To the Graduate Council:

I am submitting herewith a dissertation written by Kailayapillai Sivakumaran entitled "Expression and Function of Southern Bean Mosaic Virus Genes." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

David L. Hacker, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the council:

Associate Vice Chancellor and
Dean of The Graduate School

EXPRESSION AND FUNCTION OF SOUTHERN BEAN MOSAIC VIRUS GENES

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Kailayapillai Sivakumaran

May 1998

ACKNOWLEDGEMENTS

I wish to express my heart-felt gratitude to my advisor, Dr. David L. Hacker, for his guidance, support, encouragement, patience and friendship throughout this period of study, and for making it an invaluable learning experience.

I wish to express my appreciation to my Committee members Dr. David Brian, Dr. Brad Reddick, Dr. Stuart Riggsby, and Dr. Gary Stacey, for their valuable time, suggestions, and the liberal use of their facilities.

I also wish to thank Dr. Albrecht von Arnim, Dr. Bing Garcia, and Mr. Benjamin Fowler, for their assistance in various stages of this project.

Finally, my thanks to Douglas Trembley, Sook-Kyung Lee, Savithra Senanayake, Jennifer Black, Gwyn Williams, and Baburaj Kunnanmal, for their friendship and support, and for making my time in Knoxville memorable.

ABSTRACT

The cowpea strain of southern bean mosaic virus (SBMV-C) is a member of the sobemovirus group of positive-sense RNA viruses. A genome-length cDNA clone of SBMV-C was constructed and used as template for the *in vitro* synthesis of infectious SBMV-C RNA. Inoculation of *Vigna unguiculata* (cowpea) with the synthetic RNA resulted in a systemic infection of this host. The SBMV-C cDNA clone was used for the generation of mutations in three of the four SBMV-C open reading frames (ORFs). Synthetic transcripts generated from these mutant constructs were used to determine that the ORF1, ORF3, and coat protein genes are required for infectivity in cowpea but were not required for SBMV-C RNA synthesis in cowpea protoplasts. From these results it was concluded that these three proteins function in SBMV-C cell-to-cell movement. The requirement for the coat protein in cell-to-cell movement suggests that virus particles are transported locally. Studies were also carried out to determine the expression strategy of SBMV-C RNA. It was determined that the presence of cap analog decreased ORF1 and ORF2 expression in a cell-free extract, that the addition of AUG codons 5' to the ORF1 or ORF2 initiation codons decreased the translation of these genes *in vitro* and *in vivo*, and that deletions or insertions in ORF1, in general decreased ORF1 and ORF2 translation *in vitro* and *in vivo*. From these results it was concluded that ORF1 and ORF2 are expressed by a 5' end-dependent scanning mechanism rather than by internal ribosome entry.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	**LITERATURE REVIEW**	1
	BACKGROUND LEADING TO THE STUDY OF SOUTHERN BEAN MOSAIC VIRUS GENE EXPRESSION AND FUNCTION	1
	Introduction	1
	SBMV-C Genome Organization, Expression and Function	2
	Unconventional Translation Initiation	5
	Transport of Plant Viruses	8
II.	**IDENTIFICATION OF VIRAL GENES REQUIRED FOR CELL-TO-CELL MOVEMENT OF SOUTHERN BEAN MOSAIC VIRUS**	13
	INTRODUCTION	13
	MATERIALS AND METHODS	15
	Construction of a genome-length SBMV-C cDNA clone	15
	Construction of mutants	18
	Inoculation of plants and protoplasts	19
	Analysis of RNA from plants and protoplasts	19
	Analysis of virus from plants and protoplasts	20
	RESULTS	21
	Characterization of the genome-length SBMV-C cDNA clone	21
	Construction of SBMV-C mutants	23
	Infections of cowpea plants with wild-type and mutant SBMV-C transcripts	25
	Infections of cowpea protoplasts with wild-type and mutant SBMV-C transcripts	25
	DISCUSSION	31

III. EXPRESSION OF SOUTHERN BEAN MOSAIC VIRUS GENOMIC RNA 35

INTRODUCTION 35

MATERIALS AND METHODS 36

Construction of mutants 36
Construction of ORF2/GUS fusions 39
SBMV-C RNA isolation 39
In vitro transcription and translation 40
Transient GUS expression in onion epidermal cells 40

RESULTS 41

Effect of cap analog on SBMV-C translation 41
Effect of AUG codons on ORF1 and ORF2 translation *in vitro* 46
Effects of AUG codons on ORF2 expression *in vivo* 47
Effects of deletions and insertions on ORF2 expression *in vitro* and *in vivo* 53

DISCUSSION 55

IV. FUTURE DIRECTION OF SOUTERN BEAN MOSAIC VIRUS GENE EXPRESSION AND FUNCTION RESEARCH 61

TO DETERMINE THE ROLE OF THE 5'END LINKED VPg IN SBMV-C TRANSLATION INITIATION 61

TO FURTHER CHARACTERIZE THE ROLE OF ORF1 AND ORF3 PROTEINS IN CELL-TO-CELL MOVEMENT OF SBMV-C 61

TO DETERMINE ROLE OF SBMV-C RNA ELEMENTS IN TRANSLATION 62

REFERENCES 63

APPENDIX 75

VITA 77

LIST OF FIGURES

FIGURE		PAGE
1.	Genome organization of SBMV-C	3
2.	Diagram describing the cloning strategy and mutant constructs	16
3.	SDS-PAGE analysis of SBMV-C coat protein	22
4.	Diagram describing the mutant constructs	24
5.	Hybridization analysis of total RNA from cowpea plants	26
6.	Hybridization analysis of total RNA from cowpea protoplasts	28
7.	Electron micrographs of SBMV-C particles from cowpea protoplasts	29
8.	Diagram describing the mutant constructs	37
9.	The effect of cap analog on the translation of SBMV-C and BMV RNA	43
10.	The effect of additional AUG codons within the 5' UTR on SBMV-C translation	48
11.	The effect of additional AUG codons on SBMV-C translation	49
12.	Effects of AUG codons on ORF2/GUS expression in onion epidermal cells	52
13.	The effects of ORF1 deletions and insertions on ORF1 and ORF2 expression in wheat germ extract	54
14.	The effects of ORF1 deletions and insertions on ORF2 expression in onion epidermal cells	56

CHAPTER I

LITERATURE REVIEW

BACKGROUND LEADING TO THE STUDY OF SOUTHERN BEAN MOSAIC VIRUS GENE EXPRESSION AND FUNCTION.

Introduction

Southern bean mosaic virus (SBMV) is a member of the sobemovirus group of positive-sense RNA viruses. Four serologically-related strains of SBMV have been identified (Grogan and Kimble, 1964). The bean strain (SBMV-B) systemically infects several *Phaseolus* species including *P. vulgaris* but does not infect cowpea (*Vigna unguiculata*) (Tremaine and Hamilton, 1983). The cowpea strain (SBMV-C) infects *V. unguiculata* and *Pisum sativum*, but fails to systemically infect beans (Shepherd and Fulton, 1962). The Ghana (SBMV-G) and Mexican (SBMV-M) strains are transmissible to several cultivars of both bean and cowpea (Lamptey and Hamilton, 1974; Yerkes and Patino, 1960). The characteristic symptoms of SBMV-C infection in trifoliate leaves of *V. unguiculata* cultivar (cv) California blackeye are an initial vein clearing followed by the appearance of a mosaic pattern, mottling, rugosity, and downward cupping of the leaf margins. In contrast, the infected primary leaves may not show any symptoms (Shepherd and Fulton, 1962). The virus is transmitted mechanically (breach of the epidermal layer of leaves by injury), by chrysomelid beetles, and through seed stock (Tremaine and Hamilton, 1983).

Other members of the sobemovirus group include lucrene transient streak virus (LTSV), cocksfoot mottle virus (CfMV), turnip rosette virus (TRV), velvet tobacco mottle virus (VTMV), rice yellow mottle virus (RYMV), blueberry shoestring virus

(BBSSV), solanum nodiflorum mottle virus (SNMV), sowbane mosaic virus (SOMV), and subterranean clover mottle virus (SCMV) (Hull, 1988). The genomes of SBMV-C, SBMV-B, RYMV, LTSV, and CfMV have been sequenced (Wu et al., 1987; Othman & Hull, 1995; Ngon A Yassi et al., 1994; Jeffries et al., 1995; Mäkinen et al., 1995b; Ryabov et al., 1996).

SBMV-C Genome Organization, Expression and Function

The monopartite RNA genome of SBMV-C is 4,194 nucleotides (nt) in length (Wu et al., 1987). The 5' end of the RNA is not capped but is covalently linked to a viral protein (VPg) (Ghosh et al., 1979). The 3' end of the genome is not polyadenylated, but the 3' terminal 120 nt are predicted to fold into a secondary structure that is conserved in other sobemovirus RNAs (Wu et al., 1987; Ngon A Yassi et al., 1994). The SBMV-C genome has four overlapping open reading frames (ORF) designated ORF1 through ORF4 that may be translated to produce proteins of 21 kDa (ORF1), 105 kDa (ORF2), 18 kDa (ORF3) and 31 kDa (ORF4) (Fig.1) (Wu et al., 1987). ORFs 1-3 are translated from the genomic RNA whereas ORF4 is translated from a subgenomic RNA (sgRNA) that is coterminal with the 3' end of the genomic RNA (Rutgers et al., 1980). The 5' end of the sgRNA is also covalently bound to VPg (Ghosh et al., 1981).

The 105 kDa protein is predicted to be autoproteolytically cleaved into at least three polypeptides including VPg (12 kDa), a trypsin-like serine protease (20-25 kDa), and an RNA-dependent RNA polymerase (RdRp) (65-70 kDa) (Wu et al., 1987; Gorbalenya et al., 1988). The functions of the protease and RdRp have been predicted based on amino acid sequence homology between these proteins and other viral proteins

of known function (Wu et al., 1987; Gorbalenya et al., 1988). The functions of the ORF1 and ORF3 gene products of SBMV-C or any other sobemovirus have not been determined. Little amino acid sequence homology is observed among the sobemovirus ORF1 proteins (Ngon A Yassi et al., 1994). Even the ORF1 proteins of SBMV-C and SBMV-B are only 26% identical in amino acid sequence (Othman and Hull, 1995). In contrast, an 18 amino acid region of the SBMV-C ORF3 protein is conserved in the ORF3 proteins of other sobemoviruses, in the ORF2 proteins of the subgroup II luteoviruses, and in the ORF2 protein of pea enation mosaic enamovirus RNA 1 (Ryabov et al., 1996). The ORF2 proteins of these viruses are expressed by a –1 ribosomal frameshift. These proteins also have amino acid homology to sobemovirus RdRp protein (Miller et al., 1995).

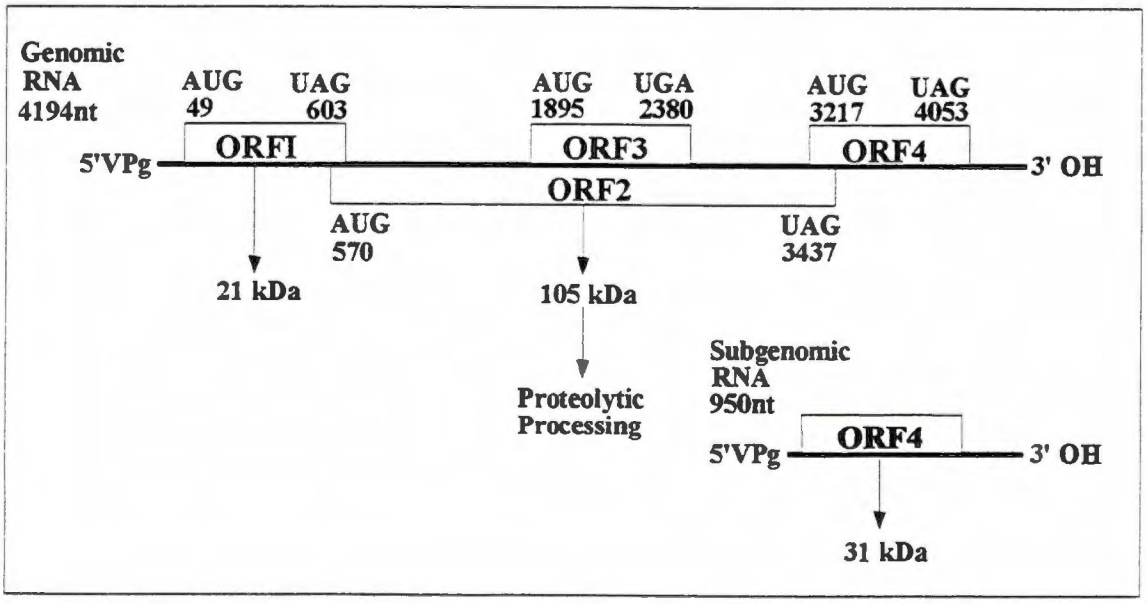


Figure 1. Genome organization of SBMV-C. The nucleotide positions of the initiation and termination codons of the open reading frames are noted (Wu et al., 1987).

The icosahedral capsid of SBMV-C is composed of 180 chemically identical subunits of the 31 kDa coat protein expressed from ORF4 (Rutgers et al., 1980; Salerno-Rife et al., 1980; Hermodson et al., 1982). The structure of the protein has been resolved by X-ray diffraction (Abad-Zapatero et al., 1980). Besides its role in encapsidation of the viral RNA, the coat protein is also required for beetle transmission of the virus (Mahmood, 1994). In addition, the RYMV coat protein has been shown to be required for efficient cell-to-cell movement of this virus (Brugidou et al., 1995).

Most of the other sobemovirus genomes that have been sequenced have an organization that is similar to that of SBMV-C, but the CfMV genome is an exception. Its ORF2 is divided into two reading frames designated ORF2a and ORF2b. The latter is translated by a -1 ribosomal frameshift in ORF2a to produce a polyprotein that may be processed to produce VPg, a serine protease, an ORF3-like protein, and an RdRp (Mäkinen et al., 1995a,b). A separate ORF3 is not present in the CfMV genome. The functional -1 ribosomal frameshift site includes the sequence 5'-UUUAAAC-3' followed by a RNA hairpin (Mäkinen et al., 1995a). These RNA elements are conserved near the 5' ends of the ORF3 coding regions of the other sobemoviruses suggesting that the sobemovirus ORF3 may be translated by a -1 ribosomal frameshift in ORF2 (Mäkinen et al., 1995a).

As is evident from this discussion, little is known about sobemovirus gene expression and function. The elucidation of the expression strategy of the SBMV-C genomic RNA, and the determination of the functions of the SBMV-C ORF1 and ORF3 proteins in the viral infection cycle are the subjects of this thesis.

Unconventional Translation Initiation

Most eukaryotic mRNAs have a 5' terminal cap (m^7GpppN) and a 3' poly(A) tail that are required for efficient initiation of translation (for review see Sachs et al., 1997). Many viral RNAs, including those of SBMV-C, lack one or both of these structural elements. As described below, viruses may use alternative translational strategies to compensate for these deficiencies. One objective of this thesis is to determine how ribosomes initiate SBMV-C translation in the absence of a cap and poly(A) tail.

According to the ribosome scanning model, translation is initiated by the recruitment of the small ribosomal subunit (43S subunit) to the mRNA followed by scanning in a 5' to 3' direction to an initiation codon where the 43S subunit pauses and allows binding of the large ribosomal subunit (60S) (Kozak, 1989; Merrick and Hershey, 1996). Efficient recruitment of the 43S subunit to the mRNA requires binding of eukaryotic initiation factor 4F (eIF4F) to the 5' cap. In most eukaryotic cells eIF4F is composed of three subunits termed eIF4E, eIF4A, and eIF4G (Merrick and Hershey, 1996). eIF4E specifically recognizes the 5' cap (Merrick and Hershey, 1996), eIF4A displays ATP-dependent RNA helicase activity and functions in mRNA unwinding (Pause et al., 1994), and eIF4G functions as an adaptor protein that binds to eIF4E, eIF4A, eIF3, and to the mRNA (Hentze, 1997; Morley et al., 1997). Binding of eIF4F to the 5' cap is followed by the unwinding of the mRNA near its 5' end by eIF4A (Pause et al., 1994). The 43S ribosomal subunit then binds at or near the 5' end of the mRNA and begins scanning (Kozak, 1989). In plant cells, two forms of eIF4F are found but neither is associated with eIF4A (Browning, 1996). eIF4F and eIF(iso)4F are composed of eIF4E homologs with molecular masses of 26 and 28 kDa, respectively and eIF4G homologs

with molecular masses of 220 and 86 kDa subunits, respectively (Browning, 1996).

The 5' cap and the 3' poly(A) tail may stabilize the RNA and may act synergistically to stimulate translation (Gallie, 1991; Tarun and Sachs, 1995; Jacobson, 1996). The synergistic effect suggests that the 5' cap structure and the poly(A) tail interact during translation initiation (Gallie, 1991). This interaction may involve both translation initiation factors and the poly(A) binding protein which may help stabilize the translational machinery and commit the mRNA to multiple rounds of translation (Gallie, 1996; Tarun and Sachs, 1996; Sachs et al., 1997).

The best studied example of a viral RNA element that substitutes for the 5' cap is the internal ribosome entry site (IRES) of poliovirus (PV), a member of the *Picornaviridae* (for review see Jackson and Kaminski, 1995). The PV genomic RNA is not capped but is covalently bound to VPg (Lee et al., 1977), and the 3' end of the RNA is polyadenylated (Yogo and Wimmer, 1972). The 5' untranslated region (UTR) of PV is about 740 nt in length, contains multiple AUG codons 5' to the initiation codon, and has a highly ordered secondary structure (Meerovitch and Sonenberg, 1993; Jackson and Kaminski, 1995). PV infection results in the specific inhibition of host but not viral protein synthesis due to the proteolytic cleavage of eIF4G by the 2A protease of PV (Sonenberg, 1990). PV RNA is therefore not translated by cap-dependent scanning as described above. Instead, 43S ribosomal subunits bind to an internal site on the RNA near the initiation codon at nt 743 (Pelletier and Sonenberg, 1988). Internal ribosome binding requires five RNA hairpins in the IRES located between nt 100-630 of the 5' UTR. IRES elements have been observed in the 5' UTRs of all other picornaviruses characterized to date and in a number of other viral and cellular mRNAs (Jackson et al., 1996; Sachs et al.,

1997).

Several cellular proteins have been shown to be required for IRES function. The C-terminal fragment of proteolytically cleaved eIF4G along with eIF4A and eIF3 stimulate ribosome binding to the IRES (Ohlmann et al., 1996; Borman et al., 1997a). The La autoantigen and the pyrimidine tract binding protein (PTB), proteins not normally associated with mRNA translation, have been shown to bind IRES elements (Meerovitch et al., 1993; Pestova et al., 1991). In addition, a protein termed poliovirus translation factor (PTF) has been isolated from human HeLa cells and shown to be necessary for IRES-mediated translation (Gamarnik and Andino, 1996).

