaf number
(old → young)
24
22
20
18
16
14
12
healthy
25 S –
18 S –

Figure 4. Analysis of Integrity of RNA Isolated from Turnip Leaves at 14 Days Postinoculation-Plant #2.

RNA was isolated from the indicated leaves (numbered as described in Materials and Methods) of an infected plant; the leaflets and shoot apex were designated leaf #15. RNA (5 μg) was glyoxalated, electrophoresed, and stained as described. An equivalent amount of RNA from an uninoculated plant was also included (healthy). The positions of the 25 S and 18 S cytoplasmic rRNAs have been indicated.

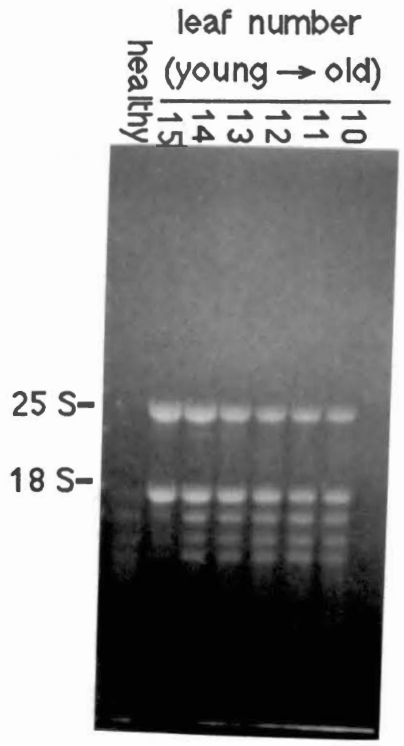

leaf number
(young → old)
healthy
15
14
13
12
11
10
25 S–
18 S–

Figure 5. Gel Blot Analysis of CaMV-specific RNA Present in Turnip Leaves at 28 Days Postinoculation-Plant #1.

The gel described in Figure 3A was transferred to a nylon membrane after staining, destaining, and photography of rRNA. CaMV-specific RNAs were detected following hybridization with radiolabeled, cloned CaMV DNA (pCS101). The X-ray film was exposed for 2 hr without an intensifying screen (Panel A) or 5 hr with an intensifying screen(Panel B).

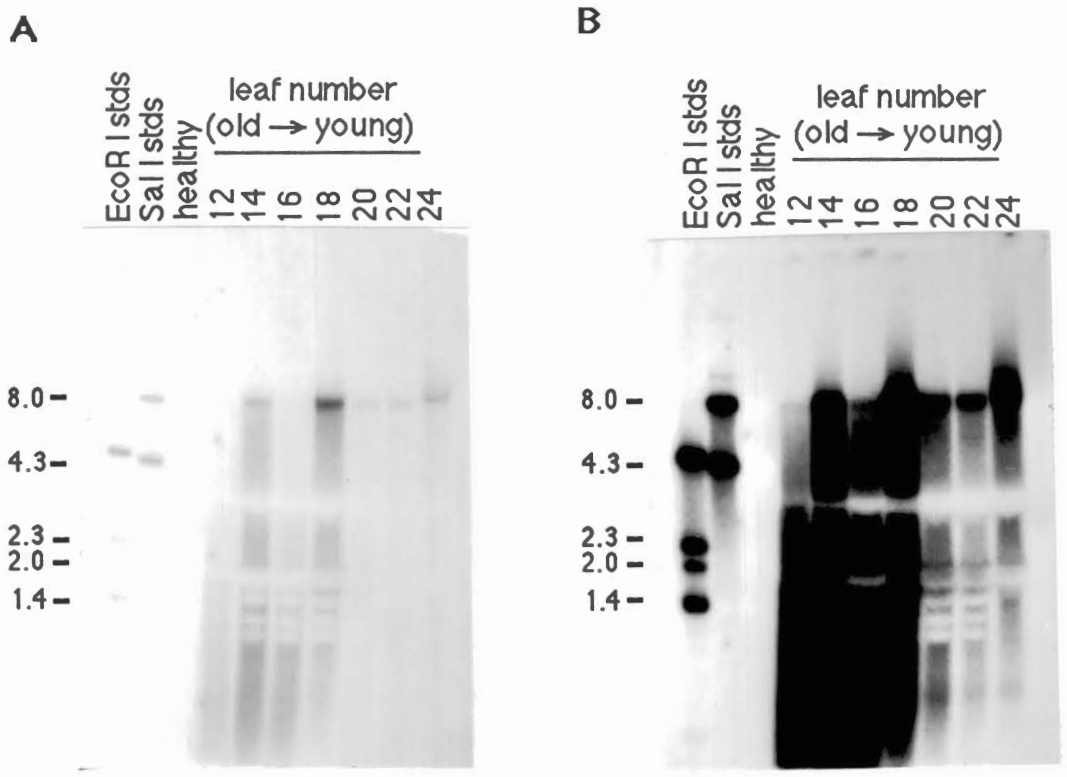

A
EcoR I stds
Sal I stds
healthy
leaf number
(old → young)
12
14
16
18
20
22
24
8.0
4.3
2.3
2.0
1.4
B
EcoR I stds
Sal I stds
healthy
leaf number
(old → young)
12
14
16
18
20
22
24
8.0
4.3
2.3
2.0
1.4

Figure 6. RNA Isolated from Turnip Leaves at 14 Days Postinoculation-Repeat of Analysis for Plant #2.

RNA was isolated from the indicated leaves (numbered as described in Materials and Methods) of an infected plant; the leaflets and shoot apex were designated leaf # 13. Oligo(dT)-cellulose chromatography was performed as described.
(A) One-half of the polyadenylated RNA fraction purified from 40 μg of RNA isolated from each leaf was applied to a 1% agarose gel. An equivalent amount of RNA isolated from an uninoculated plant (healthy) and 2 ng of unlabeled restriction fragments of pCS101 (SalI std and EcoRI std) were also glyoxalated and applied; the sizes of the restriction fragments are indicated (Kb). Gel blot analysis was performed using radiolabeled, cloned CaMV DNA (pCS101).
(B) Aliquots of nonpolyadenylated RNA fractions (6 μg) corresponding to the polyadenylated RNA fractions described above were glyoxalated, electrophoresed, and stained as described. An equivalent amount of nonpolyadenylated RNA from an uninoculated plant was also included (healthy). The positions of the 25 S and 18 S cytoplasmic rRNAs are indicated.

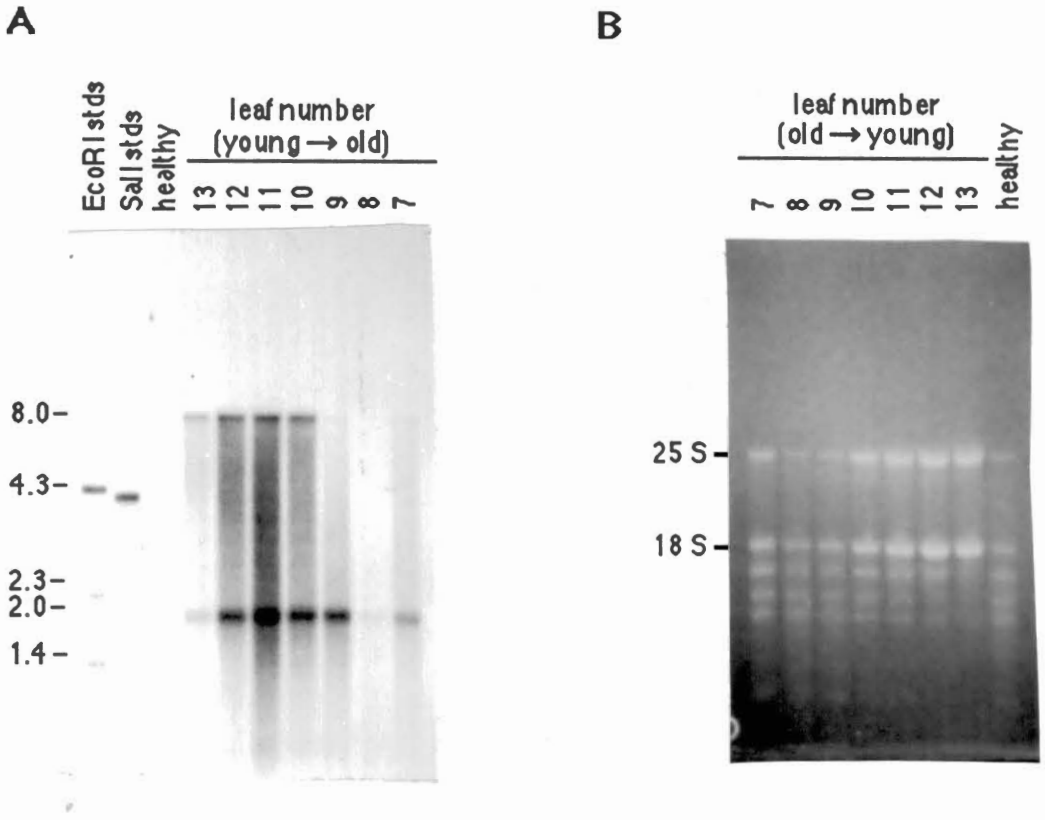

A
EcoRI stds
Sal I stds
healthy
leaf number
(young → old)
13
12
11
10
9
8
7
8.0–
4.3–
2.3–
2.0–
1.4–
B
leaf number
(old → young)
7
8
9
10
11
12
13
healthy
25 S–
18 S–

Figure 7. Gel Blot Analysis of CaMV-specific RNA Present in Turnip Leaves at 14 Days Postinoculation-Plant #2.

The gel described in Figure 4, was transferred to a nylon membrane after staining, destaining, and photography of rRNA. CaMV-specific RNAs were detected following hybridization with radiolabeled, cloned CaMV DNA (pCS101). Unlabeled restriction fragments of pCS101 (2 ng) were also glyoxalated and used as size standards (SalI std and EcoRI std). The sizes of these restriction fragments are indicated (Kb).

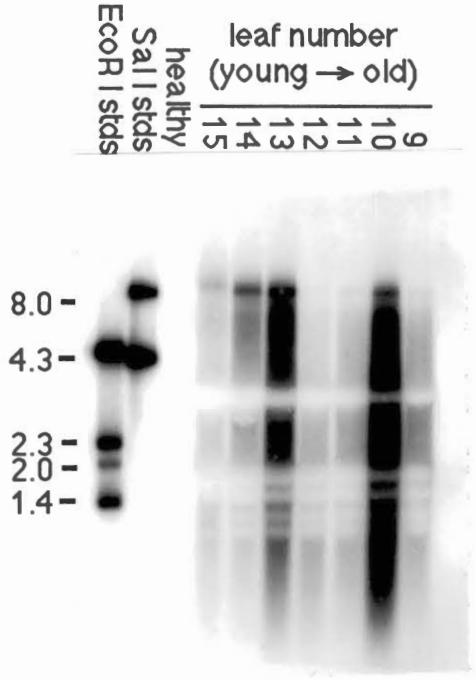

EcoR I stds
Sal I stds
healthy
leaf number
(young → old)
15
14
13
12
11
10
9
8.0
4.3
2.3
2.0
1.4

Figure 8. Gel Blot Analysis of Polyadenylated CaMV RNA Present in Turnip Leaves at 14 Days Postinoculation-Plant #3.

RNA was isolated from the indicated leaves (numbered as described in Materials and Methods) of an infected plant; the leaflets and shoot apex were designated leaf # 13. One-half of the polyadenylated fraction purified from 40 µg of each RNA sample was applied to a 1% agarose gel. An equivalent amount of polyadenylated RNA from an uninoculated plant (healthy) and 10 µg of the nonpolyadenylated RNA fraction from leaf 11 (A - leaf number 11) were also included. The positions of the CaMV 35 S and 19 S RNAs are indicated; these RNAs were identified based on their migration relative to the migration of unlabeled SalI and EcoRI fragments of pCS101 which had been gyoxalated and applied to the gel (not shown). Gel blot analysis was performed using radiolabeled, cloned CaMV DNA (pCS101).
(A) The X-ray film was exposed for 3 days with a screen.
(B) The X-ray film was exposed for 6 days with a screen.

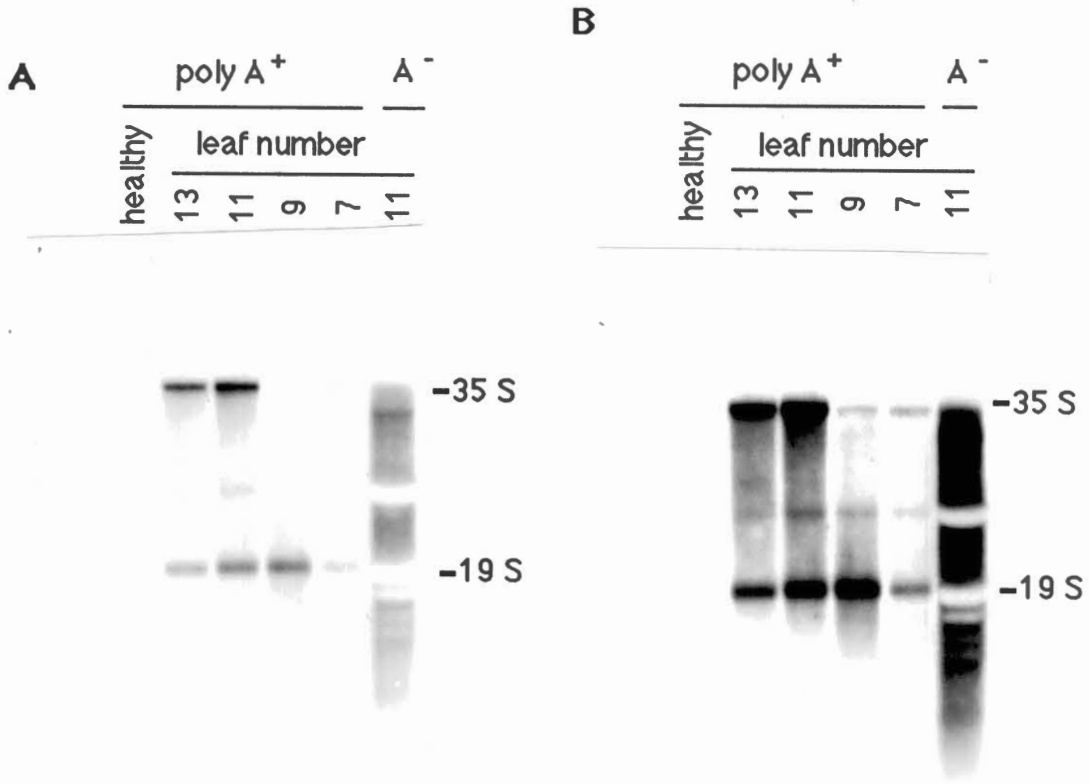

A
poly A+
A-
healthy
leaf number
13
11
9
7
11
-35 S
-19 S
B
poly A+
A-
healthy
leaf number
13
11
9
7
11
-35 S
-19 S

Figure 9. Results of Nuclease Protection Assays of Poly+ RNA Isolated at 28 and 14 Days Postinfection-Plants 1 and 2.

The assay was performed using 1/4 of the polyadenylated RNA purified from 50 μg of RNA isolated from the indicated leaves. Leaf numbering and assay conditions are described more fully in Materials and Methods. The assay was also performed using an equivalent amount of poly A+ RNA isolated from an uninoculated plant (healthy). Sample RNA was annealed with 1.4 x 106 cpm of complementary RNA probe. The hybridization reactions were then digested with S-1 nuclease to yield the indicated 35 S- and 19 S-protected fragments. The size of the undigested probe is shown (probe). This probe is described more fully in Materials and Methods. A dideoxy-C DNA sequencing reaction was used to generate DNA size standards (not shown).
(A) RNA was isolated from the indicated leaves at 28 days postinfection; the leaflets and shoot apex were designated leaf # 24. The gel was exposed overnight without an intensifying screen.
(B) RNA was isolated from the indicated leaves at 14 days post infection; the leaflets and shoot apex were designated leaf #15. The gel was exposed for 5 days with an intensifying screen.

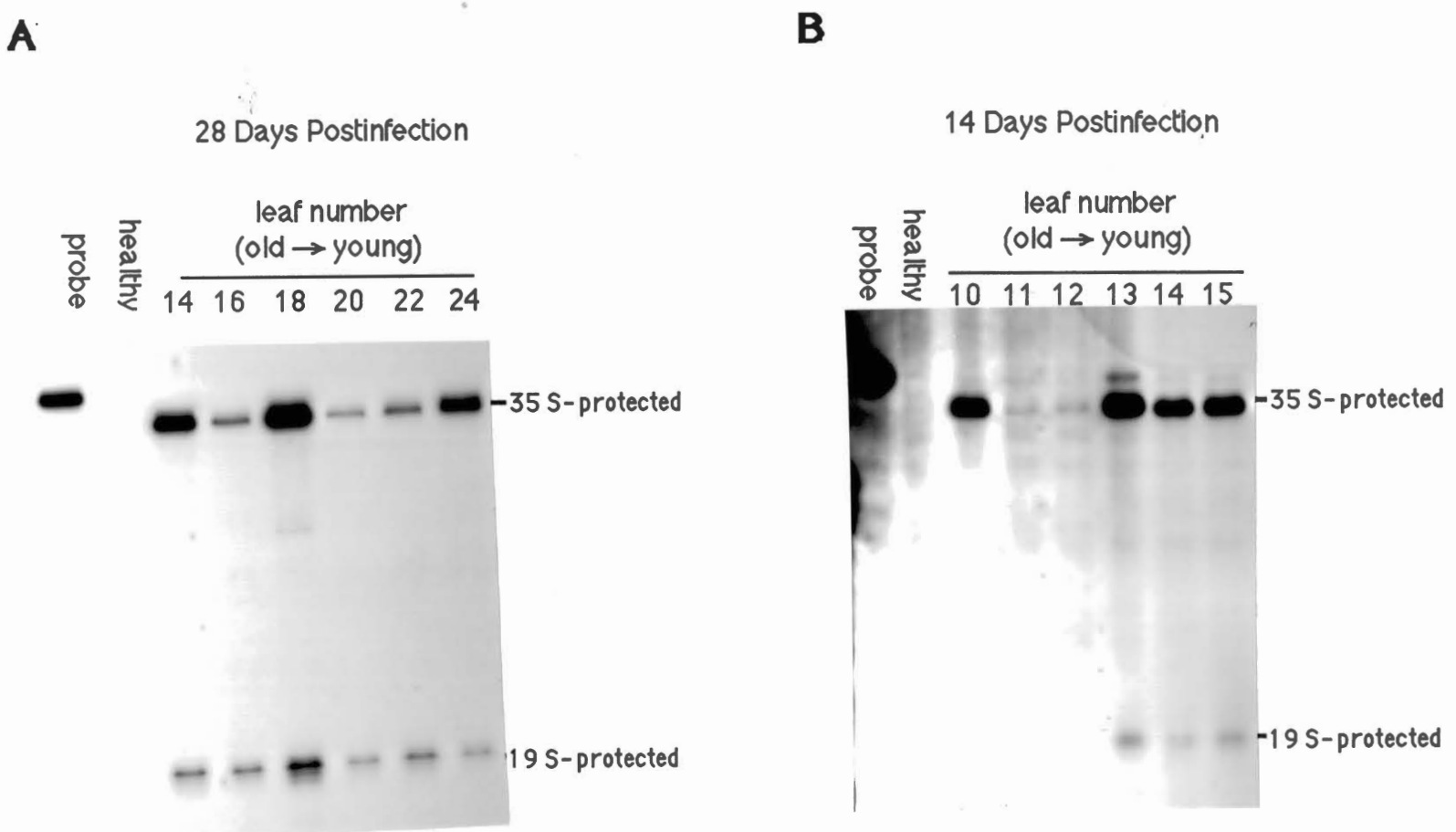
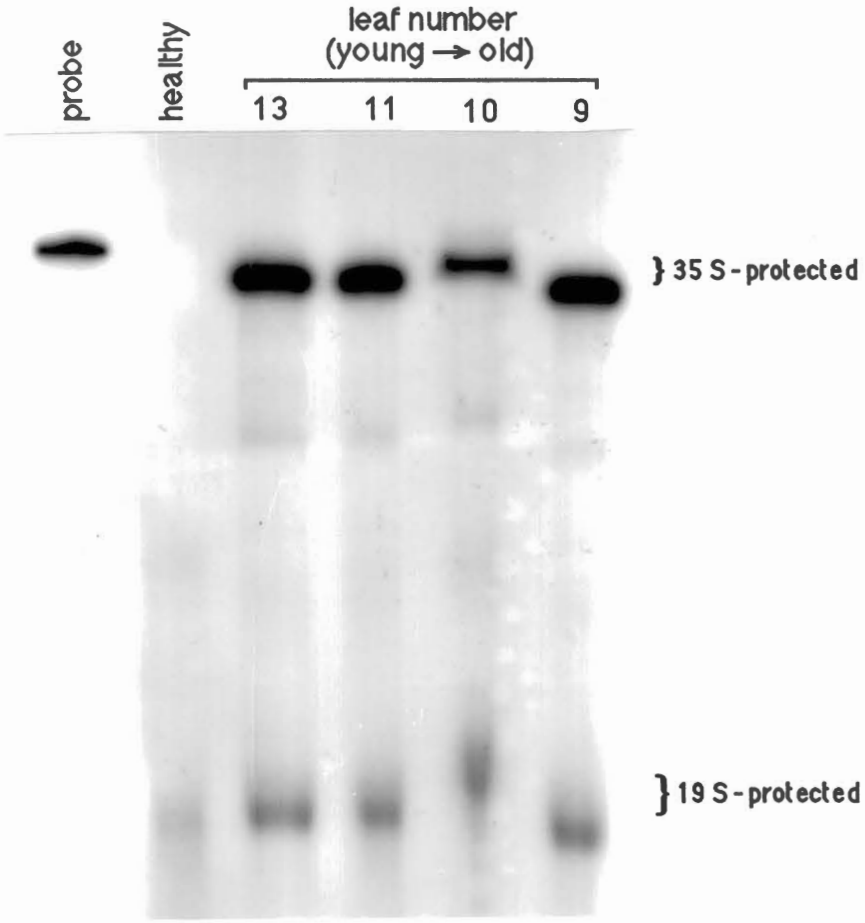

