AN ABSTRACT OF THE THESIS OF

John J. Weiland for the degree of Doctor of Philosophy in Genetics presented on April 10, 1992.

Title: The Roles of Turnip Yellow Mosaic Virus Genes in Virus Replication.

Abstract approved: Redacted for Privacy

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Turnip yellow mosaic virus is a monopartite, plus sense RNA virus infecting the *Cruciferae*, and is a model system for the study of RNA virus replication. A cDNA clone (pTYMC) representing an infectious RNA genome of the European isolate of TYMV was constructed and used to assess the importance of virus genes in virus infectivity.

Derivatives of pTYMC with alterations in open reading frame 69 (ORF-69) were made. The mutations disrupted the expression of ORF-69 *in vitro* as predicted. Although the ORF-69 mutants were competent for replication in protoplasts, none of the mutants detectably infected turnip or Chinese cabbage plants, except where reversion mutations led to the restoration of an uninterrupted ORF-69. The data suggest a role for ORF-69 expression in the cell-to-cell movement of the virus.

Mutant RNAs with a deletion or frameshift in the coat protein ORF infected protoplasts and plant leaves. No systemic infection symptoms were generated by these mutants, and no viral products were detected in young, expanding tissue of infected plants. When the coat protein deletion mutant and an ORF-69 mutant were co-inoculated onto plants, only a virus producing a coat protein of wild type size was detected in symptomatic, systemic tissue in these inoculations, emphasizing a requirement for the expression of native size coat protein for the systemic translocation of TYMV infection.

The role of ORF-206 expression in TYMV replication was examined. Three classes of mutants were made in ORF-206: those affecting the synthesis of the 150 kDa protein, those affecting the synthesis of the 70 kDa protein, and those affecting the synthesis of both the 150 and the 70 kDa proteins. All ORF-206 mutations eliminated RNA infectivity. Protoplast inoculations using

mixtures of individual ORF-206 mutant RNAs and a helper genome demonstrated that co-replication of defective genomes could occur. Moreover, inoculations in which individual 150 kDa and 70 kDa protein mutant RNAs were combined showed that complementation between these two classes of mutants was possible. The data indicate that RNAs expressing wild type 150 kDa protein are favored replication substrates in mixed infections, and suggest that the 150 kDa protein functions preferentially in *cis*.

The Roles of Turnip Yellow Mosaic Virus Genes in Virus Replication

by

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A THESIS
submitted to
Oregon State University

in partial fulfillment of the
requirements for the degree of

Doctor Of Philosophy

Completed April 10, 1992
Commencement June 1993

APPROVED:

Redacted for Privacy

Professor of Agricultural Chemistry/ Genetics Program in charge of major.

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Dean of Graduate School

Date thesis is presented April 10, 1992

Typed by John J. Weiland for John J. Weiland

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PREFACE

An edited version of Chapter I of this thesis has been published in Nucleic Acids Research (Weiland and Dreher, 1989). Not included in the published manuscript is the information in figure I.3 showing the accumulation of viral coat protein and RNA in infected plant tissue.

An edited version of Chapter II has been published in Virology (Bozarth et al., 1992). Reference in that manuscript to "data not shown" has been included in this thesis as an addendum to chapter II. Western blot analysis of p69 accumulation in infected tissue, mutagenesis of the putative N-glycosylation site and cell-free translations presented in the published manuscript are the work of Connie S. Bozarth. Construction of the ORF-69 initiaton codon mutants and premature stop codon mutants used in the study, the analysis of these mutants in protoplast inoculations and plants, and peptide competition studies with TYC69 antiserum were the work of John J. Weiland. *In vitro* translations of all initiation codon and premature stop codon mutants that were constructed for the study, the effects of Mg^{2+} concentrations on the production *in vitro* of various protein species originating from ORF-69, and a northern blot comparing the accumulation of viral RNAs in protoplasts for TYMC and TYMC-178/224 appear in the addendum to chapter II and are the work of John J. Weiland.

Manuscripts currently in preparation for publication are represented by chapters III and IV. Plasmid construct pTYMC-Δ210-1759 in chapter IV was constructed by Kay L. Bransom. Plasmid construct pTYMC-Δ4086-4587 was constructed by Ching-Hsiu Tsai. All other plasmids reported in chapters III and IV were made by J.J.W.

The Roles of Turnip Yellow Mosaic Virus Genes in Virus Replication.

INTRODUCTION

Members of the plant kingdom, as with all known living organisms, store the genetic information for their development and propagation in their chromosomal deoxyribonucleic acid (DNA). The separation of genetic information onto several DNA chromosomes is a characteristic of higher organisms, including multicellular plants. With each cell division and, hence, for the growth and reproduction of each plant, chromosomal DNA is replicated and inherited with high fidelity, yielding daughter cells with matching chromosome sets (Raven et al., 1976).

The information in the chromosome is activated via cellular transcription, in which regions of the DNA are copied enzymatically into discrete ribonucleic acid (RNA) strands. Ribonucleic acid is inherently less chemically stable than DNA and displays a higher mutation rate than copied DNA. It has been speculated that these characteristics might explain the evolution of DNA as the informational store in all known living organisms (Eigen and Biebricher, 1988). RNA transcribed from cellular DNA has three known functions: 1) to serve a structural role, as in the assembly of ribosomes; 2) to serve in an enzymatic role, as in ribozymic cleavage of other RNA molecules; and 3) to serve as the information link between the chromosome and proteins--that of the messenger RNA (mRNA) and transfer RNA (tRNA)(Adams et al., 1986). The process of accessing chromosomal information and forming the final gene product is known as gene expression (Lewin, 1990).

Viruses are obligate intracellular parasites of unicellular and multicellular organisms (Matthews, 1991). They consist generally of an RNA or DNA genome encapsidated by a protein, glycoprotein and/or lipoprotein shell. Once inside a host cell, a virus embarks on a pathway of viral genome expression and virus chromosome replication. After the cycle is completed, several progeny virus particles exist as a result of the replication of a single progenitor virus. These in turn may proceed to invade uninfected host cells, and the replication cycle begins anew.

The genome sizes of viruses are small relative to those of living organisms (Matthews, 1991). Mammals and higher plants possess genomes of 10^9-10^{11} base pairs, and that of the common laboratory bacterium *E. coli* is about 10^7 base pairs in size. By contrast, the largest genomes of entities classified as viruses are ca. 4-5 X 10^5 kilobase pairs. Genome sizes for the smallest viruses are in the range of 1-3 X 10^3 bases.

Whereas about half of the known animal virus classes have RNA as their genetic material, greater than ninety percent of the known higher plant viruses have RNA genomes (Fields and Knipe, 1990; Matthews, 1991). A viral genome, whether DNA or RNA, is often divided onto several chromosomes. In animal viruses, these chromosomes are usually packaged in a single virus particle. A large number of RNA plant viruses exist, however, which have multiple chromosomes each encapsidated in a separate particle. Viruses with multiple, separately encapsidated nucleic acids are subclassified, therefore, as being monopartite (one viral chromosome), bipartite (two viral chromosomes), tripartite (three viral chromosomes) or multipartite, based on the number of chromosomes required for full infectivity of the virus (Matthews, 1991).

A fundamental process in virus propagation is the replication of the viral nucleic acid genome. Our studies are mostly concerned with the replication of "positive (or plus) sense" RNA viruses. Designation of a viral RNA genome as "positive" denotes the ability of the RNA genome to act directly as mRNA in the synthesis of virus encoded proteins. The strand of RNA complementary to the genomic RNA serves as template in the synthesis of progeny plus sense RNA molecules, and is appropriately referred to as "negative (or minus) sense ". The crucial factors involved in the replication of a plus sense RNA virus have best been examined in the bacteriophage Qß (Blumenthal and Carmichael, 1979), and Qß continues to be a paradigm for the replication of many positive sense RNA viruses.

We have been most interested in the replication mechanisms of positive sense plant RNA viruses. Turnip yellow mosaic virus (TYMV) is one of the most well characterized plant RNA viruses in terms of replication biology and biochemistry. Reports exist on TYMV regarding replication cytology, enzymology and the detection of replicative intermediates (reviewed in Bove and Bove, 1985; Francki et al., 1985; Matthews, 1991). Detailed information on replication biochemistry of this virus is lacking, however, and the application of modern molecular biology techniques to studies of TYMV would advance our knowledge of the replication of this virus in particular and other related RNA viruses in general. Because of our interest in the replication mechanisms of positive sense RNA plant viruses, we chose to examine in greater detail the replication strategy for turnip yellow mosaic virus.

A) Turnip yellow mosaic virus.

1) General virology.

Turnip yellow mosaic disease was first attributed to a virus agent by Markham and Smith (1946). Turnip yellow mosaic virus (TYMV) was distinguished from previously characterized viruses due to the fact that it was transmissible by flea beetles rather than aphids, and displayed bright yellow symptoms as compared to the growth stunting typical of other viral infections. TYMV also exhibited a broad host range within the Brassica tribe in contrast with other known viruses of crucifers.

Since its discovery, a number of viruses related to TYMV have been reported infecting dicotyledonous plants (Koenig, 1976). This has led to the creation of a separate taxonomic group for these interrelated viruses. As TYMV was the first characterized member of the group, all relatives of TYMV are broadly classified as plant tymoviruses (turnip yellow mosaic virus) (Francki et al., 1985; Hirth and Givord, 1988; Matthews, 1991).

2) Virus Architecture.

TYMV is a 29 nanometer spherical plant virus. Virus particles contain protein, RNA, polycationic spermine and spermidine, and other ionic elements. The protein component in TYMV virions is exclusively virus encoded (Peter et al., 1972; Pleij et al., 1976). Encapsidated RNA is a mixture of TYMV genomic and subgenomic RNA, and minor amounts of tRNA (Pinck et al., 1970; Pleij et al, 1976). Cationic spermine and spermidine (Beer and Kosuge, 1970) probably serve to neutralize the net negative charge on the sugar phosphate backbone of the encapsidated RNA.

Extensive examination of the virus particle has led to a model for the structure of TYMV (Finch and Klug, 1966). The capsid shell is comprised of 180 monomer subunits of coat protein (CP). Monomers aggregate to form virion substructures called capsomeres. A single capsomere is composed of either 5 monomers (a pentamer) or 6 monomers (a hexamer). Twenty hexamers and twelve pentamers combine to form the mature virus particle. Since empty virus particles devoid of RNA can be formed, it is thought that significant protein-protein interactions, as well as RNA-protein interactions, contribute to the TYMV virion structure (Hirth and Givord, 1988; Matthews, 1991).

The entire TYMV coat protein has been sequenced (Peter et al., 1972), and matches the amino acid sequence encoded by the open reading frame (ORF) positioned at the 3'-end of the genome and in the virus subgenomic RNA (Guilley and Briand, 1978). The coat protein is 189 amino acids long and is N-acetylated on the amino-terminal methionine that is encoded by the initiating codon of the CP gene ORF (Peter et al., 1972). Results using immunochemical techniques suggest that the coat protein C-terminus and N-terminus are in close proximity to each other and are located on the virion surface with the central portion of the protein folded towards the interior of the particle (Quesniaux et al., 1983). Determination of RNA binding domains on internal amino acid stretches of the monomer by *in-situ* crosslinking studies supports this contention (Ehresmann et al., 1980).

3) **Virus Genome.**

All known tymoviruses have positive (messenger) sense RNA genomes 6.0 to 6.4 kilobases (kb) in length and are rich in cytidine monophosphate (Hirth and Givord, 1988; Matthews, 1991). The genomes of several tymoviruses, including three isolates of TYMV have been entirely sequenced using a combination of cloned cDNA sequencing and direct RNA sequencing methodologies (Morch and Haenni, 1988; Keese et al., 1989; Osorio-Keese et al., 1989; Ding et al., 1989; Ding et al., 1990a; Dreher and Bransom, 1991).

The sequenced tymovirus genomes all encode three major ORFs greater than 500 nucleotides in length (Fig. 1). By analogy to TYMV, the 3'-end ~0.6 kb ORF in all tymoviruses encodes the virus coat protein. Computer generated alignments of sequences encoded in the long ~5.0 kb ORF suggests a role for the protein product in viral RNA replication (Morch et al., 1988; Keese et al., 1989; Koonin, 1991). The ~2.0 kb 5'-end ORF initiates from the first AUG of the genomic RNA and extensively overlaps the ~5.0 kb ORF out-of-frame. Whereas the ~5.0 kb ORFs encode proteins with high sequence similarity among the tymoviruses, the lengths and encoded sequences of the ~2.0 kb ORFs significantly divergent (Table 1).

The 5'-end of the TYMV genomic RNA is capped with 7mGpppG similar to eukaryotic mRNAs (Briand et al., 1978). The 3'-end 89 nucleotides can fold into a structure resembling cellular tRNA (Dumas et al., 1987) and exhibits tRNA-like properties *in vitro* (Pinck et al, 1970; reviewed in Mans et al., 1991) and *in vivo* (Joshi et al., 1982a). The tRNA-like structure can be specifically charged with valine *in vitro* (Florentz et al., 1991) and in *Xenopus* oocytes (Joshi et al., 1978) and valylated viral RNA has been detected in infected plant extracts (Joshi et al., 1982a). Using infectious RNA from cloned cDNA of TYMV, infectivity has recently been positively correlated with valine charging *in vitro* (Tsai and Dreher, 1991). Additional evidence suggests that the tRNA-like structure contains promoter sequences directing the initiation of viral RNA replication (Morch et al., 1987).

4) Gene expression strategies.

a) Non-capsid genes.

The existence of bacterial, plant and animal derived cell-free translation systems has facilitated the analysis of cellular and viral RNA expression strategies (Kozak, 1989b). Translation of fractionated TYMV RNA provided initial evidence for the modification of encoded nonstructural proteins by autoproteolysis (Morch and Benicourt, 1980), and for the expression of the coat protein via a subgenomic RNA 3' coterminal with the genomic RNA (Pleij et al., 1976).

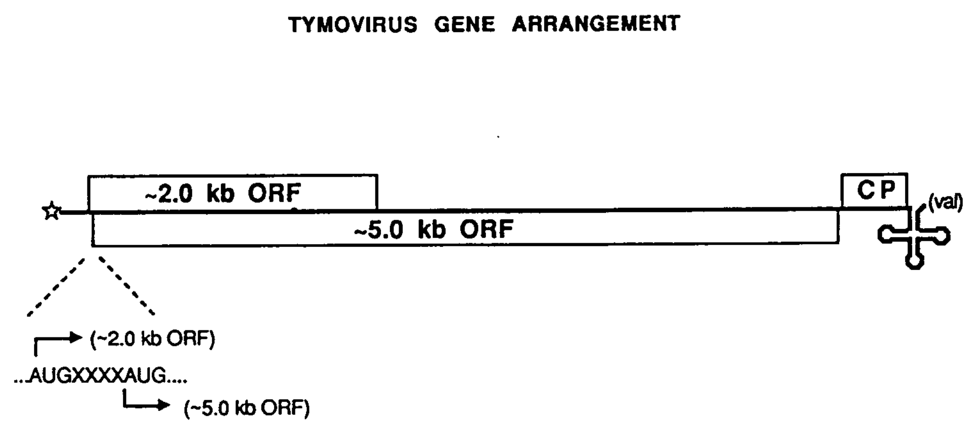


Figure 1) General gene arrangement in the tymoviruses. The star and the cloverleaf represent the 5' end cap structure and the 3' end tRNA-like structure, respectively. The solid black line represents the genomic RNA and the flanking open boxes, the encoded open reading frames (ORFs). The nucleotide spacing separating the initiation codons for the ~2.0 kb and ~5.0 kb ORFs is shown.

Table 1) Characteristics of genomes and encoded genes in sequenced tymoviruses.

		TYMC[a]	TYMV[b]	TYMV[c]	EMV[d]	OYMV[e]	KYMV[f]
Genome RNA length	(nts)	6318	6318	6319	6330	6211	6362
5'-end UTR	(nts)	87	87	88	101	171	78
~2.0 kb ORF	(nts)	1887	1887	1887	1950	1794	2262
	(codons)	629	629	629	650	598	754
~5.0 kb ORF	(nts)	5535	5535	5535	5520	5331	5625
	(codons)	1845	1845	1845	1840	1777	1875
coat protein ORF	(nts)	570	570	570	567	579	567
	(codons)	190	190	190	189	193	189
3'-end UTR	(nts)	109	109	108	132	146	84
Additional known physical features		5'-end genomic and subgenomic RNA cap structure.[g] 3'-end tRNA-like structure can bind valine.[h,i]			Valine binding 3'-end.[j]		Valine binding 3'-end.[k]

a) Dreher and Bransom, 1991.
b) Morch, Boyer and Haenni, 1988.
c) Keese, MacKenzie and Gibbs, 1989.
d) Osorio-Keese, Keese and Gibbs, 1989.
e) Ding, Keese and Gibbs, 1989.
f) Ding, Gibbs and Keese, 1990a.
g) Guilley and Briand, 1978.
h) Pinck, Yot, Chapeville and Duranton, 1970.
i) Joshi, Chapeville and Haenni, 1982.
j) Pinck and Hall, 1978.
k) Van Belkum, Bingkun, Rietveld, Pleij and Borsch, 1987a.

Abbreviations:
TYMC-Turnip Yellow Mosaic Virus (Corvallis strain)
TYMV–Turnip Yellow Mosaic Virus
EMV–Eggplant Mosaic Virus
OYNV–Ononis Yellow Mosaic Virus
KYMV–Kennedya Yellow Mosaic Virus
UTR–Untranslated Region
nts–nucleotides

The expression of TYMV RNA leads to the synthesis of three abundant high molecular weight polypeptides (Morch et al., 1989; Weiland and Dreher, 1989; Bransom et al., 1991). The long ~5.0 kb ORF (denoted ORF-206 in TYMV) directs the synthesis of two of these abundant proteins, the 206 kDa (p206) and 150 kDa proteins, with the numerical designations reflecting their relative migration in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein p206 is the full-size product encoded in ORF-206, and the 150 kDa protein is amino-coterminal with p206, but lacks the carboxyterminal (C-terminal) amino acids retained on p206. A C-terminal 70 kDa protein generated from the processing of p206 is present in low and variable amounts in translations of TYMV, and appears to be unstable in rabbit reticulocyte lysate and wheat germ translation extracts. Recent data suggest that the production of 150kDa protein results from the co-translational autoproteolysis during the decoding of ORF-206 by translating ribosomes (Morch et al, 1989; Bransom et al., 1991).

An abundant protein of Mr ~75 kDa is the product of the 5'-end ~2.0 kb ORF (ORF-69 in TYMV). Whereas the synthesis of the ORF-69 product probably initiates at the first AUG on the genomic RNA (Weiland and Dreher, 1989; Chapter I), translation of ORF-206 appears to initiate seven nucleotides 3' of the ORF-69 initiating AUG (Briand et al., 1978; Keese et al., 1989; Weiland and Dreher, 1989). "Leaky scanning" has been proposed to explain the efficient initiation of translation at several 5'-end initiation codons in an mRNA (Kozak, 1989a), and may be operative in the expression of tymoviral genomes.

b) Coat protein gene.

During TYMV replication, a 695 nucleotide RNA coterminal with the 3'-end of the genomic RNA is produced (Pleij et al., 1976; Guilley and Briand, 1978; Weiland and Dreher, 1989). This subgenomic RNA (sgRNA) is the sole subgenomic RNA detected in TYMV infections and encodes sequences for the viral coat protein (Guilley and Briand, 1978). Cell-free translation experiments indicate that the expression of TYMV coat protein directly from the genomic RNA is inefficient. Efficient coat protein expression occurs from the sgRNA *in vitro* (Pleij et al., 1976), however, and coat protein production can be controlled at the level of sgRNA transcription (Gargouli et al., 1990).

The sgRNA has a 5'-end 7mGpppA cap structure in contrast to the 7mGpppG cap structure on the genomic RNA 5'-end. Although the sgRNA contains the 3'-end tRNA-like structure, no production *in vivo* of discrete minus-sense RNA complementary to the sgRNA has been observed (Weiland and Dreher, unpublished). Thus, either sequences beyond those contained in the sgRNA are required for minus-sense RNA synthesis, or the structure of the sgRNA prohibits its use as template in RNA replication.

5) Genome replication and sgRNA transcription.

The replication strategy for TYMV has been one of the most extensively studied in plant RNA viruses (Mouches et al., 1974 ; Mouches et al., 1984; Bove and Bove, 1985; Dreher and Hall, 1988a). A model for the tymovirus replication cycle including sgRNA transcription is shown in Fig 2. This model is general, and is applicable to viruses in the Sindbis virus supergroup related to TYMV (see Section B). A review of the cytology, enzymology, and molecular genetics of TYMV replication is presented below.

a) Replication cytology.

Following the infection of Chinese cabbage protoplasts and plants with TYMV, distinct sub-cellular changes can be observed (Hatta and Matthews, 1974; Francki, 1985; Matthews, 1991). Chloroplasts in infected cells begin to aggregate and swell, and spaces between adjacent chloroplasts become filled with virus particles.

Thin sections of infected cells observed by electron microscopy reveal changes at the chloroplast membrane now considered to be characteristic of tymovirus infections. Vesicles formed along the chloroplast surface appear to be caused by the invagination of both the outer and inner membranes. Using *in situ* labelling of replicating TYMV genomic RNA and antisera raised to purified TYMV replication enzyme (replicase), a high concentration of TYMV RNA (Garnier et al., 1980) and putative replicase (Garnier et al., 1986), respectively, has been detected in association with these vesicles. The observation has prompted investigators to propose, therefore, that the vesicles constitute TYMV RNA replication centers. A model based on the results suggests that newly synthesized viral RNA is extruded from a membranous replication center, where the RNA may re-enter the expression/replication cycle or become packaged into a virus particle (Francki, 1985; Matthews, 1991).

b) Replication enzymology.

Until recently, the replicase for TYMV was the only RNA-dependent RNA replicase of a virus in the Sindbis supergroup that had been solubilized and partially purified. The replicase preparation from TYMV-infected Chinese cabbage was reportedly capable of accepting TYMV RNA as exogenous template, and synthesizing minus sense RNA, which was hydrogen bonded to the template plus sense RNA (Mouches et al., 1974). Although the replicase activity was specific for TYMV template RNA, the enzyme was not capable of catalyzing efficiently the complete cycle of viral RNA replication as has been reported for the replicase of bacteriophage Qß (Blumenthal and Carmichael, 1979). Immunological analysis of the TYMV replicase suggests that it is composed of only two subunits; a virus encoded polypeptide of Mr 115 kDa and a host plant protein of Mr 45 kDa (Mouches et al., 1984). This subunit composition would make the replicase of TYMV extremely simple relative to the proposed complexes for other positive sense RNA viruses (Dreher and Hall, 1988a; Quadt and Jaspers, 1989). The well characterized replicase of the *E. coli* bacteriophage Qß, for example, is composed of three host-encoded subunits (EF-Tu, EF-Ts and ribosomal protein S1) and one virus-encoded subunit (Blumenthal and Carmichael, 1979). It is noted, however, that the analysis of replicase activity in the reported two subunit enzyme for TYMV involved only the incorporation of radiolabelled nucleotide into acid precipitable product. No data on the forms of RNA synthesized were included in the study.

Interestingly, the recent purification of cucumber mosaic virus (CMV) replicase indicates that a likewise simple composition of virus (two subunits) and host (one subunit) encoded proteins is present in the active replication complex (Hayes and Buck, 1990a). This enzyme appears capable of initiating and completing the synthesis of both plus and minus sense genomic RNA from added viral RNA, and marks the first example of such activities reported for the replicase preparation of a eukaryotic RNA virus. These

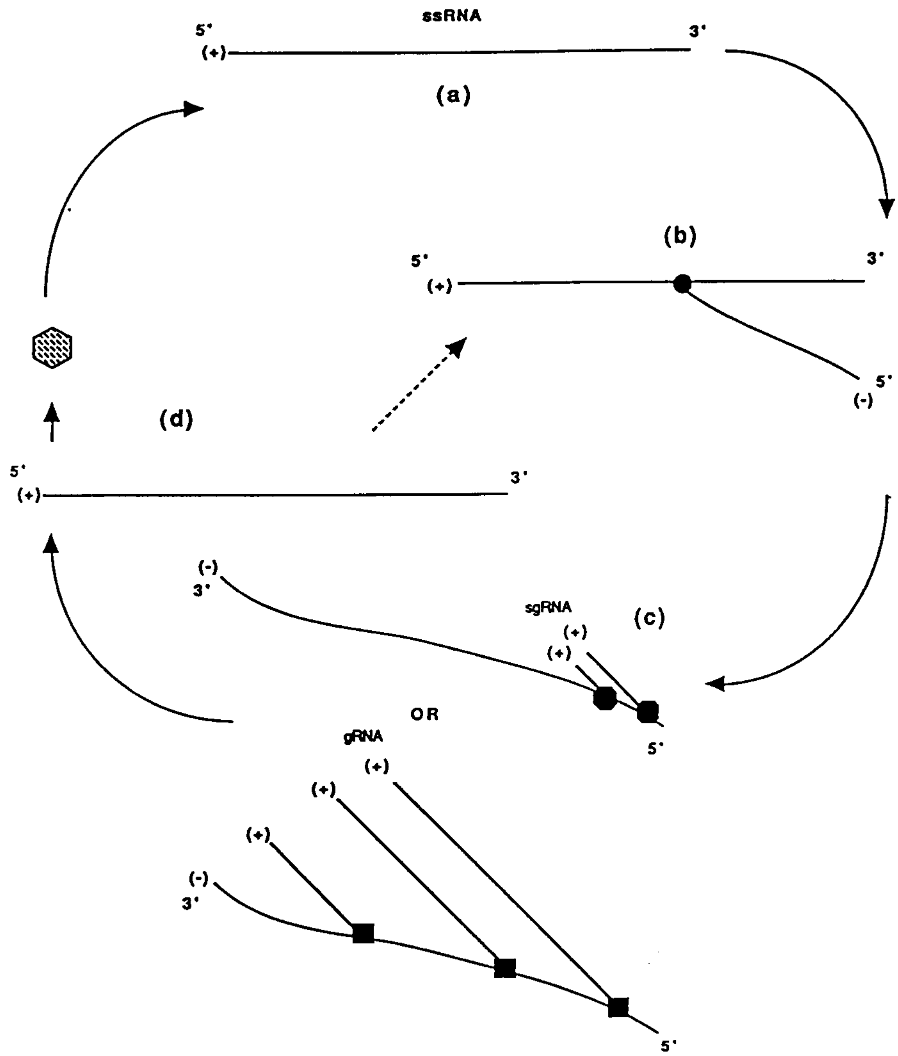


Figure 2) General replication model for the Sindbis-like viruses. Genomic plus-sense RNA (a) is copied to genomic minus-sense molecules (b). The minus-sense molecules are templates for the synthesis of plus-sense genomic RNA (gRNA) or subgenomic RNA (sgRNA) (c). Genome length progeny molecules are encapsidated, translated or re-enter the replication cycle (d). Black boxes and circle are replication complexes, the octagons are transcription complexes, and the polarity of the molecules are as labelled. The stippled hexagon represents encapsidated RNA.

results may pave the way for a more extensive re-examination of the replication enzymology of TYMV and other RNA viruses.

c) Genetic elements involved in genome RNA replication and subgenomic RNA transcription.

In general, sequences involved in the replication of TYMV are poorly characterized. *Cis*-acting sequences involved in TYMV genome replication and sgRNA transcription have tentatively been identified based on limited biochemical evidence (Morch et al., 1987), on the alignment of significantly similar nucleotide sequences between the tymoviruses (Ding et al., 1990b), and recently on biological data examining the effects of base substitutions in the tRNA-like structure on RNA infectivity (Tsai and Dreher, 1991). TYMV-encoded *trans*-acting replication factors have been designated as such based on amino acid sequence similarities of proteins encoded by TYMV to other viral proteins with known roles in RNA replication (Habili and Symons, 1989; Dreher and Bransom, 1992) and on recent mutagenesis studies using an infectious cDNA clone of TYMV (Weiland and Dreher, 1989; Chapter I; Chapter IV).

A review of our current understanding regarding the *cis*-acting and *trans*-acting replication elements in the Sindbis supergroup of viruses, of which the tymoviruses are a sub-group, is presented below. Detailed information on proposed replication factors and elements involved in the replication of TYMV is included therein.

B) Genetics of RNA replication in Sindbis virus and related plant viruses: a literature review.

1) General introduction.

Viruses have traditionally been classified based on their physical characteristics and host range (Murphy and Kingsbury, 1990). With the extensive collection of virus nucleic acid sequences, and the subsequent comparison of sequences within and between virus families, new virus phylogenies or supergroups have arisen. These phylogenies are based on similarities between virus nucleic acid sequences directly, or between amino acid sequences in virus genes predicted from the nucleotide sequences (Kamer and Argos, 1984; Gorbalenya and Koonin, 1989; Koonin, 1991). Using a combination of virus physical properties and nucleic acid sequence analysis, the eukaryotic positive sense RNA viruses have been divided into three major groups; 1) the Sindbis virus supergroup, with the animal Sindbis virus as the type member, 2) the picorna virus supergroup, of which poliovirus is the type member, and 3) the luteovirus/carmovirus supergroup, with type member carnation mottle virus (Gorbalenya and Koonin, 1989; Habili and Symons, 1989; Koonin, 1991; Goldbach et al., 1991).

Due to the extensive genetic and biochemical characterization of Sindbis virus, it is considered the type virus of the supergroup bearing its name. Members of the Sindbis virus supergroup have diverse virion morphologies (Table 2). Inclusion of a virus in the Sindbis supergroup, therefore, is based on both the structure of the virus genomic RNA (both physical and coding characteristics) and its gene expression strategies (Zimmern, 1988; Goldbach et al., 1991). All members of the group have a 5'-end cap structure on their genomic RNAs similar to that found at the 5'-end of cellular mRNAs. Furthermore, group members express a subset of their viral genes via subgenomic RNAs (sgRNA) synthesized during viral RNA replication. Subgenomic RNAs are usually 3'-end coterminal with the genomic RNA, and are efficient mRNAs for genes encoded at the 3'-end of a viral genome (Matthews, 1991). Finally, members display significant homologies among amino acid sequences encoded in genes for viral non-capsid proteins, and it is proposed that these homologies relate these viruses evolutionarily (Habili and Symons, 1989; Koonin, 1991; Goldbach et al., 1991). Although the plant tombusviruses and carmoviruses were at one time considered to be members of the Sindbis virus supergroup, they lack both a 5'-end cap structure (Carrington et al., 1989) and important encoded amino acid sequences present in all other group members (Habili and Symons, 1989; Koonin, 1991).

The ability to generate infectious transcript RNA from cloned cDNA of RNA virus genomes has rapidly advanced our understanding of viral genetic elements involved in virus multiplication and pathogenesis (Fields and Knipe, 1990; Matthews, 1991). The following sections will review

Table 2) General features of the animal Sindbis virus, and currently recognized members of the plant Sindbis virus supergroup[1,2]. The type virus for each group and its common abbreviated name is shown in parentheses. Genome sizes are given in kilobases.

Virus group	Virion morphology	Genome /size (approx.)	3'-end
Alphaviruses (Sindbis)	Spherical	Monopartite/12.0 kb	poly(A)[3]
Tobamoviruses (tobacco mosaic virus, TMV)	Rigid rod	Monopartite/6.0 kb	tRNA-like[4]
Potexviruses (potato virus X, PVX)	Flexuous rod	Monopartite/6.0 kb	poly(A)
Tymoviruses (turnip yellow mosaic virus, TYMV)	Spherical	Monopartite/6.0 kb	tRNA-like
Carlaviruses (Carnation latent virus, CarLV)	Flexuous rod	Monopartite/8.0 kb	poly(A)[7]
Tobraviruses (tobacco rattle virus, TRV)	Rigid rod	Bipartite/9.0 kb	tRNA-like[5]
Bromoviruses (brome mosaic virus, BMV)	Spherical	Tripartite/8.0 kb	tRNA-like
Cucomoviruses (cucumber mosaic virus, CMV)	Spherical	Tripartite/8.0 kb	tRNA-like
Alfalfa Mosaic Virus (AlMV)	Bacilliform	Tripartite/8.0 kb	X-OH[6]
Hordeiviruses (barley stripe mosaic virus, BSMV)	Rigid rod	Tripartite/10.0 kb	tRNA-like
Furoviruses (beet necrotic yellow vein virus, BNYVV)	Rigid rod	Quadripartite/14.0 kb	poly(A)

1) Goldbach et al., 1991
2) Koonin, 1991.
3) Polyadenosine monophosphate structure similar to cellular mRNA.
4) Structures which can be aminoacylated by aminoacyl-tRNA synthetases.
5) The TRV 3'-end has tRNA-like secondary structure, but is not efficiently aminoacylated (van Belkum et al., 1987b).
6) Lacks poly(A) tail and tRNA-like structure.
7) For potato virus M carlavirus.

recent progress in the study of *trans*-acting and *cis*-acting virus-encoded genetic elements involved in the replication of Sindbis virus and plant members of the Sindbis supergroup. Information regarding sequences required for sgRNA synthesis is included where available.

2) Trans-acting genetic elements in the replication of Sindbis and related plant viruses.

a) Monopartite viruses.

i) Sindbis virus.

Sindbis virus has a 12 kilobase (kb) RNA genome encoding two major open reading frames (ORFs) (Strauss and Strauss, 1988). The 5'-end ORF encodes a nonstuctural (ns) polyprotein with a relative molecular weight of 230 kilodaltons (Mr 230K, nsP123) and terminating at an opal (UGA) stop codon (Fig. 3). Upon suppression of this stop codon, polyprotein synthesis may be extended to yield an Mr 270K protein (nsP1234; Strauss et al.,1983; Li and Rice, 1989; deGroot et al, 1991). The major 3'-end ORF encodes the virus capsid proteins (Strauss et al., 1984).

Polyprotein nsP123 is processed by an autoprotease to generate the mature proteins nsP1, nsP2 and nsP3 (Hardy and Strauss, 1988). The extension product resulting from translational readthrough of the opal terminator is cleaved off of the polyprotein to generate nsP4 (Li and Rice, 1990). Protein nsP4 can also be cleaved from a protein subset of processed nsP1234, such as nsP34 (deGroot et al., 1990).

A large body of evidence indicates that the Sindbis virus nonstructural proteins are involved in virus RNA replication (Strauss and Strauss, 1988; Hahn et al, 1989; Barton et al., 1991; Li and Rice, 1991). Using Sindbis virus mutants temperature sensitive for RNA replication (ts RNA^- mutants) in complementation assays, mutants have been assigned to discrete complementation groups (Strauss and Strauss 1988, Hahn et al., 1989). Several of the ts mutants characterized represent multiple nucleotide changes in nsP1234. In some cases, however, a ts mutant involves a single nucleotide change (resulting in a single amino acid change) in one of the four mature nonstructural proteins (Hahn et al., 1989).

Examples of nucleotide changes, and corresponding amino acid changes, in the Sindbis virus nonstructural proteins which have an effect on virus replication are outlined below. Additional evidence for the role of a specific protein in virus replication is noted where available.

nsP1

Sindbis virus nsP1 is the aminoterminal protein cleaved from the nonstructural polyprotein precursor. Amino acid sequences encoded in this protein display significant homology with nonstructural proteins in many members of the plant Sindbis-like viruses (Kamer and Argos, 1984; Ahlquist et al., 1985; Gorbalenya and Koonin, 1989; Fig. 3). Genetic evidence from the analysis of an RNA$^-$ ts mutant (Wang et al., 1991) and of mutants which rescue Sindbis virus replication in cells deprived of methionine (Durbin and Stollar, 1985) or GTP (Malinowski and Stollar, 1981) implies that nsP1 plays a role in RNA replication and capping of the RNA genome.

The nucleotide substitution in mutant ts-11 maps to the nsP1 coding region, and results in a single amino acid change in the protein (Sawicki et al., 1981; Hahn et al., 1989; Wang et al., 1991). Genomic minus sense RNA synthesis is shut off in the replication of this mutant when the incubation temperature of infected cells is shifted from 30° C (permissive) to 40° C (non-permissive). Since plus sense genomic and subgenomic RNA synthesis are not affected by the mutation, a role for nsP1 in the initiation of viral minus sense RNA synthesis has been proposed (Wang et al., 1991).

Protein nsP1 may also be a methyltransferase. Sindbis virus replicates poorly in cells deprived of methionine (Durbin and Stollar, 1985; Mi et al, 1989), and this is thought to reflect a requirement for methyl group transfer in Sindbis virus replication. Genetic evidence has recently shown that mutant virus competent for efficient replication in methionine deprived cells had fixed two nucleotide changes resulting in amino acid changes in nsP1 (Mi et al., 1989). Since Sindbis virus genomic RNA is capped with a 7-(methyl)-guanosine structure, it is proposed that nsP1 is involved in the methylation of the cap structure. Biochemical evidence further supports a role for nsP1 as a methyltransferase. Sindbis virus nsP1 expressed in *E. coli* has been shown to induce a distinct methyltransferase activity in bacterial cell extracts (Mi et al, 1991).

A potential guanyltransferase activity may also reside on nsP1. Ribavarin and mycophenolic acid reduce cellular GTP levels by inhibiting inosine monophosphate dehydrogenase, and cells treated with these agents support Sindbis virus replication poorly (Malinoski and Stollar, 1981). Mutants recovering elevated replication levels under these conditions harbor amino acid substitutions in nsP1 (Scheidel and Stollar, 1991). The authors propose that the mutations confer an increased affinity for GTP in some component of the replication complex, perhaps in nsP1 itself, and that this binding of GTP is related to the capping of viral RNA. The combined evidence points, therefore, to a role for nsP1 in RNA capping, as well as in the initiation of minus sense RNA synthesis.

nsP2

Sindbis virus nsP2 has been divided into two domains representing distinct biochemical activities. Based on computer-generated alignments, the amino terminus of nsP2 encodes a potential purine nucleoside triphosphatase (NTPase) common to proteins exhibiting a nucleic acid helicase activity (Matson and Kaiser-Rogers, 1990). The core amino acids in this domain are ...GXGKS/T..., where X encodes an unspecified amino acid, the fifth position is a serine or threonine, and lysine is proposed to be the residue binding the purine nucleotide (Hodgeman, 1988; Gorbalenya and Koonin, 1989). This amino acid sequence core is strictly conserved in nonstructural proteins encoded by members of the Sindbis virus supergroup. Sequences surrounding and including the core constitute a domain with a high similarity to a family of putative nucleic acid helicases including *E. coli* replication and recombination proteins (Gorbalenya and Koonin, 1989; Matson and Kaiser-Rogers, 1990). Furthermore, biochemical studies have shown that a protein containing this domain encoded by a plant potyvirus may possess an ATP-dependent helicase activity (Lain et al., 1990).

Sindbis virus RNA$^-$ ts mutants mapping to nsP2 have historically been somewhat confusing. Most nsP2 mutants affect sgRNA synthesis, but appear to fall into two complementation groups (Hahn et al., 1989). For example, substitutions in ts-21 are localized to the aminoterminus of nsP2, the mutant falls into complementation group A, but programs wild-type polypeptide synthesis and processing at the non-permissive temperature (Hardy et al., 1990). Mutant ts-7 falls into complementation group G, has nucleotide changes that map to the C-terminus of nsP2, fails to process its polyprotein correctly after temperature shift, but is similar in phenotype to several group A RNA$^-$. Part of the confusion in matching a mutant phenotype to a specific complementation group has most likely resulted from the fact that many of the ts mutants isolated have multiple nucleotide changes located at several positions in the nsP1234 coding sequence. Additional sequence analysis of Sindbis mutants will no doubt reveal which mutants are confounded by changes at several sites.

The critical nucleotides affecting polyprotein processing in mutants ts7, ts17 and ts24 have been mapped to the C-terminus of nsP2 (Hardy et al., 1990). This indicated that the domain responsible for polyprotein processing might be located in the C-terminal half of nsP2. Recent protein sequence alignment (Gorbalenya et al., 1991) and biochemical data (Hardy and Strauss, 1989; Ding and Schlessinger, 1989) strongly indicate that the Sindbis virus nonstructural protein autoprotease is present on the C-terminus of nsP2. Putative catalytic cysteine and histidine residues in the protease active site flank the amino acids changed in mutants ts-7 and ts-17. Failure of the mutants to process the polyprotein after temperature shift probably account for the RNA$^-$ phenotype, although an additional substitution in nsP3 (for ts-7) that does not affect processing has been shown to contribute to the RNA$^-$ phenotype (Hahn et al., 1989). The protease functions both in *cis* and in *trans*, and processing of the polyprotein is temporally regulated (Hardy and Strauss, 1989; deGroot et al., 1990).

