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Characterization of the genome of maize chlorotic dwarf virus
and an associated satellite RNA

Ge, Xin, Ph.D.
The Ohio State University, 1990
CHARACTERIZATION OF THE GENOME OF MAIZE CHLOROTIC DWARF
VIRUS AND AN ASSOCIATED SATELLITE RNA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Xin Ge, B.A., M.A.

* * * * *

The Ohio State University
1990

Dissertation Committee: Approved by:
D. T. Gordon
R. E. Gingery
M. D. McMullen
D. J. Jackwood

Advisor, Department of Plant Pathology
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VITA

November 15, 1957 .................. Born - Harbin, P. R. China.


1985 - 1987 ....................... Teaching Assistant, Northeast Agricultural College, Harbin, China.

1987- 1990 ....................... Research Associate, Department of Plant Pathology, The Ohio State University, Wooster, Ohio.

PUBLICATIONS


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Maize chlorotic dwarf virus (MCDV), the type and only member of MCDV group, has not been studied at the molecular level. This paper presents some molecular characteristics of the MCDV genome and of a satellite RNA of MCDV, discovered during this study.

MCDV virion contains only one species of RNA of about 13 kilobases (kB) in length. The viral RNA had a poly(A) tract at the 3' end and a genome-linked protein (VPg) of about 18 kilodaltons (kDa) presumably at the 5' end. In vitro translation showed that the RNA can direct the synthesis of a series of proteins, with the largest being about 250 kDa.

Only genomic-sized MCDV RNA was found in total cellular RNA, poly(A)-containing RNA, polyribosomal RNA and
double-stranded (ds) RNA samples from MCDV-infected maize. Genomic-sized MCDV RNA was the only MCDV RNA found associated with polyribosomes from MCDV-infected maize after immunoprecipitation with antibodies to MCDV capsid proteins. It is concluded that the MCDV genome is monopartite, about 13 kb in length and positive sense, and that MCDV belongs to Picornavirus supergroup.

During this work, a 1-kB ssRNA species was found encapsidated in MCDV coat proteins. This particle was termed the slower sedimenting virus-like particle (SSVLP). Northern hybridization assays showed that SSVLP RNA had less than 10% homology with MCDV genomic RNA. SSVLP appeared dependent on MCDV for replication. In infected tissue, the presence of SSVLP RNA was correlated with decreases in the amounts of MCDV virions, MCDV RNA and MCDV capsid proteins. Multimeric ds SSVLP RNAs were found in maize tissue co-infected with MCDV and SSVLP. The 3' terminal structure of the RNA was a phosphate and the 5' terminal structure was not VPg. The SSVLP RNA was sequenced and no open reading frames for proteins larger than 10 kDa were found. The sequence obtained contained 1031 nucleotides and had sequence similarities to virusoids and viroids. The sequence could assume a so-called hammerhead structure. It is concluded that the SSVLP RNA, is a satellite RNA of MCDV, now named satellite MCDV (SMCDV), which probably replicates via a rolling circle mechanism.

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INTRODUCTION

Maize chlorotic dwarf virus (MCDV) is both a biologically distinct and an economically important plant virus. Among the more than 800 plant viruses studied so far, MCDV, along with rice tungro spherical virus (RTSV), differs from the others mainly in that it is transmitted by leafhoppers in a semipersistent relationship, i.e., transmissible virus is retained by the leafhopper for up to several days. MCDV is limited to the United States (U. S.) and Mexico and causes a severe disease on maize (Zea mays L.) in the southeastern U. S. No effective control of the disease has been developed (Gingery, 1988).

As will be discussed in the following section, viruses containing single-stranded, messenger-sense RNA genomes have been provisionally classified into two supergroups or superfamilies: the Picornavirus-like virus and Alphavirus-like virus supergroups (or superfamilies) (Goldbach and Wellink, 1988; Strauss and Strauss, 1988). Members of these two supergroups differ mainly in their genome structures and organizations. Because the structure and organization of the MCDV genome is unknown, the status of the classification
of MCDV among plant RNA viruses and the viruses of these two supergroups is unclear. Thus, information concerning MCDV genome structure and organization is crucial for the classification of MCDV.

Conventional measures for control of maize chlorotic dwarf, such as virus resistance and vector control, have not been fully successful. Among the most promising molecular approaches for virus disease control are the use of transgenic plants expressing viral capsid protein genes (Powell et al., 1986; Loesch-Fries et al., 1987) or satellite RNAs (Gerlach et al., 1987; Harrison et al., 1987).

For plants transformed to express viral capsid protein gene(s), infecting viruses are blocked, it is believed, because the high level of viral capsid protein expression inhibits virus uncoating, an essential step for virus replication (Angenent et al., 1990; Powell et al., 1990; Register and Beachy, 1988). To develop this approach, one needs to know how the capsid protein(s) is expressed.

MCDV has been found associated with a satellite-like RNA (Ge et al., 1989), so disease control through a satellite RNA is potentially possible for the maize chlorotic dwarf disease. The satellite RNA of cucumber
mosaic virus (CMV) has been used successfully in controlling CMV diseases in China (Tien and Chang, 1983; Tien et al., 1987; Yang et al., 1986). Plants transformed with satellite RNA sequences have been shown resistant to their helper virus infections (Courtice, 1987; Gerlach et al., 1987; Harrison et al., 1987). Using satellite RNAs to transform plants for resistance may have advantages over using viral capsid protein genes to transform plants (Courtice, 1987). The study of virus satellites has significance for both basic and applied science. Due to the small genome size and efficient replication, satellites (satellite viruses and satellite RNAs) are useful models to study viral replication and encapsidation. It is also possible to use satellites as vectors for foreign genes in the transformation of plants (Van Emmelo et al., 1987). The enzymatic activities of some satellite RNAs (classified as group II satellite RNAs in this thesis) and some cellular RNAs, termed ribozymes, open a new area of study (Buzayan et al., 1986b, 1986c; Cech and Bass, 1986; Forster and Symons, 1987a, 1987b; Haseloff and Gerlach, 1988). The use of the ribozyme activity of a group II satellite RNA as gene regulator in vivo has been reported (Cameron and Jennings, 1989), which suggests another potential means of controlling plant viral diseases by using satellites. However, satellites have different effects on the development of plant viral diseases, ranging from attenuation to intensification of symptoms (Francksi, 1985;
Kaper and Collmer, 1988), and due to the heterogeneity of satellite RNAs (Donis-Keller et al., 1981; Kurath and Palukaitis, 1990) and the high rate of mutation of RNAs (Domingo and Holland, 1988; Holland et al., 1982), there are potential dangers of producing untoward effects in using satellites. Therefore it is necessary to characterize a satellite before its use in application.

An objective of the present research was to provide some of the essential information needed for the classification of MCDV and the development of molecular approaches for the control of maize chlorotic dwarf by genetic engineering. The specific objectives were to determine: a) the terminal structures of the MCDV genome; b) the translation strategy of the MCDV genome; c) the characteristics of the associated satellite-like RNA; and d) the biological effects of the associated satellite-like RNA on MCDV-infection.
CHAPTER I
LITERATURE REVIEW

CHARACTERIZATION OF MAIZE CHLOROTIC DWARF VIRUS (MCDV)

MCDV is the type member of maize chlorotic dwarf virus group of plant viruses (Matthews, 1982; Gingery, 1988). Rice tungro spherical virus (RTSV) is the only other virus suspected of belonging to the MCDV group.

MCDV was first identified and characterized in 1972 (Bradfute et al., 1972) and is one of two viruses infecting maize (Zea mays L.) in the United States (U. S.) that cause diseases of major economic importance (Gordon and Nault, 1977; Gordon et al., 1981). MCDV has been reported from the U. S., where it occurs primarily in the southeastern states, and once from Mexico (Gordon et al., 1981). Effective control measures for maize chlorotic dwarf have not been developed, although tolerant maize inbreds and hybrids have been bred and tolerant hybrids are grown in areas of the U. S. where the disease causes appreciable damage. Dominant genes for resistance to maize chlorotic dwarf have not been identified in maize to date (Gingery, 1988; Rosenkranz and Scott, 1987; Scott et al., 1981).
MCDV has an isometric particle, about 30 nm in diameter and contains a single-stranded (ss) RNA genome with molecular weight (M_r) of about 3.2 x 10^6 Daltons (Da) (Gingery, 1976; Gingery et al., 1978). The virion has a sedimentation coefficient of 183 S and a density in CsCl of 1.50 g/ml. MCDV is transmitted obligately by leafhoppers in a semipersistent relationship, a relationship shared only with RTSV for plant viruses transmitted by leafhoppers (Choudhury and Rosenkranz, 1983; Gingery, 1976, 1988; Gingery et al., 1978; Nault et al., 1973). Purified MCDV virions cannot be transmitted by the vectors and a putative, virally encoded, helper component has been reported for vector transmission (Hunt et al., 1988). MCDV was located in its vector's foregut (Childress and Harris, 1989). The efficiency of MCDV transmission by different leafhopper species has been associated with their phylogenetic relatedness to *Graminella nigrifrons* (Forbes), the major field vector of MCDV (Nault and Madden 1988). MCDV has three serologically distinct capsid proteins (CP), designated CP1, CP2 and CP3, with M_r s of 34, 26 and 24 kDa, respectively (Maroon et al., 1989). Two kinds of inclusion bodies, striated sheet or fibrous inclusions and granular inclusions, have been reported for MCDV-infected cells (Ammar et al, 1987; Harris and Childress, 1981; Nault and Ammar, 1989). Symptom differences on maize have been used to differentiate MCDV isolates (Gingery and Nault 1989; Hunt
et al., 1988; Nault, personal communication).

Although the above biological, physical, chemical and serological characteristics have been reported, information on the molecular biology of the virus has not been published, which is important for further classification of the virus and control of the viral disease.

**CLASSIFICATION OF PLANT VIRUSES WITH SINGLE-STRANDED MESSENGER-SENSE RNA GENOMES**

Currently, more than 40 groups of plant viruses are recognized (Brown, 1989; Matthews, 1982). Characteristics separating these groups are the type of genomic nucleic acid, morphology of the virion and, to a lesser extent, other physical, chemical, serological and biological characteristics. The total number of described plant viruses is more than 800 (Matthews, 1982). Plant viruses contain only one type of genomic nucleic acid. The types, number of strands and polarity of the nucleic acids are: a) double-stranded (ds)DNA, b) ssDNA, c) dsRNA, d) ss(-)RNA and e) ss(+).RNA (Table 1). Ss(+)RNA functions as mRNA in vivo and in vitro, whereas for ss(-)RNA the complementary RNA strand functions as the mRNA. The majority of plant viruses have ss(+)RNA genomes (Table 1), and these RNAs are often infectious.
<table>
<thead>
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<th>Selected representative groups</th>
<th>Selected representative viruses</th>
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<td>dsDNA</td>
<td>2</td>
<td>Caulimoviruses</td>
<td>Cauliflower mosaic virus</td>
</tr>
<tr>
<td>ssDNA</td>
<td>1</td>
<td>Geminiviruses</td>
<td>Maize streak virus</td>
</tr>
<tr>
<td>dsRNA</td>
<td>1</td>
<td>Plant Reoviruses</td>
<td>Maize rough dwarf virus</td>
</tr>
<tr>
<td>ss(-)RNA</td>
<td>1</td>
<td>Plant Rhabdoviruses</td>
<td>Maize mosaic virus</td>
</tr>
<tr>
<td>ss(+)RNA</td>
<td>~30</td>
<td>Remaining groups</td>
<td>Maize chlorotic dwarf virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Maize dwarf mosaic virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Maize chlorotic mottle virus</td>
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Because the MCDV genome is ss(+)RNA, as I will demonstrate in the results of this thesis, the remaining review will be focused mainly on the genomes of ss(+)RNA viruses of animals and plants.

Recently it has been pointed out that animal and plant viruses with ss(+)RNA genomes are related (Argos et al., 1984; Domier et al., 1987; Goldbach and Wellink, 1988; Strauss and Strauss, 1988) and they may be separated into two supergroups or superfamilies based on their genome structure and organization (Goldbach and Wellink, 1988; Strauss and Strauss, 1988). They are: the Alphavirus-like viruses and the Picornavirus-like viruses. The Alphavirus-like viruses express their 3' proximal genes by producing subgenomic RNA, whereas Picornavirus-like viruses express their entire genome through a polyprotein which is proteolytically processed to generate functional proteins. Viruses with similar genome expression strategies also have similar terminal structures in their genomes and share amino acid sequence homologies in their non-structural proteins (Argos et al, 1984; Domier et al, 1987; Goldbach and Wellink, 1988; Strauss and Strauss, 1988). For example, all Alphavirus-like viruses have a "cap" structure at the 5' end of their genome, whereas the 5' end of genome of Picornavirus-like viruses is blocked with a small protein called the genomic-linked viral protein (VPg). Without
exception, the genomes of all members of the latter group are polyadenylated at the 3' end.

GENOME STRUCTURES OF SS(+)RNA VIRUSES

Structure of the 5'-terminus of the viral genome

Three major 5' end structures of viral genomes have been identified for ss(+)RNA viruses. One is the "cap", which is a 7-methyl-guanosine linked with RNA through a 5'-5' phosphodiester bond \([m^7G(5')pppN(m)pN(m)p]\) (Moss, 1984). The "cap" structure is a common feature of eucaryotic cellular mRNAs (Banerjee, 1980; Shatkin, 1976) and functions presumably to protect mRNA (Furuichi et al., 1977), to stimulate the correct initiation of mRNA translation (Kozak, 1978; Shatkin, 1985) and to permit correct and efficient splicing (Green et al., 1983; Konarska et al, 1984). At least eleven of the more than thirty ss(+)RNA plant viruses groups have been demonstrated to have a cap at the 5' terminus of their genomes. These groups are the Tobamoviruses, Hordeiviruses, Alfalfa mosaic virus (AlMV) group, Bromoviruses, Cucumoviruses, Ilarviruses, Potexviruses, Tymoviruses, Tombusviruses, Furoviruses and Tobraviruses (Dougherty and Hiebert, 1985; Goldbach, 1986; Matthews, 1981). Closteroviruses (Karasev et al., 1989) and members of the Carnation mottle virus group (Koenig, 1988) may also have capped genomes.
The second 5' terminal structure among ss(+)RNA virus genomes is a VPg (Daubert and Bruening, 1984; Lee et al., 1977; Wimmer, 1982.) which is a peptide covalently bonded to the viral genome through a phosphodiester bond to the 5' phosphate terminus of the RNA (Ambros and Baltimore, 1978; Jaegle et al., 1987; Rothberg et al., 1978). This 5' terminal structure has been found in the genomes of plant Potyviruses (Hari, 1981; Riechmann et al., 1989; Siaw et al., 1985), Comoviruses (Daubert et al., 1978; Daubert and Bruening, 1979; Stanley et al., 1978), Nepoviruses (Harrison and Barker, 1978; Mayo et al., 1979; Mayo et al., 1982a), Luteoviruses (Mayo et al., 1982b; Murphy et al., 1989), Sobemoviruses (Ghosh et al., 1981), pea enation mosaic virus (Reisman and De Zoeten, 1982) and parsnip yellow fleck virus (Murant, 1988). It has also been found in DNA animal viruses (see Wimmer, 1982), dsDNA bacterial viruses (Salas et al., 1978), dsRNA animal viruses (Persson and MacDonald, 1982), ss(+)RNA calicivirus (Burroughs and Brown, 1978) and ss(+)RNA Picornaviruses (Lee et al., 1977; Sanger et al., 1977). The size of the VPg linked to plant virus genomes ranges from 4 to 24 kDa. The location of the VPg gene for the Comoviruses and Potyviruses has been reported (Shahabuddin et al., 1988; Wellink et al., 1986; Zabel et al., 1984). While the functions of this structure are not clear, several hypotheses have been presented (Daubert and Bruening, 1984; Putnak and Phillips, 1981; Semler et al.,
1988; Wimmer et al., 1987). These are that the VPg functions as: a) a primer for viral RNA replication, as for the DNA Adenoviruses; b) a signal for nucleic acid encapsidation, as suggested for bacterial phages (Bjornsti et al., 1982); and c) protection for the RNA from 5' exonucleases. VPg is required for infectivity of some viruses (Burroughs and Brown, 1978; Chu et al., 1981; Harrison and Barker, 1978; Veerisetty and Sehgal, 1980), but it is not needed in the case of others (Daubert et al., 1978; Flanegan, 1977; Hari, 1981; Sanger et al., 1977). The function for VPg as a protease in viral RNA replication has also been suggested (Andrews and Baltimore, 1986; Semler et al., 1988; Yong et al., 1985). It is interesting that VPg is absent from polyribosomal RNA of poliovirus (Hewlett et al., 1976), a Picornavirus, and cell-free translation assays have shown that the presence or absence of VPg in viral RNA does not alter in vitro translation (Chu et al., 1981; Golini et al., 1980; Koenig and Fritsch, 1982; Stanley et al., 1978).

The third 5' terminal structure is an unmodified diphosphate or triphosphate. Tobacco necrosis virus and its satellite RNA have genomes with such a 5' terminus (Lesnaw and Reichmann, 1970).
Structure of the 3'-terminus of the viral genome

Three structures also have been reported for the 3' end of ss(+)RNA viral genomes. These structures are: a polyadenylate [poly(A)]; a tRNA-like structure; and any one of the four ribonucleotides with a 3' hydroxyl group (X-OM). The poly(A) tail is a common feature of the 3'-end of most eucaryotic mRNAs (Nevins, 1984). Some prokaryotic mRNAs have poly(A) sequence also (Edmonds and Kopp, 1970; Srinivasan et al., 1975). In cells of eucaryotic organisms, the poly(A) terminus is not transcribed directly from DNA genome, but rather is added in the nucleus post-transcriptionally (Nevins, 1984). However, for some ss(+)RNA viruses, the poly(A) tail is transcribed from the terminal poly(U) of the (-) strand viral RNA (Dorsch-Hasler et al., 1975; Frey and Strauss, 1978; Sawicki and Gomatos, 1976). Some virus genomes contain a polyadenylation signal at the 3' end, which suggests the possible existence of a post-transcriptional mechanism for adding poly(A) to viral RNA (Abouhaidar, 1988; Abouhaidar and Lai, 1989; Meyer et al., 1984). The specific function of poly(A) has not been elucidated for either cellular mRNA or viral RNA, although it appears to enhance mRNA stability (Hieter et al., 1976; Marbaix et al., 1975). For cellular mRNAs, poly(A) may also function in mRNA processing and transportation from the nucleus to cytoplasm (reviewed by Nevins, 1984). Poly(A) is
required for infection of some viruses (Goldstein et al., 1976; Sarnow, 1989; Spector and Baltimore, 1974) but not others (Baxt et al., 1979; Hari et al., 1979; Grubman et al., 1979). Besides the 3'-terminal poly(A), internal poly(A)s of about a dozen nucleotides have been documented for the Hordeiviruses (Agranovsky et al., 1978, 1982), Bromoviruses (Ahlquist et al., 1981), alfalfa mosaic virus (Langereis et al., 1986) and a fungal virus (Hannig et al., 1986). Plant Potyviruses (Hari, 1981; Hari et al., 1979), Nepoviruses (Mayo et al., 1979), Comoviruses (El Manna and Bruening, 1973; Semancik, 1974; Steele and Frist, 1978), Potexviruses (Abouhaidar, 1988; Abouhaidar and Lai, 1989; Guilford and Forster, 1986), Furoviruses (Bouzoubaa et al., 1987; Goldbach, 1986), and parsnip fleck yellow virus (Murant, 1988) have been shown to contain poly(A) at the 3'-end of their genomes. Carlaviruses may also contain a poly(A) sequence in their genomes (Tavantzis, 1984). Usually RNA viruses containing poly(A) contain both poly(A)-plus [poly(A)+] and poly(A)-minus [poly(A)-] virion RNA fractions (Frey and Strauss, 1978; Hari et al., 1979; Sagripanti, 1985).