A
28 Days Postinfection
probe
healthy
leaf number
(old → young)
14 16 18 20 22 24
35 S-protected
19 S-protected
B
14 Days Postinfection
probe
healthy
leaf number
(old → young)
10 11 12 13 14 15
35 S-protected
19 S-protected

Figure 10. Results of Nuclease Protection Assay of Total RNA-Plant 4.

The assay was performed using 10 μg of total RNA isolated from the indicated leaves. The numbering of leaves, assay conditions, and complementary RNA probe are described more fully in Materials and Methods. The assay was also performed using 10 μg of RNA isolated from an uninoculated plant (healthy). Sample RNA was annealed with 2.5 x 10 6 cpm of probe. The hybridization reactions were then digested with S-1 nuclease to yield the indicated 35 S- and 19 S-protected fragments. The size of the undigested probe is also shown (probe).


probe
healthy
leaf number
(young → old)
13
11
10
9
} 35 S-protected
} 19 S-protected

A_{260} / A_{280} ratios of 2.0 (not shown). Recovery of RNA from old leaves was complicated by the presence of insoluble or poorly soluble material in the pellets recovered following the isopropanol-precipitation step of the RNA extraction. Thus, the purity of RNA samples decreased with increasing leaf age. When analyzed by gel blot assay, CaMV-specific signal was not detected in RNA samples that were treated with RNAase, suggesting that RNA samples were significantly free of DNA (not shown).

Analysis of RNA Integrity. The intactness of RNA samples was routinely analyzed using electrophoresis and acridine-orange staining to detect discrete rRNA bands. These analyses were complicated by variation in the degree and uniformity of gel destaining (not shown). When less than seven μg of RNA was analyzed, minor RNA species, including degradation products, were difficult to detect (Fig. 6B). Similarly, the sensitivity of detection was impaired on gels that were overly destained or inadequately destained. Analysis was greatly facilitated when seven or more μg of RNA were present on an optimally-destained gel (e.g. Fig. 3A).

Electrophoresis and staining of total RNA samples revealed the presence of several discrete bands (Fig. 3A). Based upon their predominance in the RNA samples from young nongreen tissues, the two major RNAs from these tissues were tentatively identified as the 25 S and 18 S cytoplasmic ribosomal RNAs. When the molecular weights of these RNAs were estimated based upon their migration relative to glyoxalated DNA size standards (not shown), the estimated molecular weights agreed with weights reported for the cytoplasmic rRNAs of other plant species (reviewed in Leaver, 1979).

Major RNAs migrating faster than the 18 S RNA were less detectable in samples isolated from young leaves than in samples from older leaves (e.g. Fig. 3A). Since the young leaves had not yet become green, they were expected to lack a full complement of photosynthetic structures, including chloroplast rRNAs. These smaller RNAs were therefore identified as plastid rRNAs. A minor band migrating slightly ahead of the

cytoplasmic 25 S rRNA was occasionally present (e.g. Fig. 3A, healthy). This RNA was tentatively identified as intact 23 S chloroplast rRNA. The 23 S chloroplast rRNA is seldom isolated from plant tissues as an intact molecule; instead this RNA is usually isolated as three fragments (reviewed in Leaver, 1979). The four bands below the 18 S cytoplasmic rRNA band (Fig. 3A) probably therefore correspond to the chloroplast 16 S rRNA and three discrete fragments from the chloroplast 23 S rRNA.

Although discrete rRNA bands were observed following electrophoresis and staining of total RNA samples, some degree of degradation was still evident in most samples. Mild degradation was indicated by the presence of heterogeneously-sized fragments which migrated ahead of discrete bands. The intensity of signal from such fragments was typically more intense near the band and became less intense as the distance from the band became greater (e.g. Fig. 3A, leaves 14 and 16). This effect will be referred to as "smearing" of an RNA band. Undegraded or mildly degraded samples showed 25 S bands that were slightly more intense than the 18 S bands (e.g. Fig 3A, leaves 18, 20, 22, 24). This was expected as these two RNAs should be present in equimolar quantities (reviewed in Leaver, 1979). Severe degradation was indicated by perturbation in the relative intensities of the 25 S and 18 S rRNAs. In severely degraded samples, the 25 S rRNA band was less intense than the 18 S rRNA band (e.g. Fig. 3A, leaf 12). Thus, the larger 25 S rRNA appeared more susceptible to degradation than the smaller 18 S rRNA.

Using these criteria for RNA integrity, degradation appeared to be more severe in samples isolated from old leaves than in samples from young leaves (Fig. 3A, Fig. 4). Apparently, it was more difficult to isolate intact RNA from older leaves than from young leaves.

Analysis of Gel Blot Assay Technique. In gel blot assays using cloned CaMV DNA as a probe, no signal was ever detected in lanes containing RNA from healthy plants, demonstrating that these assays were specific for CaMV RNA or DNA (e.g. Fig.5,

healthy). No changes in signal intensity which ran diagonal to the direction of electrophoresis were observed, suggesting that observed changes are not artifacts of the hybridization conditions.

To assess the efficiency of transfer of DNA fragments from gels using the capillary transfer technique, a set of DNA fragments (BRL 1 Kb ladder) was radiolabeled, glyoxalated, separated by electrophoresis, and transferred from the gels by capillary transfer. After transfer, the agarose gels were dried and exposed to X-ray film. Comparison of autoradiograms of the membrane with autoradiograms of the dried gel revealed that fragments larger than 4 Kb were transferred from the gel less quantitatively than smaller fragments (not shown).

To assess the efficiency of capillary transfer on individual gel blot assays, the relative signals of the 8.0 Kb and 4.3 Kb bands from *Sal*I- digested pCS101 were compared. These fragments were present in equimolar amounts in the *Sal*I-digested plasmid DNA (Richards et al., unpublished). Based on this stoichiometry and the relative sizes of the fragments, the 8.0 Kb *Sal*I standard band should have been 1.9 times as intense as the 4.3 Kb band on gel blot assays. However, on gel blot assays the 8.0 Kb *Sal*I standard band was typically slightly less intense than the 4.3 Kb standard band (e.g. Fig. 5, lane *Sal*I std). Occasionally even lower relative intensities were observed (Fig. 6, *Sal*I std lane). The relative intensities of the two *Sal*I-standard bands were therefore used as a measure of transfer efficiency. No aberrations in the relative signals from fragments smaller than 4.3 Kb were observed (Fig. 5). Scanning densitometry of an autoradiogram from a typical assay showed that the 8.0 Kb band was only 0.64 times as intense as the 4.3 Kb band (not shown). Therefore, only one-third of the 8 Kb DNA fragment was detected by the gel blot assay. Thus, if DNA and RNA capillary transfer efficiencies were similar, only one-third of the CaMV 35 S RNA present in a given sample was detected in typical gel blot assays.

Analysis of the CaMV RNA Content of Total RNA Samples from Individual Leaves. RNA was extracted from the leaves of plant number three at 14 days postinfection and analyzed on gel blot assays. When seven μg of total cellular RNA from each leaf were applied to the gel, the amount of CaMV-specific RNA detected in the gel blot assay varied from leaf to leaf (Fig. 7). Variation was also apparent when the CaMV-specific RNA content was analyzed by dot blot assay (not shown). To examine the reproducibility of leaf-to-leaf variation in relative CaMV RNA content, RNA was isolated in separate extractions and analyzed on separate gel blot assays. Gel blot assays were reproducible independent of the RNA extraction (not shown). RNA from leaves 10 and 13 gave strong CaMV-specific signal on both gel blot assays . Similarly, leaves 9, 11, 12, and 15 gave low CaMV-specific signal on both assays. Thus, the relative CaMV RNA content in each total RNA sample appeared to be independent of RNA handling. Leaf-to-leaf variation in CaMV RNA content occurred in plant number two as well as in plant number one. Comparison of the gel blot assays of RNA from these plants suggested that variations in CaMV RNA content were variable from plant to plant (Fig. 5 versus Fig. 7).

Analysis of CaMV RNA Integrity in Total RNA Samples. The integrity of the CaMV 35 S RNA was assessed using gel blot assays to detect CaMV-specific RNA. The CaMV 35 S RNA was identified by its comigration with the 8.0 Kb DNA size standard. Some smearing of the 35 S RNA was apparent even in total RNA samples which showed little or no degradation when their rRNAs were analyzed as described above (leaves 18, 20, 22; Fig. 5 and Fig. 3A, respectively). Various degrees of smearing of the 35 S RNA are apparent in the literature (Odell et al., 1981; Covey and Hull, 1981).

Within the 35 S RNA smear, negative "bands" which showed little or no probe binding were apparent (e.g. Fig. 5). The positions of these negative bands corresponded to the positions of the rRNA bands on the stained gel (not shown). Such bands have been reported by others (Odell et al., 1981; Covey and Hull, 1981). Presumably, rRNAs

prevented the probe from binding to portions of the membrane, thereby preventing detection of RNA which comigrated with rRNA.

For each sample, the intensity of the 35 S RNA band relative to the smear from heterogeneously-sized fragments was noted. The intensities of 35 S RNA bands relative to smears decreased with increasing age of the leaf (Fig. 5, Fig. 7). This suggested that 35 S RNA isolated from older leaves was less intact than 35 S RNA from younger leaves.

Effects of Oligo(dT)-Cellulose Chromatography on RNA Quality. Oligo(dT)-cellulose chromatography (polyA selection) was used to separate nonpolyadenylated RNA from polyadenylated RNA. The integrity of rRNAs before and after oligo(dT)-cellulose chromatography was appr. equal (e.g. Fig. 3A versus Fig. 3B), suggesting that RNA samples experienced little or no degradation during oligo(dT)-chromatography. Electrophoresis and staining of polyadenylated fractions revealed small amounts of rRNA still present in the polyadenylated fractions (not shown). After chromatography, gel blot assays of polyadenylated fractions detected an RNA which comigrated with the 18 S rRNA (Fig. 8). Based on the migration of this CaMV-specific band relative to size standards, this band was identified as the CaMV 19 S RNA. A minor band which comigrated with the 23 S rRNA was also apparent (Fig. 8). This probably represents an rRNA "shadow-band" artifact (Odell et al., 1981). This band could also be detected when assays were performed using a subclone of the CabbS ORF II as a probe (not shown). The amount of CaMV-specific signal recovered after chromatography varied significantly between two lots of oligo(dT)-cellulose from different manufacturers (not shown).

Analysis of the Quality and Quantity of Polyadenylated 35 S CaMV RNA Isolated from Leaves. The polyadenylated fraction from each sample was analyzed by gel blot assay. The intensities of 35 S bands relative to 19 S bands were noted. Less 35 S signal relative to 19 S signal was detected in samples from older leaves than in samples from younger leaves (Fig. 6A, Fig. 8). In all assays, the 35 S RNA band and / or high

molecular weight smear was detectable if the 19 S band was easily detectable (e.g. Fig. 6A).

Because the relative 35 S RNA signal decreased with increasing leaf age, and the extent of RNA degradation seemed to increase with increasing leaf age, the possibility exists that changes in the relative 35 S RNA band intensities are artifacts of degradation. This possibility was supported by the observation presented earlier that the 23 S rRNA seemed more susceptible to degradation than the 18 S rRNA, suggesting that large RNAs are especially sensitive to degradation. Gel blot assays of polyadenylated RNA were therefore not suited to analysis of minor changes in the relative amounts of RNAs of different sizes.

Analysis of CaMV RNAs by Nuclease Protection. Nuclease protection assays for CaMV RNAs were developed and used to overcome the limitations of gel blot assays for estimating the relative amounts of 35 S and 19 S RNA. Nuclease protection assays measured the amounts of 19 S RNA and the amounts of CaMV RNAs which did not have a terminus near the 19 S promoter, presumably the 35 S RNA (or degradation products of the 35 S RNA).

No detectable probe was protected by polyadenlated RNA isolated from uninfected plants (Fig. 9A and B). Little (Fig. 9B) or no (Fig. 9A) undigested probe could be detected in assays of polyadenylated RNA from infected plants. Polyadenylated RNA from infected plants protected probe fragments of two different sizes. The migrations of these fragments relative to DNA size standards were consistent with expected migrations for protected probe species (not shown). For polyadenylated RNA samples from plant number two, a long exposure to X-ray film was required to detected protected probe fragments. This fact, and the very low signal from leaves 11 and 12 (Fig. 9B), suggested that under these conditions, the amount of polyadenylated CaMV RNA in these samples was barely detectable by nuclease protection assay. Probe fragments protected by the polyadenylated

RNA isolated from plant number one were detectable after an overnight exposure of the gel to X-ray film. A minor protected fragment migrating to a position between those of the 35 S-protected fragment and the 19 S-protected fragment was observed for one sample from plant number one (Fig. 9A, leaf 18). This band was also observed in other assays of polyadenylated RNA (not shown). Minor bands were present in the assay of polyadenylated RNA from plant number two(Fig. 9B), but high nonspecific and lane-specific background hindered further assessment of the validity of these bands.

Nuclease protection assay was used to estimate the amount of 35 S RNA relative to 19 S RNA in each sample. RNA samples which showed comparable degrees of degradation (e.g. Fig. 3A, leaves 14 and 16) showed very different 35 S to 19 S ratios (e.g. Fig. 9A, leaves 14 and 16). Similarly, RNA fractions which showed comparable amounts of degradation (e.g. Fig. 3A, leaves 14 and 16) showed quite different 35 S RNA contents (e.g. Fig. 9A, leaves 14 and 16). Additionally, protected probe fragments were not smeared, suggesting that only insignificant amounts of the protecting species of RNA were degraded to sizes smaller than the probe (Fig.9). These observations suggested that, unlike gel blot assays, nuclease protection assay of the 35 S RNA content relative to 19 S RNA content was not sensitive to variations in degrees of sample degradation. Similarly, nuclease protection assay of the 35 S RNA content of RNA samples did not appear to be sensitive to variations in degrees of sample degradation.

Leaf-to-leaf variation in the 35 S to 19 S ratio was apparent when total RNA (Fig. 10) or polyadenylated RNA (Fig. 9, A and B) from three different plants was analyzed by nuclease protection assay. The nuclease protection assay of total RNA from plant number four was complicated by an artifactual fragment which comigrated with the 19 S-protected fragment (Fig. 10, healthy). Additionally, poor electrophoretic resolution of protected fragments complicated this experiment. Nonetheless, the data from three different plants suggested that the 35 S RNA content relative to 19 S RNA content varied among RNA samples. This variation appeared random from plant to plant. More 35 S RNA relative to

19 S RNA was detected in leaves which had higher total CaMV RNA contents than in leaves which had relatively lower CaMV RNA contents (Fig. 9 versus Fig. 5).

Changes in the CaMV RNA Population During Viral Replication in Protoplasts

To analyze changes in the CaMV RNA population during viral replication, RNA was isolated from protoplast cultures at various times postinoculation. Cell vitality and viral replication were assessed. RNA was characterized essentially as for RNA extracted from plants (above). Nuclease protection assays were not used to characterize RNA from protoplasts.

Isolation and Culture of Protoplasts. An average of 7 X 10^6 protoplasts were recovered from each gram of stemless leaf tissue digested with cellulase and pectinase. Several media were examined to determine the optimal medium for cell survival. A greater percentage of uninfected protoplasts survived four days of culture (not shown) when cultured in modified A medium (A Δ medium; described in Materials and Methods) rather than A medium (Kao and Michayluk, 1981), MS medium (Murashige and Skoog, 1962), or K_3 medium (Kao et al., 1972). When inoculated cells were cultured in A Δ medium for one to four days, staining with FDA (Widholm, 1972) or Evan's blue (Gaff and Okong'O-ogola, 1971) typically indicated that appr. one half of the cell population was viable. Cell mortality did not increase between day one and day four (not shown). Vigorous cell wall regeneration, frequent protoplast budding, and frequent cell elongation were noted (not shown). Bilateral cell division was very rare. Thus, turnip protoplasts cultured as described in Materials and Methods showed reasonable vitality.

To further assess protoplast vitality, changes in the packed cell volumes and total cellular RNA content of cultures were examined in two experiments (the RNA time course experiments described below). When 6 x 10^6 protoplasts were cultured in 3 ml of media in

60-mm diameter petri plates, packed cell volumes increased appr. four-fold during the period of culture (Fig. 11). Under these conditions, RNA content increased to almost twice the initial content (e.g. Fig. 12). Thus, protoplasts cultured as described showed vigorous growth.