Barley yellow dwarf luteovirus (BYDV) also has an internal RNA element that functions to recruit translation initiation factors to the RNA. BYDV RNA lacks a 5' cap and a 3' poly(A) tail, but a 109 nt region present 4.5 kb from the 5' end of the genome is required for efficient cap-independent translation (Wang and Miller, 1995; Wang et al., 1997). This RNA element termed a 3' translational enhancer (3' TE) functions in conjunction with the 5' UTR to recruit eIF4F to the RNA (Wang et al., 1997). The 3' TE also functions in translation initiation when moved to the 5' UTR of the mRNA (Wang et al., 1997). The mechanism by which the 3' TE functions to recruit eIF4F is not understood.

The potyvirus genome structure is similar to that of the picornaviruses in that the RNA has a 5' VPg and a 3' poly(A) tail. The 150 nt 5' UTR of tobacco etch potyvirus (TEV) enhances translation in a cap-independent manner (Carrington and Freed, 1990). It has also been demonstrated that the TEV 5' UTR and poly(A) tail function synergistically to enhance translation (Gallie et al, 1995). These observations suggest that the ends of the

RNA interact for efficient translation in a cap-independent manner (Gallie, 1996). Recently, it was shown that the VPg of turnip mosaic potyvirus (TuMV) binds to the cap binding protein eIF(iso)4E (Wittmann et al., 1997). The functional importance of the interaction between VPg and eIF(iso)4E is not known, but it has been hypothesized that this interaction may help recruit eIF(iso)4E for viral RNA translation (Wittmann et al., 1997).

Viral RNA elements which facilitate translation by mimicking a poly(A) tail have also been described. The tobacco mosaic tobamovirus (TMV) RNA is capped but not polyadenylated (Guilley et al., 1979; Rietveld et al., 1983). The 3' UTR of TMV includes five RNA pseudoknots. The two 3' pseudoknots mimic a tRNA-like structure and are required for viral replication (Salomon et al., 1976). The other three pseudoknots constitute the upstream pseudoknot domain (UPD) and are required for the stimulation of TMV translation (Gallie and Walbot, 1990; Leathers et al., 1993). The sequence and higher-order structure of the UPD are thought to be necessary for efficient translation (Leathers et al., 1993). This RNA element is codependent on the 5' cap for function in translation initiation (Leathers et al., 1993). Similar effects on translation have been observed for an RNA pseudoknot within the 3' UTR of brome mosaic bromovirus (BMV) 3' UTR (Gallie and Kobayashi, 1994).

Transport of Plant Viruses

The second objective of this thesis is to determine the functions of the ORF1 and ORF3 proteins of SBMV-C. These two proteins may be important for virus transport. Unlike animal viruses which are released from infected cells by budding or cell lysis, plant

viruses are transported from one cell to another via cellular organelles termed plasmodesmata. These channels traverse cell walls and provide a cytoplasmic connection between neighboring cells, thereby playing an important role in cell-to-cell communication and in the transport of water and nutrients throughout the plant (Deom et al., 1992; Lucas and Gilbertson, 1994). Intercellular movement of plant viruses is facilitated by virus encoded movement proteins (MPs). Although the MPs of numerous viruses have been identified, the requirements for sobemovirus cell-to-cell movement are not known (Atabekov and Taliansky, 1990; Deom et al., 1992, Lucas and Gilbertson, 1994).

Two diverse mechanisms have been described for the cell-to-cell movement of plant viruses. TMV and cowpea mosaic virus (CPMV) serve as the models for these two forms of local movement. The TMV MP binds the TMV RNA and transports it through plasmodesmata by increasing their size exclusion limit (Wolf et al., 1989; Atkins et al., 1991). The CPMV MP induces the formation of tubular structures at the sites of plasmodesmata. These tubules allow the transport of virions from cell to cell (van Lent et al., 1990; Wellink and van Kammen, 1989). In addition to MPs, viral structural proteins or proteins involved in viral RNA synthesis may be required for cell-to-cell movement (Carrington et al., 1996).

TMV moves from cell to cell as an RNA-protein complex (Atkins et al., 1991). The TMV MP (p30) binds cooperatively with and nonspecifically to single strand nucleic acid (Citovsky et al., 1990, 1992). Nucleic acid binding ability has also been observed for the MPs of red clover necrotic mosaic virus (RCNMV), alfalfa mosaic virus (AMV), and cucumber mosaic virus (CMV) (Osman et al., 1992; Schoumacher et al., 1992; Giesman-Cookmeyer and Lommel, 1993). The intracellular transport of the TMV RNA-MP

complex to the plasmodesmata may be facilitated by interactions with the cytoskeleton (Heinlein et al., 1995). Colocalization of the ribonucleic protein complex with microtubules has been observed using an MP-green fluorescent protein (GFP) fusion protein (McLean et al, 1995). Since microtubules and microfilaments facilitate directed movement of macromolecular complexes and mRNA through the cytoplasm (Vale, 1987; Lanford, 1995; St. Johnston, 1995), the cytoskeleton may provide tracks upon which viral movement complexes are transported to the plasmodesmata (Carrington et al., 1996).

The TMV MP has also been shown to alter the size exclusion limit of plasmodesmata. The diameter of the plasmodesmata is usually about 50 nm (Lucas and Gilbertson, 1994). The space formed between the plasma membrane and the appressed ER called the cytoplasmic annulus has a radial width of approximately 8-10 nm. Proteins are thought to be embedded in the inner and outer leaflets of the plasma membrane and appressed ER, and the spatial arrangement of plasmodesma proteins divides the cytoplasmic annulus into numerous microchannels having diameters of 2.5-3 nm (Lucas and Gilbertson, 1994). Cell-to-cell transport is presumed to take place through these plasmodesmal microchannels. Under physiological conditions, the size exclusion limit of plasmodesmata restricts movement of molecules less than 1 kDa in size (Goodwin, 1983; Robards and Lucas, 1990). In the presence of the TMV MP, the size exclusion limit is increased 10-fold (Wolf et al., 1991).

In contrast to TMV, CPMV moves as a virus particle through a tubule formed by its 48 kDa MP (van Lent et al., 1990). Virus-induced tubules have also been observed in plants infected with members of the caulimoviruses, nepoviruses, bromoviruses and tospoviruses (Kasteel et al., 1997; Perbal et al., 1993; Wieczorek and Sanfaçon, 1993;

Storms et al., 1995). For CPMV, the tubules may form at the sites of plasmodesmata, but tubules are also formed in CPMV-infected cowpea protoplasts (van Lent et al., 1991). The induced tubules were found to have an internal diameter roughly the width of the virions, and virus particles have been observed inside the tubules (van Lent et al., 1990; Kasteel et al., 1993). The CPMV MP has been shown to have at least two distinct functional domains, an N-terminal domain that is involved in tubular formation and a C-terminal domain that is involved in facilitating the loading of virus particles into the tubules (Lekkerkerker et al., 1996). Interestingly, it has also been shown that the CPMV MP has the ability to induce tubules in insect cells (van Lent et al., 1991; Kasteel et al., 1993, 1996; Storms et al., 1995). This suggests that the MP interacts with cellular components that have been conserved across animal and plant kingdoms.

Compared to the amount of information on cell-to-cell movement of plant viruses, little is known about long-distance movement. This form of movement occurs through the vascular tissue and requires that the virus or viral nucleic acid enters and exits the conductive tissue (i.e., bundle sheath cells, phloem parenchyma, companion cells, and the sieve elements) (Atabekov and Taliansky, 1990). It is thought that long distance movement involves viral and host factors that are distinct from those involved in cell-to-cell movement (Carrington et al., 1996). Viral proteins have been identified which support long-distance but not cell-to-cell movement (Petty et al., 1990; Traynor et al., 1991; Nelson et al., 1993), and many viruses require the coat protein for long-distance movement (Dawson et al., 1988; Vaewhongs and Lommel, 1995; Petty et al., 1990; Suzuki et al., 1991; Boccard and Baulcombe, 1993). In addition, viral nonstructural proteins such as the p19 of tomato bushy stunt tombusvirus and the HC-Pro of

potyviruses may function in long-distance movement (Scholthof et al., 1995; Klein et al., 1994; Cronin et al., 1995). The replicase proteins of some plant viruses have also been shown to be involved in long-distance movement (Nelson et al., 1993; Gal-on et al., 1994; Weiland and Edwards,1996).

Though the requirements for cell-to-cell movement for most groups of plant viruses have been determined, no MP has been identified for the sobemovirus group. The RYMV coat protein has been shown to be required for efficient cell-to-cell movement, but it is not known if any of the RYMV nonstructural proteins are required (Brugidou et al., 1995). Chapter II describes a genetic approach to determine the requirements for SBMV-C cell-to-cell movement.

CHAPTER II

IDENTIFICATION OF VIRAL GENES REQUIRED FOR CELL-TO-CELL MOVEMENT OF SOUTHERN BEAN MOSAIC VIRUS

INTRODUCTION

Most plant viruses are known to express one or more proteins that facilitate cell-to-cell movement of the viral genome through plasmodesmata (Carrington et al., 1996). In general, the movement proteins (MPs) of the positive-sense RNA viruses have been shown to function by one of two mechanisms. The MP may bind the viral genome and transport it through the plasmodesmata. This mechanism has been described in detail for the MP of TMV (Citovsky et al., 1990). The TMV MP not only forms a complex with the TMV RNA, but it also interacts directly with plasmodesmata to increase their size exclusion limit (Wolf et al., 1989). Intracellular transport of the MP-RNA complex to the plasmodesmata may be facilitated by an interaction between this complex and the cytoskeleton (Heinlein et al., 1995; McLean et al., 1995). The second mechanism involves the formation of intercellular tubules by the MP. For example, tubules formed by the 48 kDa MP of CPMV transport CPMV particles to adjacent cells (van Lent et al., 1990, 1991; Wellink et al., 1993).

An MP has not been identified for the sobemoviruses, a group of positive-sense RNA viruses whose type member is southern bean mosaic virus (SBMV) (Hull, 1988). Little is known about the functions of the SBMV-C proteins in the viral infection cycle. Based on amino acid homology to other viral proteins of known function, the 105 kDa protein may be a polyprotein that is proteolytically processed to yield VPg, a serine protease, and an RNA-dependent RNA polymerase (RdRp) (Wu et al., 1987; Gorbalenya

et al., 1988). Functions for the ORF1 and ORF3 proteins have not been assigned. All the sobemoviruses characterized to date express an ORF1 protein, but there is little amino acid sequence conservation among these proteins (Othman and Hull, 1995; Mäkinen et al., 1995b). ORF3 is conserved in the genomes of at least two other sobemoviruses, rice yellow mottle virus (RYMV) (Ngon A Yassi et al., 1994) and lucerne transcient streak virus (LTSV) (Jeffries et al., 1995). Interestingly, a distinct ORF3 is absent from the genome of cocksfoot mottle sobemovirus (CfMV), but an ORF3-like domain is present within ORF2b, the gene that also encodes the putative RdRp of this virus (Mäkinen et al., 1995b; Ryabov et al., 1996). CfMV ORF2b is expressed by a -1 ribosomal frameshift in ORF2a to produce a 103 kDa protein (Mäkinen et al., 1995a). This protein may be proteolytically processed to yield VPg, a serine protease, an ORF3-like protein, and an RdRp (Mäkinen et al., 1995b; Ryabov et al., 1996). The -1 ribosomal frameshift site in CfMV is conserved near the 5' end of ORF3 in the genomes of the sobemoviruses described above (Mäkinen et al., 1995a).

The only other information on the functions of the sobemovirus proteins has come from experiments with RYMV. The involvement of the RYMV CP in virus transport has been demonstrated using an infectious genome-length cDNA clone of this virus (Brugidou et al., 1995). Two RYMV CP mutants, each expressing a CP with a C-terminal deletion, did not systemically infect rice and moved from cell to cell inefficiently as compared to the spread of wild-type virus. These results suggest that the RYMV CP is required for systemic movement and for efficient cell-to-cell movement.

The purpose of the present study was to determine the requirements for SBMV-C cell-to-cell movement. A full-length SBMV-C cDNA clone was constructed, and

transcripts synthesized from this clone were shown to be infectious in cowpeas. The analysis of ORF1, ORF3, and CP mutants demonstrated that all three of these genes are required for SBMV-C cell-to-cell movement.

MATERIALS AND METHODS

Construction of a genome-length SBMV-C cDNA clone

RNA was isolated from purified SBMV-C by incubation in 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA for 20 hr at 37°C followed by extraction with phenol: chloroform (1:1). Oligonucleotide SBMV22, complementary to the 3' end of the RNA (Table 1; Fig.2A), was used to prime cDNA synthesis by AMV reverse transcriptase (Boehringer Mannheim) according to the manufacturer's specifications. A nonviral SmaI site was present at the 5' end of SBMV22. SBMV-C cDNA was used as a template for the amplification of four overlapping regions of the SBMV-C genome by the polymerase chain reaction (PCR). PCR was performed with *Pfu* DNA polymerase (Stratagene) and the following pairs of oligonucleotides: SBMV2 and SBMV9 (SBMV-C nt 1-628), SBMV5 and SBMV8 (SBMV-C nt 547-1700), SBMV3 and SBMV4 (SBMV-C nt 1637-3462), and SBMV7 and SBMV22 (SBMV-C nt 3247-4194) (Table 1; Fig.2A). Oligonucleotide SBMV2 included the sequence for the bacteriophage T7 RNA polymerase promoter in addition to two nonviral G residues between the promoter and SBMV-C nt 1.

The SBMV2/SBMV9 PCR product was cloned as a blunt-end fragment into the HincII site of pUC19 to produce pORF1. The three other PCR products were cloned separately into HincII-digested pBluescript (Stratagene) to produce pORF2A

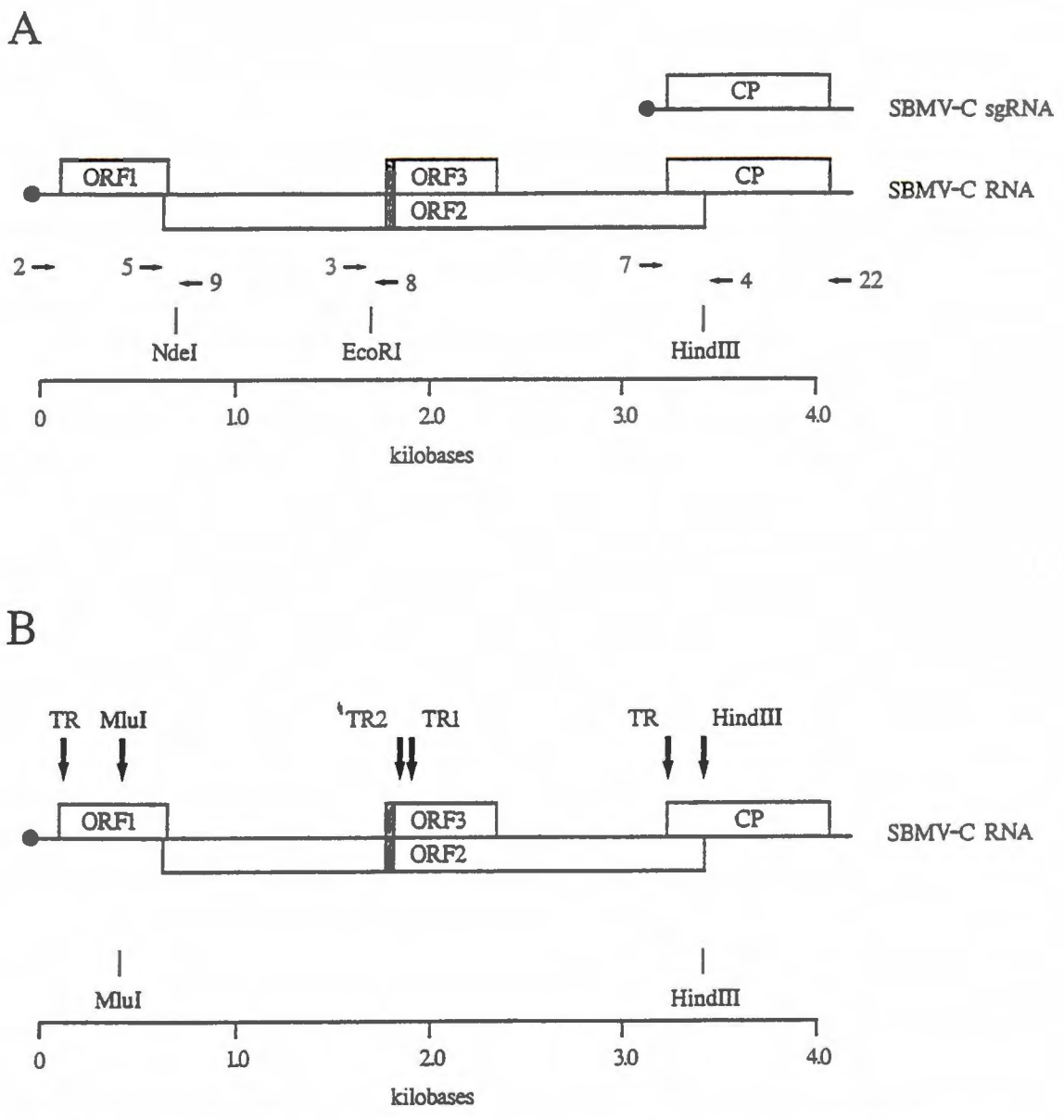


Figure 2. Diagram describing the cloning strategy and mutant constructs. **(A)** SBMV-C genome organization and cloning strategy. Diagrams of the SBMV-C genomic and subgenomic RNAs are shown. The ORFs are illustrated as boxes. The predicted site of –1 ribosomal frameshift is shown as a hatched box. VPg is illustrated as a closed circle at the 5' ends of the RNAs. The positions of restriction sites that were used for constructing the genome-length clone are indicated. The positions of the forward and reverse oligonucleotide primers used for cDNA synthesis and PCR are illustrated with arrows. The oligonucleotides are described in Table 1. **(B)** Mutations within the SBMV-C genome. The positions of the ORF1 (TR and MluI), ORF3 (TR1 and TR2), and CP (TR and HindIII) mutations are marked with arrows. The positions of the MluI and HindIII restriction sites used to construct pSBMV1-ORF1 MluI and pSBMV1-CP HindIII are marked.