Analysis of RNA synthesis for nsP2 mutants reveals that most C-terminal changes result in increased genomic minus sense RNA synthesis, and reduced sgRNA synthesis (Hahn et al., 1989). One mutant (ts-21) mapping to the aminoterminal portion of nsP2 is defective for sgRNA synthesis, but does not produce elevated levels of minus sense RNA (Sawicki and Sawicki, 1985). Physical linkage of the protease and potential helicase domains on nsP2 makes it difficult to separate the many potential roles for this protein in Sindbis virus RNA replication. Further work will be required to dissect the various observed functions of nsP2 onto sub-domains of the protein.

nsP3

Of the four mature Sindbis virus nonstructural proteins, nsP3 shows the least amino acid sequence homology to its counterpart in other alphaviruses, and no apparent similarity to sequences encoded in Sindbis-like plant viruses (Strauss and Strauss, 1988). Although a substitution mutation mapping to this protein gives an RNA$^-$ phenotype (ts-7), it was not reported which aspect of RNA synthesis was affected (Hahn et al., 1989). The assembly of replication complexes at 40° C is inhibited with this mutant, but the complexes are stable if the temperature shift occurs after subunit assembly (Sawicki et al., 1981). It must be rememberd, however, that ts-7 also harbors a mutation in nsP2. Further experimentation will be required to separate the effects of the nsP2 mutation from the nsP3 mutation in this isolate.

It has been proposed that the ratio of nsP4 to the semiprocessed nsP34 fusion protein may regulate the switch from minus sense to plus sense RNA synthesis (deGroot et al., 1990; deGroot et al., 1991). Elevated ratios of nsP34 to nsP4 are apparent ~4 hours post-inoculation at roughly the same time that minus strand synthesis normally ceases. In addition, a protein the size of nsP34 is detectable in replication complexes using anti-nsP3 and anti-nsP4 antisera (Barton et al., 1991). Biochemical tests using replication complexes prepared at different timepoints during infection should help to unravel the involvement of nsP3 in Sindbis virus replication.

nsP4

Sindbis virus nsP1234 is the product of the opal termination codon readthrough during translation, and subsequent cleavage of the polyprotein immediately downstream of the suppressor amino acid yields mature nsP4 (Li et al, 1989). Amino acid sequences in nsP4 represent some of the most highly conserved sequences within the Sindbis virus supergroup. Alignments of amino acid sequences in nsP4 with other virus encoded protein sequences (Koonin, 1991) reveals a high similarity to known RNA-dependent RNA polymerases (Morrow et al., 1987). The signature sequence for RNA-dependent polymerases is a ..GDD.. amino acid triplet, and based on the comparison of sequences surrounding this motif among all positive sense RNA viruses, phylogenetic trees showing intervirus evolutionary relationships have been constructed (Koonin, 1991).

Temperature sensitive mutants mapping to nsP4 fall into two categories: 1) total RNA$^-$ mutants defective in all forms of RNA synthesis, and 2) one characterized mutant defective in the the shutoff of minus sense RNA synthesis late in the infection cycle (Hahn et al., 1989; Sawicki et al., 1990). Total RNA$^-$ mutants in nsP4 are consistent with a putative RNA polymerase function for this protein. Elongation during RNA synthesis *in vitro* is disrupted at the non-permissive temperature in nsP4 mutant ts-6 (Barton et al., 1988) further supporting the notion that this protein is the virus RNA polymerase.

A single nsP4 amino acid mutant (a ts-24 derivative) also displays characteristics of many nsP2 mutants. After temperature shift, previously shut-off minus strand synthesis is reactivated (Sawicki et al., 1990). The authors propose that a domain of nsP4 may function in giving preference to minus sense versus plus sense template usage during normal Sindbis virus RNA replication. Thus the mutation leads to the breakdown of this preference and plus sense RNA serves as template equally well as minus sense RNA.

Biochemical studies have recently shown that the observed instability of nsP4 is due to its susceptibility to proteolysis (deGroot et al., 1991). nsP4 appears to be degraded by a ubiquitin-mediated pathway in both cell-free translation extracts and cell monolayers. By changing N-terminal amino acids of nsP4, the degradation was shown to adhere to the N-end rule (Bachmair et al., 1986). What role the rapid degradation of nsP4 plays in Sindbis virus replication is not known, but it may be operative in the regulation of RNA synthesis by controlling nsP34/nsP4 ratios as described above.

In summary, Sindbis virus is the prototype member of the Sindbis supergroup of viruses. Amino acid sequences in nsP1 are similar to a domain in the majority of the supergroup members (Goldbach et al., 1991), and nsP1 is potentially involved in viral RNA capping. Amino acid sequences in nsP2 and nsP4 show significant similarity to encoded sequences in all members of this virus supergroup (Ahlquist et al., 1985; Gorbalenya and Koonin, 1989; Koonin, 1991), and encode a putative RNA helicase and polymerase, respectively. The organization of these domains encoded in the genomes of the Sindbis-like plant viruses, and their involvment in viral RNA replication is outlined below. The domains, or core amino acids within a

Figure 3) Genome structure of Sindbis virus and the plant Sindbis-like viruses. Bold dark lines represent the genomic RNAs, boxes are genome open reading frames (ORFs) and the nature of the genomic RNA 3'-end is shown ("t" = tRNA-like structure, $(A)_n$ = polyA tail). AlMV does not have a tRNA-like structure, and the TRV tRNA-like structure does not become aminoacylated. Encoded domains conserved amongst the viruses suggestive of a methyltransferase activity (**m**), NTP-dependent helicase activity (▲), and RNA-dependent RNA polymerase activity (◆) are indicated by the shaded boxes, and the coat protein ORF for each virus is marked "cp". ORFs are labelled by nomenclature commonly used in the literature for each virus, parenthetic numbers refer to protein sizes after stop codon (open diamond) readthrough and open circles mark sites of polyprotein cleavage. Full virus names are shown in Table 1.

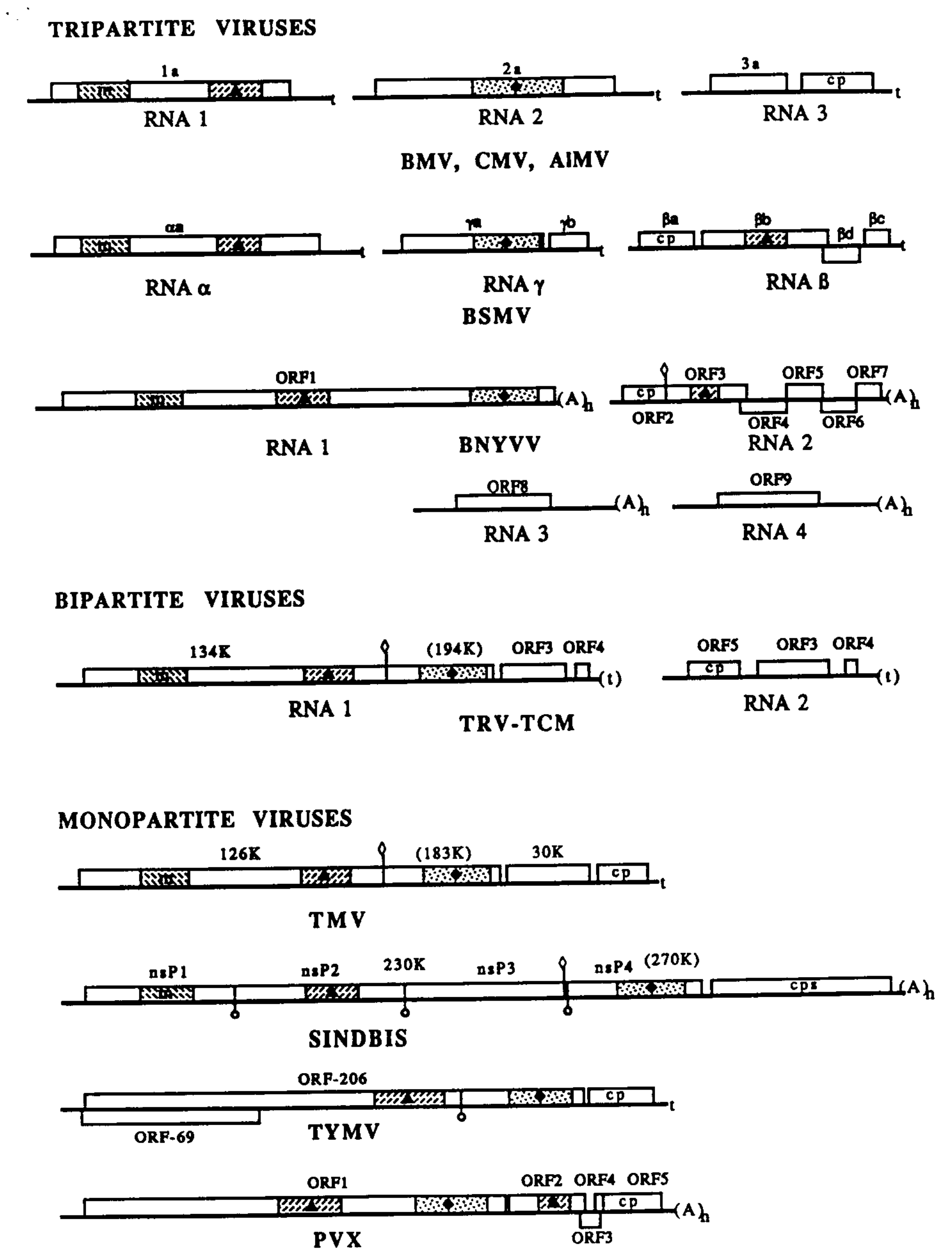

TRIPARTITE VIRUSES
1a
RNA 1
2a
RNA 2
3a
cp
RNA 3
BMV, CMV, AlMV
αa
RNA α
γa
γb
RNA γ
βa
βb
βc
βd
cp
RNA β
BSMV
ORF1
(A)n
RNA 1
BNYVV
ORF3
ORF5
ORF7
cp
ORF2
ORF4
ORF6
RNA 2
ORF8
RNA 3
ORF9
RNA 4
BIPARTITE VIRUSES
134K
(194K)
ORF3
ORF4
(t)
RNA 1
TRV-TCM
ORF5
ORF3
ORF4
cp
RNA 2
MONOPARTITE VIRUSES
126K
(183K)
30K
cp
TMV
nsP1
nsP2
230K
nsP3
nsP4
(270K)
cps
SINDBIS
ORF-206
cp
ORF-69
TYMV
ORF1
ORF2
ORF4
ORF5
cp
ORF3
PVX

Figure 3

domain, are hereafter referred to as the M-domain (methyltransferase domain) after that present on nsP1, the ..GXGKS/T.. (helicase) motif regarding that on nsP2, and the ..GDD.. (polymerase) motif for that on nsP4.

ii) Tobacco Mosaic Virus (TMV)

TMV is one of the best characterized of the plant viruses. The first evidence that an RNA molecule alone could induce a viral disease was demonstrated by inducing tobacco mosaic disease in tobacco plants via inoculation with purified TMV RNA (Gierer and Schramm, 1956). TMV virion structure, and its formation and disassembly has been extensively investigated (Fraenkel-Conrat and Williams, 1955; Wilson 1984; Matthews, 1991).

The common strain of TMV has an RNA genome of 6.4 kb. Four virus genes are encoded on the RNA in the following 5' to 3' order: 126K, 183K, 30K, and the virus coat protein gene (Goelet et al., 1982; Okada et al., 1987; Lehto and Dawson, 1990; Fig. 3). The 126K gene terminates with a UAG stop codon. Through suppression of this codon by a tyrosine-charged suppressor tRNA(Beier et al., 1984), readthrough synthesis occurs to the end of the ORF, thereby expressing the 183K gene (Pelham, 1978; Skuzeski et al., 1990). Expression of both the 30K and coat protein genes occurs via separate sgRNAs (Watanabe et al., 1984; Beck and Dawson, 1990).

Computer-generated comparisons between sequences encoded in the TMV 126/183K genes and those in other positive sense RNA viruses reveal homologies in three domains (Fig. 3). An N-terminal domain is homologous to the M-domain in Sindbis virus nsP1, the C-terminal domain on the 126K protein contains the ..GXGKS/T.. motif, and the readthrough 183K protein C-terminal sequences encode the ..GDD.. motif (Kamer and Argos, 1984; Ahlquist et al., 1985). Separation in TMV of the expression of the putative RNA polymerase gene from the 5'-end conserved domains by stop codon suppression has an organization similar to that in Sindbis virus (Fig. 3).

Deletion mutagenesis of infectious cDNA clones of TMV has demonstrated that sequences including the 30K and coat protein genes are dispensible for virus RNA amplification in protoplasts (Meshi et al., 1987; Takamatsu et al., 1987) and that sequences encoding the virus coat protein are not strictly required for the infection of plant leaves (Dawson et al, 1988). A reduced accumulation of RNA in infected protoplasts is observed for mutants with alterations in the coat protein gene (Ogawa et al., 1991). Mutations disrupting the expression of the 126/183K genes, however, have proven lethal to virus replication (Ishikawa et al, 1986; Meshi et al., 1987). In addition, the synthesis of viral RNA *in vivo* has been strongly correlated with the appearance of the 126/183K gene products (Watanabe et al., 1984; Young and Zaitlin, 1987; Ishikawa et al., 1991). The 126K gene product can bind radiolabelled purine nucleotide (preferably guanine) in infected cell extracts (Dunigan and Zaitlin, 1990), and may signify the involvment of this protein in the capping of the viral RNA.

The importance in virus replication of the UAG stop codon terminating the 126K gene has been examined. Replacement of the UAG codon with UAU specifying the insertion of tyrosine during translation of the 183K gene resulted in an RNA that was infectious in tobacco protoplasts, but at reduced levels as compared to infections with wild type transcript RNA (Ishikawa et al., 1986). Replacement of the UAG codon with UAA also resulted in infectious virus with reduced accumulation in protoplasts, but no frameshift mutants in this region were viable. The nature of the amino acid inserted during the suppression of this stop codon is not known. The accumulation of the 126K and 183K gene products *in vivo* has recently been analyzed in protoplast inoculations with both wild type and UAU mutant transcripts (Ishikawa et al., 1991). Using antiserum to the 126K gene product, both the 126/183K gene products were detectable in wild type infections, but only the 183K product was seen in UAU mutant infections. Although the total accumulation of RNA for infections with the UAU mutant was reduced, the data showed that ratios of plus to minus sense RNAs were similar in wild type or UAU mutant infections. This suggests that the 183K gene product can perform tasks of the 126K product in genome replication, albeit at reduced efficiency.

Defined ts mutations in the 126/183K genes have been shown to affect virus host range (Meshi et al., 1988) and sgRNA synthesis (Watanabe et al., 1987). No reported ts mutants in these genes have been shown to have specific effects on genome RNA replication. Mutants with temperature sensitivity for both double stranded and single stranded RNA synthesis (Dawson and White, 1978; Dawson and White, 1979) have yet to be mapped to specific regions of the genome.

iii) Turnip yellow mosaic virus (TYMV)

TYMV has a 6.3 kb RNA genome and is the type member of the tymoviruses. Specifics on the RNA and virion physical structure have been reviewed (see Section A).

The genome of TYMV encodes three major ORFs (Morch et al., 1988; Keese et al., 1989; Weiland and Dreher, 1989). Virus coat protein is encoded at the genome 3'-end and is expressed via an sgRNA (Pleij et al., 1976; Guilley and Briand, 1978). The first AUG codon at the 5'-end of the genome initiates a 1.9 kb ORF (ORF-69) and seven nucleotides 3' of this first AUG is a second out-of-frame AUG initiating a 5.5 kb ORF (ORF-206) (Briand et al., 1978; Keese et al., 1989). ORF-206 encodes both the ..GXGKS/T.. and the ..GDD.. motifs (Habili and Symons, 1989; Koonin, 1991) and its expression is essential for virus replication (Weiland and Dreher, 1989; Weiland and Dreher, in preparation; Chapter IV). Comparisons of amino acid sequences in ORF-206 with sequences encoded in other Sindbis-like viruses reveals a distant relationship between TYMV and potato virus X (Rozanov et al., 1989). ORF-69 expression appears to be dispensible for virus replication, but may instead be involved in virus movement within infected plants (Bozarth et al., in the press; Chapter III).

Protein p206 is the full-length ORF-206 expression product. Proteolysis during *in vitro* expression of ORF-206 by a virus encoded protease generates an N-terminal protein of Mr 150K and a C-terminal fragment of Mr 70K (Morch et al., 1982; Morch et al., 1989; Bransom et al., 1991). Although a ten amino acid sequence potentially harboring the cleavage site has been localized (Bransom et al., 1991), positioning of the virus-encoded protease within the 150K protein requires further mapping. Cysteine is a catalytic amino acid of the thiol proteases (Gorbalenya et al., 1991), and mutation of a cysteine codon to serine in the sequence encoding the 150K protein has recently been shown to abolish the processing of p206 to yield the 150K protein (K. Bransom, unpublished). This suggests that the cysteine residue that was mutated may be part of the catalytic center of the protease that processes p206.

The 150K protein is analogous to the TMV 126K gene product, possessing amino acid sequences found in purine NTPases (Morch et al., 1988; Habili and Symons, 1989). The protein has no apparent sequence homology, however, to the M-domain found in TMV and Sindbis virus, and potentially involved in viral RNA capping. A protein of Mr 150K which reacts with antiserum raised to a TYMV replicase preparation has been detected *in vivo* (Candresse et al., 1987).

The TYMV 70K protein harbors amino acid sequences similar to those in Sindbis virus nsP4 and is a candidate virus RNA polymerase (Morch et al., 1988; Bransom et al., 1991). The protein is unstable in cell-free translation extracts relative to other viral proteins (Bransom et al., 1991), but can be detected in infected cell extracts using antiserum specific for the protein (Connie S. Bozarth, unpublished). Instability of this protein in extracts may be due to targeted degradation by a cellular protease as seen for Sindbis virus nsP4 (deGroot et al., 1991). Whether the polymerase domain is functional in the 70K protein, or as a part of p206 is not known.

Reports investigating TYMV replication enzymology have suggested a simple subunit composition for the active replicase (Bove and Bove, 1985; Candresse et al., 1987; Quadt and Jaspars, 1989). Purified replicase prepared from TYMV infected tissue appears to contain only one major virus encoded polypeptide (Mr 115K) and one host polypeptide (Mr 45K), and the enzyme accepts TYMV RNA as template for the synthesis of acid precipitable product RNA (Mouches et al., 1984). Although the Mr 115K protein in these extracts appears to be of viral origin, the mechanism behind its expression from the genomic RNA is obscure. Use of antisera raised to regions of 150K and 70K proteins may ultimately reveal how the 115K protein is generated*in vivo.* Complementation experiments in which the 150K and 70K proteins are expressed from separate replicating RNAs might also help to determine the roles these proteins play in RNA replication (Weiland et al., in preparation; Chapter IV).

iv) Potato virus X (PVX)

The filamentous virus PVX has a 6.4 kb RNA genome. Although PVX is the type member of the potexviruses, studies on white clover mosaic virus (WClMV) and clover yellow mosaic virus (ClYMV) have provided much of the current knowledge regarding gene expression and replication in this family of viruses. Hence their contributions to the understanding of potexvirus replication will be considered where that for PVX is lacking.

Five genes are apparent from analysis of the nucleotide sequence of PVX (Skryabin et al., 1988; Fig. 3). A 4.4 kb ORF encodes protein sequences suggesting a role for the product in RNA replication, with the ..GXGKS/T.. and ..GDD.. motifs positioned centrally and C-terminally on the protein, respectively (Rozanov et al., 1990). The M-domain has not been detected on this protein. The 4.4kb ORF is followed by a block of three small ORFs which are overlapping, the middle of which is out-of-frame with the other two. Such an organization for these small ORFs has been found in the furovirus BNYVV and the hordeivirus BSMV, and has been termed the "triple gene block" (Morozov et al., 1989). Interestingly, ORF2 (the first ORF of of the triple gene block) encodes an additional ..GXGKS/T.. motif. The 3'-end ORF encodes the viral coat protein. Separate sgRNAs are involved in the expression of the triple gene block and the coat protein (Forster et al., 1987).

Cell-free translations of potexvirus RNA produce a large Mr 150K-190K protein that is presumably the product of the large 4-5 kb ORF (Bendena and Mackie, 1986). No evidence exists for virus-encoded proteolysis in the potexviruses, and no termination codon separates the conserved replication domains as in TMV. Only the apparent full length product of the long ORF in ClYMV was detectable in both rabbit reticulocyte translations and pea protoplasts (Brown and Wood, 1987).

Construction of infectious cDNA clones for PVX (Hemenway et al., 1990) and WClMV (Beck et al., 1990) has been reported, and has allowed for examination of the involvement of the triple gene block in virus infectivity. Mutations in the triple gene block abolished cell-to-cell movement of WClMV (Beck et al., 1991), but were tolerated for virus genome amplification in plant cells. Any involvement of the virus coat protein in genome amplification awaits further testing. By analogy to other Sindbis-like plant viruses, expression of the long ORF should be critical for productive infection by the virus.

2) Bipartite viruses.

i) Tobacco rattle virus (TRV).

TRV is the sole bipartite virus in the Sindbis supergroup of plant viruses. The 8.7 kb genome is divided onto two RNA chromosomes

(Cornelissen et al., 1986; Hamilton et al., 1987; Angenent et al., 1989). A 6.7 kb RNA (RNA 1) encodes a long ORF with amino acid sequences homologous to the three domains found on the TMV 183K gene (Fig. 3). Two smaller ORFs are encoded at the 3'-end of this RNA and are expressed by sgRNAs. RNA 2 encodes the virus coat protein and varies from 2.0 to 4.0 kb in size between different isolates of TRV. Size difference in these RNAs generally reflects a duplication of RNA 1 sequences on RNA 2 (Angenent et al., 1986). Subgenomic RNAs are reportedly involved in the expression of the 3'-end genes from RNA 1 (Guilford et al., 1989) and possibly the coat protein gene from RNA 2 (Robinson et al., 1983).

TRV RNA 1 is itself competent for infection of tobacco leaves, suggesting a dispensibility for coat protein sequences in both RNA replication and cell-to-cell movement of the virus (Hamilton and Baulcombe, 1989). Recent mutagenesis studies using infectious cDNA clones of TRV RNA 1 indicate that neither of the two 3'-end ORFs are required for RNA replication in protoplasts (Ziegler-Graff et al., 1991; Guilford et al., 1991). The ..GXGKS/T.. and ..GDD.. motifs encoded in the RNA 1 long ORF are separated by an amber stop codon in a manner similar to that in TMV, and suppression of the stop codon permits synthesis of the ..GDD.. -encoding domain (Pelham, 1979; Skuzeski et al., 1990). By analogy to TMV, expression of this interrupted ORF is required for TRV replication.

3) Tripartite viruses.

i) Alfalfa Mosaic Virus (AlMV).

The genome characteristics of AlMV, brome mosaic virus (BMV) and cucumber mosiac virus (CMV) are extremely similar (Dreher and Hall, 1988a; Matthews, 1991; Goldbach et al., 1991; Fig. 3). AlMV is a bacilliform virus and has a genome of 8.2 kb divided onto three RNAs of various size. RNA 1 encodes a protein bearing the M-domain and the ..GXGKS/T.. motif, and the single ORF on RNA 2 encodes the ..GDD.. signature sequence identifying it as the potential virus RNA polymerase gene. RNA 3 encodes two ORFs (Barker, et al., 1983), one encoding the coat protein in the 3' half of the RNA and a 5'-end ORF (the 32K gene) implicated in virus cell-to-cell movement (Stussi-Garaud et al., 1987). The 32K gene in AlMV is analogous to the 3a gene in

BMV and CMV (Fig. 3). The AlMV coat protein is expressed from an sgRNA (van der Kuyl, 1991).

AlMV RNA 1 and RNA 2 are necessary and sufficient to support viral RNA replication in isolated plant cells (Nassuth and Bol, 1983). Temperature sensitive mutants for RNA replication have been described which map to RNA 1 and RNA 2 (Sarachu et al., 1985). Although presence of the coat protein gene is not necessary for the replication of RNAs 1 and 2 to occur, there exists a requirement for coat protein in the RNA inoculum for infection to be initiated (Houwing and Jaspars, 1987). Until infectious cDNA clones of AlMV RNAs 1 and 2 are reported, accurate description of the nature of these ts mutants and involvement of the coat protein in RNA replication will remain obscure.

The replication of AlMV RNAs 1 and 2 in the absence of RNA 3 is characterized by reduced levels of plus sense RNA, as well as elevated levels of genomic minus sense RNA (Nassuth and Bol, 1983). Theoretically this altered balance of strand accumulation may be explained by decreased protection of the virus progeny RNA in the absence of the coat protein gene, and increased minus sense RNA resulting from the increased replication of unprotected template RNAs. Using an infectious clone of AlMV RNA 3, replication of RNA 3 derivatives has been examined in protoplasts from plants transgenic for the expression of RNA 1 and RNA 2 (Taschner et al., 1991; van der Kuyl et al., 1991a). By inoculation of such protoplasts, the involvement of the 32K and coat protein genes in the replication of RNA 3 has been investigated. Although the replication of coat protein mutants in this assay was reduced severely as compared to the 32K gene mutants, the results clearly indicate that sequences encoding the AlMV coat protein, and not the 32K gene, mediate the strand asymmetry observed during AlMV replication, and that levels of minus sense RNA actually increase in coat protein mutants (van der Kuyl, 1991b). Furthermore, a frameshift mutant removing the C-terminal 21 amino acids of the coat protein did not display the elevated minus sense RNA phenotype, suggesting that the phenotype of decreased plus sense RNA accumulation and increased minus sense RNA synthesis can be genetically separated.

Interestingly, plants transgenic for the expression of AlMV RNAs 1 and 2 can be infected by RNA 3 in the absence of added coat protein. This has led to the proposal that the role of the coat protein in the initiation of AlMV infection is to condition efficient RNA 1 and 2 gene expression (Taschner et al., 1991). Plants transgenic for the expression of either RNA 1 or RNA 2 also complemented inocula containing gel-purified RNAs 2 and 3, and RNAs 1 and 3, respectively.

AlMV replicase preparations from infected plants contain the protein products encoded on RNAs 1 and 2, and also contain virus coat protein (Quadt et al., 1991). Whether or not the coat protein is a contaminant of the preparation or is required for replicase activity is unknown. It may be that the coat protein plays a role in RNA replication beyond that of replication initiation, or by means other than stimulating the expression of RNAs 1 and 2.

ii) Brome mosaic virus.

BMV is an icosahedral virus with a gene organization highly similar to that of AlMV (Dreher and Hall, 1988a; Matthews, 1991). RNAs 1 and 2 encode proteins 1a and 2a, respectively, and RNA 3 encodes the genes for the 3a protein and coat protein in 5' to 3' order. RNA 1 coding sequences share similarities with the M-domain and the ..GXGKS/T.. stretch found in other Sindbis-like viruses (Ahlquist et al., 1985). RNA 2 encodes the putative virus RNA polymerase (Kamer and Argos, 1984; Koonin, 1991). The BMV coat protein is expressed from an sgRNA.

Evidence that RNAs 1 and 2 are sufficient for inducing virus replication in plant cells was first reported using gel purified viral RNA (Kiberstis et al., 1981) and later confirmed using infectious cDNA clones of BMV RNAs 1 and 2 (French et al., 1986). Using infectious clones of RNAs 1 and 2, defined lethal and conditional mutations have been made in the encoded 1a and 2a coding sequences, respectively (Kroner et al., 1989; Kroner et al., 1990). Upon temperature shift of inoculated protoplasts, ts mutations in the 1a protein led to cessation of all viral RNA synthesis, and mutants with separate changes in the M-domain and ..GXGKS/T.. domain could not complement each other in mixed RNA 1 inoculations. Coding and non-coding mutants of RNA 1 that are temperature sensitive for replication effect the accumulation of RNAs 2 and 3 suggesting that a wild type level of 1a protein in infections is critical for efficient BMV RNA replication.

Many RNA 2 mutants, on the other hand, displayed only moderate decreases in RNA synthesis after temperature shift (Kroner et al., 1989), and experiments in which RNA 2 was diluted or altered so as to be nonreplicative in protoplast inoculations revealed that low levels of RNA 2 can support high levels of RNA 1 and RNA 3 replication (Rao and Hall, 1990). One RNA 2 ts mutation affecting the 2a protein preferentially reduced positive versus negative strand RNA synthesis at the non-permissive temperature (Kroner et al., 1989). Results using a BMV 2a deletion mutant further suggest that domains in the 2a protein have regulatory as well as catalytic roles in BMV RNA replication. A defective RNA 2 expressing an N-terminal portion of the 2a protein appeared to inhibit in *trans* the replication of co-inoculated wild type RNA 2, but not RNA1 (Marsh et al., 1991a). From these experiments the evidence points to a more strict requirement for the wild-type expression of RNA 1 sequences than RNA 2 sequences in BMV infections.

The possibility that non-coding RNA domains may have *trans*-acting functions has also been investigated in BMV. Deletion mutants of RNA 2 that are nonreplicative in isolated protoplasts, but are quite stable in the cell, have been described. These nonreplicative deletion variants were co-inoculated with RNAs 1, 2, and 3. An inhibition of the replication of RNAs 1, 2 and 3 were seen in these inoculations suggesting that some region of the defective RNA was inhibiting RNA replication in*trans* (Marsh et al., 1991b).

In separate experiments, the effect of alterations in RNA 3 on the plus to minus sense RNA ratio was examined. Investigations have shown that in the absence of co-inoculated RNA 3, the ratio of plus to minus sense RNA 1 and 2 in infected cells is near unity (Marsh et al., 1991c), although separate work disputes this claim (French et al., 1986). Despite the fact that coat protein structural gene mutants reduced the accumulation of plus strands in infections with RNAs 1 and 2, and mutant RNA 3, not all of the plus strand RNA reduction that is observed when RNA 3 is absent was accounted for in these mutants. A point mutant that abolishes BMV sgRNA synthesis was tested, and was found to mimic the phenotype (plus vs. minus sense RNA ratio) of RNA 3 deficiency. How the sgRNA or the promoter region may function in the regulation of plus versus minus RNA synthesis is not known. The examples demonstrating apparent *trans*-acting functions for genome non-coding regions highlight the point that viral ORF products may represent only one type of *trans*-acting regulatory element involved in virus replication.

An extract from BMV-infected barley constituting a replicase preparation has been used extensively in the mapping of BMV promoters for replication and sgRNA transcription (Miller et al., 1985; Miller at al., 1986; Dreher and Hall, 1988b; Marsh et al., 1988). Proteins 1a and 2a are present in active replicase preparations, and antiserum raised to a peptide derived from protein 1a C-terminal sequences could inhibit replicase activity *in vitro* (Quadt et al., 1990). The specific roles of the two proteins in RNA replication, however, remain unknown.

iii) Cucumber mosaic virus (CMV)

CMV is a major disease problem throughout the world (Francki et al., 1985), but is suprisingly less well studied at molecular levels than AlMV and BMV. Similar to these viruses, CMV has a tripartite genome with RNAs 1 and 2 encoding potential replication proteins, and RNA 3 encoding a 3'-end coat protein gene and a 5'-end 0.9 kb ORF (Kataoka et al., 1990; Fig. 3). Protein 1a encoded on RNA 1 has both the M-domain and the ..GXGKS/T.. sequence, and the 2a gene on RNA 2 encodes sequences suggesting an RNA polymerase activity for the product. Like the coat proteins in BMV and AlMV, the CMV coat protein is expressed from a sgRNA (Schwinghammer and Symons, 1977).

Although studies on the replication of CMV have progressed more slowly than in BMV and AlMV, three laboratories have recently reported the construction of infectious CMV cDNA clones (Rizzo and Palukaitis, 1990; Hayes and Buck, 1990b; Suzuki et al., 1991). Like AlMV and BMV, CMV RNAs 1 and 2 can replicate independently in the absence of RNA 3 (Nitta et al., 1988). This has enabled the determination of sequences on RNA 3 that are dispensible for its replication in the presence of RNAs 1 and 2 (Suzuki et al., 1991; see below).

Remarkably, studies on CMV RNA replication enzymology have recently surpassed those for other eukaryotic RNA viruses. A preparation of CMV replicase has been described that is capable of accepting genomic CMV RNA and proceeding with all of the steps of virus replication *in vitro*, including progeny positive sense RNA and sgRNA synthesis (Hayes and Buck, 1990a). Only for the replicase of bacteriophage Qβ have these activities previously been observed (Blumenthal and Carmichael, 1979). Both viral proteins 1a and 2a, and a host protein of Mr 50K are present in the CMV replicase preparation. Should these proteins represent the sole constituents of a replicase with the described activities, studies clarifying the roles of the proteins in CMV RNA replication might advance rapidly.

iv) Barley stripe mosaic virus (BSMV).

The complex gene organization of BSMV sets this virus apart from the previously described tripartite Sindbis-like viruses (Goldbach et al., 1991; Jackson et al., 1991; Fig. 3). Three RNAs of the BSMV genome, α, ß and γ, are packaged into rod-shaped particles. RNA α encodes a polypeptide with the M-domain and ..GXGKS/T.. domains. RNA ß encodes, in 5' to 3' order, the virus coat protein and a triple gene block (Morozov et al., 1989), and the first ORF of the triple gene block encodes a second ..GXGKS/T.. motif. RNA γ encodes a 5'-end ORF with the ..GDD.. sequence and other conserved amino acids indicating a role for this gene as an RNA polymerase. The 3'-end ORF on this RNA is expressed from an sgRNA (Petty et al., 1990b).

Experiments using infectious cDNA clones of BSMV have led to the determination of genes strictly required for virus replication (Petty et al., 1990b). Replication of RNAs α and γ can occur in isolated plant cells without the presence of RNA ß. Expression of both the RNA αa gene encoding the ..GXGKS/T.. motif, and the γa gene product bearing the ..GDD.. motif is necessary to support virus replication, analogous to the gene requirement in the other tripartite viruses. Interestingly, the short RNA γ 3'-end ORF appears to encode a factor modulating the replication of RNAs α and ß; disruption of this gene led the to decreased accumulation of RNAs α and ß relative to RNA γ in infections of barley plants (Petty et al., 1990b).

v) Beet necrotic yellow vein virus (BNYVV).

As well as having a cumbersome name, BNYVV displays a complex gene arrangement and gene requirement for its propagation (Bouzoubaa, et al., 1987; Brunt and Richards, 1989; Fig. 3). Natural isolates of BNYVV have four genomic RNAs. RNA 1 encodes an ORF for a potential 237K polypeptide whose sequences show, in 5' to 3' order, similarity to the M-domain, the

..GXGKS/T.. sequence and the ..GDD.. motif found in other Sindbis-like viruses (Goldbach et al., 1991). Encoded in the 5'-end of RNA 2 is the virus coat protein gene. If the stop codon terminating the coat protein gene is suppressed during translation, an Mr 95K product can be synthesized (Ziegler et al., 1985). Analysis of the coding in the middle of RNA 2 reveals a triple gene block found only in filamentous virus members of the Sindbis supergroup (Morozov et al., 1989). Finally, a short ORF is encoded at the 3'-end of the RNA. RNAs 3 and 4 are associated with fungal transmission of the virus, and appear to encode one polypeptide each (Lemaire et al., 1988; Brunt and Richards, 1989).

Experiments with infectious cDNA clones of BNYVV have shown that RNAs 1 and 2 are necessary and sufficient to generate systemic infections in inoculated plants (Quillet et al., 1989; Jupin et al., 1990a). Although sequences encoded in RNA 1 suggest that this RNA alone should be sufficient for infection of protoplasts, no report of this has appeared. The uninterrupted nature of the domains encoded on RNA 1 and conserved in the Sindbis-like viruses is unusual. Members of the supergroup that encode all three of these domains tend to separate them by proteolysis, by a suppressible termination codon or by physical separation of the ORFs encoding these domains onto individual genomic RNAs. The availability of infectious clones of the virus will doubtless aid in delineating the domains required to support infectivity of the virus. Antisera raised to domains of the RNA 1 encoded product should help to determine whether processing of the polypeptide occurs *in vivo*, as none apparently occurs *in vitro* (Jupin et al., 1988).

3) *cis*-Acting sequences in replication of Sindbis virus and related plant viruses.

Introduction.

Cis-acting genetic elements are those operative on the DNA or RNA template in which they are encoded (Lewin, 1991). An example of a *cis*-acting genetic element is a transcription promoter. In theory, a transcription promoter affects only the gene to which it is physically linked. Hence, it does not affect in *trans* the expression of a separate gene.

Origins of replication are *cis*-acting replicative elements of cellular and viral DNA chromosomes (Adams et al., 1986). Because the model for the replication of linear RNA viruses is similar to the act of DNA transcription, the term "promoter" has been chosen to define the *cis*-acting regions required for the initiation of viral RNA replication (Dreher and Hall, 1988a). In the Sindbis virus superfamily, "promoter" is also used to define sequences required for the initiation of sgRNA synthesis.

For many of the Sindbis-like plant viruses, sequences necessary for the recognition and replication of genomic RNAs have been defined by studying the replicative potential of deletion derivatives of genomic RNAs in the presence of active replication complexes (French and Ahlquist, 1987; Ogawa et al., 1991; Marsh et al., 1991a; van der Kuyl et al., 1991b). In addition, necessary *cis*-acting sequences required for efficient replication in RNA viruses can be deduced from the structure of defective interfering RNAs (DIs). DIs are deletion mosaics of a virus genome which are efficiently co-replicated and often co-packaged in the presence of helper virus (Levis et al., 1986; Holland 1990).

Current knowledge of *cis*-acting genetic elements involved in the replication and subgenomic RNA transcription of Sindbis virus and Sindbis-like plant viruses is reviewed in the following sections. Experiments defining *cis*-acting replication elements based on studies with DIs are included where available. Because satellite RNAs often have no sequences in common with the helper virus (Matthews, 1991), they are not included in this text.

Monopartite viruses

i) Sindbis virus.

Sindbis virus genomic RNA has a 3'-end polyA tail following a block of 19 nucleotides that is highly conserved among alphavirus genomes. The polyA tail is also present on the Sindbis sgRNA. Information on the important *cis*-acting sequences involved in Sindbis virus RNA replication has been gleaned from investigations on the replication of Sindbis virus DIs (Monroe and Schlessinger, 1983; Levis et al., 1986), from computer generated nucleotide sequence comparisons within the alphaviruses (Ou et al., 1983), and from mutagenesis studies using infectious cDNA clones of Sindbis virus (Kuhn et al., 1990; Niesters and Strauss, 1990). The sequences of the DIs examined show the retention of blocks of nucleotides highly conserved between alphavirus genomes (Ou et al., 1983)

It has been demonstrated that transcripts of a cloned Sindbis virus DI can be co-inoculated with standard virus and accumulation of the DI throughout the infection monitored by standard methods (Levis et al., 1986). By combining the co-inoculation studies with deletion mutagenesis of a cloned DI, it was found that only 19 of the 3'-end terminal nucleotides were required for the replication of the DI RNA molecule (Levis et al., 1986). These 19 nts are highly conserved within the genomic RNAs of the alphaviruses (Strauss and Strauss, 1988). Point substitutions placed in the 3' end 19 nucleotide region of infectious Sindbis virus transcripts revealed scattered positions in the block that are important for virus replication, and showed differential replication phenotypes between mosquito and chick cell monolayers (Kuhn et al., 1990).

Although all Sindbis virus DIs retain a 5' 51 nucleotide stretch highly conserved amongst the alphaviruses, some isolates also possess cellular tRNA sequences at their 5'-terminus (Monroe and Schlesinger, 1983). These sequences can be replaced by bona fide Sindbis virus 5' end sequences without a significant decrease in DI replication (Strauss and Strauss, 1988). Deletion analysis of a cloned DI showed that only 162 nucleotides from the 5' end of the molecule were required for efficient replication of the RNA to occur (Levis et al., 1986). That the 5'-terminal tRNA sequences are nonhomologous to the Sindbis virus genome suggests that RNA secondary or tertiary structure is more important than primary nucleotide sequence in determining an active substrate for replication. Mutagenesis studies on the 51 nucleotide block in an infectious clone of Sindbis virus showed varying infection phenotypes in mosquito versus chick cells, a phenotype similar to that seen for 3' end substitution mutants of the virus (Niesters and Strauss, 1990). Substitutions were designed to disrupt hairpin structures predicted by the sequence in the block. Most mutants constructed were viable and showed wild type plaque morphologies, but virus accumulation was variable and not correlated with plaque size. Temperature shift experiments were not performed in this study and may reveal phenotypes not uncovered at the optimal incubation temperature.