Characterization of CaMV Replication in Turnip Protoplasts. When the procedure of Maule (1983) for PEG-mediated infection of protoplasts was scaled up four-fold, typically one-third to one-half of the cell population was recovered after inoculation and washing (not shown). To assess the effect of culture conditions on viral replication, inoculated cells were mixed well and cultured by two different methods. Cells were cultured in either Costar® tissue culture plates or in petri plates. After four days of culture, appr. one-half of the cells cultured in tissue culture plates were viable (not shown). The viability of cells cultured in petri plates was not examined in this experiment. Viral replication was assessed as described in Materials and Methods. Aliquots of cells sampled from the tissue culture plates showed only small variations in the amount of CaMV-specific probe which was bound (Fig. 13). However, for the cells cultured in petri plates, appr. 3-fold more probe was bound by the samples collected at 2.5 days postinoculation than by the samples collected at 1 day postinoculation (Fig. 13). Similarly, an increase in the amount of bound probe was noted for the samples harvested from petri plates at four days postinoculation. Thus, cells cultured in petri plates supported viral replication whereas cells cultured in tissue culture plates did not. Culture conditions affected viral replication. Because cells cultured in petri plates supported more vigorous viral replication than cells cultured in tissue culture plates, petri plates were the preferred culture system in all subsequent experiments.

To determine the effects of inoculum concentration on viral replication, cells (8×10^6) were inoculated with either 10 μg or 50 μg of virions and cultured in petri plates (1×10^6 cells / 3 ml). Cells were sampled every 12 hr. When samples were assayed for CaMV replication, very little or no retained virion inoculum was detected immediately following

Figure 11. Changes in Packed Cell Volume During Growth of Turnip Protoplasts.

Freshly-isolated protoplasts were infected and cultured as described in Materials and Methods. Two cultures were harvested at the indicated times postinoculation. Each point represents the estimated packed cell volume from two cultures. Experiments one and two were done with protoplasts isolated in separate experiments.

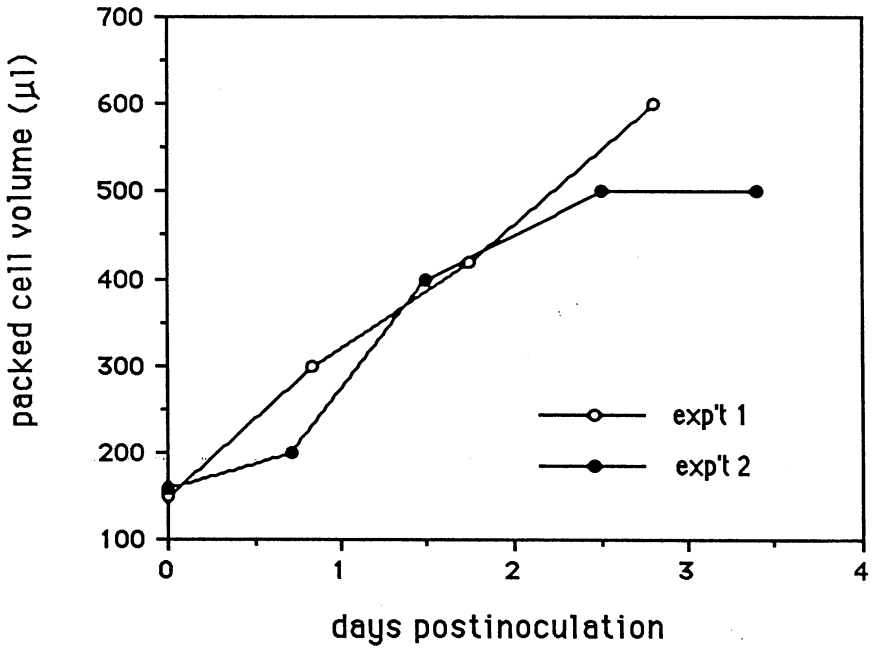

700
600
500
400
300
200
100
packed cell volume (µl)
0
1
2
3
4
days postinoculation
exp't 1
exp't 2

Figure 12. Changes in Cellular RNA Content During Culture of Protoplast Cultures.

Freshly-isolated protoplasts were infected and cultured as described in Materials and Methods. Two cultures were harvested at the indicated times postinoculation. Each point represents the estimated total cellular RNA recovered from two cultures. Experiments one and two were done with protoplasts isolated in separate experiments.

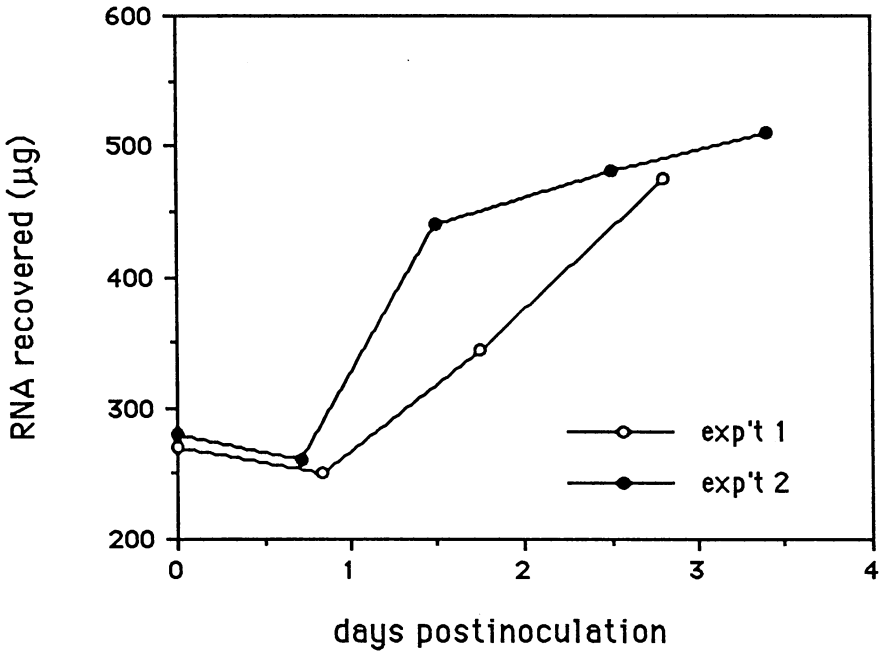
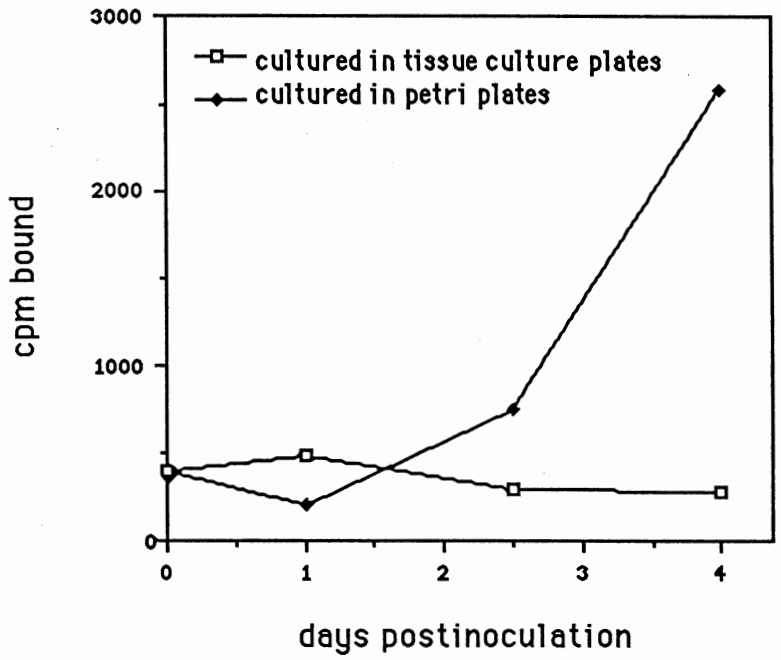

600
500
400
300
200
RNA recovered (µg)
0
1
2
3
4
days postinoculation
exp't 1
exp't 2

Figure 13. Effects of Culture Conditions on Replication of CaMV.

Freshly-isolated protoplasts were infected (50 µg of CabbS virions / 107 cells) and cultured as described in Materials and Methods. Cells were cultured in either Costar® 24 - well tissue culture plates (0.7 ml of culture / 16 mm- diameter well) or in disposable petri plates (3 ml of culture / 60 mm-diameter plate), as indicated. Quadruplicate aliquots from the cultures (104 cells) were spotted onto nitrocellulose at the indicated times postinoculation. After all samples had been spotted, the membrane was treated with NaOH, neutralized, baked, and probed using 1 x 106 cpm of radiolabeled, cloned CaMV DNA (pCS101) per ml of hybridization solution. After four low stringency washes with 2X SSC / 0.1 % SDS at 25o C, the final stringency wash was performed at 65o C using 0.1X SSC / 0.1% SDS. The washed membrane was exposed to X-ray film. After autoradiography, each spot was excised and the bound radioactivity determined by liquid scintillation counting. Plotted points represent the sums of quadruplicate samples.


3000
2000
1000
0
cpm bound
cultured in tissue culture plates
cultured in petri plates
0
1
2
3
4
days postinoculation

inoculation and washing (Fig. 14A). This is in contrast to other experiments, in which significant amounts of inoculum were retained (e.g. Fig. 15). Cells inoculated with either 10 μg or 50 μg of virions bound increasing amounts of probe between 1.5 and 2.5 days postinoculation (Fig. 14A). However, the amount of CaMV DNA detected in the cells which were infected with 10 μg of virions appeared to decline at 4 days postinoculation relative to 3.5 days postinoculation (Fig. 14A). This decline may have been artifactual. When this culture was sampled four days postinoculation, samples tended to bead up on the nitrocellulose membrane rather than wetting the membrane quickly and uniformly, as was usually observed (not shown). Cells inoculated with 50 μg of virions were therefore spotted to a different area of the nitrocellulose membrane for the day-four sampling. A similar decline in CaMV DNA content at day four was not observed for these cells. Therefore, in assessing the relative rates of replication in these two populations of cells, only samples collected before day four were considered.

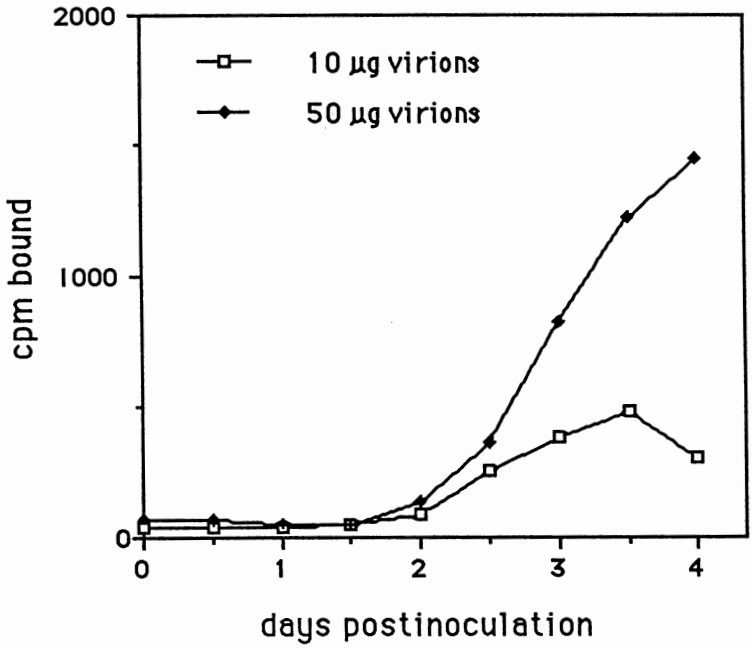
At the resolution of this experiment, replication first became detectable simultaneously in both cultures after a lag period of appr. two days (Fig. 14A). In cells infected with 10 μg of virions, CaMV DNA accumulated at only appr. 40 % the rate observed for cells that were inoculated with 50 μg of virions (Fig. 14A). Thus, the concentration of the inoculum had little or no effect on the lag period, but determined the rate of viral replication subsequent to this lag.

To determine whether turnip protoplasts could be infected with unencapsidated DNA, protoplasts from the experiment described above were also inoculated with DNA isolated from virions. DNA and virion inocula contained comparable concentrations of CaMV genome equivalents. Protoplasts which were inoculated with DNA isolated from CaMV virions appeared to retain the inoculum, but did not show increases in CaMV DNA content over the four day period of the experiment (Fig. 14B). Thus, under these conditions, virions, but not CaMV DNA, could be used to infect turnip protoplasts. Virions, rather than viral DNA, were therefore the inoculum of choice for further experiments.

Figure 14. Response of Turnip Protoplasts to Inoculation with Different Amounts of CaMV DNA or Virions.

Freshly-isolated protoplasts (8 x 106 cells) were infected and cultured (3 x 106 cells / 3 ml in a petri plate) as described in Materials and Methods. Quadruplicate aliquots of the culture (appr. 1 x104 cells) were spotted onto nitrocellulose at the indicated times postinoculation. After all samples had been spotted, the membrane was treated with NaOH, neutralized, baked, and probed using 1 x 106 cpm of radiolabeled, cloned CaMV DNA (pCS101) per ml of hybridization solution. After four low-stringency washes with 2X SSC / 0.1% SDS at 25 o C, the final stringency wash was performed at 65 o C using 0.1X SSC / 0.1% SDS. The washed membrane was exposed to X-ray film; after autoradiography, spots were excised and bound radioactivity was determined by liquid scintillation counting. Shown are the total cpm for each time point; the radioactivity bound by untransfected cells (background) has been subtracted. Protoplasts were inoculated with CabbS virions (A) or with CaMV CabbS DNA (B).

A


2000
1000
0
cpm bound
10 µg virions
50 µg virions
0
1
2
3
4
days postinoculation

B

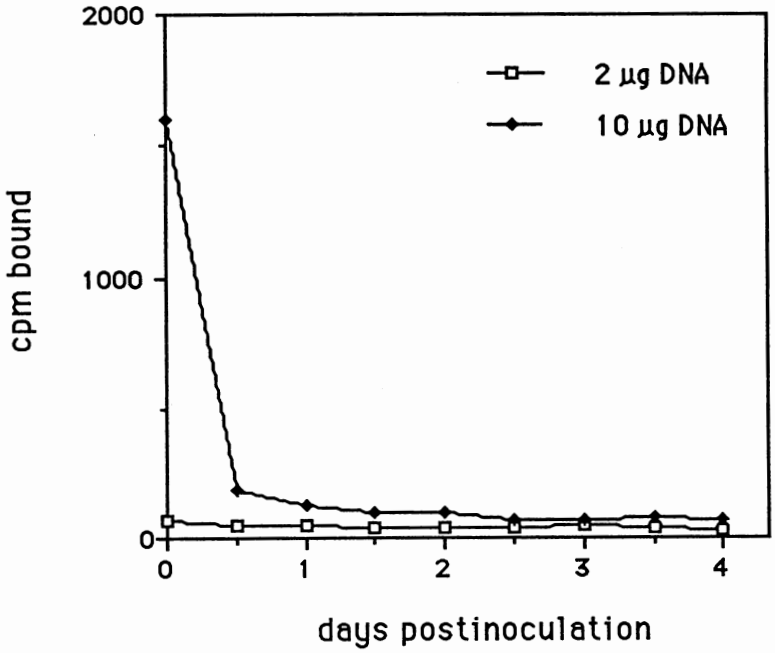
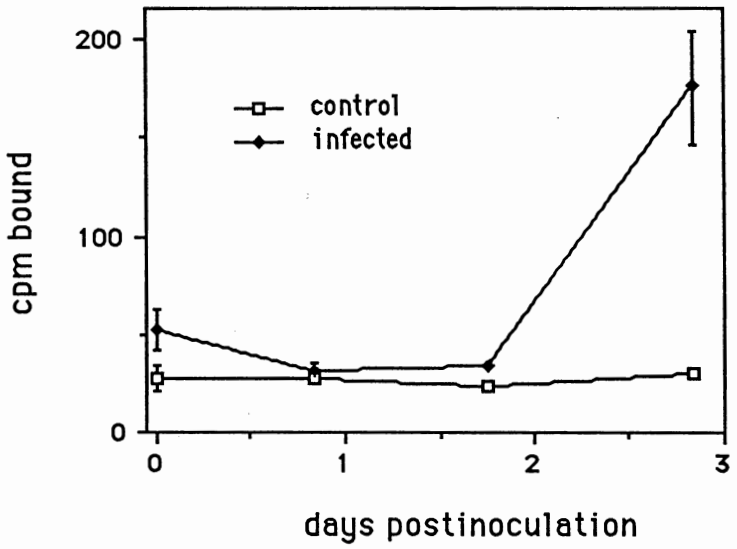

2000
1000
0
cpm bound
2 µg DNA
10 µg DNA
0
1
2
3
4
days postinoculation

Figure 15. Replication of CaMV in Turnip Protoplasts-RNA Time Course One.

Freshly-isolated protoplasts were infected and cultured as described in Materials and Methods. Triplicate aliquots of the culture (appr. 104 cells) were spotted onto nitrocellulose at the indicated times postinoculation. After all samples had been spotted, the membrane was treated with NaOH, neutralized, baked, and probed using 1 x 106 cpm of radiolabeled, cloned CaMV DNA (pCS101) per ml of hybridization solution. After three low stringency washes with 2X SSC / 0.1 % SDS at 25o C, the final stringency wash was performed at 25o C using 0.1X SSC / 0.1% SDS. The washed membranes were exposed to X-ray film; after autoradiography, each spot was excised and the bound radioactivity determined by liquid scintillation counting. Plotted points represent the means of triplicate samples.


200
100
0
cpm bound
control
infected
0
1
2
3
days postinoculation

Viral replication was also followed for the cultures used in the characterization of CaMV RNA accumulation (see below). Comparison of low-resolution viral replication curves from the RNA time course experiments suggested that in these experiments, the observed lag times and replication rates were comparable to those observed in previous experiments (c.f. Figs. 13 and Fig. 15).

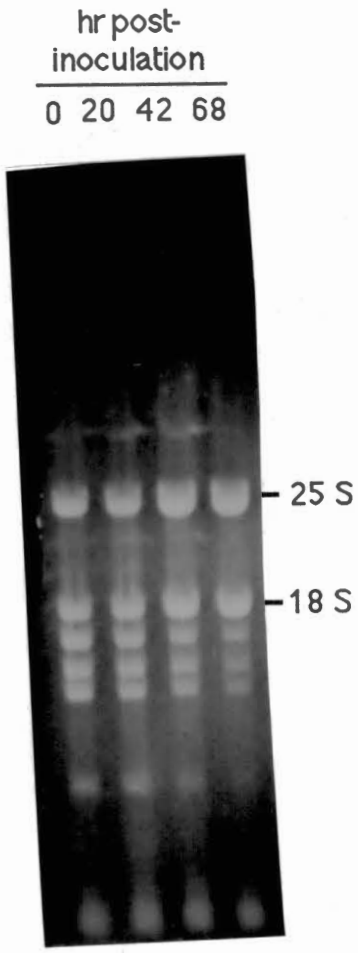
Time Course of RNA Synthesis. To characterize changes in the CaMV RNA population during viral replication, protoplasts were infected, mixed, and cultured in individual petri plates. At various times postinfection, cultures were harvested and aliquots of these cells were spotted to nitrocellulose to assay viral replication. RNA was isolated from the rest of the harvested cells. RNA pellets isolated from protoplasts contained little or no insoluble material. Crude RNA pellets were not reprecipitated before spectrophotometry and oligo(dT)-cellulose chromatography. The A_{260} / A_{280} ratio of each sample fell in the range of 1.7 to 1.9 (not shown). No correlation between the A_{260} / A_{280} ratios and the age of the harvested cultures was noted.