The 3' ends of some ss(+)RNA viruses are considered to have a tRNA-like structure for the following reasons (Haenni et al., 1982; Weiner and Maizels, 1987): a) the last three nucleotides at 3'-terminus of these viruses are CCA-OM, as
with tRNA; b) the secondary and higher degree structure of the last 100-200 nucleotides at the 3'-end of these RNAs resemble tRNAs (Pleij et al., 1985); and c) the RNAs containing this structure serve as substrates for enzymes in tRNA metabolism, especially aminoacyl-tRNA synthetase (Briand et al., 1976, 1977; Hall, et al., 1987; Kohl and Hall, 1974; Weiner and Maizels, 1987). It has been shown that the tRNA-like structure may interact with viral replicase to initiate (-) strand RNA synthesis (Bujarski et al., 1986; Dreher and Hall, 1988; Hall et al., 1986, 1987; Miller et al., 1986). The tRNA-like structure may also have other functions such as protection of the RNA from enzymatic degradation (Dreher and Hall, 1988; Hall et al., 1987). It has been suggested that the tRNA structure is a molecular fossil of the original RNA world (Weiner and Maizels, 1987). Tymoviruses, Tobamoviruses, Bromoviruses, Cucumoviruses and Hordeiviruses have tRNA-like structures at the 3'-terminus of their genomes (Dougherty and Hiebert, 1985; Goldbach, 1986; Matthews, 1981).

Ss(+)RNA viruses that have a X-oh 3'-terminus are the Sobemoviruses, Luteoviruses, Tombusviruses, Tobraviruses, Ilarviruses, tobacco necrosis virus (TNV) and AlMV (Dougherty and Hiebert, 1985; Goldbach, 1986; Matthews, 1982).
Some plant satellite RNAs possess a free hydroxyl at the 5' terminus and a 2', 3' cyclic phosphodiester at the 3' terminus, which result from the autocatalytic cleavage of the RNA precursor (Bruening, 1989; Forster et al., 1990).

**GENOME EXPRESSION STRATEGIES OF SS(+) RNA VIRUSES**

Different genome expression strategies are shown by ss(+)-RNA viruses. Among these are: a) the production of a polyprotein which is proteolytically processed to generate functional proteins; b) expression of part of the genome, usually the 3' proximal end, via smaller mRNAs, called subgenomic RNAs; c) a combination of the above two strategies; d) protein production involving a "readthrough" mechanism; e) protein production involving a frame shift; and possibly f) protein production involving internal initiation and/or polycistronic translation.

The monopartite Potyviruses (Allison et al., 1986; Dougherty and Carrington, 1988; Robaglia et al., 1989; Vance and Beachy, 1984a, 1984b) and bipartite Comoviruses (Goldbach and Van Kammen, 1985) and Nepoviruses (Jobling and Wood, 1985) express their whole genome from monocistronic mRNA by producing a large protein precursor (polyprotein) from each segment of their genome. The polyprotein is further processed in cis and/or trans proteolytically by a proteinase(s) encoded by the viral RNA to generate
functional proteins (Dougherty and Hiebert, 1985; Dougherty and Carrington, 1988; Goldbach and Van Kammen, 1985; Krausslich and Wimmer, 1988). Although factors from the host plants are speculated to have a role in the proteolytic processing (Carrington et al., 1990), no positive evidence for this has been reported. With this strategy, viruses produce viral proteins in equal molarity. The disadvantages of this strategy are that there is no obvious regulation of the timing or level of gene expression. However, post-translational regulation of gene products by differential processing has been reported (Dougherty and Parks, 1989). Animal Picornaviruses also use this mode of genome expression (Wimmer et al., 1987; Semler et al., 1988).

Many plant viruses express the 5' proximal end of their genome directly from genomic-sized RNA, but express the 3' proximal portion by producing one or more smaller mRNAs or subgenomic RNAs. For example, the genome of the brome mosaic virus consists of three segments, the larger two segments of which are monocistronic, each producing a mature protein. The smallest segment is bicistronic with the 5' terminal gene being expressed directly from this segment and the 3' terminal gene (capsid protein gene) being expressed via a subgenomic RNA (Dougherty and Hiebert, 1985). Members of the Tobamoviruses, Cucumoviruses, Bromoviruses, Ilarviruses, AlMV, Sobemoviruses, Tymoviruses,
Potexviruses, Tombusviruses, Carnation mottle virus group, Luteoviruses, Tobraviruses and Hordeiviruses express their genomes in this manner (Dougherty and Hiebert, 1985; Koenig, 1988). With this strategy, the expression of genes for different functions can be regulated: replicase genes are translated first, and then, structural proteins. Usually, the subgenomic RNAs are translated more efficiently than genomic viral RNAs, due possibly to their smaller size, more efficient ribosome binding (Pyne and Hall, 1979) or possibly to more RNA molecules being available for translation.

Tymoviruses and possibly Sobemoviruses (Dougherty and Hiebert, 1985; Hull, 1988) produce both polyproteins and subgenomic RNAs. Again, subgenomic RNA is used to express the 3' terminal portion of the genome and polyprotein is translated from the 5' portion.

Some viruses have developed strategies in which more than one protein is expressed from the same RNA region. In this case, RNA translation sometimes continues through a "leaky" stop codon (termed "readthrough") to produce a larger protein (Pelham, 1978). Since the readthrough of the stop codon is a rare event, this strategy has advantages of both "genetic economy" and control of protein levels. TMV genomic expression is a classical example of the readthrough strategy (Pelham, 1978).
A frame shift involves synthesis of a single polyprotein from two separate reading frames on a RNA template. The resulting polyprotein is later cleaved proteolytically into mature proteins (Craigen and Caskey, 1987). In this strategy, the stop codons are sometimes bypassed because ribosomal frame shifts during translation. Like readthrough, a frame shifts occur only occasionally, so the virus can differentially regulate the abundance of different proteins. This mode of gene expression was originally found in procaryotes and later in eucaryotes (Craigen and Caskey, 1987). Retroviruses (Hizi et al., 1987; Jacks and Varmus, 1985) and Coronaviruses (Brierley et al., 1987) follow this strategy in regulating the expression of their polymerases. Expression of the polymerase genes of the barley yellow dwarf virus (Miller et al., 1988) and red clover necrotic mosaic virus (Xiong and Lommel, 1989) genomes may also follow this strategy.

Unlike procaryotic mRNA, most eucaryotic mRNAs are monocistronic and are translated by a ribosome-scanning mechanism (Kozak, 1978, 1989). In this mechanism, the ribosome first binds to mRNA via a cap-binding protein at or near the 5' end and then the ribosome scans the mRNA downstream until it finds the first AUG (translation initiation codon) with proper context to begin translation. This mechanism works well with most mRNAs (including viral RNAs).
containing a cap structure at the 5' end, but not with mRNAs from viruses which do not have a cap (Carrington and Freed, 1990; Jang et al., 1989; Pelletier and Sonenberg, 1988, 1989). For these viruses, an internal initiation mechanism has been suggested, in which the ribosome initiates translation by recognizing an internal sequence at the 5' nontranslating region upstream from the AUG in a cap-independent manner. The internal initiation model is even supported by evidences from cap-containing mRNAs. Sendai virus, a virus which contains a ss(-)RNA as its genome, expresses one of its mRNAs in a cap-dependent, scanning independent manner, and this mRNA is translated polycistronically (Curran and Kolakofsky, 1988, 1989). Vesicular stomatitis virus, another ss(-)RNA virus, has a polycistronic mRNA and one polypeptide was shown in vitro to be synthesized by internal initiation (Herman, 1986). An mRNA of a dsRNA virus also has been reported to be a polycistronic (Mertens and Dobos, 1982), and a dsDNA virus expresses its polymerase by internal initiation (Hassin et al., 1986). The internal initiation and polycistronic translation has also been reported in a maize chloroplast transcription unit in vivo (Barkan, 1988). If this mechanism really exists, which is doubted (Kozak, 1989), it is possible that some plant viruses express their genomes multicistronically. It has been suggested that some ss(+)RNA plant viruses use this strategy (Dougherty and
Hiebert, 1985; Dougherty et al., 1985), but there has been only one such report (Mirkov et al., 1989). However, this was for a satellite virus in an \textit{in vitro} system.

From the above review, several conclusions may be stated: a) viruses with a capped genome usually produce subgenomic RNAs; b) viruses with genomes having a 5\' VPg and 3\' poly(A) produce polyproteins; and c) only monopartite ss(+)RNA viruses produce both polyproteins and subgenomic RNAs, although only some of them do so.

TECHNIQUES FOR THE STUDY OF RNA VIRUS GENOME EXPRESSION

There have been two main approaches to the study of RNA virus genome expression strategies, namely cell-free translation of viral RNA and detection of RNA species in virions and in infected tissue. Although the best results are usually obtained by using both approaches, controversial reports after using both approaches still exist (Otal and Hari, 1983; Dougherty, 1983). While cell-free translation assays give \textit{in vitro} results, detection of viral RNA in virions and in infected-tissue shows \textit{in vivo} situations. However, artifactual results concerning the presence of subgenomic RNA are not rare in the latter approach, making the use of appropriate controls necessary.

An alternative approach to the study of viral genome expression strategy is by nucleic acid sequencing and
analysis (Allison et al., 1986; Miller et al., 1988; Robaglia et al., 1989). Once again, the result obtained with this technique should be checked with the results obtained with the above approaches (Dougherty et al., 1985; Xiong et al., 1988).

DEPENDENT VIRUSES AND RNAs

Many plant RNA viruses have associated dependent viruses or RNAs (Murant and Mayo, 1982; Francki, 1985, Kaper and Collmer, 1988). Dependent agents include satellite viruses, satellite RNAs, virusoids, defective interfering (DI) particles and DI RNAs. The common characteristics among them are a ssRNA genome and dependence on their helper virus for replication. Satellite viruses have genomes which encode their capsid proteins. On the other hand, satellite RNAs are usually too small to have a capsid protein gene and are encapsidated in the capsid protein of their helper virus, either separately or together with the helper virus genome. A virusoid is a satellite RNA with a circular ssRNA genome that forms highly base-paired secondary structure, like a viroid. There are no extensive sequence homologies between the genomes of the satellite viruses or satellite RNAs and their helper viruses. In contrast, DI particles and DI RNAs have genomes of deleted (defective) helper virus genomes. The presence of DI particles is usually associated with attenuated helper virus symptom expression (Burgyan et
al., 1989; Huang, 1973, 1988; Hillman et al., 1985, 1987). Although the presence or absence of sequence homology between the dependent agent and the helper virus separates DIs from satellites, there are reports of a dependent agent which has a RNA genome containing a part of helper virus genome and a part of sequence unrelated to its helper virus (Li and Simon, 1990; Li et al., 1989; Simon and Howell, 1986; Simon et al, 1988).

**SATELLITES OF PLANT RNA VIRUSES**

Satellites of plant RNA viruses include satellite viruses and satellite RNAs. Four satellite viruses have been identified, and three of their genomes have been sequenced (Masuta et al., 1987; Mirkov et al., 1989; Ysebaert et al., 1980). Compared to satellite viruses, satellite RNAs occur more commonly (Francki, 1985; Kaper and Collmer, 1988; Murant and Myro, 1982) and more than 20 satellite RNAs have been discovered, most of them associated with viruses from the Nepovirus, Sobemovirus and Tombusvirus groups (see Table 2 for references). Although it is difficult to generalize the structure and organization of satellite RNAs, a review of the literature reveals that it is possible to separate them into two groups, based on their configurations, terminal structures and genome replication modes (Table 2).
The first group of satellites has linear ssRNA genomes with a blocked or phosphorylated 5'-end and a hydroxyl 3'-end. In infected tissue, only one species of dsRNA corresponding to the satellite RNA is found. These RNAs probably replicate like the linear RNAs of their helper viruses. The four satellite viruses (STMV, SPMV, STNV and SMWLMV) all have these characteristics (Table 2).

The second group of satellites are circular ssRNAs, or in some cases linear forms are found (usually in encapsidated virions), the 5' terminus is a free hydroxyl group, whereas the 3' terminus is a 2', 3'-cyclic phosphodiester (Bruening, 1989; Buzayan et al., 1986a). In infected tissue, dsRNAs corresponding to multimeric RNAs are found. The satellite RNAs of this group probably replicate by a rolling circle model (Branch and Robertson, 1984, 1985; Bruening et al., 1988; Hutchins et al., 1985; Symons et al., 1987), which states that the circular RNA is used as template for replication to generate multimeric RNAs, and the latter, like viroids (Owens and Hammond, 1988), are cleaved by the RNAs themselves to produce monomeric RNAs (Bruening, 1989; Bruening et al., 1988; Buzayan et al., 1986b, 1986c; Forster and Symons, 1987a, 1987b; Prody et al., 1986). Sequence and structure similarities between the satellite RNAs of this group and viroids, as well as group I introns, have been noticed, which suggests an intron origin.
for virusoids, viroids and group II satellite RNAs (Bruening et al., 1988; Dinter-Gottlieb, 1986; Kingsbury, 1988).

Another feature which seems to separate the two groups is the size of the RNAs. Group I satellite RNAs are usually more than 500 nucleotides, whereas group II satellite RNAs are usually about 300-400 nucleotides (Table 2).

Hepatitis delta virus (HDV) shares some similarities with group II satellite RNAs, although its helper virus is an animal dsDNA virus (Wang et al., 1986; Kingsbury, 1988). HDV contains a ss(+) circular RNA genome, of about 1700 nucleotides (Chen et al., 1986; Wang et al., 1986), and apparently replicates by the rolling circle model (Chen et al., 1986; Wang et al., 1986; Wu and Lai, 1989; Wu et al., 1989). It has been demonstrated that the RNA can undergo self-cleavage and ligation reactions (Kuo et al., 1988; Sharmeen et al., 1988; Wu and Lai, 1989; Wu et al., 1989). HDV also has some similarities with viroids and virusoids in nucleotide sequence and structure (Wang et al., 1986).

Satellite RNAs associated with Cucumoviruses (CARN 5 and PARNA 5) have features of both satellite groups. Like their helper viruses, the satellite RNAs are capped at the 5' ends and contain hydroxyl groups at the 3' ends, the characteristics of group I satellite RNA (see Table 2 for
references). Analysis of dsRNA of CARNA 5 and PARNA 5 showed that both CMV RNA and CARNA 5, as well as PSV and PARNA 5, contained an unpaired guanosine at the 3' end of the minus strand, suggesting that the satellite RNAs might replicate like their helper viruses (Collmer and Kaper, 1985; Collmer et al., 1985). However, multiple dsRNA species of PARNA 5 and CARNA 5 have been found in infected tissue (Linthorst and Kaper, 1984b; Yong et al., 1986), and PARNA 5 even has some structural similarities with viroids and self-splicing introns (Collmer et al., 1985), indicating that they might be able to replicate like group II satellite RNAs. Also, they are small molecules about the size of group II RNAs. However, it was suggested that multimeric dsRNAs could also result from a self cleavage-ligation reaction, as happens with introns (Zaug et al., 1983), instead of rolling circle replication (Collmer et al., 1985). It is possible that CARNA 5 and PARNA 5 represent an evolutionary linkage between group I and group II satellites.

Satellite RNA of cymbidium ringspot virus (SCRSV) is another example of a satellite RNA with characteristics of both groups in that while its 5' end is blocked and its 3' end is a free hydroxyl, multiple dsRNA forms have been detected in infected tissue (Burgyan and Russo, 1988).
Although the satellite RNA of groundnut rosette virus forms multimeric dsRNAs in infected tissue, the pattern of the dsRNAs seems irregular, and its terminal structures are unknown (Murant et al., 1988). Thus, it is not placed in group II satellite RNA.

EFFECTS OF DEPENDENT VIRUS AND RNA INFECTIONS ON DISEASES AND THEIR HELPER VIRUS REPLICATION

Different effects of dependent virus and RNA infections on viral diseases have been observed, ranging from disease attenuation, to no detectible modification, to disease exacerbation. This subject has been reviewed extensively (Francki, 1985; Kaper and Tousignant, 1984; Kaper and Collmer, 1988; Murant and Myro, 1982).

In some instances, different strains or isolates of a satellite have different effects on disease expression. For example, one isolate of cucumber mosaic virus satellite RNA (CARNA 5) (Kaper and Tousignant, 1977) causes necrosis in tomato (Kaper and Waterworth, 1977), whereas another isolate ameliorates the same disease (Mossop and Francki, 1979). It was suggested that RNA sequence differences might be responsible for the different effects (Mossop and Francki, 1979). Indeed, sequence variations do exist between necrosis-inducing and non-necrosis-inducing isolates of CARNA 5 (Collmer et al., 1983; Kaper and Tousignant, 1984;
Kaper et al., 1988; Palukaitis and Zaitlin, 1984), with the necrosis-inducing domain being located in the 3' half of the satellite RNA (Kurath and Palukaitis, 1989). A satellite RNA of turnip crinkle virus contains two domains: a 5' domain which resembles other satellites and a 3' domain which is basically a part of its helper's genome (Simon and Howell, 1986). This satellite RNA intensifies the symptoms induced by its helper virus (Li and Simon, 1990; Li et al., 1989; Simon and Howell, 1986) with the 3' domain of the satellite RNA determining virulence of exacerbation (Simon et al., 1988).

Host plants also influence the effects of a satellite. Whereas the CARNA 5 causes necrosis in tomato, it attenuates symptoms induced by CMV in peppers and maize (Waterworth et al., 1979). However, there is the possibility that the population of CARNA 5 used might have been heterogenous (Kurath and Palukaitis, 1990). Host effects are apparent in cases where satellite RNAs replicate to different extents in different hosts (Garcia-Luque et al., 1984; Kaper, 1976; Kaper and Tousignant, 1977).

Although it is generally assumed that dependent viruses and RNAs interfere with helper virus replication, the latter has been studied for only a few helper viruses. For example, CARNA 5 decreases the amount of CMV genomic
RNA, and reduces the infectivity of CMV (Habili and Kaper, 1981; Kaper, 1982; Kaper and Tousignant, 1977, 1984; Piazzolla et al., 1982; Yang et al., 1986) and the yield of virion (Jacquenmond and Leroux, 1982). Satellite tobacco necrosis virus decreased the amount of TNV, although only infectivity on a local lesion host was measured (Kassanis, 1968). Satellite tobacco ringspot virus RNA can interfere with helper virus encapsidation (Schneider, 1971). The mechanisms of the various interferences are still a mystery, but the following hypotheses have been advanced: a) satellite RNA may have higher affinity for viral replicase so its replication overtakes its helper's replication (Hanada and Francki, 1989); b) satellite RNA interferes with its helper virus' replication by binding directly to helper RNA (Rezaian et al., 1985; Rezaian and Symons, 1986); and c) satellite RNA has a unique structure which helps it escape from controlled replication (Kaper and Collmer, 1988).