Sindbis virus structural proteins are synthesized from a 4,106 nucleotide sgRNA (Rice and Strauss, 1981). Recent studies have been undertaken to define the necessary elements in the genomic RNA which allow for the efficient synthesis of the sgRNA. Using the standard notation of +1 for the first nucleotide of the sgRNA transcript, investigators found that sequences between -19 and +5 were necessary and sufficient to promote sgRNA synthesis from a Sindbis DI vector RNA *in vivo* (Levis et al., 1990). The result is consistent with computer-generated alignments between several alphavirus genomes, showing a block of nucleotides conserved within the alphaviruses between -19 and +2 with respect to the sgRNA start site (Ou et al., 1982).

ii) TMV

The genomic RNA of TMV has a 3'-end non-coding region that exhibits tRNA-like properties (Rietveld et al., 1984), and a 5'-end non-coding region that functions as a strong translational leader sequence (Gallie et al., 1987). Only recently have *cis*-acting sequences involved in TMV replication and sgRNA synthesis been analyzed. Thus, experiments have been reported in which large deletions removing most of the 126/183K genes have been made, and the defective RNAs successfully replicated by helper virus (Raffo and Dawson, 1991; Ogawa et al., 1991). Co-inoculations of deletion derivatives of TMV with helper virus in protoplasts have shown that the 5'-end 206 nucleotides are sufficient to allow replication of the defective replicon by helper virus (Ogawa et al., 1991). Mutations in the TMV genomic RNA leader sequence have also defined nucleotides important for virus replication (Takamatsu et al., 1991), although the possibility that a deleterious effect on 126/183K gene expression affected the observed accumulation of the mutant RNAs can not be ruled out. Coat protein and 30K gene deletion mutants have previously been shown to be infectious in protoplasts, including a construct retaining only 196 3'-end nucleotides (Takamatsu et al., 1987). By inference from the two studies, it may be postulated that these 5' and 3' sequences are necessary and sufficient for RNA recognition and replication in the presence of helper virus. The actual construction and testing of such a molecule, however, has not been reported.

The existence of RNA pseudoknot structures has been a recent discovery originating with work on plant RNA viruses (Pleij et al., 1985). The structure is characteristic of regions within, and immediately 5' to, the 3'-end tRNA-like structures present in several plant RNA virus genomes (Dreher and Hall, 1988a; Mans et al., 1991). Pseudoknots have been found in association with RNA coding regions which promote translational frameshifting (Wills et al., 1991), and in RNA non-coding regions which interact with specific RNA binding proteins (Guttel and Woese, 1990). Five pseudoknots are predicted from the sequence of the TMV common strain (van Belkum et al., 1985). Using site directed mutagenesis of an infectious TMV cDNA clone, the pseudoknot located nearest the 3'-end tRNA-like structure was found to be essential for virus replication (Takamatsu et al., 1990).

Subgenomic RNAs are synthesized in TMV infections, allowing for the efficient expression of the 30K and coat protein genes (Dawson and Lehto, 1990). Interestingly, no clear conservation of nucleotide sequence exists between regions immediately 5' of the 30K and coat protein sgRNA start sites. Experiments attempting to delimit the coat protein sgRNA promoter regions have been performed. Deletions to within 96 nucleotides of the coat protein initiation codon from the 5' side still permitted the transcription of sgRNA but at a lower rate than wild type (Meshi et al., 1987).

Recent data have shown that sequence blocks harboring the subgenomic promoters for both the coat protein and 30K genes can be independently repositioned in the genome and still retain activity (Lehto et al., 1990). A 235 nucleotide fragment including the start site of the coat protein mRNA was sufficient to direct sgRNA synthesis when substituted for sequences in the 30K gene. The wild type 30K gene sgRNA is not capped, unlike the genomic and coat protein sgRNA, and it has been proposed that this difference is related to the dissimilarity in the putative 30K and coat protein gene promoters (Watanabe et al., 1987). Curiously, replacement of the 30K gene promoter with the 253nt sequence block containing the coat protein gene promoter resulted in the synthesis of apparently un-capped 30K sgRNA (Lehto et al., 1990). This suggests an unusual dependance of promoter sequences on position in the genome in determining the nature of the sgRNA 5'-end terminal structure.

iii) TYMV

The 3'-end of the TYMV RNA genome can fold into a tRNA-like structure (Mans et al., 1991), can be charged with valine *in vitro* (Pinck et al., 1970) and *in vivo* (Joshi et al., 1982a), and may encompass promoter sequences involved in the initiation of TYMV RNA replication (Tsai and Dreher, 1991; Morch et al., 1987). Although 3' end fragments of TYMV RNA derived from cloned cDNA are acceptable template for TYMV RNA replicase *in vitro* (Morch et al., 1987), the mapping of minimal promoter elements involved in viral minus sense RNA synthesis has not been reported.

With the construction of an infectious cDNA clone of TYMV RNA (Weiland and Dreher, 1989), an initial analysis of *cis*-acting promoter elements involved in TYMV RNA replication and sgRNA synthesis has begun (Tsai and Dreher, 1991; Weiland and Dreher, manuscript in preparation, Chapter IV). The valylation of the TYMV RNA 3'-end has been shown to require specific nucleotides in the anticodon loop of the tRNA-like structure (Florentz et al., 1991; Tsai and Dreher, 1991). In contrast with results obtained in similar studies with BMV (Dreher et al, 1990), the ability to valylate 3'-end mutant RNA transcripts of TYMV is positively correlated with the replication of infectious RNA transcripts bearing the same mutations (Tsai and Dreher, 1991). The monopartite nature of TYMV may prove advantageous in such studies, as recombination between mutant and wild type genomic RNAs in multipartite viruses like BMV can potentially obscure conclusions drawn from quantitative promoter studies *in vivo* (Bujarski et al., 1986).

Computer generated comparisons between sequenced tymoviruses have revealed a highly conserved core nucleotide sequence near the start site of tymovirus sgRNA transcription. The core region, called the "tymobox" is proposed to be involved in sgRNA transcription, and is the most highly conserved nucleotide block in these viruses (Ding et al., 1990b). This high conservation has allowed for the specific detection of tymoviruses in infected plant extracts using oligonucleotide probes specific for the sequence. Whether the tymobox in itself constitutes the TYMV sgRNA promoter remains to be tested. Although a report postulating a mechanism for TYMV sgRNA synthesis has appeared, no functional delineation of the nucleotide sequences required for the event was presented (Gargouri et al., 1989).

iv) PVX

PVX and the closely related viruses, WClMV and ClYMV have 3'-end polyA tails. Using infectious cloned cDNA of WClMV (Beck et al., 1990) it has been demonstrated that the presence in the RNA of a putative sequence directing the addition of a polyA tail is important both for polyA tail length and for RNA replication (Guilford et al., 1991). Further disruptions in the triple gene block near the 3'-end of the RNA pointed to sequences in this region dispensible for RNA replication in isolated plant cells (Beck et al., 1991).

The discovery in ClYMV infected tissue of short defective RNAs derived from viral genome sequences is an important step in defining the promoter sequences involved in the replication of this virus (White et al., 1991). All defective RNAs cloned and sequenced had fusions between the 5'-end terminal ~700 nts and the 3'-end terminal ~400 nts of ClYMV genomic RNA. The defective RNAs also fused in-frame the 5'-end of ORF1 and the 3'-end of the coat protein ORF. Additional work should eventually delimit regions on the defective RNAs essential for their co-replication by ClYMV helper virus, and will determine whether the ORF1/coat protein gene fusion is required for defective RNA replication.

Bipartite viruses

i) TRV

The 3'-end of TRV folds into a tRNA-like structure, but is not aminoacylated as seen for TMV, TYMV, and several tripartite viruses (van Belkum et al., 1987b). By comparing the nucleotide sequences of TRV RNAs 1 and 2, scattered homologies between the two are apparent (Angenent et al., 1986). These are proposed to be important *cis* elements for the replication of TRV.

Using infectious cDNA clones of TRV, deletion mutagenesis has been performed on RNA 2, and the competence for replication of these molecules in the presence of RNA 1 tested (Angenent et al., 1989). The results showed a requirement for the 5'-end 340 nucleotides and the 3'-end 405 nucleotides for the replication of TRV RNA 2 to occur. No analysis of TRV sgRNA promoters has been reported.

Tripartite viruses

i) AlMV

Although the replication of AlMV has been extensively studied in the recent past, the construction of infectious cDNA clones of AlMV RNAs 1 and 2 remains elusive (van der Kuyl, 1991b). Nonetheless, studies using tobacco plants transgenic for the expression of RNA 1, RNA 2 and both RNAs 1 and 2 have allowed for elegant complementation analysis in this virus to be performed (Taschner et al., 1991).

Initial experiments suggested that the presence of coat protein was required for AlMV RNA to be infectious, and that a region of secondary structure at the 3' end of all three AlMV RNAs was the site of coat protein binding (Houwing and Jaspars, 1987). The engineering of tobacco plants expressing both AlMV RNAs 1 and 2 (P12 plants) has demonstrated that AlMV coat protein is not strictly required for the initiation of RNA 3 replication in protoplasts and plants (van der Kuyl et al., 1991a, van der Kuyl, 1991b). This has led to experiments in which the replication competence of RNA 3 deletion mutants was tested.

Using infectious cDNA of AlMV RNA 3, sequences within the 5'- end 169 nucleotides were shown to be required for the amplification of this RNA in protoplasts prepared from P12 plants (van der Kuyl et al., 1991a). Interestingly, 3'-end terminal sequences were not strictly required for RNA 3 amplification in these assays, although the accumulation of these mutant RNAs was severely reduced as compared to wild type. The result was consistent with data from *in vitro* experiments in which AlMV replicase preparations were programed with mutant RNAs bearing 3' terminal deletions (van der Kuyl et al., 1990).

Deletions in the 32K gene (Fig 3) did not prevent RNA 3 replication, but mutations reducing sgRNA synthesis or otherwise disrupting coat protein expression replicated to levels 100 fold below wild type. Using an *in vitro* replication system, the sgRNA promoter was mapped to between -55 and -8 nucleotides relative to the start site of sgRNA transcription (van der Kuyl et al., 1990), but results from protoplast inoculations showed that additional elements in the sgRNA leader region were important for efficient transcription (van der Kuyl et al., 1991a). The definition of a 5' border to the 3'-end terminal sequences required for RNA 3 replication was not reported.

ii) BMV

The 3'-end terminal 200 nucleotides of the three BMV genome RNAs are nearly identical (Ahlquist et al., 1981), include a subset of terminal sequences exhibiting tRNA-like properties (Loesch-Fries and Hall, 1982; Dreher and Hall, 1988a,b), and encompasses the promoter for the initiation of viral RNA replication *in vitro* (Bujarski et al., 1985; Dreher and Hall, 1988b) and *in vivo* (Dreher et al., 1989; Rao et al., 1990). Using infectious cDNA clones of BMV, the *cis* acting elements involved in the replication of BMV RNA 3 have been examined by deletion analysis (French and Ahlquist, 1987), as well as by point mutantional analysis in the tRNA-like structure (Dreher et al., 1989). Mapping of the promoter regions required for the initiation of sgRNA synthesis *in vitro* and *in vivo* has also been described (Marsh et al., 1988; French et al., 1988).

Internal deletion analysis of BMV RNA 3 has indicated that at least 117 5'-end nucleotides and at least 200 3'-end nucleotides are required for the efficient amplification of this molecule in the presence of RNAs 1 and 2 (French and Ahlquist, 1987). In addition, a stretch of 150 nucleotides present between the two ORFs encoded on RNA 3 is required, suggesting a role for this sequence in either template conformation or recognition of the template by the viral replicase. This intergenic sequence shows homology to nucleotides near the 5'-ends of all bromoviral RNAs (French and Ahlquist, 1987). Curiously, this sequence in CCMV, a related bromovirus, is located near the 5'-end of RNA 3, and deletion of the intergenic region does not interfere with the replication of CCMV RNA 3 deletion mutants (Pacha et al., 1990).

Substitutions and small deletions have been placed into the BMV RNA3 tRNA-like structure and the effects of the changes tested in cell-free aminoacylation and replication assays (Dreher et al., 1984; Bujarski et al., 1985; Dreher and Hall, 1988b), and in protoplast inoculations with RNAs 1 and 2 (Dreher et al., 1989; Rao and Hall, 1990). In general, both stem and loop regions exist in the tRNA-like structure which, if altered, reduce replication below 50% of wild type. In addition, regions within the structure were isolated that affected distinct functions; thus some aminoacylation deficient mutants were competent for replication *in vitro* and *in vivo*, and certain replication deficient mutants could tyrosylate efficiently *in vitro* (Hall et al., 1990). Overall the evidence from these studies indicated that efficient tyrosylation *in-vitro* is not required for efficient RNA replication in protoplasts. Finally, evidence supports a telomeric role for tRNA-like structures in plant viral RNAs (Rao et al., 1989). Since tRNA nucleotidyl transferase is capable of repairing lost nucleotides to the ..CCA terminus of viral tRNA-like structures, the activity is proposed to lend structural integrity to the RNA chromosomes *in vivo*.

The 5'-ends of many Sindbis-like plant virus RNAs contain nucleotide sequences reminiscent of those found in tRNA genes (Marsh and Hall, 1987; Marsh et al., 1989). Since tRNA genes possess transcription promoters nested within the tRNA structural gene sequence, the promoter elements have been dubbed internal control regions (ICRs) (Hall et al., 1990). Using a deletion mutant of BMV RNA 2, mutations in the ICR boxes present in the 5' end of the RNA were shown to debilitate the co-replication of this mutant in the presence of BMV RNAs 1 and 2 (Pogue et al., 1990). In what form of genome RNA (plus sense, minus sense, double stranded RNA) the ICRs might be active in is currently unknown, although it has been argued that a specific secondary structure may be important in plus sense RNA synthesis (Pogue and Hall, 1992). The work also demonstrated that sequences encoding the C-terminus of the 2a protein were dispensible for the replication of RNA 2 (Pogue et al., 1990; Marsh et al., 1991a).

BMV RNA 3 deletions were used to analyze sequences important in sgRNA production. In a cell-free transcription assay, the complete sgRNA promoter was mapped to 62 nucleotides (-50 to +12) (Marsh et al., 1988). Characterization of the sequences required for sgRNA synthesis *in vivo* suggested an extension of this promoter to -74 (French et al., 1988). Since the transcription of sgRNA in BMV likely occurs via the internal initiation of transcription on single stranded minus sense RNA 3 (Miller et al., 1985), it should be noted that conclusions regarding the sgRNA/RNA 3 ratio from inoculation studies did not take into account potential differences in the levels of minus sense RNA 3 for the mutants described.

iii) CMV

The 3'-ends of the CMV genome RNAs can be tyrosylated *in vitro* (Kohl and Hall, 1974), and can assume a secondary structure similar to that in BMV (Ahlquist et al., 1981). Only recently has the construction of infectious cDNA clones of CMV allowed for an examination of *cis* acting sequences involved in CMV RNA replication (Hayes and Buck, 1990b; Rizzo and Palukaitis, 1990; Suzuki et al., 1991).

Infectious transcripts of CMV RNA 3 have been used to map *cis* acting regions in this RNA that are important for replication (Suzuki et al., 1991). Deletions on RNA 3 to within ~130 nucleotides from the 5'-end and ~350 nucleotides of the 3'-end of the RNA were tolerated for the replication of the mutant RNAs, although deletions in the coat protein gene (Fig. 3) led to drastic reductions in plus sense RNA accumulation. The importance of the intergenic region in replication was not examined in this study. The authors propose that the reduced accumulation of RNA 3 coat protein mutants is due to the lack of RNA protection normally afforded by the coat protein.

iv) BSMV

The BSMV RNA 3'-end can be tyrosylated *in vitro* (Agranovsky et al., 1981) and *in vivo* (Loesch-Fries and Hall, 1982), and can potentially fold into a tRNA-like structure similar to that found in BMV (Kozlov et al., 1984; Fig 3). Since RNA ß is dependent on RNAs α and γ for replication, experiments have recently been performed to examine the involvement of RNA ß genes in BSMV replication and pathogenesis. Unfortunately, experiments using deletion mutants of RNA ß were only performed with whole barley, tobacco and *Chenopodium* plants (Petty and Jackson, 1990a). Since RNA ß genes b, c and d are required for infectivity of BSMV on plants, the involvement of *cis* acting regions on RNA ß in RNA replication alone could not be determined in these experiments. Coat protein deletions in RNA ß, however, were competent for systemic infection of barley plants in the presence of RNAs α and γ, suggesting that encapsidation of BSMV RNAs is not required for systemic translocation of the virus.

Inoculations of barley plants with complementing RNA γ deletion mutants have indirectly led to the determination of sequences dispensible in the replication of RNA γ (Petty et al., 1990). Greater than 80% of the γb gene and ~60% of the γa gene can be deleted, and replicated mutant RNA recovered from infected barley plants. The inoculation of protoplasts with BSMV mutant RNAs should further the knowledge of RNA regions required for replication, as studies in whole plant infections can obscure factors affecting replication versus virus movement.

v) BNYVV

BNYVV RNAs posses polyA tails and lack the tRNA-like structures found at the 3'-ends of most multipartite positive strand plant RNA viruses (Brunt and Richards, 1989). Although the gene organization in BNYVV suggests that co-replication studies should be possible using only RNA 1 as a helper virus RNA, no report of independent RNA 1 replication in protoplasts has been issued.

Manipulation of infectious cloned cDNA of BNYVV RNA 3 has allowed investigators to determine the minimum 5'-end and 3'-end regions required for the amplification of this RNA in the presence of co-inoculated RNAs 1 and 2 in plants (Jupin et al., 1990a). Deletion analysis showed that only 73 3'-end nucleotides and 300 5'-end nucleotides were required for replication of this RNA to occur. The deletions described originated from the center of the RNA, and it is not known if an RNA bearing only the 3' 73 nucleotides and 5' 300 nucleotides is competent for replication. It has further been shown that RNA 3 transcripts lacking a polyA tail can be replicated, but only after reacquisition of the tail *in vivo* (Jupin et al., 1990b).

CHAPTER I

Infectious TYMV RNA from cloned cDNA: Effects *in vitro* and *in vivo* of point substitutions in the initiation codons of two extensively overlapping ORFs.

ABSTRACT

Full-length cDNA of the 6.3 kb turnip yellow mosaic virus (TYMV) genome was placed between a T7 promoter and a unique Hind III site. *In vitro* transcription of Hind III-linearized DNA of clone pTYMC yielded full-length RNA transcripts. In inoculations of Chinese cabbage protoplasts and plants, capped transcripts and virion RNA had similar specific infectivities and produced similar systemic symptoms. We have used the pTYMC clone in studies of the expression of two overlapping open reading frames (1.9 kb and 5.5 kb ORFs) by making mutants with alterations in the initiation codons. Evidence is presented from *in vitro* translations of mutant and wild type RNAs that both ORFs are expressed from TYMV RNA. A mutant in the initiation codon of the 5.5 kb ORF did not replicate in protoplasts, while mutants in the initiation codon of the 1.9 kb ORF replicated at low levels. The two groups of mutants were not able to complement each other.

INTRODUCTION

Turnip yellow mosaic virus (TYMV) is a small icosahedral virus with a plus-sense RNA genome that infects members of the Cruciferae (Matthews, 1980). The c. 6.3 kb genomic RNA possesses a 5' ^{7}mGpppG cap structure and a 3' end exhibiting several tRNA-like properties. TYMV has been the subject of many studies investigating the cytology of infection (Matthews, 1981; Garnier et al., 1986), the enzymology of replication (Mouches et al., 1974; Candresse et al., 1986), and the properties of the tRNA-like structure (Rietveld et al., 1982; Dumas et al., 1987). The mode of genome expression remains poorly understood, although *in vitro* translation studies have suggested proteolytic maturation of primary translation products (reviewed in Haenni et al., 1987). In order to fully exploit this accumulated information for further studies on viral replication, an important priority is the establishment of cDNA clones from which infectious transcripts can be generated. This approach has proven useful for a number of positive strand RNA viruses, including brome mosaic virus (Ahlquist et al., 1984; Dreher et al., 1989), tobacco mosaic virus (Dawson et al., 1986; Meshi et al., 1986), poliovirus (ven der Werf et al., 1986), Sindbis virus (Rice et al., 1987), and bacteriophage Qß (Shaklee et al., 1988). We report here the isolation of a cDNA clone, representing the entire TYMV genome placed under the control of the bacteriophage T7 promoter, from which capped, infectious RNA transcripts can be made *in vitro*.

We also report the application of this cDNA clone to further investigations on the expression of the nonstructural genes of TYMV. Previous translational studies have resulted in the assignment of all nonstructural gene products to a c. 5.5 kb open reading frame (ORF) (Haenni et al., 1987; Morch et al., 1982b). However, the recent completion of the nucleotide sequence of the genomic RNA has revealed the existence of an ORF of 1884 nt capable of encoding a 69 kd protein, beginning at nucleotide 88 (ORF-69). This ORF extensively overlaps an ORF of 5532 nt encoding a 206 kd protein (ORF-206) beginning at nucleotide 95 in a different reading frame (Morch et al., 1988). The codon usage of ORF-69 suggests that this previously unrecognized ORF may indeed be expressed (Morch et al., 1988), although an early preliminary study had suggested that ribosomes predominantly initiate at AUG-95 (Briand et al., 1978). If initiation occurs at the AUGs of both of the extensively overlapping ORFs, TYMV RNA represents an unusual eukaryotic messenger. Only a few similar examples have been reported from animal viruses (Valle and Morch, 1988; Curran and Kolakofsky, 1988). We report studies that demonstrate that both overlapping ORFs of TYMV RNA are expressed in cell-free translations. We also describe initial experiments on the consequences, *in vitro* and *in vivo*, of mutations in the initiation codon of each of these ORFs.

MATERIALS AND METHODS

Materials

TYMV, type strain, obtained from Dr. C. Florentz (Strasbourg, France) had been multiply passaged through turnips (*Brassica rapa*). Virus was prepared from fresh leaf tissue according to Lane (1986) and viral RNA was purified from virions by double phenol extraction. Chinese cabbage (*Brassica pekinensis* cv. Wong Bok) plants were grown in a growth chamber under 16 hr day-length at 20°C. Reverse transcriptase was purchased from U.S. Biochemicals, T7 RNA polymerase from BRL, InhibitAce ribonuclease inhibitor from 5 Prime-3 Prime, Inc. (West Chester, PA, USA), and m^{7}GpppG cap analogue from New England Biolabs. Macerase™ and Cellulysin™ were from Calbiochem, and PEG-8000 from Baker Chemical Co. Synthetic deoxyoligonucleotides were purified on 20% urea-polyacrylamide gels.

cDNA synthesis and cloning

First strand cDNA synthesis was primed with a 5'-phosphorylated synthetic deoxyoligonucleotide [d(CTGGTTCCGATGACCCTCG)], which was complementary to the 3' end of virion RNA (Guilley and Briand, 1978). A short 5' extension was designed to create a BstN I restriction site at the 3' end of the viral sequence, after ligation of the cDNA to a Sma I-cut vector. The coincidence of the BstN I cleavage position with the 3' end of the TYMV cDNA would facilitate any future reengineering of the sequence at this position. Virion RNA (20 µg) was reverse transcribed in a reaction containing low specific activity [α-^{32}P]dATP, a 13-fold molar excess of priming oligomer, 1 unit/µl ribonuclease inhibitor, and 1 u/µl of reverse transcriptase for 2 hr at 42°C (Murray et al., 1983). Full length DNA/RNA duplexes were purified via native low melting point agarose gel electrophoresis, and the RNA strand was removed by ribonuclease A treatment.

Second strand cDNA synthesis was specifically primed at the 3' end of full-length first strands with the phosphorylated oligomer [d(AATTAATACGACTCACTATA-GTAATCAACTACCAATTCC)]. This primer comprised the class III T7 promoter sequence upstream of the initiation site (Dunn and Studier, 1983) adjacent to the 5' sequence of TYMV genomic RNA (Briand et al., 1978; see Fig. I.1). Second strand was made using the Klenow fragment of DNA polymerase I, with a subsequent incubation with T4 DNA polymerase to ensure the presence of blunt ends. Double stranded full-length TYMV cDNA was ligated to Sma I-linearized vector pUC8, and used to transform competent *E. coli* DH-5α cells. Three full-length cDNA clones with about 6.5 kb inserts were selected for further study and shown by dideoxy sequencing (Chen and Seeburg, 1985) to have correct 5' and 3' non-coding sequences (clones pT7YH1, -I2, -1A), except that each clone had a C in place of U at position 10 (Briand et al., 1978; Guilley and Briand, 1978). This substitution appeared to have been accidentally introduced by the second strand primer (McClain et al., 1986), since only U was detected at position 10 of the virion RNA (not shown). All three clones were inserted in the (+) orientation in the vector, with the BamH I site of the pUC8 polylinker positioned adjacent to the 3' end of the viral cDNA.

In vitro mutagenesis and 3'end fragment replacement

A Hind III site (absent from the TYMV cDNA) was positioned at the 3' -CCA terminus (Fig. 1) of each of the clones. This site was initially introduced into a cDNA clone of the 3' 258 nucleotides of the TYMV genome (mpTYSma) (Dreher et al., 1988) by mutagenesis directed by the oligomer d(TTCGAGCTCAAGCTTGGTTCCGATG), and using dU-containing template DNA produced in *E. coli* strain CJ236 (Kunkel et al., 1987). Double digestion of the resulting construct (mpTYSma-H) with Sma I and Hind III yielded a c. 260 bp fragment that was used to replace the Sma I/Hind III fragment of the full length clones. Transcripts from template DNA (pT7YH1-H, -I2-H, -1A-H) linearized at the unique 3' Hind III site were expected to contain 5 extra nucleotides (-AAGCU) beyond the normal -CC terminus of TYMV virion RNA (Fig. 1).

In order to increase the genetic diversity of the cloned sequences, 5' and 3' halves of the three full-length clones were shuffled by substitution of the 3'

Sac I-Hind III fragments (Fig. 1). This created clones pT7YT1, -2, -3, -4, -5 and -6; pT7YT4 (5'H1, 3'I2) was one of the clones shown to yield infectious transcripts. Finally, the C10 of pT7YT4 was altered to the wild type U10 to create pTYMC, which we define as the Corvallis strain of TYMV. The U10 substitution was accomplished by site-directed mutagenesis using the deoxyoligonucleotide d(ATTGGTAGTTGATTAC).

Mutagenesis to disrupt the initiation codons of ORF-69 and ORF-206

The initiating codons of the two overlapping ORFs (AUG-88, ORF-69; AUG-95, ORF-206) were altered by introducing point substitutions into pTYMC. The 1.3 kb EcoR I-Pst I fragment from pTYMC was subcloned into M13mp19 for site-directed mutagenesis. Two deoxyoligonucleotides were used to create point mutations within the initiating codons: d(GCAAATGAGTAACGGCCTT) yielded mutant 206ACG, and d(ATTGCAAA(A/G)GAGTAAT) yielded mutants 69AAG and 69AGG. After verifying the sequences of all mutants between the 5'-EcoR I and Nco I^{210} sites, the EcoR I/Nco I fragments harbouring the mutations were shuttled back into the full length clone pTYMC.

In vitro transcriptions

Template DNA linearized with Hind III (10 µg) was incubated in a 100 µl reaction containing 40 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 5 mM DTT, 0.5 mg/ml bovine serum albumen, 1 mM each ATP, UTP and CTP, 0.5 mM GTP, 2 mM cap analogue (^{7}mGpppG), 1 u/µl ribonuclease inhibitor and 1500 u/ml T7 RNA polymerase for 2 hr at 37°C. Transcripts were labelled by the inclusion of [α-^{32}P]-UTP at 0.1 Ci/mmol. The reactions were terminated with 20 mM EDTA and 0.1% SDS, phenol extracted, and ethanol precipitated in the presence of ammonium acetate. Template DNA was removed by treatment with deoxyribonuclease I (Macaloid-treated to remove ribonucleases, Maniatis et al., 1982), followed by precipitation with 3 M ammonium acetate (incubation for 15 min at -80°C prior to centrifugation). Pellets were resuspended in 30 µl water, and analyzed on phosphate/agarose gels after glyoxalation (Maniatis et al., 1982). High quality T7 RNA polymerase was found to be essential in obtaining transcripts with high infectivity. Typical yields estimated by Cerenkov counting were 10-15 µg of RNA from 10 µg of DNA template.

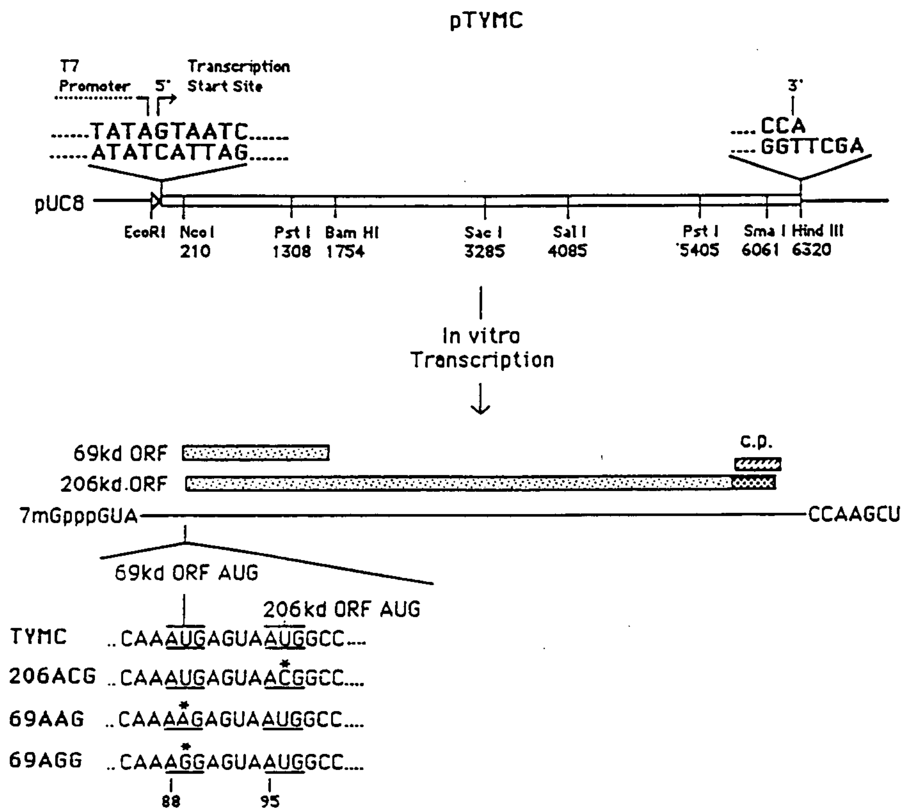


Fig. I.1) Diagrammatic representation of pTYMC, a pUC-derived clone containing a full-length insert of TYMV genomic cDNA (upper panel). The TYMV insert, its 5' and 3' termini and significant restriction sites marked, is flanked at the 5' by a bacteriophage T7 promoter (arrow head) and at the 3' end by a unique Hind III site. The 3'-structure of Hind III-linearized pTYMC is shown at upper right. When this linearized template is transcribed with T7 RNA polymerase in the presence of cap analogue, full-length capped transcripts beginning at the viral 5' terminus as indicated in the lower panel are synthesized. These transcripts are fully infectious in protoplasts and result in systemic symptoms in Chinese cabbage plants. The two overlapping ORFs encoded by TYMV RNA, 69 kd beginning at AUG-88 and 206 kd beginning at AUG-95, are shown in the lower panel, together with the mutations introduced into the initiation codons. The amber-suppressible read-through extension of ORF-206 is shown, as is the remaining significant ORF of TYMV, which encodes the coat protein (c.p.).

Inoculation of Chinese cabbage protoplasts

Leaves were taken from Chinese cabbage plants held in the dark for two days. Protoplasts were released from finely sliced leaves by incubation in the presence of 2% cellulysin™, 0.1% macerase™ and 0.1% bovine serum albumin in 0.1% MES [2-(N-morpholino)ethanesulphonic acid] containing 0.55 M mannitol for 2 hr at 30°C. Protoplasts were inoculated with virion or transcript RNA in the presence of polyethylene glycol 8000 containing 3 mM $CaCl_2$, at pH 5.3 (Samac et al., 1983). Transcripts (5 μg) free of DNA were used as inocula. The inoculated protoplasts were held in MES-buffered 0.55 M mannitol under constant light, and harvested 20-24 hr post inoculation. Harvested protoplasts were used for analysis of viral coat protein and viral RNAs in western and northern blots, respectively, or to inoculate Chinese cabbage plants.

Inoculation of Chinese cabbage plants

Four week-old plants were held in the dark for 24 hr prior to inoculation. The two oldest leaves were lightly dusted with carborundum and mechanically inoculated using gloved fingertips. The inocula, resuspended in 5 mM sodium acetate (pH 4.5) containing 0.8 mM magnesium acetate and 3 mg/ml bentonite, were 10 μg (5 μl) of full-length transcript RNA or 20 μl containing 10^5 lysed, infected protoplasts (infections resulting from inoculations with transcript or virion RNA). Mock and positive control inoculations using dried infected tissue or TYMV RNA (5 μg) or virions were performed in parallel. The plants were returned to the growth chamber and scored for symptoms 7-10 days post inoculation.

Analysis of viral products in plant tissue

(i) Western assays for viral coat protein Antiserum raised in rabbits against TYMV virions was used to detect TYMV coat protein in extracts from protoplasts or plants. Protoplasts (10^5) were gently pelleted and resuspended for lysis in 20 μl of 5 mM sodium acetate (pH 4.5) containing 0.8 mM magnesium acetate. After treatment in loading buffer at 65°C for 20 min, samples were separated by SDS-PAGE on 14% gels (Laemmli, 1970), and electroblotted onto nitrocellulose (Schleicher & Schuell). Blots were incubated in a 10^{-3} dilution of antiserum in MTPBS (5% non-fat dried milk and 1% Tween 20 in PBS) for 16 hrs at 23°C. Detection utilized horseradish peroxidase-linked second antibody. As little as 2 ng of coat protein could be detected.

For analysis of inoculated plants, leaf tissue was ground in a mortar and pestle with SDS-PAGE electrophoresis buffer (Laemmli, 1970) (3 ml/g fresh weight). Extracts were clarified by centrifugation at 16,000g for 8 min at 4°C. Samples were separated by SDS-PAGE and blotted as above.

(ii) Northern assays for viral RNA

RNA extraction, electrophoresis and blotting Protoplasts were resuspended to 0.38 ml in 50 mM Tris-HCl (pH 7.2) containing 100 mM NaCl, 1 mM EDTA, 1% SDS and 3mg/ml bentonite. The extract was immediately deproteinized with phenol/chloroform (twice), and the nucleic acids were ethanol precipitated in the presence of ammonium acetate. Samples were glyoxalated and electrophoresed through 1% agarose/phosphate buffer gels (Maniatis et al., 1982). Northern transfer of the nucleic acid was by a modified procedure of Parker and Li (1988). The gel was placed onto Whatman 1MM paper supported by a sponge soaked in 0.2 N NaOH. A pre-wetted sheet of Zeta-Probe nylon transfer membrane (Bio-Rad) was placed over the gel, followed by two sheets of Whatman paper and several highly absorbant paper towels. After transfer for 30 min, the membrane was immediately neutralized in 2X SSPE (20 mM sodium phosphate, pH 7.5, 0.3 M NaCl, 2.2 mM EDTA) for 15 min and air dried.

RNA probe preparation and hybridization The c. 0.9 kb Pst I/Hind III 3'-end fragment of pTYMC (Fig. I.1) was subcloned into pIBI 76 (International Biotechnologies, Inc.), a plasmid containing SP6 and T7 promoters. After linearization with Pst I, this construct was transcribed with T7 RNA polymerase to produce a minus sense RNA complementary to the 3'-terminal 0.9 kb of the TYMV genome. Probes with specific activities of about 10^8 dpm/µg were produced by transcription in the presence of 10 µM [α-^{32}P]UTP.

Blots were prehybridized at 60°C for 4 hr in 4X SSPE, 1% SDS, 0.5% non-fat dried milk (Carnation), and 100 µg/ml tRNA or total yeast RNA. Hybridization was in the above solution containing 5×10^6 dpm of the RNA probe for 12 hr at 60°C. The membranes were then washed in 0.5X SET (15 mM Tris-HCl, pH 8.0, 75 mM NaCl, 1 mM EDTA) containing 0.1% SDS and 0.1% sodium pyrophosphate at 65°C, and finally autoradiographed. The detection limit was 1 ng of virion RNA.

In vitro translations

Virion RNA and full-length genomic transcripts of pTYMC and its derivatives were translated in a rabbit reticulocyte translation system (BRL; 0.5 μg of RNA per assay). Products were labelled with ^{35}S-methionine (690 Ci/mmol) or ^{3}H-leucine (130 Ci/mmol), and analyzed by SDS-PAGE and fluorography.

RESULTS

Cloning of full-length TYMV cDNA

TYMV (type strain) was purified from turnips (*Brassica rapa*) and had been previously maintained in that host. The virus was highly infectious to both turnips and Chinese cabbage (*Brassica pekinensis* cv. Wong Bok), the latter host being used in all the studies reported here. The procedure we employed for obtaining cDNA clones of the TYMV genome made use of synthetic deoxyoligonucleotide primers to direct the synthesis of both cDNA strands, as described in Materials and Methods. This strategy ensured the recovery of full-length cDNA, and provided flanking sequences important in controlling the subsequent generation of TYMV RNA by *in vitro* transcription. Fig. I.1 describes the essential elements of clone pTYMC, a pUC-derived plasmid containing a full-length insert of TYMV cDNA. A modified T7 promoter has been fused to the 5' sequence of the TYMV cDNA, such that the expected start of transcription coincides with the 5'-G residue of the viral sequence. A unique Hind III restriction site has been placed immediately adjacent to the 3' end of the viral sequence for use in linearizing DNA templates for transcription with T7 RNA polymerase.

Transcription of pTYMC and demonstration of transcript infectivity

Transcription of Hind III-linearized pTYMC template with T7 RNA polymerase in the presence of cap analogue generated capped RNAs that comigrated with virion RNA on denaturing gels (Fig. I.2). RNA yields were significantly decreased relative to those generated from the wild type T7 promoter (Dunn and Studier, 1983), due to the substitution of nucleotides downstream from the initiation site (GGGAGA- replaced by GTAATC-). This attenuated promoter resulted in unacceptably low yields under the usual conditions employed for direct capping during transcription [50 μM GTP and 0.5 mM cap analogue m^7GpppG (Contreras et al., 1982)]. By transcribing in the presence of 0.5 mM GTP and 2 mM m^7GpppG, we have been able to synthesize about 1-1.5 µg RNA /µg of DNA template. Under these conditions, about 50% of transcripts were capped (not shown), as estimated by transcribing pTYMC template linearized at Rsa I[57] and analyzing the short transcripts on sequencing gels.

Transcripts resulting from the Hind III-linearized pTYMC templates (Fig. I.1) were designed to have correct 5' termini and five extra bases (-$AAGCU_{OH}$) beyond the 3'-terminal -$AACC_{OH}$ of virion RNAs (Briand et al., 1977). The correct 5' sequence of the TYMC transcripts was verified by primer extension sequencing using reverse transcriptase (not shown). Accuracy of the 5'-terminal sequences of infectious *in vitro* transcripts has been crucial in other RNA viral systems, while short 3' extensions are commonly present in infectious transcripts (Dreher et al., 1989; Ahlquist et al., 1987).

The infectivity of capped TYMC transcripts was tested by inoculating Chinese cabbage leaf protoplasts and plants. After inoculation of 2-4x10^5 protoplasts with 5 µg of transcript RNA and incubation in the light for 20-24 hr, protoplasts were analyzed for the presence of viral coat protein and viral RNAs in western and northern blots, respectively. TYMC transcripts were highly infectious: the accumulations of viral products were similar to those present in protoplasts inoculated in parallel with 2 µg of TYMV virion RNA (Fig. I.3a). In at least five experiments with independently transcribed RNAs, the specific infectivity of TYMC RNA transcripts was 2-5-fold lower than that of authentic virion RNA. The infectivities of TYMC and TYMC-C10 (a variant with C at position 10 that was initially isolated as a result of cDNA cloning; see Materials and Methods) transcripts were not significantly different from each other (Fig. I.3). These high infectivity rates of TYMC transcripts were markedly dependent on the quality of T7 RNA polymerase used.

Systemic infections could be established in Chinese cabbage plants when inoculated directly with RNA transcripts or with protoplasts carrying infections arising from TYMC or TYMC-C10 RNA inocula. These infections were comparable to those arising from inoculation with TYMV RNA or from infected protoplasts inoculated with virion RNA. Symptoms appeared 7-9 days post inoculation; they were evident as chlorotic local lesions on the inoculated leaf and were systemic in the younger leaves. Extracts of symptomatic young leaves with infections derived from cDNA clones and from virions had similar amounts of virion RNA and coat protein (Fig I.3 b,c). These analyses demonstrate that the infections derived from cDNA clones pTYMC and pTYMC-C10 have wild type phenotype, and confirm that the TYMC genotype is representative of the type (Strasbourg) strain from which it was generated. Clone pTYMC has been newly defined as the Corvallis strain of TYMV. The sequence of the pTYMC cDNA will be reported elsewhere.