Polyadenylated RNA was purified from 200 μg of total RNA from protoplasts by oligo(dT)-cellulose chromatography. Nonpolyadenylated fractions were also collected. To examine RNA integrity, the nonpolyadenylated fractions of RNA were analyzed by electrophoresis and acridine-orange staining. Ribosomal RNAs showed very little smearing (Fig. 16). The relative intensities of the 23 S to 18 S RNA bands showed that these RNAs were present in the expected ratios. These observations suggested that these RNA samples had suffered little or no degradation.

Gel blot assays were used to characterize CaMV RNA in the polyadenylated fraction from each sample. Although this assay has limitations (discussed above), it allowed RNAs to be detected and sized and gave a rough estimate of transcript levels. RNA from infected protoplasts was transferred from gels to nylon membranes using electrotransfer rather than capillary transfer. Initially, the efficiency of electrotransfer was estimated using

Figure 16. Analysis of Integrity of RNA Isolated from Turnip Protoplasts at Various Times Postinoculation -RNA Time Course One.

RNA was extracted from the protoplast cultures described in Figure. 15. Polyadenylated and nonpolyadenylated RNAs were separated by oligo(dT)-cellulose chromatography. Aliquots (appr. 15 μg) of the nonpolyadenylated fractions were glyoxalated, separated by electrophoresis, and stained as described. The positions of the 25 S and 18 S cytoplasmic rRNAs are indicated.


hr post-
inoculation
0 20 42 68
25 S
18 S

radiolabeled DNA size standards. The electrotransfer of radiolabeled 8-Kb DNA fragments appeared to be quantitative (not shown). For the gel blot assay, transfer efficiency was estimated by examining the relative intensities of the bands produced by unlabelled DNA restriction fragments after hybridization. Although the DNA fragments showed smearing toward the wells, comparison of the relative signal from the 8 Kb and 4 Kb *Sal*I-standard fragments confirmed that the electrotransfer of DNA to nylon membranes (Fig.17, *Sal*I std) was as efficient or more efficient than capillary transfer. No aberrations in the relative intensities of the *Eco*RI-standard bands were noted (Fig. 17, EcoRI std). However, the sensitivity of the assay was impaired by the presence of background at the top of the membrane which was not lane-specific. Even so, detection of the 0.4 Kb restriction fragment (not shown) in 2 ng of *Eco*R I- digested pCS101 showed that as little as 0.07 ng of glyoxalated DNA could be detected.

A gel blot assay for tubulin RNA was used to assess RNA integrity and to determine the relative amounts of polyadenylated RNA present in each lane. After hybridization with the CaMV-specific probe and exposure to X-ray film, the membrane was stripped of the CaMV-specific probe and rehybridized with radiolabeled, cloned *A. thaliana* α3 tubulin DNA (pSRL 51; Ludwig et al., 1987). Using the tubulin-specific probe, the DNA standards gave a single major band with smearing toward the well (not shown). Thus, the vector-containing band of the DNA standards hybridized with vector sequence in the tubulin probe. The gel blot assay with the tubulin-specific probe detected a major RNA species in all of the RNA samples (Fig. 18). The migration of this RNA relative to size standards was consistent with its identification as an authentic tubulin transcript (Ludwig et al., 1987). In addition, a minor band of appr. four Kb was detectable. Because this band was present at the appr. position expected for the 25 S rRNA, this band was tentatively identified as a shadow-band artifact. Minor bands comigrating with the 25 S rRNA on gel blot assays of tubulin RNA have been observed by others (Ludwig et al., 1988). Very little tubulin RNA could be detected immediately after protoplast isolation and inoculation

Figure 17. Gel Blot Analysis of CaMV-specific RNA Present in Turnip Protoplasts at Various Times Postinoculation -RNA Time Course One.

RNA was isolated from turnip protoplasts at the indicated times after inoculation with CaMV virions. The polyadenylated fractions purified from 200 μg of RNA were glyoxalated and applied to the gel. Unlabeled restriction fragments of pCS101 (2 ng) were also glyoxalated and applied to the gel (SalI std and EcoRI std). Sizes (Kb) of the fragments are indicated. Gel blot analysis was performed using radiolabeled, cloned CaMV DNA(pCS101). The position of the 19 S CaMV RNA is indicated. Although the CaMV 35 S RNA is not visible, its expected position has also been indicated [-(35 S)].

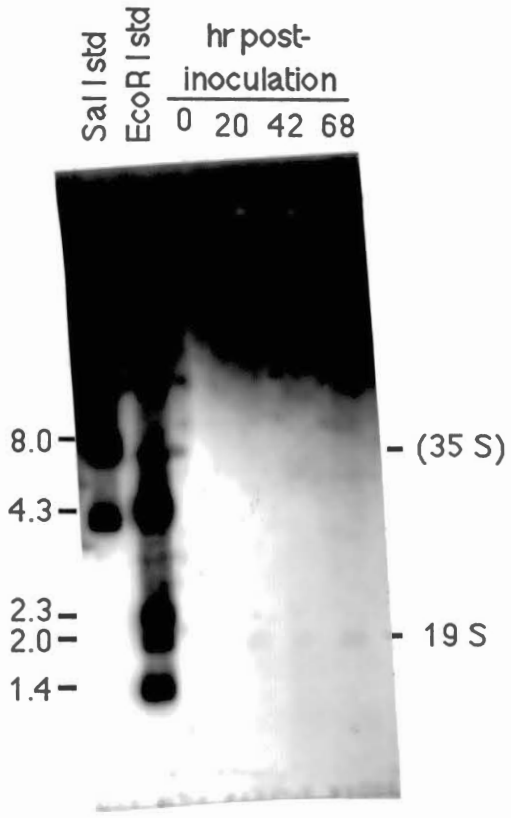
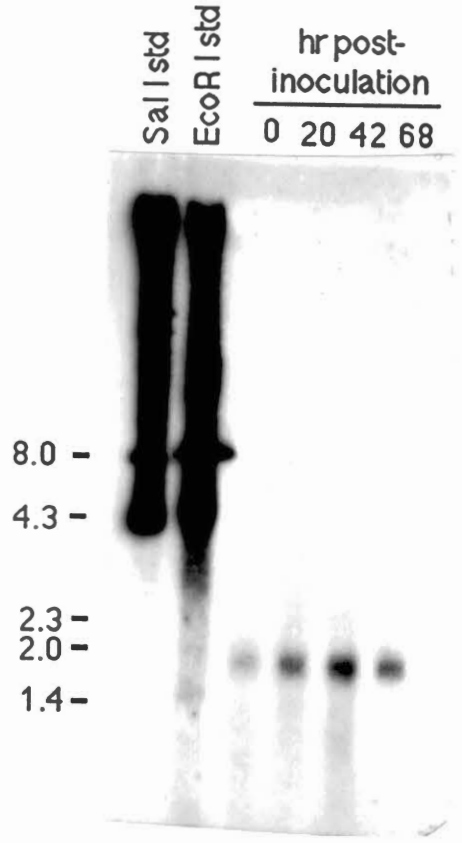

Sal I std
EcoR I std
hr post-
inoculation
0 20 42 68
8.0
4.3
2.3
2.0
1.4
(35 S)
19 S

Figure 18. Analysis of Tubulin RNA Isolated from Turnip Protoplasts at Various Times Postinoculation-RNA Time Course One.

The membrane described in Figure 17 was stripped of the CaMV-specific probe and reprobed using radiolabeled, cloned *Arabidopsis* α3-tubulin DNA (pSRL51). The sizes (Kb) and estimated positions of bands from the glyoxalated pCS101 restriction fragments (EcoRI std and SalI std; Figure 17) are indicated.


Sal I std
EcoR I std
hr post-inoculation
0 20 42 68
8.0
4.3
2.3
2.0
1.4

(Fig. 18). In RNA fractions isolated during subsequent culture, the tubulin band in the RNA sample isolated 42 hr postinoculation was more intense than the tubulin bands in the RNA samples isolated at 20 hr and 68 hr postinoculation. Although slight lane-specific smearing was noted above and below the tubulin-specific band, the tubulin RNA in each sample appeared to be reasonably intact (Fig. 18).

When radiolabeled cloned CaMV DNA was used to detect CaMV RNA in samples isolated from infected protoplasts, no signal was detected immediately following inoculation (Fig. 17, 0 hr post inoculation). Thus, the assay with this probe did not detect endogenous cellular transcripts or viral inoculum. CaMV-specific RNA was first detected 20 hr postinoculation and at all subsequent time points (Fig. 17). This RNA was identified as the CaMV 19 S RNA based upon its migration relative to DNA size standards. Comparison of the relative signals from the 0.4 Kb *Eco*RI band (not shown) and the 19 S RNA band suggested that appr. 0.07 ng of viral RNA were present in the lanes containing RNA isolated at 20, 42, and 68 hr postinoculation. The 19 S band in the RNA sample isolated at 68 hr postinoculation was slightly more intense than the bands observed in the RNA samples isolated at 20 and 42 hr postinoculation. The 19 S RNA was detected prior to the onset of DNA replication (c.f. 20 hr postinoculation in Fig. 17 and Fig. 15).

In this experiment, the CaMV 35 S RNA was not detected in any of the RNA samples isolated postinoculation (Fig. 17). Perhaps failure to detect the 35 S RNA in this experiment was due to the high background present at the top of the membrane. To determine if the CaMV 35 S RNA could be detected in RNA samples isolated from infected protoplasts, RNA was isolated in a separate experiment from 8×10^6 protoplasts at 24 hr postinfection and subjected to oligo(dT)-cellulose chromatography. Using a gel blot assay, the 35 S RNA, albeit slightly degraded, was detected in this sample (not shown). The CaMV 19 S RNA was also present at 24 hr postinoculation. To further confirm that the 35 S RNA was produced in infected protoplasts, RNA was again isolated in a separate experiment and subjected to oligo(dT)-cellulose chromatography. Although no CaMV

RNA was detected in RNA samples isolated at 24 hr postinoculation, both the CaMV 35 S and 19 S RNA were detected in RNA samples isolated at 60 hr postinoculation (not shown).

Because there seemed to be discrepancies regarding the detection of CaMV RNAs following inoculation with virions, a second analysis of the changes in the CaMV RNA population during viral replication in protoplasts was undertaken. When cells were inoculated, cultured, and assayed for viral replication, the low-resolution replication curves suggested that the lag time and replication rate were comparable to those observed in other experiments (c.f. Figs. 13, 15, and 19). RNA yield and quality were also comparable, although inadequate destaining of the gel may have prevented the detection of mild degradation (Fig. 20).

In the repeat, the gel blot assay with the CaMV-specific probe revealed that most of the *Sal*I DNA size standards were retained in the wells during electrophoresis. Therefore the estimation of transfer efficiency was not feasible (Fig.21, *Sal*I std). No aberrations in the relative intensities of the *Eco*RI-standard bands were noted (Fig. 21, *Eco*RI std).

As for the previous experiment, the gel blot assay with the tubulin-specific probe detected an authentic tubulin RNA in all samples (Fig. 22). Also consistent with previous observations, a minor tubulin-specific band of appr. four Kb was again detected. Comparison of the relative intensities of the signal from the *Eco*RI size standard and tubulin-specific signal from both experiments suggested that more polyadenylated RNA was bound to the membrane in the second experiment than in the first (Figs. 22 and 18, respectively). In both experiments, very little tubulin RNA could be detected immediately after protoplast isolation and inoculation (Fig. 18 and Fig. 22). For the repeat , a dramatic increase in tubulin band intensity was noted for the RNA sample isolated 60 hr postinoculation (Fig. 22). The samples isolated at 60 hr post inoculation and from an infected plant appeared to have 2 to 4 times more tubulin RNA than the samples from 17,

Figure 19. Replication of CaMV in Turnip Protoplasts-RNA Time Course Two.

Freshly-isolated protoplasts were infected and cultured as described in Materials and Methods. Triplicate aliquots of the culture (appr. 104 cells) were spotted onto nitrocellulose at the indicated times postinoculation. After all samples had been spotted, the membrane was treated with NaOH, neutralized, baked, and probed using 1 x 106 cpm of radiolabeled, cloned CaMV DNA (pCS101) per ml of hybridization solution. After three low stringency washes with 2X SSC / 0.1 % SDS at 25o C, the final stringency wash was performed at 25o C using 0.1X SSC / 0.1% SDS. The washed membranes were exposed to X-ray film; after autoradiography, each spot was excised and the bound radioactivity determined by liquid scintillation counting. Plotted points represent the means of triplicate samples.

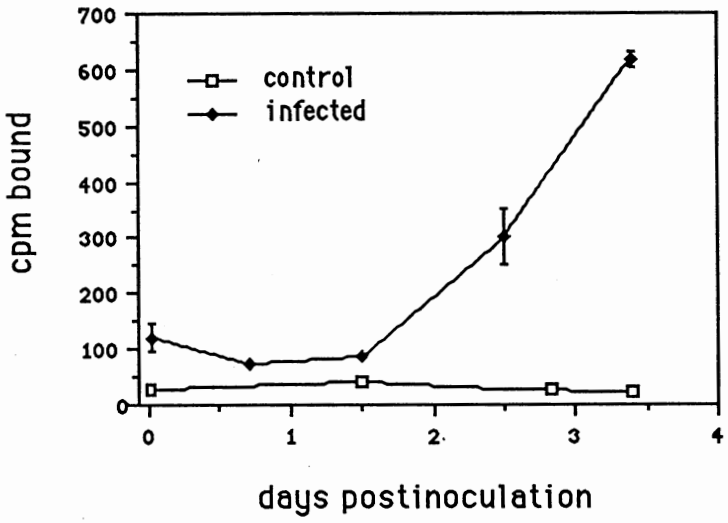
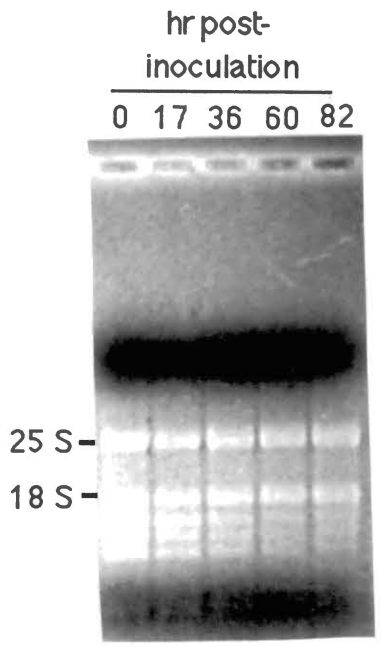

700
600
500
400
300
200
100
0
cpm bound
control
infected
0
1
2
3
4
days postinoculation

Figure 20. Analysis of Integrity of RNA Isolated from Turnip Protoplasts at Various Times Postinoculation -RNA Time Course Two.

RNA was extracted from the protoplast cultures described in Figure. 19. Polyadenylated and nonpolyadenylated RNA were separated by oligo(dT)-cellulose chromatography. Aliquots (appr. 7 μg) of the nonpolyadenylated fractions were separated by electrophoresis and stained as described. The positions of the undenatured 25 S and 18 S cytoplasmic rRNAs are indicated.


hr post-
inoculation
0 17 36 60 82
25 S
18 S

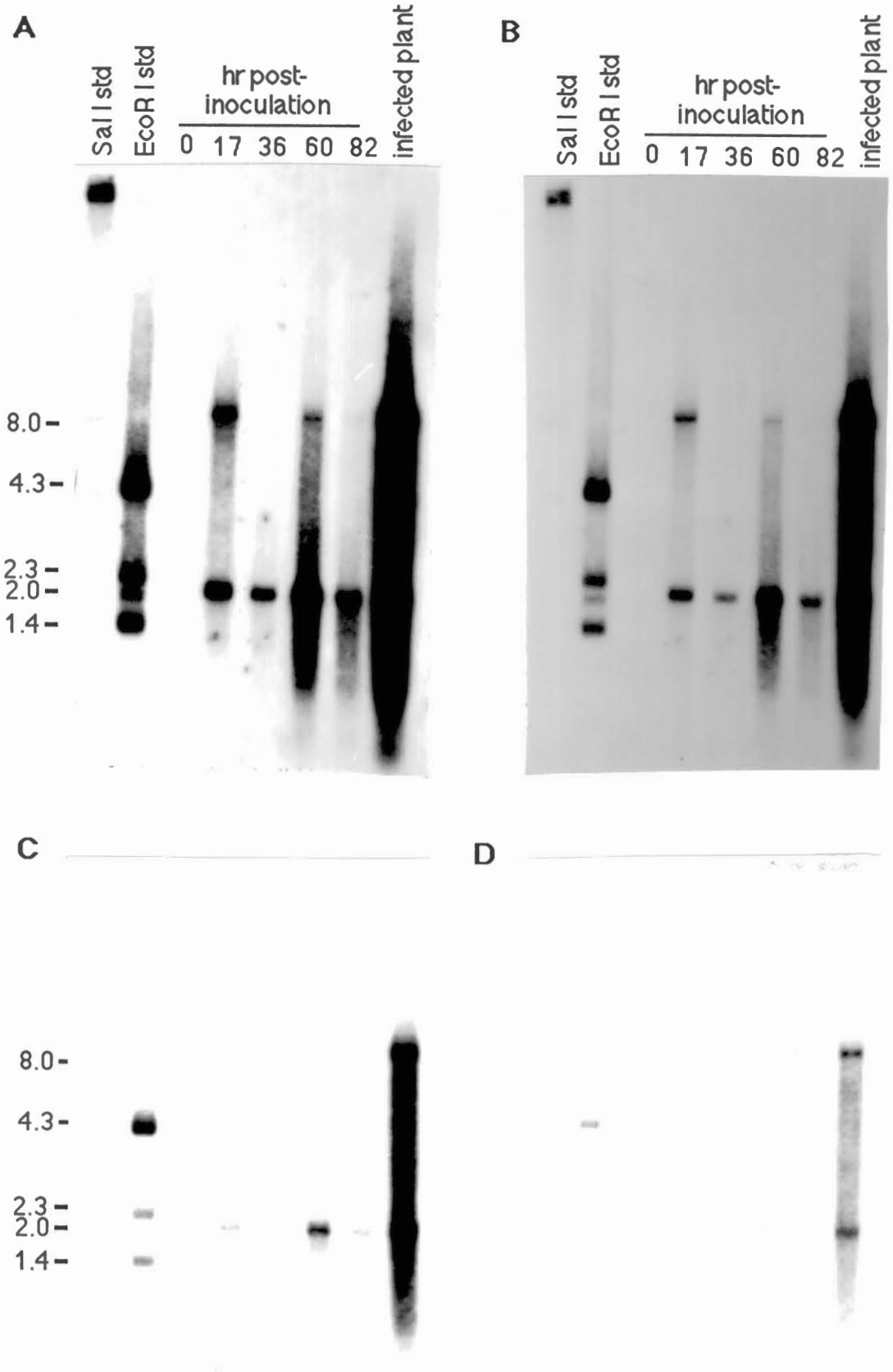
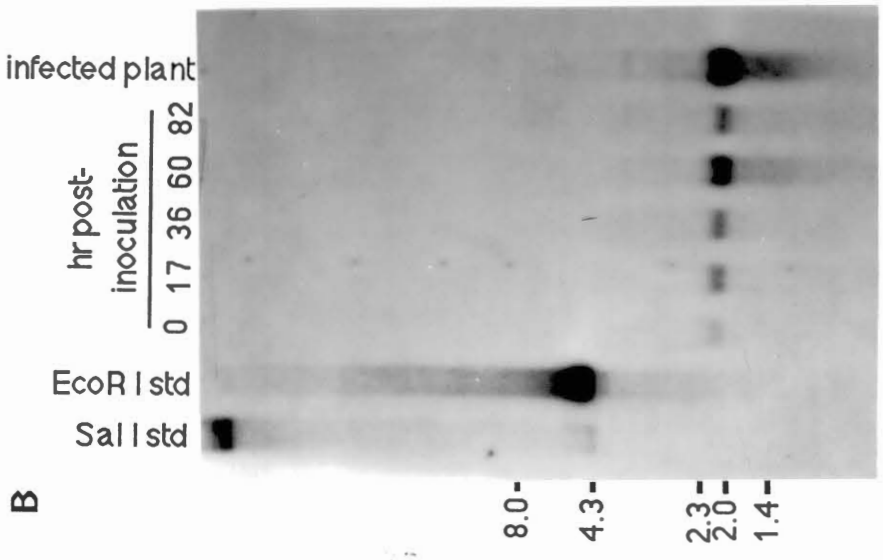
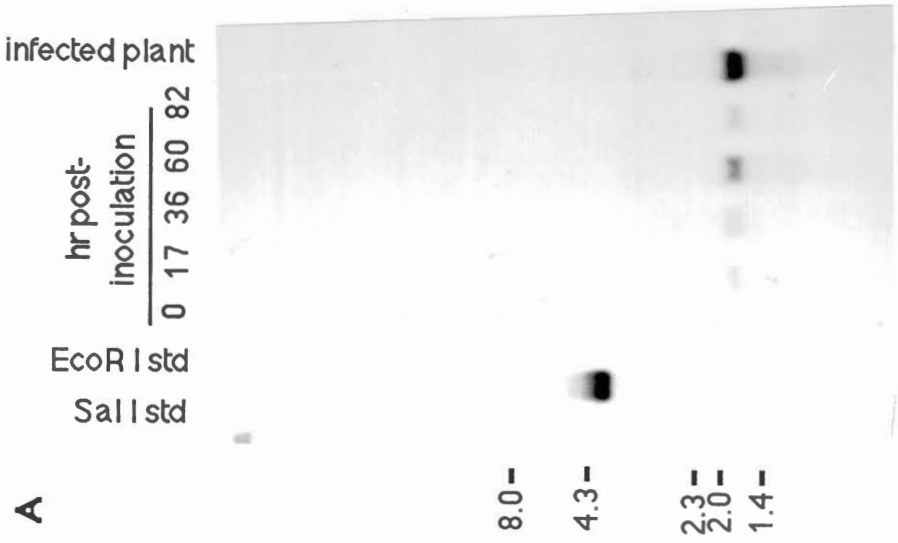