The use of satellite RNAs as biocontrol agents for plant viral diseases has been successful in some cases (Tien and Chang, 1983; Tien et al., 1987; Yang et al., 1986; Yoshida et al., 1985). Recently, plants transformed with satellite RNA cDNAs have been shown resistant to virus infections (Gerlach et al., 1987; Harrison et al., 1987). Satellites, especially the group II satellites, have become more and more useful in both basic and applied sciences.
<table>
<thead>
<tr>
<th>Satellite</th>
<th>Helper virus group or virus</th>
<th>Size of satellite RNA (NT)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>GROUP I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGFV&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>Nepovirus</td>
<td>1114</td>
<td>Fuchs et al., 1989; Pinck et al., 1988.</td>
</tr>
<tr>
<td>SCYMV(500K)</td>
<td>Nepovirus</td>
<td>~1500&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Piazzolla et al., 1989.</td>
</tr>
<tr>
<td>STBRV</td>
<td>Nepovirus</td>
<td>1375</td>
<td>Fritsch et al., 1984; Hemmer et al., 1987; Meyer et al., 1984.</td>
</tr>
<tr>
<td>SPEV</td>
<td>Pea enation mosaic virus</td>
<td>~1000</td>
<td>Demler &amp; De Zoeten, 1989.</td>
</tr>
<tr>
<td>STNV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Tobacco necrosis virus</td>
<td>1239</td>
<td>Ysebaert et al., 1980.</td>
</tr>
<tr>
<td>STMV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Tobamovirus</td>
<td>1059</td>
<td>Valverde &amp; Dodds, 1986, 1987; Mirkov et al., 1989.</td>
</tr>
<tr>
<td>SPMV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Panicum mosaic virus</td>
<td>826</td>
<td>Buzen et al., 1984; Masuta et al., 1987.</td>
</tr>
</tbody>
</table>
Table 2. (continued).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Host Name</th>
<th>Molecular Size</th>
<th>References</th>
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</thead>
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<tr>
<td>SMWLMV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Maize white line mosaic virus</td>
<td>~1400</td>
<td>Gingery &amp; Louie, 1985.</td>
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<tr>
<td>Group II</td>
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<tr>
<td>STRSV</td>
<td>Nepovirus</td>
<td>359</td>
<td>Kiefer et al., 1982; Buzayan et al., 1986a, 1986b.</td>
</tr>
<tr>
<td>SAMV</td>
<td>Nepovirus</td>
<td>300</td>
<td>Davies &amp; Clark, 1983; Kaper et al., 1988b.</td>
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<tr>
<td>SCYMV(170K)</td>
<td>Nepovirus</td>
<td>~500</td>
<td>Piazzolla et al., 1989.</td>
</tr>
<tr>
<td>SVTMV</td>
<td>Sobemovirus</td>
<td>366</td>
<td>Randles et al., 1981; Haseloff &amp; Symons, 1982; Chu et al., 1983.</td>
</tr>
<tr>
<td>SLTSV</td>
<td>Sobemovirus</td>
<td>322</td>
<td>Abouhaidar &amp; Paliwal, 1988; Jones et al., 1983.</td>
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<tr>
<td>SSN MV</td>
<td>Sobemovirus</td>
<td>377</td>
<td>Haseloff &amp; Symons, 1982; Franck et al., 1985.</td>
</tr>
<tr>
<td>SSCMV</td>
<td>Sobemovirus</td>
<td>327 &amp; 388</td>
<td>Franck et al., 1985.</td>
</tr>
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Table 2. (continued).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genus</th>
<th>Code</th>
<th>References</th>
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<tbody>
<tr>
<td>HDV</td>
<td>Hepatitis</td>
<td>1700</td>
<td>Chen et al., 1986; Wang et al., 1986.</td>
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<td><strong>OTHERS</strong></td>
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<tr>
<td>CARNA 5</td>
<td>Cucumovirus</td>
<td>335</td>
<td>Collmer et al., 1983; Collmer &amp; Kaper, 1985; Piazzolla et al., 1982.</td>
</tr>
<tr>
<td>PARNA 5</td>
<td>Cucumovirus</td>
<td>393</td>
<td>Collmer et al., 1985; Linthorst &amp; Kaper, 1984b.</td>
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<td>SGRV</td>
<td>Luteovirus</td>
<td>900</td>
<td>Murant et al., 1988.</td>
</tr>
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Table 2. (continued).

<table>
<thead>
<tr>
<th>Reference Name</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMV = Arabis mosaic virus</td>
<td></td>
</tr>
<tr>
<td>BYDV = Barley yellow dwarf virus</td>
<td></td>
</tr>
<tr>
<td>CARNA 5 = Cucumber mosaic virus-associated RNA 5</td>
<td></td>
</tr>
<tr>
<td>CRSV = Cymbidium ringspot virus</td>
<td></td>
</tr>
<tr>
<td>CYMV = Chicory yellow mottle virus</td>
<td></td>
</tr>
<tr>
<td>GFV = Grapevine fanleaf virus</td>
<td></td>
</tr>
<tr>
<td>GRV = Groundnut rosette virus</td>
<td></td>
</tr>
<tr>
<td>HDV = Hepatitis delta virus</td>
<td></td>
</tr>
<tr>
<td>LTSV = Lucerne transient streak virus</td>
<td></td>
</tr>
<tr>
<td>MWLMV = Maize white line mosaic virus</td>
<td></td>
</tr>
<tr>
<td>PARNA 5 = Peanut stunt virus-associated RNA 5</td>
<td></td>
</tr>
<tr>
<td>PEMV = Pea enation mosaic virus</td>
<td></td>
</tr>
<tr>
<td>PMV = Panicum mosaic virus</td>
<td></td>
</tr>
<tr>
<td>SCMV = Subterranean clover mottle virus</td>
<td></td>
</tr>
<tr>
<td>SNMV = Solanum nodiflorum mottle virus</td>
<td></td>
</tr>
<tr>
<td>TBRV = Tomato black ring virus</td>
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</tr>
<tr>
<td>TBSV = Tomato bushy stunt virus</td>
<td></td>
</tr>
<tr>
<td>TCV = Turnip crinkle virus</td>
<td></td>
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<tr>
<td>TMV = Tobacco mosaic virus</td>
<td></td>
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<tr>
<td>TNV = Tobacco necrosis virus</td>
<td></td>
</tr>
<tr>
<td>TRSV = Tobacco ringspot virus</td>
<td></td>
</tr>
<tr>
<td>VTMV = Velvet tobacco mottle virus</td>
<td></td>
</tr>
</tbody>
</table>

"S" signifies satellite and for most satellite viruses and RNAs the name is derived from the name of the helper virus with the prefix "satellite" added. Some satellites have names that differ from that of the helper virus, e.g. groundnut rosette virus.
Table 2. (continued).

c  The sizes with mark "-" were calculated from the molecular weights of these RNAs, assuming the average molecular weight for each ribonucleotide is 330 daltons.

d  Satellite virus.
CHAPTER II
MATERIALS AND METHODS

VIRUS ISOLATES, MAINTENANCE AND PROPAGATION

MCDV strains-T (Type) and -WS (White stripe) (Hunt et al., 1987) were propagated in maize inbred Oh28 and transmitted with the leafhopper, *Graminella nigrifrons*, from infected plants to healthy, young maize Oh28 seedlings, as previously described (Gingery, 1976; Nault et al., 1973).

VIRUS PURIFICATION AND VIRAL RNA ISOLATION

MCDV was purified as described by Hunt et al. (1987). Viral RNA was isolated by resuspending purified MCDV in 1 x NETS [10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM ethylene diamine tetraacetate (EDTA); 0.5% sodium dodecyl sulfate (SDS)] and extracting twice with phenol-chloroform-isoamyl alcohol (PCIA) (25:24:1). The aqueous phase was mixed with 0.1 volume of 3 M sodium acetate (pH 6.0) and 2.5 volumes of 95% cold ethanol. After standing at -20°C overnight, the RNA was pelleted by centrifugation for 15 min in a microcentrifuge at 4°C. The RNA pellet was washed with 70% ethanol, air dried, resuspended in water and stored at -20°C. Since viral RNA from MCDV virions was degraded to
some extent in most cases, rate zonal sucrose gradient centrifugation was used to select the full-length, genomic RNA according to procedure described by Otal and Hari (1983).

The purification of maize dwarf mosaic virus (MDMV) strains A and 0 were performed according to McDaniel and Gordon (1989), and the purification of brome mosaic virus (BMV) was based on the protocol of Hull (1985). The RNAs of MDMV and BMV were extracted by the same method as used for MCDV.

**ISOLATION OF TOTAL TISSUE CELLULAR RNA AND POLY(A)-CONTAINING RNA**

Total tissue RNA was isolated according to Chirgwin et al. (1979) using guanidine isothiocyanate as the tissue extraction buffer. Poly(A)-containing RNA was selected by oligo(dT) chromatography (Aviv and Leder, 1972). For the reconstitution assay, purified MCDV virions or MCDV RNA was mixed with healthy maize tissue and both total tissue RNA and poly(A)-containing RNA were isolated as described above. Only apical tissue of maize seedlings about 1 to 2 wk post-inoculation were extracted for RNA isolation.
ISOLATION OF DOUBLE-STRANDED RNA (dsRNA)

MCDV-infected maize tissue, about 2 to 3 wk post-inoculation, was harvested. Total tissue nucleic acids were isolated by the phenol extraction method of Otal and Hari (1983). The nucleic acids were fractionated into 2 M LiCl-insoluble and -soluble fractions (Diaz-Ruiz and Kaper, 1978). The latter was chromatographed twice on a CF-11 cellulose column (Dodds et al., 1987). The dsRNA was precipitated with ethanol. The dsRNA identity of the preparations was confirmed with RNase A digestion test: dsRNA samples were incubated with RNase A (100 μg/ml) in high salt buffer (3X SSC) (1X SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) or low salt buffer at 37°C for 30 min. After deproteination with phenol, the samples were checked by agarose gel electrophoresis.

ISOLATION OF POLYRIBOSOMES AND POLYRIBOSOMAL RNA

Total cellular polyribosomes were purified from maize, either healthy or MCDV-infected, mainly as described by Larkins (1986) with modifications. Briefly, young, apical plant tissue, harvested 7 to 10 days after inoculation, was ground in liquid nitrogen with a pestle in a mortar, and the powder was homogenized in four volumes (v/w) of tissue extraction buffer (TEB) (200 mM Tris-HCl, pH 8.5; 400 mM KCl; 200 mM sucrose; 35 mM MgCl₂; 25 mM ethylene glycol-bis-(2-amino ethyl ether)-N,N'-tetra acetic acid (EGTA); 1% 2-
mercaptopethanol). The extract was strained through two-layers of cheesecloth, and the filtrate was centrifuged at 2000g for 5 min. The supernatant was mixed with 1/10 volume of 10% Triton X-100 in TEB and centrifuged again at 30,000g (16,000 rpm) in a Type 35 rotor (Beckman) for 10 min at 4°C. The supernatant was then layered over 4 ml of a 1.75 M sucrose cushion in 40 mM Tris-HCl, pH 8.5, 200 mM KCl, 30 mM MgCl₂, 5 mM EGTA in SW41 rotor tubes (Beckman) and centrifuged at 225,000g (36,000 rpm) for 90 min at 4°C. The pellets were considered to be polyribosomes. To isolate polyribosomal RNA, the polyribosome pellets were resuspended in EDTA-containing buffer (40 mM Tris-HCl, pH 7.5; 150 mM KCl; 15 mM EDTA; 0.2 mg/ml heparin) or puromycin-containing buffer (50 mM Tris-HCl, pH 7.5; 500 mM KCl; 5 mM MgCl₂; 1 mM puromycin; 0.2 mg/ml heparin) (Blobel, 1971). Mg²⁺-containing buffer (25 mM Tris-HCl, pH 7.5; 150 mM KCl; 7.5 mM MgCl₂; 0.2 mg/ml heparin) was used as the control. Release of polyribosomal RNA using puromycin was achieved by Blobel's (1971) method. The suspensions were centrifuged in a microcentrifuge for 5 min at 4°C, and the supernatants were layered on 1.75 ml sucrose cushions in SW 60 tubes (Beckman), the same cushions as used to pellet polyribosomes. The samples were centrifuged at 225,000g (41,000 rpm) in a SW60 (Beckman) for 90 min at 4°C, and the upper phase above the cushion and the pellet were collected, adjusted to 15 mM EDTA and 1% SDS and extracted with PCIA.
RNAs from each fraction were precipitated with ethanol.

NORTHERN HYBRIDIZATION ASSAY

The Northern Blot protocol described by Williams and Mason (1985) was followed, and 1.0% agarose gels were usually used to separate RNAs. Prehybridization and hybridization buffers were 3X SSC (1X SSC: 0.15 M NaCl; 15 mM sodium citrate, pH 7.0), 5X Denhardt's solution, 0.1% SDS, 50 μg/ml yeast tRNA, and 50 μg/ml boiled salmon sperm DNA. The nitrocellulose filters were hybridized at 65°C for 18 to 24 hr and washed in 3X SSC and then 0.1X SSC containing 0.1% SDS at 65°C five times each, with a 10 min incubation between washings.

PREPARATION OF 32P-LABELED cDNA PROBES

Sucrose gradient purified, full-length MCDV RNA was primed with oligo(dT) and random primers and reverse transcribed by AMV reverse transcriptase (Bio-Rad, Richmond, CA) at 37°C for 2 hr in the presence of 32P-dCTP. The RNA template was degraded with 50 mM sodium hydroxide at 65°C for 1 hr, and unincorporated 32P-dCTP was separated from cDNA by chromatography through a Nick-column (Pharmacia, Piscataway, NJ).

In addition to cDNA probes reverse transcribed from MCDV genomic RNA, cloned MCDV capsid protein (CP) 1 and CP2
genes were also used to prepare DNA probes. These clones were cloned in lambda gt11, screened by antisera to MCDV CP1 and CP2, respectively, and the clones hybridized to MCDV cDNA probes reverse transcribed from MCDV RNA (M. D. McMullen, personal communication). The templates were denatured by boiling and then mixed with random primers, nucleotides, $^{32}$P-dCTP and the large fragment of E. coli DNA polymerase I (Klenow fragment), as described by Sambrook et al. (1989). The incubation was at room temperature for 2 hr, and the reaction was terminated by adding EDTA to 50 mM final concentration. Unincorporated $^{32}$P-dCTP was separated from synthesized DNA by chromatography on the Nick-column (Pharmacia, Piscataway, NJ). The probes were denatured by boiling before hybridization.

RNA QUALITY AND QUANTITY ASSAY

The quality of RNA was judged by mini-agarose gel electrophoresis and UV-absorbance $A_{260/280}$ ratio. Only RNAs with $A_{260/280} > 1.8$ were used. RNA quantity was determined by using an extinction coefficient = 24.

CELL-FREE TRANSLATION ASSAYS

Viral RNAs were translated in both rabbit reticulocyte lysate (RRL) and wheat germ extract (WGE) cell-free translation systems (BRL, Gaithersburg, MD).
1. Translation in WGE: The translation of MCDV RNA in WGE was conducted as described by the manufacturer. $^{35}$S-methionine (250 $\mu$Ci/ml final concentration) was used to label the synthesized proteins. The incubation was at 25°C for 2 hr.

2. Translation in RRL system: 30 $\mu$l of reaction mixture contained 1.2 mM Mg$^{2+}$; 220 mM K$^+$; 23 mM NaCl; 25 mM HEPES (pH 7.2); 0.33 mM calcium chloride (CaCl$_2$); 0.7 mM EGTA; 0.017 mM EDTA; 0.17 mM dithiothreitol; 8.3 $\mu$M hemin; 17 $\mu$g/ml creatine kinase; 10 mM creatine phosphate; 50 $\mu$M of each amino acid (minus methionine); 330 $\mu$Ci/ml $^{35}$S-methionine; and various amounts of mRNA. The incubation was at 30°C for 60 min, and the reaction was stopped by adding 1 $\mu$g of boiled RNase A and incubating at 30°C for 15 min.

3. Incorporation assay, immunoprecipitation assay, polyacrylamide gel electrophoresis, and fluorography: These assays were conducted according to the procedures described by Davis et al. (1986). Antisera used were provided by Dr. D. T. Gordon.

POLYRIBOSOME IMMUNOPRECIPITATION ASSAY

1. Preparation of RNase-free IgGs from antisera: IgGs were isolated from whole rabbit sera by protein A-Sepharose chromatography. Briefly, antisera (D. T. Gordon, personal
communication) were diluted with 1 volume of TN buffer (20 mM Tris-HCl, pH 8.0; 100 mM NaCl) and added to a protein A-Sepharose column. The column was washed with TN buffer, and the IgG attached to the column eluted with 100 mM glycine (pH 3.0). The eluted IgG was immediately neutralized with 0.5% (v/v) 1 M Tris and NaN₃ was added to 0.2%. The IgG was stored at 4°C.

2. Immunoprecipitation: Polyribosomes were purified as described above. All the following manipulations were done in cold room unless otherwise indicated. Polyribosome pellets were resuspended in polyribosome buffer (PB) (25 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM MgCl₂, 0.2 mg/ml heparin; and 1 mg/ml cycloheximide) and rocked for 20 min. The suspension was centrifuged in microcentrifuge for 5 min and the supernatant recovered. 20 μg of protein A column-purified IgG from antisera to MCDV, maize dwarf mosaic virus (MDMV) virions or preimmune serum were added to aliquots of the polyribosome supernatant (about 0.5 A₂₆₀ unit in 300 μl) and incubated for 45 min. 75 μl of 10% protein A-Sepharose in PB was added to the mixture, and the latter was rocked for 1 hr. The immune-complex was separated from the other components by centrifugation for 1.5 min in a microcentrifuge. The "supernatant" was recovered. The immune-complex pellet was washed with PB five times, and polyribosome dissociation buffer (PDB) (40 mM Tris-HCl, pH
8.5, 15 mM EDTA) was added. The mixture was rocked in cold room for 20 min and centrifuged for 1.5 min. The supernatant was recovered. This step was repeated and the resulting supernatant was designated "polyribosomal RNA". The pellet after EDTA treatment was washed using a vortex mixer with PDB plus 1% SDS at room temperature three times, with 5 min between washes, and then centrifuged for 1.5 min. The supernatant, designated "pellet", was recovered. The "supernatant", "polyribosomal RNA" and "pellet" were treated with EDTA and SDS (final concentration 20 mM and 1%, respectively) and extracted with PCIA. The RNAs were precipitated from the aqueous phases with ethanol. MCDV RNAs in these fractions were detected by Northern hybridization assay.

**ELECTRON MICROSCOPY**

1. **Direct electron microscopy (EM)**

   **Polyribosome**: Copper EM grids covered with carbon were floated on polyribosome preparations in polyribosome buffer (PB) (see above) for a few min. The excess liquid was drawn off with a piece of filter paper, and the polyribosomes on the grid were fixed with 2% glutaraldehyde in 50 mM sodium cacodylate (pH 7.2) for 2 min (Larkins, 1986). The grid was rinsed with water, stained with 2.5% uranyl acetate for 5 min and viewed under EM.
**Virus:** EM grids were floated on virus suspensions in 1X NET buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 1 mM EDTA) or 0.1 M potassium phosphate buffer for a few min. The samples were stained with 2.5% uranyl acetate for 5 min.