In vitro translation of initiation codon mutants suggests that ORF-69 is expressed

The establishment of an infectious cDNA clone makes possible new approaches to studying TYMV gene expression. We have been interested in determining which of the two overlapping reading frames present at the 5' end of TYMV genomic RNA (ORF-69 initiating at AUG-88 and ORF-206 initiating at AUG-95) are expressed and necessary for replication. Three mutants with single base substitutions in the initiation codons were constructed using site-directed mutagenesis. In mutants pTYMC-69AAG and pTYMC-69AGG, the initiating codon of ORF-69 was altered, while in mutant pTYMC-206ACG, the initiating codon of ORF-206 was altered (Fig. I.1). The latter substitution left the amino acid sequence encoded by ORF-69 unchanged (AAU was replaced with AAC, both encoding asparagine).

In order to verify that the substitutions in the initiation codons blocked translation of the relevant ORF, full-length, capped mutant and wild type (TYMC) transcripts were translated in parallel with virion RNAs in a rabbit reticulocyte translation system. Because the ORF-69 encodes only three methionines but many leucines (Morch et al., 1988), translations were allowed to incorporate either ^{3}H-leucine or ^{35}S-methionine. When products directed by virion RNA were labelled with ^{3}H-leucine, three major products (c. 200, 150 and 75-80 kDa) and many less intensely labelled products were observed after SDS-PAGE and fluorography (Fig. I.4a). When labelled with ^{35}S-methionine, the pattern of bands was similar except that the 75-80 kDa product was less prominent. The c. 200 and 150 kDa species are commonly recognized translation products arising from TYMV RNA (Haenni et al., 1987), and are presumably encoded by the ORF-206. Except for the 20 kDa coat protein, some of the smaller products typically observed have been interpreted as proteolytic maturation products arising from the long ORF (Haenni et al., 1987). The prominent 75-80 kDa product has not been recognized, probably because previous workers have exclusively labelled products with ^{35}S-methionine. Experiments described below argue that the 75-80 kDa protein arises from ORF-69.

Translation of the mutant TYMV RNAs verified that the mutations were effective in preventing efficient expression of the relevant ORF. The 75-80 kDa product was absent from mutants TYMC-69AGG and -69AAG, while other bands were unchanged (Fig. I.4b). This suggests that the 75-80 kDa product is indeed derived from ORF-69, and that this ORF may be normally expressed from TYMV RNA. The markedly differential incorporation of labelled methionine and leucine into the 75-80 kDa product supports the above assignment. Its aberrant migration in SDS-PAGE is presumably due to its highly basic character (pI= 11.5; Morch et al., 1988).

Reflecting the loss of the AUG-95 initiation codon, the predominant c. 200 and 150 kDa products were not translated from the 206ACG mutant. In contrast, synthesis of the 75-80 kDa product was enhanced. Several minor unidentified products, some larger than 100 kDa, were synthesized similarly from mutant TYMC-206ACG and from wild type RNA (Fig. I.4b). Since caps do not enhance the translation of TYMV RNAs in the rabbit reticulocyte extract (not shown), many of the minor products present in all lanes may arise from the translation of uncapped RNA fragmentation products.

Infectivity of initiation codon mutant RNAs

The effect of the initiation codon mutations on viral replication was studied in protoplasts, using northern blots to detect viral RNA and western blots to detect coat protein. At least five experiments employing independently synthesized transcripts were conducted. No viral products were seen in protoplasts inoculated with mutant 206ACG. However, inoculations with mutants 69AAG and 69AGG did give rise to infections. Both mutants behaved similarly, producing about 50-fold less viral RNA and coat protein than wild type transcripts during the 20-24 hr incubation following inoculation (Fig. I.5).

The ability of the ORF-69 and ORF-206 mutants to complement each other and give rise to a normal infection was investigated. Protoplasts were inoculated with combinations of the 206ACG and 69AGG or 69AAG mutants (2.5 or 5 µg of each). No complementation was observed, and the accumulations of both viral RNA and coat protein for the mutant mixtures were the same as for the ORF-69 mutants alone (Fig. I. 5).

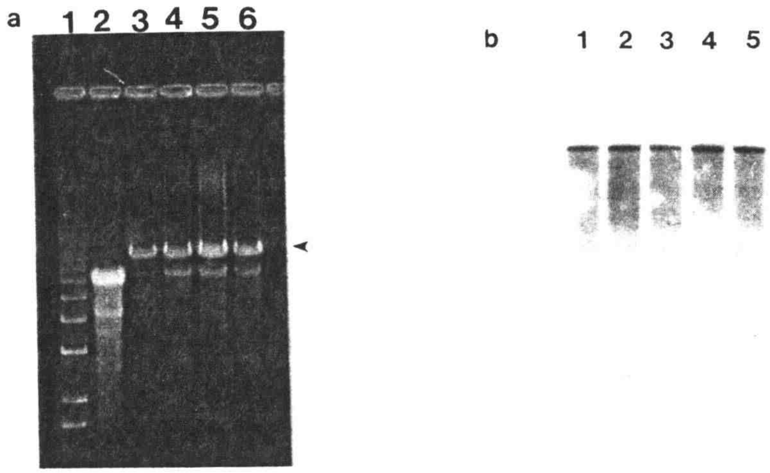


Fig. I.2) Electrophoretic analysis on 1% agarose gels of TYMC transcripts and TYMV virion RNA denatured by treatment with glyoxal. **(a)** Comparison of virion RNA (lane 2) with transcripts from four clones containing full-length TYMV inserts (lanes 3-6), visualized by staining with ethidium bromide. The DNA templates (arrow) have not been removed from these transcripts. All RNAs (6.3 kb long) comigrate, between the 6 and 7 kb bands of the 1 kb DNA marker (BRL). **(b)** Autoradiographic analysis of ^{32}P-labelled full-length transcripts, freed from template DNA by treatment with deoxyribonuclease and precipitation with ammonium acetate. These RNAs were used to inoculate protoplasts. Lane 1, TYMC transcripts; lane 2, TYMC-C10; lane 3, TYMC-69AAG; lane 4, TYMC-69AGG; lane 5, TYMC-206ACG.

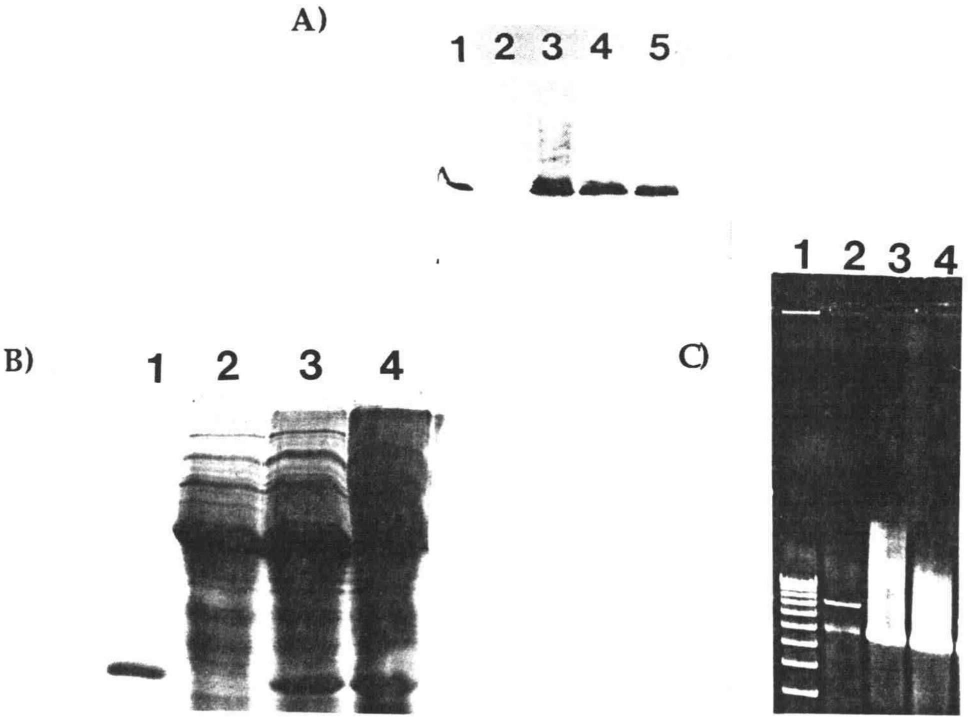
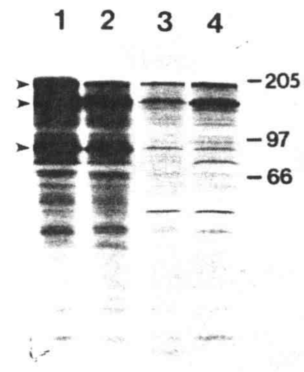


Fig. I.3) Infectivity comparison in protoplasts and plants of *in vitro* transcripts and virion RNA. A) Western blot detection (14% SDS-PAGE) of TYMV coat protein in extracts of protoplasts (10^5) mock-inoculated (lane 2), or inoculated with 2 µg virion RNA (lane 3), 5 µg TYMC transcripts (lane 4), 5 µg TYMC-C10 transcripts (lane 5). TYMV virions (50ng) were run as coat protein marker in lane 1. B) Coat protein accumulation in TYMC and TYMV infected Chinese cabbage plants. Extracts from systemic, symptomatic tissue were fractionated by 14% SDS-PAGE, and the gel was subsequently stained with Coommasie brilliant blue. TYMV virions were loaded as a control (5 µg, lane 1), followed by extracts from a mock inoculated plant (lane 2), a plant inoculated with TYMC capped transcript RNA (lane 3) and TYMV RNA (lane 4). C) Accumulation of viral RNA in TYMC and TYMV infected plants. Virus was prepared from symptomatic tissue according to Lane (1986). RNA was extracted from the virus preparation and electrophoresed in a 1% Tris-acetate agarose gel. Standards [1 kb ladder (BRL), lane 1; and a TYMC transcript, lane 2] were loaded, as was RNA prepared from symptomatic tissue after inoculation with TYMC capped transcript RNA (lane 3) and TYMV RNA (lane 4).

a)



b)

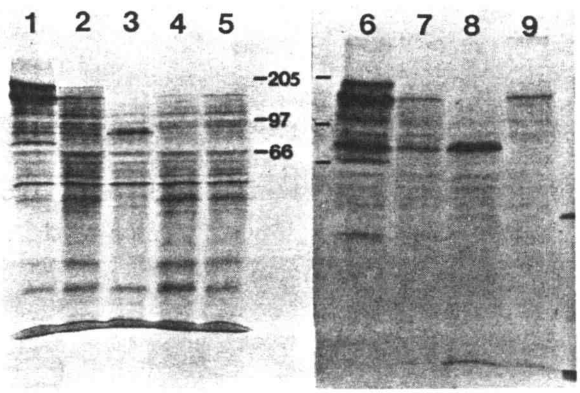


Fig. I.4) Translation of TYMV virion RNA, and wild type and mutant transcript RNAs (0.5 µg) in rabbit reticulocyte cell-free systems. The radiolabelled products were separated by 10% SDS-PAGE and fluorographed. **(a)** Translation products made in two different reticulocyte extracts programmed by TYMV virion RNA, labelled with ^{3}H-leucine (lanes 1 and 2) or ^{35}S-methionine (lanes 3 and 4). The main translation products, 195 kDa and 150 kDa (arising from ORF-206) and 75-80 kDa (arising from ORF-69), are arrowed. Migration position of standards is marked (kd). **(b)** Products labelled with ^{35}S-methionine (lanes 1-5) or ^{3}H-leucine (lanes 6-9) in reactions programmed by TYMV virion RNA (lanes 1,6), TYMC transcript (lanes 2,7), 206ACG mutant transcript (lanes 3,8), 69AGG transcript (lane 4), 69AAG transcript (lanes 5,9).

Fig. I.5) Viral replication in protoplasts inoculated with initiation codon mutant and wild type transcripts. Viral products in protoplasts (10^5) incubated for 20-24 hr after inoculation were analyzed by western blotting using anti-TYMV serum and 14% SDS-PAGE to detect coat protein (a,b), or by northern blotting from 1% agarose gels to detect viral genomic (G) or subgenomic (S) RNA (c,d). A normal degradation product present in virion RNA preparations is arrowed. Some ribosomal RNAs hybridize in all lanes, perhaps because the probe is G-rich. **(a)** Proteins extracted from protoplasts inoculated with: no RNA (lane 2); 2 µg TYMV RNA (lane 3); and 5 µg of transcripts TYMC-C10 and 1/10 dilution (lanes 4 and 5); TYMC (lane 6); 206ACG (lane 7); 69AAG (lane 8); 69AGG (lane 9). Lane 1 contains 50 ng of TYMV virions. **(b)** Complementation tests for ORF-69 and ORF-206 mutants (see also Fig. 5d). Proteins extracted from protoplasts inoculated with: 2 µg TYMV RNA (lane 2); no RNA (lane 3); and 5 µg of transcripts TYMC and 1/10 dilution (lanes 4 and 5); 206ACG (lane 6); 69AAG (lane 7); 2.5 µg each of 69AAG and 206ACG (lane 8). Lane 1 contains 50 ng of TYMV virions. **(c)** RNAs extracted from protoplasts inoculated with: 2 µg TYMV RNA (lane 2); no RNA (lane 3); and 5 µg of transcripts TYMC-C10 (lane 4); TYMC (lane 5); 206ACG (lane 6); 69AGG (lane 7); 69AAG (lane 8). Lane 1 contains 50 ng of TYMV RNA. **(d)** RNAs extracted from protoplasts inoculated with: 5 µg of 206ACG transcript (lane 4); 2 µg of TYMV RNA (lane 5); 5 µg of TYMC transcript (lane 6); 5 µg of 69AAG transcript (lane 7); 2.5 µg each of 69AAG and 206ACG (lane 8). Lanes 1-3 contain 50, 5 and 1 ng, respectively, of TYMV RNA.

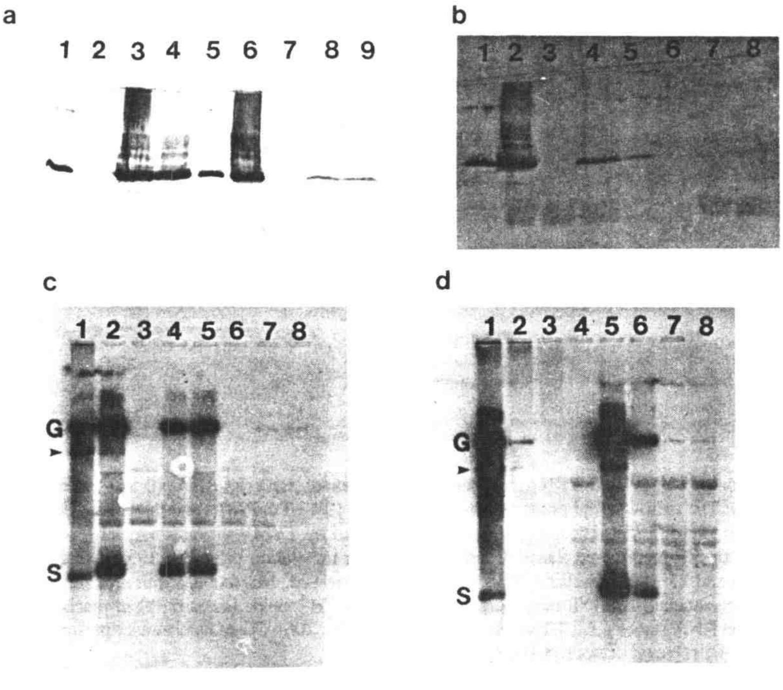


Figure I.5

DISCUSSION

pTYMC-derived infection in Chinese cabbage protoplasts and plants

By establishing a cDNA clone of the entire TYMV genomic RNA (pTYMC), we have been able to generate RNA transcripts *in vitro* that produce wild type infections in protoplasts and give rise to wild type systemic infections in Chinese cabbage plants. The transcripts are highly infectious, displaying a specific infectivity in protoplasts only 2-5 fold lower than virion RNA, and yielding virus accumulations in whole plants similar to those resulting from inoculation with RNA. The ability to synthesize such biologically active TYMV transcripts from cDNA clones will permit the molecular biology of TYMV to be more rapidly investigated.

Translation of ORF-69 *in vitro*

In vitro translations programmed with the wild type and mutant RNAs have indicated for the first time that ORF-69 is translated from genomic RNA, giving rise to a product that migrates somewhat anomalously in SDS-PAGE at 75-80 kDa . The strongest evidence that the 75-80 kDa product arises by translational initiation at AUG-88 comes from its absence from the translation products of mutants 69AAG and 69AGG. This conclusion is consistent with ribosomal initiation normally occurring at the 5'-most AUG of an mRNA and with the statistical arguments (Morch et al., 1988) suggesting that ORF-69 is expressed. It will nevertheless be important to verify this conclusion by immunoprecipitation with antibodies reactive with polypeptides derived from ORF-69, and by direct protein sequencing. The antiserum would also be useful for determining whether ORF-69 is expressed *in vivo*.

Several factors have contributed to the failure to recognize the presence of the 75-80 kDa product in earlier translation studies (Benicourt et al., 1978; Haenni et al., 1987): (i) prior to the determination of the genomic sequence of TYMV (Morch et al., 1989), there was no precedent for the presence of extensively overlapping ORFs among related plant viruses; (ii) because ORF-69 is exceptionally scarce in methionine codons (0.5%), its product is only obvious when labelled with ^{3}H-leucine (10.2% of codons) rather than ^{35}S-methionine (Fig. I.4a); (iii) the 69 kDa product was probably interpreted as one of the products of the proteolytic maturation of the 206 kDa translation product (Morch et al., 1980); (iv) apparently misleading preliminary experiments (Briand et al., 1978) gave no indication of initiation at AUG-88.

<u>Effects of base substitutions in initiation codons of ORF-69 and ORF-206</u>

The alteration of the initiation AUG codon of each ORF prevented the expression *in vitro* of that ORF, but not the overlapping ORF (Fig. I.4b). It is thus most reasonable to conclude that the two ORFs are expressed by separate initiation events. The ability of some ribosomes to escape initiation at AUG-88 in favour of AUG-95 in wild type TYMV RNA would be consistent with ribosomes scanning the mRNA 5'-3', with initiation rates being determined by the context at each AUG codon (Curran and Kolakofsky, 1988). The relatively poor context at AUG-88 (C at -3, A at +4) and better context at AUG-95 (G at -3, G at +4) (Kozak, 1986) might allow some ribosomes to pass the first AUG, resulting in initiation at AUG-95 (leaky scanning) (Kozak, 1987).

Other properties of the variant RNAs are not readily explainable in terms of usual ribosomal behaviour. Alteration of the AUG-95 initiation codon resulted in greater expression of the upstream ORF-69 (Fig. I.4b), while mutations within the AUG-88 initiation codon (mutants 69AGG and 69-AAG) failed to significantly increase the expression of the downstream ORF-206 (both 150 and 206 kDa products). In general, the removal of an upstream AUG increases initiation at the nearest downstream AUG (Kozak, 1987), while the removal of a downstream AUG would not be expected to alter initiation upstream (Curran and Kolakofsky, 1989). All of the sequence changes of the initiation codon mutants fall outside the accepted context positions (-3 to +4 relative to the A of the AUG codon) (Kozak, 1987) that might be expected to influence the ribosomal initiation of the overlapping ORF. Perhaps a mechanism exists whereby an interdependence of initiation at the two closely-spaced AUG codons (AUG-88 and AUG-95) controls the relative rate of use of each ORF.

None of the pTYMC variant clones produced infection in Chinese cabbage plants. Viral products could not be detected in protoplasts inoculated with mutant 206ACG. This is consistent with the fact that ORF-206 encodes a product with sequence elements common to known RNA-dependent polymerases and other essential nonstructural proteins of RNA viruses (Kamer and Argos, 1984; Morch et al., 1988).

Mutants 69AAG and 69AGG, which were unable to support the synthesis of normal levels of 75-80 kDa product *in vitro*, did replicate in protoplasts, but only poorly. The role or function of ORF-69 and its putative product is unknown and there is no analogue in either molecular weight, amino acid composition or position of the ORF in the genome in other plus-stranded RNA plant viruses. Although the *in vitro* translations discussed above and the fact that ORF-69 is initiated by the 5'-most AUG of the viral RNA suggest that this ORF is expressed *in vivo*, studies with specific antisera are needed to establish this point. It is therefore uncertain whether the poor replication of mutants 69AAG and 69AGG is due to the absence of the putative ORF-69 product. The failure of mutant 206ACG to rescue the poor replication of the ORF-69 mutants may reflect a dispensibility of ORF-69 for replication. Alternatively, the poor replication may be due to the production of the essential ORF-206 translation products in limiting amounts from mutants 69AAG and 69AGG, or due to a direct interference with replication by (+) strand promoter attenuation resulting from the sequence alterations. A further, provocative possibility suggested by the observations that initiation of the overlapping ORFs is not independent *in vitro*, is that ORF-69 and ORF-206 may need to be expressed in *cis* for efficient replication.

In order to further investigate the functions of the two overlapping ORFs, it will be necessary to pursue experiments that determine whether ORF-69 is expressed *in vivo*, and if so to make constructions that permit complementation studies in protoplasts and plants. The infectious transcripts described in this report make such investigations readily feasible.

CHAPTER II

Expression of ORF-69 of turnip yellow mosaic virus is necessary for viral spread in plants.

ABSTRACT

Turnip yellow mosaic virus RNA has two extensively overlapping open reading frames (ORFs) encoding non-capsid proteins. The longest of these, ORF-206, is essential for RNA replication. We have investigated the expression and role of ORF-69 (encoding a Mr 69 kDa protein) using specific antiserum and mutant RNAs designed to interrupt ORF-69 expression. TYC69 antiserum immunoprecipitated a protein of apparent Mr 75 kDa (p69) from *in vitro* translations of TYMV RNA. Mutant RNAs with stop codons inserted at nucleotides 139, 178 and 178/224 in ORF-69 expressed ORF-69 at very low levels *in vitro*. These mutants replicated to wild type levels in Chinese cabbage protoplasts, but were not recovered from any leaves of inoculated plants. These results suggest that ORF-69 products are dispensible for replication, but are required in facilitating viral spread. A revertant and pseudorevertant were recovered from plants showing delayed onset of symptoms after inoculation with two of the above mutants, indicating the importance of ORF-69 expression in establishing a systemic infection in plants. ORF-69 expression could be detected using western blots in extracts from young, symptomatic leaves of infected plants. A single band of apparent MW 75-80K was detected in leaves infected with cDNA-derived viral RNAs, while a doublet was detected after infection with Type strain viral RNA, suggesting the presence of two ORF-69 alleles or differential post-translational modification.

INTRODUCTION

Turnip yellow mosaic virus (TYMV) is a monopartite, plus-stranded RNA plant virus which infects members of the Cruciferae family. The TYMV genomic RNA (6.3 kb) contains overlapping open reading frames (ORFs) encoding two non-capsid proteins with calculated molecular weights (Mrs) of 206 kDa (ORF-206) and 69 kDa (ORF-69; Morch *et al.*, 1988; Keese *et al.*, 1989). A third ORF encodes the 20-kDa coat protein, which is expressed from a subgenomic RNA. Recently, *in vitro* experiments have shown that the 206-kDa protein (p206) is proteolytically processed in *cis* to give proteins of Mr 150 kDa (N-terminal) and 70 kDa (C-terminal) (Bransom *et al.*, 1991). Expression of ORF-206 is required for RNA replication (Weiland and Dreher, 1989).

Prior to availability of the genomic sequence of TYMV RNA (Morch *et al.*, 1988; Keese *et al.*, 1989), *in vitro* translation studies did not account for expression of ORF-69, and its product may have been incorrectly designated as a cleavage product arising from ORF-206 (Haenni *et al.*, 1987). By mutagenesis of the initiation codons of ORF-206 and ORF-69 and subsequent translation of the mutant RNAs *in vitro*, it was shown that a protein with apparent Mr 75 kDa is expressed from ORF-69 (Weiland and Dreher, 1989). The role of this protein is not known, but it as been suggested to be a movement protein supporting systemic spread of the virus in plants (Morch *et al.*, 1988; Hull, 1989). However, no sequence similarity between the ORF-69 product and any known or putative movement proteins has been detected (Atabekov and Taliansky, 1990).

Here we use immunological detection to verify that ORF-69 is expressed *in vitro* and *in vivo*, and demonstrate that ORF-69 products accumulate in TYMV-infected Chinese cabbage and *Arabidopsis thaliana* plants. Experiments with substitution mutants show that, although RNA replication in protoplasts does not require wild-type levels of expression of ORF-69, efficient ORF-69 expression is necessary in producing a systemic infection in the plant.

MATERIALS AND METHODS

Virus and plant material.

Chinese cabbage (*Brassica pekinensis* cv. Wong Bok), turnips (*Brassica rapa*, cv. Just Right) and *Arabidopsis thaliana* (ecotype Bencheim) plants were grown in a growth chamber under 16 hour day-length at 21°C. Purified virions or ground tissue samples from lyophylized Chinese cabbage plants infected with TYMV (Strasbourg type strain; Weiland and Dreher, 1989) were resuspended in 10 mM Tris-HCl (pH7.2), 0.9 mM KH_2PO_4, 3 mM Na_2HPO_4, 14 mM NaCl and 3 mg/ml bentonite (virus inoculation buffer) and used to mechanically inoculate the first and second leaves of 4-week-old plants.

Extraction of proteins from plants.

Individual leaves were harvested, pulverized to a powder in liquid nitrogen, and then further ground in an extraction buffer containing 70 mM Tris-HCl (pH 7.2), 2M urea, 1% SDS, 0.6% ß-mercaptoethanol, 2 mM EDTA and 1mM phenylmethylsulfonyl fluoride. The samples were placed in a boiling water bath for 3 min, vortexed vigorously until cool, and then centrifuged (14,000 x *g*) for 15 min to remove cellular debris. Protein content of the supernatant was determined using a Coomassie Blue G-250 dye-binding protein assay kit (Pierce) with bovine serum albumin as a standard. Samples were diluted with SDS-PAGE loading buffer before fractionation on SDS-polyacrylamide gels.

Antiserum production and western blots.

Antiserum reactive with TYMV coat protein has been described elsewhere (Weiland and Dreher, 1989). Antiserum to a synthetic peptide corresponding to the C-terminal 14 amino acids of ORF-69, with an additional N-terminal cysteine for coupling to keyhole limpet hemocyanin (peptide C-69; Multiple Peptide Systems, San Diego CA) was raised as described (Bransom *et al.*, 1991), producing antiserum TYC69.

For western blot detection, plant extracts were separated by SDS-PAGE. Electroblotting of proteins onto nitrocellulose and western blot development were as described (Weiland and Dreher, 1989), except that some western blots were developed with an enhanced chemiluminescence kit (Amersham). Keyhole limpet hemocyanin (5 µg/ml) was included in the antipeptide antibody incubation solutions to reduce background.

***In vitro* mutagenesis.**

In vitro mutagenesis was conducted as previously described (Kunkel *et al.*, 1987; Weiland and Dreher, 1989), using dU-containing single stranded DNA templates from M13 subclones of pTYMC. Plasmid pTYMC contains a 6.3 kbp DNA copy of the TYMV genome. Transcripts TYMC generated *in vitro* from this cDNA clone are infectious on Chinese cabbage protoplasts and plants (Weiland and Dreher, 1989). In this paper, we differentiate these transcripts from non-clonal inoculum (TYMV). All mutants involved substitutions that left the amino acids encoded by the overlapping ORF unchanged. The mutations were created in fragments of pTYMC, which were completely sequenced before subcloning back into pTYMC using restriction enzyme sites marked in Fig. II.1. The following mutants were used in this study: 1) TYMC-69AAG in which the AUG initiation codon of ORF-69 was changed to AAG (Weiland and Dreher, 1989); 2) TYMC-139UAG, in which the ORF-69 codon at nucleotide 139 (CAG) was changed to a stop codon (UAG); 3) TYMC-178UGA, in which the ORF-69 codon at nucleotide 178 (GGA) was changed to a stop codon (UGA); 4) TYMC-178/224, in which ORF-69 codons at nucleotides 178 (GGA) and 224 (GAA) were changed to stop codons (UGA and UAA respectively); 5) TYMC-206ACG, in which the AUG initiation codon of ORF-206 was changed to ACG (Weiland and Dreher, 1989); 6) TYMC-838G, in which a potential N-glycosylation site (Asn-Ser-Thr) at codons 249-251 of ORF-69 was altered by substituting A with G at nucleotide 838 (Thr to Ala). A summary of the location of point changes is shown in Fig. II.1.

***In vitro* transcription and inoculation of transcripts onto plants and protoplasts.**

Transcription of *Hind*III-linearized template DNA was as described (Weiland and Dreher, 1989). Capped transcripts (5 µg) were mechanically inoculated onto plants as described above for virion inoculations. The plants were visually scored for systemic symptoms 7-10 days after inoculation, andleaves were harvested and treated as described above to assess the presence of virus encoded proteins by western blot analysis.

Protoplasts were isolated from Chinese cabbage or turnip leaves and 2-4×10^5 were inoculated with either 2 µg of TYMV RNA or 5 µg of capped RNA transcript as described (Weiland and Dreher, 1989). Inoculated protoplasts

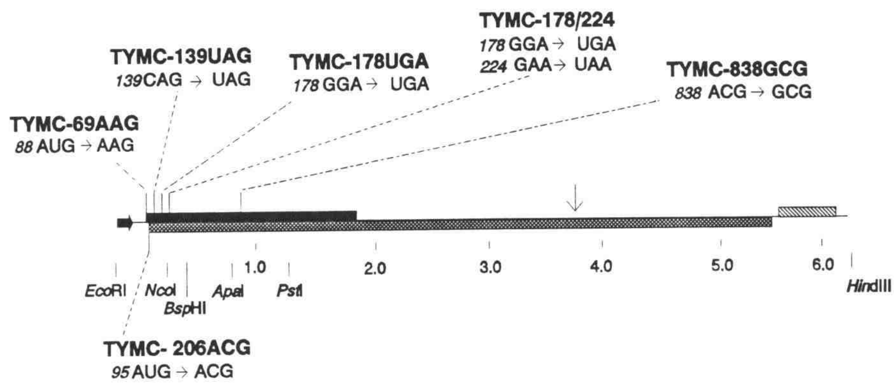


Fig. II.1) Diagram of pTYMC, a genomic cDNA clone of TYMC, indicating the encoded open reading frames and the mutations studied. The filled arrow at left indicates the T7 promoter, and the remainder of the diagram comprises the TYMV genome. The three ORFs encoded by TYMV RNA are indicated (ORF-69, filled; ORF-206, cross-hatched; coat protein ORF, hatched), and the location of the proteolytic cleavage of p206 to form the 150-kDa and 70-kDa proteins (Bransom *et al.*, 1991) is marked by a vertical arrow. The names of the mutant RNAs studied is given together with their nucleotide substitutions. The substitutions had no effect on the coding of the overlapping ORF. Relevant restriction sites are marked, and a nucleotide scale (in kbp) is given.

were used as a source of amplified mutant RNA in inoculations of some plants. In this case, a fraction of the harvested, inoculated protoplasts was resuspended in virus inoculation buffer and mechanically inoculated onto plants.

***In vitro* translations and immunoprecipitations.**

In vitro translations were performed in micrococcal nuclease-treated rabbit reticulocyte lysates as described (Bransom *et al.*, 1991). Two µl of each reaction mixture were diluted with SDS-PAGE sample buffer and fractionated on a SDS-polyacrylamide gel (10%).

For immunoprecipitations, the translation mixture was brought to 2% SDS and boiled for 1 min. Fifteen µl of the boiled translation products were mixed with 1.5 µl of antiserum TYC69 and 60 µl of 50mM Tris-HCl (pH7.5), 150mM NaCl, 1% Triton X-100, and 0.1% SDS. For peptide competition experiments, 1-20 µg/ml of either C-69 or C-206 peptide (C-206 corresponds to the C-terminus p206; Bransom *et al.*, 1991) was incubated with the diluted antiserum for 30 min prior to addition of the translation sample. The antigen/antibody complexes were precipitated with Protein A-Sepharose (Pharmacia). The immunoprecipitates were removed from the Protein A-Sepharose by boiling in SDS-PAGE sample buffer, and loaded onto SDS-polyacrylamide gels (10%). Gels used to separate translation and immunoprecipitation products were soaked in 1M sodium salicylate for 5 min prior to drying and product detection by fluorography (Sambrook *et al.*, 1989).

RESULTS

Immunological identification of ORF-69 *in vitro* translation products.

The major products detected when TYMV RNA is translated *in vitro* in the presence of [^{3}H]leucine are 3 non-capsid products of apparent Mr 206 kDa, 150 kDa and 75 kDa (Fig. II.2A, lane TYMC) and the 20-kDa coat protein (not shown in Fig. II.2A). In a previous study, RNAs with mutant initiation codons were created in order to assign the non-capsid proteins to the correct ORFs (Weiland and Dreher, 1989). *In vitro* translation of the ORF-206 initiation codon mutant (TYMC-206ACG) showed an absence of p206 and the

150-kDa protein (which are N-coterminal), and enhanced levels of the 75-kDa protein; *in vitro* translation of the ORF-69 initiation codon mutant (TYMC-69AAG) showed an absence of the 75-kDa protein (Fig. II.2A; Weiland and Dreher, 1989). Two other ORF-69 mutants, in which stop codons were created at nucleotide 178 (TYMC-178UGA) or at nucleotides 178 and 224 (TYMC-178/224), also showed an absence of the 75-kDa protein (Fig. II.2A).

To verify the identity of the 75-kDa protein as a product of ORF-69, immunoprecipitations using antiserum TYC69 were performed on translations directed either by wild-type TYMC or the mutant transcripts described above. Immunoprecipitations of products directed by TYMC and TYMC-206ACG RNAs contained a 75-kDa protein as the major product (Fig. II.2B), which was absent for all of the ORF-69 mutants. The immunological reaction of antiserum TYC69 with this protein was specific: no protein was immunoprecipitated with preimmune serum, and addition of peptide C-69 as a competitor (1-20 μg/ml) to immunoprecipitation reactions prevented recovery of the 75-kDa protein in immunoprecipitates (Fig. II.2C). Another peptide (C-206; Bransom *et al.*, 1991) had no effect on immunoprecipitation with antiserum TYC69. These results show that the 75-kDa protein is clearly the full-length ORF-69 product, initiating at nucleotide 88 and containing the C-terminal amino acids reactive with antiserum TYC69; we have termed this protein p69. As noted previously (Weiland and Dreher, 1989), p69 migrates slower than expected in SDS-PAGE, perhaps due to its high pI of 12.1.

Immunoprecipitation of translation products directed by TYMC, TYMC-178UGA and TYMC-178/224 RNAs with antiserum TYC69 yielded low amounts of proteins that migrated slightly faster than p69 (Fig. II.2B). These bands were not consistently detected in immunoprecipitates of translation products made from TYMC-206ACG or -69AAG RNAs, and for all RNAs were less prominent when carried out at higher Mg^{2+} concentration (3.65 mM cf. 1.65 mM total Mg^{2+}). The immunoprecipitation of these products was specific by the same criteria described above for p69 (not shown).

Detection of ORF-69 products in TYMV-infected plants

The expression of ORF-69 during TYMV infection in Chinese cabbage and *Arabidopsis* plants was studied using antiserum TYC69 in western blot analyses of leaf extracts. A variety of extraction conditions were tried, but

TYC69-reactive products were detected only when strongly dissociating and denaturing conditions (1% SDS, 2M urea, 0.6% ß-mercaptoethanol) were employed. Four-week-old Chinese cabbage plants were inoculated with TYMV and each leaf of the plants was harvested separately after 7 days. When western blots of these extracts were incubated with TYC69 antiserum, a doublet of Mr 75-80K was detected (Fig. II.3A); binding of antiserum to both bands of the doublet was prevented by competition with peptide C-69 but not with other peptides (not shown), verifying the identity of both as ORF-69 products. The lower band of the ORF-69 doublet expressed *in vivo* appeared to co-migrate with p69 expressed *in vitro*. No signal was detected in extracts from mock-inoculated Chinese cabbage plants (Fig. II.3A). The ORF-69 products were most abundant in the young, partially expanded systemically infected leaves (Fig. II.3A), but were not detected in inoculated leaves (not shown). TYMV-inoculated *Arabidopsis* leaves showed a similar distribution of ORF-69 products, but the intensities of the two bands of the doublet varied with leaf age: the lower band was predominant in older leaves while both bands were of equal intensity in younger leaves (Fig. II.3B).

Chinese cabbage plants inoculated with TYMC had symptoms identical to plants inoculated with TYMV. However, the TYC69-reactive product was a single band which corresponded in size to the upper band seen in TYMV-inoculated plants (not shown). The presence of a single band in tissues infected with the clonally derived TYMC suggested that TYMV RNA contains a population of RNAs with more than one ORF-69 allele. Alternatively, incomplete post-translational modification of the ORF-69 product could occur in the case of TYMV but not TYMC, accounting for the doublet seen in TYMV-inoculated plants. Inspection of ORF-69 from TYMC (EMBL accession No. X16378) revealed a potential N-glycosylation site Asn-Ser-Thr at codons 249-251. This site was also present in an Australian isolate, but not in the sequence reported for a TYMV isolate (Morch *et al.*, 1988) originally derived from the same source as the isolate used in this laboratory. Utilization of the putative N-glycosylation site of TYMC might account for the results described above.

We designed mutant TYMC-838G to eliminate the potential N-glycosylation site and thus determine whether it is utilized *in vivo*, and also whether N-glycosylation is a post-translational modification important for the function of ORF-69 products. Chinese cabbage and *Arabidopsis* plants inoculated with TYMC-838G transcripts showed systemic symptoms indistinguishable from those of plants inoculated with either TYMV or TYMC. In addition, there was no difference in the timing of onset of symptoms arising from the different inocula. As with extracts from plants inoculated with TYMC, only the higher Mr band was detected with antiserum TYC69 in western blots of extracts from TYMC-838G-infected plants (not shown). Thus, N-glycosylation of ORF-69 products does not have an essential role in TYMV infection, and perhaps does not even occur.

Is ORF-69 expression necessary for viral replication or systemic spread?

In previous studies (Weiland & Dreher, 1989), transcript RNA in which the ORF-206 initiation codon was altered (TYMC-206ACG) did not replicate in protoplasts, and the ORF-69 initiation codon mutant (TYMC-69AAG) replicated poorly. Because of the close proximity of the ORF-206 and ORF-69 initiation codons, it was not clear from this study whether the poor replication of TYMC-69AAG was due to limiting amounts of p69, or to interference in the expression of the essential ORF-206. In order to restrict expression from ORF-69 but avoid changes near the initiation codon of ORF-206, the following mutants were constructed: TYMC-139UAG and -178UGA, in which stop codons were placed downstream from the ORF-69 initiation site (at nucleotides 139 and 178), and TYMC-178/224, a double mutant with the nucleotide 178UGA codon as well as a stop codon (224UAA) downstream from a potential in-frame initiation site (AUG codon at nucleotide 211). The only AUG codon downstream of nucleotide 224 is at nucleotide 1609.

The replication of wild type TYMC and the above mutants was studied in protoplasts by western blot analyses of coat protein accumulation following inoculation with transcript RNA (Fig. II.4); coat protein is produced from subgenomic RNA that is generated by replication and is absent from the inoculum. Similar results were obtained with Chinese cabbage and turnip protoplasts; results from the turnip experiments are shown in Fig II.4. Accumulation of coat protein from TYMC was 20-50 times that of mutant TYMC-69AAG 24 hr after inoculation of turnip protoplasts. Mutant viruses TYMC-139UAG (not shown), -178UGA and -178/224 (Fig. II.4) produced levels of coat protein similar to that for infections with TYMC. Likewise, northern blots detecting genomic and subgenomic RNAs present in protoplasts inoculated with mutant TYMC-178/224 showed that viral RNAs were present at wild type levels (see Chapter II addendum). These results indicate that sufficient replication to support normal coat protein and RNA synthesis occurs even when ORF-69 expression is severely interrupted. The poor replication of TYMC-69AAG is probably not due to interference with ORF-69 expression, and may be the result of a direct effect on RNA replication by (+) strand promoter mutation, or to a perturbation of the expression of ORF-206 (Weiland and Dreher, 1989; Osorio-Keese *et al.*, 1989).

Mutant RNA transcripts and, in some cases, a fraction of the harvested, infected protoplasts were inoculated onto Chinese cabbage plants. TYMV and TYMC were the only RNAs that gave rise to systemic infections. RNAs TYMC-69AAG, -139UAG, -178UGA and -178/224 failed to generate systemic symptoms or local lesions, and attempts to detect viral coat protein by western blot analysis of tissue taken from the site of inoculation in these plants were unsuccessful. Thus, although ORF-69 expression is not required at wild-type levels for viral replication in plant cells, it is needed for cell-to-cell movement of the virus.