Table 1. Description of Oligonucleotides Used in This Study

Designation	Sequence (5' to 3')	Description
SBMV2	GGGGG**TAATACGACTCACTATA**GGCACAAAA-TATAAGAAGGAAAAGTGC	Equivalent to SBMV-C nt 1-25 (underlined), T7 RNA Polymerase promoter
SBMV3	GGGCAAAGTGAAGTTGGG	Equivalent to SBMV-C nt 1637-1654
SBMV4	CGACGAGCGTAGCATGGGAGG	Complementary to SBMV-C nt 3442-3462
SBMV5	CGCTTCCAGTACACCTGCCG	Equivalent to SBMV-C nt 547-566
SBMV7	CCGCGCGAGATAAGAGC	Equivalent to SBMV-C nt 3247-3263
SBMV8	GTCCGCCCAGGCTTTCCC	Complementary to SBMV-C nt 1683-1700
SBMV9	GCGAAGCTCCACATGTTCGCAACTAGG	Complementary to SBMV-C nt 602-628
SBMV22	CG**CCCGGG**CCATTCGGATAGCGCTCGG	Complementary to SBMV-C nt 4176-4194 (underlined), SmaI restriction site (bold)
SBMV48	CAAGCGGGTAGCCGTTACAAATGCTC	Complementary to SBMV-C nt 3260-3285, mutagenesis of nt 3272 (underlined)
SBMV50	GCTTAAAACCAATCATCAATAAATCATGAAAC	Complementary to SBMV-C nt 44-75, mutagenesis of nt 58-62 (underlined)
SBMV52	GCAAGTCGATGATCAACAATTTCAGAAGGGCA-TGCC	Complementary to SBMV-C nt 1892-1927, mutagenesis of nt 1904 and 1913 (underlined)
SBMV74	CATGCCACCTGTTTAGTGGGTGGGTCCAC	Complementary to SBMV-C nt 1869-1897, mutagenesis of nt 1883 (underlined)

(SBMV5/SBMV8), pORF2B (SBMV3/SBMV4), and pORF4 (SBMV7/SBMV22). The EcoRI/HindIII fragment (SBMV-C nt 1662-3433) from pORF2B was cloned into EcoRI/HindIII-digested pORF2A to produce pORF2. The NdeI/HindIII fragment (SBMV-C nt 593-3433) from pORF2 was then cloned into NdeI/HindIII-digested pORF1 to produce pORF1/2. Finally, the HindIII/ClaI fragment from pORF4 (including SBMV-C nt 3433-4194) was cloned into HindIII/EcoRI-digested pORF1/2. In this step, the ClaI and EcoRI sites were present in the multiple cloning sites of the respective vectors. Both of these restriction sites were made blunt with Klenow fragment prior to ligation. The resulting genome-length SBMV-C clone was designated pSBMV1. Digestion of this clone with SmaI produced a 4.2 kbp DNA fragment which contained the SBMV-C cDNA and the T7 RNA polymerase promoter. This fragment was cloned into the SmaI site of pUC129 (a gift from Dr. Noel Keen) to produce pSBMV2 which was subsequently used for oligonucleotide-directed mutagenesis.

Construction of mutants

pSBMV1-ORF1 MluI and pSBMV1-CP HindIII were constructed by digestion of pSBMV1 with MluI (SBMV-C nt 359) and HindIII (SBMV-C nt 3433), respectively. The ends were made blunt with Klenow fragment and religated. Oligonucleotide-directed mutagenesis to produce pSBMV2-ORF1 TR, pSBMV2-ORF3 TR1, pSBMV2-ORF3 TR2 and pSBMV2-CP TR was performed as described by Kunkel (1985) using oligonucleotides SBMV50, SBMV52, SBMV74, and SBMV48, respectively (Table 1). All mutations were confirmed by DNA sequencing.

Inoculation of plants and protoplasts

Capped transcripts were synthesized from SmaI-linearized plasmid DNA (1μg) in the presence of GpppG (Pharmacia) using the Ribomax kit (Promega) according to the manufacturer's recommendations. Following a 2 hr incubation at 37°C, plasmid DNA was digested with DNase I (Pharmacia) at 37°C for 15 min. For plant inoculations, the transcription reactions were diluted with one volume of 20 mM sodium phosphate (pH 7.0) and one volume of inoculation buffer [10 mM sodium phosphate (pH 7.0), 1% (w/v) bentonite, and 1% (w/v) celite] prior to mechanical inoculation of the cotyledons of 8-10 day old *V. unguiculata* cv. California blackeye (cowpea). Plants were maintained under greenhouse conditions for 3 weeks.

Protoplasts were prepared from the cotyledons of 8-10 day old cowpea plants as described by Jones et al. (1990). A BioRad Gene Pulser was used to electroporate 2×10^6 protoplasts with 6 μg of capped transcript RNA in cold 0.6 M mannitol and 10 mM $CaCl_2$ with a pulse of 1,550 V from a 25 μF capacitor. The protoplasts were then incubated on ice for 15 min prior to centrifugation at 75 x g for 5 min in a clinical centrifuge. The pelleted protoplasts were suspended in incubation buffer containing 0.6 M mannitol, 10 mM $CaCl_2$, and 1X Aoki salts (Aoki and Takebe, 1969) and incubated at room temperature under fluorescent light for 40 hr.

Analysis of RNA from plants and protoplasts

Inoculated and systemic cowpea leaves were collected at 14 and 21 days postinoculation, respectively. Total RNA was extracted from 0.5g of tissue as described by Hillman et al. (1987), and 20 μg of RNA was blotted onto a nylon membrane using a

Schleicher and Schuell slot-blot manifold. Hybridization was performed in 6X SSC and 1X Denhardt's solution (Sambrook et al., 1989) at 65° C for 16 hr using ^{32}P-labeled pSBMV1 DNA as a probe.

Total RNA was extracted from protoplasts 40 hr after electroporation as described by Jones et al. (1990). Following two extractions with phenol:chloroform (1:1) and precipitation with ethanol, the RNA was treated with DNase I for 15 min at 37°C to remove residual plasmid DNA. RNA (15 μg) was blotted onto a nylon membrane using a slot-blot manifold. Hybridization was performed as described above.

Analysis of virus from plants and protoplasts

Inoculated and systemic cowpea leaves were collected as described above. Virus was isolated from 0.5 g of tissue by precipitation with polyethylene glycol (PEG) as described by Lommel et al. (1982). After the second PEG precipitation, virus was resuspended in 50 μl of 10 mM NaOAc (pH 5.5). To detect SBMV-C coat protein, virus preparations were analyzed by 12.5% SDS-PAGE. Proteins were detected by staining with Coomassie brilliant blue.

Protoplasts were collected 40 hr after electroporation by centrifugation at 75 x g for 3 min. After removal of the supernatant, the cells were suspended in 250 μl of 20 mM NaOAc (pH 5.5) and lysed by sonication. Cell debris was removed by centrifugation at 14,000 rpm for 15 min in a microcentrifuge. Virus in the clarified lysates was detected by immunosorbent electron microscopy as described by Fuentes and Hamilton (1993) using an anti-SBMV-C antiserum (American Type Culture Collection). Virus immunosorbed to copper grids was visualized using a Hitachi 600 transmission electron microscope.

RESULTS

Characterization of the genome-length SBMV-C cDNA clone

A genome-length SBMV-C cDNA clone designated pSBMV1 was constructed in pUC19 from four overlapping SBMV-C PCR products as described in Materials and Methods (Fig.2A). The cloned SBMV-C cDNA was flanked at its 5' end by the bacteriophage T7 RNA polymerase promoter and at its 3' end by a SmaI restriction site. To facilitate transcription by the T7 RNA polymerase two nonviral G residues were included between the promoter and SBMV-C nt 1. After the construction of this clone it was demonstrated that the published sequence for SBMV-C included a 5' terminal C residue that was not present in the viral RNA (Hacker and Sivakumaran, 1997). Transcript RNA synthesized from SmaI-digested pSBMV1 using T7 RNA polymerase therefore contained three nonviral nucleotides at both its 5' end (GGC) and its 3' end (GGG). The 3' terminal nonviral nucleotides were contributed by a portion of the SmaI restriction site.

Both capped and uncapped transcripts synthesized *in vitro* from pSBMV1 were infectious in cowpea plants. Mosaic symptoms were initially observed on systemic leaves 10-14 days postinoculation. For plants inoculated with SBMV-C RNA, systemic symptoms were observed 6-7 days postinoculation. Both the inoculated and systemic leaves of transcript-inoculated plants yielded SBMV-C (Fig.3). Primer extension analysis of the 5' end of the viral RNA recovered from this virus demonstrated that the three 5' nonviral nucleotides were not retained during SBMV-C replication in cowpea (data not shown).

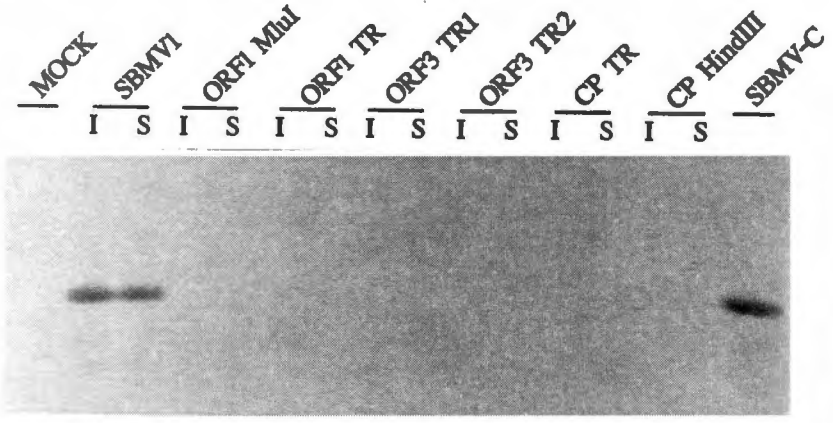


Figure 3. SDS-PAGE analysis of SBMV-C coat protein. Virus was isolated from the inoculated (I) and systemic (S) leaves of cowpea plants following inoculation with RNA transcripts synthesized from plasmid DNA as indicated. The virus preparations were electrophoresed on a 12.5% denaturing polyacrylamide gel, and protein was detected with Coomassie blue. Purified SBMV-C was used as a standard in the lane marked SBMV-C. An extract from an uninfected cowpea plant was used as a control (MOCK).

Construction of SBMV-C mutants

The genome-length SBMV-C clones pSBMV1 and pSBMV2 were used to generate mutations in the ORF1, ORF3, and CP genes. A total of six mutations were constructed, two in each gene (Fig.2B). To generate the ORF1 mutant pSBMV1-ORF1 MluI an insertion of four base pairs was made at the MluI site (SBMV-C nt 359). This mutant was expected to produce a truncated ORF1 protein of 105 aa (Fig.4). The wild-type ORF1 protein is 184 aa in length (Wu et al., 1987). pSBMV2-ORF1 TR was generated by the substitution of nonsense codons for the third and fourth codons of ORF1 (Fig.4). Nonsense codons were also substituted for the third and sixth codons 3' to the potential ORF3 initiation codon (SBMV-C nt 1895) to generate the ORF3 mutant pSBMV2-ORF3 TR1 (Fig.4). To construct the second ORF3 mutant pSBMV2-ORF3 TR2, a Glu codon (GAA; SBMV-C nt 1883-1885) between the predicted site of the -1 ribosomal frameshift (nt 1796-1802) and the potential ORF3 initiation codon (SBMV-C nt 1895) was changed to a nonsense codon (UAA) (Fig.4). This mutation was expected to terminate ORF3 translation if this gene was translated by a -1 ribosomal frameshift in ORF2. The CP mutant pSBMV1-CP HindIII was constructed by the insertion of four base pairs at the HindIII site (SBMV-C nt 3433) (Fig.4). A truncated CP containing aa 1-55 was expected to be translated from this mutant. pSBMV2-CP TR was constructed by making a T to C substitution at SBMV-C nt 3272 that changed the CP initiation codon to ACG (Fig.4). Translation of CP was therefore expected to initiate at the AUG codon at SBMV-C nt 3403 to produce a truncated CP containing aa 44-260. None of these mutations affected the amino acid sequence of p105 expressed from ORF2.

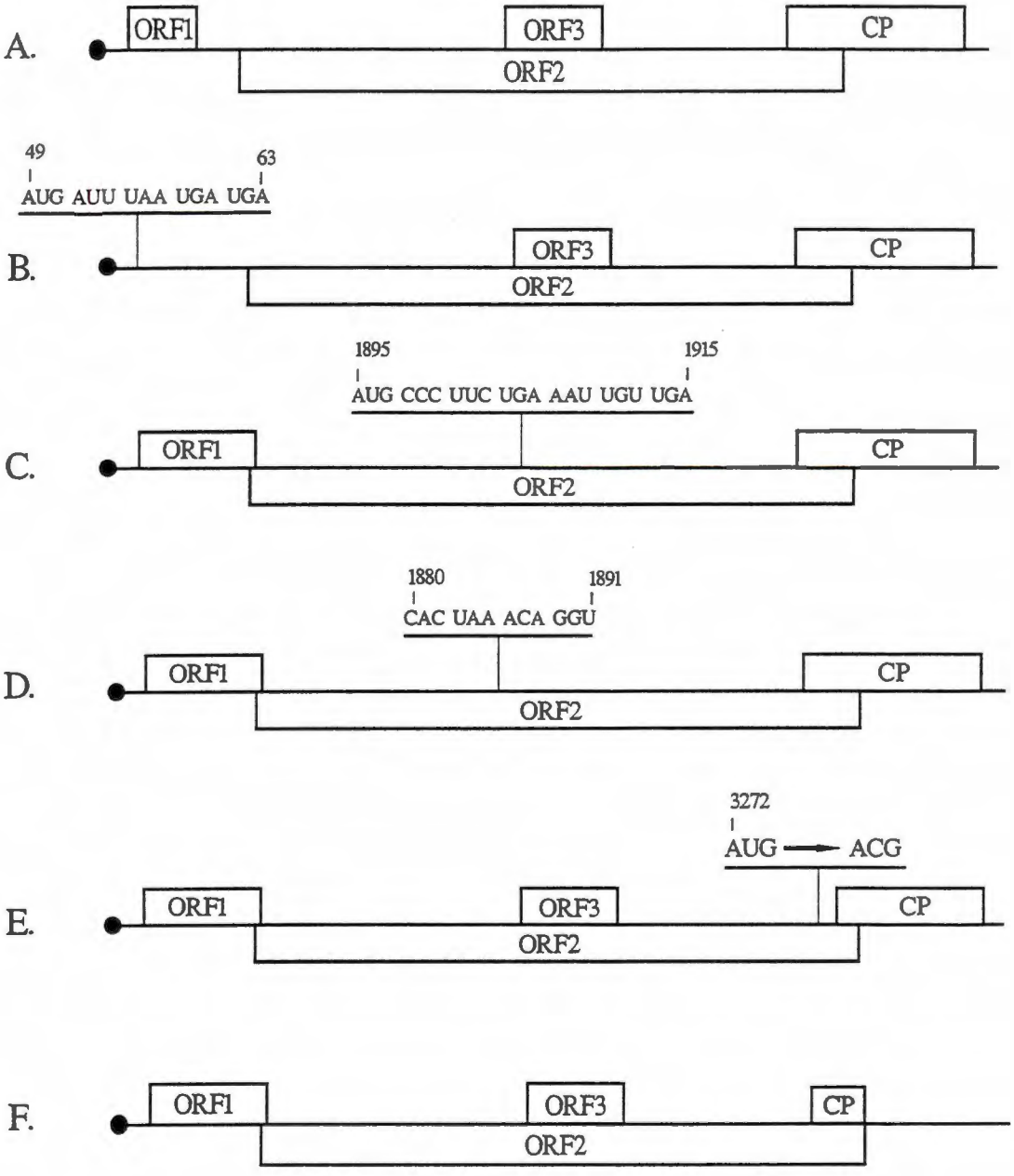


Figure 4. Diagram describing the mutant constructs. **(A)** pSBMV1-ORF1 MluI has a four base pair insertion at the MluI site and is expected to produce a truncated ORF1 protein. **(B)** pSBMV2-ORF1 TR has stop codons for the third and fourth codons of ORF1. **(C)** pSBMV2-ORF3 TR1 has stop codons for the third and sixth codons of ORF3. **(D)** pSBMV2-ORF3 TR2 has a stop codon inserted between the –1 ribosomal frameshift site and the ORF3 initiation site. **(E)** pSBMV2-CP TR has the CP initiation site at SBMV-C 3272 changed to ACG, and is expected to initiate from the AUG at SBMV-C 3403. **(F)** pSBMV1-CP HindIII has a four base pair insertion at the HindIII site and is expected to produce a truncated CP.

Infections of cowpea plants with wild-type and mutant SBMV-C transcripts

Capped transcripts synthesized *in vitro* from pSBMV1 and the six mutant constructs were individually inoculated onto the cotyledons of 8-10 day old cowpea plants. The inoculated and systemic leaves of the plants were harvested at 14 and 21 days postinoculation, respectively. Virus was only isolated, however, from plants inoculated with pSBMV1 RNA (Fig.3). To determine if viral RNA was present in plants inoculated with the mutant transcripts, total RNA was extracted from the inoculated and systemic leaves of these plants, blotted to a nylon membrane using a slot-blot manifold, and hybridized to a radiolabeled SBMV-C probe. Viral RNA was only detected in the inoculated and systemic leaves of plants inoculated with pSBMV1 RNA (Fig.5).

Infections of cowpea protoplasts with wild-type and mutant SBMV-C transcripts

To determine if the mutations in the ORF1, ORF2, and CP genes affected SBMV-C RNA synthesis, transcripts from pSBMV1 and the six mutant constructs were electroporated individually into cowpea protoplasts. As controls, protoplasts were electroporated with either SBMV-C RNA or transcript RNA synthesized from EcoRI-digested pSBMV1 (Fig.2A). ΔEcoRI RNA was 1662 nt in length and was not expected to replicate since it did not encode the SBMV-C RdRp. After electroporation with SBMV-C RNA, viral RNA accumulation reached a maximum at 24-40 hr and then declined (data not shown). Based on this result, total RNA was extracted from protoplasts 40 hours after electroporation with transcript RNA. Equal amounts of RNA were blotted to a nylon membrane using a slot-blot manifold and hybridized to a radiolabeled SBMV-C probe. All of the synthetic full-length SBMV-C RNAs replicated in

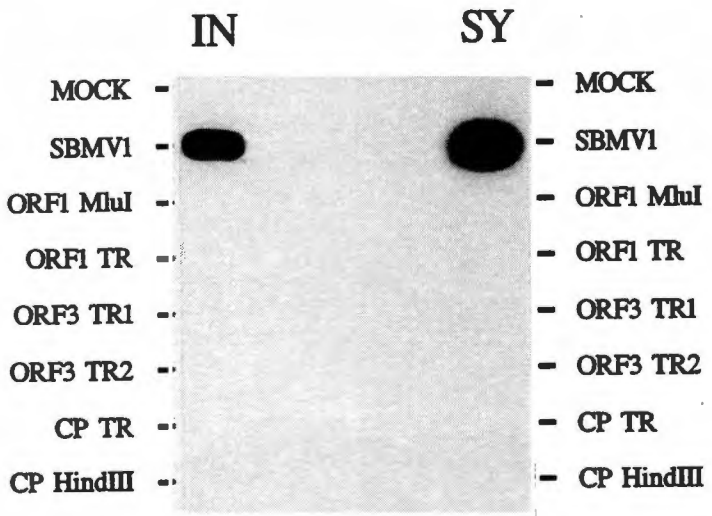


Figure 5. Hybridization analysis of total RNA from cowpea plants. Plants were inoculated with RNA transcripts synthesized from plasmid DNA as indicated. Total RNA was isolated from the inoculated (IN) and systemic (SY) leaves of inoculated cowpeas and analyzed as described in Materials and Methods. Total RNA from an uninfected cowpea plant was used as a control (MOCK).

cowpea protoplasts as judged by the accumulation of viral RNA over background levels (Fig.6). In contrast, no viral RNA was detected in cells electroporated with the nonreplicating ΔEcoRI RNA or in cells receiving no SBMV-C RNA (Fig.6). Among the SBMV-C mutants, only ORF1 MluI RNA consistently accumulated to lower levels in protoplasts than did SBMV1 RNA. The level of viral RNA accumulation was significantly higher in protoplasts electroporated with SBMV-C RNA as compared to those electroporated with SBMV1 RNA. This difference in the accumulation of RNA may be due to the presence of nonviral nucleotides at the 5' and 3' ends of the synthetic SBMV1 RNA and may account for differences observed in the appearance of SBMV-C symptoms in cowpeas inoculated with SBMV-C RNA versus SBMV1 RNA.