A
Sal I std
EcoR I std
hr post-inoculation
0 17 36 60 82
infected plant
8.0
4.3
2.3
2.0
1.4
B
Sal I std
EcoR I std
hr post-inoculation
0 17 36 60 82
infected plant
C
8.0
4.3
2.3
2.0
1.4
D

Figure 22. Analysis of Tubulin RNA Isolated from Turnip Protoplasts at Various Times Postinoculation -RNA Time Course Two.

The membrane described in Figure 21 was was stripped of the CaMV-specific probe and reprobed using radiolabeled, cloned Arabidopsis a3-tubulin DNA (pSRL51). The sizes (Kb) and estimated positions of bands from gyloxalated pCS101 restriction fragments are indicated (EcoRI std and SalI std). Panels A and B represent different exposures of the membrane to X-ray film to facilitate analysis of all samples.


B
infected plant
hr post-inoculation
0 17 36 60 82
EcoR I std
Sal I std
8.0
4.3
2.3
2.0
1.4


A
infected plant
hr post-inoculation
0 17 36 60 82
EcoR I std
Sal I std
8.0
4.3
2.3
2.0
1.4

36, and 82 hr postinoculation. Smearing characteristic of degradation was evident in the samples isolated at 60 and 82 hr and in the sample from the infected plant.

The increase in tubulin RNA content at 60 hr postinfection cannot be explained as an anomalously high recovery of polyadenylated RNA after oligo(dT)-cellulose chromatography, since the amount of polyadenylated CaMV RNA recovered from four aliquots of a single RNA sample varied only slightly (not shown). Similarly, in the RNA samples isolated from leaf tissues, the relative amount of CaMV RNA in each polyadenylated RNA fraction (Fig. 9) accurately reflected the relative amount of total CaMV RNA in each total RNA fraction (Fig. 5 and 7). Also, while an explanation for the loss of a portion of a polyadenylated fraction can be envisioned, it is more difficult to conceive of an explanation for greatly enhanced recovery. Thus, the increase in tubulin RNA content (Fig. 22) was probably not artifactual.

As in the previous experiment, the gel blot assay with the CaMV-specific probe detected no CaMV-specific RNA immediately following inoculation, although the CaMV 19 S RNA was detected at all times subsequent to inoculation (Fig. 21). The relative signals from the 2.3 Kb and 4.3 Kb *Eco*RI bands of the size standards and from the 19 S RNA band were compared visually (Fig. 21B). Such comparison suggested that appr. 0.3 to 2 ng of 19 S RNA were present on the membrane in each lane. Therefore more CaMV 19 S RNA, like the tubulin RNA, was detected in the polyadenylated RNA isolated from cells during the repeat of the experiment than in the RNA isolated during the original experiment. Expression of the 19 S RNA was slightly elevated at 17 hr postinoculation relative to 36 and 82 hr postinoculation (Fig. 21). In the RNA samples isolated at 60 hr postinoculation, expression of the 19 S RNA was greatly elevated. This increase was coincident with a large increase in tubulin RNA content (Fig. 22). Although these increases might be due to enhanced recovery of polyadenylated RNA following oligo(dT)-cellulose chromatography, the magnitude of the 19 S increase appeared greater than the magnitude of the tubulin increase (c.f. Fig. 21 and Fig. 22), suggesting that the trivial

explanation is insufficient to explain the change in 19 S level. The 19 S RNA, like the tubulin RNA, showed smearing characteristic of degradation in the samples isolated at 60 hr and 82 hr postinoculation and in the sample from an infected plant (c.f. Fig. 21 and Fig. 22). The 19 S RNA was detected before the onset of viral replication and did not accumulate relative to total cellular RNA (c.f. Fig. 17 and 21).

In contrast to the first experiment, the CaMV 35 S RNA was detected in the repeat of the experiment (Fig. 21). Estimates of the amounts of 35 S RNA present in the sample were precluded by the absence of a reliable estimate of transfer efficiency. The 35 S RNA, like the 19 S RNA, was first observed at 17 hr postinoculation, 19 hr before the onset of detectable DNA replication. However, by 36 hr postinoculation and at all subsequent time points, considerably less 35 S RNA was detected. The 35 S signal in the RNA sample isolated at 60 hr postinoculation was greater that the signal observed in the samples isolated at 36 hr and 82 hr postinoculation. In the RNA isolated at 60 and 82 hr postinoculation, the 35 S RNA showed smearing characteristic of degradation (Fig. 21). Therefore, the relative lack of 35 S RNA in these samples might be attributed to degradation. However, for the RNA isolated at 36 hr postinoculation, neither the 35 S RNA nor high molecular weight fragments characteristic of degraded 35 S RNA were detected (Fig. 21). Nor did the CaMV 19S and tubulin-specific bands in these lanes show smearing characteristic of degradation (Fig. 21 and Fig. 22, resp). Thus, the absence of the 35 S RNA in the sample isolated at 36 hr postinoculation was not due to degradation or loss of polyadenylated sample. In a separate experiment, the 35 S RNA could be detected at 60 hr postinoculation, but not at 24 hr postinoculation (not shown). In this experiment, the 35 S band was appr. twice as intense as the 19 S band, suggesting that, for a given time postinoculation, the amount of 35 S RNA relative to the amount of 19 S RNA varied from experiment to experiment.

Two minor CaMV-specific bands which migrated slightly ahead of the 35 S RNA band were detected in the repeat of the experiment (Fig. 21A). These bands were detected

in the RNA isolated at 17 hr postinoculation, but not in the RNA isolated at subsequent time points during this experiment. Visual inspection suggested that these minor bands were appr. one-tenth as intense as the 35 S band. These bands were not observed in the RNA sample isolated immediately after inoculation, suggesting that they were, in fact, due to CaMV-specific RNAs rather than endogenous cellular transcripts or inoculum DNA. Comparison of the migration of these minor bands to the migration of glyoxalated DNA size standards suggested that the large and small minor CaMV RNAs were appr. 7.4 and 6.6 Kb long, respectively. Because their migration was much slower than the migration expected for rRNA bands (not shown), these minor bands could not be explained as rRNA shadow-band artifacts.

The tubulin RNA content relative to total RNA was compared between inoculated protoplasts and infected plants. Visual inspection of the autoradiograms suggested that systemically infected cells had two- to six-fold more tubulin RNA relative to cellular RNA than did infected protoplasts (Fig. 22). The expression of 19 S RNA was also greater in the tissues isolated from infected plants (Fig. 21).

In the sample from an infected plant, the degradation observed for 35 S RNA and tubulin RNA were compared. While the tubulin-specific bands showed only minor smearing, the 35 S RNA showed extensive degradation (Fig. 22 and Fig. 21, respectively). This observation supported the hypothesis that the longer 35 S RNA was more susceptible to degradation than shorter RNAs.

Discontinuation of Experiments. Further analysis of changes in the CaMV RNA population during viral replication in protoplasts was not done. Difficulties with plant materials and time considerations forced the discontinuation of experiments. Analysis of the CaMV RNA populations isolated from protoplasts was therefore not repeated beyond the observations presented above.

Discussion

Two Novel CaMV RNAs ?

Both the CaMV 35 S and 19 S RNA are produced in infected protoplasts (Fig. 21, 17 hr postinoculation). Additionally, two novel CaMV-specific bands were observed (Fig. 21, 17 hr postinoculation). The relationship between these two minor RNAs and minor CaMV RNAs reported by other authors is unclear. The two minor RNAs reported here migrated at 7.4 and 6.6 Kb (Fig. 21), suggesting that they are not the minor RNA species of 4.9, 4.5, and 4.3 Kb which were reported by Condit et al. (1983). Similarly, the 7.4 and 6.6 Kb RNAs (Fig. 21) are too large to be the 22 S RNA hypothesized by Plant et al. (1985) and Hohn et al. (1990). Nor do the 7.4 and 6.6 Kb RNAs (Fig. 21) correspond to those reported by Covey and Hull (1981).

However, the 7.4 and 6.6 Kb RNAs (Fig. 21) may coincide with the 6.7 and 6.2 RNAs isolated from viral replication complexes by Thomas et al. (1985). The differences between the size estimates of Thomas et al. (1985) and my estimates for the sizes of these RNAs could be explained as minor variations in apparent electrophoretic mobilities. Although the RNA isolation procedure does not disrupt CaMV virions (Fig. 2), it is unclear as to whether or not viral replication complexes would be disrupted by this RNA isolation procedure. Some DNA replication intermediates can be recovered from infected protoplasts following phenol extraction in the absence of proteinase digestion, (Maule, 1985a; Thomas et al., 1985). Thus, RNA intermediates in the process of reverse transcription might be isolated during the phenol extraction step of the RNA extraction method used here. These 7.4 and 6.6 Kb RNAs might therefore correspond to RNA replication intermediates. Other minor species of 4.7, 3.4, and 2.6 Kb were also isolated from replication complexes (Thomas et al., 1985). Although such species were not detected in my work (Fig. 21), perhaps lane-specific background and rRNA shadow-banding prevented the detection of CaMV RNAs in these areas of the lanes.

Alternatively, the 7.4 and 6.6 Kb bands (Fig. 21) may represent minor mRNAs which code for CaMV gene products. These two minor RNAs were polyadenylated, consistent with a role in translation. Such messenger RNAs might be transcribed from novel subgenomic promoters. Alternatively, these minor RNAs could be mRNAs arising from splicing of the 35 S RNA, although splicing has no known role in caulimovirus gene expression.

Rather than representing authentic mRNAs, the 7.4 and 6.6 Kb RNA species (Fig. 21) might have arisen by spurious splicing of the 35 S RNA to generate nonfunctional minor RNA species smaller than the 35 S RNA. A strain of CaMV which accumulates a noninfectious variant representing appr. 25 % of the viral population has been described (Hirochika et al., 1985). This variant is thought to arise from reverse transcription of a spliced RNA intermediate. For FMV, a caulimovirus which shares many properties with CaMV, a noninfectious mutant representing 15 % of the viral population apparently arises from reverse transcription of a spliced version of the full-length RNA (Scholthof et al., 1991). Other CaMV mutants arising from reverse transcription of spliced RNAs have also been reported (Hohn et al., 1986; Melcher et al., 1986b; Vaden and Melcher, 1990; Pennington and Melcher, submitted). For CabbS, the CaMV strain used in my work, deletion mutants do not represent a major portion of the viral population. Thus, splicing of the CabbS 35 S RNA is infrequent or spliced RNAs are not normally utilized as templates for reverse transcription during CabbS replication. The failure to detect these minor RNAs in RNA samples isolated from whole plants might reflect differences in RNA splicing efficiency in protoplasts versus in plants. Perhaps imperfect intron consensus sequences are recognized with less specificity in protoplasts than in plants.

The failure to detect these RNAs in samples from whole plants might have other explanations. If these RNAs are expressed only in the early stages of viral replication, they would be difficult, if not impossible, to detect in RNA samples isolated from whole plants. A third explanation suggests that these minor bands might be obscured by the slight

smearing of the 35 S RNA band frequently observed in RNA samples isolated from plants (e.g. Odell et al., 1981). The significance of the 7.4 and 6.6. Kb minor RNA species (Fig. 21) must remain speculative, however, until these RNAs can be better documented and characterized.

Characterization of Cell Growth

Turnip protoplasts provided a system in which synchronous viral replication could be studied. However, it is important to acknowledge the unique physiological status of the cell populations utilized in these and other studies. Protoplasts undergo a number of major physiological changes during isolation and culture. Removal of the cell wall and osmotic shock induce responses similar to those induced by wounding (reviewed in Gould and Daines, 1985; Meyer , 1985). Subsequent exposure to growth hormones results in dedifferentiation and resetting of the cell clock, ie, synchronization of the cell population (reviewed in Meyer , 1985).

Increases in packed cell volumes of protoplast cultures were noted (Fig. 11), but the mitotic activity of these cells was not characterized. However, these cultures may have proceeded through at least a portion of the cell cycle. When explants of Jerusalem artichoke were cultured in the presence of growth hormones, the amount of total RNA in each explant increased appr. three-fold before the first synchronous division (MacLeod et al., 1979). However, if growth regulators were omitted from the medium, the total RNA content of each explant increased only slightly and cells did not divide. Thus, significant increases in RNA content occurred only in explants that were mitotically active. Therefore, the observation that turnip protoplast cultures accumulated RNA (Fig. 12) suggests that these cells were mitotically active.

A second observation supports the argument that the cells used here were synchronized and active in cellular DNA replication. These cells showed a sudden increase in tubulin RNA content after 60 hr of cultivation (Fig. 22). A similar, but less dramatic,

increase was also seen at 42 hr postinoculation in a separate experiment (Fig. 20). During the naturally synchronous mitosis of *Physarum polycephalum* , α - tubulin mRNA content begins to increase during G_2 , peaks during metaphase, and decreases rapidly thereafter (Schedl et al., 1984). In synchronized Zinnia mesophyll cells, tubulin content increases four-fold during DNA synthesis (Iwasaki et al., 1988). Similarly, in cucumber cotyledons, expression of β - tubulin RNA increases during cytokinin-induced cell division and elongation (Peng et al., 1990). These observations suggest that plant cells, like Physarum plasmodia, undergo transient increases in tubulin mRNA content during the cell cycle. Thus, in protoplast cultures, the increase in tubulin RNA content (Fig. 22) is consistent with the hypothesis that these cells were undergoing synchronous division. The differences in the magnitude of the increases observed in the two experiments (c.f. Fig. 20 and Fig. 22) could reflect a difference in the point of the mitotic cycle at which RNA samples were collected. The timing of mitosis, in turn, could vary among cells isolated and cultured in separate experiments.

The significance of the protoplast buds observed in these experiments is uncertain. The predominant viewpoint is that protoplast budding is an artifact due to the extrusion of the cytoplasm through a weak portion of the regenerating cell wall (Hanke and Northcote, 1974; Horine and Ruesink, 1972). However, Meyer and Abel (1975) reported that budding can involve the migration of nuclear material to the daughter bud. Turnip protoplast preparations extensively incorporate ^{3}H-thymidine into their DNA (Maule, unpublished-cited in Maule, 1985b). Also, turnip protoplasts produced buds in which CaMV antigen was detected, thereby suggesting that buds are competent to replicate CaMV (Maule, 1983). Thus, it is seems quite probable that the protoplast buds observed in my experiments were authentic cells rather than anucleate subprotoplasts.

Are Viral Replication and Cell Division Coordinated ?

The results presented here, and the results of other researchers, prompt me to propose that CaMV replication and gene expression are controlled by host mechanisms integral to cell division and the cell cycle. Regulation might act at transcriptional, posttranscriptional, translational, and / or posttranslational levels to stimulate CaMV replication in actively dividing cells. Alternatively, such regulation could function to repress CaMV replication in the absence of cell division.

The proposed coordination between the cell cycle of the host and CaMV replication and gene expression may reflect the presence of CaMV infection in the young dividing tissues of the host. Several observations suggest that CaMV replicates very close to the meristematic apex of systemically-infected hosts (reviewed in Maule, 1985b). During systemic infection, even very small leaves are fully symptomatic. CaMV symptoms are often sectored on the first leaves that show systemic symptoms (Melcher, 1989). Such sectoring is reminiscent of chimeric tissues observed by developmental biologists. CaMV antigen can be detected in 90-100 % of the protoplasts isolated from systemically infected leaves (Yamaoka et al., 1982; Maule, 1983). Results from cross-protection experiments (Zhang and Melcher, 1989-see Chapter III, discussion) are also consistent with the hypothesis that CaMV replicates very near the meristematic apex of systemically infected hosts.