Two exceptional cases were observed, in which a Chinese cabbage plant inoculated with protoplasts infected with TYMC-139UAG or TYMC-178UGA developed systemic symptoms after a delay (12 days post inoculation) relative to normal symptom appearance (8 days). Sequence analysis of the virion RNA extracted from these plants revealed a reversion mutation in the former case (wild type = ^{139}CAG^{141}; inoculated = UAG; recovered = CAG), and a pseudoreversion in the latter (wild type = ^{178}GGA^{180}; inoculated = UGA; recovered = CGA). The latter mutation resulted in a substitution of an arginine in place of a glycine codon in ORF-69, but did not alter the coding of ORF-206. The strong selective advantage favoring the absence of a stop codon interrupting ORF-69 emphasizes that efficient ORF-69 expression plays an important role in a successful viral infection of plants.

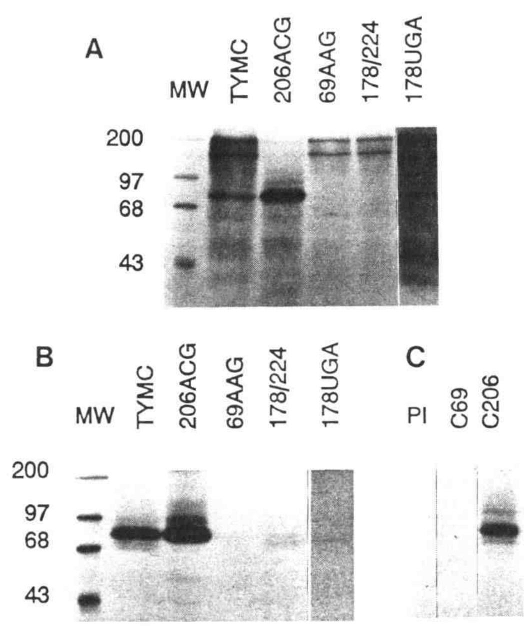


Fig. II.2) *In vitro* translation products and antiserum TYC69-specific immunoprecipitates synthesized from wild-type and mutant TYMC transcript RNAs. A. Rabbit reticulocyte lysates were programmed in the presence of [^{3}H]leucine and 1.7mM Mg^{2+} by the transcripts indicated on each lane. The products were separated by SDS-PAGE (10%). The three major labelled products in lane TYMC are p206, the 150-kDa protein and p69 (apparent Mr 75 kDa). The 70-kDa protein arising from p206 processing is poorly labelled under the conditions used (Bransom *et al.*, 1991). Mr, molecular weight markers. B. Translation products made from the indicated RNAs and shown in A were immunoprecipitated with antiserum TYC69 and protein A-Sepharose, and separated by SDS-PAGE (10%). Careful alignment shows that the bands in lanes 178/224 and 178UGA have migrated further than the major band (p69) in lane TYMC. C. Specificity of immunoprecipitation. Immunoprecipitations were carried out on translations of TYMC with pre-immune serum (PI) or with antiserum TYC69 in the presence of 5µg/ml of either peptide C-69 or C-206.

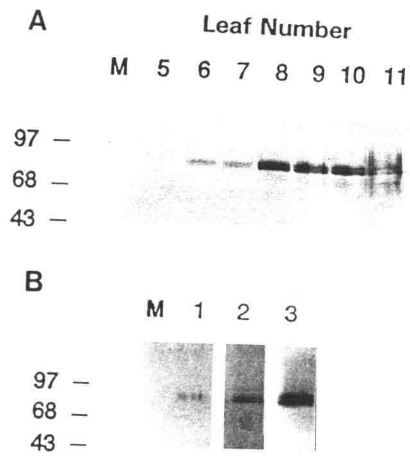


Fig. II.3) Western detection of ORF-69 expression in plants infected with TYMV using antiserum TYC69. A. Expression in Chinese cabbage. Leaves from a plant inoculated with TYMV RNA were separately harvested 11 days post-inoculation and extracted for SDS-PAGE separation and western blotting. Horseradish peroxidase-linked second antibodies and color detection with 4-chloro-1-naphthol were used. Lane headings refer to leaf numbers, which begin with the first true leaf (1). Leaves 1 and 2 were inoculated and leaf 5 was the first symptomatic leaf. M, mock-inoculated. B. Expression in *Arabidopsis thaliana*. Extracts of leaves from a plant inoculated with TYMV RNA (or mock-inoculated, M) were separately analyzed as in A. The extracts were from a mature leaf above the inoculated leaf (lane 1), a half-expanded leaf (lane 2) and a small emerging leaf (lane 3); all three leaves were symptomatic.

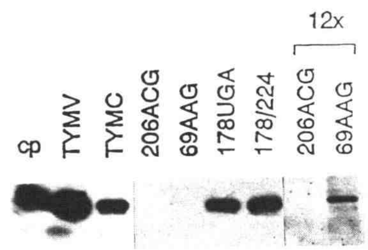


Fig. II.4) Replication of TYMC mutants in turnip protoplasts. Protoplasts were inoculated with the RNA transcripts indicated, or with TYMV virion RNA, and harvested after 24 hr. Extracts of 0.2 x 10^6 protoplasts were separated by SDS-PAGE (14%) and western blots were probed with coat protein-specific antiserum and developed with a horseradish peroxidase-linked chemiluminescent procedure. The lane marked cp contains coat protein marker (50 ng of virions). Other lanes are marked with the name of the inoculated RNA. The last two lanes represent a 12-fold longer reexposure of two of the lanes from the main part of the figure.

DISCUSSION

Expression of ORF-69

Using specific immunological detection, we have shown that ORF-69 of TYMV RNA is expressed efficiently both *in vitro* and *in vivo*. *In vitro* translations directed by wild-type RNA yielded p69, the full-length ORF-69 product, which migrates with an apparent Mr 75 kDa in SDS-PAGE. Other, minor ORF-69 products migrating faster than p69 in SDS-PAGE were also observed. Since these proteins are produced from the double-stop-codon mutant TYMC-178/224 RNA, they must either initiate in ORF-206 and frame-shift at some point to ORF-69, or initiate at non-AUG codons within ORF-69 (Peabody, 1989) shortly downstream of nucleotide 224. The amounts of the minor products can be varied by altering the Mg^{2+} concentration during translation (not shown), suggesting that they arise by artifactual ribosomal initiation (Kozak, 1989). It remains to be determined whether these minor ORF-69 proteins play roles in the viral life cycle or are merely experimental artifacts.

ORF-69 products accumulated *in vivo* to levels readily detectable on western blots. The products were not freely soluble, and required SDS, urea and ß-mercaptoethanol for extraction. ORF-69 products were readily detectable on western blots of TYMV-infected plant extracts made from young symptomatic leaves, but only low levels were detected in older, fully expanded symptomatic leaves. In plants inoculated with clonal sources of virus (TYMC, TYMC-838G), a single ORF-69 product of Mr 75-80K was detected, while plants inoculated with our isolate of TYMV showed two ORF-69 products (Fig. II.3). It could be that our TYMV isolate contains two alleles of ORF-69 encoding two electrophoretic variants, but it is also possible that the two ORF-69 products arise from differential post-translational modification. By mutationally removing a potential N-glycosylation site, we have shown that variable N-glycosylation does not explain the presence of two ORF-69 products, but other modifications may be involved.

Role of ORF-69 products

Although the initation codon mutant TYMC-69AAG showed reduced replication in protoplasts (Weiland and Dreher, 1989: Fig. II.4), mutants with an interrupted ORF-69 but wild-type initiation codons (TYMC-19UAG, -

178UGA, -178/224) supported wild-type levels of replication in protoplasts. All of the above mutants failed to make detectable amounts of p69 *in vitro*. These results indicate that normal replication can occur even when ORF-69 expression is severly interrupted. however, it is premature to conclude that ORF-69 has no role in replication, since the stop codon muants supported the synthesis of low amounts of the faster migrating ORF-69 products *in vitro*. The presence of extensively overlapping ORFs, which preclude a deletion analysis, has complicated these investigations. The poor replication of TYMC-69AAG (Weiland and Dreher, 1989; Fig. II4) is probably not due to interference with ORF-69 expression, but may be the result of a direct effect on RNA replication by (+) strand promoter mutantion, or or to a perturbation of ORF-206 expression resulting from an altered nucleotide context surrounding its initiation codon (Weiland and Dreher, 1989; Osorio-Keese et al., 1989).

Despite being capable of RNA replication that supported efficient coat protein synthesis in protoplasts, the above mutants were not recovered after inoculation onto plants unless a mutation that reconstituted an intact ORF-69 occurred after inoculation. The strong selective advantage favoring the absence of a stop codon interrupting ORF-69 emphasizes that efficient ORF-69 expression plays an important role in a successful viral infection. This suggests that ORF-69 products are needed for viral movement in plants, functioning analogously to the so-called movement proteins of other plant viruses (Hull, 1989; Atabekov and Taliansky, 1990). The ORF-69 products have several characteristics in common with movement (or putative movement) proteins of other viruses. These proteins are expressed early in infection (Stussi-Garaud *et al.*, 1987; Albrecht *et al.*, 1988; Shanks *et al.*, 1989; Lehto *et al.*, 1990; van Lent *et al.*, 1990) and in most cases, the level of detectable protein declines steadily as young symptomatic leaves expand fully and mature. Movement proteins (as also the ORF-69 products) are typically difficult to extract from plant tissues, perhaps due to their localization at the cell wall or plasmodesmata (Stussi-Garaud *et al.*, 1987; Hull, 1989). The highly basic nature of the ORF-69 products (p69 has a pI = 12.1) also suggests that they may possess nucleic acid binding properties, as has been demonstrated recently for the movement protein of tobacco mosaic virus (Citovsky *et al.*, 1990) and the putative movement protein of cauliflower mosaic virus (Citovsky *et al.*, 1991).

ADDENDUM TO:

Expression of ORF-69 of Turnip Yellow Mosaic Virus is Necessary for Viral Spread in Plants.

INTRODUCTION

The following addendum reports results obtained in studies on a full set of ORF-69 mutants that were referred to, but not included in the published manuscript with the above title (Chapter II). This section includes additional experiments examining the synthesis of p69 when Mg^{2+} concentrations in the translation mixes are altered. Northern blot analysis of RNA replication in protoplasts for mutant TYMC-178/224 as compared TYMC to is also presented. Finally, the nucleotide sequence analysis of revertant viral RNAs extracted from plants inoculated with mutants TYMC-139UAG and TYMC-178UGA, but displaying systemic symptoms is reported. Unless otherwise mentioned, all procedures, materials and methods were according to those detailed in Chapter II.

RESULTS AND DISCUSSION

Expression of ORF-69 minor products *in vitro* is Mg^{2+}-dependent.

Figure II.5 diagrams the complete set of mutants used in the study addressing the role of ORF-69 in TYMV RNA replication and virus infectivity. The alterations were designed to affect the codign only in the reading frame in question, and not the coding in the overlapping ORF.

Immunoprecipitations of total products from cell-free translations of wild type and ORF-69 mutant RNA's confirm that the Mr 75 kDa protein is the full-size product encoded by ORF-69 (Fig II.6A,B; Chapter II). The major protein species precipitated by 69C antiserum from translations programmed with TYMV, TYMC and 206ACG (ORF-206 initiator AUG->ACG) is Mr 75 kDa. No Mr 75 kDa protein was produced in translations programmed with ORF-69 mutants. The results are consistent with the disruption of ORF-69 expression by interfering with translation inititation (for TYMC-69AAG and TYMC-69AGG) or by premature translation termination (for the stop codon mutants).

Several products of lower abundance, but with a faster migration than the 75 kDa protein during SDS-PAGE, were specifically precipitated by TYC69 antiserum from both mutant and wild type translation mixes (Fig II.6B; Chapter II). The faster migrating species may be products resulting from translation initiation in ORF-69 at non-AUG codons or from ribosomal frameshifting into ORF-69 following translational initiation in a reading frame other than ORF-69. Previous work has characterized a hierarchy for the initiation of translation at non-AUG codons *in vitro* (Peabody, 1989). In rabbit reticulocyte lysate translation systems, ACG and CUG codons could be used to initiate translation at rates of 80% relative to that of AUG, when these codons were substituted for the natural initiator AUG in a mouse dihydrofolate reductase mRNA. Codons AAG and AGG were the poorest codons (17% relative to AUG) from which translation could be inititiated in this assay.

Magnesium (Mg^{2+}) has been shown to influence the efficiency of translation initiation *in vitro* from alternative AUG or non-AUG start sites (Atkins et al., 1974; Kozak, 1989b). It has further been suggested that adjusting Mg^{2+} concentrations in cell-free translation systems can often result in gene expression *in vitro* that mimics expression *in vivo* (Kozak, 1989b). To test whether Mg^{2+} concentrations affected the production of the smaller products shown to react with TYC69-antiserum, or indeed led to the expression of ORF-69 *in vitro* mimicking the expression *in vivo*, $Mg(OAc)_2$ concentrations were altered in the translations of TYMV and TYMC transcript RNAs. Varying the concentrations of $Mg(OAc)_2$ altered the overall yield of translation products (Fig II.7A), and a clear shift towards the production of a protein the size of full-size p69 was seen when translation was carried out in the presence of increasing concentrations of Mg^{2+} (Fig II.7B). Whether or not the multiple forms of protein reactive with the TYC69 antisera are related to the doublet seen on Western blots of TYMV-infected plants (Fig. II.3) is not known. Consistent alignment of the bands observed on SDS-PAGE fluorographs with the bands detected by western blotting was not possible.

Replication of point mutants in protoplasts.

As shown in Chapters I and II point mutant 69AAG which has an altered ORF-69 initiation codon, fails to direct the synthesis of the Mr 75 kDa protein in cell-free translations and replicates to ~2% of wild type in Chinese cabbage protoplasts (Weiland and Dreher, 1989; Chapter II). Additional stop codon mutants in ORF-69 were constructed for reasons presented in Chapter II, and a western blot showing the accumulation of virus coat protein for the additional mutants not presented in Chapter II is shown in Fig II.8A.

All stop codon mutants tested in Chinese cabbage or turnip protoplasts replicated to levels similar to wild type (Fig II.8A). Replication levels were determined by the accumulation of coat protein on western blots of harvested protoplast extracts. In addition northern blot analysis confirmed wild type levels of progeny plus-strand, template minus strand and subgenomic RNA accumulation for inoculations of mutant TYMC-178/224 (Fig II.8B).

Variability in the signal between inoculations of a clone is controlled for by performing multiple inoculations of a clone within a single experiment. During the course of the thesis work, this researcher found turnips (cv "Just Right") to be a more consistent source of high quality protoplasts for inoculations, and the accumulation of viral RNA in these isolated protoplasts as compared to protoplasts from Chinese cabbage plants appeared slightly higher as well (Fig. II.10). Turnip protoplasts were

Fig. II.5) Map of TYMC and ORF-69 mutants used in this study. A) General map of pTYMC showing the T7 RNA polymerase promoter (dark arrow) and the Hind III site used to linearize template DNA prior to transcription. Black bars symbolize proteins p69, p206 and the virus coat protein encoded in the genomic RNA. The ..GKT.. and ..GDD.. tri-amino acids represent regions implicated in nucleoside triphosphate binding and RNA dependent RNA polymerase activity, respectively, and the open diamond marks the site where p206 is cleaved by a virus encoded protease. B) ORF-69 substitution mutants in pTYMC. Open bars are ORFs and the central line represents the transcript RNA. Inverted triangles represent AUG codons in ORF-69. In two initiation codon mutants (69AAG, 69AGG) the AUG initiating ORF-69 has been changed to AAG and AGG, respectively. The AUG initiating ORF-206 has been changed to ACG in mutant 206ACG. Nucleotide substitutions introduced into ORF-69 that create stop codons are shown with the nucleotide altered presented in italics and numbered as to the position of the change in the genomic RNA sequence. The stippled region denotes the blocking of sequence expression downstream of the introduced mutations. None of the mutations alter the amino acids encoded in the overlapping reading frame.

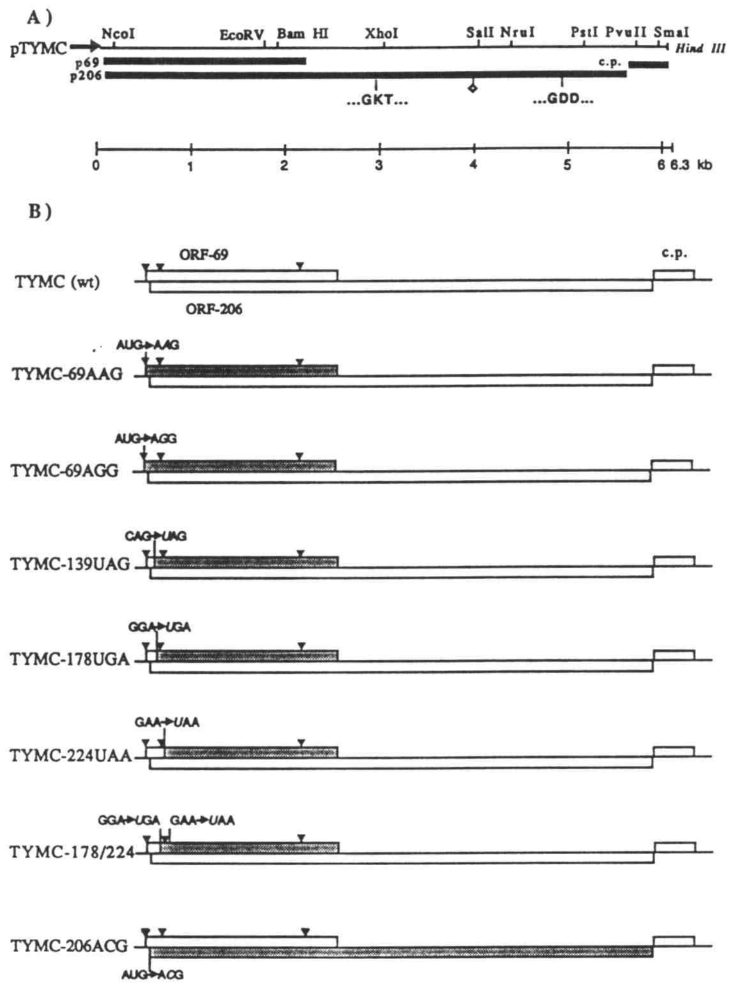

A)
pTYMC
NcoI
EcoRV
Bam HI
XhoI
SalI
NruI
PstI
PvuII
SmaI
Hind III
p69
p206
c.p.
...GKT...
...GDD...
0
1
2
3
4
5
6
6.3 kb
B)
ORF-69
c.p.
TYMC (wt)
ORF-206
AUG→AAG
TYMC-69AAG
AUG→AGG
TYMC-69AGG
CAG→UAG
TYMC-139UAG
GGA→UGA
TYMC-178UGA
GAA→UAA
TYMC-224UAA
GGA→UGA
GAA→UAA
TYMC-178/224
TYMC-206ACG
AUG→ACG

Figure II.5

subsequently used in all investigations in Chapters III and IV, unless otherwise indicated.

The combined western blot and northern blot results for inoculations involving TYMC, TYMC-69AAG (Chapter I) and TYMC-178/224 supports the use of coat protein accumulation in these western blots as a measure of the replication levels for all mutants tested. The accumulation in protoplasts of virus-specific RNAs for the TYMC-178/224 mutant (which was similar that for to TYMC) as compared to the ORF-69 initiation codon mutants (which replicate 50-fold lower than TYMC) underscores the importance of using a number of mutants harboring changes at multiple distant sites when attempting to assess the involvment of a gene in a specific biological process. From the results in Chapter II and those presented here, the expression of the full-size ORF-69 product appears to be dispensible for TYMV replication in isolated plant cells.

Point mutants fail to replicate *in planta.*

Plant protoplasts infected with TYMV are potent inoculum for infecting Chinese cabbage or turnip plants, probably due to the infectious nature of mature virus as compared to purified viral RNA. Protoplasts infected with TYMC and mutant RNAs were therefore used in the inoculation of Chinese cabbage and turnip plants. With the exception of two plants in at least 3 trials (three plants per clone) using detectably infected protoplasts, no symptoms arose on plants infected with ORF-69 stop codon mutants (see below). The results of these trials are tabulted in Table II.1. In all cases plants inoculated with TYMV or TYMC RNA inoculated protoplasts became infected and produced wild type lesions and systemic symptoms. A similar result was obtained in at least 5 inoculation trials (3 plants per clone) with naked TYMV, TYMC and ORF-69 mutant RNA's, but no infection was ever generated by the ORF-69 mutant RNAs in these inoculations.

It was possible that the ORF-69 mutants were infecting plants locally or systemically but were symptomless. To test this possibility, tissue from inoculated, symptomless leaves and young expanding tissue was assayed for viral coat protein several weeks post-inoculation. No viral antigen was ever detected from these symptomless plants, and no evidence of viral lesions of any kind were ever observed on inoculated leaves.

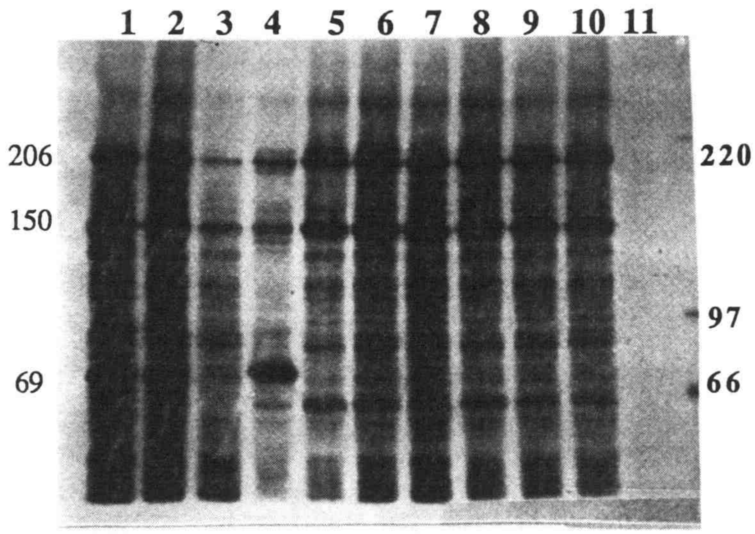
Recovery of point revertents from symptomatic plants

In two cases alluded to above and in Chapter II, Chinese cabbage plants inoculated with protoplasts infected with TYMC-139UAG and TYMC-178UGA RNAs developed systemic symptoms. Symptoms only appeared on one of the three plants inoculated within a trial for both mutants. Although delayed in onset (12-14 days versus the normal 7-10 days post-inoculation), the symptoms from these infections were indistinguishable from wild type.

Virus was prepared from these plants and viral RNA subsequently purified using standard methods (Lane, 1986). The 5'-end 0.6 kb were reverse transcribed and amplified by PCR using the conditions described in Chapter III. The oligonucleotides used in the analysis were TYSEQ526(-) (for reverse transcription) and TYMV5'(+) + TYSEQ526(-) for PCR amplification. Nucleotide sequence analysis of PCR products revealed that the 139UAG mutant had reverted back to CAG, thereby restoring both the wild type amino acid and full ORF-69 (Fig. II.9). Mutant 178UGA, however, had been replaced by a pseudorevertant [wild type GGA(Gly)->mutant UGA(Stop)->pseudorevertant CGA(Arg)]. Although an arginine codon replaced the original glycine codon, a full ORF-69 was restored and the virus was able to move systemically throughout the plant, indicating the viable replacement of glycine by arginine at this position. The nucleotide changes presumably resulted from polymerase error during replication of the RNA. Passaging of symptomatic tissue for these two cases onto healthy plants resulted in the appearance of symptoms indistiguishable from wild type inoculum. The recovery of both revertants underscores, therefore, the importance of a functional ORF-69 in producing systemic, symptomatic TYMV infection.

Fig. II.6) Cell-free translation of TYMV RNA, and TYMC and mutant transcript RNAs. A) Translation products (2 µl) were fractionated by 7% SDS-PAGE and fluorographed. Translations wre programmed with TYMV RNA (lane 1), and transcript RNA of TYMC (lane 2,3), TYMC-206ACG (lane 4), TYMC-69AAG (lane 5), TYMC-69AGG (lane 6), TYMC-139UAG (lane 7), TYMC-178UGA (lane 8), TYMC-224UAA (lane 9), TYMC-178/224 (lane 10) and no added RNA (lane 11). Positions of molecular weight standards are marked in bold type and positions of TYMV encoded proteins 206, 150 and 69 are labelled. B) Proteins from 15 µl of translation were immunoprecipitated with TYC69 antiserum as described in the manuscript. After separation of precipitated proteins by 7% SDS-PAGE, the gel was fluorographed. Total translation of TYMV RNA was included on the gel (lane 1) as were immunoprecipitations from translations programmed with TYMV RNA (lane 2), and transcript RNAs from TYMC (lane 3), TYMC-206ACG (lane 4) TYMC-69AAG (lane 5), TYMC-139UAG (lane 6), TYMC-178UGA (lane 7), TYMC-224UAA (lane 8), and TYMC-178/224 (lane 9).

A)



B)

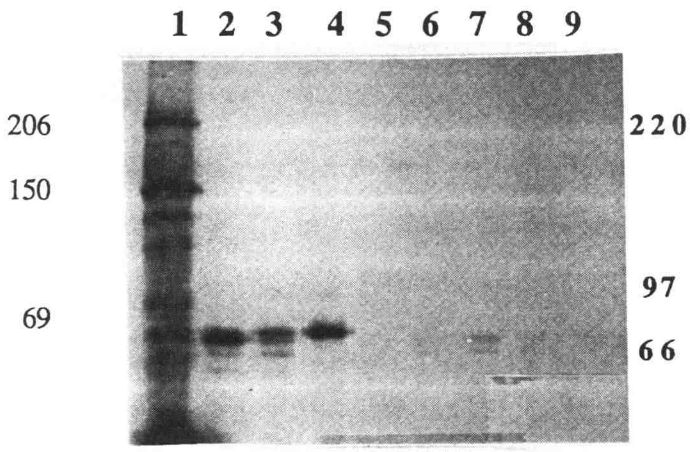


Figure II.6

Fig. II.7) Dependence of ORF-69 product synthesis on Mg^{2+} concentration. Translation products were fractionated by 7% SDS-PAGE and fluorographed. A) TYMV RNA (lanes 1-3) or capped TYMC transcript RNA (lanes 4-6) was translated in a rabbit reticulocyte lysate with $Mg(OAc)_2$ adjusted to 1.1 mM (lanes 1 and 4), 1.5 mM (lanes 2 and 5) or 3.5 mM (lanes 3 and 6) concentration in the reaction mix. B) The same reactions as in A, following immunoprecipitiation with TYC69 antiserum. Note the decreased abundance of faster migrating cross-reactive proteins with increased Mg^{2+} in the synthesis reaction. Molecular weights of marker proteins are in bold type on the left of the picture, and the position of the p69 is in plain type on the right of the picture.

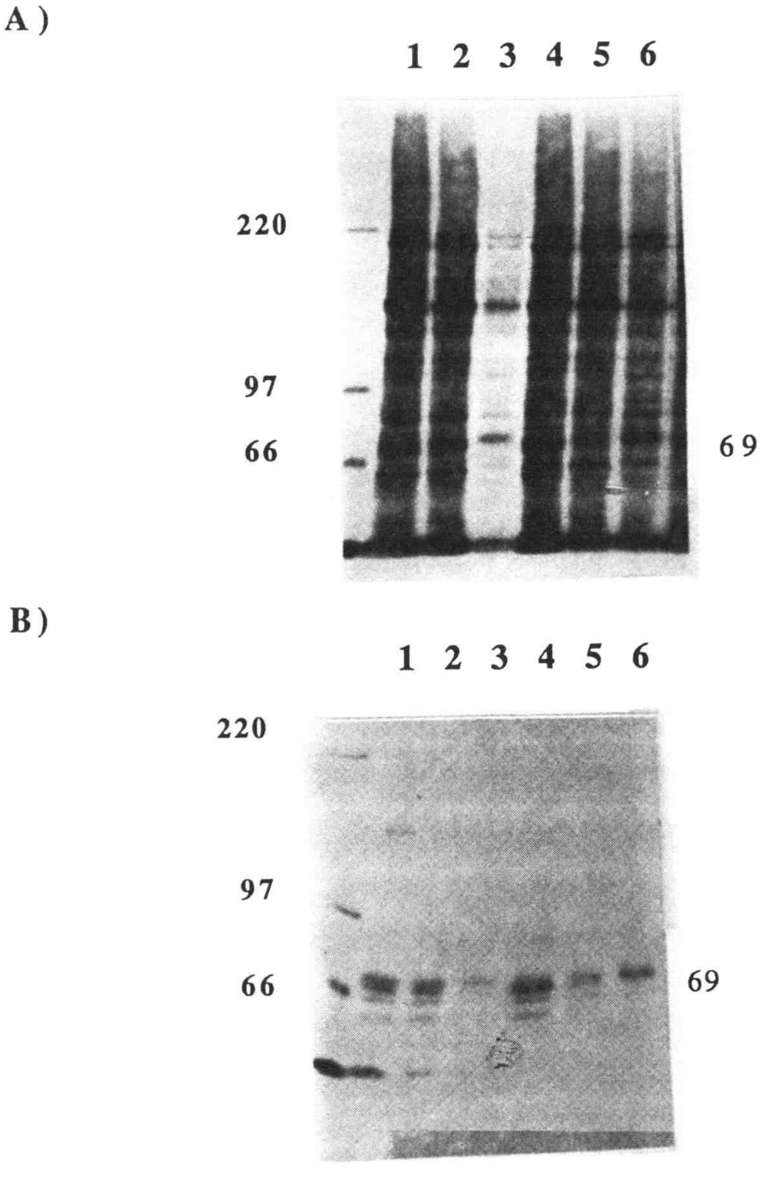
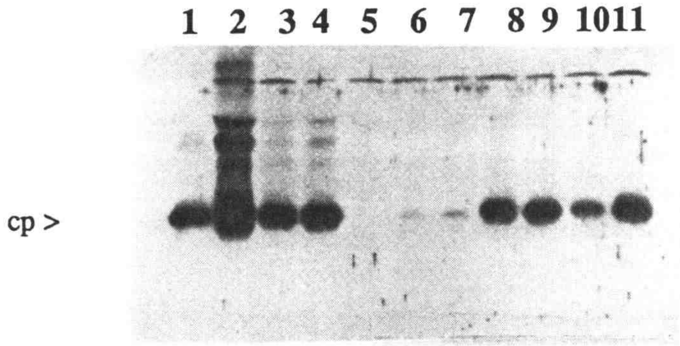


Figure II.7

Fig. II.8) Replication of ORF-69 mutants in turnip protoplasts. A) Protoplasts (4 X 10^5) were inoculated with TYMV (2 μg) or TYMC transcript (5 μg) RNAs. After harvesting, cell extracts were separated by 14% SDS-PAGE, western blotted as described in the manuscript and incubated with anti-TYMV antiserum. TYMV virions (30 ng) were loaded as a control (lane 1), and extracts from cells inoculated with TYMV RNA (lane 2), transcript RNA from TYMC (lanes 3 and 4, two separate preparations), TYMC-206ACG (lane 5), TYMC-69AAG (lane 6), TYMC-69AGG (lane 7), TYMC-139UAG (lane 8), TYMC-178UGA (lane 9), TYMC-224UAA (lane 10), TYMC-178/224 (lane 11). The "cp>" marks the postion of the coat protein on these blots. B) Comparison of TYMC RNA to TYMC-178/224 in the accumulation of viral RNAs in protoplasts. Total nucleic acids extracted from protoplasts were separated by denaturing agarose gel electrophoresis and blotted to a nylon membrane. The membrane was hybridized to a radiolabelled RNA probe complementary to the TYMV 3'-end terminal 259 nts. This gel shows the result of two independent experiments (lanes 3-4, and 5-8). TYMV RNA (50 ng) was loaded as a control (lane 1) as was TYMC-178/224 RNA (10 ng; lane 2). Extracts from protoplasts inoculated with TYMC RNA (lanes 3, 5 and 6) or TYMC-178/224 (lanes 4, 7 and 8). The g and sg mark the positions of the genomic and subgenomic RNAs, respectively.

A)



B)

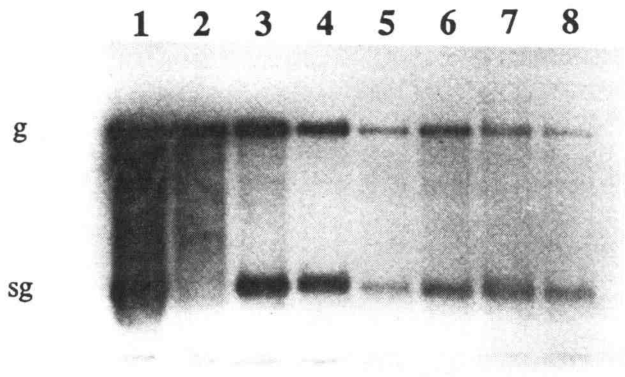


Figure II.8

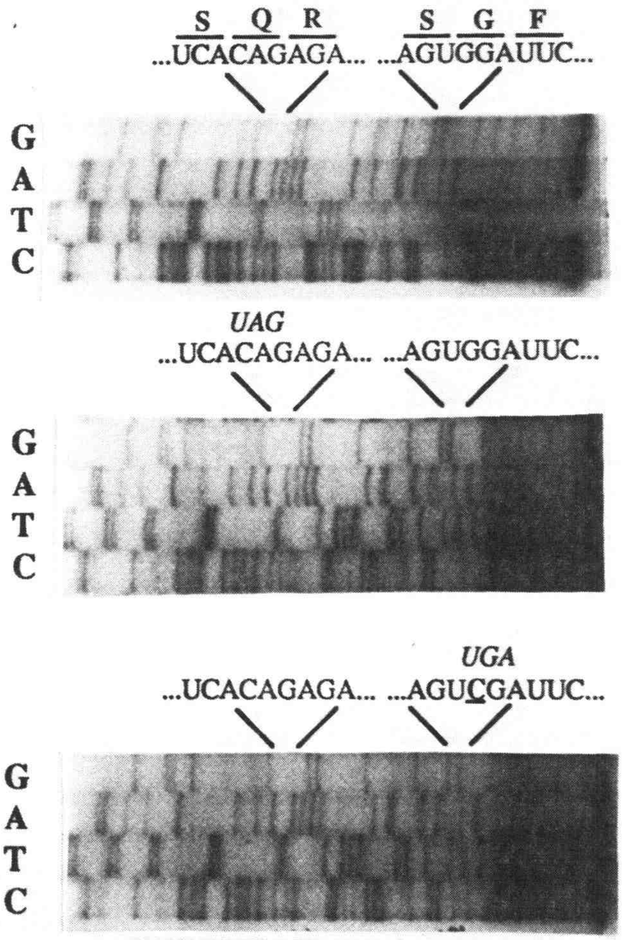


Fig. II.9) Sequence analysis of viral RNAs extracted from systemically infected leaf tissue in plants inoculated with protoplasts that were infected with RNAs TYMC-139UAG and TYMC-178UGA. An extract of total nucleic acids were prepared from infected tissue as described for northern blot analysis, and the RNA was reverse transcribed after priming with an oligomer complementary to sequences between nucleotides 526 and 545 in the 5' region of the genomic RNA. PCR amplification followed using this same oligomer and an oligomer homologous to TYMV RNA 5'-end terminal nucleotides. The resultant DNA fragments were sequenced using standard chain terminating methodologies. Treatment of infected tissue from inoculations with TYMC, TYMC-139UAG and TYMC-178UGA and the corresponding sequences are shown. The wild type sequence in TYMC is the plain type and the italicized type represents the stop codon created by the mutation. The underlined "C" is the reverted postion present in the viral RNA from the infected tissue.

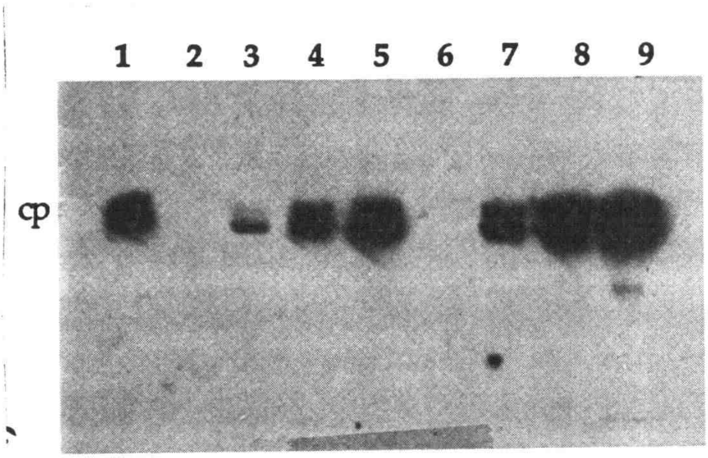


Fig. II.10) Infection of Chinese cabbage and turnip protoplasts with TYMV RNA. Protoplasts (10^6) from each plant type were inoculated and incubated for 20 hr at 25°C under constant light. Extracts from harvested protoplasts were separated by 14% SDS-PAGE, electroblotted to nitrocellulose and reacted with antisera as described in Chapter II. The blot was exposed to X-ray film after incubation in chemiluminescenent substrates of horseradish peroxidase. TYMV virions (30 ng, lane 1) were loaded as a control, followed by extracts from cells inoculated with sterile water (lanes 2,6), and with 0.05 µg (lanes 3,7), 0.5 µg (lanes 4,8) and 5 µg (lanes 5,9) of TYMV RNA. Lanes 2-5 and 6-9 represent extracts from Chinese cabbage and turnip protoplasts, respectively. The "cp" indicates the position of the cross-reactive coat protein on the blot.

Table II.1) Summary of ORF-69 mutants in cell-free expression and infectivity assays.

	Translation *in vitro*		Infectivity *in vivo*	
	p69 PRODUCT[1]	p206 PRODUCT[2]	PROTOPLAST INFECTION[3]	PLANT INFECTION[4]
TYMC	+	+	+	+
TYMC-206ACG	++	-	-	-
TYMC-69AAG	-	+	(+)	-
TYMC-69AGG	-	+	(+)	-
TYMC-139UAG	-	+	+	(-)
TYMC-178UGA	-	+	+	(-)
TYMC-221UAA	-	+	+	-
TYMC-178/221	-	+	+[a]	-

1 Represents immunodetection of full-size p69 in at least 3 independent rabbit reticulocyte lysate translations for each mutant listed. A "+" means detectable at the level produced in translations of TYMC transcript RNA, and a ++ indicates higher accumulation of the p69 product than observed in translation of TYMC transcript RNA.

2 Proteins characteristic of ORF-206 expression (p206 and the 150 kDa protein). A "+" indicates expression of both proteins to levels similar to that observed in the expression of TYMC RNA for at least 3 independent translations.

3 Replication of RNAs in Chinese cabbage (4 reps) or turnip (3 reps) protoplasts as estimated by western blot analysis of accumulated virus coat protein. A "+" indicates replication similar to that of TYMC (within a factor of 2) in at least 4 independent trials after a 24 hr incubation. A "(+)" indicates replication at ~2% that of TYMC.
a Indicates parallel results in both western and northern blot analysis for at least 4 independent inoculations.

4 Generation of infection in Chinese cabbage or turnip plants. A "+" denotes symptomatic, systemic infection characteristic of TYMV. A "-" indicates no systemic or local symptoms in at least 25 plants (16 Chinese cabbage and 9 turnip for each mutant) inoculated, and no detectable coat protein on inoculated or systemic leaves in these cases. The "(-)" indicates one case (out of 25 plants) for each mutant noted where reversion to wild type symptoms occurred (see chapter II).

CHAPTER III

Disruption of native coat protein synthesis debilitates long distance transport of TYMV in infected plants.