To determine if the mutations in the ORF1, ORF3, and CP genes affected SBMV-C assembly, transcripts from pSBMV1 and the six mutant constructs were electroporated individually into cowpea protoplasts. As a control, protoplasts were electroporated with SBMV-C RNA. At 40 hr after electroporation cells were lysed by sonication. Virus particles present in the lysates were trapped on copper grids using antibody to the SBMV-C coat protein and viewed by electron microscopy. Spherical particles measuring 27.7 nm ± 2.6 nm in diameter were observed in lysates from cells electroporated with SBMV-C RNA (Fig.7A) and in lysates from cells electroporated with transcript RNA from pSBMV1 (Fig.7B), either of the two ORF1 mutants (Fig.7C,D), or either of the two ORF3 mutants (Fig.7E,F). No virus particles were observed in lysates from cells electroporated with CP HindIII or CP TR RNA or from cells which received no SBMV-C RNA (data not shown).

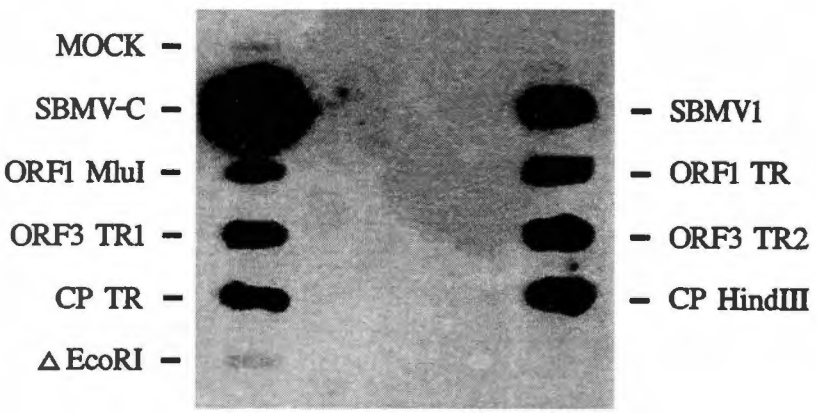


Figure 6. Hybridization analysis of total RNA from cowpea protoplasts. Protoplasts were electroporated with SBMV-C virion RNA (SBMV-C) or with RNA transcripts synthesized from plasmid DNA as indicated. Protoplasts were also electroporated in the absence of SBMV-C RNA (MOCK). Total RNA from protoplast extracts were analyzed as described in Materials and Methods.

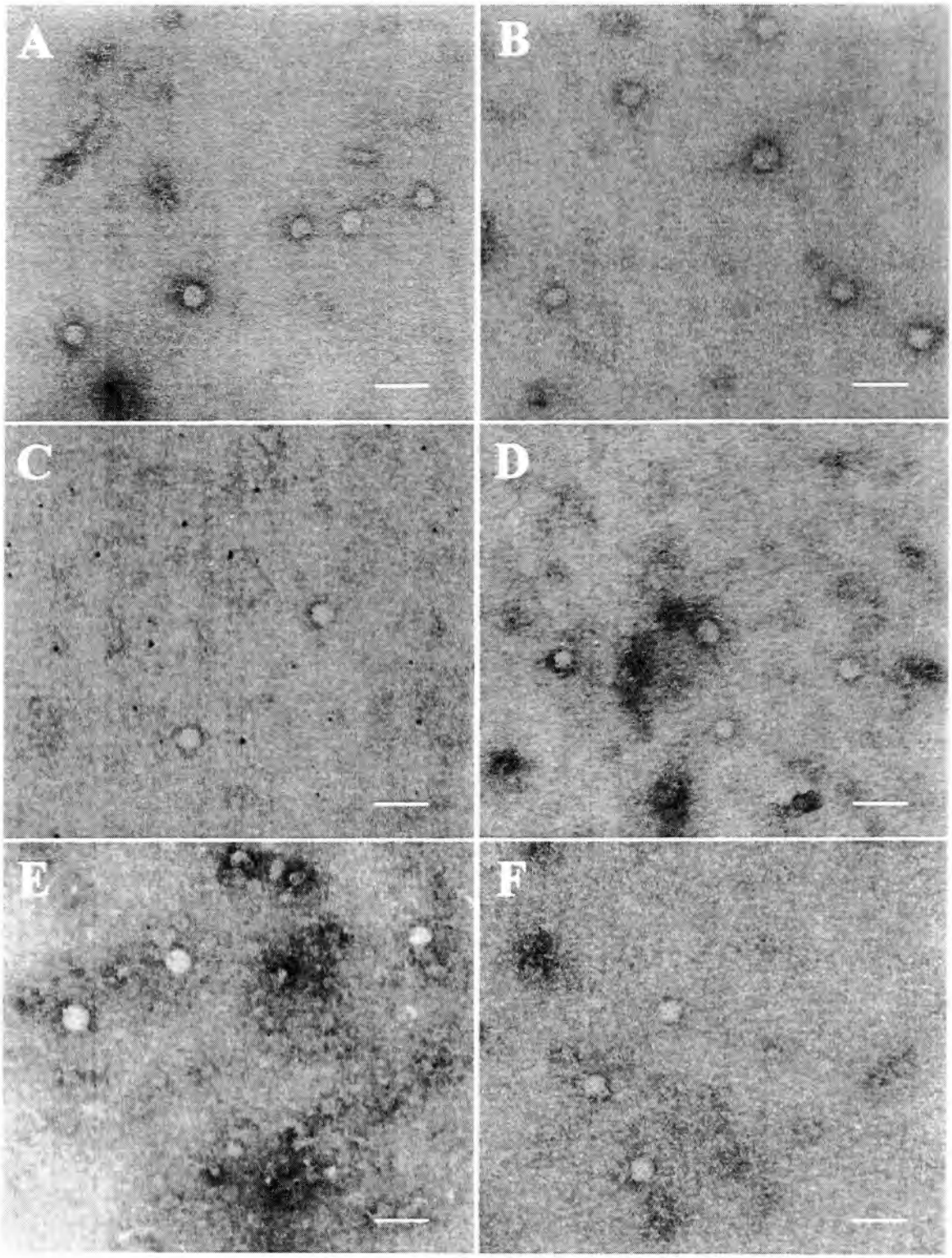

A
B
C
D
E
F

Figure 7. Electron micrographs of SBMV-C particles from cowpea protoplasts. Protoplasts were electroporated with (A) SBMV-C virion RNA or with RNA transcripts synthesized from the following plasmids: (B) pSBMV1, (C) pSBMV1-ORF1 MluI, (D) pSBMV2-ORF1 TR, (E) pSBMV2-ORF3 TR1, and (F) pSBMV2-ORF3 TR2. Lysates from the protoplasts were analyzed by immunosorbent electron microscopy. Bar markers represent 50 nm. (Immunosorbent electron microscopy data and electron micrographs provided by Mr. Benjamin Fowler)

DISCUSSION

This is the second report of the synthesis of infectious RNA from a genome-length cDNA clone of a sobemovirus. Previously, Brugidou et al. (1995) constructed an infectious RYMV cDNA clone and described the phenotypes of two CP mutants. The SBMV-C cDNA clone was used to determine the roles of three viral proteins in the SBMV-C infection cycle. It was demonstrated that the ORF1, ORF3, and coat proteins are required for SBMV-C infection of cowpea. The three viral proteins are not, however, required for SBMV-C RNA synthesis or assembly. These results suggest that the SBMV-C ORF1 and ORF3 proteins along with CP are required for SBMV-C cell-to-cell movement.

Since ORF1 and ORF3 proteins are nonstructural proteins required for cell-to-cell movement, these proteins may be tentatively classified as MPs. The mechanism by which these proteins function and their subcellular locations are currently unknown. The ORF1 protein is acidic with a calculated pI of 5.0, while the ORF3 protein is basic with a calculated pI of 9.5. Computer analysis of the amino acid sequences of the sobemovirus ORF1 and ORF3 proteins using the Kyte-Doolittle algorithm did not reveal any conserved hydrophobic regions in either of these proteins that were indicative of transmembrane domains (data not shown) (Kyte and Doolittle, 1982).

The requirement for CP in SBMV-C cell-to-cell movement suggests that virus particles rather than unencapsidated RNA are transported from cell to cell. Alternatively, the CP may have distinct functions in assembly and cell-to-cell movement as described for the tobacco etch potyvirus CP (Dolja et al., 1994; 1995). One argument against separable assembly and cell-to-cell transport functions for the SBMV-C CP is that the CP mutants

were each expected to express a truncated form of the protein. The SBMV-C CP may be divided into two domains, the N-terminal random (R) domain (amino acids 1-42) and the C-terminal shell (S) domain (amino acids 65-260) that are connected by a flexible arm (amino acids 43-64) (Abad-Zapatero et al., 1980). The R domain and part of the arm (amino acids 1-55), and the arm and the S domain (amino acids 44-260) were expressed by CP HindIII and CP TR, respectively. The inability of these mutants to move from cell to cell suggests that neither the R nor the S domain alone is sufficient for cell-to-cell transport. It should be pointed out, however, that it was not possible to detect either of these truncated forms of CP in infected cowpea.

The results presented here differ from those of Brugidou et al. (1995). The RYMV CP is required for infection of rice, but RYMV does move cell-to-cell, albeit very inefficiently, in the absence of CP. It remains a possibility that the level of cell-to-cell spread of SBMV-C RNA in the absence of full-length CP was undetectable by the slot-blot assay. Certainly, it may be stated for both SBMV-C and RYMV that wild-type levels of cell-to-cell movement do not occur in the absence of full-length CP.

Others have noted that there is little amino acid conservation in the ORF1 proteins of the sobemoviruses whose genomes have been characterized (Mäkinen et al., 1995b; Ngon A Yassi et al., 1994; Othman and Hull, 1995). For example, the ORF1 proteins of the C and B strains of SBMV are only 25.7% identical and 47.6% similar in amino acid sequence (Othman and Hull, 1995). The only suggestion that the sobemovirus ORF1 proteins are related to the MPs of other positive-sense RNA viruses is the observation that a region of CfMV ORF1 has nucleic acid identity to the beet yellows closterovirus p65 gene that encodes the putative MP of this virus (Mäkinen et al., 1995b).

In contrast to the ORF1 protein, amino acid homology to the sobemovirus ORF3 proteins has been observed in other viral proteins (Ryabov et al., 1996). The amino acid sequence GWPERGSGAEIGSLLLQA from SBMV-C ORF3 (encoded by SBMV-C nt 2009-2062) is conserved in ORF3 of the subgroup II luteoviruses and in ORF3 of pea enation mosaic virus RNA1 (Ryabov et al., 1996). For these viruses and for the sobemovirus CfMV the conserved domain is translated as part of a polyprotein that also includes the RdRp domain. It will be of interest to determine if the luteovirus and CfMV ORF3-like domains function in cell-to-cell movement.

The results presented here also suggest that the SBMV-C ORF3 protein may be expressed by a -1 ribosomal frameshift in ORF2 as previously predicted (Mäkinen et al., 1995a). The ORF3 TR2 mutant has an in-frame stop codon between the predicted frameshift site (nt 1796-1802) and a potential initiation codon in the ORF3 reading frame (SBMV-C nt 1895). The inability of this mutant to infect cowpea suggests that the ORF3 protein was not expressed, possibly as the result of premature termination at the in-frame stop codon following a -1 ribosomal frameshift. It is also possible that the ORF3 TR2 mutation may have affected initiation of translation at the ORF3 AUG codon (nt 1895). However, conservation of the frameshift site in RYMV, SBMV-B, and LTSV and demonstration of a -1 ribosomal frameshift to express ORF2b of CfMV further support the conclusion that SBMV-C ORF3 may be expressed by a -1 ribosomal frameshift in ORF2 (Mäkinen et al., 1995a).

This is the first report of the identification of an MP for a sobemovirus. The mechanism by which the sobemoviruses move from cell to cell, however, is not known. The possible requirement for particles in cell-to-cell transport suggests that SBMV-C may

move via intercellular tubules as observed for the como- and nepoviruses and the *Bromoviridae* (van Lent et al., 1990; Wieczorek and Sanfaçon, 1993; Kasteel et al., 1997). Additional experiments will be necessary to determine the precise functions and subcellular locations of the ORF1 and ORF3 proteins. For this reason, recombinant ORF1 and ORF3 proteins have been purified and used to raise antisera. With these reagents it should be possible to gain a better understanding of SBMV-C cell-to-cell movement in the near future.

CHAPTER III

EXPRESSION OF SOUTHERN BEAN MOSAIC VIRUS GENOMIC RNA

INTRODUCTION

SBMV-C is a member of the sobemovirus group of positive-sense RNA viruses (Hull, 1988). The monopartite genome of SBMV-C is 4.2 kb in length and encodes four ORFs (Fig.1) (Wu et al., 1987). The genomic RNA serves as the mRNA for ORFs 1-3, and the coat protein (CP) gene is expressed from a subgenomic RNA (sgRNA) that is synthesized in the infected cell (Rutgers et al., 1980; Salerno-Rife et al., 1980). The sgRNA is approximately 950 nt in length and is co-terminal with the 3' end of the genomic RNA (Rutgers et al., 1980; Hacker and Sivakumaran, 1997). The two RNA species are not capped at their 5' ends or polyadenylated at their 3' ends. Instead, the 5' ends are covalently bound to a viral protein VPg (Ghosh et al., 1979, 1981), and 120 nt at the 3' end of the genome are predicted to fold into an RNA hairpin that is conserved in the genomes of other sobemoviruses (Ngon A Yassi et al., 1994; Ryabov et al., 1996). These structural features suggest that translation initiation on SBMV-C RNAs may be mechanistically different from that observed for capped and polyadenylated mRNAs.

Numerous studies have shown that cap-dependent translation requires the binding of eukaryotic initiation factor 4F (eIF4F) to the capped 5' end of the mRNA followed by binding of the 43S ribosomal complex at or near the 5' end. Subsequent 5' to 3' scanning of the 43S complex to an AUG codon occurs in conjunction with RNA unwinding by eIF4A and eIF4B (Jackson et al., 1995). Alternatively, cap-independent translation has been documented for several viral and cellular mRNAs (Jackson and Kaminski, 1995).

Translation initiation on these mRNAs requires an internal ribosome entry site (IRES) located within the 5' UTR. These mRNAs are characterized by long 5' UTRs that contain multiple AUG codons and extensive RNA secondary structure that is required for IRES function. Cap-independent internal ribosome entry, however, has been shown to require eIF4F and other translation initiation factors that are necessary for cap-dependent translation (Anthony and Merrick, 1991; Pause et al., 1994; Ohlmann et al., 1996; Pestova et al., 1996).

Given the unusual nature of the 5' and 3' ends of SBMV-C RNA, a study was initiated to determine the mechanism(s) involved in its translation. The results described in this chapter demonstrate that ORF1 and ORF2 are translated by a 5' dependent scanning mechanism rather than by cap-independent internal ribosome entry.

MATERIALS AND METHODS

Construction of Mutants

A full-length SBMV-C cDNA clone pSBMV2 (see chapter II) was used for oligonucleotide-directed mutagenesis according to the method of Kunkel (1985). Mutants pSBMV-G22U/U23G, pSBMV-A15U, pSBMV-Py29-35Pu, pSBMV-U231A, pSBMV-G500U, pSBMV-U50C/G51C were constructed using the oligonucleotides SBMV64, SBMV72, SBMV49, SBMV66, SBMV67, and SBMV85 respectively. pSBMV-G22U/U23G/U231A was constructed from pSBMV-G22U/U23G using oligonucleotide SBMV66 (Fig.8A). Descriptions of the oligonucleotides used in this study are provided in Table 2. All oligonucleotides were synthesized by the Marshall University DNA Core Facility. All mutations were confirmed by DNA sequencing.

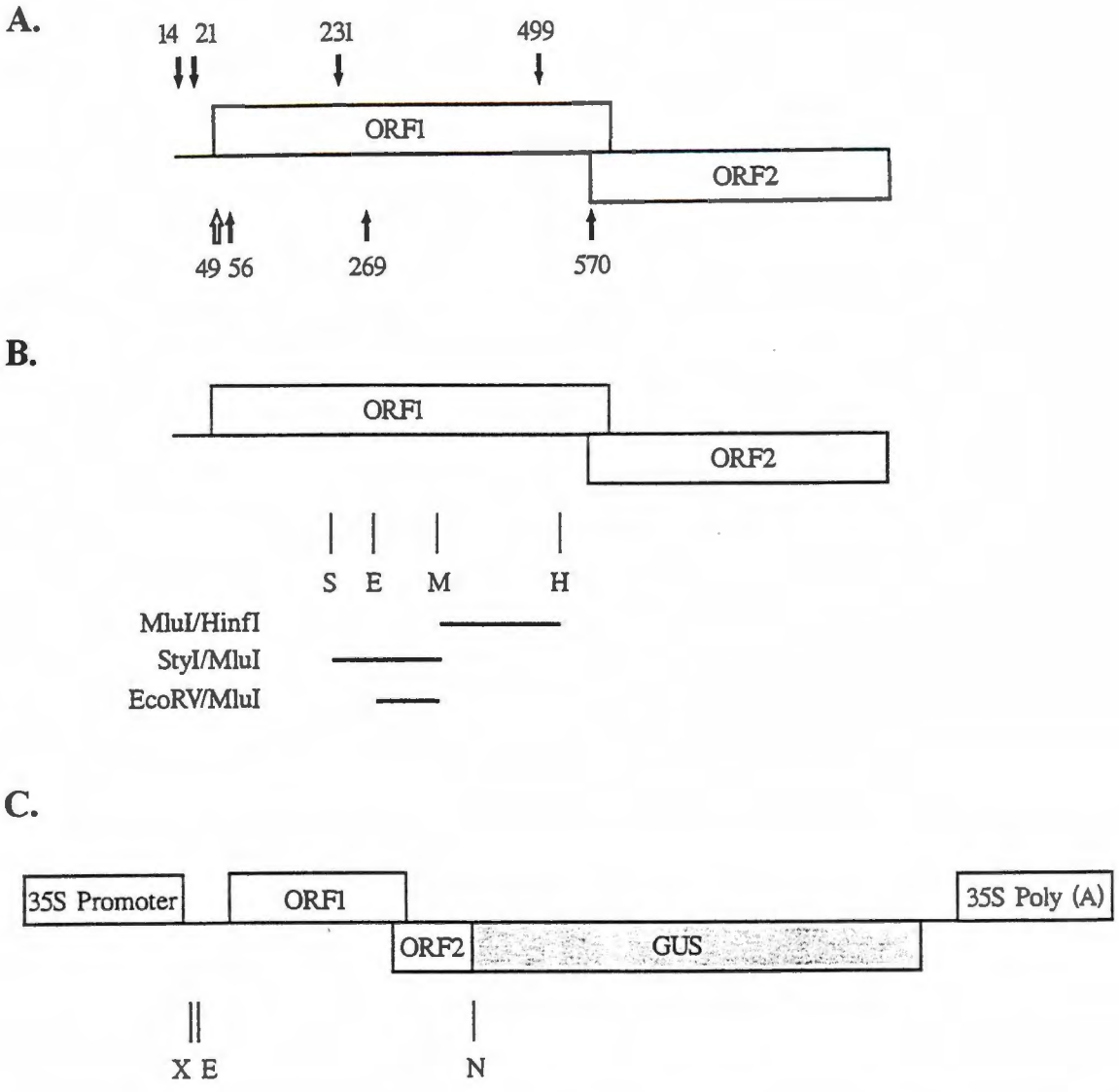


Figure 8. Diagram describing the mutant constructs. **(A)** A diagram of the 5' end of the SBMV-C genome showing the positions of AUG codons described in the text. The positions of AUG codons in wild-type SBMV-C are marked by arrows below the diagram. The positions of AUG codons introduced into the SBMV-C genome and described in the text are marked by arrows above the diagram. The open arrow marks the position of the ORF1 initiation codon that is eliminated in pSBMV-U50C/G51C. The numbers refer to the positions of the AUG codons (Wu et al., 1987). ORF2 is not drawn to scale. **(B)** A diagram of the 5' end of the SBMV-C genome showing the positions of ORF1 deletions. The positions of the StyI (S), EcoRV (E), MluI (M), and HinfI (H) restriction sites are shown. The postions of the deletions are represented as bars and are identified by name. **(C)** A diagram of pRTL2-GUS/SBMV WT is shown. The positions of the XhoI (X), EcoRI (E), and NcoI (N) restriction sites are marked. The GUS gene is shown in gray. The CaMV 35S promoter and poly(A) signal are illustrated with boxes. The diagram is not drawn to scale.