The activity of the 35 S promoter may be linked to the cell cycle. Expression of reporter genes driven by the 35 S promoter, and by variants of the 35 S promoter constructed in vitro, has been examined using transient expression assays and transgenic plants. Although expression of the 35 S promoter was originally thought to be maximal in a variety of cell types, or "constitutive" (Odell et al., 1985), subsequent work has demonstrated that this promoter has a more complicated pattern of expression that is regulated by a combination of cis-acting elements (Benfey and Chua, 1990 and references

therein). Expression from this promoter is strongest in actively dividing tissues (Benfey et al., 1989; Fromm et al., 1989; Nehra et al., 1990) and young tissues (Williamson et al., 1989; Schneider et al., 1990). The 35 S promoter and the promoter of the wheat histone gene contain hexamer motifs for the binding of transcription factors (Lam et al., 1989; Mikami et al., 1989). These motifs and the transcription factors that bind to them are speculated to be important in cell cycle-dependent expression of histone genes (Kawata et al., 1990; Tabata et al., 1991). Nagata et al. (1987) and Fujiwara et al. (1991) have used transient assays of 35 S promoter-driven reporter genes to demonstrate that transcription from the 35 S promoter is cell-cycle dependent.

The observation that the 35 S promoter might be cell-cycle dependent could explain the observation that detection of the 35 S RNA at given times postinoculation was variable among experiments (c.f. Fig. 19 and Fig. 21). Additionally, in other experiments, the 35 S RNA was detected at 24 hr or 60 hr postinoculation (not shown). It is well accepted that the physiological state of a leaf is an important determinant of cell proliferation during *in vitro* culture (for references see Fitter and Drikorian, 1982; Peirik, 1987). Hence, the timing of cell division during protoplast culture might vary in response to the physiological state of individual plants or leaves. If expression of the 35 S RNA were cell-cycle dependent, then this variability might affect the expression of the 35 S RNA. Thus, the variability among experiments might be attributable to variable tissue response *in vitro* .

The transient increase in 35 S RNA content at 60 hr postinoculation (Fig. 21) may be related to cell-cycle specific 35 S promoter activity. The increase in 35 S RNA content was coincident with a very strong increase in tubulin RNA content (Fig. 22). Since tubulin RNA content might well be expected to increase during the S or G_2 phases of the plant cell cycle (discussed above), these results suggest that 35 S RNA content also increased during or near the S or G_2 phase of cell division. Such an interpretation is consistent with the observations of Nagata et al. (1987) and Fujiwara et al. (1991), who suggested that transcription from the 35 S promoter is activated during the S phase of cell division.

Expression of the 19 S RNA also appears to be linked to the protoplast cell cycle. A slight increase in 19 S RNA content was noted at 68 hr postinoculation (Fig. 19). In a separate experiment, a much larger, transient increase in 19 S RNA content was noted at 60 hr postinoculation (Fig. 21). These results imply that the levels of CaMV 19 S RNA, like the levels of CaMV 35 S RNA and tubulin RNA, are regulated in concert with the cell cycle.

The nature of the lag preceding CaMV replication can also be interpreted in light of a model in which CaMV replication is coordinated with the cell cycle. CaMV replication curves (Fig. 14), consistent with those observed by others (Maule, 1983), demonstrated that the onset of detectable CaMV replication is preceded by a lag period of appr. 48 hr (e.g. Fig. 14). Detection of CaMV RNA as early as 17 hr postinoculation (Fig. 21) demonstrates that transcription preceded the onset of replication by at least 19 hr. Kobayashi et al.(1990) detected CaMV proteins as early 12 hr after infecting protoplasts with CaMV virions. Maule (1985a) detected free CaMV DNA lacking the gaps characteristic of viral DNA as soon as 5 hr following inoculation of turnip protoplasts with virions, thereby suggesting that uncoating began well before the onset of replication. Although is not clear if the DNA species observed in Maule's work was actively transcribed, the detection of viral RNA as early as 17 hr postinoculation in the work presented here demonstrates that the lag duration does not simply represent the time required for uncoating of virions.

Although the lag preceding the onset of CaMV replication might be the minimum time required to accumulate the viral gene products necessary for the onset of replication, the data presented here and in other studies suggest an alternative explanation. An increase in the concentration of the viral inoculum did not decrease the two-day lag preceding the onset of viral replication (Fig. 14). Thus, the rate of accumulation of viral gene products was not the limiting factor responsible for the length of the lag, suggesting that the duration of the lag was instead determined by a host factor(s). Hussain et al. (1987) demonstrated that

different tissue culture media could have dramatic effects on the duration of the lag. However, the actual rate of CaMV DNA accumulation was unaffected by the different media. Thus, the CaMV replication curve could be separated into two components; the lag component and the replication component. The observation that the exaggerated lag was not simply due to inhibited CaMV replication suggests that this lag reflected a change in the expression of some critical factor for viral replication. Since the growth of cells in different tissue culture media varies widely, I speculate that the critical factor missing during the exaggerated lag time observed by Hussain et al. (1987) was related to cell division. A viral requirement for a cell factor related to cell division is consistent with the observation that cell viability was not sufficient for CaMV replication, since infected cells cultured in tissue culture plates were viable, but not competent for replication (Fig. 13). Although it has been suggested that CaMV replication in protoplasts might require active host DNA synthesis (Maule, unpublished-cited in Nagata et al., 1987), this possibility and its implications have not been explored.

Arguments against a relationship between the CaMV replication cycle and replication of host cells center on three points (Maule et al., 1989): CaMV replicates in turnip protoplasts which are thought to be mitotically inert, the patterns of accumulation of CaMV in leaf tissue (Maule et al., 1989) do not resemble those of the gemini virus ACMV (Fargette et al., 1987), and CaMV replicates in mature host tissues. The first point has been discussed above. The second point concerns the observation by Maule et al. (1989) that CaMV accumulated throughout the growth and expansion of systemically infected leaves and that older systemically infected leaves have greater CaMV contents than younger systemically-infected leaves. This was contrasted (Maule et al., 1989) with work of Fargette et al. (1987) who observed that in infected *Datura stramonium* L., younger leaves contained more ACMV than older leaves. Because the replication of geminiviruses is thought to be regulated in concert with the cell cycle of their hosts (Townsend et al., 1986), the difference between the patterns of CaMV and ACMV accumulation was taken as

evidence that CaMV replication is not regulated in concert with host cell division (Maule et al., 1989). However, this comparison seems rather arbitrary, since the replication cycles of these viruses are probably quite different. Additionally, subsequent work suggests that the DNA of a different gemini virus, TYLCV, accumulates throughout the growth and expansion of the leaf (Ber et al., 1990). Therefore, a comparison between caulimovirus and geminivirus replication in infected plants does not constitute evidence against a correlation between CaMV replication and host cell division.

Other types of regulation, such as tissue-specific response and photoinduction, are not excluded by the hypothesis that CaMV replication and gene expression are coordinated with the cell cycle of host cells. Similarly, this hypothesis does not rule out replication in nondividing tissues, since it must also be acknowledged that CaMV spreads through, and replicates in (Melcher et al., 1981) mature leaf tissues. Although many of the observations made during my experiments, as well as observations reported by other workers, can be explained in the context of this hypothesis, it should be stressed that they do not prove the hypothesis.

Other Mechanisms Controlling CaMV Gene Expression

The CaMV 19 S RNA content of cultures increased over the three- to four-day period of the experiments since CaMV 19 S RNA content did not decline relative to total RNA content during culture (Fig. 21) and the total RNA content of cultures increased during the period of culture (Fig. 12). Howell and Hull (1978), also concluded that the CaMV RNA content of infected protoplast cultures increases with time. If the amount of CaMV RNA detected at given times represents steady state levels, then increases in CaMV RNA content could result from increases in transcriptional template or increases in transcriptional activity of a constant amount of template. Additionally, such increases could result from both mechanisms. Alternatively, the increases in CaMV RNA content might reflect the accumulation of CaMV RNA under conditions in which the rates of RNA production and

degradation were not equal. However, the molecular basis of the observed increases in the CaMV RNA content of protoplast cultures cannot be addressed by the data presented here.

In infected protoplasts, expression of CaMV RNAs may be controlled by different mechanisms at various times postinfection. Expression of the 19 S RNA decreased slightly between 17 hr and 36 hr postinoculation (Fig. 21). Expression of the 35 S RNA also decreased at this time. A similar decrease was not seen between these time points for tubulin expression (Fig. 22), suggesting that the cell cycle is not the only determinant of CaMV RNA levels. Candidates for alternative mechanisms include those that govern cell wall regeneration or wound responses. The observation that 35 S promoter is wound-inducible in transgenic plants (Barnes, 1990) might be related the the observation that 35 S RNA levels were greatly elevated at 17 hr postinoculation.

The level of expression of the 35 S RNA relative to the 19 S RNA was not invariable. Expression of the 35 S RNA in infected protoplasts dropped dramatically between 17 and 36 hr postinoculation (Fig. 21). However, expression of the 19 S RNA decreased only slightly. Differences in 35 S RNA expression relative to 19 S expression also occur within a single host plant (Fig. 9). Since changes in the relative amounts of these two RNAs occur, the expression of these two RNAs are not controlled by identical mechanisms.

Covey et al. (1990) demonstrated that the amount of 35 S RNA relative to 19 S RNA in RNA samples isolated from infected turnip leaves was quite different from the relative amounts of these two RNAs in samples from infected rape leaves. Higher 35 S to 19 S ratios were characteristic of RNA isolated from turnips, the more susceptible host. Shewmaker et al. (1985) transformed host and nonhost plants with CaMV DNA sequences. They demonstrated differences in the level of "35 S" RNA relative to "19 S" RNA in transgenic hosts versus nonhosts. Thus, the relative levels of the two major CaMV RNAs vary from host to host.

Although Covey et al. (1990) and Shewmaker et al. (1985) demonstrated host-specific differences in the relative expression of the 35 S and 19 S RNAs, differences in the

relative expression of the two major CaMV RNAs can be affected by factors other than the genome of the host, since such differences occur within infected protoplasts (Fig. 21) and among individual leaves of infected plants (Fig. 9). The mechanisms responsible for determining the levels of expression of the 35 S and 19 S RNA remain unknown. Although work with transgenic plants has failed to demonstrate "random" changes in 35 S promoter strength (Williamson et al., 1989; Barnes, 1990; Schneider et al., 1990), perhaps a factor not characterized in my study, such as nonuniform shading or undetected wounding, affected transcription of the 35 S RNA *in planta* . Alternatively, 35 S and / or 19 S RNA levels might be controlled posttranscriptionally. Cytoplasmic levels of histone and tubulin RNAs, both of which are expressed in concert with the cell cycle, are subject to posttranscriptional control (reviewed in Cleveland, 1988 and Marzluff and Pandey, 1988, respectively). Additionally, although there appeared to be no correlation with changes in CaMV RNA levels and the onset of viral replication, viral processes might function in determining the varying levels of 35 S and 19 S RNA expression. Furthermore, changes in the amounts of 35 S and 19 S RNA that were detected might reflect changes in the polyadenlyation state of these transcripts.

The mechanisms controlling the expression of the 35 S and 19 S RNA, while not identical, may share overlapping features such as transcriptional factors. The observation that the relative expression of these two RNAs is variable suggests that such variation might have functional significance. The function of this variability remains open to speculation.

Summary

Many observations regarding the biology of CaMV can be interpreted in light of a model in which the CaMV replication cycle is linked to the cellular replication cycle. Although such a model is speculative, it can be tested. To address this model, the mitotic index of the infected cells would need to be correlated with the time course of CaMV RNA

production in protoplasts. The system described here, particularly the introduction of a nuclease protection assay that differentiates between the major CaMV RNAs, is suited to such an investigation. Additionally, cell culture systems which inhibit cell division, particularly growth media lacking plant growth hormones or cell cultures in which specific stages of the cell cycle are blocked with inhibitors, could be used to investigate this theory. Additionally, the differential expression of the two major CaMV RNAs *in planta* can now be reliably investigated using the nuclease protection assay developed here.

CHAPTER III

CROSS PROTECTION BETWEEN STRAINS OF CAMV

Introduction

Definitions of Cross Protection

Cross protection can be broadly defined as the protection of a plant from viral disease by a previous inoculation with a related virus. In this review, cross protection will be defined by two requirements: the protecting virus and the challenge must be closely related and the protecting strain must be present in the same tissues or cells as the challenge to interfere with the replication of the challenge (Palukaitits and Zaitlin, 1984). However, the effects of nonspecific interference during "true" cross protection should probably not be disregarded when considering hypotheses for the mechanisms of cross protection.

Cross protection has been used to protect a number of crop plants from severe viral disease (reviewed in Palukaitis and Zaitlin, 1984). Increased understanding of the principles underlying cross protection might enhance the application of cross protection as an economical and practical method for protecting crops and might provide valuable insights into viral biology. Although very little cross-protection work has been done with CaMV, this virus presents unique opportunities for enhancing our understanding of the mechanisms by which cross protection functions. Additionally, studies of cross protection between strains of CaMV might increase our understanding of CaMV biology.

Some Basic Observations from Other Systems

Cross protection has been extensively reviewed (Hamilton, 1980; Fulton, 1982; Sherwood, 1987). The quantitative and qualitative aspects of protection are highly dependent upon the identities of the viruses and hosts in question. Protection may or may not be reciprocal between two given strains. Additionally, protection between two strains may act by different mechanisms in different hosts (Rezende et al., submitted). Protection from disease may involve the partial or complete suppression of challenge replication. Protection may be temporary: although the challenge is inhibited initially, upon prolonged cultivation the host may develop symptoms.

Models for the Mechanisms of Cross Protection.

Several mechanisms might prevent the establishment and spread of the challenge (reviewed in Palukaitis and Zaitlin, 1984; Sherwood, 1987). These mechanisms might operate individually or in concert. Some possible mechanisms of cross protection include: specific inhibitors of viral replication, altered cellular metabolism, exhaustion of cellular resources, sequestration of viral molecules, inhibition of virion uncoating, and inhibition of the transport of the infectious entity. These mechanisms will be discussed in more detail below.

Protecting inoculations might induce changes in cell metabolism, including the production of molecules that are directly inhibitory or which signal general changes in the host (reviewed in Sherwood, 1987; White and Antinow, 1991). Examples might include the induction of pathogenesis-related proteins or phytoalexins, the production of a"dark green agent" observed in the systemic mosaic symptoms of TMV-infected tobacco, or the production of other molecules. Such molecules might act to inhibit viral replication in a nonspecific or specific fashion.

Infection by the challenge virus might also be inhibited if the protecting virus depleted factors essential for viral replication (Kohler and Hauschild, 1950). Candidates for such limiting factors include metabolic precursors, ribosomes (Ross, 1974), or auxiliary host proteins (Palukaitis and Zaitlin, 1984).

Alternatively, molecules of the challenge virus that are essential for challenge replication might be sequestered by the preexisting molecules of the protecting virus. Models invoking sequestration of the challenger's RNA by the protecting virus's coat protein (de Zoeten and Fulton, 1975) or complementary "antisense" RNA (Palukaitis and Zaitlin, 1984) have been proposed. Irreversible binding between the replicase of the protecting strain and the challenger's RNA might also inhibit the replication of the challenger's RNA (Gibbs, 1969).

Sherwood and Fulton (1982) demonstrated that uncoating of the challenge was inhibited during cross protection between strains of TMV. On cross-protected plants, viral RNA was more infectious than RNA encapsidated in coat protein. This observation led to development of the field of coat protein-mediated cross protection (for references see Wisniewski et al., 1990).

The mechanisms which prevent initial infection of a protected leaf or cells may also function to prevent spread of the challenge when protection is only partial or breaks down. The failure of TMV to spread within a systemically infected leaf from light green areas to dark green areas suggests a blockage of transport (reviewed in Sherwood, 1987). Additionally, transgenic plants which produce TMV coat protein seem to show impaired virus transport which may be due to the inhibition of virus replication (Register et al., 1989; Wisniewski et al., 1990).

One aspect of cross protection that has received little attention is the mechanisms by which viruses limit their own replication within host cells. The mechanisms which down-regulate replication of the protecting strain, or the division of viral replication into discrete stages, might function to prevent replication of challenge strains (Ishikawa et al., 1991).

Observations with CaMV.

Cross protection between strains of CaMV has been documented at the symptom (Tomlinson and Shepherd, 1978) and DNA levels (Zhang and Melcher, 1989). Incomplete protection due to poor infection of the host or weak replication of the protecting strain was demonstrated using mechanical or aphid-mediated transmission of infection on brussell sprouts (Tomlinson and Shepherd, 1978). Such protection was incomplete after 20 days of propagation of the protecting strain but was absolute after a 25 day incubation before challenge. Zhang and Melcher (1989) isolated CaMV DNA from infected turnips to examine cross protection between two isolates of CaMV. Absolute protection from challenge was demonstrated when leaves above the initially inoculated leaf were challenged between 2 and 8 days after the initial inoculation. Cross protection between strains of CaMV was not temporary since the symptoms (Tomlinson and Shepherd, 1978) or DNA (Zhang and Melcher, 1989) of the challenge virus remained suppressed upon prolonged cultivation.

Statement of Purpose

CaMV has several unusual features which provide unique opportunities in the field of cross protection, including: the unique mode of replication, the ease of detecting strain-specific differences between isolates, the variety of molecules that can be assayed, the ability to very easily differentiate between encapsidated and free nucleic acid, the documented time course of CaMV DNA uncoating and synthesis (Maule, 1985a), and the ubiquitous presence of CaMV in systemically infected leaves. These features might provide unique insight into the mechanisms which underly cross protection. Additionally, cross protection phenomena might illuminate mechanisms which control CaMV gene regulation. These possibilities justified an initial investigation of cross protection between strains of CaMV at the whole plant and single-cell levels. This study hypothesized that

cross protection between strains could be demonstrated using a simplified assay system. Since CaMV particles are present in most or all cells of systemically infected leaves (reviewed in Maule, 1985b), it was also expected that protoplasts isolated from systemically infected leaves could be used to examine whether or not cross protection operates at the cellular level.

Materials and Methods

Reagents

The highest quality reagents available in the laboratory were used. Specific sources are noted where such sources are considered to be especially important for the success of the experimental technique.

Plant Inoculation and Growth.

Turnip plants were grown and inoculations with virions were performed as previously described (Gardner et al., 1980). Alternatively, inocula for Ca-NB2 was prepared by inoculating turnip plants with CaMV DNA cloned in bacterial plasmids and linearized immediately before inoculation (Zhang and Melcher, 1989). Lysates of systemically symptomed leaves from these plants were used as inocula to generate plants that were cross-protected by this viral strain. For cross-protection challenge, infected turnip plants (45 days old) were inoculated with virions (Gardner et al., 1980) 17 days after the initial inoculations.

For inoculation with viral RNA, a single 3 cm leaf of a 4 week-old turnip was inoculated with 20 µl of TVCV RNA at a concentration of 0.3 µg / µl in celite and phosphate buffer, as described for CaMV virion inoculations (Gardner et al., 1980).

Purification of Virions and Viral RNA.

CaMV virions were prepared and purified as described by Hull et al. (1976). TMV 204 virions were the generous gift of William Dawson's lab. Purified TVCV virions were prepared by the method of Thompson et al. (1988) for the preparation and purification of potyviruses. RNA was isolated from the virions of TVCV or TMV by the Proteinase K / SDS method for CaMV DNA isolation (Gardner et al., 1985). Viral RNA (5 µg) was treated with 1 U/µl RNAase-free DNAase (BRL) following the recommendations of the enzyme supplier before being used as inocula on plants or as template for cDNA synthesis. RNA digestions were done with 10 µg / ml RNAase in 10 mM Tris HCl, pH 7.5, 10 mM EDTA, for 20 min at 37° C.