ABSTRACT

A frameshift mutant (TYMC-5985^{fs}) and a deletion mutant (TYMC-Δ5708-6062) were constructed in the coat protein gene of pTYMC, an infectious cDNA clone of TYMV. The sequence alterations in the frameshift mutant create a truncated coat protein (cp) open reading frame (ORF) lacking 46% of the codons in the 3' end of the gene. The deletion mutant lacks 354 nucleotides (nts) of sequence within the cp ORF, and has the 5' end 21 codons and the 3' end 49 codons of the ORF fused in-frame. Cell-free translation confirmed that the mutant RNAs were competent for directing the expression of the nonstructural proteins essential for virus replication. In inoculated plant protoplasts, both of the cp mutant RNAs induced the accumulation of plus sense genomic RNA to levels 10% of that observed for parallel inoculations with TYMC RNA. The accumulation of minus sense genomic RNAs for both mutants was similar to that for inoculations with TYMC. Although the ratio of accumulated subgenomic RNA (sgRNA) to genomic RNA for the deletion mutant was similar to that observed in infections with TYMC RNA (~1.2), the sgRNA/genomic RNA ratio for the frameshift mutant (~ 0.3) was reduced. Chinese cabbage and turnip plants inoculated with the mutant RNAs developed chlorotic yellow lesions on inoculated leaves typical of infection with TYMC RNA, although the onset of lesion development was delayed (2-3 days). The signal on western blots resulting from the detection of mutant coat protein antigen in infected lesion extracts was at least 100-fold less than that produced by antigen detected in lesions induced by TYMC RNA. The migration of the cross-reactive antigen in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was

consistent with the sizes predicted for the encoded mutant proteins (12.8 kDa and 7.6 kDa for the frameshift and deletion mutants, respectively). For a leaf infected by TYMC-5985^{fs} RNA, cp antigen was detected as far removed from the site of infection as the inoculated leaf petiole. No systemic symptoms were associated with infections of either mutant, and no cp antigen was detected in emerging leaf tissue. Wild type systemic symptoms were generated on a turnip plant inoculated with protoplasts that had been co-inoculated with TYMC-5708-6062 RNA and an ORF-69 mutant RNA (TYMC-178/224). TYMC-178/224 produces wild type levels of cp and viral RNA in protoplasts, but is defective for cell-to-cell movement. Analysis of the viral RNA in the systemically infected tissue showed only the presence of virus expressing the full-size 20 kDa cp. Taken together the data show the following: 1) the synthesis of native TYMV cp is dispensible for viral RNA amplification in plant cells, but the plus/minus ratio of accumulated genomic RNAs is reduced in infections as a result of the mutations, 2) the synthesis of native TYMV cp is not required for the induction of symptomatic lesions on leaves inoculated with the mutant RNAs, and is apparently dispensible for the cell-to-cell movement of the viral RNA, and 3) synthesis of native TYMV cp appears to be required for the efficient systemic translocation of the virus.

INTRODUCTION

Turnip yellow mosaic virus (TYMV) is a plus sense RNA virus infecting the members of the Cruciferae. Extensive biochemical and biophysical studies have been performed on the viral RNA and virus particle respectively (Hirth and Givord, 1988; Matthews, 1991). With the early studies on the virus particle by Finch and Klug (1966), TYMV became one of the first viruses to be characterized by X-ray diffraction and electron microscopy combined.

TYMV is the type member of the plant tymoviruses, and the nucleotide sequences of three isolates of TYMV have been reported (Morch et al., 1988; Keese et al., 1989; Dreher and Bransom, 1991). Three major ORFs are encoded in the TYMV RNA genome (see Fig III.1). ORF-69 is initiated from the first AUG of the genomic RNA and the ORF-69 gene product appears to be involved in the cell-to-cell movement of the virus (Bozarth et al.,1992). ORF-206 encodes proteins with amino acid sequence motifs highly conserved amongst the plus sense RNA viruses, and alignments of these motifs with those in other RNA viruses place TYMV within the Sindbis supergroup of plant viruses (Goldbach et al., 1991). Expression of ORF-206 is required for TYMV RNA replication to occur (Weiland and Dreher, 1989; Chapter IV). Finally, the ORF for the virus cp is encoded at the 3'-end of the genome and the cp is expressed from a sgRNA (Pleij et al., 1976; Guilley and Briand, 1978).

Although virus capsids are necessary to protect virus genomes from degradative extracellular and intracellular environments, a number of viruses have been shown to be competent for replication in the absence of cp production. In several cases where it has been examined, however, the accumulation of plus sense genomic RNA is often (Marsh et al. 1990; Suzuki et al., 1991; Ogawa et al., 1991), but not always (French et al., 1986) reduced in such mutants. Nevertheless the dispensibility of cp sequences in the replication of several plus strand RNA viruses has added new insight into the replication strategies of these viruses. Tobacco mosaic virus (TMV; Dawson et al, 1988), brome mosaic virus (BMV; French et al., 1986), tobacco rattle virus (TRV; Hamilton and Baulcombe, 1989) and barley stripe mosaic virus (BSMV; Petty et al., 1990) are a few of several plant viruses demonstrating the dispensibility of cp sequences on the amplification of viral RNA in plant cells. Furthermore, cp sequences in BMV (French et al., 1986), TMV (Takamatsu et al., 1987; Dawson et al., 1989) and BSMV (Joshi et al., 1990) have been successfully replaced by "reporter" genes, resulting in viruses that express active heterologous genes in place of the cp gene.

We were interested in examining the effects on TYMV RNA replication and virus infectivity of mutations designed to disrupt native cp expression. Using an infectious cDNA clone of TYMV (pTYMC; Weiland and Dreher, 1989), a deletion and frameshift mutant affecting sequences in the cp ORF were constructed and tested for replication in protoplasts and plants. The results suggest that cp mutants in TYMV can replicate in isolated plant cells and induce the formation of infected, chlorotic lesions on inoculated plant leaves, but imply that native cp sequences are required for efficient systemic translocation of TYMV in infected plants.

MATERIALS AND METHODS

Plant materials, virus stocks and deoxyoligonucleotides.

Turnip (*Brassica rapa* cv. Just Right) and Chinese cabbage were grown in a controlled chamber at 21° C with a 16 hr daylength. Plasmid pTYMC, a cDNA clone containing an infectious TYMV sequence, has previously been described (Weiland and Dreher, 1989). TYMC virus is that prepared from an infected plant that was inoculated with TYMC transcript RNA, whereas TYMC transcript represents RNA synthesized *in vitro* from cloned DNA. TYMC virus and viral RNA were prepared by standard methods (Lane, 1986).

Deoxyoligonucleotides used in PCR amplification and sequencing were as follows; their polarity with respect to the TYMC genome is indicated:

Oligo TYMV5'(+)	5'-TACGTAATCAACTACCAATTCC-3'
Oligo TYCSEQ526(-)	5'-ATGGTAATACATCAGG-3'
Oligo TYPCR/MUT-5644(+)	5'-TTCACTACGTCCAGTACCAATCAGCCCCAACATG-3'
Oligo TYMV3'(-)	5'-CTGGTTCCGATGACCCTCG-3'
Oligo TYMUT-96C(+)	5'-GCAAATGAGTAACGGCCTT-3'

<u>Plasmid DNAs, and *in vitro* transcription and translation.</u>

Construct pTYMC-Δ5708-6062 was made by deleting sequences in pTYMC between the *Pvu*II^{5708} restriction enzyme site (*Pvu*II site at nucleotide 5708) and the *Sma*I^{6062} site (Fig III.1). Plasmid pTYMC-5985^{fs} was constructed by end-filling of *Bgl*II-digested pTYMC DNA with DNA polymerase I (Klenow fragment, USB) followed by re-ligation of the blunt ends (Maniatis et al., 1982).

DNA templates were linearized at the unique *Hind*III site and were transcribed as reported (Weiland and Dreher, 1989) to generate capped transcript RNAs. Addition of radiolabelled [α-^{32}P]UTP (0.1 Ci/mmol; DuPont-NEN) to the transcriptions allowed for estimation of transcript yield by scintillation counting. RNAs were translated in a rabbit reticulocyte lysate according to the manufacturers instructions (Gibco-BRL), in the presence of 1.0 mM $Mg(OAc)_2$. Translation mixtures contained transcript RNA at 0.01 μg/μl and 0.33 μCi/μl of [^{3}H]-leucine (130 Ci/mmol, DuPont-NEN) in a reaction volume of 30 μl. Products were separated by SDS-PAGE and fluorographed.

<u>Protoplast and plant inoculation.</u>

Protoplasts were prepared from turnip plants and inoculated as previously reported for Chinese cabbage (Weiland and Dreher, 1989), with minor modifications. Inoculations were carried out in glass culture tubes (Kimax), which had been autoclaved under distilled water, drained and baked at 260°C overnight. Protoplasts were released from turnip leaf tissue as described in Chapter I, and resuspended in 0.55 M mannitol/ 0.1% MES-KOH (pH 5.7) ("MM"). Resuspended cells (4 X 10^5) were added to 1 ml of MM in a 1.5 ml microfuge tube and gently pelleted at 55 X g in a clinical centrifuge for 10 min. The glass culture tubes contained 150 μl of 40% PEG8000/3 mM $CaCl_2$ (pH 5.8). MM was removed from the pelleted cells and transcript RNA (5 μg) was added. The cells and RNA were immediately pipetted into the PEG solution and gently mixed. Aliquots of MM (200 μl) were then added to the mixture and gently mixed until a total volume of 1.6 ml MM had been added. Inoculated cells were incubated on ice for 15 min, gently pelleted and washed in MM. Cells were finally resuspended to 0.5 ml in MM containing complex

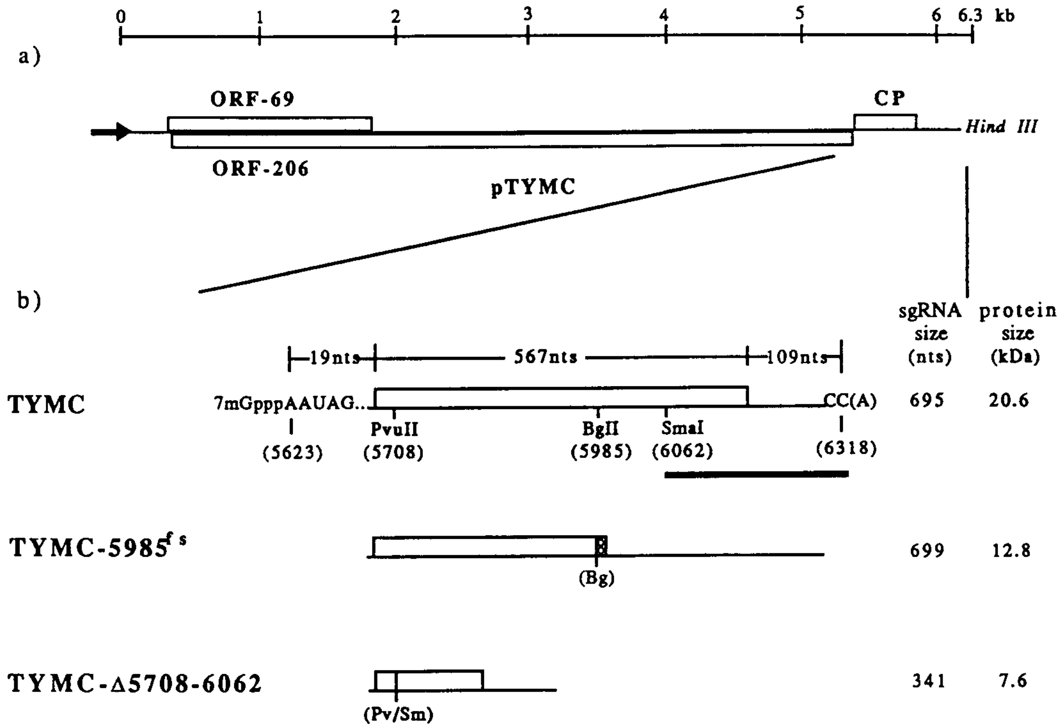


Fig. III.1) Map of TYMC and coat protein mutants. a) ORFs (open bars) of the TYMV genome showing the coding of the coat protein near the 3'-end of the RNA, and ORF- 206 and ORF-69 encoding nonstructural proteins. The arrow represents the T7 RNA polymerase promoter and the italicized *Hind* III site is used to linearize the cloned DNA prior to transcription. b) Coding structure of the TYMC sgRNA showing sizes of the coding and non-coding regions. Restriction sites used in the construction of mutants are marked. Nucleotide numbers in parentheses are relative to the genomic RNA. The "(A)" denotes the non-templated nucleotide on sgRNAs (Guilley and Briand, 1978) putatively added by cellular nucleotidyl transferase. The open bar is the coat protein ORF and the stippled block is the short reading frame after shifting from the coat protein ORF. Predicted sizes of the sgRNAs and coat proteins resulting from the mutations are shown. The solid bar spans sequences present in the riboprobe used in northern blot studies.

salts and antibiotics [0.2 mMKPO_4 pH6.5, 10 mM $CaCl_2$, 1 mM KNO_3, 1 mM $MgSO_4$,1 µM KI, 1 µM $CuSO_4$, 0.3 mg/ml Cephaloridine (Sigma) and 10 µg/ml Gentamycin sulfate (Sigma)] and incubated in the culture tubes for 30 hrs under constant light at 25° C unless otherwise indicated.

Harvested protoplasts or transcript RNA were used to inoculate Chinese cabbage and turnip plants. Protoplasts (1 X 10^5) or RNA (10 µg) were resuspended and diluted with concentrated inoculation buffer (1X IB = 10 mM Tris-HCl pH 7.2, 0.9 mM KH_2PO_4, 3 mM Na_2HPO_4, 14 mM NaCl and 3 mg/ml bentonite) to a final concentration of 1X in 20 µl. Sterile, blunted Pasteur pipets were used to inoculate the outer leaves of 2 week-old Chinese cabbage or turnip plants.

Tissue extraction, and western and northern blotting.

Protoplasts and plant tissue were harvested and extracted using a modification of a procedure which allows for the simultaneous recovery of nucleic acids and proteins (Wolpert and Dunkle, 1983). Protoplasts (3-4 X 10^5) were resuspended in 0.4 ml extraction buffer [TE (10 mM Tris-HCl pH 8.0 and 1 mM Na_2EDTA), including 100 mM NaCl, 1% SDS and 3 mg/ml bentonite], and immediately extracted twice with an equal volume of buffer-saturated phenol/chloroform (Sambrook et al., 1989). Plant tissue was ground in extraction buffer at a ratio of 0.4 ml buffer to 0.1 g fresh tissue, and was subsequently processed in the same manner as the protoplasts. For analysis of lesion extracts, a 10 mm leaf disc was punched which had the lesion at the center of the disc. Each disc was ground in extraction buffer and processed as described for other plant tissues. After phenol/chloroform extraction, total nucleic acids were precipitated from the aqueous phase with ethanol and resuspended in 20 µl TE (for protoplasts) or 60 µl TE (for leaf tissue). A portion (250 µl) of the organic phase from the first phenol/chloroform extraction was mixed with 1.2 ml of cold methanol containing 0.25M ammonium acetate, the mixture was incubated on ice for 15 min and the precipitated proteins were collected by centrifugation. The pellet was washed with cold methanol, dried *in vacuo* and resuspended in protein loading buffer [PLB: 4 X = 62.5 mM Tris-HCl pH 6.8, 8%(w/v) SDS, 20%(v/v) ß-mercaptoethanol, 40% (w/v) sucrose, and 0.2 mg/ml bromophenol blue].

Resuspended proteins were separated by 14% SDS-PAGE. Electrotransfer of separated proteins to nitrocellulose and subsequent antisera incubations were as described (Weiland and Dreher, 1989). Blots were exposed to autoradiographic film after incubation in chemiluminescent substrates of horseradish peroxidase (ECL, Amersham) according to the manufacturer.

Nucleic acids harvested from protoplasts were glyoxalated, electrophoresed in 1% agarose gels (Sambrook et al., 1989) and blotted to Zeta Probe^{TM} nylon membranes as described (Weiland and Dreher, 1989). Hybridization of blots was according to the procedure of Singh and Jones (1984) with minor modifications. The neutralized blot was air dried and then incubated in hybridization solution [HybS: 4X SSPE (pH 7.5) (Sambrook et al., 1989) including 0.2 mg/ml polyanetholesulphonic acid (PAES; Calbiochem), 0.6% SDS, 0.25% sodium pyrophosphate and 100 µg/ml yeast RNA] at 65° C for 2 hr. After prehybridization, the solution was replaced with 10 ml HybS containing 10^6 cpm RNA probe complementary to TYMV 3'-end sequences (Weiland and Dreher, 1989; Fig III.1) and incubated at 65° C for at least 10 hr. Blots were washed in #5 wash [0.25 X SET (7.5 mM Tris-HCl pH8.0, 30 mM NaCl, 0.5 mM EDTA) including 0.1% SDS and 0.1% sodium pyrophosphate] at 65° C and autoradiographed using pre-flashed film. Band intensities on autoradiographs were quantitated by scanning densitometery (Zeineh laser densitometer).

<u>cDNA synthesis, PCR and sequencing.</u>

Tissue from symptomatic emerging leaves was ground in nucleic acids extraction buffer [10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA, 100 mM NaCl, 1% SDS, 3 mg/ml bentonite] in the presence of an equal volume of buffer saturated phenol/chloroform. Total nucleic acids were recovered from the aqueous phase by ethanol precipitation and were resuspended in sterile water. Reverse transcription of a fraction of the nucleic acid was performed using oligomer TYMV3'(-) complementary to the 3'-end 19 nucleotides of the TYMV genome, and (in a separate reaction) with oligomer TYCSEQ526(-) complementary to sequences near the 5'-end of the genomic RNA and priming at nucleotide 526 on the genomic RNA. Reverse transcription reactions (20 µl) contained 5 µg of sample nucleic acid, 50 mM Tris-HCl pH8.3, 20 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each dGTP, dATP, dTTP, dCTP and 5 units of AMV reverse transcriptase (Life Sciences), and were incubated at 42°C for 2 hr. Following cDNA synthesis, the samples were subjected to

PCR using oligomer TYMV5'(+) which corresponds to the TYMV 5' terminal 19 nucleotides, and oligomer TYPCR/MUT-5644(+) corresponding to sequences near the start of the sgRNA and primes at nt 5644 in the TYMC sequence. PCR reactions (100 µl) contained 2 µl of reverse transcription reaction, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton-X100, 1 unit Taq DNA polymerase and 200 ng of each of the opposing primers. Thermocycling parameters were 94°C, 1 min; 55°C, 1 min; 72°C, 3 min., and were performed in a Perkin-Elmer Cetus DNA Thermocycler. Following PCR, the products were analyzed by agarose gel electrophoresis in Tris-borate buffer (Sambrook et al., 1989).

Asymmetric PCR at the 5' sequences employed only the TYCSEQ526(-) oligomer (200 ng) in a 100 µl reaction. Amplification conditions were the same as for the symmetric PCR, and used 10 µl of the dsDNA generated by the above PCR reaction as atemplate in the reaction. The ssDNA products were sequenced using standard methodologies (Sequenase kit, USB).

RESULTS

Expression of coat protein mutant RNAs *in-vitro.*

Experiments have shown that several plus sense RNA viruses can tolerate cp gene disruptions and remain competent for the infection of isolated plant cells (summarized in Takamatsu et al., 1990b). We have examined the possibility that TYMV might also replicate in the absence of native cp synthesis. The TYMV cDNA in clone pTYMC is 6318 nucleotides long (Dreher and Bransom, 1992) and the sgRNA start site is at nucleotide 5624 relative to the 5' end of the viral coding strand (Fig. III.1). The wild type TYMV sgRNA is 695 nucleotides in length: a 19 nucleotide leader sequence is followed by 567 nucleotides coding for the cp and 109 nucleotides of 3'-end non-coding sequence (Guilley and Briand, 1978). An in-frame deletion mutant (TYMC-Δ5708-6062) was constructed, and lacks sequences from the $PvuII^{5708}$ to the $SmaI^{6062}$ restriction sites in the cp gene. The deletion results in the fusion in-frame of the N-terminal 21 codons and the C-terminal 49 codons of the cp gene. Frame shift mutant TYMC-5985^{fs} has 4 additional nucleotides at the BglII site at nt 5985. The mutation cases a shifting of reading frames from that of the cp ORF to a short out-of-frame ORF terminating after 19 codons at codon UGA^{6045}.

Cell-free translation of wild-type and mutant RNA's were performed to ensure the functionality of the nonstructural ORFs in TYMC (Fig III.2). Protein products characteristic of wild type ORF-206 and ORF-69 expression *in vitro* were synthesized from the mutants similar to that for TYMC. Barring any unforseen silent mutations in these ORFs accruing from the cloning procedure, the mutant clones possess the non-structural protein coding information required for RNA replication. Since genome-length RNA is a poor mRNA for the synthesis of cp *in vitro,* mutant cps were not observed in these translations. Proteins of the expected sizes for the mutant cps, and cross-reactive with anti-TYMV antiserum are observed in lesions generated on leaves by inoculation with the cp mutant RNAs (FigIII.4)

<u>Replication of coat protein gene mutants in plant protoplasts.</u>

Capped RNAs transcribed from linearized templates were inoculated onto turnip protoplasts and products of virus replication were subsequently analyzed. Northern blot analysis confirmed the production of plus and minus strand genomic length RNA, and sgRNA for inoculations with TYMC-Δ5708-6062 and TYMC-5985^{fs} (Fig III.3). The accumulation of positive strand RNA was ~10% for the cp mutants relative to TYMC as determined by scanning densitometry of autoradiographs (Fig. III.3B,C). Interestingly, the levels of minus-strand RNA accumulation were similar between wild-type TYMC, TYMC-Δ5708-6062 and TYMC-5985^{fs} infections (Fig III.3A). The ratio of accumulated sgRNA/genomic RNA for the TYMC-Δ5708-6062 mutant was similar to that in infections with TYMC RNA (~1.2), but the amount of sgRNA produced in TYMC-5985^{fs} infections was visibly reduced (Fig. III.3B,C). Consistent detection of mutant cp on western blots of infected protoplast extracts was not possible (not shown) as accumulation of the mutant cps in protoplasts inoculations was probably near the threshold of detection. The detection of these proteins in inoculated leaf lesions by anti-TYMV antiserum was estimated to be less than 100 fold below that for the presence of wild type cp in infected lesions. The reduced detectability may be the result of lost epitopes for antibody binding on the mutant cps, the reduced accumulation of the mutant cps in the cell, or both. Antibody binding probably occur at N-terminal amino acid sequences in the mutant cps, since exposed epitopes of TYMV virions include the cp N-terminus (Quesniaux et al., 1983).

Infection of plants with coat protein gene mutants.

Inoculation of Chinese cabbage or turnip plants with TYMC-Δ5708-6062 or TYMC-5985^{fs} RNA resulted in the appearance of lesions on inoculated leaves that were similar to those induced by TYMC RNA. Development of lesions (3-5 per inoculated leaf) for these mutants was delayed by 2-3 days as compared to inoculations with TYMC RNA, appearing 9-11 days post-inoculation. Lesions from the deletion and frameshift mutants were individually harvested and extracted in order to examine the accumulation of virus cp antigen. Western blots of extracts from lesions infected with TYMC-Δ5708-6062 or TYMC-5985^{fs} infected lesions showed the presence of cross-reactive protein of a size consistent with the alteration made in the cp gene (Fig III.4A).

No systemic symptoms were seen on at least 20 plants displaying lesions from TYMC-Δ5708-6062 or TYMC-5985^{fs} inocula, and no cp antigen was detected in young expanding leaf tissue. Although western blots indicated that the detection of the mutant cps was reduced relative to wild type size cp, mutant cp was readily detectable in lesions generated by inoculation of the mutant RNAs. It would seem likely that if viral spread had translocated to young, rapidly dividing tissue, detection of mutant cp should not be problematic. Nevertheless, it is possible that these plants were in fact systemically infected, but at levels below detection on western blots.

To test the extent to which mutant cp could be detected in the infected plant, leaf lesions infected with TYMC-5985^{fs} and connected plant tissues were harvested and analyzed for the presence of cp antigen. Tissues extracted to test for the presence of virus antigen included the inoculated leaf midrib, inoculated leaf petiole, plant stalk adjoining the petiole, root tissue immediately below the plant stalk and a young expanding leaf. Virus antigen was detected in the inoculated leaf lesion, leaf midrib and leaf petiole (Fig III.4B). No antigen was detected in stalk, root or young leaf tissue. The results imply that viral infection for the cp gene mutants progresses only as far as the inoculated leaf petiole.

Systemic symptoms are accompanied by the apparent regeneration of wild type virus.

Attempts were made to restore the ability of the cp mutants to spread systemically *in planta* by complementation using a second co-infecting RNA. Mutant TYMC-178/224 encodes a disrupted ORF-69 that is disrupted by termination codons positioned in the 5' end of the ORF. The mutant replicates to wild-type levels in isolated plant cells but does not appear to move cell-to-cell in inoculated plants (Bozarth et al., 1992; Chapter II). Mixtures of TYMC-Δ5708-6062 RNA and TYMC-178/224 RNA were inoculated onto plants using various inoculation schemes. In at least 20 plants involving equimolar mixed RNAs of the two mutants (10 μg each), an average of 3 to 5 lesions per leaf were generated on the inoculated leaves. All lesions resulting from this inoculation scheme, however, contained only TYMC-Δ5708-6062 infection as determined by western blot analysis (not shown).

Since we have observed that infected protoplasts are a more potent inoculum than naked transcript RNA for infecting plants, complementation attempts were made using protoplasts co-infected with TYMC-178/224 and TYMC-Δ5708-6062 as a source of inoculum. In all but one case (out of 18 plants inoculated), the lesions that were induced by the inoculation with infectedprotoplasts contained only TYMC-Δ5708-6062 infection as determined by western blot analysis.

In the exceptional case, an unusually high number of lesions appeared on the inoculated leaf (>20 versus an average of 8 per leaf) with a timing similar to that for inoculations with protoplasts infected with TYMC, and systemic symptoms developed on the plant. The inoculated leaf was divided into eight rectangular sections and the tissue harvested and analyzed for viral products. Systemic tissue was likewise harvested, and comparative western blot analysis was performed on the samples using anti-TYMV antiserum. In a fraction of the sections harvested from the inoculated leaf, small amounts of a cross-reactive protein with a size similar to wild type cp were detected (Fig III.5). The predominant protein detected, however, was a cross-reactive species consistent with that predicted for the mutant cp produced from the TYMC-Δ5708-6062 infection. Western detection revealed the products in systemically infected tissue to be exclusively of wild-type size. Sequence determination of TYMV-specific PCR products amplified from the

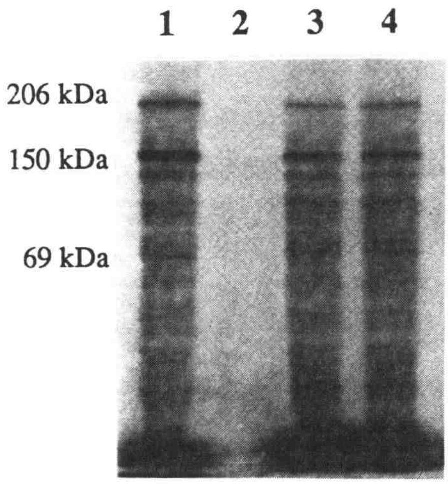


Fig. III.2) Cell-free translations of TYMC and mutant RNAs. RNA (30 ng) was translated in a rabbit reticulocyte lysate (30 µl) in the presence of [^{3}H] leucine, and 2 µl of the reaction was separated by10% SDS-PAGE. The products shown represent translation of ORF-69 (asterisk, p69) and ORF-206 (arrows, p206 and the 150 kDa protein). TYMC RNA (lane 1), no added RNA (lane 2), TYMC-Δ5708-6062 RNA (lane 3), and TYMC-5985^{fs} RNA (lane 4).

Fig. III.3) Replication of TYMC and coat protein mutants in turnip protoplasts. Transcript RNA (5 µg) was inoculated onto 4 X 10^5 turnip protoplasts and incubated for 36 hrs at 25° C. Total nucleic acids harvested from the cells and samples representing 1 X 10^5 cells were northern blotted as described in the text, hybridized to a ^{32}P-labelled probe and the blot was autoradiographed. The g=genomic, sg=subgenomic and sg*=Δ5708-6062 subgenomic RNAs are marked.

A) Detection of minus sense RNAs. A plus sense probe of TYMC 3'-end sequences (5708-5985) was used. Control lanes contained TYMV RNA (10 ng, lane 1) and Δ5708-6062 RNA (10 ng, lane 2). Other lanes were loaded with infected cell extracts from inoculations with TYMC RNA (lanes 3,4), Δ5708-6062 RNA (lanes 5,6), and 5985^{fs} RNA (lanes 7,8). The blot was exposed for 48 hrs with an intensifying screen.

B) The same blot as in (a) but hybridized (without removal of previous label) with a minus sense 3'-end RNA probe (nts 6062-6318). The blot was exposed for 10 hrs without an intensifying screen.

C) Comparative plus sense RNA accumulation for TYMC and coat protein mutant RNAs in protoplasts. Inoculations of 4 X 10^5 protoplasts were performed in triplicate for TYMC and the mutant RNAs. The northern blot was hybridized to radiolabelled minus sense 3'-end sequences. Lane 1, TYMV RNA (10 ng) and extracts from protoplasts inoculated with TYMC RNA (lanes 2-4), TYMC-Δ5708-6062 RNA (lanes 5-7), and TYMC-5985^{fs} RNA (lanes 8-10). The blot was exposed for 18 hr without an intensifying screen.

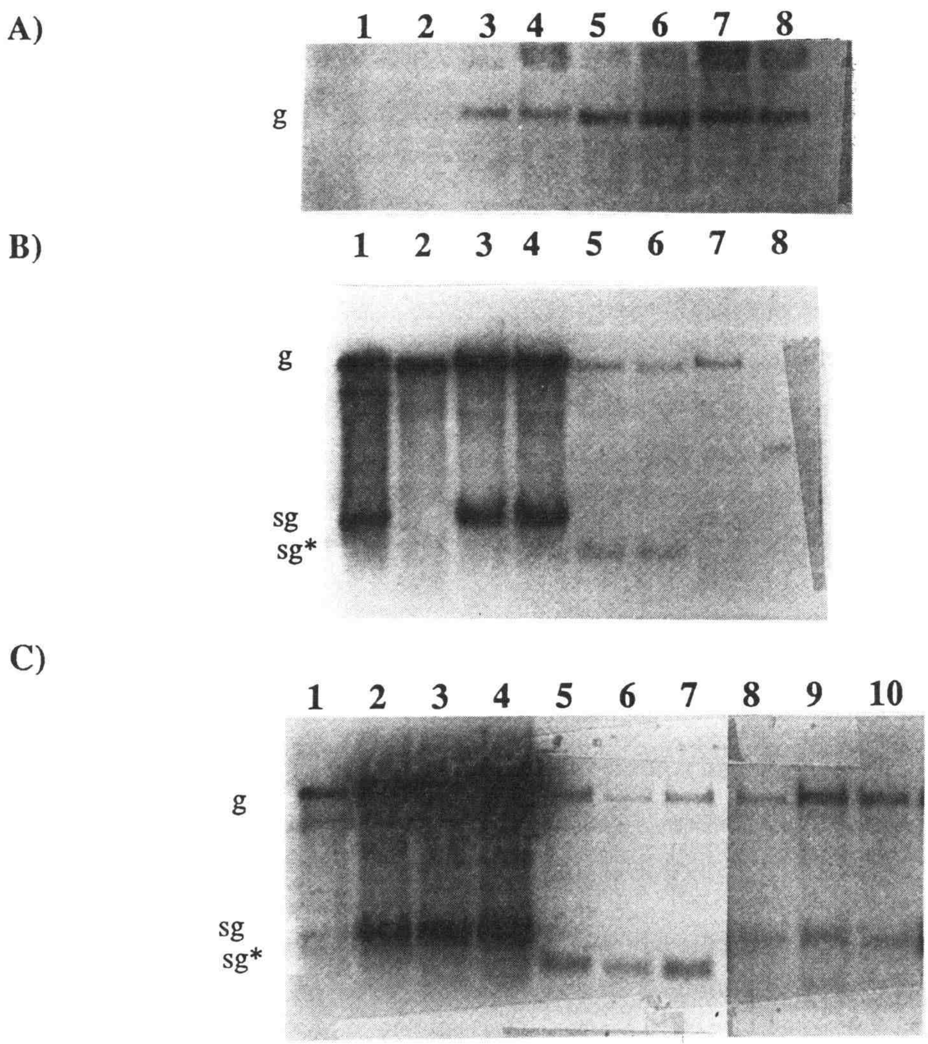


Figure III.3

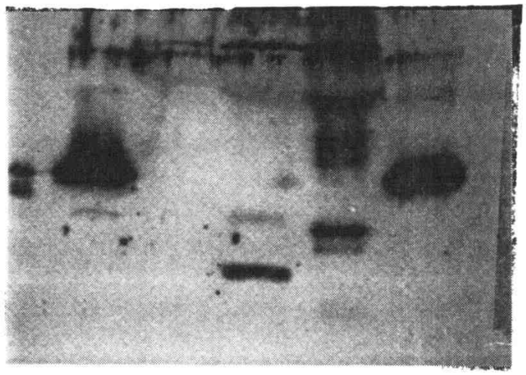
A)

1 2 3 4 5

cp (wt)

cp (5985fs)

cp (Δ5708-6062)



B)

1 2 3 4 5 6 7

*

c p

cp (5985fs)

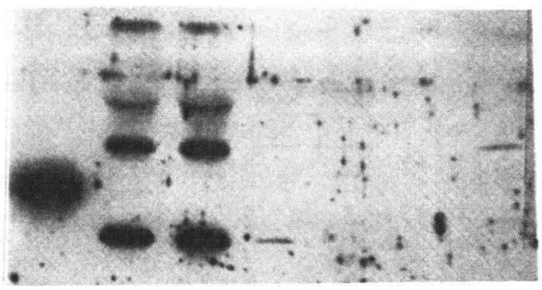


Fig. III.4) Accumulation of coat protein antigen in plants inoculated with TYMC and coat protein mutant RNAs. Transcript RNA (10 μg) was inoculated onto 2 week old Chinese cabbage plants and the resultant lesions were harvested for extraction 21 days post-inoculation. Denatured extract (15 μl) was separated by 14% SDS-PAGE, and western blotted as described in the text. The positions of the wild type and mutant coat proteins are marked.

A) Lanes were loaded with TYMV virus (50 ng; lane 1), and extracts from mock inoculated leaf tissue (lane 2), a TYMC-Δ5708-6062 induced lesion (lane 3), a TYMC-5985fs induced lesion (lane 4), and a TYMC induced lesion (extract diluted 1/10) (lane 5).

B) Accumulation of coat protein antigen in various plant tissues induced by TYMC-5985fs RNA. Lnes contained TYMV virus (30 ng; lane 1), and extracts from a virus lesion (lane 2), leaf mid-rib adjacent to lesion (lane 3), petiole of inoculated leaf (lane 4), plant stalk adjoining inoculated leaf petiole (lane 5), root tissue adjoining stalk (lane 6) and emerging young leaf (lane 7). The slower migrating cross-reactive band (lanes 2,3) may represent dimerized mutant cp.

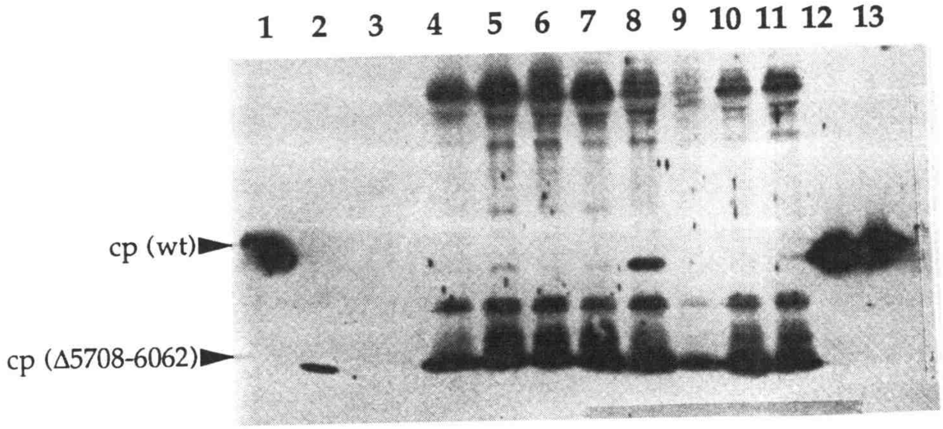


Fig. III.5) Accumulation of wild-type and mutant coat protein in leaf tissue inoculated with protoplasts infected with a mixture of TYMC-178/224 and TYMC-Δ5708-6062 RNAs. A leaf with >20 lesions was divided into eight sections of equal mass, and the proteins were extracted and separated by 14% SDS-PAGE. Following transfer of proteins to nitrocellulose, the blot was incubated with anti-TYMV antisera. Lanes contain TYMV virions (30 ng, lane 1) and extracts from various tissues of the infected plant: a lesion induced by TYMC-Δ5708-6062 alone (lane 2), mock inoculated leaf tissue (lane 3), the eight sections of symptomatic, inoculated tissue from the mixed inoculation (lanes 4-11), systemically infected tissue from the same plant (diluted 1->100; lane 12) and systemically infected tissue from a TYMC-inoculated control plant (diluted 1->100; lane 13). The positions of the wild type and mutant cps are marked.

sample also shows the presence of only wild type sequences (not shown). Re-passaging of this systemically infected tissue resulted in the development and display of symptoms indistinguishable from an infection induced by TYMC.

DISCUSSION

Using an infectious cDNA clone of TYMV RNA we have demonstrated that the expression of native TYMV cp is not required for TYMV RNA replication and sgRNA transcription. An in-frame cp deletion mutant (TYMC-Δ5708-6062) that lacked 354 bases in the cp structural gene performed similar to TYMC at the level of minus-strand accumulation in protoplast infections, but displayed reduced genomic plus strand accumulation (10% relative to that for TYMC). A cp ORF mutant having a frame-shift caused by a 4 nucleotide insertion (TYMC-5985^{fs}) displayed a similar phenotype, indicating that the alteration of +/- RNA ratios is probably a function of the encoded protein, and not the genomic or sgRNA. Curiously, the level of sgRNA relative to genomic RNA was reduced in the frame-shift mutant. This might be explained by the recent finding that the abundance of certain ORF-encoding RNA's is reduced when translation has been prematurely disrupted (Atwater et al., 1990).

Recent experiments on several Sindbis-like RNA viruses have demonstrated the general dispensibility of cp sequences in viral RNA amplification (Nassuth and Bol, 1983; French and Ahlquist, 1986; Takamatsu et al., 1987; Petty et al., 1990a). It has been presumed that the cp serves only in protection of the viral genome RNA. More in-depth investigation, however, has revealed a potential role for the cp gene in the regulation of virus plus and minus strand RNA synthesis . Experimentation in BMV suggests that the removal of cp sequences can cause the normally elevated plus to minus sense RNA ratio to decrease (Marsh et al., 1991c). In addition, elevated absolute levels of minus-sense RNA are produced in infections with cp gene mutants of AlMV, suggesting that the cp plays some role in the shut-off of minus-sense RNA synthesis in this virus (van der Kuyl, 1991). It is noted, however, that AlMV RNA replication exhibits an unusual dependence on the cp in its replication strategy as compared to related viruses (Houwing and Jaspars, 1987). Whether or not the cps of other plus strand RNA virus are involved in the asymmetry of genomic RNA production remains to be seen.

Reports demonstrating the decreased accumulation of plus-sense RNA in infections with cp disruption mutants for other Sindbis-like plant viruses (Suzuki et al., 1991; Ogawa et al., 1991) did not include a quantitative analysis of differences between the accumulation of plus and minus sense viral RNAs generated in infections.

The results presented show that TYMV mutants with disruptions in the cp ORF can replicate in plant cells and form virus lesions. This suggests that, whereas expression of ORF-69 appears to be required for the cell-to-cell movement of TYMV (Bozarth et al., 1992; Chapter II), the expression of native cp is dispensible for this event. The dispensibility of native cp synthesis in TYMV for lesion formation differs from results for CMV, where only one (an N-terminal in-frame deletion mutant) of four cp gene deletion mutants induced local lesions (Suzuki et al., 1991). Although the remaining mutants (which also had frameshifts in the cp C-terminal coding regions) were not competent for lesion formation, they were competent for replication in plant protoplasts, albeit at reduced levels as compared to that induced by wild type infectious transcript RNA.

Neither of the TYMC cp mutants were competent for systemic spread as examined by western blot analysis of emerging leaf extracts sampled from infected plants. Although cp antigen for the TYMC frameshift mutant was detected in the inoculated leaf petiole, this may reflect mutant cp originating from the infected lesions that was subsequently translocated to the petiole. In contrast to these results, TMV (Dawson et al., 1988) and BSMV (Petty et al., 1990) mutants with disrupted cp genes have been reported that can move cell-to-cell in inoculated plant leaves and can translocate systemically in infected plants although less efficiently than wild type virus. It may be that icosahedral viruses (like TYMV, BMV and CMV) have a more strict requirement than the filamentous viruses (like TMV and BSMV) for encapsidation in order to move systemically within a plant.