Table 2. Description of Oligonucleotides used in this study

DESIGNATION	SEQUENCE (5' TO 3')	DESCRIPTION
SBMV49	GAAACACAAAGGTCCTTCCTTTTTCC	Complimentary to SBMV-C nt 16-48, mutagenesis of nt 28-35 (underlined)
SBMV64	GGAAAATCAGCCATTTTCCTTCTTATA	Complimentary to SBMV-C nt 8-34, mutagenesis of nt 22-23 (underlined)
SBMV66	GGTTGAGGCCATGCCCAACAAGCTC	Complimentary to SBMV-C nt 218-242, mutagenesis of nt 231 (underlined)
SBMV67	GGTGAATGTTCATGTCGTCGTAGAC	Complimentary to SBMV-C nt 487-511, mutagenesis of nt 500 (underlined)
SBMV72	CAGCACTTTTCCATCTTATATTTTGTGCC	Complimentary to SBMV-C nt 1-27, mutagenesis of nt 15 (underlined)
SBMV83	GGAC**GAATTC**ACAAAATATAAGA	Complimentary to SBMV-C nt 1-14, has a EcoRI restriction site (bold)
SBMV84	CCGT**CCATGG**ACGCATTTGCGCAC	Complimentary to SBMB-C nt 621-645, has a NcoI restriction site (bold)
SBMV85	CCAATGTCTCATAAATGGTGAAACACAAAGG	Complimentary to SBMV-C nt 37-67, mutagenesis of nt 50-51 (underlined)

The deletion mutants pSBMV1-ORF1 EcoRV/MluI, pSBMV1-ORF1 StyI/MluI, and pSBMV-ORF1 MluI/HinfI (Fig.8B), were generated by digestion of pSBMV1 with MluI (SBMV-C nt 359) and either EcoRV (SBMV-C nt 271), StyI (SBMV-C nt 213), or HinfI (SBMV-C nt 520), respectively. The restricted ends were made blunt with Klenow fragment (New England Biolabs) and religated. The insertion mutant pSBMV1-ORF1 MluI was generated by digestion of pSBMV1 with MluI and the restricted ends were made blunt with Klenow fragment and religated. The deletions were confirmed by DNA sequencing.

Construction of ORF2/GUS fusions

Oligonucleotides SBMV83 and SBMV84 (Table 2) were used to amplify SBMV-C nt 1-645 from pSBMV1 and the mutant constructs described above by the polymerase chain reaction (PCR). NcoI and EcoI restriction sites were positioned at the 5' ends of SBMV84 and SBMV83, respectively. The PCR products were digested with EcoRI and NcoI and cloned into EcoRI/NcoI-digested pRTL2-GUS (a gift of Dr. Albrecht von Arnim, University of Tennessee) (Fig.8C). PRTL2-GUS/SBMV-XhoI was constructed by cloning a 1.4 kbp XhoI fragment from pSBMV1 (SBMV-C nt 2096-3168) into XhoI-digested pRTL2-GUS/WT.

SBMV-C RNA isolation

VPg-containing SBMV-C RNA was prepared by incubation of SBMV-C in 100 mM NaCl. 10mM Tris-HCl (pH 8.0), and 1mM EDTA for 20 min at 37°C followed by extraction with phenol:chloroform (1:1) and the RNA was recovered by ethanol

precipitation.

In vitro transcription and translation

Genome-length SBMV-C transcripts were synthesized from SmaI-linearized pSBMV1 and the mutants described above. Brome mosaic virus RNA3 was synthesized from EcoRI-linearized pB3TP8 (gift of Dr. Chris Cheng Kao, Indiana University). Transcription was performed in a 25 μl reaction containing 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 0.5 mM ATP, GTP, CTP, and UTP, 1 μg DNA, 25 units of T7 RNA polymerase (New England Biolabs), and 10 units of Rnasin (Promega). For capped transcripts, the GTP concentration was reduced to 0.25 mM and m^7GpppG (Ambion) was added to 0.5 mM. The reactions were incubated at 37°C for 1 hr. The template DNA was digested with Dnase I (Pharmacia), and RNA was separated from unincorporated nucleotides using either a Chromospin 10 column (Clontech) or by precipitation of the RNA in 2.5 M ammonium acetate.

Translation reactions were performed in wheat germ extract (Promega) in a final volume of 25 μl in the presence of [^{35} S] methionine (1000 Ci/mmol; ICN) for 1 hr at 25°C using 200 ng of RNA. When the cap analog m^7GpppG (Ambion) was included in the reaction, an equimolar amount of $MgCl_2$ was also added. The translation products were separated by 15% SDS-PAGE. The gels were dried, and radiolabeled proteins were quantitated using the AMBIS Radioimaging System.

Transient GUS expression in onion epidermal cells

Epidermal peels of onion bulbs were placed on agar plates containing MS basal

medium (Murashige and Skoog, 1962). Tungsten particles coated with the specified plasmid DNA (2 μg per bombardment) were bombarded onto the onion epidermal peels using the Bio-Rad Biolistic PDS-1000/He system as previously described (von Arnim and Deng, 1994). The bombarded onion peels were incubated in the dark at 22°C for 20 hr. The peels were then frozen in liquid nitrogen, ground with a mortar and pestle, and lysed in extraction buffer containing 50 mM NaH_2PO_4 (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10mM β-mercaptoethanol. GUS assays were performed as previously described (Jefferson et al., 1987). Briefly, 100 μl of the onion extract was incubated with 1 mM 4-methyl umbelliferyl glucuronide (MUG) in a reaction volume of 1 ml at 37°C for 1 hr. Following incubation 200 μl of the reaction was added to 800 μl 0.2 M Na_2CO_3. The fluorescence of 4-methyl umbelliferone (4-MU) was measured with excitation at 365 nm and emission at 455 nm on a Perkin Elmer LS50B spectrofluorimeter. Total protein was determined by Bradford (1976) assay using a Bio-Rad kit. The results were calculated in terms of specific activity (pmol 4-MU/min/μg total protein), and presented as relative amounts of GUS specific activity with GUS expression from pRTL2-GUS/SBMV WT being equal to 100%.

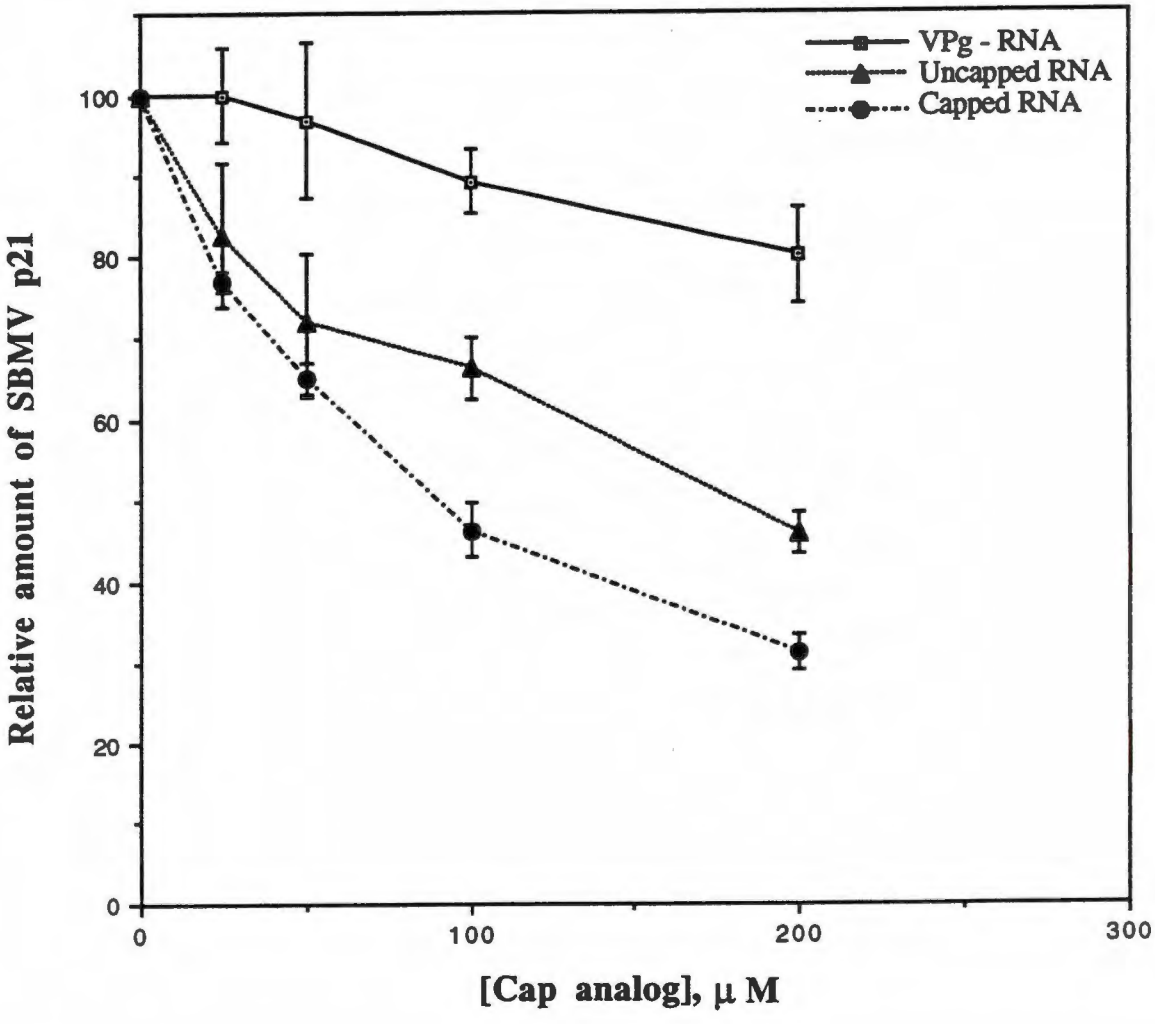
RESULTS

Effect of cap analog on SBMV-C translation

The mechanism of SBMV-C translation initiation was initially investigated by comparing the effect of cap analog (m^7GpppG) on the translation of capped, uncapped, and VPg-containing genomic RNA in wheat germ extract to its effect on the translation of the 3a protein from capped and uncapped BMV RNA3, a mRNA that is translated by

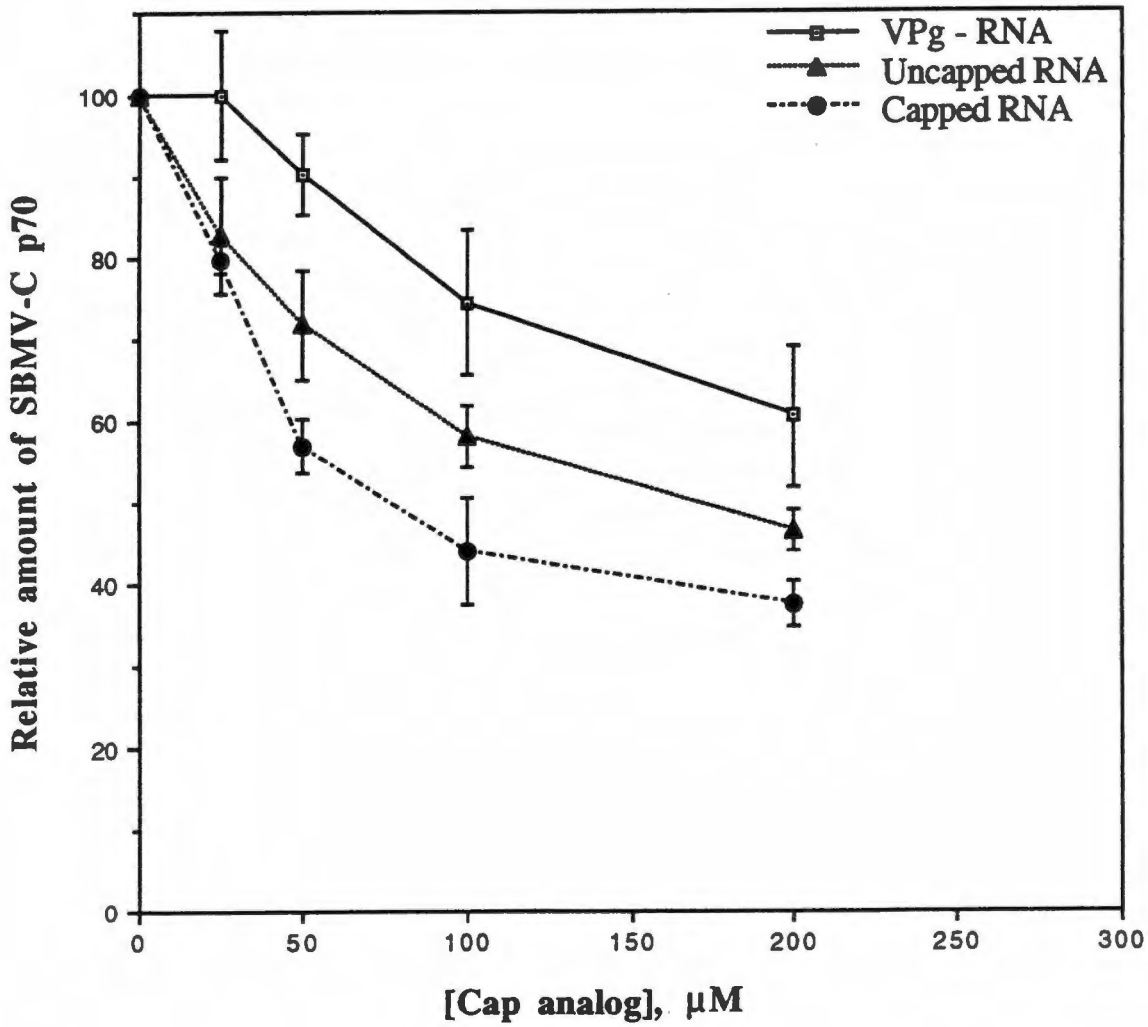
scanning ribosomes in a cap-dependent manner (Shih et al., 1976; Sasavage et al., 1979). If SBMV-C translation is initiated by ribosomes scanning from the 5' end of the RNA, then cap analog is expected to significantly reduce translation of p21 and possibly p105. On the other hand, if SBMV-C translation is initiated by internal ribosome binding, then inhibition of translation is not expected to occur in the presence of cap analog (Anthony and Merrick, 1991). The capped and uncapped RNAs used in the experiment were synthesized *in vitro* from cloned DNAs. It should be noted that the synthetic SBMV-C genomic RNA contains two additional 5' G residues that are not present in the native RNA.

For capped and uncapped SBMV-C genomic RNAs, 50% inhibition of p21 translation was observed at approximately 100 μM and 200 μM m^7GpppG, respectively (Fig.9A). In contrast, translation of p21 from VPg-containing genomic RNA was reduced only 20% at the highest concentration of cap analog (200 μM). Similar results were obtained for translation of these RNAs at KCl concentrations which ranged from 50 to 125 mM (data not shown). The effect of the cap analog (m^7GpppG) on the translation of ORF2 from capped and uncapped SBMV-C transcripts and from VPg-containing SBMV-C RNA was determined indirectly by quantitating the level of RdRp (p70), a proteolytic product of p105. For capped and uncapped SBMV-C RNAs, 50% inhibition of ORF2 translation was observed at approximately 80 and 180 μM m^7GpppG (Fig.9B), respectively. In contrast, VPg-containing SBMV-C RNA was inhibited only 40% at the highest concentration of cap analog (200 μM) and little effect on translation was observed at 25 μM m^7GpppG. For capped and uncapped BMV RNA3, translation of protein 3a was inhibited 50% at approximately 90 and 170 μM cap analog, respectively (Fig.9C). In



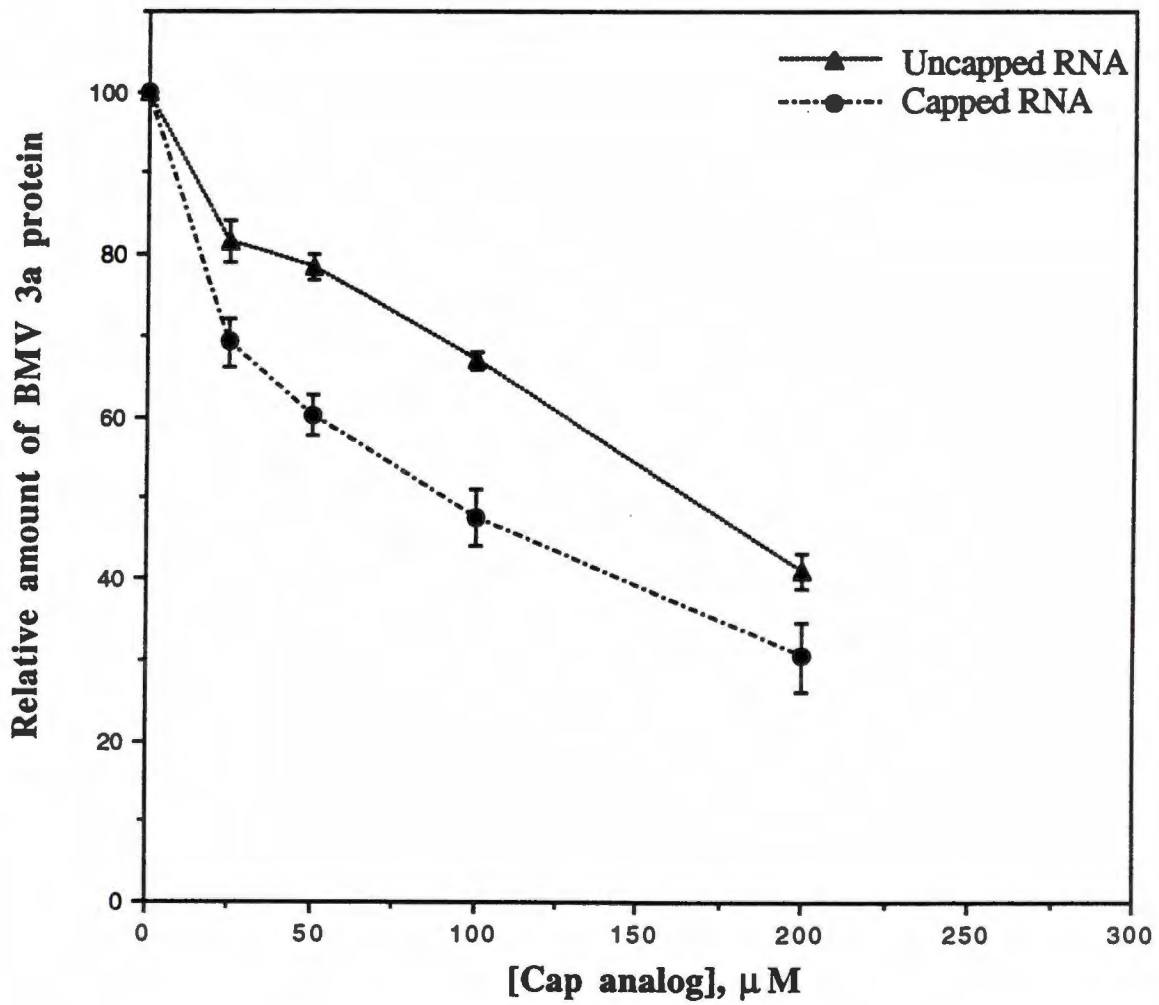
A. The effect of cap analog on the translation of SBMV-C ORF1 protein. Capped, uncapped, or VPg-containing SBMV-C genomic RNA (400 ng) was translated in the presence of various concentrations of $m^7G(5')ppp(5')G$. The relative amounts of p21 were determined using the AMBIS Radioimaging System following separation of the translation products by SDS-PAGE.