2. **Immunosorbent electron microscopy (ISEM)**

   **Polyribosome:** All following operations were performed at room temperature. EM grids were first coated with protein A-sepharose purified rabbit IgG at the concentration of 10 ng/ml in TN buffer (20 mM Tris-HCl, pH 8.0; 100 mM NaCl) for 10 min. The grids were then rinsed with 20 drops of water and floated on polyribosome suspension in PB for 10 min. The grids were washed with 30 drops of PB and then fixed, rinsed with water and stained with uranyl acetate as described above.

   **Virus:** The same procedure as above was used except that the virus was in 1X NET buffer instead of PB. The fixation step was omitted.

**IODINATION OF VIRAL RNA TO DETECT VPg**

Chloramine T method of iodination described by Harlow and Lane (1988) was used with some modification as follows. RNA preparations (about 300 µg for MCDV RNA and 50 µg for SSVLP RNA) were adjusted to 40 mM sodium phosphate (pH 7.5) in a final volume of 25 µl and 500 µCi of Na\(^{125}\)I (1.5 µl)
(carrier free) (New England Nuclear, Boston, MA) was added. 25 μl of chloramine T (1 mg/ml) was then mixed with the sample and the mixture was incubated at room temperature for 2 min. The reaction was stopped by adding 50 μl of stopping buffer (2.4 mg/ml sodium metabisulfite, 10 mg/ml L-D-tyrosine, 10% glycerol, 10 mM sodium phosphate, pH 7.5). Free 125I was separated from labeled RNA by chromatography on a Nick Column (Pharmacia, Piscataway, NJ) using a buffer of 10 mM Tris-HCl, pH 7.5, plus 0.5% SDS. The labeled RNA fraction was precipitated with ethanol and re-extracted three times with PCIA at 65°C. The RNA in the aqueous phase was precipitated with ethanol. RNase A and proteinase K digestions were performed in 1X NET and 1X NETS, respectively (1X NET: 10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA) (1X NETS: 1X NET plus 0.5% SDS) at 42°C for 3 hr with RNase A (100 μg/ml) and proteinase K (50 μg/ml). 15% SDS-PAGE was used to separate proteins. The gel was dried and autoradiographed.

**LABELING THE 3' END OF RNA WITH [5'−32P]pCp**

Both poly(A)+ and unfractionated MCDV RNA were labeled with [5'−32P] 3', 5'-diphosphate cytidine (pCp) (New England Nuclear, Boston, MA) by T4 RNA ligase (Pharmacia, Piscataway, NJ) (England and Uhlenbeck, 1978) in a reaction containing 50 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 10 mM DTT; 1 mM ATP; 100 μg/ml; 20 μCi [32P] pCp; 400 μg/ml RNA; and 600
units/ml T4 RNA ligase. The reaction was incubated at 37°C for 45 min. The unincorporated nucleotides were separated from labeled RNA by chromatography on a Nick Column.

**cDNA CLONING AND SEQUENCING**

1. **SSVLP RNA preparation:** SSVLP was isolated from MCDV-T-infected maize using the same method used to purify MCDV. The RNA was isolated by dissociating virions with 1% SDS, heating at 65°C for 5 min and sedimenting on 5-20% sucrose gradient in a buffer of 10 mM Tris-HCl, pH 7.5, 20 mM sodium acetate, 1 mM EDTA, and 0.1% SDS in SW60 rotor (Beckman) at 55,000 rpm for 4 hr at 4°C. The RNA band was collected with an Instrumentation Specialities Co. (ISCO) fractionator, precipitated with ethanol and centrifuged as above. The purified RNA was polyadenylated with *E. coli* poly A polymerase as described by Sipple (1973). The polyadenylated RNA was extracted with PCIA and precipitated with ethanol. The RNA was then used for cDNA synthesis.

2. **cDNA synthesis and cloning:** cDNA synthesis and cloning were done with kits purchased from Amersham (United kingdom). First strand-DNA was synthesized with AMV reverse transcriptase using oligo(dT)$_{12-18}$ primers. The second strand-DNA was synthesized with DNA polymerase I, RNase H and *E. coli* ligase. Adapters (5'-AATCGAGGATCCTGATGTTCTCCGATGTTGATTCCGTTGACTGAG-3') were
added to the ends of the cDNAs, and the latter ligated into lambda gt10's EcoRI sites, packaged and plated as described by the manufacturer. Clones were selected by the plaque hybridization method using cDNA probes reverse transcribed from SSVLP RNA. After secondary screening, five clones of various sizes were selected. The recombinant DNAs were isolated from twice CsCl-purified gt10 virus, digested with EcoRI and electrophoresed in 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing ethidium bromide (Sambrook et al., 1989). The inserts were purified from agarose with the GENE CLEAN kit (Bio 101, La Jolla, CA), and then ligated into the dephosphorylated, linearized phagemid vector pUC119's EcoRI (poly-linker) site. The ligated pUC119 was used to transform competent E. coli MV1190 cells. Clones were selected by plasmid minipreparation followed by restriction enzyme (EcoRI) digestion, agarose gel electrophoresis and Southern hybridization to SSVLP cDNA probes.

3. Nucleic acid sequencing: Single-stranded DNA templates were generated by superinfection with helper phage M13K07. DsDNA templates were prepared from phagemid minipreparations, which were further cleaned with either the GENE CLEAN kit (Bio 101, La Jolla, CA) or RNase A digestion followed by phenol:chloroform extraction. Double-stranded DNA templates were denatured with 0.1 N NaOH, precipitated
with ethanol, annealed with either forward primer (5'-GTTTTCCCAGTCACGAC-3') or reverse primer (5'-AACAGCTATGACCATG-3') and sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a DNA sequencing kit (Sequenase version 2.0, US Biochemicals, Cleveland, OH).

A clone (No. 12) of about 1 kb was further digested with "four-cutter" restriction enzymes (AluI, HaeIII and Sau3AI) to generate smaller fragments for sequencing. The fragments were ligated into pUC119's SmaI (AluI and HaeIII fragments) or BamHI (Sau3AI fragments) site and used to transform E. coli MV1190.

4. Sequence of 5' terminus of SSVLP RNA: The 5' terminal sequence of SSVLP RNA was determined by sequencing the RNA with synthetic primer (3'-GGTGGCCTTTTAGAAGGTTA-5'), which was derived from clone No. 12. A RNA sequencing kit (Boehringer, Indianapolis, IN) was used for the sequencing reaction.

TRANSMISSIBILITY OF SSVLP BY LEAFHOPPER Graminella nigrifrons

The protocol used was that described by Hunt et al. (1988). G. nigrifrons from oats were fed on maize, either healthy or MCDV-T infected, for 2 to 3 days. Leafhoppers
then were allowed to feed on purified SSVLP in 10% sucrose or 10% sucrose alone in 10 mM potassium phosphate buffer, pH 7.0, through a Parafilm membrane for 0.5 to 2 hr. These leafhoppers were used to inoculate either healthy or previously MCDV-T-inoculated maize for 2 days. The leafhoppers were killed with insecticide, and the plants were placed in a greenhouse. SSVLP infection was detected by either nucleic acid filter hybridization (see below) or sucrose gradient assay, the same method as used to purify MCDV.

PREPARATION OF SAMPLES FOR NUCLEIC ACID FILTER HYBRIDIZATION AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Plant tissue (0.4 g) was ground in liquid nitrogen and then briefly in 1 ml of extraction buffer (40 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) using a mortar and pestle. One ml of chloroform was added and the mixture was ground again. The extract was centrifuged in a microcentrifuge in a cold room for 10 min, and the supernatant was used for ELISA. For dot blot nucleic acid hybridization, 0.2 ml of the supernatant was adjusted with SDS to 1%, and extracted with equal volume of a phenol:chloroform mixture (1:1). The extracted supernatant was spotted on a nylon membrane and hybridized with the same probes as used for Northern blots. For quantitative assay, the spots were cut from the membrane after hybridization and the radioactivities (cpm) of each
sample were counted with a scintillation counter.

For ELISA, F(ab')$_2$ units of MCDV antiserum (D. T. Gordon, personal communication) were used to coat microtiter plates. The test samples were diluted to 50 fold (v/w) with TBS-T (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), which was the best dilution for determining the concentrations of the antigens as tested by serial dilution. Biotinylated protein A-horseradish peroxidase streptavidin system (Amersham, UK) was used to detect the presence of antigens and antibodies. The substrate was 1 mM 2, 2'-azinobis (3-ethylben-thiazoline sulfonic acid) diammonium salt (ABTS) in 0.1 M citric acid, pH 4.2, plus 0.1% H$_2$O$_2$. The results were read in a Microplate Autoreader (EL309) (Bio-Tek Instruments, Inc.) at a wavelength of 405 nm. The average value of four replicate wells of each sample was taken and the average values of the samples in different treatments were statistically analyzed by t-test according to Bryant (1960).
CHAPTER III

RESULTS

A. MCDV RNA

COMPONENTS AND SIZE OF MCDV GENOMIC RNA

1. Components of MCDV genome

Agarose gel electrophoresis of RNA extracted from purified MCDV virions revealed one predominant band with a smear of faster migrating RNA (Fig. 1A, lane 1). Rate zonal sucrose density gradient centrifugation of SDS-dissociated purified MCDV virions also revealed a major peak with slower sedimenting fragments (Fig. 1B). The shape of the absorbance curve of the fractionated MCDV RNA was essentially the same as that of RNA obtained by phenol extraction of purified MCDV (Fig. 1B). The RNA from the major peak was collected and showed a discrete band when examined in a minigel (Fig. 1A, lane 2). The RNA from the major peak was considered full-length, genomic MCDV RNA and was used as template to prepare cDNA probes.
Figure 1. Components of maize chlorotic dwarf virus (MCDV) RNA in virions. MCDV RNA from purified virions was electrophoresed in agarose minigel in TBE buffer (Sambrook et al., 1989) (A) and centrifuged on a rate zonal sucrose density gradient (B). A: lane 1: about 200 ng MCDV RNA extracted from purified virions; lane 2: about 200 ng MCDV RNA collected from the major absorbance peak from centrifuged gradient shown in B. B: ultraviolet scanning profile of centrifuged MCDV RNA. Solid line: 300 μg MCDV virions dissociated with 1% sodium dodecyl sulfate (SDS) by heating at 65°C for 5 min and RNA centrifuged on gradient; dash line: MCDV RNA extracted from 300 μg MCDV virions by SDS-phenol, precipitated by ethanol, solubilized and centrifuged on gradient.
Figure 1.
2. The size of MCDV genomic RNA

The size of MCDV genomic RNA was measured under denaturing conditions by agarose gel electrophoresis. Phage lambda DNA HindIII fragments of 23130, 9416, 6557, 4361, 2322 and 2027 base pairs (bp) [in denaturing gel, the sizes were referred as nucleotides (NT)], respectively (Sambrook et al, 1989) were used as size markers (Williams and Mason, 1985). The validity of using DNA fragments as size markers for RNA was tested by coelectrophoresis of the markers with two maize ribosomal RNAs of 3600 and 2000 NT, respectively (Pring and Thornbury, 1975). Results showed that denatured RNA and DNA fragments behaved similarly in the electrophoresis (Fig. 2), and the average correlation coefficient between the distances of migration and the sizes of the RNA and DNA fragments was 0.992 for four separate assays. Under the experimental conditions, the sizes of lambda DNA HindIII fragments and the distances of their migration in the gel fit a double logarithmic regression better than a semi-logarithmic regression formula (Fig. 2; Table 3), but for the nucleic acids smaller than 10 kB, the shapes of the two regression curves were almost the same (Fig. 2; Table 3). The inclusion of the largest fragment (23130 NT) of the lambda DNA HindIII digest into the regression usually biased the regression curve by increasing the standard deviation and decreasing the correlation coefficient (Table 3; Fig. 2), implying that the large
fragment migrated differently from the smaller ones in the gels used. Thus the size of MCDV RNA was estimated in two ways: a) using the regression formulae obtained with size markers including the largest fragment and b) using the formulae obtained with size markers excluding the largest fragment. The average size of MCDV genomic RNA calculated from twelve independent determinations in the first way was 14,900 NT (SD=1196, SD: standard deviation) and 12,669 (SD=3529), by the double-log and semi-log regression formula, respectively, while the average size calculated in the second way was 12,980 NT (SD=918) and 10,649 (SD=673), by double-log and semi-log formula, respectively. Since it was impossible to determine which formula reflected the real relationship between the sizes of nucleic acids and their migration distances in the electrophoresis, the average value of 12800 or about 13 kB calculated from the both ways was accepted as the size of MCDV genome.
Figure 2. Relationships between the sizes of denatured DNA fragments and RNAs and their migration distances in agarose gel electrophoresis. Lambda DNA HindIII fragments and maize ribosomal RNAs were denatured with glyoxal and dimethyl sulfoxide (DMSO) and electrophoresed in 1% agarose gel in 10 mM sodium phosphate buffer (pH 6.5). The gel was neutralized with 0.5 M Tris-HCl, pH 8.5, and stained with ethidium bromide. The nucleic acids were visualized under ultraviolet light. The logarithm of sizes of the nucleic acids was plotted vs. the distance of their migration and the logarithm of the distances of their migration.
Figure 2.

Migration distance (mm)

Length of the size markers (NT)

Logarithm of migration distance [ln(mm)]

ln(NT) = a + b(mm)
Table 3. Analysis of relationship between size of nucleic acids and their mobilities in electrophoresis using double-logarithmic and semi-logarithmic formula

<table>
<thead>
<tr>
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<th>Double-logarithm</th>
<th>Semi-logarithm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>r</td>
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<tr>
<td>N=6</td>
<td>0.992 (0.004)</td>
<td>0.948 (0.010)</td>
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<tr>
<td></td>
<td>0.091 (0.020)</td>
<td>0.232 (0.022)</td>
</tr>
<tr>
<td>N=5</td>
<td>1.000 (0.001)</td>
<td>0.993 (0.002)</td>
</tr>
<tr>
<td></td>
<td>0.017 (0.005)</td>
<td>0.063 (0.010)</td>
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</tbody>
</table>

a Average of twelve separate assays.

b Double-logarithm: ln(Y) = a + bln(X); semi-logarithm: ln(Y) = a + bX; where Y is the length of nucleic acid expressed in nucleotides (NT), X is the distance the nucleic acid migrated in gel in millimeter (mm); a is a constant; b is a factor.

c Correlation coefficient. Numbers in parenthesis are the standard deviations of the r value.

d Numbers of size markers (23,130, 9416, 6557, 4361, 2322 and 2027 NT, respectively) used to determine the regression, namely, N=6 includes the largest fragment (23,130 NT) of lambda DNA HindIII digest; N=5 excludes the largest fragment.

e Standard deviation. Numbers in parenthesis are the standard deviations of the SD value.
TERMINAL STRUCTURES OF MCDV GENOME

1. 5' Terminus

1). Effect of "cap" analogue on the translation of MCDV RNA in vitro.

The 5' terminus of MCDV RNA was first studied using a cell-free translation assay in rabbit reticulocyte lysate (RRL) containing a cap analogue, 7-methyl-guanosine 5'-diphosphate (Sigma, St. Louis, MO) to determine whether or not a cap structure was present. Maize dwarf mosaic virus (MDMV) RNA, a potyviral RNA probably having a VPg at the 5' terminus as other RNAs of potyviruses (Hari, 1981; Riechmann et al., 1989; Siaw et al., 1985), and brome mosaic virus (BMV) RNAs and globin mRNA, both with a cap structure at their 5' termini (Cho et al., 1978; Symons, 1985), were used as controls. Results demonstrated that MCDV RNA translation in RRL was not inhibited by the cap analogue (Fig. 3). For the controls, MDMV RNA translation was not inhibited, while the translations of BMV RNAs and globin mRNA were reduced to about 20% in the presence of the cap analogue (Fig.3). Thus, MCDV RNA appears not to be capped.
Figure 3. Effects of cap analogue (7-methylguanosine 5'-diphosphate) on the relative translation efficiency of various RNAs in cell-free rabbit reticulocyte lysate (RRL). RRL contained 0, 0.5, 1.0 and 1.5 mM cap analogue, respectively, S-methionine, and RNAs of maize chlorotic dwarf virus (MCDV), maize dwarf mosaic virus (MDMV), brome mosaic virus (BMV) and globin mRNA, respectively. The reactions were incubated at 30°C for 1 hr, and terminated by adding RNase A and incubating for another 15 min at the same temperature. Trichloracetic acid (TCA) (5%) insoluble products were precipitated onto filter paper and counts (cpm) of S-methionine were made with a scintillation counter. The relative efficiency of translation was calculated by the following formula:

Efficiency of translation =

\[
\frac{T_n}{B_n} \times 100\% + \frac{T_0}{B_0}
\]

where \(T_n\) and \(T_0\) = cpm for TCA precipitates of the translation products with or without cap analogue, respectively; \(B_n\) and \(B_0\) = cpm for TCA precipitates of background (no RNA control) with or without cap analogue, respectively.
Figure 3.
2). Detection of VPg in MCDV genome.

To test for the presence of a VPg, MCDV RNA was purified by a guanidine isothiocyanate-CsCl method (Chirgwin et al., 1979) to eliminate non-covalently bonded capsid proteins. The RNA was then labeled with Na\(^{125}\)I (carrier free) (New England Nuclear, Boston, MA) by the chloramine T method as described in MATERIALS AND METHODS. A broad protein band with a molecular weight of about 18 kDa was found associated with MCDV RNA after vigorous efforts to separate non-covalently bonded proteins (Fig. 4). The protein identity of this band was confirmed by its resistance to RNase but sensitivity to proteinase.

2. 3' Terminus

1). Presence of poly(A) tract in MCDV RNA.

About 80% of MCDV RNA end-labeled with \([5'-32P] 3', 5'-diphosphate cytidine (pCp) (New England Nuclear, Boston, MA) by RNA ligase was bound to the oligo(dT) column at high salt condition (Fig. 5A), implying that most of the MCDV RNA with an intact 3' terminus contains poly(A) sequence long enough to bind the column, since RNA ligase cannot ligate pCp to the degraded ends of RNA (England and Uhlenbeck, 1978). As a control, almost all labeled maize ribosomal RNA was eluted off the column with high salt buffer (Fig. 5B).
Figure 4. Detection of viral protein covalently linked to the genome (VPg) of maize chlorotic dwarf virus (MCDV). MCDV RNA was purified by the guanidine isothiocyanate-CsCl method, and proteins associated with the RNA were labeled with Na$^{125}$I with chloramine T. The labeled RNA-protein complex was further extracted with phenol/chloroform mixture three times at 65°C in the presence of 1% sodium dodecyl sulfate (SDS). The proteins were separated by electrophoresis in a 15% polyacrylamide gel containing SDS. Lane 1: acetone precipitated proteins from the phenol phase of the RNA extraction (note the presence of MCDV CP1, CP2 and CP3, with M,s of 34, 26 and 24 kDa, respectively) (Maroon et al., 1989); lane 2: RNA digested with RNase A followed by proteinase K; lane 3: RNA digested with RNase A only.
Figure 5. Detection of a poly(A) sequence in maize chlorotic dwarf virus (MCDV) genomic RNA. MCDV RNA (A) and maize ribosomal RNA (B) were end-labeled with [5'-$^{32}$P]3', 5'-diphosphate cytidine (pCp) by T4 RNA ligase, and then chromatographed through an oligo(dT) cellulose column first with a high salt buffer (40 mM Tris-HCl, pH 7.5; 0.5 M NaCl, 1 mM EDTA, 0.5% SDS), followed by a low salt buffer (40 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.5% SDS). The elutions were collected in aliquots and the amount of $^{32}$P was counted with a scintillation counter.
Figure 5.