This notion is strengthened by results reported here for mixed inoculations of the cp deletion mutant and an ORF-69 mutant of TYMC. Both mutants can replicate in isolated plant cells, and both are deficient for plant systemic infection. Only a derivative virus arising from the co-inoculation that had acquired wild type size cp could translocate systemically and generate wild type TYMV symptoms. The result suggests that either recombination between co-inoculated RNAs or double reversion of the

TYMC-178/224 mutant to wild type gave rise to the virus detected in the systemically infected tissue. The lack of complementation in these trials may reflect the inefficiency in the generation of multiple lesions on leaves inoculated with TYMV, since only ~5 lesions were routinely generated by inoculation with RNA transcripts of the cp mutants. The probability that the two potentially complementing RNAs were delivered to the same or adjacent cells, therefore, might have been too low. Alternatively, *trans*-complementation for the defects in ORF-69 or the cp may not readily occur. Transgenic plants separately expressing the TYMV cp and ORF-69 genes should prove useful in determining the extent to which these mutations can be complemented *in vivo*. The *trans* complementation of TMV mutant RNAs for defects in cell-to-cell spread (30K gene; Deom et al., 1987) and long distance transport (cp gene; Holt and Beachy, 1991) by the expression of the corresponding wild type genes in trangenic plants has been described.

It is noteworthy that a potential readthrough ORF accessed by suppression *in vitro* of the ORF-206 termination codon (Morch et al., 1983; Morch et al., 1988; Keese et al., 1989) is altered in both cp mutants. The evidence presented here suggests that this ORF is not required for TYMV RNA replication or virus cell-to-cell movement. Furthermore, no equivalent ORF is present in the genome sequences of at least 4 related tymoviruses (see Ding et al., 1990a). The similarity of the phenotypes of the mutants described above with cp disruption mutants of other plus-strand RNA viruses argues that the affects seen can be attributed to disruption of the cp gene. Nevertheless, experiments will need to be performed in which expression of the readthrough domain, but not the cp ORF, is abolished and the affects of such mutations on RNA infectivity analyzed before a firm conclusion can be drawn.

CHAPTER IV

Efficient replication of synthetic turnip yellow mosaic virus defective RNAs requires the expression of the 150 kDa protein in *cis*.

ABSTRACT

Two highly conserved amino acid sequence elements encoded in the putative replication proteins of Sindbis-like RNA viruses, ..GXGKS/T.. and ..GDD.. , are found in the 150 and 70 kDa proteins, respectively, encoded by TYMV ORF-206. Using an infectious cDNA clone of TYMV (pTYMC) we have shown that RNAs having amino acid substitutions and deletions in or near these domains do not replicate to detectable levels in plant protoplasts. Three classes of ORF-206 mutants were designed: 1) one mutant that would block the expression of both the 150 and 70 kDa proteins, 2) mutants that would express wild type 150 kDa protein, but would produce mutant 70 kDa protein, and 3) mutants that would express wild type 70 kDa protein, but would produce mutant 150 kDa protein. The ability of these defective RNAs to be complemented in *trans* by either a helper genome deficient in coat protein synthesis (TYMC-Δ5708-6062) or by a second defective RNA has been investigated. Replication of the 150 and 70 kDa protein mutants (classes 2 and 3) in the presence of co-inoculated TYMC-Δ5708-6062 helper genome was inefficient, resulting in the accumulation of the defective RNAs and their subgenomic RNAs to reduced levels (<1% - 10%) as compared to RNA accumulation when wild type TYMC RNA was inoculated. The co-replication of the 70 kDa protein mutants was higher than for the 150 kDa protein mutants tested in this assay. No co-replication of a defective RNA designed to block the synthesis of both the 150 and the 70 kDa proteins (class 1 RNA) was detected. Defective RNAs with mutations in the 150 kDa and 70 kDa proteins were also co-inoculated with each other in an attempt to establish an infection by complementation. Co-inoculation of an RNA having an amino acid substitution in the ..GXGKT.. motif (TY-K982S) with an

RNA bearing a substitution in the ..GDD.. motif (TY-G1663R) led to complementation between the RNAs in protoplasts, accumulating progeny RNA and virus coat protein to levels ~1% of that in a parallel TYMC inoculation. Complementation of a deletion mutant lacking most of the 70 kDa protein coding region (TYMC-Δ4086-5406) by TY-K982S resulted in the accumulation of viral products to 50 - 70% the level observed in infections with TYMC. Mutant RNAs designed to express unaltered 150 kDa protein accumulated to greater levels in these mixed infections than RNAs with mutations in the 150 kDa protein. For the complementation between TYMC-Δ4086-5406 + TY-K982S RNAs, a 10-fold greater accumulation of the former mutant RNA over the latter was observed. The data imply that TYMV-encoded replication proteins function preferentially in *cis*, favoring the RNA from which they were translated as a replication template. The poor replication of 150 kDa protein mutants, and the absence of detectable replication for a frameshift mutant in the 150 kDa protein, suggest that this *cis* effect is mediated by the 150 kDa protein.

INTRODUCTION

Turnip yellow mosaic virus (TYMV) is a small plus-strand RNA plant virus infecting the Cruciferae, and is the type member of the plant tymoviruses. The biology of TYMV replication and the biochemistry of the TYMV RNA genome have been recently reviewed (Matthews, 1991).

The 6318 nucleotide (nt) TYMV genome encodes three major ORFs (Fig IV.1). The virus coat protein is encoded at the 3'-end of the genome and is expressed via a subgenomic RNA (sgRNA)(Guilley and Briand, 1978). ORF-69 initiates at the first AUG of the genomic RNA (nt 88) and encodes a protein with a calculated molecular weight of 69 kDa (p69; Morch et al., 1988; Dreher and Bransom, 1992) which is expressed *in vitro* (Weiland and Dreher, 1989) and *in vivo* Bozarth et al., 1992). Expression of the complete ORF-69 is not required for TYMV RNA replication in protoplasts, but appears to play a role in the cell-to-cell movement of the virus (Bozarth et al., 1992).

TYMV ORF-206 initiates at nt 95, extensively overlaps ORF-69 out-of-frame, and encodes a protein of 206 kDa (p206) which is expressed *in vitro* (Morch and Benicourt, 1980; Weiland and Dreher, 1989). During *in vitro* translation of ORF-206, protein p206 is cleaved by a virus encoded protease to generate N-terminal and C-terminal proteins of 150 and 70 kDa, respectively, as estimated by the migration of these proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Morch et al., 1989; Bransom et al., 1991). The 70 kDa C-terminal protein resulting from this processing is not present in stoichiometric amounts with the 150 kDa protein, apparently due to instability of the 70 kDa protein in rabbit reticulocyte and wheat germ translation extracts. The protease responsible for the processing of the 150/70 kDa protein junction has been tentatively mapped to a region between the Bam HI^{1755} and the Kpn^{3248} restriction sites that are encoded in the viral sequences of pTYMC (Bransom et al., 1991).

Amino acid sequence motifs encoded in ORF-206 show high conservation with domains encoded in the nonstructural proteins of other RNA viruses and these similarities place TYMV in the 'Sindbis supergroup' of RNA viruses (Habili and Symons, 1989; Gorbalenya and Koonin, 1989; Koonin, 1991; Goldbach et al., 1991). One sequence element ..GXGKS/T.. (here abbreviated ..GKT..) is encoded near the C-terminus of the 150 kDa protein, and has been found in several prokaryotic and eukaryotic proteins possessing a demonstrated NTP-dependent helicase activity (Gorbalenya and Koonin, 1989; reviewed in Matson and Kaiser-Rogers, 1990). A second element (..GDD..) is encoded in the 70 kDa protein and is considered a signature sequence for RNA-dependent polymerases (Koonin, 1991). Separation of these domains onto individual virus chromosomes (Goldbach et al., 1991) or by proteolysis of a precursor polyprotein (Strauss, 1991) is a common theme in the expression strategies of plus sense RNA viruses.

Studies using infectious transcripts derived from cloned cDNA have demonstrated that the expression of virally encoded proteins bearing these conserved regions is required for RNA replication to occur (French and Ahlquist, 1986; Ishikawa et al., 1986; Weiland and Dreher, 1989; Kroner et al., 1990; Kroner et al., 1991). The replication in the presence of helper virus, however, of defective RNAs that are derived from genomic RNA sequences but that lack the above conserved domains has been reported for several monopartite 'Sindbis-like' RNA viruses (Levis et al., 1986; Raffo et al., 1990; Ogawa et al., 1991; White et al., 1991). The co-replication of genomic RNAs is furthermore obligatory for the propagation of many of the multipartite RNA viruses *in planta* (Dreher and Hall, 1988a; Matthews, 1991). The natural existence of defective RNAs has facilitated studies examining the genetic elements required in *cis* for the replication of viral RNA genomes (see thesis introduction). In the course of our studies on the replication of TYMV it was observed that defective RNAs having in-frame and out-of-frame deletions, as well as frameshift mutations resulting from minute deletions in ORF-206, were not replicated to detectable levels when co-inoculated with wild type helper virus and assayed by northern blot or ribonuclease protection analysis. In an attempt to unravel the basis for this lack of co-replication, we have constructed ORF-206 mutants with amino acid substitutions and deletions in or near the highly conserved domains. Neither of the two mutant types alters the proteolytic cleavage of the 150/70 kDa protein junction *in vitro*.

Here we show that defective RNAs with mutations in the 150 kDa protein or the 70 kDa protein are inefficiently co-replicated in the presence of a helper genome deficient in coat protein synthesis. In contrast to this, an amino acid substitution mutant in the ..GKT.. motif of the 150 kDa protein (TY-K982S) can efficiently complement RNAs bearing deletions in the 70 kDa protein, resulting in the accumulation of viral RNAs and coat protein in protoplasts to 50% the levels in wild type inoculations. The data indicate that the replication of defective TYMV RNA genomes in the presence of a helper genome is less efficient than the replication generated by complementation between two defective TYMV RNA genomes. Defective RNAs expressing native 150 kDa protein are preferentially replicated in these assays, suggesting a *cis*-acting role for the 150 kDa protein in TYMV RNA replication.

MATERIALS AND METHODS

Plant materials, virus stocks and oligonucleotides.

Turnip (*Brassica rapa* cv. Just Right) plants were grown in a controlled chamber at 21° C with a 16 hr daylength. Plasmid pTYMC, a cDNA clone containing an infectious TYMV sequence, has previously been described (Weiland and Dreher, 1989). TYMC virus is that prepared from an infected plant that was inoculated with TYMC transcript RNA, whereas TYMC transcript represents RNA synthesized *in vitro* from cloned DNA. TYMC virus and viral RNA were prepared by standard methods (Lane, 1986).

Deoxyoligonucleotides used in mutagenesis, in polymerase chain reaction (PCR) amplification and in sequencing included the M13 universal sequencing primers and the following TYMC specific primers (the +\- polarity of the oligo is with respect to the TYMV genomic RNA):

Oligo TYPCR/MUT3049(+)	5'-GCTGGCTGTGGG*T*CGACATATCCG-3'
Oligo TYPCR/MUT5071(-)	5'-GTGAGTCGTC*T*C*T*AGAAACCATG-3'
Oligo TYPCR2966(+)	5'-GAACGGATCATCCAGATAG-3'
Oligo TYPCR3330(-)	5'-TGGGAGTACTCGC-3'
Oligo TYPCR/MUT4838(+)	5'-CAAAATCGCAAACGC*G*TACACCGCTT-3'
Oligo TYSEQ5687(-)	5'-TAGAACGGTGGCGACGG-3'
Oligo TYPCR5156(-)	5'-GAGTTCAAGTTTGAAGCG-3'

Plasmid DNAs and *in-vitro* mutagenesis.

Plasmid pTYMC was the parent clone for the derivatives described in this work. Deletion, frameshift and substitution mutants were constructed in pTYMC directly or in subclones of pTYMC (Fig IV.1). Sequence analysis across mutated regions confirmed in each case the sequence of deletion junctions or substitutions that were created. Deletions were constructed as follows with enzymatic manipulations according to Sambrook et al. (1989):

To construct pTYMC-Δ5708-6062, clone pTYMC was digested at the PvuII^{5708} and SmaI^{6062} restriction enzyme sites followed by religation of the blunt ends. To construct pTYMC-Δ210-1759, pTYMC was digested at the NcoI^{210} site followed by fill-in of the 5' overhang with deoxynucleotides in the presence of DNA polymerase I (Klenow fragment; USB). After further digestion of the DNA at the BamHI^{1755} site, the ends were blunted by digestion with mung bean nuclease (Pharmacia) and the plasmid was recircularized. To construct pTYMC-Δ1537-1759, pTYMC was digested at BamHI^{1755} followed by mung bean nuclease blunting of the DNA ends. The DNA was further digested at EcoRV^{1537} and the plasmid recircularized. To construct mutants pTYMC-Δ3279-3283 and pTYMC-Δ4086-4090, plasmid pTYMC was digested at the XhoI^{3279} and SalI^{4086} sites, respectively, the ends blunted with mung bean nuclease and the plasmids recircularized. Mutants pTYMC-Δ4086-4587, pTYMC-Δ4086-5406, and pTYMC-Δ4086-6062 were all made by digestion of pTYMC at SalI^{4086} followed by mung bean nuclease treatment to blunt the ends of the molecules. The DNA was further digested at NruI^{4587} (pTYMC-Δ4086-4587) or SmaI^{6062} (pTYMC-Δ4086-6062) and the DNA recircularized. For pTYMC-Δ4086-5406, the blunted SalI-cut DNA was further digested at the PstI^{5406} site followed by mung bean nuclease blunting of the overhangs and recircularization of the DNA.

Construction of the amino acid substitution mutants pTY-K982S and pTY-G1663R was by PCR-mediated 'megaprimer' site-directed mutagensis (Sarkar and Sommer, 1990). To construct pTY-K982S, subclone pTY-Ba/St which contains pTYMC sequences from the BamHI^{1755} site to the SstI^{3283} site cloned into pUC118 (Vieira and Messing , 1987) was made. This subclone DNA was employed as template in the PCR amplification of a mutant DNA fragment using the M13 universal sequencing primer and oligo TYPCR/MUT3049(+). PCR amplification had a temperature profile of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min for a total of 25 cycles. The mutagenesis directed the alteration of two nucleotides in the pTYMC sequence (the mutant nucleotides in the oligo are italicized) and the substitutions change the amino acid at codon 982 from lysine to serine (Fig IV.1). Plasmid pTY-Ba/St was linearized at the unique HindIII site, and 1 μg of this DNA was mixed with 0.5 μg of mutant fragment DNA. The DNA mixture was adjusted to 1X Taq DNA polymerase buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton-X100) in 90 µl, boiled for 5' and immediately placed at 75°C. A cocktail (10 µl) containing 1X Taq DNA polymerase buffer, 2mM each dGTP, dATP, dTTP and dCTP, and one unit Taq DNA polymerase (Promega) was added to the tube and the reaction was incubated for 40 min at 75°C. After termination of the reaction by the addition of Na_2EDTA to 10 mM, a fraction of this megaprimed template was used as substrate in a PCR reaction. Megaprimed template (2 µl) was added to 100 µl of a reaction containing 1X Taq DNA polymerase buffer, 0.2 mM each dGTP, dATP, dTTP and dCTP, 200 ng each of M13 universal forward and reverse sequencing primers and Taq DNA polymerase (1 unit). The sample was subjected to 25 cycles of PCR as described above. After digestion of the amplified DNA with BamHI and SstI, the DNA was subcloned into pUC118 for sequencing. The efficiency of recovery of the correct mutants was 20% from this procedure and the mutant fragment was resubstituted into pTYMC after complete sequencing.

Construction of the mutant pTY-G1663R employed the same methodology. Subclone pTY-Bg/Pt contains pTYMC sequences between the $BglII^{4579}$ site and the $PstI^{5406}$ site cloned into pUC118. In this case, the mutagenic megaprimer was made by PCR amplification from template pTY-Bg/Pt using the M13 universal reverse sequencing primer and oligo TYPCR/MUT5071(-) as the opposing primers. After after extension of the megaprimer annealed to EcoRI-linearized pTY-Bg/Pt DNA, products of the reaction were used as template in a PCR reaction as described above using the M13 universal forward and reverse primers. After sequence analysis confirmed the presence of the directed mutation and the absence of fortuitous mutations, a subfragment ($NsiI^{4972}$-$PstI^{5406}$) was resubstituted into pTYMC.

Transcription and translation *in vitro*

DNA templates were linearized at the unique HindIII site and were transcribed as reported (Weiland and Dreher, 1989) to generate capped transcript RNAs. Addition of radiolabelled [α-^{32}P]UTP (0.1 Ci/mmol) to the transcriptions allowed for estimation of transcript yield by scintillation counting. RNAs were translated in a rabbit reticulocyte lysate according to the manufacturers instructions (Gibco-BRL). Translation mixtures contained transcript RNA at 0.01 µg/µl and 0.33 µCi/µl of [^{3}H] leucine (130 Ci/mmol;

Fig IV.1) Map of pTYMC and mutants used in this study. A) Restriction map of pTYMC showing the T7 RNA polymerase promoter (dark arrow) and the unique *Hind*III site used to linearize the plasmid. ORF-69, ORF-206 and the coat protein ORF (open bars) are labelled, and the black triangle denotes the site of p206 proteolytic cleavage. The ..GKT.. and ..GDD.. are the core amino acids of the putative helicase and polymerase domains, respectively, and the checkered boxes mark the sequences in the riboprobe used in northern blotting unless otherwise noted. B) TYMC mutants described in this work. Only ORF-206 and the coat protein ORF are shown. Mutant nomenclature reflects the genome nucleotide number where alterations take place, except for TY-K982S and TY-G1331R, which are are single codon substitutions and are named by the codon number changed in ORF-206. Black bars represent deleted sequence and stippled bars are out of frame sequences prior to reading frame termination. ORF-206 and coat protein ORF sequences are fused in mutant TYMC-Δ4086-6062.

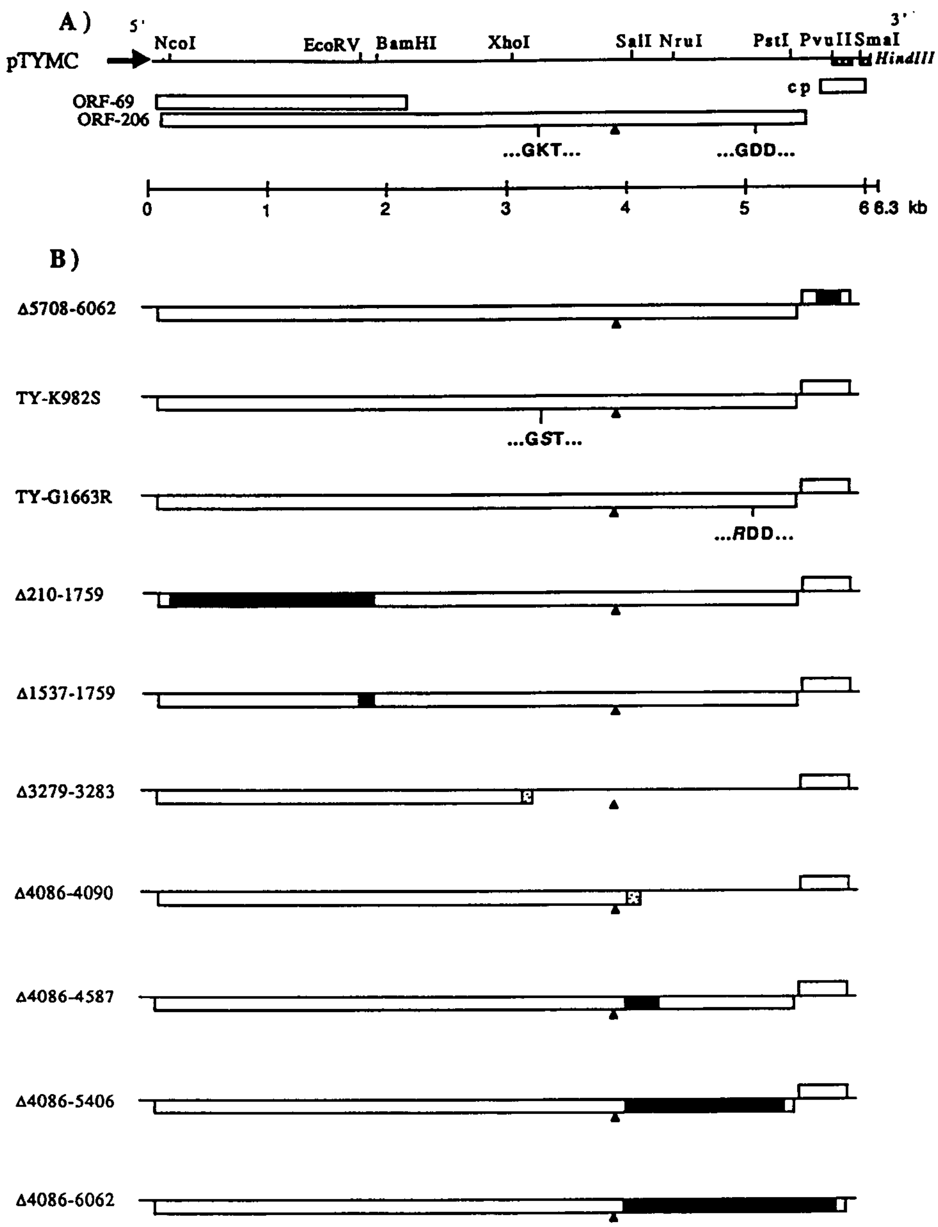

A)
5'
3'
NcoI
EcoRV
BamHI
XhoI
SalI
NruI
PstI
PvuII
SmaI
HindIII
pTYMC
cp
ORF-69
ORF-206
...GKT...
...GDD...
0
1
2
3
4
5
6
6.3 kb
B)
Δ5708-6062
TY-K982S
...GST...
TY-G1663R
...RDD...
Δ210-1759
Δ1537-1759
Δ3279-3283
Δ4086-4090
Δ4086-4587
Δ4086-5406
Δ4086-6062

Figure IV.1

NEN) in a reaction volume of 30 µl. Products were separated by SDS-PAGE and fluorographed.

Protoplast and plant inoculation

Protoplasts were prepared from turnip plants and inoculated as previously reported in Chapter III. MM was removed from the pelleted cells and transcript RNA (3 µg) was added. For co-inoculation of protoplasts 3 µg of the larger RNA, and an equimolar amount of the smaller RNA, was present in the inoculum. Inoculated cells were finally resuspended to 0.5 ml in MM containing complex salts and antibiotics [0.2 mMKPO_4 pH6.5, 10 mM $CaCl_2$, 1 mM KNO_3, 1 mM $MgSO_4$,1 µM KI, 1 µM $CuSO_4$, 0.3 mg/ml Cephaloridine (Sigma) and 10 µg/ml Gentamycin sulfate (Sigma)] and incubated in the culture tubes for 30 hrs under constant light at 25° C unless otherwise indicated.

A fraction of the protoplasts harvested after the incubation was used to inoculate turnip plants. Protoplasts (4 X 10^4) were resuspended in concentrated inoculation buffer (1X IB = 10mM Tris-HCl pH7.2, 0.9mM KH_2PO_4, 3mM Na_2HPO_4, 14mM NaCl and 3 mg/ml bentonite) to a final concentration of 1X in 20 µl. The inoculum was applied to the carborundum-dusted leaves of 2 week-old turnip plants using a blunted, sterile glass pipet.

Tissue extraction, and western and northern blotting.

Protoplasts and plant tissue were harvested and extracted, and total nucleic acids and proteins were recovered as described in Chapter III. Western blots of proteins separated by 14% PAGE were exposed to autoradiographic film after incubation in chemiluminescent substrates of horseradish peroxidase according to the manufacturer (ECL, Amersham). For coat protein quantitation, sample extracts were diluted so that western blot signals detected on pre-flashed film (Kodak-XAR-5) fell within a linear range on a standard curve generated by serial dilution of a preparation of TYMV. Signals on film were scanned by a Zeineh soft-laser scanning densitometer.

Nucleic acids harvested from protoplasts were glyoxalated, electrophoresed in 1% agarose gels (Sambrook et al., 1989) and blotted to Zeta Probe™ nylon membranes as described (Weiland and Dreher, 1989). Hybridization of blots was according to the procedure detailed in Chapter III.

RNA probe represented either sequences located between the Sma I^{6062} site and the 3'-end of the RNA, or those located between the Pvu II^{5708} and Bgl II^{5859} sites (Weiland and Dreher, 1989; Fig IV.1). Blot incubation was performed at 65°C for at least 10 hr. Blots were washed in #5 wash [0.25 X SET (7.5 mM Tris-HCl pH8.0, 30 mM NaCl, 0.5 mM EDTA) including 0.1% SDS and 0.1% sodium pyrophosphate] at 65°C and autoradiographed. Laser densitometric scanning of pre-flashed film that had been exposed to northern blots allowed for the quantitation of blotted RNAs.

All quantitation in Table IV.1 reflects the densitometric scanning of autoradiographs for the accumulation of virus genomic and subgenomic RNAs on northern blots separately, and for virus coat protein on western blots except for TY-K982S + TYMC-Δ4086-6062 RNA inoculations. For inoculations of TY-K982S + TYMC-Δ4086-6062, quantitation was based on autoradiographs of northern blots alone. Values in Table IV.1 represent the mean of at least 4 independent inoculations.

Analysis of progeny RNA: cDNA synthesis, PCR and nucleotide sequencing.

A fraction of the nucleic acids extracted from the inoculated protoplasts was adjusted to 3 M ammonium acetate and frozen at -80°C for 15 min. The thawed samples were centrifuged to collect the precipitated RNA and the pellet was rinsed with cold 100% ethanol. The pellet was dried *in vacuo* and resuspended in sterile water and a fraction was used for reverse transcription. Oligo TYPCR3330(-) (for ..GKT.. analysis) or oligo TYSEQ5687(-) (for ..GDD.. analysis) was mixed with 2 µl of sample RNA and adjusted to 50 mM Tris-HCl pH8.3, 20 mM KCl, and 10 mM $MgCl_2$ in 10 µl. Samples were heated to 95°C for 2 min and ice quenched followed by incubation at 42°C for 10 min. A cocktail [50 mM Tris-HCl pH8.3, 20 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each dGTP, dATP, dTTP, dCTP and 5 units of AMV reverse transcriptase(Life Sciences)] was then added to each sample and the reaction incubated at 42°C for 2 hr. A fraction of this reverse transcription (2 µl) was added to a 100 µl reaction containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton-X100, 1 unit Taq DNA polymerase and 200 ng of each of the opposing primers, and the sample was subjected to PCR amplification. For analysis of the ..GKT.. region, the opposing primers were oligo TYPCR2966(+) and oligo TYPCR3330(-). For analysis of the ..GDD.. region, the opposing primers were oligo TYPCR/MUT4838(+) and oligo

TYSEQ5687(-). The profile of the thermocycler (Perkin-Elmer Cetus) incubations was 94°C, 1 min; 55°C, 1 min; and 72°C, 3 min. Following amplification, 10 µl of the reaction was analyzed on a 1% Tris-acetate-buffered agarose gel (Sambrook et al., 1989). A portion of the amplified material (5 µl) was subjected to asymmetric PCR (Perkin-Elmer Cetus technical brochure). A single oligomer (200ng; TYPCR3330(-) for the..GKT.. region and TYPCRMUT4838(+) for the ..GDD.. region) was used in the synthesis of single stranded DNA in a 100 µl reaction containing 10 µl of the double stranded PCR product generated form the above reaction, and the single stranded DNA was sequenced by standard methods (Sequenase kit, USB). Oligo TYPCR2966(+) was used for sequencing near the ..GKT.. region and oligo TYPCR5156(-) was used to sequence near the ..GDD.. region.

RESULTS

Rationale of mutant RNA construction

Early efforts in our laboratory to co-replicate deletion derivatives of TYMC were based on the observation in other virus systems that the presence of terminal sequences on a defective RNA (often of only several hundred nucleotides) were sufficient to support the replication of that RNA in the presence of a homologous helper virus (French and Ahlquist, 1987; Raffo et al., 1991; White et al., 1991). The co-inoculation of TYMC derivatives having deletions in ORF-206 with TYMC virion RNA, however, resulted in detection of only the helper virus sequences on northern blots. Prolonged exposure of the blots failed to detect the accumulation of defective RNA above the background signal originating from the helper virus (data not shown). These results led us to speculate that either the replication apparatus of TYMV does not operate efficiently in *trans*, or that the defective RNAs used in the attempts lacked important sequences for their amplification in the presence of the helper virus.

ORF-206 encodes proteins of 150 kDa and 70 kDa that are expessed *in vitro*. We decided to test the possibility that either the expression of these two proteins, or the transit of ribosomes decoding ORF-206, were somehow involved in the co-replication of defective TYMC RNAs. Mutations were made in ORF-206 that were designed to a) alter the production of the 150 or 70 kDa proteins separately, while maintaining both the processing at the 150/70 kDa protein junction and the transit of ribosomes to the end of ORF-206, b) alter both the synthesis of the 70 kDa protein and the ribosomal transit along ORF-206, while maintaining the synthesis of the 150 kDa protein, and c) abolish the synthesis of both the 150 and 70 kDa proteins and alter the ribosomal transit along ORF-206. Mutants falling into these categories are depicted in Fig. IV.1.

Representative mutants in each class were translated in a rabbit reticulocyte system in order to verify that processing at the junction separating the 150 kDa and 70 kDa protein domains was normal (Fig. IV.2). Previous work has shown that sequences between $NcoI^{210}$ and Bam HI^{1755}, and sequences 3' of the $SalI^{4086}$ restriction sites were dispensible for protein cleavage at the 150/70 kDa protein junction (Bransom et al., 1991). The results of Fig IV.2 show that the ORF-206 products of all mutant RNAs processed to a normal extent, producing proteins of the expected size.

Replication of coat protein gene deletion mutant

Attempts to co-replicate defective RNAs derived from pTYMC in the presence of helper virus have been hampered by the difficulty in distinguishing between replication products arising from the helper virus and those originating from the defective RNA. In order to circumvent this problem, a coat protein gene deletion mutant (pTYMC-Δ5708-6062) was constructed from pTYMC (see also Chapter III). Such a mutant applied to our co-replication studies would allow for a size discrimination on northern blots between the subgenomic RNA (sgRNA) originating from the helper genome and that originating from the ORF-206 mutant having a wild type coat protein gene, and further allow for specific detection of co-replicated ORF-206 mutant RNAs using a radiolabelled probe that contained sequences absent from the coat protein deletion mutant. A number of plus sense RNA plant viruses defective for native coat protein synthesis have been shown to be competent for replication in isolated plant cells, although such mutants RNAs often accumulate in infections to lower levels as compared to the wild type virus (Marsh et al., 1991c; van der Kuyl et al., 1991; Ogawa et al., 1991; Suzuki et al., 1991).

Mutant TYMC-Δ5708-6062 was constructed which lacks 354 nts from both the genomic and subgenomic RNA, and results in the translational fusion of the N-terminal 21 and the C-terminal 49 amino acids of the coat protein (Fig. IV.1 and Chapter III). Mutant TYMC-Δ5708-6062 replicates in isolated plants cells, accumulating plus sense genomic RNA to levels 10% that of wild type TYMC infections, but accumulating minus sense genomic RNA to levels similar to that in TYMC infections (Weiland and Dreher, in preparation; Chapter III). Since the accumulation of minus sense genomic RNA in inoculated protoplasts is similar between TYMC and TYMC-Δ5708-6062, the decreased accumulation of plus sense genomic RNA for this mutant is thought to reflect the increased degradation of unencapsidated genomic RNA rather than an aberration of the formation of replication complexes or the rate of genomic RNA synthesis. Experiments investigating the levels of ORF-206 product accumulation or induced replicase activity in protoplasts infected with TYMC-Δ5708-6062 as compared to TYMC might resolve this issue.

Subgenomic RNA produced by mutant TYMC-Δ5708-6062 was clearly resolved from wild type size sgRNA on northern blots (Weiland and Dreher, in preparation; Chapter III). Wild type size subgenomic RNA will be referred to below as 0.7kb subgenomic RNA. The ability to specifically probe for defective RNAs using sequences absent from the Δ5708-6062 helper genome greatly facilitated the analysis of co-replication as described below, since the genomic RNAs of the coinoculated RNAs could not be readily resolved by gel electrophoresis.

<u>Co-replication of mutants in the 150 kDa and 70 kDa protein domains by helper genome.</u>

None of the mutant RNAs with alterations in ORF-206 listed in Fig. IV.1 replicated to detectable levels when independantly inoculated onto plant protoplasts (shown here for TY-K982S and TY-G1663R in Fig IV.5, and for TYMC-Δ4086-5406 in Fig IV.3). When mutants TYMC-Δ1537-1759, TY-K982S, TY-G1663R, and TYMC-Δ4086-5406 were each separately co-inoculated with TYMC-Δ5708-6062, however, sgRNA of a size predicted to originate from defective RNA replication was detected on northern blots probed with minus-sense RNA sequences absent from TYMC-Δ5708-6062 (Fig. IV.3). Genomic RNA of a size representative of the co-inoculated ORF-206 mutant was also detected with this probe.

Quantitation of the RNA signal on northern blots, shows at least a 5-fold higher co-replication of the TY-G1663R mutant than the TY-K982S mutant when co-inoculated with TYMC-Δ5708-6062 (Fig IV.3). The results further show that mutant TYMC-Δ4086-5406 is the RNA most efficiently co-replicated in this assay, with genomic and subgenomic RNA accumulating to 10% the levels observed in infections with TYMC RNA. No rescue of a mutant with a frameshift in ORF-206 prior to the 150 kDa protein cleavage point (TYMC-Δ3279-3283) was observed when this RNA was co-inoculated with TYMC-Δ5708-6062. The results show that ORF-206 mutants designed to express either wild type 150 kDa protein or 70 kDa protein could be co-replicated in the presence of a helper genome, and that the 70 kDa protein mutant RNAs were more readily rescued than RNAs with mutations in the 150 kDa protein (Fig. IV.3). With the exception of the TYMC-Δ4086-5406 RNA, however, the accumulation of the defective RNAs in these mixed infections was considerably lower than that of the helper genome in these assays (Fig IV.3B).

Complementation between RNAs encoding mutant 150 kDa and 70 kDa proteins.

Several members of the Sindbis-like plant RNA viruses encode proteins bearing the ..GKT.. and the ..GDD.. motifs on separate viral chromosomes that by themselves are non-infectious (Dreher and Hall, 1988a; Matthews, 1991); only by co-inoculation of the two RNA chromosomes is viral RNA replication observed. We decided, therefore, to examine whether defective RNAs derived from pTYMC with mutations in the 150 kDa and 70 kDa proteins could complement each other, thereby generating infections in protoplasts. RNAs with mutations in the 150 kDa and 70 kDa proteins were co-inoculated onto turnip protoplasts and the extracts of the protoplasts were examined for the accumulation of viral replication products in northern and western blots.

Since results from the co-replication experiments demonstrated that helper genome TYMC-Δ5708-6062 rescued the 150 kDa mutant RNA TY-K982S and the 70 kDa mutant TYMC-Δ4086-5406 most efficiently within their respective classes, these mutants were used in initial complementation studies. A time course examining viral RNA synthesis after the co-inoculation of protoplasts with these two mutants showed the accumulation of 6.3 and 5.0kb genomic RNAs expected for the replication of TYMC-K982S and TYMC-Δ4086-5406 RNAs, respectively. The accumulation of a subgenomic RNA of 0.7kb predicted to arise from the infection was also seen (Fig. IV.4A). Subgenomic RNA was first detectable at 18 hr post-inoculation, and both genomic RNAs were detected at 30 hr post-inoculation. Furthermore, in triplicate inoculations of protoplasts with TY-K982S + TYMC-Δ4086-5406, minus-sense RNAs of a size similar to the RNAs in the inoculum were detectable (Fig. IV.4B). The accumulation of virus-specific RNAs over time, the production of sgRNA and the accumulation of minus-sense RNAs representative of the inoculum RNAs strongly suggest that the protoplasts in these experiments were infected by complementing defective RNAs.

Additional 150 kDa and 70 kDa protein mutants were tested for the ability to complement each other in protoplasts. Parallel inoculations with TYMC RNA were performed in order to compare the accumulation of RNA in the complementations relative to that for wild type RNA. TY-K982S was first co-inoculated with a series of 70 kDa protein deletion mutants. The mutants encoding deletions in the 70 kDa protein (TYMC-Δ4086-4090, Δ4086-4587, and Δ4086-5406) replicated progressively better with deletion size, and for mixtures of TY-K982S + TYMC-Δ4086-5406 the accumulation of progeny plus sense RNA and coat protein was 50 - 70% of wild type (Fig IV.5, Table IV.1). The co-inoculation of TY-K982S + TY-G1663R RNAs resulted in the accumulation of virus genomic RNA, sgRNA and virus coat protein to levels ~1% of that for parallel inoculations of TYMC RNA. Mutant TYMC-Δ4086-6062 represents a special case, since this mutant lacks both the highly conserved "tymobox" nucleotide sequence present near the start site of sgRNA transcription (Ding et al., 1990b) and the majority of the coat protein gene. The decreased amount of sgRNA, and hence coat protein, in co-inoculations of this mutant with TY-K982S may account for the low accumulation of plus sense RNA relative to TYMC (~0.2%). The poor rescue of this mutant may alternatively reflect the absence from this mutant of important *cis*-acting sequences required for efficient replication of the molecule.

Since the highest level of replication in the complementation assays was achieved for the 70 kDa deletion mutant TYMC-Δ4086-5406, this mutant was used in experiments comparing the efficiency of complementation for 150 kDa protein mutants TYMC-Δ210-1759, TYMC-Δ1537-1759 and TY-K982S. Northern blots of extracts from inoculated protoplasts showed that replication for the complementing infection of TYMC-Δ4086-5406 + TYMC-Δ1537-1759 RNAs was reduced ~10-fold as compared to that for TYMC-Δ4086-5406 + TY-K982S RNAs, and that the TYMC-Δ4086-5406 RNA accumulated to higher levels than the TYMC-Δ1537-1759 RNA in these infections (Fig IV.6). No detectable replication for the TYMC-Δ4086-5406 + TYMC-Δ210-1759 RNA co-inoculation was observed. Although the deletions in the 150 kDa protein also affect the ORF-69 reading frame, evidence indicates that the expression of ORF-69 is not required for TYMV RNA replication to occur (Bozarth et al., 1992). The results of these inoculations suggest that deletions in regions coding for the 150 kDa protein are more debilitating to RNA replication than deletions in the 70 kDa protein, and imply that the expression of the 150 kDa protein is required for efficient co-replication of that RNA.

TY-K982S mutant RNA was used in all inoculations above, and this RNA has the same migration in agarose gel electrophoresis as TYMV and TYMC RNAs. Thus it might be that the replication observed is due to a revertant or recombinant wild type virus supporting the replication of the defective deletion mutant RNAs. To rule out this possibility, a fraction of all harvested, inoculated protoplasts were themselves inoculated onto turnip plants and the plants analyzed for symptoms two weeks post-inoculation. TYMC-infected protoplasts are a more potent plant inoculum as compared to purified viral or transcript RNA (J.J.W. and T.W.D., personal observation). For all cases (at least 4 experiments) involving inoculations with wild type TYMC-infected protoplasts, wild type systemic symptoms were evident on inoculated plants (at least 6 inoculations). In only one case (TY-K982S + TYMC-Δ4086-5406) for the mixed inoculations did an infection produced by complementing RNAs generate systemic symptoms; all other inoculations failed to induce the formation of local lesions or systemic symptoms. The local lesions induced by the protoplasts infected with TY-K982S + TYMC-Δ4086-5406 RNAs were delayed in onset relative to those for the wild type virus (visible at 14 days versus 8 days post-inoculation), and were necrotic versus the chlorotic lesions induced by protoplasts infected with TYMC. Systemic symptoms produced by the mixed infection were etched versus the mosaic pattern normally caused by TYMC. Northern blot analysis revealed that the viral RNA present in the systemic tissue remained bipartite, and further experiments have shown that wild type virus out-competes this bipartite virus in mixed plant inoculations (Weiland and Dreher, in preparation). The evidence strongly suggests, therefore, that a revertant or recombinant genome was not supporting the replication of the defective RNA, and that the viral replication induced by the co-inoculation of TY-K982S with the 70 kDa protein mutant RNAs originated from genetic complementation between the defective RNA genomes.

The replication of complementing RNAs is biased.

Although data from the northern blots confirmed that the co-inoculated defective RNAs were being replicated, it was noted that RNAs with deletions in the 70 kDa protein accumulated to higher levels in the infection than the complementing TY-K982S RNA (Figs IV.4 and IV.5). We wished to know whether or not this phenomenon was due to the faster replication of the deleted RNA as a result of its shorter size. Sample extracts from protoplasts inoculated with TY-K982S + TY-G1663R (two complementing RNAs of equal size) were therefore subjected to nucleotide sequence analysis. Sample RNA was primed with minus-sense oligonucleotide primers near the region of the mutations, and RNAs were reverse transcribed. PCR amplified DNA encompassing the mutated regions was further treated by asymmetic PCR, and the resulting single stranded molecules were sequenced. For three independent inoculations analyzed by this technique a clear excess of the TY-G1663R RNA is apparent (Fig IV.7). No detectable product DNA was generated using the PCR regime described when this method was applied to extracts inoculated with the defective RNAs alone. The results suggest that in mixed inoculations of 150 kDa protein and 70 kDa protein mutant RNAs of equal size, the 70 kDa protein mutant RNAs are preferentially replicated.