Figure 9. The effect of cap analog on the translation of SBMV-C and BMV RNA.



B. The effect of cap analog on the translation of SBMV-C ORF2 protein. Capped, uncapped, or VPg-containing SBMV-C genomic RNA (400 ng) was translated in the presence of various concentrations of $m^7G(5')ppp(5')G$. The relative amounts of p70 were determined using the ABMIS Radioimaging System following separation of the translation products by SDS-PAGE.

Figure 9 (continued)



C. The effect of cap analog on the translation of BMV 3a protein. Capped and uncapped BMV RNA3 (50 ng) was translated in the presence of various concentrations of $m^7G(5')ppp(5')G$. The relative amounts of 3a protein were determined using the ABMIS Radioimaging System following separation of the translation products by SDS-PAGE.

Figure 9 (continued)

summary, the effect of m^7GpppG on the translation of capped and uncapped SBMV-C RNA is very similar to its effect on the translation of the 3a protein from BMV RNA3. Furthermore, VPg-containing SBMV-C RNA remains susceptible to inhibition by cap analog but to a lesser extent than the capped and uncapped forms of the RNA. These results are consistent with a 5' end-dependent scanning mechanism of translation initiation on the SBMV-C RNA.

Effect of AUG codons on ORF1 and ORF2 translation *in vitro*

The effects of additional out-of-frame AUG codons 5' to the ORF1 and ORF2 initiation codons on translation of these two genes was determined in wheat germ extract. If the 43S ribosomal complex binds SBMV-C RNA near its 5' end and scans to the ORF1 initiation codon, then the addition of an AUG codon to the 5' UTR is expected to decrease translation of p21 (Kozak, 1989). Similarly, the addition of one or more AUG codons 5' to the site of ORF2 translation initiation (SBMV-C nt 570) is expected to reduce translation of this gene (Kozak, 1989). In contrast, elimination of one or more of the AUG codons between the 5' end of the RNA and the ORF2 initiation codon is expected to increase ORF2 expression. If translation of one or both of these genes occurs by internal ribosome binding, then the additional AUG codons are not expected to affect their translation. The genome-length SBMV-C cDNA clone was used to generate several mutants including additional AUG codons at SBMV-C nt 14 (pSBMV-A15U), nt 21 (pSBMV-G22U/U23G), nt 231 (pSBMV-U231A), and nt 499 (pSBMV-G500U) (Fig.8A), and a double mutant with additional AUG codons at nt 21 and nt 231 (pSBMV-G22U/U23G/U231A) was constructed (Fig.8A). Finally, the ORF1 AUG was eliminated

in the construct pSBMV-U50C/G51C (Fig.8A). To eliminate the possibility that these mutations affected the secondary structure of the SBMV-C RNA, the mutant pSBMV-Py28-35Pu in which pyrimidines at nt 28-35 were replaced by purines was generated (Fig.10).

Uncapped wild type and mutant SBMV-C RNAs were synthesized *in vitro* and translated in wheat germ extract. Addition of an AUG codon in the 5' untranslated region (5' UTR) at nt 14 (pSBMV-A15U) reduced translation of p21 (Fig.10), and addition of an AUG codon at nt 21 (pSBMV-G22U/U23G) reduced translation of p21 by nearly 80% (Fig.10). The substitution of purines for pyrimidines at nt 28-35 increased ORF1 expression (Fig.10). The results from these experiments support translation of ORF1 by a scanning mechanism. The addition of an AUG codon in the 5' UTR at nt 14 (pSBMV-A15U) or at nt 21 (pSBMV-G22U/U23G) reduced the translation of p70 (Fig.11A,B). The addition of an AUG codon within ORF1 at nt 231 (pSBMV-U231A) or at nt 500 (pSBMV-G500U) reduced translation of p70 but not p21 (Fig.11A,B). The introduction of two AUG codons, one in the 5' UTR and the other within ORF1 (pSBMV-G22U/U23G/U231A) resulted in reduced levels of both p21 and p70 translation (Fig.11A,B). The elimination of the ORF1 initiation codon (pSBMV-U50C/G51C) resulted in a 70% increase in the level of ORF2 translation (Fig.11A,B). The results from these experiments support translation of ORF1 and ORF2 by scanning ribosomes.

Effects of AUG codons on ORF2 expression *in vivo*

To determine if the addition or elimination of AUG codons affected ORF2 expression *in vivo*, SBMV-C ORFs 1 and 2 were placed under the control of the

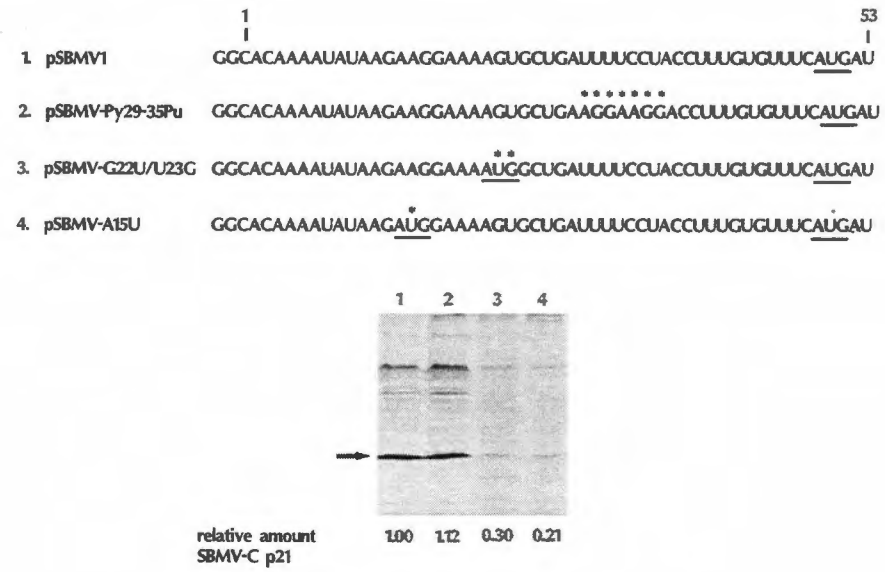
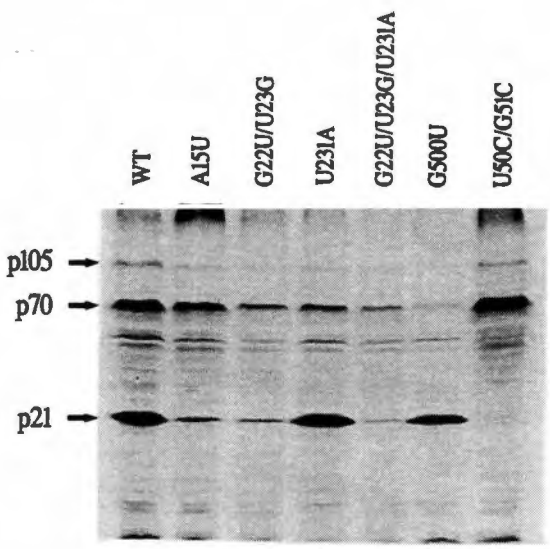
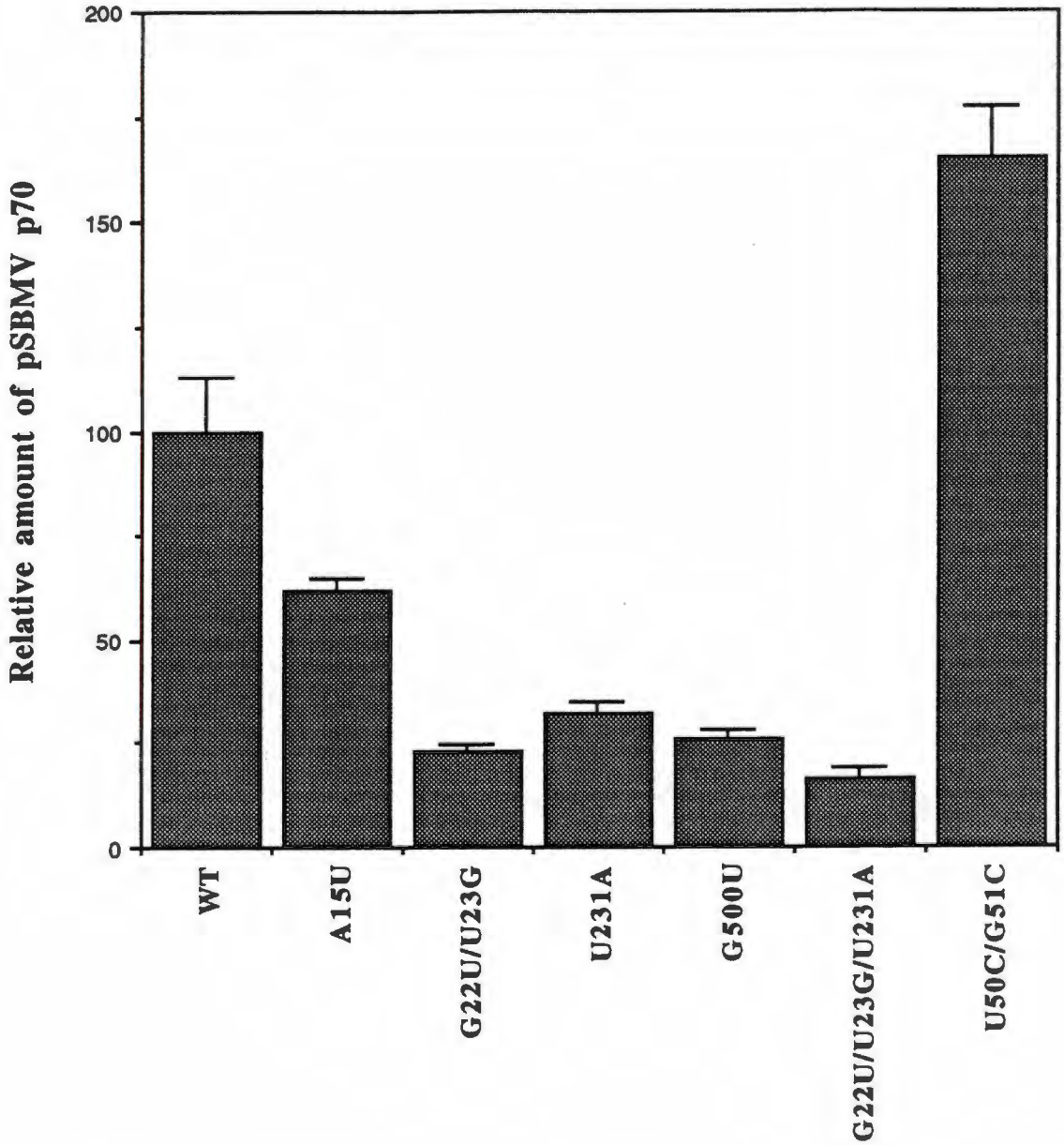


Figure 10. The effect of additional AUG codons within the 5' UTR on SBMV-C translation. Uncapped SBMV-C genomic RNA was synthesized from the plasmids listed. The sequences of the 5' UTRs of the RNAs are shown adjacent to the plasmid names. The nucleotide numbers are those of Wu et al., (1987). AUG codons are underlined, and the positions of the nucleotide changes within the 5' UTRs of the mutant RNAs are marked with asterisks. Following translation of 400 ng of genomic RNA proteins were analyzed by SDS-PAGE. The relative amount of p21 was determined using the AMBIS Radioimaging System. The arrow marks the position of SBMV-C p21.



A. Transcript RNAs synthesized from pSBMV1 (WT) and the SBMV-C mutants indicated were translated in wheat germ extract. The radiolabeled proteins were separated by SDS-PAGE. The positions of p105, p70, and p21 are marked.

Figure 11. The effect of additional AUG codons on SBMV-C translation.



B. Bar graph of p70 expression in wheat germ extract. The results from two independent experiments are shown. The levels of p70 expression were normalized to the level of its expression from pSBMV1 RNA (WT).

Figure 11 (continued)

cauliflower mosaic virus (CaMV) 35S promoter in the GUS expression vector pRTL2-GUS (Fig.8C). Initiation of translation at SBMV-C nt 570 will result in the synthesis of an ORF2/GUS fusion protein whose enzymatic activity may be detected using a flourometric assay. SBMV-C nt 1-645 from pSBMV1 and the mutant constructs shown in Fig. 8A were cloned into pRTL2-GUS. In addition a 1.4 kbp XhoI restriction fragment was cloned between the CaMV 35S promoter and SBMV nt 1 in the construct pRTL2-GUS/WT (Fig.8C). The mRNA from this construct has 19 AUG codons 5' to the ORF1 initiation codon. The plasmids were bombarded into onion epidermal cells. The specific activity of GUS was determined for each construct and normalized to the level from the wild-type ORF2/GUS protein.

The addition of a single AUG codon in the 5' UTR (pRTL2-A15U and pRTL2-G22U/U23G) reduced ORF2/GUS expression by 25-35% compared to its expression from pRTL2-WT (Fig.12). Similar results were observed for the addition of a single AUG codon in ORF1 (pRTL2-U231A and pRTL2-G500U) (Fig.12). The addition of two AUG codons between the 5' end of the mRNA and the ORF2 initiation codon (pRTL2-G22U/U23G/U231A) reduced ORF2/GUS expression by 65% relative to expression from pRTL2-WT (Fig.12). On the other hand, elimination of the ORF1 initiation codon (pRTL2-U50C/G51C) resulted in a 40% increase in the level of ORF2/GUS expression (Fig.12). Addition of 19 AUG codons 5' to the ORF1 initiation codon (pRTL2-XhoI) reduced the level of ORF2 expression to near background levels. The effects of AUG codons on the initiation of translation from the ORF2 initiation codon are similar to those observed *in vitro* except the effects were more pronounced *in vitro* than *in vivo*. The reason(s) for the differences are not known, however. The results from these studies

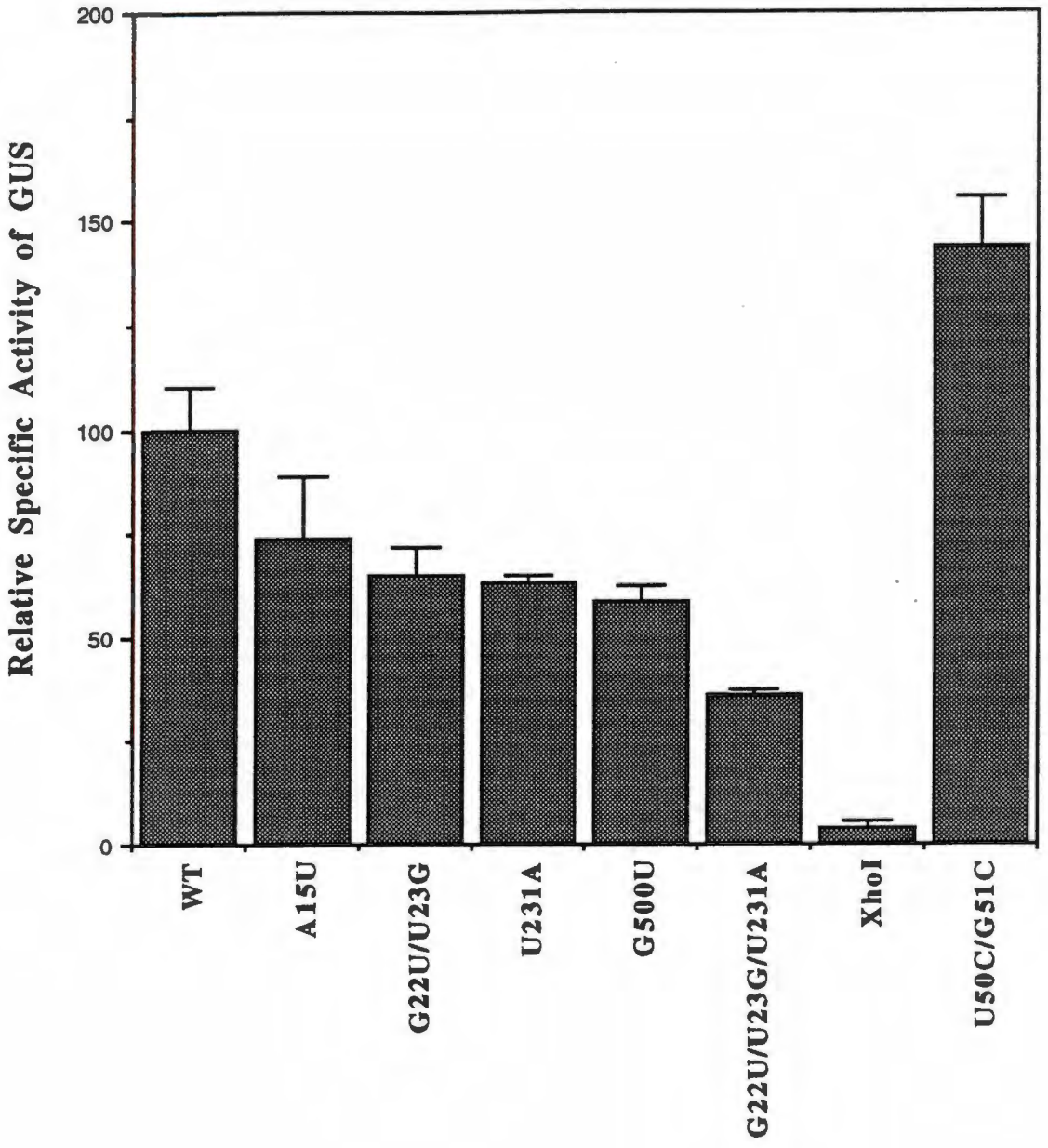


Figure 12. Effects of AUG codons on ORF2/GUS expression in onion epidermal cells. Onion peels were bombarded with tungsten pellets coated with 2 μg of the specified plasmid DNAs. The specific activity of GUS was calculated as described in Materials and Methods, and the specific activities were normalized to that of GUS expressed from pRTL2-GUS/SBMV WT (WT). The results from four independent experiments are presented.

support initiation of translation of ORF2 by scanning ribosomes.