Percentage of radioactivity

Volume of elution

High salt

Low salt

A

B

Volume of elution

Percentage of radioactivity

High salt

Low salt
2). Localization and estimation of the length of poly(A) in MCDV RNA.

In an experiment to demonstrate that the poly(A) tract is 3' terminal, MCDV RNA was extracted from MCDV virions and poly(A)⁺ MCDV RNA was selected by oligo(dT) chromatography. The poly(A)⁺ MCDV RNA was labeled with [5'-³²P]pCp by T₄ RNA ligase (Pharmacia) (England and Uhlenbeck, 1978). The labeled RNA was digested with RNase T₁ (Pharmacia) (100 units/ml) in a buffer of 16 mM sodium citrate, pH 5.0, 0.8 mM EDTA, and 3.5 M urea at 50°C for 1 hr, and the reaction mixture was electrophoresed in a 6% polyacrylamide sequencing gel containing 7 M urea and autoradiographed. Poly(A) tails with length of 30 NT to 150 NT were detected, with the strongest labeling at about 90 residues (Fig. 6).

3). Sequences of MCDV RNA adjacent to poly(A) tail:

A cDNA clone of about 250 NT in length was selected from a MCDV cDNA library constructed in phage lambda gt10 (M. D. McMullen, personal communication). This clone hybridized to the cDNA probes to MCDV RNA. The clone was subcloned into phagemid pUC119, and the double-stranded DNA template was sequenced using a reverse primer to pUC119 vector. The clone contained about 100 adenine residues at the 3' end [poly(A)], and 168 adjacent nucleotides. The sequence of the nucleotides is listed in Fig. 7. The sequence (excluding the poly(A) tail) contained 44 (26.2%)
adenine, 37 (22.0%) cytosine, 42 (25.0%) guanine and 45 (26.8%) uridine residues. A sequence (AGAUAAA) which resembles the polyadenylation signal (AAUAAA) was 11 nucleotides upstream from the poly(A) sequence. The last 38 NT adjacent to the poly(A) tail contained 64% U and A residues.

CELL-FREE TRANSLATION OF MCDV RNA

1. Translation of MCDV RNA in wheat germ extract (WGE)

MCDV RNA (12 μg/ml) was translated in WGE (BRL, Gaithersburg, MD) using potassium concentrations of 40, 120, 200 and 280 mM. Although the incorporation of 35S-methionine into TCA-insoluble products varied with [K+]i, namely 1.3, 1.7, 1.8 and 1.4 times higher than the no RNA control, respectively, the patterns of proteins translated were basically the same (Fig. 8). Six major polypeptides (Fig. 8, indicated by arrows) and a series of minor polypeptides were detected in SDS-PAGE after fluorography (Fig. 8; Table 4), with the largest ones being over 200 kDa. No translated proteins comigrated with MCDV-capsid proteins.

Compared with rabbit reticulocyte lysate (RRL), WGE gave a higher background (Fig. 8, lane 2, Fig. 10, lane 1). Thus, most cell-free translations were conducted in RRL.
Figure 6. Localization and size of the poly(A) tract in maize chlorotic dwarf virus (MCDV) RNA. MCDV RNA end labeled with [5'-32P] 3', 5'-diphosphate cytidine by T4 RNA ligase was digested with RNase T1 in a buffer of 16 mM sodium citrate, pH 5.0, 0.8 mM EDTA and 3.5 M urea as suggested by manufacture (Pharmacia) for 1 hr at 50°C. The digest was electrophoresed in a 6% polyacrylamide sequencing gel containing 7 M urea. The arrow points to the 90th nucleotide.
Figure 7. Nucleotide sequence of maize chlorotic dwarf virus (MCDV) genomic RNA adjacent to the poly(A) sequence. A MCDV cDNA clone of about 250 NT containing a poly(A) sequence was screened out from MCDV cDNA library (M. D. McMullen, personal communication) in lambda gt10, and subcloned into pUC119 phagemid. The double-stranded phagemid was sequenced directly with a reverse primer using Sanger's chain termination method (Sanger et al., 1977).
5' CCACACUUCU CCACGGGUU GUGCUGCAGU AUUAAUAUC AUUAAGGUAC
UGUGCUAUAG CCGAGAAAUU ACAAAGCGUU GAAACAUAUG ACGAUGGGGC
CCAAUGCGCA CCCGGAUGUG UUACGCACCG UUUUCUCUG UGUCACUAUA
AGAUAAAAGU GGGGUAGC(A)\textsubscript{100} 3'

Figure 7.
Figure 8. Translation of maize chlorotic dwarf virus (MCDV) RNA in wheat germ extract cell-free translation systems. Lanes from left to right: Brome mosaic virus (BMV) RNA translation products; no RNA control; and MCDV RNA translation products at 40, 120, 200 and 280 mM potassium ion. The translated products were separated by electrophoresis in a 10% polyacrylamide gel containing sodium dodecyl sulfate. The sizes of BMV RNA translation products were indicated to the left side of the figure according to Symons (1985) and Pyne and Hall (1979); major polypeptides synthesized from MCDV RNA were indicated by arrows to the right side. BMV RNA and no RNA control were in the reaction mixtures containing 200 mM potassium.
Figure 8.

BMV RNA
No RNA
MCbV RNA

280 mM
200 mM
120 mM
40 mM

120 mM
200 mM
40 mM

1
2
3
4
Table 4. Molecular weights of polypeptides synthesized from maize chlorotic dwarf virus (MCDV) RNA in rabbit reticulocyte lysate (RRL) and wheat germ extract (WGE) cell-free systems8

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>RRL</th>
<th>WGE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Majorb</td>
<td>Minorc</td>
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<td>1</td>
<td>209d</td>
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<td>2</td>
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<tr>
<td>6</td>
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<td>31</td>
</tr>
</tbody>
</table>

a The molecular weights of the polypeptides synthesized from MCDV RNA were determined from Figure 10 for RRL and Figure 8 for WGE. The conditions for the assays were described in the MATERIALS AND METHODS.

b "Major polypeptide" means stronger bands detected.

c "Minor polypeptide" means weaker bands detected.

d Molecular weight in kilodaltons (kDa).
2. Translation of MCDV RNA in RRL

1). Incorporation of $^{35}$S-methionine into polypeptides.

The incorporation of $^{35}$S-methionine into TCA-precipitable peptides was positively proportional to the concentration of MCDV RNA, with the correlation coefficient $r = 0.788$ (N=18) for viral RNA concentrations from 6 to 150 $\mu$g/ml (Fig. 9A). The highest incorporation was six times above the no RNA control (background). In parallel experiments, MDMV-A RNA gave similar levels of incorporation, MDMV-O RNA a little more, and BMV RNAs much higher incorporation (Table 5).

The incorporation of $^{35}$S-methionine for MCDV RNA translation was greatest at about 45 min after incubation at 30°C (Fig. 9B).

2). Molecular weights of polypeptides synthesized in RRL.

More than a dozen polypeptide bands were revealed after separating the translation products by SDS-PAGE (Fig. 10). The sizes of the polypeptides were determined by using a regression formula derived from co-electrophoresed protein size-markers (Table 4).
Figure 9. Factors affecting the translation of maize chlorotic dwarf virus (MCDV) virion RNA in rabbit reticulocyte lysate (RRL). A: Relative protein synthesized at various MCDV RNA concentrations incubated at 30°C for 1 hr. B: Relative protein synthesis at various incubation times for MCDV RNA (33 μg/ml) at 30°C. The relative yield of proteins synthesized were determined from the counts (cpm) of 35S-methionine in trichloracetic acid (5%) insoluble fraction of the translation products divided by those of no RNA control. The concentrations of Mg²⁺ and K⁺ were 1.2 and 220 mM, respectively, for both assays.
Figure 9.
Table 5. Incorporation of $^{35}$S-methionine into polypeptides by different viral RNAs in rabbit reticulocyte lysate cell-free system

<table>
<thead>
<tr>
<th>RNA</th>
<th>Concentration of viral RNA (μg/ml)</th>
<th>Incorporation (times over background)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCDV</td>
<td>37.5</td>
<td>2.3</td>
</tr>
<tr>
<td>MDMV-A</td>
<td>37.5</td>
<td>2.3</td>
</tr>
<tr>
<td>MDMV-O</td>
<td>37.5</td>
<td>7.8</td>
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<tr>
<td>MCDV</td>
<td>67.0</td>
<td>4.1</td>
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<td>MDMV-O</td>
<td>73.0</td>
<td>9.2</td>
</tr>
<tr>
<td>BMV</td>
<td>12.5</td>
<td>54.2</td>
</tr>
</tbody>
</table>

* The conditions of translation assays were described in the MATERIALS AND METHODS.

b MCDV: maize chlorotic dwarf virus; MDMV: maize dwarf mosaic virus: A: strain A; 0: strain O; BMV: brome mosaic virus.
3). **Immunoprecipitation of polypeptides synthesized in RRL with MCDV antisera.**

Cell-free translation products of MCDV RNA in RRL, up to 2,250,000 cpm per reaction, were immunoprecipitated by antisera (As) to MCDV virion and CP1. Preimmune serum and As to MDMV-A and MDMV-O virions (D. T. Gordon, personal communication) were used as controls. No MCDV-As precipitable polypeptides were detected among the translation products of MCDV RNA. In controls, MDMV-A and MDMV-O As precipitated a series of proteins from translation products of MDMV RNAs, including capsid protein-sized proteins (data not shown).

4). **Effect of dithiothreitol (DDT) on post-translation processing of polypeptides synthesized in RRL.**

To test for the presence of proteolytic processing of large polypeptides, which could be facilitated by the presence of DTT (Xiong et al., 1988), MCDV RNA was translated in RRL for 60 min at 30°C, and the translation products was divided into two aliquots. DTT was added to the first aliquot to give a final concentration of 10 mM, while an equal volume of water was added to the second aliquot. The incubation was continued for another 60 min at 30°C. SDS-PAGE and autoradiography analysis of the treatments showed that there were no differences in protein patterns between the two treatments (Fig. 10).
Figure 10. Effects of dithiothreitol (DTT) on post-translational processing of the in vitro translation products of maize chlorotic dwarf virus (MCDV) RNA. MCDV RNA was translated in rabbit reticulocyte lysate (RRL) for 1 hr and the translation products were incubated with 10 mM DTT added (lane 2) or without DTT added (lane 3) at 30°C for another hr. Equal amounts of products were electrophoresed in 10% polyacrylamide gel containing sodium dodecyl sulfate. Lane 1: no RNA control. Molecular weight markers were indicated to the right side of the figure, they were, from top to bottom: myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrase.
Figure 10.
MCDV RNA IN INFECTED TISSUE

1. Total tissue cellular RNA and poly(A)-containing RNA from MCDV-infected tissue

Total tissue cellular RNA and poly(A)-containing RNA were isolated from both healthy and MCDV-infected tissue and analyzed by Northern hybridization. Only full-length MCDV RNA was detected in the preparations from MCDV-infected tissue with cDNA probes reverse transcribed from MCDV genomic RNA (Fig. 11A, lanes 4 and 6) and cloned cDNAs for CP1 and CP2 (M. D. McMullen, personal communication) (Fig. 16A and 16B, lanes 3 and 6). The probes did not hybridize to RNAs from healthy tissue (Fig. 11A, lanes 3 and 5; Fig. 16, lanes 2 and 5). No subgenomic-sized MCDV-specific RNAs were detected by these probes.

2. Double-stranded (ds) RNA from MCDV-infected tissue

Double-stranded RNA was isolated from MCDV-infected tissue and analyzed by Northern hybridization assay. The dsRNA identity was confirmed by its resistance to DNase, and RNase A at high salt (3X SSC), but its susceptibility to RNase A degradation at low salt (0.1X SSC). Denatured dsRNA comigrated with full-length MCDV RNA in agarose gel and hybridized to the cDNA probes reverse transcribed from MCDV viral RNA (Fig. 11B). No smaller sized dsRNAs were detected.
Figure 11. Detection of maize chlorotic dwarf virus (MCDV) RNA in virions (V), total cellular RNA (TC), poly(A)+ RNA (A+) and double-stranded (ds) RNA from MCDV-infected maize tissue. RNA samples were denatured with glyoxal and dimethyl sulfoxide and electrophoresed in 1% agarose gel. Separated RNAs were blotted onto nitrocellulose membrane and hybridized to MCDV cDNA probes. A: Lane 1: poly(A)+ MCDV virion RNA; lane 2: poly(A)+ MCDV virion RNA; lane 3: total cellular RNA from healthy maize tissue; lane 4: total cellular RNA from MCDV-infected maize tissue; lane 5: poly(A)+ RNA from healthy maize tissue; and lane 6: poly(A)+ RNA from MCDV-infected maize tissue. B: Lane 1: MCDV virion RNA; lane 2: dsRNA from MCDV-infected plants. Genomic-sized MCDV RNA (g) was indicated by arrows to the left sides of the panels.
Figure 11.
3. Polyribosomal RNA from MCDV-infected tissue

1) The absence of MCDV virions in polyribosome samples purified from MCDV-infected tissue.

Usually, it is assumed that polyribosome preparations purified from virus-infected tissue contain virus particles because most viruses have sedimentation coefficients larger than monosomes of 80 S (Vance and Beachy, 1984; Palukaitis, 1984). However, it was found that MCDV was not present in our polyribosome preparations. The following experiments were performed to test for the presence or absence of virions in polyribosome preparations: a) Immunosorbent electron microscopy (ISEM), with a sensitivity sufficient to detect MCDV virions at the concentration of less than 90 ng/ml (Fig. 12A-C), did not detect MCDV virions in polyribosome preparations from MCDV-infected tissue. Instead, large polyribosomes with more than fifty ribosomes from the preparation (Fig. 12D) were specifically absorbed on the grids coated with the IgGs of MCDV-As (Fig. 12E); b) When purified MCDV virions were added to healthy tissue extracts and polyribosomes and polyribosomal RNAs were purified, almost none of the viral RNA was found in the polyribosomes and polyribosomal RNA preparations. Rather, most MCDV RNA was recovered in the post-polyribosome supernatant fraction of centrifuged tissue extracts, as assayed by Northern hybridization (Fig. 13); c) Polyribosomes from healthy tissue were isolated and mixed
with MCDV virions. The mixture was treated with EDTA- or puromycin-containing buffers and then centrifuged through the sucrose cushions again (see MATERIALS AND METHODS). Mg\(^{++}\)-containing buffer was used as a control. Again, MCDV RNA remained in the upper phases above the cushions regardless of the presence or absence of polyribosome dissociation factors (Fig. 14). So, at this point, MCDV RNA in virions differs from that associated with polyribosomes in that the latter can go through the cushion in the presence of Mg\(^{++}\) (see following and Fig. 15).

2). Full-length MCDV RNA associated with polyribosomes in infected tissue.

Polyribosomal RNA was released from polyribosomes isolated from MCDV-infected tissue with EDTA or puromycin treatment. As a control, Mg\(^{++}\)-containing buffer was also used to treat the polyribosomes. Northern hybridization results showed that MCDV RNA was released by EDTA or puromycin treatment, but not by Mg\(^{++}\) (Fig. 15). Only full-length MCDV RNA was detected with cDNA probes reverse transcribed from MCDV genomic RNA (Fig. 15) and to cloned CP1 and CP2 genes (Fig. 16).
Figure 12. Immunosorbent electron microscopy (ISEM) of maize chlorotic dwarf virus (MCDV) virion and polyribosomes from MCDV-infected plants using polyclonal antiserum to intact MCDV virions. A–C: Sensitivity of ISEM for detecting MCDV virions. Purified MCDV was diluted to 8.7 (A), 0.87 (B) and 0.087 (C) μg/ml and assayed by ISEM. D–E: Polyribosome preparation detected by direct electron microscopy (D) and ISEM (E). Magnifications: A–C: 60,000X; D: 90,000X; E: 210,000X.
Figure 12. (continued).
Figure 13. Reconstitution assay for polyribosome isolation. 10 μg of MCDV virions were mixed with 8 gram of healthy maize tissue and polyribosomes were isolated from the mixture by pelleting through a sucrose cushion (see MATERIALS AND METHODS). Polyribosomal RNAs were isolated by resuspending the polyribosome pellet in EDTA-containing buffer and centrifuging through another sucrose cushion. Samples both above (polyribosomal RNA, lane 3) and below the cushion (nucleoprotein, lane 5) were collected and the presence of MCDV RNA in each part was tested by Northern hybridization using cDNA probes reverse transcribed from MCDV RNA. The supernatant above the first cushion (post-polyribosome fraction, lane 4) was used to isolate RNA by guanidine isothiocyanate-CsCl method. Samples in lanes 3-5 were about 7%, 2%, and 30% of total RNAs recovered from each fraction. Lane 1: MCDV virion RNA; lane 2: RNAs from polyribosome pellet of healthy maize tissue. Genomic-sized MCDV RNA (g) was indicated by an arrow to the left of the figure.
Figure 14. Reconstitution assay for polyribosomal RNA isolation. Polyribosomes isolated from healthy maize were mixed with maize chlorotic dwarf virus (MCDV) virion, and the mixed polyribosome samples were treated with EDTA (E), puromycin (Pu) or Mg$^{++}$ (Mg). The treated polyribosome samples sedimented through another sucrose cushion. Samples both above the cushion (A) and under the cushion (U) were collected and RNAs were extracted. V: MCDV virion RNA; H: polyribosomal RNA from healthy maize tissue; I: polyribosomal RNA from MCDV-infected maize tissue. MCDV RNA was detected by Northern hybridization assay using cDNA probes reverse transcribed from MCDV RNA. Genomic-sized MCDV RNA (g) was indicated by an arrow.
Figure 15. Maize chlorotic dwarf virus (MCDV) RNA associated with polyribosomes in MCDV-infected plant. Polyribosome sample from MCDV-infected maize tissue was treated with EDTA (E), puromycin (Pu) or Mg$^{++}$ (Mg). The treated polyribosome samples sedimented through a sucrose cushion. Samples both above the cushions (A) and under the cushions (U) were collected and RNAs were extracted. V: MCDV virion RNA; H: polyribosomal RNA from healthy maize tissue; I: polyribosomal RNA from MCDV-infected tissue. RNAs in lane H and I were extracted directly from polyribosome pellets. MCDV RNA was detected by Northern hybridization assay using cDNA probes reverse transcribed from MCDV RNA. Genomic-sized MCDV RNA (g) was indicated by an arrow to the left side of the figure.
Figure 15.
Figure 16. Detection of maize chlorotic dwarf virus (MCDV) RNA species in MCDV-infected tissue with probes prepared from MCDV capsid protein (CP) 1 and CP2 genes. MCDV RNA cDNA library was constructed in lambda gt11 and clones expressing MCDV CP1 and CP2 were selected by antisera to MCDV CP1 and CP2, respectively, and Southern hybridization to probes to MCDV RNA (M. D. McMullen, personal communication). RNA samples were denatured, electrophoresed and blotted onto nitrocellulose membranes. MCDV-specific RNAs were detected by probing the membranes with cDNA probes from randomly primed CP1 (A) and CP2 (B) genes. Lane 1: MCDV virion RNA; lane 2: total cellular RNA from healthy maize tissue; lane 3: total cellular RNA from MCDV-infected maize tissue; lane 4: total cellular RNA isolated from the healthy maize tissue mixed with MCDV virion RNA; lane 5: poly(A)* RNA from healthy maize tissue; lane 6: poly(A)* RNA from MCDV-infected maize tissue; lane 7: poly(A)* from the sample in lane 4; lane 8: polyribosomal RNA from healthy maize tissue; lane 9-10: polyribosomal RNAs from MCDV-infected maize tissue released by EDTA (lane 9) and puromycin (lane 10). Part of sample in lane 3 panel A was lost. MCDV genomic-sized RNA (g) and ribosomal RNA zones (r) were indicated by arrows. Note the presence of ribosomal zones in lane 4.
Figure 16.
3). Polyribosome immunoprecipitation assay.