Infection and Superinfection of Protoplasts

Protoplasts were isolated as described in Chapter II from the youngest fully expanded leaves of healthy or infected plants three to four weeks after inoculation. Freshly isolated protoplasts were transfected with virions of CaMV or TMV by the method of Maule (1983) and cultured as described in Chpt. II.

Development of Dot Blot Assays of Superinfection
in Whole Plants or in Protoplasts

A simple dot blot assay was developed to detect CaMV CabbS or RNA-virus superinfection in turnip plants. Differences among three CaMV isolates were exploited to develop strain-specific plasmid-DNA probes. CabbS DNA (Franck et al., 1980) carried the gene which codes for the wild-type aphid-transmission factor in its ORF II. In contrast, the DNA of the Ca-NB2 isolate of CaMV carried a bacterial DHFR gene in place of the wild-type ORF II (Brisson et al., 1984). The CM4-184 strain of CaMV had a deletion of most of its ORF II (Howarth et al., 1981). Lysates of plant tissue were assayed

for viral nucleic acids as described by Maule et al. (1981) using the probes described below. These same probes were also used to detect viral replication in protoplasts by the method described by Hussain et al. (1985).

Except where otherwise noted, all plasmids were constructed using the cloning techniques recommended by Maniatis et al. (1982). Recombinant plasmids were identified by restriction analysis and / or limited DNA sequencing.

Specific Detection of CabbS DNA. A plasmid subclone of the CabbS ORF II formed the basis for a simple assay of CabbS superinfection. The plasmid pSH113 was created by inserting the 129 nt *Hin*dIII / *Ava*I fragment of pCS101 into the corresponding sites of pGEM3Z. The plasmid pSH113 also contained a short repeat at the *Ava*I site resulting from the blunt-end ligation of two end-filled *Ava*I termini. pSH113 therefore contained 133 bp of pCS101-derived DNA corresponding to nt 1513 through nt 1646 of the CabbS viral DNA (numbering of Franck et al., 1980). The orientation of the insert was such that synthesis of RNA or DNA primed from the T-7 promoter / primer would result in a a fragment of RNA / DNA complementary to the viral coding (+) strand.

Hybridizations were done with 5 x 10^6 cpm / ml of radiolabeled (Feinberg and Vogelstein, 1983) pSH113 DNA to detect CabbS-specific signal. Hybridization conditions were the same as those described in Chpt. II. Stringency washes were done at room temperature using 0.1 X SSC / 0.1 % SDS.

Specific Detection of Ca-NB2 DNA. A plasmid subclone of the Ca-NB2 ORF II formed the basis for a simple assay of Ca-NB2 infection. The plasmid pSH107 contained the 345 bp *Bam*HI / *Xho*I fragment of pCa-NB2 (Brisson et al., 1984) cloned into pGEM3Z at the *Sal*I / *Bam*HI sites. This fragment included the DHFR gene which replaced the CaMV ORF II in pCa-NB2. pSH107 was digested with *Ava*I and *Sac*I and then digested with exonuclease III from the vector-derived *Ava*I site through the *Bam*HI site to remove appr. two-thirds of the insert, including all of the CaMV ORF III. After

digestion with S1 nuclease and religation, the resultant plasmid, pSH111, contained 108 nt of sequence derived from pCa-NB2 corresponding to the 5 ' portion of the Ca-NB2 viral ORF II. The first nucleotide of the *Xho*I recognition site just 5 ' of the DHFR initiation codon in pCa-NB2 was arbitrarily defined as nt 1; pSH111 carried a portion of the pCa-NB2 derived DNA from nt 2 to nt 109. The orientation of the insert was such that synthesis of RNA / DNA primed from the T-7 promoter / primer would result in a fragment of RNA / DNA complementary to the viral coding (+) strand.

Hybridizations were done with 5 x 10^6 cpm / ml of radiolabeled pSH111 DNA to detect signal specific for Ca-NB2. Hybridization conditions were the same as those described in Chpt. II. Stringency washes were done at room temperature using 0.1 X SSC / 0.1 % SDS.

Specific Detection of the Nucleic Acids of RNA Viruses. RNA viruses were detected as described by Maule et al. (1981). Nitrocellulose membranes were soaked in 10 X SSC and air-dried. Lysates of leaves were prepared and applied to the treated membranes and the membranes were baked, prehybridized, and hybridized as described in Chpt. II. Radiolabeled cDNA complementary to TVCV RNA was prepared by reverse transcription. Reverse transcription reactions were performed by annealing 80 ng of random hexamer (Promega) with 500 ng of RNA in 12 µl of RNAase-free water for 5 min at RT. RNA was then reverse transcribed with 10U / µl MMLV reverse transcriptase (BRL) in the presence of 1 x reverse transcripase buffer (BRL), 1 U / µl RNAasin (Promega), unlabeled deoxynucleotides (dATP, dGTP, and dTTP at 0.5 mM each) and [^{32}P] α-dCTP (0.6 µM, 800 Ci / mmole) in a final volume of 20 µl. After a 1 hr incubation at RT, the reaction was brought to 100 µl with water and extracted with one volume of buffered phenol / chloroform (5:1 v/v) and precipitated with 25 µg of tRNA, 1/2 volume of 4 M ammonium acetate and 2.5 volumes of ethanol. The probe was dissolved in water, denatured with 0.1 N sodium hydroxide for 5 min at 42 °C, and used in hybridizations at concentrations of 1 x

10^6 to 2 x 10^6 cpm per ml of hybridization solution. Alternatively, the nucleic acid of TMV 204 (Dawson et al., 1986) was detected using a radiolabeled (Feinberg and Vogelstein, 1983) plasmid subclone of the TMV 204 (Dawson et al., 1986) 30 K gene at 5 x 10^6 cpm per ml of hybridization solution. This subclone contains the TMV 30 K protein inserted between the *Xho* I and *Pst* I sites of pUC129 and was a generous gift from the lab of William Dawson. Hybridization conditions were the same as those described in Chpt. II. Stringency washes were done at room temperature using 0.1 X SSC / 0.1 % SDS.

Results

Detection and Characterization of a Contaminating Virus

During cross-protection experiments using a preparation of virions (designated R59) thought to be the Ca-NB2 strain of CaMV, a discrepancy between spectrophotometric data and dot blot hybridization signal was noted. Although material which absorbed light at 260 nm was abundant, nucleic acid hybridizations detected little or no CaMV-specific nucleic acid (not shown). To address this discrepancy, undisrupted virions were subjected to electrophoresis through agarose. Virions from this preparation migrated considerably faster than authentic CabbS virions (Fig. 23) or authentic CM4-184 virions (not shown). This suggested that, while the preparation contained virions, these virions were not CaMV. Agarose gel electrophoresis of undisrupted virions from preparations of earlier passages (preparations designated U865 and U967) allowed the presence of the contaminant to be easily traced back through two previous rounds of virus isolation and passage (not shown).

The preparation examined in detail (designated R59) was almost homogeneous (Fig. 23). Thus, the nucleic acid and coat protein of the predominant virus in this preparation could be characterized without further purification of the virus. SDS-PAGE was used to size the major capsid protein of the contaminant. The apparent MW of the contaminant's coat protein was 17.5 kD (Fig. 24B). The coat protein of the contaminant

Figure. 23. Electrophoretic Mobility of the Contaminant Virions.

The relative mobilities of CaMV CabbS virions (CaMV), contaminant virions (contaminant), and BRL "1-Kb ladder" DNA size standards (stds) were analyzed by electrophoresis through 0.75 % agarose gels which were prepared and run with 1 X TAE buffer. Two µg of virions were loaded / lane and electrophoresis was performed for 30 min at a field strength of 7.7 V / cm using a MINNIE Submarine Agarose Gel Unit(Hoefer Model HE 33). Following electrophoresis, the gel was stained for 30 min with 1 µg / ml ethidium bromide and photographed under UV excitation. The size (Kb) of selected bands of the size standards is shown.

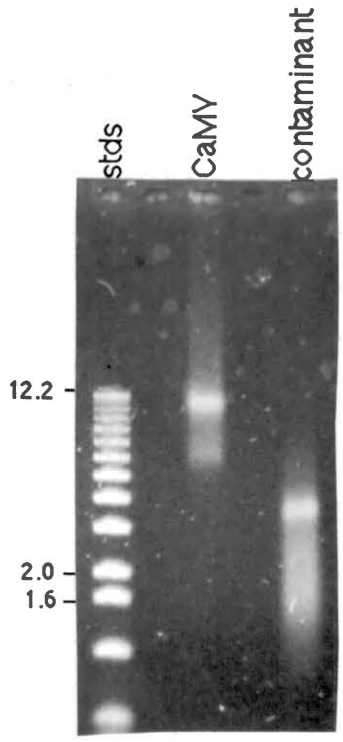
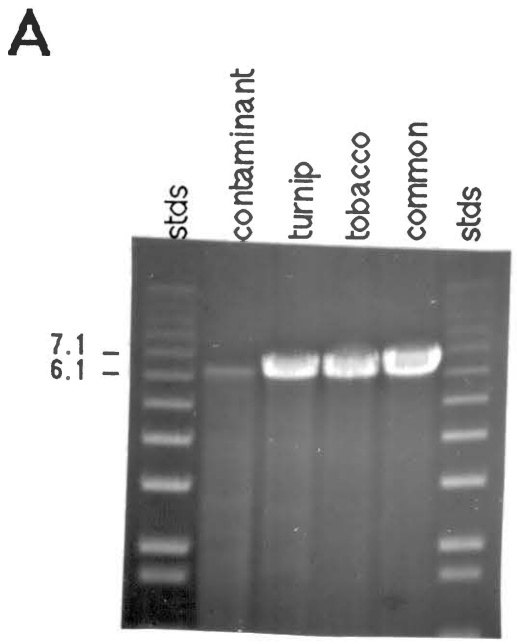
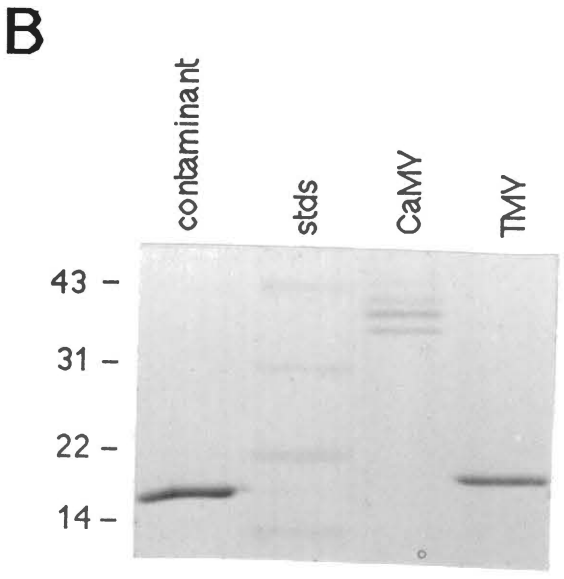

stds
CaMV
contaminant
12.2
2.0
1.6

Figure. 24. Characterization of the Contaminating Viral Nucleic Acid and Capsid Protein.

(A) Nucleic acid was isolated from virions by digesting the virions with Proteinase K in the presence of SDS. Nucleic acids were purified by extracting with phenol / chloroform (5:1) and precipitated with ethanol (Gardner and Shepherd, 1980). The nucleic acid from the original contaminated preparation (contaminant), the nucleic acid from the common strain of TMV (common), and linear DNA fragments (stds; BRL "1 Kb ladder") were analyzed on 1 % agarose gels using glyoxal and acridine orange as described by McMaster and Carmichael (1977). Purified viral RNA was also treated with DNAase and used to inoculate turnips. Homogenates of leaves which developed systemic symptoms were then used to inoculate turnips or tobacco. Virions were then isolated from this second group of plants after the development of systemic symptoms. The nucleic acid component of these passaged virions was then analyzed (turnip and tobacco, resp.). The sizes (Kb) of selected bands of the size standards are shown.
(B) The protein component of the virion was analyzed by SDS-PAGE as described in Materials and Methods. Appr. 3 μg of purified virions from the contaminated preparation (contaminant) were boiled in the presence of SDS and subjected to electrophoresis on discontinuous 12 % polyacrylamide gels. An equivalent amount of the CM4-184 strain of cauliflower mosaic virus(CaMV), the common strain of tobacco mosaic virus (TMV), and SDS-PAGE size standards (stds) were also applied to the gel. The gel was stained with Coomassie blue and destained to visualize the protein bands. The MWs (kD) of the size standards are shown.


A
stds
contaminant
turnip
tobacco
common
stds
7.1
6.1


B
contaminant
stds
CaMV
TMV
43
31
22
14

migrated slightly faster than the coat protein of the U-1 strain of TMV (TMV 204-Dawson et al., 1986). When the nucleic acid of the virus was purified and analysed by agarose gel electrophoresis and acridine-orange staining, an orange band typical of single-stranded nucleic acids (McMaster and Carmichael, 1977) was observed (not shown). This nucleic acid was RNAase sensitive (not shown). The nucleic acid of the contaminant was glyoxalated and separated by electrophoresis through agarose. The migration of this RNA relative to glyoxalated DNA size standards suggested that the single-stranded RNA of the contaminant was 6.4 Kb long (Fig. 24A). The migration of this RNA was indistinguishable from that of RNA isolated from TMV U-1 virions (Fig. 24A).

The nucleic acid of the contaminant was resistant to DNAase treatment (not shown). To obtain a source of the contaminant free of CaMV virions, the DNAase-treated nucleic acid from the contaminated preparation was used to inoculate a turnip plant. Unlike turnip plants inoculated with the contaminated preparation, this plant did not develop typical CaMV symptoms. Instead, this plant appeared symptomless until four weeks postinoculation. At this time, mild vein-clearing symptoms became apparent (not shown). These symptoms became more pronounced after cultivation of the plant for an additional four weeks. Inoculum containing the purified virus was preserved by freezing young symptomed leaves taken from this plant at 8 weeks postinoculation. RNA was extracted from virions that were isolated from symptomed plants that had been inoculated with this frozen inoculum. The RNA from passaged virions appeared to be homogeneous and comigrated with the RNA extracted from the original contaminant (Fig. 24A). Thus the frozen inoculum contained the causal agent of vein-clearing symptoms.

A different virion preparation was isolated from infected turnip plants that had been inoculated with an inoculum thought to be TuMV. These plants showed vein-clearing symptoms rather than the mosaic expected for TuMV. These vein-clearing symptoms were identical to those observed on plants inoculated with the RNA of the contaminant described above. The concentration of this putative TuMV preparation, estimated by its absorbance at

260 nm, suggested a yield which was three times the expected (Thompson et al., 1988) yield for TuMV. Because the possibility existed that this TuMV inoculum was present in growth chambers coincident with the first appearance of contaminated CaMV preparations (June and July of 1985, experiments designated U733, U755, and U865), the relationship between the virus present in the contaminated CaMV preparation and the virus prepared following inoculation with the TuMV inoculum was examined.

RNA was isolated from the virions present in the contaminated CaMV preparation and from the virions prepared following inoculation with the TuMV inoculum. These nucleic acids showed indistinguishable migrations following glyoxalation, agarose gel electrophoresis, and acridine-orange staining (not shown). These nucleic acids were transferred to nylon membranes and analyzed by hybridization with radiolabeled cDNA prepared from the RNA isolated from the contaminated CaMV preparation. This cDNA probe hybridized with nucleic acid from the TuMV-inoculated preparation and to lysates of the original TuMV inoculum that had been dot blotted to nitrocellulose by the technique of Maule et al. (1983), but did not hybridize to the RNA of TMV 204 (not shown).

Additionally, the electrophoretic mobilities of the coat proteins of the virions within these two preparations were compared using SDS-PAGE. The coat proteins of these viruses had indistinguishable electrophoretic mobilities (not shown).

Thus, the nucleic acids and coat proteins of these two viruses were indistinguishable by the methods used here. The contaminant purified from the CaMV preparation is referred to hereafter as turnip vein-clearing virus, isolate A ($TVCV_A$) while the virus isolate prepared from the TuMV inoculum is referred to as $TVCV_B$.

Characterization of Cross Protection in Plants

To detect replication of the challenging virus strain in plants protected by previous infection with Ca-NB2 or CM4-184, a simple dot blot assay was developed to detect CaMV CabbS or $TVCV_B$ superinfection in turnip plants. Lysates from CabbS-inoculated

plants gave strong signal when hybridized with radiolabeled pSH113 (Fig. 25). However, uninoculated plants or plants inoculated with Ca-NB2 gave negligible signal with this probe (Fig. 25). Thus, detection of CabbS infection with the plasmid subclone was specific. Specific detection required large concentrations of the probe during hybridizations. Hybridizations done with less than 5×10^6 cpm / ml of radiolabeled pSH113 gave little or no CabbS-specific signal (not shown). Radiolabeled $TVCV_B$ cDNA was used to detect systemic $TVCV_B$ infection (Fig. 26).

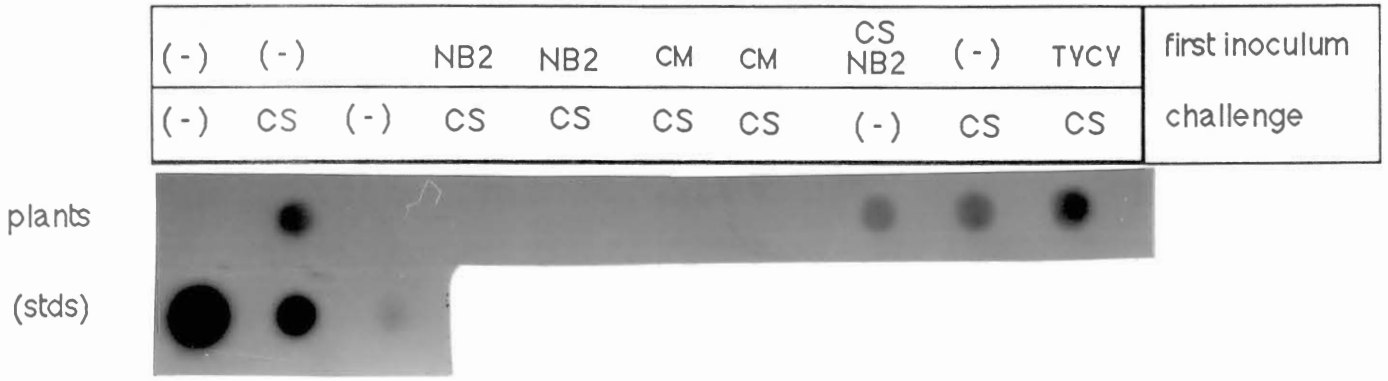
The identity of the viral strain present in the initial, protecting infections was confirmed based on the symptoms observed on the plant and the restriction patterns of viral DNA, when applicable (not shown). The identity of the virus responsible for initial infection following Ca-NB2 inoculation was confirmed using hybridization probes specific for the Ca-NB2 ORF II (not shown). After the protecting infection was allowed to develop for 17 days, the symptomed plants were challenged with either CabbS for $TVCV_B$. After challenge inoculations, plants were cultivated for 30 days and then analyzed for superinfection.