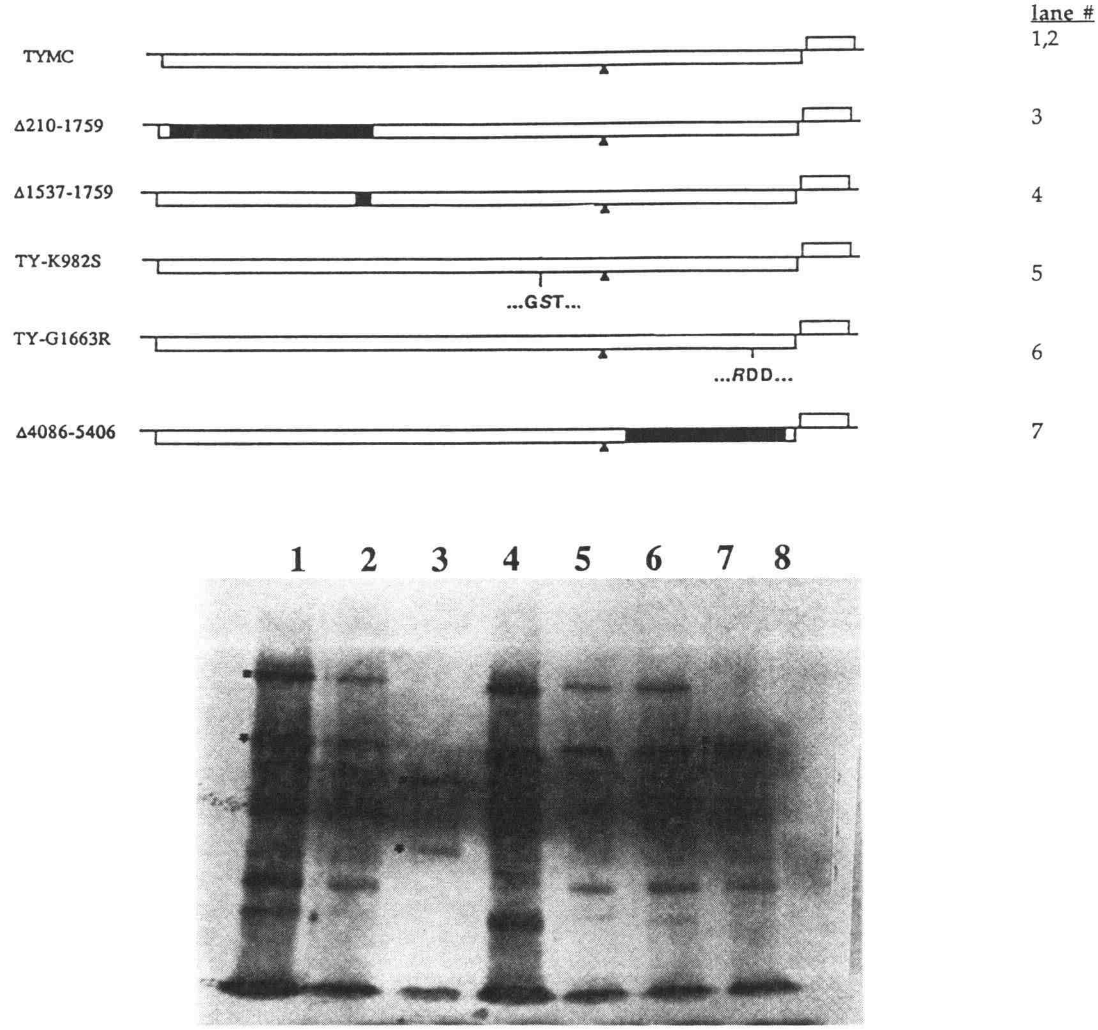


Fig IV.2) Translation *in vitro* of TYMV and TYMC transcript RNAs. A depiction of the mutant RNAs and the lane numbers to which they correspond is shown at the right of the diagram. Translations were terminated by boiling in loading buffer, a fraction of the mixture was separated by 7% SDS-PAGE after which the gel was fluorographed. Protein synthesis programmed by TYMV RNA (lane 1), TYMC RNA (lane 2), TYMC-Δ210-1759 (lane 3), TYMC-Δ1537-1759 (lane 4), TY-K982S (lane 5), TY-G1331R (lane 6), TYMC-Δ4086-5406 (lane 7), and no RNA (lane 8). The dot and the asterisk mark the 206 kDa and 150 kDa proteins, respectively, resulting from translation of wild-type ORF-206, and also mark their counterparts in the other lanes as produced by translation of the mutant RNAs. In other work (Bransom et al., 1991) processing of the TYMC-Δ210-1759 was shown to be similar to wild type RNA.

Fig IV.3) Co-replication of TYMC RNAs encoding mutant 150 kDa and 70 kDa proteins in the presence of TYMC-Δ5708-6062 helper genome. The mutants used in this assay are depicted at the top of the diagram. Turnip protoplasts (4 X 10^5) were inoculated, incubated for 30 hr at 25°C and harvested. Nucleic acid samples representing 4 X 10^4 protoplasts were glyoxalated, separated on a 1% agarose gel and blotted to a nylon membrane as described. A) Northern blots were hybridized to an RNA probe complementary to sequences between the Pvu II and SmaI restriction sites in the coat protein gene (see Fig. IV.1). The "g" and "sg" mark the genomic and subgenomic RNAs, respectively. Shown are extracts from cells inoculated with TYMC RNA (lane 1), TYMC-Δ5708-6062 RNA alone (lane 2), a mixture of TYMC-Δ5708-6062 +TYMC-Δ3279-3283 RNAs (lane 3), TYMC-Δ4086-5406 RNA alone (lane 4), and TYMC-Δ5708-6062 RNA+TYMC-Δ1537-1759 (lanes 5-6), or + TY-K982S (lanes 7-8), + TY-G1331R (lanes 9-10), + TYMC-Δ4086-5406 (lanes 11-12) RNAs. Accumulation of viral RNAs (%) is relative to that for the TYMC inoculation. B) The same blot as in (A) except hybridized to a minus sense RNA probe representing the 3' terminal 259 nts. The sg* indicates the postion of the sgRNA produced by TYMC-Δ5708-6062.

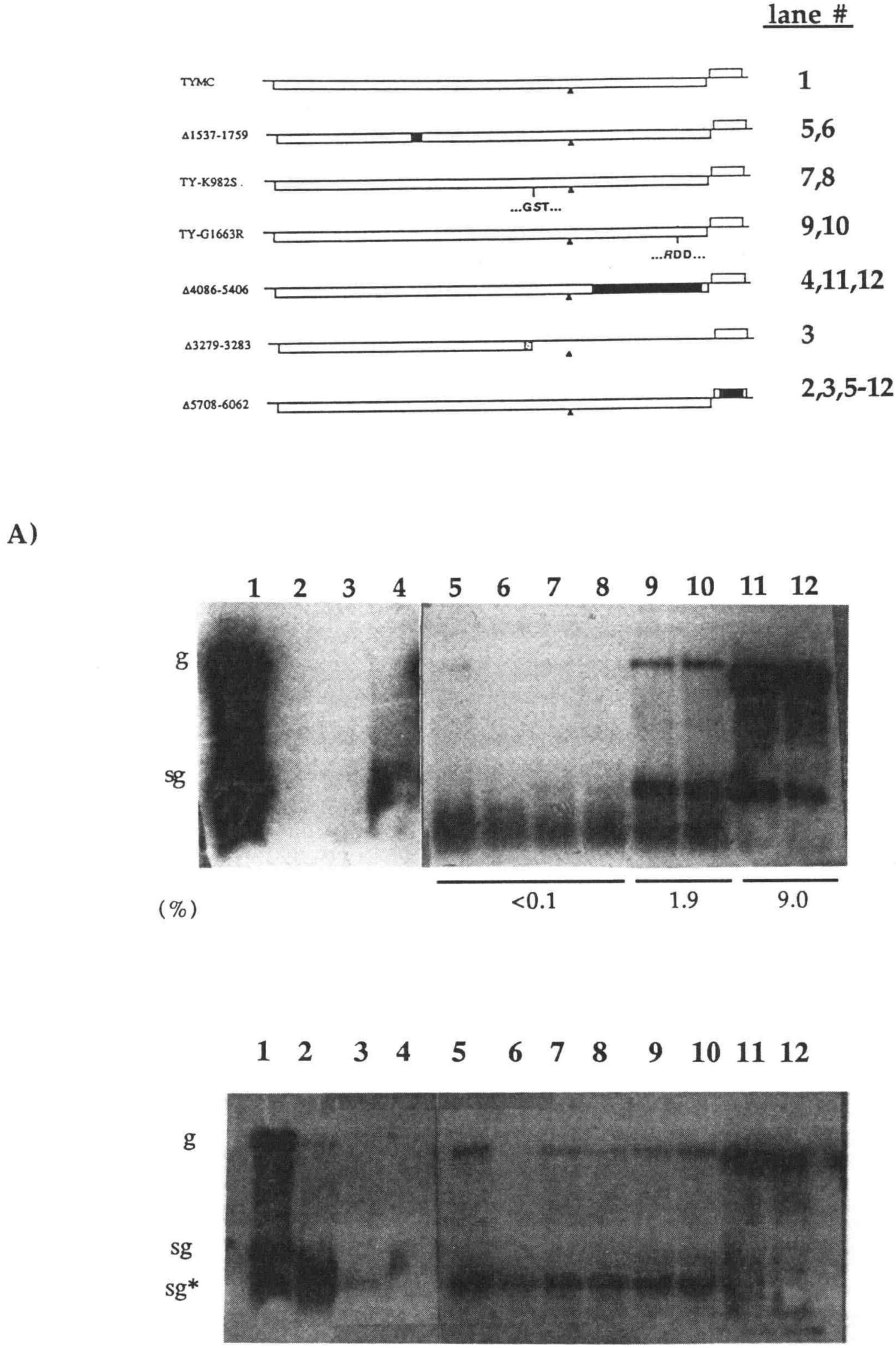

lane #
TYMC
1
Δ1537-1759
5,6
TY-K982S
7,8
...GST...
TY-G1663R
9,10
...RDD...
Δ4086-5406
4,11,12
Δ3279-3283
3
Δ5708-6062
2,3,5-12
A)
1 2 3 4 5 6 7 8 9 10 11 12
g
sg
(%)
<0.1
1.9
9.0
1 2 3 4 5 6 7 8 9 10 11 12
g
sg
sg*

Figure IV.3

Fig IV.4) Complemementation between ORF-206 mutants: Time course of RNA accumulation and minus sense RNA detection. A) Time course showing accumulation of viral RNAs in protoplasts inoculated with TY-K982S +TYMC-Δ4086-5406 transcripts (depicted at the top). Protoplasts (1 X 10^6) were inoculated with a mixture containing 10 μg of each RNA. Cells were subdivided into 5 culture tubes for incubation and harvesting at the desired time point. Extracts representing 8 X 10^4 cells were separated on a 1% denaturing agarose gel and northern blotted. The radiolabelled probe used was complementary to the 3'-end terminal 259 nts (Sma I to Hind III region). Mixed RNA (TYMV + TYMC-Δ4086-5406, 10 ng each) was used as a gel marker (lane 1), and all other lanes contain extracts from cells harvested at 1 hr (lane 2), 12 hr (lane 3), 18 hr (lane 4), 24 hr (lane 5), and 30 hr (lane 6) post inoculation. Lane M is an extract from cells mock-inoculated with sterile water. The "g" indicates the position of the TY-K982S RNA and the asterisk denotes the size of the TYMC-Δ4086-5406 RNA. This blot was exposed to film for 10 hr without an intensifying screen. B) Accumulation of minus sense RNA for TYMC RNA inoculation versus mixed inoculation of TY-K982S with TYMC-Δ4086-5406. Protoplasts (4 X 10^5) were inoculated in triplicate using separately prepared batches of transcript RNA, incubated for 30 hr and the resultant extracts were electrophoresed and northern blotted. The radiolabelled probe was identical (plus sense) to sequences in the coat protein gene (see Fig. IV.1). Extracts from cells inoculated with TYMC RNA (lanes 1-3) and TY-K982S plus TYMC-Δ4086-5406 (lanes 4-6). TYMV RNA (10 ng) was loaded as a control in lane 7. The "g" indicates the size of the TY-K982S RNA and the asterisk denotes the size of the TYMC-Δ4086-5406 RNA. This blot was exposed to film for 48 hr with an intensifying screen.

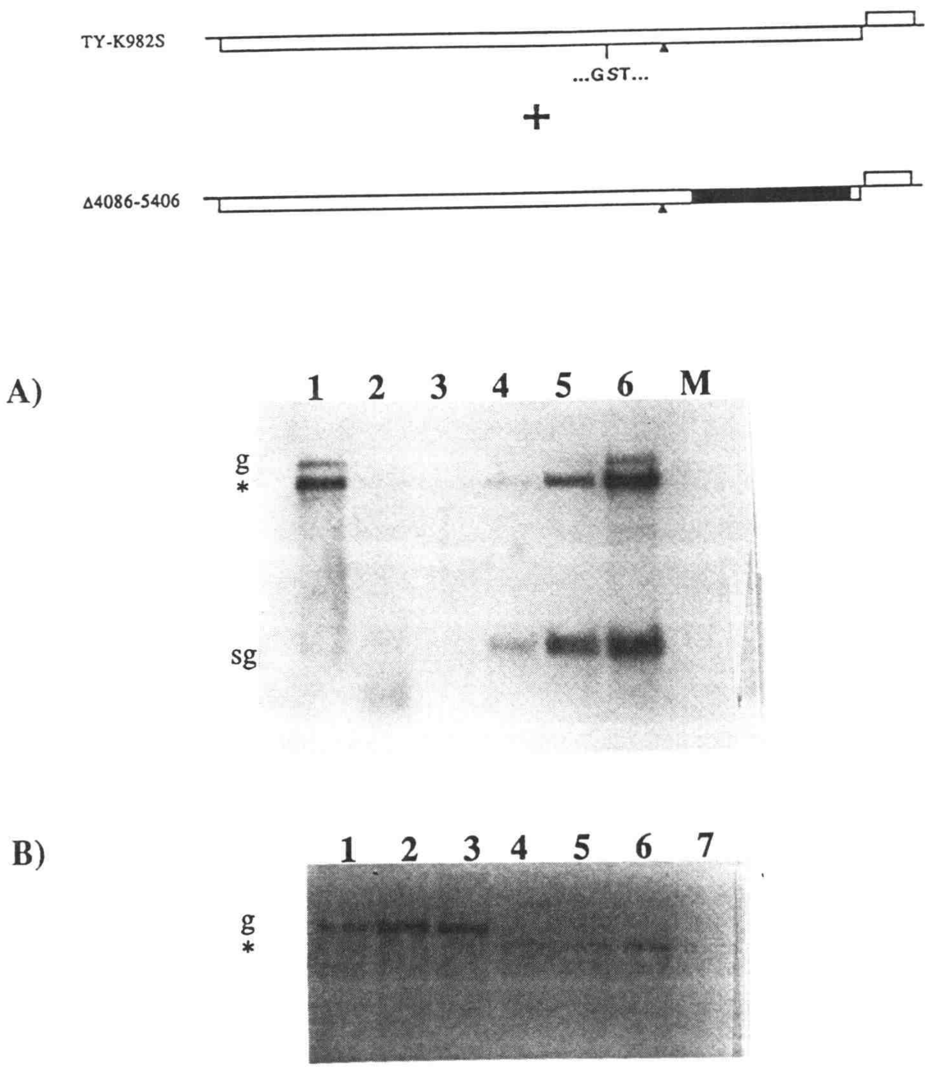
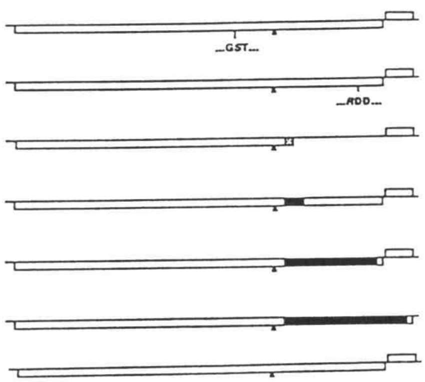


Figure IV.4

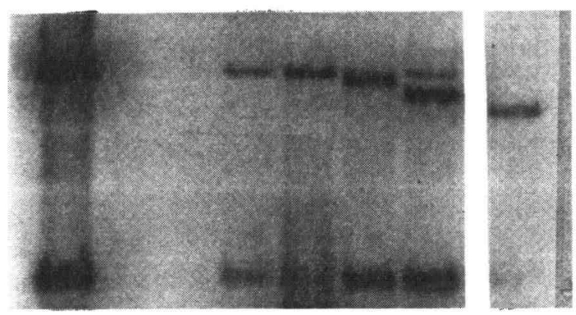
Fig IV.5) Complementation between TY-K982S mutant RNA and various RNAs encoding mutations in the 70 kDa protein. The mutants used in this study are depicted at the top of the diagram. Accumulation of RNAs and cp (%) is relative to that for TYMC. A) RNA extracted at 30 hr p.i. from 4 X 10^4 protoplasts was glyoxalated, separated on a 1% agarose gel and blotted to a nylon membrane. The radiolabelled probe used was complementary to the 3'-end 259 nts of the genome. Lanes represent extracts from cells inoculated with transcript TYMC (lane 1), TY-K982S (lane 2), TY-G1331R (lane 3), and transcript mixtures of TY-K982S + TY-G1331R (lane 4), + TYMC-Δ4086-4090 (lane 5), + TYMC-Δ4086-4587 (lane 6), + TYMC-Δ4086-5406 (lane 7), and + TYMC-Δ4086-6062 (lane 8). The signal in lane 8 is the result of a 36 hr incubation, and this RNA replicates to ~0.2% the level of TYMC after a 30 hr incubation. The positions of the genomic (g) and subgenomic (sg) RNA are indicated. B) Western blot of infected protoplasts. Extracts representing 4 x 10^4 protoplasts were separated by 14% SDS-PAGE, electroblotted to nitrocellulose, the blot reacted with anti-TYMV antiserum and developed with chemiluminescent reagents (ECL; Amersham). TYMV virus (30 ng) was loaded as a control (lane 1) and the remaining lanes loaded with extracts from cells inoculated with RNA from TYMC (lane 2), K982S (lane 3), G1663R (lane 4), or co-inoculations of K9482S + G1663R(lane 5), + Δ4086-4090 (lane 6), or + Δ4086-5406 (lane 7).



	lane #	
	A	B
TY-K982S	2,4-8	3,5-7
TY-G1663R	3,4	4,5
Δ4086-4090	5	6
Δ4086-4587	6	-
Δ4086-5406	7	7
Δ4086-6062	8	-
TYMC	1	2

A) 1 2 3 4 5 6 7 8

g



sg

(%) 100.0 1.0 4.0 22.0 47.0

B) 1 2 3 4 5 6 7

cp->

(%) 100.0 0.9 3.2 67.0

Figure IV.5

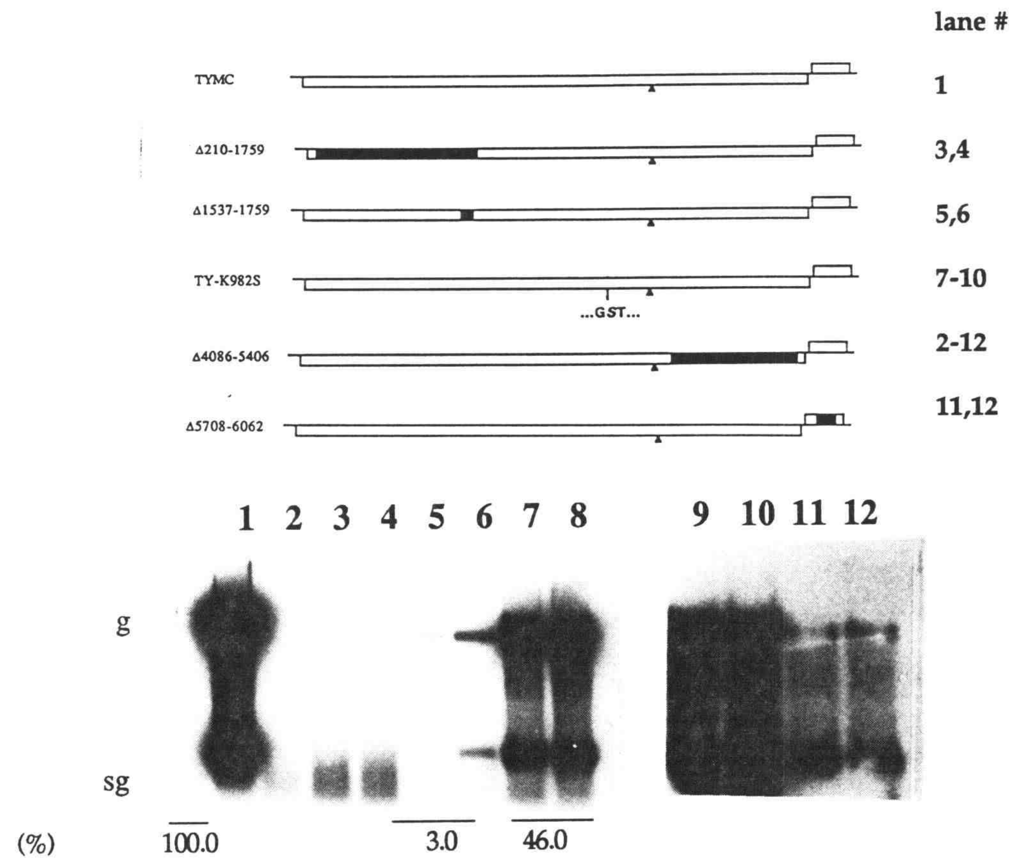


Fig IV.6) Tests for complementation between the TYMC-Δ4086-5406 70 kDa protein mutant and three 150 kDa protein mutant RNAs, and comparison of the rescue of TYMC-Δ4086-5406 by TY-K982S versus by TYMC-Δ5708-6062. Mutants used in the test are depicted at the top of the diagram. Protoplasts were inoculated, incubated and harvested as described in the previous figures. Northern blot reflects the accumulation of viral nucleic acid in 8 X 10^4 cells. The radiolabelled probe used was complementary to the 3'-end terminal 259 nts of the TYMC genome. Extracts are from cells inoculated with transcript RNA from TYMC (lane 1), TYMC-Δ4086-5406 (lane 2) and mixed inoculation of TYMC-Δ4086-5406 RNA + TYMC-Δ210-1759 (lanes 3-4), + TYMC-Δ1537-1759 (lanes 5-6), and + TY-K982S (lanes 7-8) RNA. In a parallel experiment, protoplasts were inoculated with TYMC-Δ4086-5406 + TY-K982S (lanes 9-10), and with TYMC-Δ4086-5406 + TYMC-Δ5708-6062 RNAs (lanes 11-12). The positions of the genomic (g) and subgenomic (sg) RNAs are indicated. Accumulation of viral RNAs is relative to that for TYMC.

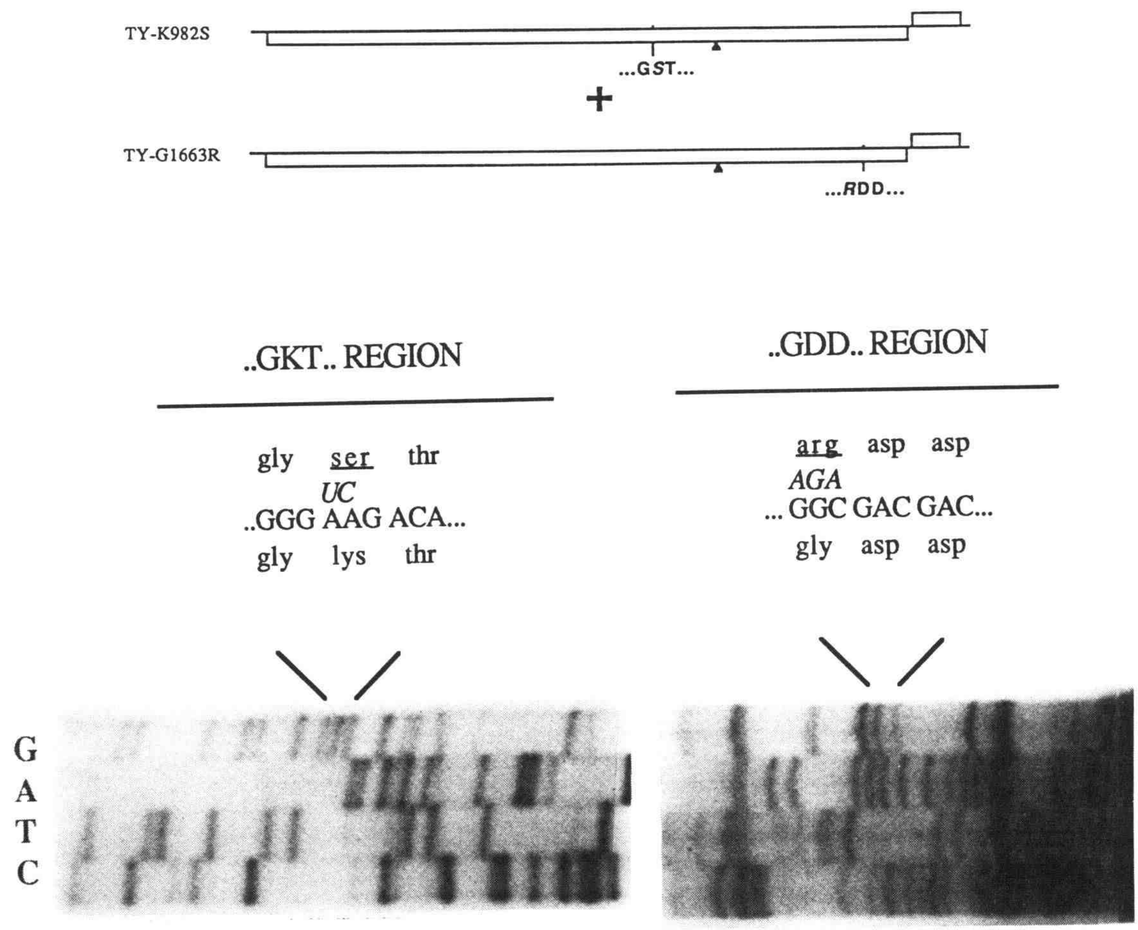


Fig IV.7) Sequence analysis of progeny RNAs resulting from TY-K982S + TY-G1331R mixed infections. Harvested sample RNAs were reverse transcribed, PCR amplified and sequenced. The sequence above was from one complementing infection, and was typical for three such inoculations examined. Plain type is the wild type TYMC sequence, and italicized type is the sequence of the respective mutants. The amino acids encoded in the region are shown in lower case and the amino acid altered by the mutation is underlined. Note the greater abundance of the TY-G1331R mutant in these mixed infections as determined by this assay, and the faint bands representing the sequence in the mutant region of the complementing genome.

Table IV.1) Replication levels for complementing RNAs and co-replicated RNAs relative to TYMC infections. Numbers reflect the area in peaks from the densitometric scanning of autoradiographs for at least three independant inoculations for each mutant.

Western Blots		peak area (avg. of three inoc.)	%[1]
Exp. #1	TYMC	3140	100
K982S +	Δ4086-5406	1246	39
K982S +	Δ4086-4587	176	6
K982S +	Δ4086-4090	30	1
K982S +	G1663R	18	0.6
Exp. #2	TYMC	342	100
K982S +	Δ4086-5406	241	70
K982S +	Δ4086-4090	11	3.2
K982S +	G1663R	3	0.9

Table IV.1 (cont.)

Northern Blots		peak area (avg. of three inoc)	%
	TYMC	70	100
K982S +	Δ4086-5406	34	49
K982S +	Δ4086-4587	15	21
K982S +	Δ4086-4090	3	4.3
K982S +	G1663R	1	1.4
Exp. #4	Δ5708-6062 (helper genome)		ratio of G1663R/K982S
	+G1663R	8.5	5.7X
	+K982S	1.5	
Exp. #5	+K982S	2.7	
	+G1663R	13	4.8X
Exp. #6	+G1663R	6.5	4.3X
	+K982S	1.5	

Differential rescue of Δ4086-5406 by K982S vs Δ5708-6062. K982S/Δ5708-6062

Exp. #7	by K982S	40	5.7X
	by Δ5708-6062	7	

Ratio of Δ4086-5406 RNAto K982S RNA within an extract: Δ4086-5406/K982S

Experiment #						
A	K982S	(2)	Δ4086-5406	(27)	14X
B	K982S	(6)	Δ4086-5406	(44)	7X
C	K982S	(3)	Δ4086-5406	(32)	11X

[1] Values are relative to TYMC (100%).

DISCUSSION

The TYMV ORF-206 encodes protein sequences implicated in viral RNA replication (Morch et al., 1988; Habili and Symons, 1989). We have demonstrated that frameshift and deletion mutations in ORF-206, a substitution in the ORF-206 initiation codon (Weiland and Dreher, 1989), and amino acid substitutions in domains of ORF-206 highly conserved within the plus strand RNA viruses are lethal to TYMV replication. Previous experiments had shown that RNAs transcribed from random internal deletion mutants of pTYMC were not efficiently replicated in the presence of helper TYMV RNA (C.-H. Tsai and J.J. Weiland, unpublished). The mutants in this study were designed, therefore, to test whether ORF-206 expression plays some role in the replication of a defective RNA when co-inoculated with a helper genome.

Co-replication of defective RNAs in the presence of helper genome is inefficient

The results presented here clearly show that defective RNAs with mutations in ORF-206 can be rescued for replication when co-inoculated with a replication-competent helper genome that has an internal deletion in the coat protein gene (TYMC-Δ5708-6062). Northern blots probed with minus-sense sequences complimentary to the 3' terminus of the TYMV genome show that production of full-length sgRNA distinguishable from the sgRNA originating from the helper virus was evident, and is an indirect indicator of the process of genomic RNA replication. Furthermore, a signal appropriate for the replication of defective RNA and the synthesis of 0.7kb subgenomic RNA were both detected on northern blots when probed with sequences that are absent in the helper genome.

Although it was clear that co-replication of the defective RNAs in the presence of a replication-competent helper genome had occurred, rescue of the defective RNAs was inefficient, especially when compared to the replication resulting from the co-inoculation of two defective RNAs (Fig. IV.6, lanes 9-12). In addition, previous attempts to co-replicate defective RNAs with wild type helper virus had failed to detect replication of the defective RNAs in these similar assays. The data suggest that the TYMV replication apparatus does not function efficiently in *trans*, preferring to replicate the RNA from which the essential virus-encoded replication proteins were translated. Contrasting these results are examples in other monopartite plus sense RNA viruses, where the co-replication of defective RNAs can occur efficiently. The tombusviruses tomato bushy stunt virus (Hillman et al., 1987) and turnip crinkle virus (Li et al., 1989) have well characterized defective interfering RNAs associated with them. Efficient replication of defective RNAs, that are deletion mosaics of the genome supporting their replication, has been described for the monopartite Sindbis virus (Levis et al., 1986), and the Sindbis-like plant virus clover yellow mosaic virus (a potexvirus; White et al., 1991). Defective RNA replication for clover yellow mosaic virus may require the maintenance of a nonstructural/coat protein ORF fusion, implying a role for defective RNA translation in efficient RNA co-replication.

<u>RNA replication is skewed in complementing infections</u>

Northern blot analysis of infected protoplasts extracts clearly shows that complementation between defective TYMC genomes can occur. The accumulation of defective RNAs in the complementing infections, however, was not equivalent between the two defective RNAs. Within the class of mutants having alterations in the 70 kDa protein region, deletion mutant RNAs replicated better than an amino acid substitution mutant. Although this might be due to the kinetics of a shorter RNA accumulating faster than a larger one when the replication rates applied to both RNAs are equal, results whereby the accumulation of two complementing RNAs of equal size was monitored argues against this as the sole cause of the bias. In fact the greater accumulation (~4 fold) of frameshift mutant TYMC-Δ4086-4090 as compared to substitution mutant TY-G1663R in inoculations with TY-K982S suggests that the 70 kDa protein sequences of the G1663R mutant may play some inhibitory role in TYMV RNA replication. That the frameshift mutant (TYMC-Δ4086-4090) does not replicate to the levels observed for the large 70 kDa protein deletion mutant (TYMC-Δ4086-5406) may reflect a partial kinetic effect resulting in higher relative accumulation of the shorter RNA in this comparison, or may alternatively reflect a greater instability of the TYMC-Δ4086-4090 RNA *in vivo*.

The biased rescue of the 70 kDa protein mutant RNAs over that of the 150 kDa protein mutants can best be explained by the preferential replication of RNAs expressing wild type 150 kDa protein. A *cis*-acting function for the 150 kDa protein in TYMV RNA replication is supported by four observations: 1) RNAs expressing wild type 150 kDa protein accumulate to higher levels than RNAs expressing mutant 150 kDa protein when replication of the respective mutants is rescued by either a helper genome or a complementing defective RNA, 2) helper RNAs (both wild type and TYMC-Δ5708-6062) are poor at rescuing the replication of defective TYMC genomes, implying a *cis*-preference in the activity of the replicase for RNA templates from which all of the essential replication proteins were expressed, 3) *trans*-complementation of RNAs expressing mutant 150 kDa protein by abundant RNAs expressing wild type 150 kDa protein in mixed inoculations is poor, and 4) an RNA encoding an ORF-206 frameshift prior to the 150/70 kDa protein cleavage junction was not detectably co-replicated. Taken together the evidence suggests a preference in the replication TYMV for those RNAs expressing wild type 150 kDa protein, and may further indicate that only defective RNAs expressing the 150 kDa protein are efficient substrates *in vivo* for the TYMV replicase.

The high accumulation of 70 kDa protein mutant RNAs relative to 150 kDa protein mutants in mixed infections may be explained by an absence of *cis*-acting sequences that normally function to limit RNA replication in the presence of the *trans* acting 70 kDa protein. Protein-mediated inhibition of RNA replication has been postulated as the mechanism behind the repression of BMV RNA 2 replication when this RNA is co-inoculated with BMV RNA1 and a mutant of RNA 2 lacking the C-terminal region of the 2a protein (Marsh et al., 1991). Interestingly, the BMV-encoded 2a protein possesses amino acid sequences homologous to sequences in the TYMV 70 kDa protein (Koonin, 1991). One argument against the existence of *cis*-acting repressor sequences lies in the poor co-replication of mutant RNAs with deletions in the 70 kDa protein when co-inoculated with wild type helper virus. Attempts to detectably replicate TYMC-Δ4086-5406 RNA in the presence of TYMC helper virus RNA were unsuccessful (not shown). It is not anticipated that the helper RNA (encoding the inhibitory sequences) would so thoroughly out-compete an RNA lacking the regions inhibitory to RNA replication.

Cis-acting replication proteins have been reported for only a few RNA and DNA viruses to date, most notably for poliovirus and bacteriophage ϕX174. Recent experiments with poliovirus indicate a requirement for viral nonstructural protein expression in the replication of poliovirus defective interfering RNAs (Hagino-Yamigishi and Nomoto, 1989). Furthermore, the poliovirus P2 protein encodes amino acid sequences that are similar to regions in the TYMV 150 kDa protein, and mutations in the 2B protein (a sub-domain of P2) are poorly complemented in *trans* (Johnson and Sarnow, 1991). In bacteria, the efficient replication of the DNA bacteriophage øX174 requires the activity of the cisA protein, a factor that derives its name from its *cis*-activity *in vivo* (Francke and Ray, 1972), and the transposase encoded by the insertion element IS50 operates preferentially on the repeats flanking the element from which it was expressed (DeLong and Syvanen, 1991). To our knowledge, however, the TYMV 150 kDa protein is the only replication protein encoded in the positive stand RNA plant viruses that is reported to display a preferential *cis* activity.

Future experiments will be aimed at determining the domains of the 150 kDa protein that mediate the *cis* preference in RNA replication. The establishment of a bipartite TYMC infection system as presented above should prove invaluable in furthering these studies.

THESIS SUMMARY

Members of the Sindbis virus supergroup have diverse particle morphologies and genome coding characteristics (Goldbach et al., 1991), but share extensive homologies in their encoded nonstructural proteins. Many of the plant Sindbis-like viruses are multipartite; viral factors required for the complete propagation of the virus in nature are encoded on separate virus chromosomes. In effect, complementation for various activities in the propagation of multipartite viruses is obligatory for the survival of the virus species. Thus, RNAs that are not competent for replication on their own, complement one another for replication. Likewise, RNAs that can replicate in a plant cell, but cannot move from cell-to-cell require the presence (often) of an aditional RNA that expresses some factor for movement to an adjacent cell.

Monopartite viruses like TYMV on the other hand encode all of the information for their propagation in nature on a single viral RNA chromosome. Yet the structure of the RNAs and the amino acid sequences encoded in the genes of both the monopartite and multipartite viruses are strikingly similar. At the outset of this work, therefore, I wished to examine the ability for a Sindbis-like monopartite RNA virus to be complemented by a second RNA genome, thereby approaching the situation found in the multipartite viruses. The construction in the course of this work of an infectious cDNA clone of the TYMV genome was a prerequisite towards the commencement of these studies.

Three aspects of genetic complementation between TYMV synthetic RNAs were examined: 1) Complementation for RNA replication between two defective RNAs, 2) complementation for cell-to-cell spread using a replication-competent mutant RNA deficient for movement between cells, and 3) complementation for systemic translocation of a virus that was competent for RNA replication and lesion formation (and by extension, for cell-to-cell spread), but was defective for efficient systemic translocation in the infected plant. RNAs were co-inoculated onto plant protoplasts and plants using various schemes in an attempt to reconstitute the deficiencies of each separate RNA by genetic complementation.

The results of the work described in the preceding pages suggest that the replication of TYMV may be characterized by the inablity for certain viral functions to be efficiently complemented in *trans*. Only in the experiments in which defective RNA replication was restored due to the *trans* complementation of co-inoculated genomes did the system mimic that for a multipartite virus. Indeed, the results from these co-replication experiments indicate that although virus multiplication in TYMV can occur as the result of *trans*-complementation for replication functions, a significant *cis*-effect was observed in the replication of RNAs expressing one of the essential replication proteins (the 150 kDa protein). Thus genomic RNAs that express wild type 150 kDa protein are preferred replication substrates in mixed infections, and the phenomenon may account for the poor rescue of defective TYMV RNA genomes in the presence of wild type helper virus. If this effect is general for other aspects of TYMV infectivity (e.g. cell-to-cell movement, systemic translocation, etc.), complementation for these various functions may work only inefficiently.

Why would the evolution of a replication complex with *cis*-preference be advantageous in a monopartite RNA virus? One explanation relates to the mutational load of an RNA virus population. When functional replication proteins are synthesized from a viral RNA and preferably replicate that RNA, they act to propagate a functional RNA genome. Efficient *trans*-replication might allow for the selection and amplification of defective interfering RNAs which could debilitate the replication of the genomic RNA (Schlesinger, 1988) or may simply increase the number of defective non-interfering RNAs which create a mutational load on the RNA population (Chao, 1988). Monopartite viruses with high recombination rates and multipartite viruses circumvent the latter problem via the continued reconstruction of functional genomes. One would predict that a virus whose replication complex functions preferentially in *cis* would display a low recombination rate, since template switching appears to be the most common mechanism by which RNA recombination occurs (Jarvis and Kirkegaard, 1991). It would be necessary for such a virus to maintain high fidelity in the replication of its genomic RNA, or the mutational load would threaten its survival.

ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
tRNA	transfer ribonucleic acid
mRNA	messenger ribonucleic acid
gRNA	genomic ribonucleic acid
sgRNA	subgenomic ribonucleic acid
dsRNA	double-stranded ribonucleic acid
ssRNA	single-stranded ribonucleic acid
cp	coat protein
ORF	open reading frame
UTR	untranslated region
nt	nucleotide
bp	basepair
kb	kilobase
kDa	kilodalton
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
AlMV	alfalfa mosaic virus
BMV	brome mosaic virus
BNYVV	beet necrotic yellow vein virus
BSMV	barley stripe mosaic virus
CMV	cucumber mosaic virus
EMV	eggplant mosaic virus
KYMV	kennedya yellow mosaic virus
OYMV	ononis yellow mosaic virus
PVX	potato virus X
TRV	tobacco rattle virus
TYMV	turnip yellow mosaic virus
TYMC	turnip yellow mosaic virus--Corvallis strain

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