Since the polyribosome samples purified from MCDV-infected maize contained no MCDV virions (Fig. 12, 13 and 14), it was assumed that any MCDV CPs associated with polyribosomes would be nascent peptides. Polyribosomes from MCDV-infected tissue were immunoprecipitated with MCDV-specific IgG. RNA was then dissociated from the immunoprecipitated polyribosomes with EDTA, followed by EDTA plus SDS. Genomic-sized MCDV RNA was detected by Northern hybridization assay of polyribosomal RNA samples precipitated by MCDV-specific IgG, but not from the samples precipitated by preimmune IgG or MDMV-A-specific IgG (Fig. 17). The results showed that MCDV CPs were associated with MCDV polyribosomal RNA in vivo.
Figure 17. Immunoprecipitation of polyribosomes purified from maize chlorotic dwarf virus (MCDV)-infected tissue. Polyribosomes purified from MCDV-infected maize tissue were resuspended and immunoprecipitated with rabbit IgGs from antiseras (As) to maize dwarf mosaic virus strain A (MDMV As), As to MCDV virions (MCDV As) or preimmune antiserum (Pre-As). MCDV RNA from unpprecipitated polyribosomes supernatant (spt) and immunoprecipitated polyribosomes released by EDTA (ppt-E), and followed by EDTA-sodium dodecyl sulfate (SDS) (ppt-S) were detected by Northern hybridization with cDNA probes reverse transcribed from MCDV RNA. MCDV genomic-sized RNA (g) and ribosomal zones (r) were indicated by arrows.
B. THE SATELLITE RNA OF MCDV

CHARACTERIZATION OF THE SLOWER SEDIMENTING VIRUS-LIKE PARTICLES (SSVLP)

1. Discovery of SSVLP

During Autumn, 1988, a slower sedimenting peak was detected for the first time in our laboratory in partially purified MCDV preparations by rate zonal sucrose density gradient centrifugation (Fig. 18). The peak was collected and pelleted, and the pelleted material was observed by electron microscopy. The pelleted material contained MCDV-like particles, except that their cores were stained by uranyl acetate (Fig. 19 A and B). The particle was referred to as the "slower sedimenting virus-like particle" (SSVLP).

2. Capsid proteins of SSVLP

Immunosorbent electron microscopy (ISEM) using antiserum to MCDV virions demonstrated that the SSVLP was serologically related to MCDV (Fig. 19 C and D). Purified SSVLP samples were dissociated with SDS and 2-mercaptoethanol, and the proteins were analyzed by SDS-PAGE. Three protein bands were detected by staining with Coomassie blue G250 in SSVLP samples, and these proteins coelectrophoresed with the three capsid proteins of MCDV (Fig. 20). No other proteins were detected in SSVLP samples.
3. **Nucleic acid of SSVLP**

Nucleic acids were extracted from SSVLP samples and electrophoresed in agarose gel and stained with ethidium bromide. A nucleic acid of about one kilobase (1 kB) was detected. The nucleic acid was resistant to DNase, but completely degraded by RNase A in high salt buffer (Fig. 21). The nucleic acid was a substrate of poly(A) polymerase, reverse transcriptase and T4 RNA ligase (see below).

4. **SSVLP particles**

Sometimes the SSVLP peaks showed multi-components upon rate zonal sucrose density gradient centrifugation (Fig. 22A). The heterogeneous peaks were collected together, and centrifuged in Cs₂SO₄ to equilibrium. Three components were detected with densities of 1.280, 1.295 (dominant component) and 1.310 gram/ml (Fig. 22B).
Figure 18. Detection of slower sedimenting virus-like particle (SSVLP): Ultraviolet (UV) absorbance profiles of rate zonal sucrose density gradients of preparations from MCDV-infected plants. MCDV was partially purified by high-speed centrifugation and low-molarity salt precipitation and centrifuged on sucrose density gradients. The centrifuged gradients were fractionated with an Instrumentation Specialities Co. (ISCO) fractionator and scanned with an ISCO ultraviolet monitor at 254 nm. A: Profile of a preparation before discovery of the SSVLP. B: Profile of a preparation after the discovery of SSVLP.
Figure 18.

OD254 nm

Depth in gradient

A

B
Figure 19. Electron micrograph of slower sedimenting virus-like particles (SSVLP) and maize chlorotic dwarf virus (MCDV) particles. Purified SSVLP (A and C) and MCDV (B and D) were absorbed to EM grids directly (A and B) or to the EM grids pre-coated with MCDV-specific antiserum (C and D). The samples were stained with 2.5% uranyl acetate. Magnifications of A and B: 180,000X; C and D: 110,000X.
Figure 20. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of slower sedimenting virus-like particle (SSVLP) and maize chlorotic dwarf virus (MCDV) capsid proteins (CPs). Purified SSVLP (lane 2) and MCDV (lane 3) were mixed with SDS and 2-mercaptoethanol, boiled for 5 min and electrophoresed in a 12.5% polyacrylamide gel containing SDS. The protein bands were stained with Coomassie Blue G-250. Molecular weight markers are shown in lane 1, which are, from top to bottom, phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The fine bands (arrow) in lane 2 and lane 3 were not consistently detected.
<table>
<thead>
<tr>
<th>Markers</th>
<th>SSVLP</th>
<th>MCDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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- 92
- 66
- 45

31
- [CP1]
- CP2
- CP3
21
14

Figure 20.
Figure 21. Nucleic acid of slower sedimenting virus-like particle (SSVLP). RNAs were electrophoresed in a 1% agarose minigel in TBE buffer (Sambrook et al., 1989) and stained with ethidium bromide. The RNAs were visualized under ultraviolet light. Lane 1: maize ribosomal RNAs as size markers [sizes (in kilobase) are indicated to the left side of the figure]; Lanes 2 and 3: nucleic acid was extracted with phenol from purified SSVLP, precipitated with ethanol and treated with DNase I (lane 2) and RNase A in 3X SSC (1X SSC: 0.15 M NaCl; 0.015 M sodium citrate, pH 7.0) (lane 3). After deproteination with phenol, the treated samples were electrophoresed and stained. Lane 4: MCDV virion RNA.
Figure 21.
Figure 22. Multi-components of slower sedimenting virus-like particles (SSVLP) in rate zonal sucrose density gradient centrifugation (A) and Cs₂SO₄ equilibrium centrifugation (B). Maize chlorotic dwarf virus (MCDV) was included in the centrifugations as a marker. The gradients were fractionated with an Instrumentation Specialities Co. (ISCO) fractionator with an ISCO ultraviolet monitor at wavelength of 254 nm. The peaks in B had densities of 1.280 (peak 1), 1.295 (peak 2), 1.310 (peak 3) and 1.442 g/ml (MCDV peak) in Cs₂SO₄, respectively.
Figure 22.
CHARACTERIZATION OF SSVLP RNA

1. Relationship between SSVLP and MCDV RNAs

Serially diluted MCDV RNA and SSVLP RNA were electrophoresed and blotted. The blots were hybridized to cDNA probes reverse transcribed from MCDV RNA (Fig. 23A) or from SSVLP RNA (Fig. 23B). Results showed that MCDV and SSVLP RNAs hybridized weakly to the heterologous probes (Fig. 23). The percentage homology was calculated to be less than 10% in the following manner: In Fig. 23A, 6.3 ng of MCDV RNA (lane 13) and 1000 ng of SSVLP RNA (lane 1) showed a similar intensity of label when hybridized to MCDV-cDNA probes. Since the size of SSVLP RNA is about 8% of MCDV genome (1000/13,000) and assuming that MCDV-cDNA probes covered the entire genome with equal intensity, the percentage sequence homology (X%) between the two RNAs would be: (X%) (8%) (1000) = (100%) (6.3). The calculation shows that the X% equals 8%.

2. Terminal structures of SSVLP RNA

The 5' terminus of SSVLP RNA was tested for the presence of VPg by iodination of the RNA with $^{125}$I. The experiment was conducted in parallel with MCDV RNA (Fig. 4). About 50 μg of SSVLP RNA was used for iodination, which is about two times more RNA in terms of moles than the amount of MCDV RNA used. No covalently linked protein was found associated with SSVLP RNA, whereas VPg was readily detected
in association with the genome of MCDV.

The possible presence of poly(A) sequence in SSVLP RNA was tested by oligo(dT) chromatography of SSVLP RNA 3' end labeled with [5'-\^32P]pCp. MCDV RNA and maize ribosomal RNAs were used as controls (see Fig. 5). No poly(A) sequence was found associated with SSVLP RNA (Fig. 24).

The 3' end of SSVLP RNA was tested for the presence of phosphate. SSVLP RNA was treated with or without calf intestinal alkaline phosphatase (0.1 unit/10 μl) in 50 mM Tris-Cl, pH 8.5. After incubation at 37°C for 30 min, the reaction mixtures were diluted with one volume of water and extracted with a phenol:chloroform mixture (1:1). The RNA in aqueous phase was labeled with [5'-\^32P] 3', 5'-diphosphate cytidine by RNA ligase directly. The incorporation of \^32P was tested after Nick-column separation of free nucleotide from RNA. Three independent experiments showed that dephosphorylated SSVLP RNA was labeled 1.6, 2.2 and 5.7 times more than SSVLP RNA incubated without the enzyme. As controls, phosphatase treatments of yeast tRNA and maize ribosomal RNA decreased labeling to 30 and 70%, respectively, compared with the RNAs incubated without the enzyme. The label was attached to the intact SSVLP RNA rather than a degraded form (Fig. 25).
Figure 23. Relationships between RNAs of slower sedimenting virus-like particle (SSVLP) and maize chlorotic dwarf virus (MCDV) as determined by Northern hybridization assays. Sucrose-gradient-purified RNAs from SSVLP and MCDV were electrophoresed in agarose gels after denaturation with glyoxal. The RNAs were transferred to a nitrocellulose membrane and hybridized with $^{32}$P-labeled cDNA probes reverse transcribed from MCDV RNA (A) or SSVLP RNA (B). The blots were washed at a high stringency conditions [0.1X SSC, (1X SSC: 0.15 M NaCl; 0.015 M sodium citrate, pH 7.0) 65°C] after hybridization. A: RNAs were probed with cDNA probes reverse transcribed from randomly primed MCDV RNA template. Lanes 1-6: SSVLP RNA of 1000, 500, 250, 125, 63 and 31 ng per lane, respectively; lane 7, 10,000 ng of total cellular RNA from healthy maize tissue; lane 8: no RNA (blank); lanes 9-14: MCDV RNA of 100, 50, 25, 12.5, 6.3 and 3.1 ng per lane, respectively. B: RNAs were probed with cDNA probes reverse transcribed from randomly primed SSVLP RNA template. Lanes 1-6: MCDV RNA of 1000, 500, 250, 125, 63 and 31 ng per lane, respectively; lane 7: 10,000 ng of total cellular RNA from healthy maize tissue; lane 8: no RNA (blank); lanes 9-14: SSVLP RNA of 100, 50, 25, 12.5, 6.3 and 3.1 ng per lane, respectively.
Figure 23.
Figure 24. Absence of poly(A) sequence in slower sedimenting virus-like particle (SSVLP) RNA. SSVLP RNA was end-labeled with $[5'-^{32}P]3'$, 5'-diphosphate cytidine (pCp) by T4 RNA ligase and then chromatographed through an oligo(dT) cellulose column first with a high salt buffer (40 mM Tris-HCl, pH 7.5; 0.5 M NaCl, 1 mM EDTA, 0.5% SDS), followed by a low salt buffer (40 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.5% SDS). The elutions were collected in aliquots and the amount of $^{32}P$ was counted with a scintillation counter. MCDV RNA and maize ribosomal RNAs were used as controls which was shown in Fig. 5.
Figure 24.
Figure 25. Presence of phosphate at the 3' end of slower sedimenting virus-like particle (SSVLP) RNA. SSVLP RNA was treated with (lane 1) or without (lane 2) calf intestinal alkaline phosphatase (CIAP), and then labeled with [\(5'\text{--}^{32}P\)]5' 5'-diphosphate cytidine by T4 RNA ligase. Equal amounts of reactions were loaded onto a 3.5% polyacrylamide gel. Full-length SSVLP RNA (F) is indicated by an arrow.
SSVLP RNA-specific dsRNAs were detected in dsRNA samples isolated from MCDV-infected tissue containing SSVLP by Northern hybridization with reverse transcribed cDNA probes from SSVLP RNA. At least six bands with estimated length of 1041 NT (monomer), 1952 NT (dimer), 3153 NT (trimer), 4804 NT (tetramer), 6779 NT (hexamer) and 8808 NT (octamer) were detected after Northern hybridization (Fig. 26A, lane 7). In addition to the multimeric SSVLP dsRNAs, at least five bands of RNA specific to SSVLP RNA were detected in total tissue RNA samples purified from MCDV-infected tissue containing SSVLP (Fig. 26A, lanes 3 and 5).

Multiple RNA bands were also detected in SSVLP by Northern hybridization (Figure 26B).

A plot of the logarithm of multimeric dsRNAs vs. the logarithm of their migration distances resulted a straight linear regression line (Fig. 26C), as expected for multimers of SSVLP RNA.
Figure 26. Multimeric RNAs of slower sedimenting virus-like particle (SSVLP) from maize chlorotic dwarf virus (MCDV)-infected tissue and SSVLP. A: Total cellular RNA (lanes 2, and 4-7) and dsRNA (lane 9) were isolated from healthy (lane 2) and MCDV-infected (lane 4-7, and 9) tissue. The RNA samples were denatured with glyoxal, electrophoresed in 1% agarose gel, and blotted onto nitrocellulose membrane. The blot was probed with $^{32}$P-labeled cDNA reverse transcribed from randomly primed gel-purified SSVLP RNA (lane 1). Lanes 3 and 8: no RNA (blanks). Samples in lanes 4-7 were from different isolates of MCDV with (lanes 4 and 6) or without (lanes 5 and 7) SSVLP. B: The same probe from panel A was used to hybridize RNA extracted from SSVLP. Note the presence of two faint bands (indicated by arrows). C. Plot of multimer sizes [ln(unit)] of SSVLP RNAs in lane 9 of panel A vs. the distances of their migration [ln(mm)] in gel electrophoresis.
Figure 26.
Figure 26. (continued).
4. Molecular cloning and sequencing of the SSVLP RNA

SSVLP RNA, purified by two sequential sucrose density gradient centrifugations and polyadenylated with *E. coli* poly(A) polymerase, was used to synthesize cDNA for cloning. The percentage of nucleotide incorporation in cDNA synthesis reaction, as monitored by the presence of $^{32}$P-dCTP in the reaction, was 0.8%. The efficiency of the first strand cDNA transcribed to the second strand DNA was 90.4%. The total amount of dsDNA synthesized was 110 ng and the yield was approximately 5.5% of SSVLP RNA template.

The DNA was ligated to *EcoRI* adapters (see MATERIALS AND METHODS) and cloned into the lambda gt10 *EcoRI* site. Five clones, having inserts of estimated lengths 1080 (No. 5), 870 (No. 10), 680 (No. 11), 1040 (No. 12) and 770 (No. 15) base pairs (bp), including adapters, were selected by Southern hybridization using $^{32}$P-labeled cDNA probes to SSVLP RNA. These clones were subcloned into phagemid vector pUC119.

Clone No. 12 was digested with *AluI*, *HaeIII* and *Sau3AI* (Fig. 27), and the resulting fragments were subcloned into pUC119.
Figure 27. Restriction enzyme digestions of slower sedimenting virus-like particle (SSVLP) RNA cDNA clone No. 12. The cloned SSVLP RNA cDNA was cut from pUC119 vector with EcoRI and purified with the GENECLEAN Kit (Bio 101, La Jolla, CA). The insert thus purified (undigested) was digested with AluI (AluI), HaeIII (HaeIII) and Sau3AI (Sau3AI). Lambda DNA HindIII fragments (left lane) and pUC18 DNA Sau3AI fragments (right lane) were used as size markers. The sizes of pUC18 fragments were indicated according to Sambrook et al. (1989). The electrophoresis was conducted in a native agarose gel.
Figure 27.
Attempts at ssDNA template sequencing of the five clones subcloned in pUC119 (No. 5, 10, 11, 12 and 15) all gave ambiguous results. This occurred with all five clones and happened again after retransformation and cloning. However, when the restriction enzyme fragment subclones of No. 12 were used to prepare ssDNA templates, the problem was overcome. Nevertheless, ssDNA sequencing (conducted by M. Jones and M. D. McMullen) was used to confirm the questionable regions of SSVLP cDNA sequence obtained from dsDNA sequencing, and the entire sequence of clone No. 12 was obtained by dsDNA sequencing of the restriction enzyme fragment subclones. A clone (No. 5) larger than clone No. 12 by having 37 and 51 more NT beyond the 5' and 3' ends, respectively, of clone No. 12 was also sequenced from the both ends by dsDNA sequencing. The 5' proximal sequence of SSVLP RNA was obtained from sequencing the RNA directly with a synthetic primer complementary to the sequence 90–109 NT from the 5' end of the RNA, which was derived from the sequence of clone No. 12 (see MATERIALS AND METHODS) using an RNA sequencing kit (Boehringer). The sequencing strategy was shown in Fig. 28. The sequence of SSVLP RNA is shown in Fig. 29.
Figure 28. Diagram of sequencing strategy of slower sedimenting virus-like particle (SSVLP) cDNA and RNA. SSVLP cDNA clone No. 12 (Top) contained 924 nucleotides (NT) and its restriction enzyme digested subclones (indicated) were sequenced from both directions. A, H and S stand for restriction enzymes AluI, HaeIII and Sau3AI, respectively. Clone No. 5 (1012 NT) (Middle) containing 37 and 51 more NT beyond the 5' and 3' ends of clone No. 12, respectively, was sequenced from both ends. The 5' end of SSVLP RNA (Bottom) was obtained by sequencing the RNA with a primer (the thick bar) complementary to the sequence 90-109 NT from the 5' end of the RNA.
Figure 28.
Figure 29. Nucleotide sequence of slower sedimenting virus-like particle (SSVLP) RNA. SSVLP RNA cDNA clones in pUC119 were sequenced by dideoxynucleotide chain termination method (Sanger et al., 1977) using both reverse primer and forward primer for dsDNA sequencing reactions. The 5' extremity of SSVLP RNA was obtained by sequencing the RNA with an internal primer and reverse transcriptase. The 3' end of the sequence was from the nucleotide to which poly(A) tail was added (clone No. 5). Sequence boxed showed 60% homology with the central conserved region of viroids. The Xs in position 1, 10, 17 and 18 were the undetermined nucleotides.
Figure 29.
The SSVLP RNA sequence obtained contained 1031 nucleotides, including the first nucleotide at the 5' end. Whether the 3' end of SSVLP RNA was represented in the sequence was uncertain, because polyadenylation added poly(A) at different positions of SSVLP RNA 3' terminus (Fig. 28). The sequence contained 22.3% adenine, 24.4% cytosine, 28.7% guanine and 24.2% uridine. Sequence analysis showed that there were no open reading frames (ORF) encoding proteins larger than forty amino acid residues in SSVLP RNA. In the complementary strand of SSVLP RNA, an ORF of seventy two amino acid residues was found.