After challenge inoculations, CabbS symptoms could be detected in plants that had not been given protecting inoculations (not shown). Dot blot hybridization detected CaMV-specific sequences in the young, systemically-infected leaves of these plants (Fig. 25). Thus, these plants could be infected even though they were quite mature.

When plants showing systemic CM4-184 or Ca-NB2 symptoms were challenged with $TVCV_B$ virions, systemic $TVCV_B$ symptoms could not be detected in the background of CaMV symptoms at 30 days postchallenge. However, $TVCV_B$ infection could be detected in young leaves from positions above the inoculated leaf by dot blot hybridization with the $TVCV_B$ - specific probe (Fig. 26). Similarly, plants that had been previously inoculated with $TVCV_B$ developed symptoms characteristic of systemic CaMV infection following challenge with Ca-NB2 or CM4-184. Thus, cross protection was not observed between unrelated viruses. When plants showing symptoms of systemic Ca-NB2 or CM4-184

Figure 25. Dot Blot Assay of Cross Protection between Strains of CaMV.

Initial inoculations and subsequent challenge inoculations were performed as described in Materials and Methods. Plants were inoculated as previously described (Gardner et al., 1980) with CM4-184 (CM), Ca-NB2 (NB2), or TVCVB (Lartey et al., in preparation) virions, as indicated. At 17 days post-inoculation, the three youngest leaves longer than 7 cm were challenged with the CabbS strain of CaMV (CS). At 30 days post-challenge, one to two of the youngest leaves 6 to 12 cm in length were assayed for CabbS DNA. The spot blot was hybridized as described in Materials and Methods using a CabbS-specific probe (pSH113) to detect systemic CabbS infection (plants) or to detect CabbS DNA in lysates from healthy plants to which CabbS virions had been added (stds). Standards (stds) contained (L to R) 1 μg, 0.1 μg, 0.01 μg, or 0.001 μg of virus / spot. Following overnight hybridization, the membrane was washed three times with 2X SSC / 0.1 % SSC at 25 o C followed by a final stringency wash with 0.1 X SSC / 0.1 % SDS at 25 o C for 30 min. The washed membrane was exposed to X-ray film overnight without an intensifying screen.


(-) (-) NB2 NB2 CM CM CS NB2 (-) TVCV
first inoculum
(-) CS (-) CS CS CS CS (-) CS CS
challenge
plants
(stds)

infection were challenged with CabbS, CabbS-specific sequences could not be detected in young emerging leaves at 30 days post challenge (Fig. 25). Thus, initial inoculations with CaMV CM4-184 or with Ca-NB2 prevented subsequent systemic infection with CaMV CabbS.

CabbS infection was not prevented by Ca-NB2 following coinoculation with CabbS and Ca-NB2 (Fig. 25). Thus, the prevention of superinfection was not due to competition between CabbS and Ca-NB2. Protection was specific to related viruses, ie, strains of CaMV, as previous inoculation with $TVCV_B$ did not prevent the appearance of CabbS in the young leaves of plants that were previously infected with $TVCV_B$ (Fig. 25).

Development of an Assay for Cross Protection in Protoplasts

The assay described above demonstrated that strain-specific differences in CaMV DNA could be exploited to examine superinfection in plant tissues. To examine if such an approach was also feasible in protoplasts, protoplasts were isolated from systemically-infected plants. The yield of protoplasts from systemically-infected leaves was appr. 50 % of the yields obtained from healthy leaves. Recovery of cells after PEG treatment and washing was appr. 50 % of the recovery observed for protoplasts isolated from healthy leaves. Following PEG-mediated infection and washing, light microscopy revealed that cells from healthy plants were almost free from debris at this step. In contrast, cells from infected leaves were appr. 50 % debris by volume following the final wash. Although protoplast preparations isolated from infected leaves were less stable during manipulations and contained debris, upon subsequent culture these cells showed survival rates comparable to those obtained from healthy cells. In three initial experiments, the yields and stabilities of protoplasts from infected tissue were adequate for the experiments that were planned.

However, in subsequent experiments, the yields of viable cells obtained following digestion and PEG-mediated inoculation of infected tissue were much lower. The inability

to recover useful amounts of viable cells led to the decision to terminate subsequent attempts at examining cross protection between strains of CaMV in protoplasts.

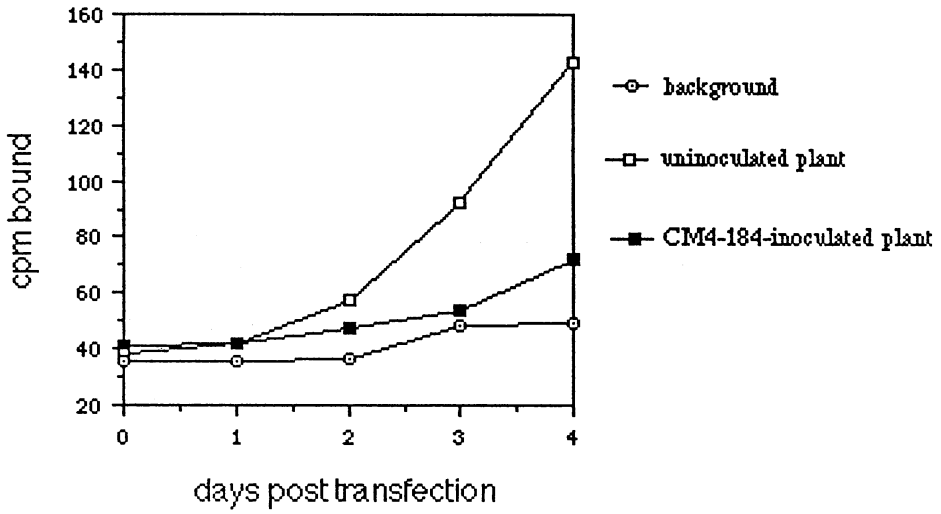
Results are presented from one experiment designed to assay viral replication in cells that were isolated from healthy or infected turnip leaves (Fig. 27). Because protoplast yields were quite low, the cells were cultured in Costar® tissue culture plates. To prevent the deleterious effects of inadequate aeration in this culture system (discussed in Chpt. II), protoplasts were cultured at 0.5 x 10^6 to 1 x 10^6 cells / ml in 0.4 ml of media. Nucleic acid replication in these cells was assayed as described in Chpt. II, but using the strain-specific probes described above. Protoplasts were isolated from healthy plants and infected with either TMV U-1 (Dawson et al., 1986) or the CabbS strain of CaMV. In the cells inoculated with TMV, TMV-specific signal showed a steady increase throughout the 4 day period of the experiment (Fig. 27A). However, when this assay of TMV U-1 replication in protoplasts was repeated using a different preparation of cells, TMV signal showed no increase during the first day of culture, increased appr. 10-fold between day one and day two, and plateaued after day two (not shown). Thus, although the shapes of the replication curves for TMV were variable, replication of TMV in turnip protoplasts was demonstrated in both experiments. In the cells inoculated with CaMV, the rate of CaMV replication was comparable to those observed in other experiments until day four, at which time replication seemed to be slightly repressed (c.f. Fig. 19 and Fig. 27B).

Protoplasts from leaves of plants infected with CM4-184 were inoculated with either TMV U-1 or CaMV CabbS. In the cells infected with TMV, TMV-specific signal appeared to increase slightly during culture (Fig. 27A). However, this signal was only slightly above background. In the cells inoculated with CaMV CabbS, CabbS-specific signal slowly declined during subsequent culture (27B). Thus, in cells isolated from infected plants, replication of the superinfecting viruses was not unequivocally demonstrated.

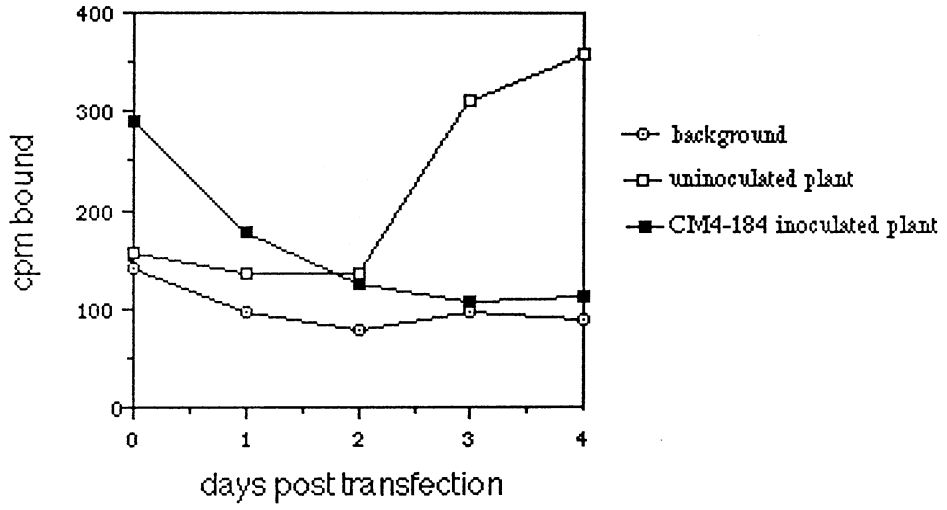
Figure 27. Infection of Protoplasts Isolated from Healthy or Infected Plants

Protoplasts were isolated from the youngest expanded leaves of turnip plants four weeks after the plants had been inoculated with CaMV CM4-184 virions; protoplasts were also isolated from equivalent leaves of a plant which had not been inoculated. Freshly-isolated protoplasts (107 cells) were infected with 40 μg of virions and cultured as described in Materials and Methods. Aliquots (appr. 104 cells) of each culture were spotted onto nitrocellulose at the indicated times postinfection; aliquots of an uninoculated culture of cells which were isolated from an infected plant were also spotted (background). The membranes were treated as described below and probed using 5 x 106 cpm of radiolabeled, cloned DNA per ml of hybridization solution. After three low stringency washes with 2X SSC / 0.1 % SDS at 25o C, the final stringency wash was performed at 25o C using 0.1X SSC / 0.1% SDS. The washed membranes were exposed to X-ray film; after autoradiography, each spot was excised and the bound radioactivity determined by liquid scintillation counting. Points plotted represent the means of duplicate (background) or triplicate (infected) aliquots from the indicated cultures.
(A) Protoplasts were infected with TMV virions (common strain). After all culture aliquots were applied, the membrane was baked and probed with pTMV30, a cDNA clone of the TMV (common strain) 30K region.
(B) Protoplasts were infected with CaMV CabbS virions. After all culture aliquots were applied, the membrane was treated with NaOH, neutralized, baked, and hybridized with a CabbS-specific probe, pSH113.

A


160
140
120
100
80
60
40
20
cpm bound
0
1
2
3
4
days post transfection
background
uninoculated plant
CM4-184-inoculated plant

B


400
300
200
100
0
cpm bound
0
1
2
3
4
days post transfection
background
uninoculated plant
CM4-184 inoculated plant

Discussion

Characterization of a Contaminating Virus

$TVCV_A$ was the causal agent of vein clearing symptoms. Although vein-clearing symptoms were not observed during $TVCV_A$ / Ca-NB2 coinfection, such symptoms would probably have been masked by the more severe Ca-NB2 symptoms. Following inoculation of a plant with DNAase-treated inoculum, vein-clearing symptoms were observed, thus demonstrating that these symptoms could be induced by the single-stranded RNA of $TVCV_A$. Because CaMV DNA is infectious (Lebeurier et al., 1980), failure to observe typical Ca-NB2 symptoms following inoculation of turnips with DNAase-treated nucleic acid suggested that this treatment removed most, if not all, of the CaMV DNA. The observation that $TVCV_A$ RNA could be reisolated from symptomed tissue after passage (Fig. 24A) confirmed that this virus was, in fact, the causal agent of the symptoms.

The observations that $TVCV_B$ induced symptoms identical to those of $TCVC_A$, contained RNA and coat protein indistinguishable from those of $TVCV_A$, and was present in the growth chamber coincident with $TVCV_A$, suggest that these two preparations of virions contain the same virus. Substantiation of this hypothesis would require a more detailed comparison of the viruses in these two preparations, such as characterization of the immunological properties or amino acid compositions of the coat proteins of the virions. Other approaches could be used to demonstrate homologies at the RNA level.

The coat protein of $TVCV_A$ has an apparent molecular weight of 17.5 kD (Fig. 24A). The genome of this virus is a 6.4 Kb - long, single-stranded RNA (Fig. 24B). The molecular weights of the protein and nucleic acid components of $TVCV_A$ are consistent with its tentative identification as a member of the tobamovirus group (Francki et al., 1985). Electron microscopy and immunological characterization (Robert Lartey, in preparation) have confirmed this identification. Because $TVCV_A$ and $TVCV_B$ infect turnip, a crucifer, they may be strains of Ribgrass Mosaic Virus or Youcai Mosaic Virus (Oshima

and Harrison, 1975). However, characterization of symptoms on select hosts has suggested that this virus may be a novel tobamovirus (Robert Lartey , in preparation).

Although the undisrupted $TVCV_A$ virions appear homogeneous during agarose gel electrophoresis (Fig. 23), the possibility that the purified preparation contains two or more populations of tobamoviruses or tobamovirus strains cannot be ruled out. However, as very few tobamoviruses infect crucifers, it seem reasonable to assume that the virus population is, in fact, relatively homogeneous.

The novel host range of $TVCV_A$ may be of considerable utility for investigating the molecular mechanisms which determine viral host range. Other than the exceptions noted above and the symptomless infection of Arabidopsis with TMV-C and TMV-P (Urban et al., 1988), crucifers are not systemic hosts for tobamoviruses (Francki et al., 1985). Although TMV U-1 (TMV 204-Dawson et al., 1986) does not systemically infect turnips, both TMV U-1 and $TVCV_A$ infect tobacco (Robert Lartey, in preparation). Thus, TMV U-1 and $TVCV_A$ have overlapping, but distinct, host ranges. TMV U-1 and $TVCV_A$ are probably quite different at the sequence level since their nucleic acids do not hybridize with each other. Partial sequence analysis has confirmed that the sequences of TMV U-1 and $TVCV_A$ are different (Robert Lartey, in preparation). These differences could be exploited to investigate the molecular basis for the distinct host ranges observed. Significantly, TMV U-1 cannot replicate in turnip plants, but can replicate in turnip protoplasts (Fig. 27A). Thus, the basis for the distinct host ranges of TMV U-1 and $TVCV_A$ may reflect differences in cell-to-cell transport (30K) function. Although such an interpretation might be overly simplistic (reviewed in Atabekov and Dorokhov, 1984), it could provide a starting point for an investigation into the mechanism(s) by which the host ranges of these two viruses are determined. Additionally, an attempt to complement TMV U-1 infection of turnips with $TVCV_A$ might prove insightful.

Development of an Assay of Cross Protection

The assay of cross protection between strains of CaMV developed in the work presented here has several advantages over assays that have been utilized previously. Tomlinson and Shepherd (1978) documented cross protection between strains of CaMV by observing inoculated host plants for the development of symptoms specific for the challenge. However, observations from other virus systems demonstrate that infections may be symptomless (reviewed in Atabekov and Dorokhov, 1984) and that the challenger may replicate without inducing symptoms (Cassells and Herrick, 1977). Thus, symptoms may not accurately reflect the absence of superinfection. Zhang and Melcher (1989) extracted CaMV DNAs and utilized restriction fragment length polymorphisms between the protecting and challenge strains to demonstrate systemic protection of the host from the challenge strain. This method, while unambiguous, is rather labor intensive. As described here, utilization of the dot blot assay technique coupled with the use of strain-specific hybridization probes resulted in successful strain-specific detection of CaMV infection (Fig. 25). The exploitation of differences in the DNA sequences of three strains of CaMV allowed cross protection among these strains to be assayed in a manner that was convenient and unambiguous. This assay could easily be applied to large numbers of plants to generate statistically meaningful results.

The utility of this assay in detecting CaMV replication in infected protoplasts was also demonstrated (Fig. 27). Because it is questionable as to whether or not TMV replicated in cells from infected leaves (Fig. 27A), the observation that CabbS failed to infect this same preparation of cells (Fig. 27B) has little significance. The poor TMV replication suggests that the protoplasts isolated from infected leaves were not growing vigorously. Thus, the inhibition CaMV replication in these cells may have been due to poor cell viability or poor cell vigor rather than cross protection. The speculation that CaMV replication in protoplasts requires cell division (presented earlier) is also consistent with this interpretation.

Alternatively, the poor replication of the TMV challenge may have been related to the presence of two infecting viruses within individual cells.

Thus, this system could, in theory, be applied to investigate cross protection at the single-cell level. However, difficulties with plant materials prevented such an investigation. Plant cell stability and viablility declined with each successive attempt to isolate protoplasts from infected leaf tissues. Other authors have reported the isolation and culture of protoplasts from systemically symptomed leaves of turnip plants infected with CaMV (Howell and Hull, 1978; Furusawa et al., 1980; Yamaoka et al, 1982; Maule et al., 1983; Thomas et al., 1985). Thus, the examination of cross protection between strains of CaMV in turnip protoplasts isolated from systemically infected leaves is practical in theory. However, successful demonstration of its application was not accomplished in the work presented here.

Cross Protection Among Strains of CaMV

Observations regarding cross protection between strains of CaMV suggest that CaMV is widely distributed among the cells in the leaf tissues and very young tissues of the host. In many host / virus systems, prolonged cultivation of the challenged, cross-protected host can result in the establishment of the challenge infection (reviewed in Hamilton, 1980). It has been suggested that such breakdown in cross protection involves replication of the challenge in plant tissues that are free of the protecting infection (Kunkel, 1934). However, the results presented here, and results from other authors (Tomlinson and Shepherd, 1978; Zhang and Melcher, 1989), suggest that cross protection among strains of CaMV continues even during extended cultivation of the challenged plant. Because CabbS, an unusually competitive strain of CaMV (Zhang and Melcher, 1989), was used as the challenger in the study presented here, the observation that protection does not break down with time suggests the protecting strain is present in most if not all, of the cells of the challenged tissues.

Zhang and Melcher (1989) challenged cross-protected plants by inoculating healthy leaves from positions above the previously inoculated leaf. They observed that the protecting inoculation given as little as 2 days before challenge was adequate to protect the host from the subsequent challenge. Their results suggest that protection did not function by preventing the establishment of infection in the inoculated leaf, but rather acted at some later point in the spread of systemic infection. Candidates for the point at which cross protection against the spread of CaMV infection is manifested include long distance transport and / or infection of the quickly growing tissues at the shoot apex. These results are consistent with the theory that CaMV is ubiquitously distributed among the very young tissues of the host.

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VITA

Steven D. Hartson

Canditate for the Degree of

Doctor of Philosophy

Thesis: REGULATION OF CAULIFLOWER MOSAIC VIRUS GENE EXPRESSION

Major Field: Biochemistry

Biographical:

Personal Data: Born Riverside, California, May 1, 1961 to Wayne and Elizabeth Hartson.

Education: Graduated from U.S. Grant High School, Oklahoma City, Oklahoma in May 1979; received Bachelor of Science Degree in Biochemistry from Oklahoma State University, in May of 1985; completed requirements for the Doctor of Philosophy degree in December, 1991.

Professional Experience: Research Assistant, Department of Biochemistry, Oklahoma State University, September, 1985 to September, 1991.