Effects of deletions and insertions on ORF2 expression *in vitro* and *in vivo*

If translation of SBMV-C ORF2 takes place by internal ribosome binding, then a substantial portion of the region 5' to the ORF2 initiation codon may function as the IRES. On the other hand, if expression of SBMV-C ORF2 is by a scanning mechanism, deletions and insertions within ORF1 were not expected to decrease the expression of ORF2. To address this question deletions of SBMV-C nt 218-359 (pSBMV1-ORF1 StyI/MluI), nt 274-359 (pSBMV1-ORF1 EcoRV/MluI), and nt 364-520 (pSBMV1-ORF1 MluI/HinfI) were made in pSBMV1. The StyI/MluI deletion eliminated the AUG codon at SBMV-C nt 269. In addition to these deletion mutants, a four base pair duplication was made at the MluI site at SBMV-C nt 359 (pSBMV1-ORF1 MluI). For the analysis of ORF2 expression in onion epidermal cells, SBMV-C nt 1-645 from these plasmids were cloned into pRTL2-GUS as described above.

Uncapped wild type and mutant SBMV-C RNAs were synthesized *in vitro* and translated in wheat germ extract. The levels of both p21 and p70 were quantitated following separation of the radiolabeled proteins by SDS-PAGE. ORF1 expression from the four mutants was reduced 50-75% relative to ORF1 expression from pSBMV1 RNA (Fig.13). The EcoRV/MluI deletion and the MluI insertion resulted in 50% reductions in ORF2 expression, and the StyI/MluI deletion resulted in a 20% reduction in ORF2 expression (Fig.13). The elimination of an AUG codon in the StyI/MluI deletion may have contributed to higher ORF2 expression from this RNA. In contrast, deletion of SBMV-C nt 364-520 (pSBMV1-ORF1 MluI/HinfI) resulted in a 65% increase in ORF2

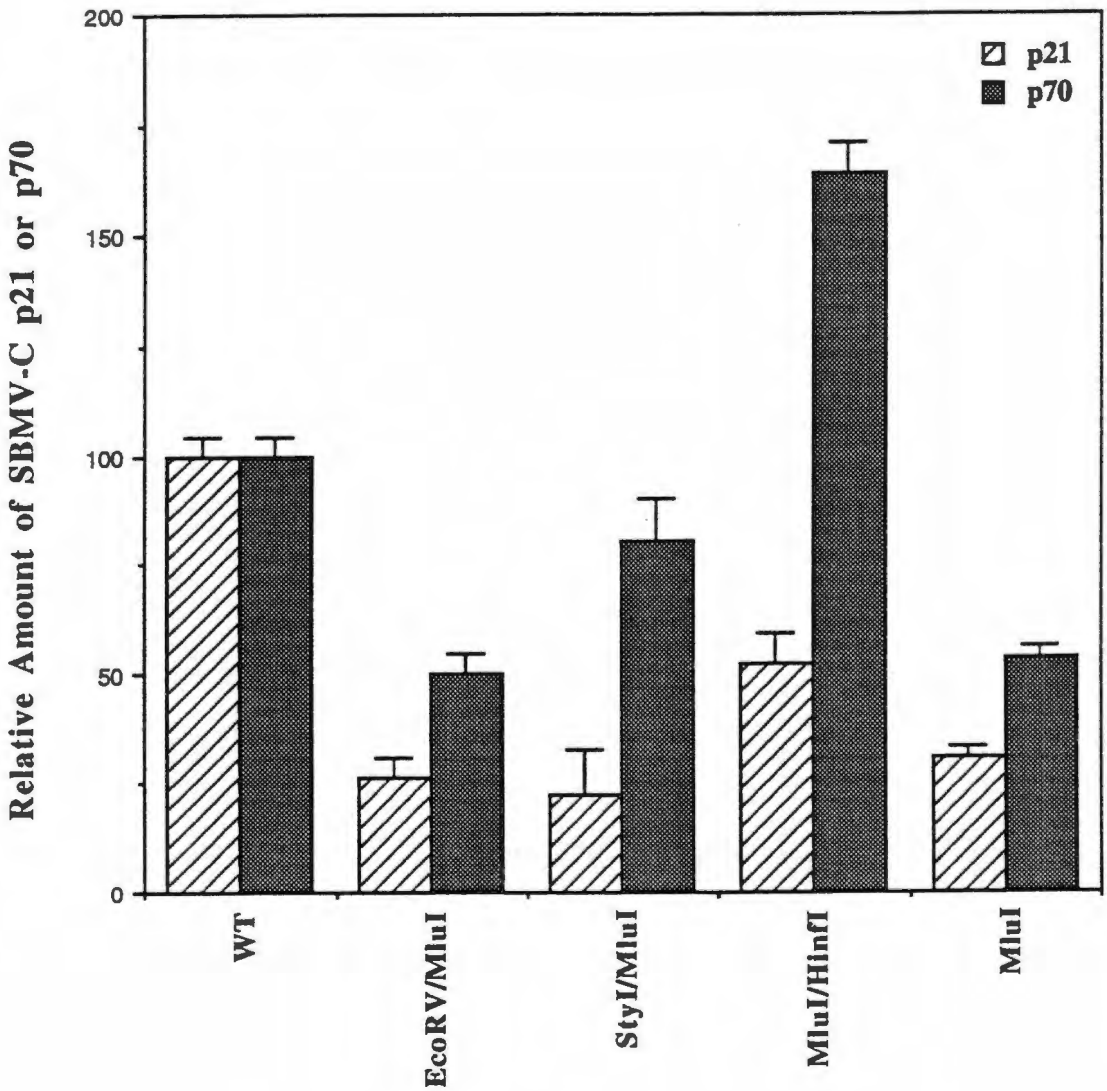


Figure 13. The effects of ORF1 deletions and insertions on ORF1 and ORF2 expression in wheat germ extract. A bar graph of p21 and p70 expression is shown. Transcript RNAs (200 μg) synthesized from pSBMV1 and four mutants designated in the figure were translated in wheat germ extract. The radiolabeled proteins were separated by SDS-PAGE, and the amount of p21 and p70 were determined using the AMBIS Radioimaging System. The levels of p21 and p70 were normalized to the levels of these proteins expressed from pSBMV1 RNA (WT). The results from two independent experiments are presented.

expression (Fig.13). Elimination of possible secondary structures in this region may be a reason for the increased level of ORF2 expression.

The effects of the ORF1 mutations on ORF2/GUS expression in onion epidermal cells were determined as described in the previous section. As in wheat germ extract, the EcoRV/MluI deletion and the MluI insertion resulted in reductions in ORF2/GUS expression relative to its expression from pRTL2-GUS/SBMV WT (Fig.14). Deletion of SBMV-C nt 218-359 (pRTL2-GUS/SBMV StyI/MluI) resulted in a 10% increase in ORF2/GUS expression (Fig.14). As above, this effect may have been due to the elimination of the AUG codon at SBMV-C nt 269. The MluI/HinfI deletion resulted in a 40% reduction in ORF2/GUS expression (Fig.14). The decreased level of ORF2/GUS expression may be due to sequence or structural elements present within the deleted region. These results are in contrast to the ones obtained from translation in wheat germ extract (Fig.13). With the exception of the MluI/HinfI mutant, the expression of ORF2 from the ORF1 deletion and insertion mutants was qualitatively similar in wheat germ extract and in onion epidermal cells.

DISCUSSION

The 5' cap and the 3' poly(A) tail of eukaryotic mRNAs are involved in the recruitment of 43S ribosomal subunits (Sachs et al., 1997). Although the genomic RNA of SBMV-C has neither of these structural components, the results presented here suggest that 43S ribosomes bind to the 5' end of the RNA and scan in a 5' to 3' direction to the ORF1 initiation codon. Some ribosomes, however, bypass this AUG and two others to initiate ORF2 translation.

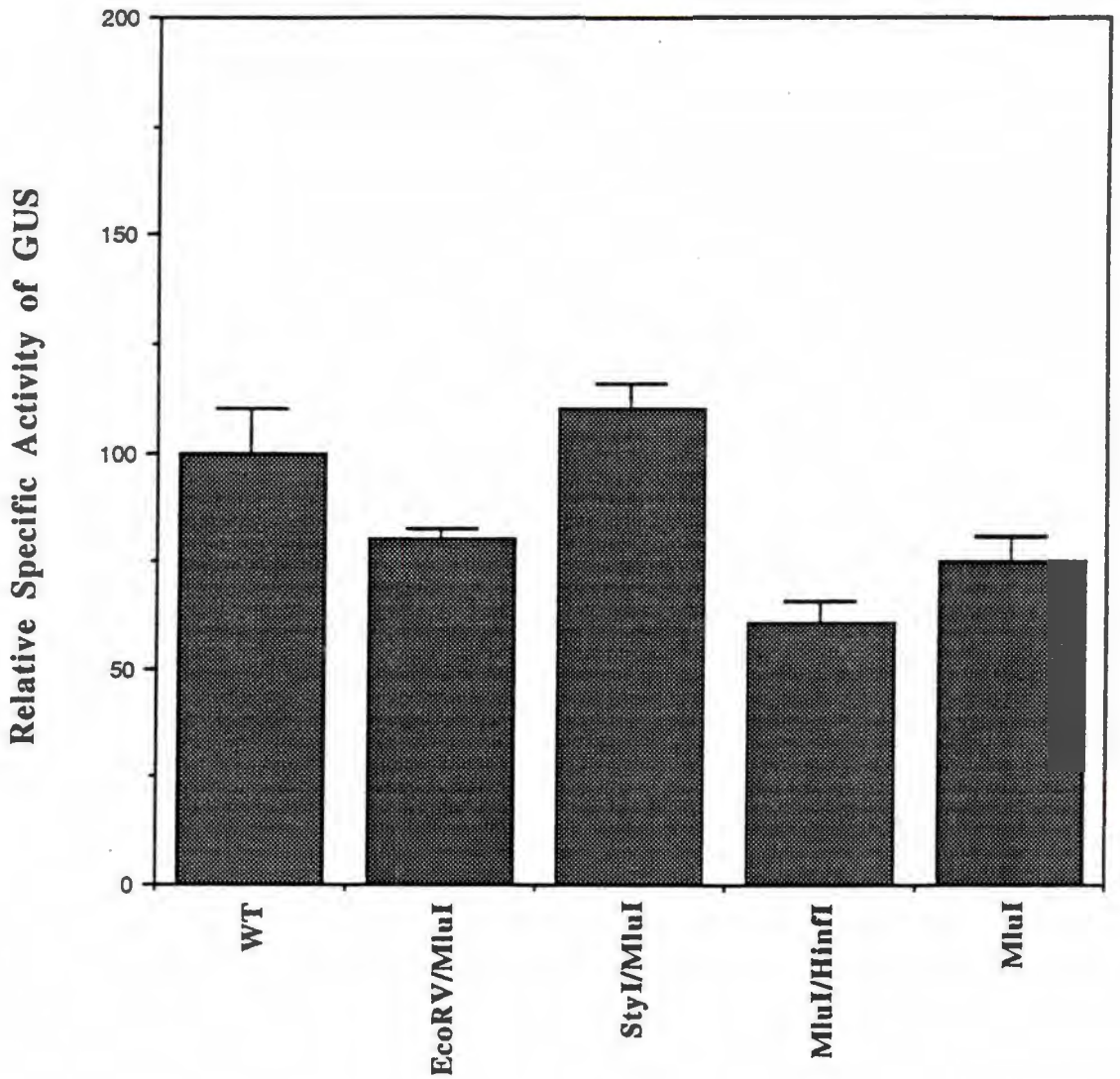


Figure 14. The effects of ORF1 deletions and insertions on ORF2 expression in onion epidermal cells. pRTL2-GUS/SBMV WT and the four mutants designated in the figure were bombarded into onion epidermal peels. The specific activity of GUS was calculated as described in Materials and Methods and normalized to that of GUS expressed from pRTL2-GUS/SBMV WT. The results from four independent experiments are presented.

Translation of p21 from the genomic RNA appears to occur by 5' end-dependent ribosomal scanning. This conclusion is based on the sensitivity of p21 and coat protein translation to the presence of cap analog and to the addition of AUG codons to the 5' UTRs of the SBMV-C RNAs. In contrast, the cell-free translation of PV RNA by cap-independent internal ribosome entry has been shown to be unaffected by the presence of cap analog (Pelletier and Sonenberg, 1989). Furthermore, the presence of multiple initiation codons in the PV 5' UTR does not prevent translation of the PV ORF (Ehrenfeld and Semler, 1995). Although the cell-free translation experiments presented here do not support cap-independent internal ribosome entry as the mechanism of ORF1 translation initiation, it is important to consider two potential problems with the experimental approach. The first is the possibility that translation of the SBMV-C RNAs in wheat germ extract does not reflect the mechanism by which they are translated *in vivo*. The second concerns the reliance of these experiments upon capped and uncapped synthetic RNAs whose 5' ends differ from those of the viral RNAs. Additional *in vivo* experiments with VPg-containing RNAs will be needed to confirm the conclusions drawn from the cell-free translation studies.

Several lines of evidence support leaky scanning as the mechanism of ORF2 expression. First, the presence of cap analog decreased the level of ORF2 translation from capped, uncapped, and VPg-containing SBMV-C RNA in wheat germ extract. Second, the addition of one or more AUG codons between the 5' end of the RNA and the ORF2 initiation codon reduced ORF2 expression, whereas the elimination of the ORF1 initiation codon increased ORF2 expression. Likewise, the elimination of the AUG codon at SBMV-C nt 269 in the StyI/MluI deletion may have been responsible for increased ORF2

expression relative to its expression from RNAs containing the EcoRV/MluI deletion. The addition or elimination of AUG codons was not expected to affect ORF2 translation by internal ribosomal binding (Jackson et al., 1995). Third, deletions or insertions within ORF1 either reduced ORF2 expression 20-50% or enhanced it. If an RNA element within ORF1 served as an IRES for ORF2, then some or all of these changes in ORF1 were expected to abolish ORF2 translation by affecting RNA secondary structure (Jackson et al., 1995).

With these results, the general expression strategy for SBMV-C has now been defined. ORF1 is translated by scanning ribosomes in a 5' end-dependent manner. Ribosomes that bypass the first three AUG codons on the genomic RNA may initiate translation at the ORF2 initiation codon. None of the first three AUG codons is present in a strong context for translation, $^{A}/_{G}$NNAUGG, as defined by Kozak (1987) and Lűtcke et al. (1987). All have a U residue at the –3 position and either an A or U at the +4 position, while the ORF2 initiation codon has an A at the –3 position and a U at the +4 position (Wu et al., 1987). The CP is also expressed by 5' end-dependent scanning from the sgRNA (Hacker and Sivakumaran, 1997). Finally, there is evidence that ORF3 is translated by a –1 ribosomal frameshift in ORF2 (Mäkinen et al., 1995; chapter II). Similarities to the SBMV-C expression strategy have been observed for the subgroup II luteoviruses and of pea enation mosaic enamovirus RNA1. The RdRps of these viruses are evolutionarily related to the sobemovirus RdRp (Koonin and Dolja, 1993), and they have a 5' VPg but lack a poly(A) tail (Miller et al., 1995). For these viruses the 5' terminal ORF (ORF 0) overlaps with the 5' end of ORF1, and ORF2 is translated by a –1 ribosomal frameshift in ORF1 resulting in the synthesis of a polyprotein that is

proteolytically cleaved to produce VPg, protease, and RdRp (Miller et al., 1995). ORFs 0 and 1 are predicted to be translated by scanning ribosomes (Miller et al., 1995).

Although the general SBMV-C expression is now known, it is not clear how ribosomes are recruited to the RNA. The VPg and specific regions of the viral RNA may be utilized to fulfill the roles normally performed by a 5' cap and a 3' poly(A) tail. In this regard, the presence of VPg has been shown to reduce the inhibition of ORF1 and ORF2 by cap analog, suggesting that VPg may enhance the efficiency of SBMV-C translation in wheat germ extract. Recently, the VPg of turnip mosaic potyvirus (TuMV) has been shown to interact with the eukaryotic initiation factor (iso) 4E [eIF(iso)4E] of *Arabidopsis thaliana* in the yeast two-hybrid system (Wittmann et al., 1997). The TuMV VPg may play a direct role in recruiting translation initiation factors and/or 43S ribosomes, but the importance of this interaction in TuMV translation has not been demonstrated.

Several examples of viral RNA elements, other than the IRES, that function in translation have been described. The uncapped, non-polyadenylated genomic RNA of barley yellow dwarf luteovirus (PAV strain) has a translational enhancer near its 3' end that functions like a 5' cap to recruit eIF4F (Wang et al., 1997). Tobacco mosaic tobamovirus RNA is capped but not polyadenylated. The 3' end of the RNA contains several RNA pseudoknots and serves as a substitute for a poly(A) tail (Gallie and Walbot, 1990). Tobacco etch potyvirus also has a 5' VPg and a poly(A) tail. An RNA element within the 5' UTR, however, functions in conjunction with the poly(A) tail for efficient translation (Carrington and Freed, 1990; Gallie et al., 1995). Specific regions of the SBMV-C genome that facilitate its translation have not been described. It is interesting, however, that mutations at the MluI site of the SBMV-C genome generally had a negative

effect on both ORF1 and ORF2 expression. Further experimentation will be required to determine if an RNA element within ORF1 functions in SBMV-C translation.

CHAPTER IV

FUTURE DIRECTION OF SOUTHERN BEAN MOSAIC VIRUS GENE EXPRESSION AND FUNCTION RESEARCH

TO DETERMINE THE ROLE OF THE 5' END-LINKED VPg IN SBMV-C TRANSLATION INITIATION

The VPg containing SBMV-C RNA was shown to be minimally inhibited by the presence of cap analog (see chapter III). The VPg may interact with translation initiation factors to facilitate their recruitment to the viral RNA.

1. It is not known if the VPg remains bound to the SBMV-C RNA during translation. Experiments should be designed to determine if the VPg is present on polysome-bound SBMV-C RNA.
2. SBMV-C RNA may require low amounts of the initiation factor complex for translation. Experiments using antibodies to deplete the initiation factors should be carried out in order to determine this.
3. Experiments using the yeast two-hybrid system should be carried out to determine possible interactions between the initiation factor complex and VPg.

TO FURTHER CHARACTERIZE THE ROLE OF ORF1 AND ORF3 PROTEINS IN CELL-TO-CELL MOVEMENT OF SBMV-C.

It was demonstrated that the ORF1, ORF3, and coat proteins are required for SBMV-C infection of cowpea (see chapter II). The mechanism by which these proteins function and their subcellular localization are presently not known. The availability of

antisera against ORF1 and ORF3 will help in determining the role of these proteins in cell-to-cell movement. Immunocytochemical electron microscopy using the ORF1 and ORF3 antisera should be carried out to determine the subcellular localization of these proteins.