The 5' end of SSVLP RNA contained the highly conserved self-cleaving sequences GAAAC (Fig. 29, NT 44-48) and CUGANGAU (Fig. 29, NT 20-27) of virusoids and small satellite RNAs (Bruening, 1989; Symons et al., 1985, 1987). Virusoids and small satellite RNAs are classified as group II satellites in this thesis (Table 2). The conserved sequence of SSVLP RNA could form a so-called "hammerhead" structure (Fig. 30A), which was proposed for group II satellites (Fig. 30B) (Bruening, 1989; Forster and Symons, 1987a, 1987b).

SSVLP RNA also contains a region (nucleotides 456-475, Fig. 29, boxed) which shared 60% homology with the central conserved region of viroids (Fig. 30C) (Diener, 1983, 1986;
Haseloff et al., 1982; Symons et al., 1987), which was suspected to be involved in viroid self-processing reactions (Diener, 1986).

SSVLP RNA contained a 22-NT sequence (nucleotide 382-403, Fig. 29, underlined), which, if in double-stranded form, would be a palindrome. The same sequence forms a stable hairpin structure \( G = -16.7 \). The GGAAAA hexamer repeated three times (NT 95-100, 443-448 and 762-767). SSVLP RNA has ACCCCGCGC repeats (Fig. 29, NT 888-896 and 921-929), which have the potential to base-pair with the putative central conserved region of SSVLP RNA (Fig. 29, underlined). There was also a symmetry sequence GCGUGCCU at positions 700-707 and 738-745 (Fig. 29, indicated by arrows).
Figure 30. Schematic presentation of a "hammerhead" structure of SSVLP RNA (A) as compared with those in virusoids and small satellite RNAs (B) (Bruening, 1989). A and B: The Watson and Crick base-pairings are shown as solid lines; the potential partial G-U pairing (Haseloff et al., 1982) are shown as colons; dashed line: possible matches. The stem III was shown tentatively, because of the uncertainty of the 3' end of the RNA, so was the 5' terminal hydroxyl of SSVLP RNA. X: nucleotide unidentified. Figures beside nucleotides in A were the number of the nucleotides from 5' end. Sequences CUGANGAU and GAAAC between stem I and II and II and III, respectively, are conserved in all virusoids and self-cleaving small satellite RNAs (B). B: redraw from Fig. 7 of Bruening (1989). N: any one of four ribonucleotides; R: purine; Y: pyrimidine. C: sequence homology between SSVLP RNA and viroid central conserved region (potato spindle tube viroid) (Diener, 1986). The same nucleotides in the two sequences were indicated by colons.
**SSVLPE RNA (456-465)**

AGAGAACCCCGGCGGCUCCUC

AGAGAUCACCAGGGGAAACCU

Viroid central conserved region

Figure 30.
BIOLOGICAL PROPERTIES OF SSVLP RNA

1. Dependence of SSVLP on MCDV

Purified SSVLP was transmitted by *G. nigrifrons* to: a) demonstrate that SSVLP is transmitted by the same vector species as MCDV; b) determine whether a helper component (Hunt et al., 1988) is needed for the transmission of SSVLP by the leafhoppers; and c) If the answer to b) is no, demonstrate the dependence of SSVLP RNA on MCDV for replication. The third objective would have not been achieved if the transmission of SSVLP by the leafhopper required the helper component, because that the putative helper component has not been purified (R. E. Gingery, personal communication). SSVLP RNA-free MCDV isolates, as determined by RNA filter hybridization, were used for vector feeding and inoculation. The experimental design was described in the MATERIALS AND METHODS. The results showed that purified SSVLP was transmitted by *G. nigrifrons* to and replicated in MCDV-infected maize (Table 6, A), but not in the healthy (Table 6, B), without the aid of putative helper component. Since SSVLP RNA can replicate in MCDV-infected plants, but not in the healthy, presumably purified SSVLP was transmitted to both MCDV-infected and healthy maize, SSVLP RNA apparently cannot replicate without the help from MCDV.
2. Strain dependence of SSVLP RNA

Leafhopper vectors previously given an acquisition access period on MCDV-T-infected maize (SSVLP-free) were membrane fed to SSVLP purified from MCDV-WS infected maize. The leafhoppers were then given an inoculation access to healthy maize. The plants showing symptoms of MCDV-T infection were assayed for the presence of SSVLP RNA. Results showed that SSVLP RNA from plants infected with MCDV-WS can replicate in MCDV-T infected plants (Table 6, C).

3. Effect of SSVLP RNA on MCDV symptoms

SSVLP RNA containing maize plants infected with MCDV-WS or -T strains showed no observable symptom differences compared to SSVLP RNA-free plants infected with MCDV-WS or -T, respectively.

4. Effect of SSVLP RNA on MCDV replication

To test the effects of SSVLP RNA on MCDV replication, the yield of MCDV virions, the concentration of MCDV CPs and the concentration of MCDV RNA were compared between MCDV-infected plants with and without the SSVLP RNA. Leafhoppers from MCDV-infected plants (SSVLP-free) were membrane fed with purified SSVLP in 10% sucrose in 10 mM potassium phosphate buffer, pH 7.0 (PP). The control was 10% sucrose in PP without SSVLP. The leafhoppers were then given an
inoculation access to healthy maize plants. Plants showing MCDV symptoms were tested individually 3 wk after the inoculation for the presence of SSVLP RNA by an RNA hybridization assay. SSVLP RNA decreased the yield of MCDV virion (Fig. 31), MCDV coat proteins (Fig. 32) and MCDV RNA (Fig. 33) in MCDV-T infected plants about 70%, 80% and 50% respectively, whereas the decreases were 40%, 30%, and 30%, respectively, for MCDV-WS (Fig. 31-33). Statistical analyses conducted according to Bryant (1960) showed that the differences in MCDV virion yields, CP concentrations and RNA contents were significant (P <0.05) between MCDV-T-infected maize plants with and without SSVLP RNA. None of the differences for MCDV-WS were significant.
Table 6. Transmissibility of purified slower sedimenting virus-like particle (SSVLP) by *Graminella nigrifrons*.

<table>
<thead>
<tr>
<th>G. nigrifrons plants</th>
<th>Membrane feeding</th>
<th>IAP plants</th>
<th>SSVLP transmission Experiment No.</th>
<th>Transmission rate (%) (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP</td>
<td>SSVLP-WS</td>
<td>MCDV-WS</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td>5/10²</td>
<td>7/9</td>
</tr>
<tr>
<td>H-oats</td>
<td>SSVLP-WS</td>
<td>MCDV-WS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-oats</td>
<td>Sucrose</td>
<td>MCDV-WS</td>
<td>-</td>
<td>0/9</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td>H-maize</td>
<td>0/12³</td>
<td>0/8³</td>
</tr>
<tr>
<td>H-oats</td>
<td>SSVLP-WS</td>
<td>H-maize</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
<td>H-maize</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCDV-T</td>
<td>SSVLP-WS</td>
<td>H-maize</td>
<td>3/5</td>
<td>3/9</td>
</tr>
<tr>
<td>MCDV-T</td>
<td>Sucrose</td>
<td>H-maize</td>
<td>0/5</td>
<td>0/9</td>
</tr>
</tbody>
</table>

³ G. nigrifrons from various sources (AAP plants) were fed through membrane on either purified SSVLP in 10% sucrose or 10% sucrose alone. The membrane-fed leafhoppers were then allowed to inoculate (IAP) (5-8 adult G. nigrifrons /plant) either healthy maize seedlings or ones previously inoculated with MCDV (SSVLP RNA-free). Three wk after inoculation, plants were tested for the presence of SSVLP RNA by RNA dot blot hybridization.
Table 6. (continued).

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP</td>
<td>Acquisition access period; H-oats: healthy oats (non-host of MCDV); MCDV-T: MCDV-T-infected maize. The duration of AAP on oats was longer than 4 days and on MCDV-infected maize plants was about 2-3 days.</td>
<td></td>
</tr>
<tr>
<td>SSVLP-WS</td>
<td>SSVLP purified from MCDV-WS-infected maize. The SSVLP preparation for membrane feeding was in 10% sucrose in 10 mM potassium phosphate, pH 7.0 (PP). Sucrose: 10% sucrose in PP. The duration of membrane feeding was 0.5-2 hr.</td>
<td></td>
</tr>
<tr>
<td>IAP</td>
<td>Inoculation access period; MCDV-WS: maize plants inoculated with MCDV-WS (SSVLP RNA-free) 2 days before being inoculated with the membrane-fed leafhoppers; H-maize: healthy maize. The duration of IAP was 2 days.</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>Number of plants tested positive for SSVLP RNA by RNA dot blot hybridization/number of plants with MCDV symptoms tested.</td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>Not tested.</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>Number of plants tested positive for SSVLP RNA/Number of plants tested.</td>
<td></td>
</tr>
</tbody>
</table>
Figure 31. Effect of slower sedimenting virus-like particle (SSVLP) RNA on the yield of maize chlorotic dwarf virus (MCDV) virions. Maize plants of the same age with or without SSVLP RNA were tested individually for the relative yield of MCDV virion by sucrose density gradient centrifugation. Equal weights of tissue from the same parts of plants were used for all tests. The area under the MCDV peaks recorded by Instrumentation Specialities Co. (ISCO) ultraviolet monitor were calculated by formula $Y = (a \times b)/2$, where $Y$ = the area under a peak, $a$ = the height of the peak, $b$ = the width of the peak at the half-height. The numbers below the bars were the numbers of plants tested. The vertical line on each bar represents the standard deviation. Adjacent bars: replications.
Figure 31.
Figure 32. Effect of slower sedimenting virus-like particle (SSVLP) RNA on the concentration of maize chlorotic dwarf virus (MCDV) capsid proteins in MCDV-infected tissue. The same part of maize plants (the same age) infected with MCDV with and without SSVLP RNA were tested individually for the relative concentration of MCDV capsid proteins (CPs) by ELISA. The numbers below the bars were the numbers of plants tested. The vertical line on each bar represents the standard deviation. Adjacent bars: replications.
Figure 32.
Figure 33. Effect of slower sedimenting virus-like particle (SSVLP) RNA on the concentration of maize chlorotic dwarf virus (MCDV) RNA in MCDV-infected tissue. Maize plants of the same age and infected with MCDV with and without SSVLP RNA were tested individually for the relative concentration of MCDV RNA by RNA filter hybridization (dot blot hybridization) 3 wk after inoculation. Nucleic acid samples from equal amounts of tissue of same part of plants were spotted onto nylon membranes and probed with $^{32}$P-labeled SSVLP cDNA probes prepared from clone No. 12 or MCDV cDNA probes prepared from randomly primed MCDV RNA. After hybridization, each dot was cut from the membrane probed with MCDV cDNA probes and counted for the relative amounts of $^{32}$P (cpm) with a scintillation counter. The numbers below the bars were the numbers of plants tested.
Amounts of radioactivity (cpm) (Thousands)

MCDV-T

SSVLP+  SSVLP-

MCDV-WS

SSVLP+  SSVLP-

Figure 33.
MCDV GENOME IS MONOPARTITE, OF ABOUT 13 KB IN LENGTH

Based on the results of gel electrophoresis (Fig. 1A) and rate zonal sucrose density gradient centrifugation of MCDV virion RNA (Fig. 1B), MCDV appears to have a monopartite genome. This conclusion is in agreement with the report of Gingery (1976). The possibility that more than one RNA segment of the same size is encapsidated in MCDV virions appears unlikely. Although the pathogenicity of the MCDV virions has not been proven by Koch's postulates, an indirect demonstration of the pathogenicity involving neutralization of infectivity using antiserum to the 183 S virion has been reported (Hunt et al., 1988). Repeated purification of MCDV from infected plants has revealed only the 183 S MCDV virion (R. E. Gingery and D. T. Gordon, personal communication), except for the SSVLP which has been found to encapsidate a satellite RNA (see below). There is no evidence that the MCDV genome or any part of it is encapsidated in capsid proteins other than those associated with the 183 S virion.
The size of the MCDV genome (ca. 13 kB) as determined in this study is appreciably larger than the 3.2 X 10^6 kDa (ca. 10 kB) reported previously by Gingery (1976) which was estimated by linear-log sucrose gradient centrifugation and polyacrylamide gel electrophoresis. In the present study, the size of the RNA was estimated by electrophoresis of glyoxal-denatured nucleic acids in agarose gel using DNA size markers. The electrophoresis of glyoxylation RNA in agarose gels was reported to be better than other electrophoresis methods for determining the size of RNAs larger than 5 kB (Murant et al., 1981). On the other hand, denaturing PAGE usually underestimates nucleic acid size if they are larger than 7 kB (Murant et al., 1981; Reijnders et al., 1974). Further, it was found in this study that under the experimental conditions a more linear relationship between nucleic acid size and electrophoretic migration distance was obtained by a double-logarithmic plot than by a semi-logarithmic plot, especially when a large nucleic acid (more than 10 kB) was included (Fig. 2; Table 3). The latter finding suggests that a semi-log transformation tends to underestimate molecular weight when the nucleic acid molecule is large (Fig. 2). The validity of using DNA as size markers for estimating the size of RNA in denaturing agarose gel electrophoresis was suggested by Williams and Mason (1985) and shown to be valid in the present study by using DNA markers to estimate the sizes of ribosomal RNAs.
whose sizes were already known (Fig. 2). Another reason for using DNA markers was that no RNA markers of the size of MCDV RNA were available. Since the large linear nucleic acid fragments moved at a different migration rate from that for smaller nucleic acids under the experimental conditions (Fig. 2), the inclusion of a size marker larger than MCDV genomic RNA seemed important for accurately determining the size of MCDV RNA. Calculation of the MCDV RNA size without reference to the size marker larger than MCDV RNA might underestimate the size, whereas inclusion of the larger marker might overestimate the size. On the other hand, the double-log formula could overestimate the size, whereas the semi-log formula could underestimate it. The final estimate of about 13 kB represents a compromise among these potential estimation errors. For this size RNA, the potential coding capacity would be for a protein of more than 450 kDa, which is almost 50% more than the coding capacity of the Potyvirus genome (Allison et al., 1986; Dougherty and Carrington, 1988; Robaglia et al., 1989), and almost twice as much as that of the animal poliovirus (Semler et al., 1988; Wimmer et al., 1987).

**MCDV GENOME IS POSITIVE SENSE**

Based on the following results it is concluded that the MCDV genome has a positive polarity. First, MCDV RNA was translated in cell-free protein synthesis systems to
produce proteins of high molecular weights. Secondly, full-length MCDV RNA of the same polarity as the genomic RNA was associated with polyribosomes in infected plants. Lastly, MCDV RNA has a poly(A) tract at the 3' terminus.

The genomic RNAs of most ss(+)RNA viruses are infectious, either as isolated genomic RNA itself or associated with capsid proteins (e.g., alfalfa mosaic virus and Ilarviruses). Further, the RNAs of the Luteoviruses, which like MCDV are obligately insect transmitted, are infectious when used to inoculate protoplasts (Kubo and Takanami, 1979; Myro et al., 1982b; Young et al., 1989). In contrast, neither MCDV genomic RNA nor the virion was shown to be infectious in maize protoplasts (R. A. Gomez-Luengo and D. T. Gordon, personal communication) and attempts to inoculate maize seedlings with MCDV RNA using the Particle Gun (Klein et al., 1987, 1988) were unsuccessful, whereas controls with brome mosaic virus RNA were successful (Ge, X. et al., unpublished). It is speculated that the apparent lack of infectivity of MCDV RNA is attributable to the failure to place MCDV RNA in susceptible sites in the inoculated maize, to the susceptibility of MCDV RNA to RNase degradation or to a combination of the above.
MCDV GENOME HAS TERMINAL STRUCTURES SIMILAR TO PICORNAVIRUSES

Based on the present findings, MCDV RNA appears to have a VPg at the 5' terminus and a poly(A) tail at the 3' terminus. The 5' terminal location of the VPg has not been demonstrated positively yet. However, the lack of cap structure of MCDV RNA (Fig. 3) and the absence of internally located VPg in any viral RNA genomes (Daubert and Bruening, 1984; Wimmer, 1982) indicate the 5' terminal location of this VPg. The size of the VPg (18 kDa) is larger than VPgs of the Picornaviruses and Comoviruses, but is similar to those of the Potyviruses and pea enation mosaic virus (see INTRODUCTION for information on the size of these VPgs). The presence of a 5' VPg and a 3' poly(A) is a characteristic of the Picornaviruses, Potyviruses, Comoviruses, Nepoviruses (Goldbach and Wellink, 1988; Strauss and Strauss, 1988) and parsnip yellow fleck virus (Murant, 1988), indicating that MCDV is taxonomically related to the proposed Picornavirus supergroup, which includes these plant virus groups.

The extent of MCDV virion RNA that was polyadenylated was about 80% (Fig. 4A), indicating that most of the MCDV RNA with an intact terminus contained poly(A). The 3' terminal sequence of MCDV RNA has a possible poly(A) addition signal (AGAUAAA) (Fig. 7). Although the
polyadenylation signal (AAUAAA) is highly conserved among eucaryotic mRNAs, some RNAs have the signal with a single base change (Nevins, 1984). In addition, the putative signal sequence of SSVLP RNA was at the correct position relative to the poly(A) (Nevins, 1984). It will be interesting to test whether a poly(U) sequence exists in the minus-sense MCDV RNA.

**NO MCDV SUBGENOMIC RNA WERE DETECTED IN MCDV-INFECTED TISSUE**

Only full-length MCDV RNA was detected in both total cellular RNA and poly(A)-containing RNA from MCDV-infected plants (Fig. 11A), indicating that the predominant MCDV RNA in infected tissue is the full-length, genomic RNA, and the absence of subgenomic RNA. It is not known how much of this MCDV RNA came from the virions or from other structures, such as viral polyribosome, striposomes (Wilson, 1984, 1985), informosomes (Dorokhov et al., 1983; Preobrazhensky and Spirin, 1978), replication complex (Dorssers et al., 1983; Wimmer et al., 1987) or other undescribed structures. However, such RNA was obtained from young, newly infected maize tissue which might minimize the amount of MCDV RNA from virions. Sometimes, less-than-full-length MCDV-specific RNAs were detected (Fig. 16, for example). There are two reasons to exclude the possibility that they are the subgenomic RNAs of MCDV. First, the bands were also present in the reconstituted RNA sample (Fig. 16, lane 4), which was
prepared from the mixture of healthy maize tissue and purified MCDV virions. The RNA from MCDV virions has never shown such bands when electrophoresed alone. So these bands might have resulted from degradation of MCDV RNA during RNA purification. Secondly, these bands were at the positions of ribosomal RNAs. Therefore it is thought they were artifacts of electrophoresis of high amounts of ribosomal RNAs and degraded MCDV RNA, which seems a general problem with this technique (Otal and Hari, 1983; Dougherty, 1983).

MCDV-specific dsRNA corresponding in size to full-length genomic RNA was the only dsRNA detected in MCDV-infected tissue (Fig. 11B), indicating the absence of subgenomic RNA. The significance of this MCDV-specific dsRNA is unknown since it may be authentic replication form (RF) of MCDV RNA or an artifact of RNA purification (for a review, see Libonati et al., 1980). Because virtually all ssRNA viruses are associated with RF or dsRNA in infected tissue, dsRNA is a reliable diagnostic indicator of virus infection (Dodds et al., 1984), but its value in studying viral genome expression is uncertain. DsRNA corresponding to the subgenomic RNAs has been detected for some (Dawson, 1983; Palukaitis et al., 1983) but not other ssRNA viruses (Zelcer et al., 1981; Hayes et al., 1984). To further complicate the interpretation, there have been reports showing that the subgenomic RNAs of some viruses are
generated by internal initiation transcription of the negative strand of the genomic RNA (Gargouri et al., 1989; Miller et al., 1985), suggesting the absence of autonomous replication of subgenomic RNA. However, the presence of the subgenomic-sized dsRNA usually indicates subgenomic RNA exists (Dawson, 1983; Palukaitis et al., 1983).

The direct method for determining the size and number of MCDV RNAs expressed in infected tissue is to isolate polyribosomes and identify the associated MCDV RNAs (Palukaitis, 1984). In the isolation of polyribosomes from virus-infected tissue, two precautions must be observed. First, the polyribosome must be free of other viral RNA-protein complexes, especially virions. Secondly, all sizes of polyribosomes must be isolated so that none, including the smallest are overlooked. For this study, the first precaution appears satisfied in that no MCDV virions were detected in polyribosomes isolated from infected tissue. This was demonstrated by the following: a) MCDV virions did not sediment through the sucrose cushion under conditions which allowed polyribosomes to sediment (Fig. 13); b) the sensitivity of the nucleoproteins (polyribosomes) to EDTA and puromycin, and their requirement of Mg"++ for integrity (Fig. 15), compared with MCDV virions which responded to EDTA, puromycin, and Mg"++ similarly (Fig. 14); and c) MCDV virions were not detected in the polyribosome samples from
infected tissue by ISEM (Fig. 12). Concerning the second precaution, polyribosomes containing about five ribosomes were revealed by electron microscope (Fig. 12D), suggesting that small mRNAs were not overlooked.

The $^{32}$P-cDNA probes used were prepared from a) full-length MCDV genomic RNA primed with random and oligo(dT) primer and b) randomly primed cloned MCDV CP1 and CP2 genes. The reason for using the first cDNA probe was to ensure that the probe would detect subgenomic RNAs from any portion of the MCDV genome. Since plant viruses which produce subgenomic RNAs usually express their capsid proteins from subgenomic RNA (Dougherty and Hiebert, 1985; Palukaitis, 1984), the second probes were used to detect subgenomic RNAs in RNA samples from MCDV-infected tissue. These probes failed to detect subgenomic-sized MCDV-specific RNA in Northern hybridization assays (Fig. 16), thus confirming the results obtained with cDNA probes to full-length MCDV genomic RNA that no subgenomic RNAs were produced by MCDV.

**MCDV CAPSID PROTEINS ARE TRANSLATED FROM FULL-LENGTH MCDV GENOMIC RNA**

The total weight of the proteins synthesized in cell-free translation systems with full-length MCDV RNA exceeded the coding capacity of the RNA (Fig. 8, Fig. 10; Table 4). Thus, some of these proteins must have been either premature
termination products or products of proteolytic processing. However, no proteolytic processing was observed after incubation of the translation products with added DTT (Fig. 10). The lack of evidence for proteolytic processing and the absence of detectible MCDV capsid proteins in the proteins synthesized from full-length MCDV genomic RNA in the cell-free translation systems are at variance with predictions of the Picornavirus hypothesis. The following speculations are presented to explain this inconsistency between predictions and findings. First, MCDV RNA may not have been expressed fully or efficiently in cell-free translation systems because of its unusually large size and its sensitivity to RNase degradation, so ribosomes would have rarely reached the genes located at the 3' end of the genome, which are usually capsid protein genes for plant RNA viruses (Dougherty and Hiebert, 1985). Secondly, the conditions for cell-free translation (e.g., RNA and salt concentrations) may not have been optimal for MCDV genomic RNA expression. Thirdly, the proteolytic processing of the large proteins might have occurred simultaneously as the proteins were translated. Fourthly, the concentration of MCDV capsid proteins synthesized may have been too low to have been detected. Lastly, polyproteins containing synthesized MCDV CPs may not have been properly processed for serological detection.
Although cell-free translation failed to provide evidence that MCDV CPs were expressed from full-length MCDV genomic RNA, results from immunoprecipitation of polyribosomes from MCDV-infected tissue clearly indicated that MCDV CPs were expressed from such RNA (Fig. 17). Polyribosomes containing full-length MCDV genomic RNA were immunoprecipitated specifically with IgGs from antiserum to intact MCDV virions. It is assumed that the IgGs reacted with nascent MCDV CPs present in the polyribosomes. The conclusion drawn from these findings is that MCDV CPs are translated from full-length MCDV genomic RNA *in vivo* and that the nascent MCDV CPs are antigenic. That the polyribosomes were contaminated with MCDV virions thereby leading to immunoprecipitation of the virions seems unlikely as discussed above. Furthermore, the specific release of MCDV RNA by EDTA (Fig. 17) confirmed the polyribosomal RNA identity.

Compared with conventional techniques for studying viral genome expressions, polyribosome immunoprecipitation assay offers several advantages. First, unlike cell-free translation in which viral RNA is usually expressed in a foreign or non-host environment, this assay assesses the expression of viral RNA in the host cell environment. Secondly, this assay allows for the association of an RNA species with certain polypeptides to which the antibodies
react. In other words, this technique can identify the RNA species from which a protein or proteins was translated, which is impossible to do with the Northern hybridization assay for total cellular or polyribosomal RNA. Thirdly, this assay offers less risk of artifacts because the immunoprecipitation step would eliminate most ribosomal RNAs associated with plant mRNAs. Lastly, this assay selectively releases polyribosomal RNAs from immunoprecipitated polyribosomes with EDTA, so even though polyribosomes may be contaminated with virions, the virions still would not interfere with the results, providing that the virions are not structurally sensitive to EDTA.

**MCDV BELONGS TO PICORNAVIRUS SUPERGROUP**

Results reported here suggested that full-length MCDV genomic RNA is the only MCDV RNA in infected maize. The association of this RNA with polyribosomes indicated that it was acting as an mRNA in the expression of its genome. In addition, polyproteins with sizes up to 200 kDa were translated *in vitro* under the direction of MCDV RNA (Fig. 8 and 10), suggesting that MCDV expresses its genome through a polyprotein, which is probably proteolytically processed to smaller proteins later. Although internal initiation of translation of viral RNAs has been reported for some plant viruses (Carrington and Freed, 1990; Jang et al., 1989; Pelletier and Sonenberg, 1988, 1989), the initiation sites
are limited to the 5' uncoding region of the mRNA. Also, all alleged polycistronic eucaryotic mRNAs contain a cap structure at their 5' end, unlike viral mRNAs with a VPg 5' terminus (Curran and Kolakofsky, 1988, 1989; Hassin, 1986; Herman, 1986; Merten and Dobos, 1982). Further, no viral RNAs containing VPg and poly(A) termini have been demonstrated to be polycistronic, nor has any plant RNA virus been shown to express its genomic RNA as polycistronic mRNA in vivo.

The plant Potyviruses, Comoviruses and Nepoviruses and the animal Picornaviruses all produce polyproteins as their genome expression strategy (Dougherty and Hiebert, 1985; Dougherty and Carrington, 1988; Goldbach and Van Kammen, 1985; Goldbach and Wellink, 1988). They all share the following additional characteristics (Goldbach and Wellink, 1988; Strauss and Strauss, 1988): a) the 5' terminus of the genome is a VPg; b) the 3' terminus of the genome is polyadenylated; c) their genomes have a similar gene order; d) they express their genomes in the form of polyproteins, i.e. they do not employ subgenomic RNAs to express their genome; and e) they have amino acid sequence homologies in their non-structural proteins. The MCDV genome has been shown in this study to possess several of these characteristics, namely a putative 5' VPg, 3' poly(A), expression of a polyprotein and an absence of subgenomic
RNA. In addition, MCDV contains three serologically distinct capsid proteins (Maroon et al., 1989), which resembles the animal Picornaviruses which produce four capsid proteins, two of them from same precursor (Wimmer et al., 1987). Based on these similarities between MCDV and the Picornavirus-like viruses, it is proposed that MCDV be classified as a Picornavirus-like virus. If this proposal is adopted, MCDV would be the first isometric, monopartite, ss(+)RNA plant virus to be classified in the Picornavirus supergroup.

SSVLP RNA IS A SATELLITE RNA OF MCDV

The dependence of SSVLP RNA on MCDV for replication was demonstrated in this study. G. nigrifrons, after feeding on purified SSVLP, transmitted SSVLP to maize plants previously infected with MCDV but not to healthy maize (Table 6). In addition, the encapsidation of SSVLP RNA in MCDV CPs (Fig. 19, 20) suggested indirectly the dependence of the RNA on MCDV. The satellite nature of this RNA was shown by cross hybridization in that SSVLP RNA had less than 10% homology with MCDV (Fig. 23), which excludes the possibility of SSVLP RNA being a defective interfering (DI) RNA or subgenomic RNA of MCDV.

Analysis of sequence data of SSVLP RNA revealed that SSVLP RNA resembles virusoids and small plant virus
satellite RNAs in that it contains the consensus sequences of these RNAs (CUGANGAU and GAAAC) (Bruening, 1989; Symons et al., 1985, 1987) and has the potential to form the "hammerhead" secondary structure (Fig. 30), which is involved in RNA self-processing (Bruening, 1989; Forster and Symons, 1987a, 1987b; Forster et al., 1990; Symons et al., 1987). Although the stem I of the hammerhead of SSVLP RNA looks speculative (Fig. 30A), the presence of this stem in SSVLP RNA was implied by RNA sequencing reactions in which reverse transcriptions were abruptly stopped at the 3' portion of the proposed hairpin, a reason why the 17th and 18th nucleotides were not determined (Fig. 30A). There were four nucleotides (positions 1, 10, 17 and 18) not determined in the sequence (Fig. 29), all of them were in stem I (Fig. 30A). The 17th and 18th nucleotides were most probably C and A, respectively, assuming they could be base-paired with the third and second nucleotides (Fig. 30A). The third stem (stem III) of SSVLP RNA (Fig. 30A) is proposed tentatively, because the 3' end has not been identified positively yet.

In addition to the similarities of SSVLP RNA to virusoids and small satellite RNAs, SSVLP RNA contains a region (Fig. 30C) that shares 60% sequence homology with the central conserved region of viroids, which is supposed to be involved in RNA self-processing (Diener, 1986; Haseloff et al., 1982; Symons et al., 1985, 1987). Other regions (882
to 901 and 917 to 936) also have some similarity to the opposite part of the region. The significance of the similarity and the structural and functional relationships of this region with the hammerhead structure remain to be determined.

The hypothesis that SSVLP RNA replicates via a rolling circle mechanism was supported by the following evidence. a) The RNA has terminal structures different from those of its helper virus genome; namely, it does not have a 5' VPg and 3' poly(A), indicating it might replicate differently from MCDV. The dissimilarity in terminal structures from the helper virus genome is a characteristic of group II satellites (Table 2). b) The RNA possesses a 3' phosphate (Fig. 25), an unique feature of self-processing satellite RNA (Bruening 1989; Bruening et al., 1988; Forster et al., 1990). 3) There are sequence homologies and structure similarities between RNAs of SSVLP and group II satellites and viroids (Fig. 30), the latters presumably replicate by the rolling circle model. 4) The RNA forms double-stranded multimers in infected cells (Fig. 26), which is strong evidence for a rolling circle replication (Hutchins et al., 1985; Symons et al., 1987). Although it has been suspected that the multimeric RNA of peanut stunt virus satellite (PARNA 5) could be formed from self-ligations instead of rolling circle replication (Collmer et al., 1985), like the
cyclization reaction of an intron (Zaug et al., 1983), this suspicion does not apply to SSVLP RNA in that the intron-like cyclization needs a 5' phosphate and 3' hydroxyl (Cech and Bass, 1986; Zaug et al., 1983). However the 3' end of SSVLP RNA is a phosphate. As discussed before, PARNA 5 has a 5' cap and 3' hydroxyl (Collmer et al., 1985). Since capping is a post-transcriptional reaction (Moss, 1984), PARNA 5 basically has both the termini and autocatalytic sequences necessary for the intron-like self-ligation to produce multimeric RNAs (Collmer et al., 1985). In addition, the multimeric RNA profiles of PARNA 5 (Linthorst and Kaper, 1984b) look different from those of group II satellites (Kiefer et al., 1982; Hutchins et al., 1985; Symons et al., 1985) and those of SSVLP RNA (Fig. 26) in that for the latter the abundance of multimeric RNAs decrease gradually from the smaller to the larger ones, whereas for PARNA 5 there seems no such abundance gradient: dimeric RNA is the major multimer (Linthorst and Kaper, 1984b). Another possibility for SSVLP RNA to form multimeric RNA is by the self-ligation reactions of tobacco ringspot virus satellite RNA (Buzayan et al., 1986c) or hepatitis delta virus RNA (Wu and Lai, 1989). However, there have been no reports showing that this reaction can form multimers larger than the dimer (Buzayan et al., 1986c; Wu and Lai, 1989).
It is concluded that SSVLP RNA is a satellite RNA of MCDV and belongs to the group II satellites which probably replicate through a rolling circle mechanism. It turns out that satellite maize chlorotic dwarf virus (SMCDV), as it will now be named, is the largest plant virus satellite in group II, and MCDV has the largest genome of a plant virus associated with a satellite RNA. In many respects, SMCDV RNA is like the satellite RNA of tobacco ringspot virus (STRSV) in that both satellites share the following characteristics. They have: a) multidensity components of encapsidated RNA (Fig. 22) (Schneider et al., 1972); b) multimeric RNAs (Fig. 26) (Kiefer et al., 1982; Sogo and Schneider, 1982); c) encapsidated linear RNA (Buzayan et al., 1986a); and d) RNA terminal structures unlike those of their helper viruses (Fig. 24, 25) (Buzayan et al., 1986a). Finally, both RNAs decrease the encapsidation of their helper viruses (Fig. 31) (Schneider, 1971).

The linear configuration of SMCDV RNA was suggested when an internal primer to the 5' end region of the RNA was used to reverse transcribe the RNA in that the transcription stopped strongly at a certain position. In addition, the RNA served as a substrate for RNA ligase. It is possible that there are circular RNAs in SMCDV in low proportion, which needs to be further tested. The presence of circular-
form monomeric ssRNA in vivo, however, is implied by a rolling circle model (Bruening, 1989; Bruening et al., 1988; Symons et al., 1987).

The 3' terminus of SSVLP RNA has not been identified positively yet. Of the five cDNA clones selected, three clones had the poly(A) added at the different positions in SMCDV RNA (Fig. 28, clone Nos. 12 and 5, for example). Since SMCDV RNA has a 3' terminal phosphate (Fig. 25), it is thought that the polyadenylations were on the broken ends of the RNA which happened to have a 3' hydroxyl, because poly(A) polymerase needs a free hydroxyl at the 3' position of ribose (Sippel, 1973). Based on the size of SMCDV RNA (1041 NT for monomer, Fig. 26), the sequence shown in Fig. 29 might be very close to the 3' end of the RNA, if it is not the end.

SSVLP RNA HAS POTENTIAL VALUES IN RESEARCH AND APPLICATIONS

It was reported above that G. nigrifrons can transmitted SMCDV without the presence of the putative helper component (Table 6). Since SMCDV RNA is encapsidated with only MCDV capsid proteins and the interactions are likely between the helper component and capsid proteins of virions, the independence of SMCDV transmission from the putative MCDV helper component suggests that the binding mechanism proposed for the Potyviruses (Berger and Pirone,
1986) seems unlikely for MCDV transmission. The distinct particle morphology of SMCDV (Fig. 19), its high transmission rate by the leafhoppers (Table 6) and the availability of cDNA clones to SMCDV RNA make it a good tool for the study of the mechanism of MCDV transmission.

Despite the interference of MCDV replication by SMCDV RNA, no symptom differences were observed for MCDV-infected plants with or without SMCDV RNA. The same situation has been noted for satellite tobacco necrosis virus (STNV), which interferes with TNV replication, but has almost no effect on symptoms of TNV-infected plants (Kassanis, 1962, 1981).

In the study of the effects of SMCDV RNA on MCDV infection and replication, it has been shown that SMCDV RNA had a greater influence on MCDV-T (a mild strain) replication than on that of MCDV-WS (a severe strain) (Fig. 31-33). Since the SMCDV RNA used in these experiments originated in association with an isolate of MCDV-WS, the different effects probably resulted from an interaction between SMCDV RNA and MCDV isolates. The mechanism of the interaction is unknown, but the low homology between SMCDV RNA and MCDV RNA suggests that both sequence interaction or antisense RNA regulation (Inouye, 1988; Rezaian et al., 1985; Rezaian and Symons, 1986) and competition for
replicase and coat proteins (Hanada and Francki, 1989) are possibly involved. It has been demonstrated that for some viruses certain primary sequences and secondary structures of the viral genome are required for them to initiate encapsidation (Turner and Butler, 1986; Turner et al., 1988) and replication (Bujarski et al., 1986; Miller et al., 1986). The encapsidation of SMCDV RNA with MCDV CPs and the dependence of SMCDV RNA on MCDV for replication, together with the existence of a low degree homology between the two RNAs, imply that the homology sequences might be involved in these processes. In addition, the possible association of ribozyme activity with SMCDV, which is implied by the sequence and structure of the RNA (Fig. 30) and required for rolling circle replication, indicates that ribozyme action mechanism is not impossible (Cameron and Jennings, 1989; Haseloff and Gerlach, 1988).

Whether the low yield of MCDV-virion induced by SMCDV RNA infection is the result of low yield of MCDV RNA synthesis (or the reverse) needs to be determined. It should be noted that the assays for testing the effects of SMCDV RNA on MCDV replication were conducted 3 wk after inoculation, at which time MCDV was probably being synthesized at its highest level during the infection of a plant, thus the interference might have been underestimated.
It is not known whether SMCDV RNA occurs in field infections. However, it is possible that SMCDV RNA could be used to control maize chlorotic dwarf disease in the field. To explain, after the mechanism of interference is understood, genetically engineered cDNA clones of SMCDV RNA could be constructed and integrated into the maize genome (Baulcomb et al., 1986; Gerlach et al., 1987; Harrison et al., 1987) or be used as a biocontrol agent as has been done with other satellites (Tien and Chang, 1983; Tien et al., 1987).

In summary, SMCDV RNA has potential value in the research of MCDV transmission, MCDV encapsidation, MCDV replication, ribozyme activity and biocontrol of maize chlorotic dwarf disease.
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