TO DETERMINE ROLE OF SBMV-C RNA ELEMENTS IN TRANSLATION

It was demonstrated that mutations within ORF1 resulted in reduced levels of ORF1 and ORF2 translation (chapter III), suggesting that ORF1 sequences may be important in translation of ORF1 and ORF2. In addition, the predicted pseudoknot structure present at the 3' end may also be important in translation of SBMV-C RNA. Experiments using regions within the ORF1 coding region fused to a reporter system should be carried out in cowpea protoplasts to investigate its possible role in SBMV-C translation.

REFERENCES

REFERENCES

Abad-Zapatero, C., Abdel-Meguid, S.S., Johnson, J.E., Leslie, A.G.W., Rayment, I., Rossmann, M.G., Suck, D., and Tsukihara, T. (1980). Structure of southern bean mosaic virus at 2.8 Å resolution. Nature 286:33-39.

Anthony, D.D., and Merrick, W.C. (1991). Eukaryotic initiation factor (eIF)4F. Implications for a role in internal initiation of translation. J. Biol. Chem. 266:10218-10266.

Aoki, S., and Takebe, I. (1969). Infection of tobacco mesophyll protoplasts by tobacco mosaic virus ribonucleic acid. Virology 39:439-448.

Atabekov, J.G., and Taliansky, M.E. (1990). Expression of a plant virus-coded transport function by different viral genomes. Adv. Virus Res. 38:201-248.

Atkins, D., Hull, R., Wells, B., Roberts, K., Moore, P., and Beachy, R.N. (1991). The tobacco mosaic virus 30K movement protein in transgenic tobacco plants is localized to plasmodesmata. J. Gen. Virol. 72:209-211.

Balsham, G.J., and Sonenberg, N. (1996). RNA-protein interacting in regulation of picornavirus RNA translation. Microbiol. Rev. 60:499-511.

Boccard, F., and Baulcombe, D.C. ((1993). Mutational analysis of cis-acting sequences and gene function in RNA3 of cucumber mosaic virus. Virology 193:563-578.

Borman, A.M., Kirchweger, R., Ziegler, E., Rhoads, R.E., Skern, T., and Kean, K.M. (1997a). eIF4G and its proteolytic cleavage products: effect on initiation of protein synthesis from capped, uncapped, and IRES-containing mRNAs. RNA 3:186-196.

Browning, K.S. (1996). The plant translation apparatus. Plant Mol. Biol. 32:107-144.

Brugidou, C., Holt, C., Ngon A Yassi, M., Zhang, S., Beachy, R., and Fauquet, C. (1995). Synthesis of an infectious full-length cDNA clone of rice yellow mottle virus and mutagenesis of the coat protein. Virology 206:108-115.

Carrington, J.C., and Freed, D.D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. J. Virol. 64:1590-1597.

Carrington, J.C., Kassachau, K.D., Mahajan, S.K., and Schaad, M.C. (1996). Cell-to-cell and long-distance transport of viruses in plants. The Plant Cell 8:1669-1681.

Citovsky, V., Knorr, D., Schuster, G., and Zambryski, P. (1990). The p30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. Cell 60:637-647.

Citovsky, V., Womg, M.L., Shaw, A.L., Prasad, B.M.V., and Zambryski, P. (1992). Visualization and characterization of tobacco mosaic virus movement protein binding to single-stranded nucleic acids. Plant Cell 4:397-411.

Cronin, S., Verchot, J., Haldeman-Cahill, R., Schaad, M.C., and Carrington, J.C. (1995). Long-distance movement factor: A transport function of the potyvirus helper component proteinase. Plant Cell 7:549-559.

Dawson, W.O., Bubrick, P., and Grantham, G.L. (1988). Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement and symptomatology. Phytopathology 78:783-789.

Deom, C.M., Lapidot, M., and Beachy, R.N. (1992). Plant virus movement proteins. Cell 69:221-224.

Dolja, V.V., Haldeman, R., Robertson, N.L., Dougherty, W.G., and Carrington, J.C. (1994). Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants. EMBO J. 13:1482-1491.

Dolja, V.V., Haldeman-Cahill, R., Montgomery, A.E., VandenBosch, K.A., and Carrington, J.C. (1995). Capsid protein determinants involved in cell-to-cell and long distance movement of tobacco etch potyvirus. Virology 207:1007-1016.

Ehrenfeld, E., and Semler, B.L. (1995). Anatomy of the poliovirus internal ribosome entry site. *In* "Current Topics in Microbiology-203". (Sarnow, P. Ed). pp.65-83. Springer-Verlag, New York.

Fuentes, A.L., and Hamilton, R.I. (1993). Failure of long-distance movement of southern bean mosaic virus in a resistant host is correlated with lack of normal virion formation. J. Gen. Virol. 74:1903-1910.

Gallie, D.R. (1991). The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev. 5:2108-2116.

Gallie, D.R. (1996). Translational control of cellular and viral mRNAs. Plant Mol. Biol. 32:145-158.

Gallie, D.R., and Walbot, V. (1990). RNA pseudoknot domain of tobacco mosaic virus can functionally substitute for a poly(A) tail in plant and animal cells. Genes Devel. 4:1149-1157.

Gallie, D.R., and Kobayashi, M. (1994). The role of the 3'-untranslated region of non-polyadenylated plant viral mRNAs in regulating translational efficiency. Gene 142:159-165.

Gallie, D.R., Caldwell, C., Pitto, L. (1995). Heat shock disrupts cap and poly(A) tail function during translation and increases mRNA stability of introduced reporter mRNA. Plant Physiol. 108:1703-1713.

Gal-On, A., Kaplan, I., Rossinck, M.J., and Palukaitis, P. (1994). The kinetics of infection of zucchini squash by cucumber mosaic virus indicate a function for RNA 1 in virus movement. Virology 205:280-289.

Gamarnik, A.V., and Andino, R. (1996). Replication of poliovirus in Xenopus oocytes requires two human factors. EMBO J. 15:5988-5998.

Ghosh, A., Dasgupta, T., Salerno-Rife, T., Rutgers, T., and Kaesberg, P. (1979). Southern bean mosaic virus RNA has a 5' linked protein but lacks 3' terminal poly(A). Nucl. Acids Res. 7:2137-2146.

Ghosh, A., Rutgers, T., Mang, K.Q., and Kaesberg, P. (1981). Characterization of the coat protein mRNA of southern bean mosaic virus and its relationship to the genomic RNA. J. Virol. 39:87-92.

Giesman-Cookmeyer, D., and Lommel, S.A. (1993). Alanine scanning mutagenesis of a plant virus movement protein identifies three functional domains. Plant Cell 5:973-982.

Giesman-Cookmeyer, D., Silver, S., Vaewhongs, A.A., Lommel, S.A., and Deom, C.M. (1995). Tobamovirus and dianthovirus movement proteins are functionally homologous. Virology 213:38-45.

Goodwin, P.B. (1983). Molecular size limit for movement in the symplast of the Elodea leaf. Planta 157:124-130.

Gorbalenya, A.E., Koonin, E.V., Blinov, V.M., and Donchenko, A.P. (1988). Sobemovirus genome appears to encode a serine protease related to cysteine proteases of picornaviruses. FEBS Lett. 236:287-290.

Grogan, R.G., and Kimble, K.A. (1964). The relationship of severe bean mosaic from Mexico to southern bean mosaic virus and its related strain from cowpea. Phytopath. 54:75-78.

Guilley, H., Jonard, G., Kukla, B., and Richards, K.E. (1979). Sequence of 1000 nucleotides at the 3' end of tobacco mosaic virus RNA. Nucl. Acids Res. 6:1287-1295.

Hacker, D.L., and Sivakumaran, K. (1997). Mapping and expression of southern bean mosaic virus genomic and subgenomic RNAs. Virology 234:317-327.

Heinlein, M., Epel, B.L., Padgett, H.S., and Beachy, R.N. (1995). Interaction of tobamovirus movement proteins with the plant cytoskeleton. Science 270:1983-1985.

Hentze, M.W. (1997). EIF4G: a multipurpose ribosome adapter? Science 275:500-501.

Hermodson, M.A., Abad-Zapatero, C., Abdel Meguid, S.S., Pundak, S., Rossmann, M.G., and Tremaine, J.H. (1982). Amino acid sequence of southern bean mosaic virus coat protein and its relationship to the three-dimensional structure of the virus. Virology 119:133-149.

Hillman, B.I., Carrington, J.C., and Morris, T.J. (1987). A defective interfering RNA that contains a mosaic of a plant virus genome. Cell 51:427-433.

Hull, R. (1988). The sobemovirus group. *In* "The plant viruses" (Koenig, R., Ed), 3:113-146. Plenum, New York.

Jackson, R.J. (1996). A comparative view of initiation site selection mechanisms. *In* "Translational control" (Hershey, J.W.B., Matthews, M.B., and Sonenberg, N. Eds). pp. 71-112. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Jackson, R.J., and Kaminski, A. (1995). Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. RNA 1:985-1000.

Jackson, R.J., Hunt, S.L., Reynolds, J.E., and Kaminski, A. (1995). Cap-dependent and cap-independent translation: Operational distinctions and mechanistic interpretations. Curr. Topics Micro. Immunol. 203:1-29.

Jacobson, A. (1996). Poly(A) metabolism and translation: the closed-loop model. *In* "Translational Control" (Hershey, J.W.B., Matthews, M.B., and Sonenberg, N. Eds). pp. 451-480. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-glucurodinase as sensitive and versatile gene fusion marker in higher plants. EMBO. J. 6:3901-3907.

Jeffries, A.C., Rathjen, J.P., and Symons, R.H. (1995). Lucerne transcient streak virus complete genome. GenBank Accession Number U31286.

Jones, R.W., Jackson, A.O., and Morris, T.J. (1990). Defective interfering RNAs and elevated temperatures inhibit replication of tomato bushy stunt virus in inoculated protoplasts. Virology 176:539-545.

Kasteel, D., Wellink, J., Verver, J., van Lent, J., Goldbach, R., and van Kammen, A. (1993). The involvement of cowpea mosaic virus M RNA-encoded proteins in tubule formation. J. Gen. Virol. 74:1721-1724.

Kasteel, D.T.J., Perbal, C.M., Boyer, J.C., Wellink, J., Goldbach, R.W., Maule, A.J., and van Lent, J.W.M. (1996). The movement proteins of cowpea mosaic virus and cauliflower mosaic virus induce tubular structures in plant and insect cells. J. Gen Virol., 77:2857-2864.

Kasteel, D.T.J., van der Wel, N.N., Jansen, K.A.J., Goldbach, R.W., and van Lent, J.W.M. (1997). Tubule-forming capacity of the movement proteins of alfalfa mosaic virus and brome mosaic virus. J. Gen. Virol. 78:2089-2093.

Klein, P.G., Klein, R.R., Rodriguez-Cerezo, E., Hunt, A.G., and Shaw, J.G. (1994). Mutational analysis of the tobacco vein mottling virus genome. Virology 204:759-769.

Koonin, E.V., and Dolja, V.V. (1993). Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences. Crit. Rev. Biochem. Mol. Biol. 28:375-430.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. 15:8125-8148.

Kozak, M. (1989). The scanning model for translation: an update. J. Cell. Biol. 108:229-241.

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82:488-492.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105-132.

Lamptey, P.N.L., and Hamilton, R.I. (1974). A new cowpea strain of southern bean mosaic virus from Ghana. Phytopath. 64:1101-1104.

Langford, G.M. (1995). Actin- and microtubule-dependent organelle motors: Interrelationships between the two motility systems. Curr. Opin. Cell Biol. 7:82-88.

Leathers, V., Tanguay, R., Kobayashi, M., and Gallie, D.R. (1993). A phylogenetically conserved sequence within viral 3' untranslated RNA pseudoknots regulates translation. Mol. Cell Biol. 13:5331-5347.

Lee, Y.F., Nomoto, A., Detjen, M.B., and Wimmer, E. (1977). A protein covalently linked to poliovirus genomic RNA. Proc. Natl. Acad. Sci. USA. 74:59-63.

Lekkerkerker, A., Wellink, J., Yuan, P., van Lent, J., Goldbach, R., and van Kammen, A.B. (1996). Distinct functional domains in the cowpea mosaic virus movement protein. J. Virol. 70:5658-5661.

Liu, D.X., and Inglis, S.C. (1992). Internal entry of ribosomes on a tricistronic mRNA encoded by infectious bronchitis virus. J. Virol. 66:6143-6154.

Lucas, W.J., and Gilbertson, R.L. (1994). Plasmodesmata in relation to viral movement within leaf tissues. Annu. Rev. Phytopathol. 32:387-411.

Lütcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kern, H.F., and Scheele, G.A. (1987). Selection of AUG initiation codons differs in plants and animals. EMBO J. 6:43-48.

Mahmood, T. (1994). Role of viral coat protein and plant binding components in the transmission of plant viruses by leaf feeding beetles. Ph.D. Dissertation. University of Arkansas.

Mäkinen, K., Naess, V., Tamm, T., Aaspollu, A., and Saarma, M. (1995a). The putative replicase of the cocksfoot mottle sobemovirus is translated as a part of the polyprotein by –1 ribosomal frameshift. Virology 207:566-571.

Mäkinen, K., Tamm, T., Næss, V., Truve, E., Aaspõllu, A., and Saarma, M. (1995b). Characterization of cocksfoot mottle sobemovirus genomic RNA and sequence comparison with related viruses. J. Gen. Virol. 76:2817-2825.

McLean, B.J., Zupan, J., and Zambryski, P.C. (1995). Tobacco mosaic virus movement protein associates with the cytoskeleton in tobacco cells. Plant Cell 7:2101-2114.

Meerovitch, K., and Sonenberg, N. (1993). Internal initiation of picornavirus RNA translation. Sem. Virol. 4:217-227.

Meerovitch, K., Svitkin, Y.V., Lee, H.S., Lejbkowicz, F., Kenan, D.J., Chan, E.K.L., Agol, V.I., Keene, J.D., and Sonenberg, N. (1993). La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. J. Virol. 67:3798-3807.

Merrick, W.C., and Hershey, J.W.B. (1996). The pathway and mechanism of eukaryotic protein synthesis. *In* "Translational Control". (J.W.B. Hershey, M.B. Matthews, and N. Sonenberg, Eds.) pp. 31-69. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Miller, W.A., Dinesh-Kumar, S.P., and Paul, C.P. (1995). Luteovirus gene expression. Crit. Rev. Plant Sci. 14:179-211.

Morley, S.J., Curtis, P.S., and Pain, V.M. (1997). eIf4G: Translation's mystery factor begins to yield its secrets. RNA 3:1085-1104.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nelson, R.S., Li, G., Hodgson, R.A.J., Beachy, R.N., and Shintaku, M. (1993). Impeded phloem-dependent accumulation of the masked strain of tobacco mosaic viurs. Mol. Plant-Microbe Interact. 6:45-54.

Ngon A. Yassi, M., Ritzenthaler, C., Brugidou, C., Fauquet, C.M., and Beachy, R.N. (1994). Nucleotide sequence and genome characterization of rice yellow mottle virus RNA. J. Gen. Virol. 75:249-257.

Ohlmann, T., Rau, M., Morley, S.J., and Pain, V.M. (1996). The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E. EMBO J. 15:1371-1382.

Osman, T.A., Hayes, R.J., and Buck, K.W. (1992). Cooperative binding of the red clover necrotic mosaic virus movement protein to single-stranded nucleic acids. J. Gen. Virol. 73:223-227.

Othman, Y., and Hull, R. (1995). Nucleotide sequence of the bean strain of southern bean mosaic virus. Virology 206:287-297.

Pause, A., Methot, N., Svitkin, Y., Merrick, W.C., and Sonenberg, N. (1994). Dominant negative mutants of mammalian translation initiation factor eIF4A define a critical role for eIF4F in cap-dependent and cap-independent initiation of translation. EMBO J. 13:1205-1215.

Pelletier, J., and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 334:320-325.

Pelletier, J., and Sonenberg, N. (1989). Internal binding of eukaryotic ribosomes on poliovirus RNA: Translation in HeLa cell extracts. J. Virol. 63:441-444.

Perbal, M.C., Thomas, C.L., and Maule, A.J. (1993). Cauliflower mosaic virus gene 1 product (P1) forms tubular structures which extend from the surface of infected protoplasts. Virology 195:281-285.

Pestova, T.V., Hellen, C.U.T., and Wimmer, E. (1991). Translation of poliovirus RNA: role of an essential cis-acting oligopyrimidine element within the 5' nontranslated region and involvement of a cellular 57 kDa protein. J. Virol. 65:6194-6204.

Pestova, T.V., Hellen, C.U.T., and Shatsky, I.N. (1996a). Canonical eukaryotic initiation factors determine initiation of translation by internal ribosome entry. Mol. Cell. Biol. 16:6859-6869.

Pestova, T.V., Shatsky, I.N., and Hellen, C.U.T. (1996b). Functional dissection of eukaryotic initiation factor 4F; the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. Mol. Cell. Biol. 16:6870-6878.

Petty, I.T.D., French, R., Jones, R.W., and Jackson, A.O. (1990). Identification of barley stripe mosaic virus genes involved in viral RNA replication and systemic movement. EMBO J. 9:3453-3457.

Rietveld, K., Pleij, C.W.A., and Bosch, L. (1983). Three-dimensional models of the tRNA-like 3' termini of some plant viral RNAs. EMBO J. 2:1079-1085.

Robards, A.W., and Lucas, W.J. (1990). Plasmodesmata. Ann. Rev. Plant Physiol. Plant Mol. Biol. 41:369-419.

Rutgers, T., Salerno-Rife,T., and Kaesberg, P. (1980). Messenger RNA for the coat protein of southern bean mosaic virus. Virology 104:506-509.

Ryabov, E.V., Krutov, A.A., Novikov, V.K., Zheleznikova, O.V., Morozov, S.Y., and Zavriev, S.K. (1996). Nucleotide sequence of RNA from the Sobemovirus found in infected cocksfoot shows a luteovirus-like arrangement of the putative replicase and protease genes. Phytopath. 86:391-397.

Sachs, A.B., Sarnow, P., and Hentze, M.W. (1997). Starting at the beginning, middle and end: Translation initiation in eukaryotes. Cell 89:831-838.

Salerno-Rife, T., Rutgers, T., and Kaesberg, P. (1980). Translation of southern bean mosaic virus RNA in wheat embryo and rabbit reticulocyte extracts. J. Virol. 34:51-58.

Salomon, R., Sela, I., Soreq, H., Giveon, D., and Littauer, U.Z. (1976). Enzymatic acylation of histidine to tobacco mosaic virus RNA. Virology 71:74-84.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sasavage, N.L., Friderici, K., and Rottman, F.M. (1979). Specific inhibition of capped mRNA translation in vitro by m^7G5'ppp5'G and m^7G5'ppp5'm^7G. Nucleic Acids Res. 6:3613-3642.

Scholthof, H.B., Scholthof, K.B.G., Kikkert, M., and Jackson, A.O. (1995). Tomato bushy stunt virus is regulated by two nested genes that function in cell-to-cell movement and host-dependent systemic invasion. Virology 213:425-438.

Schoumacher, F., Erny, C., Berna, A., Godefroy-Colburn, T., and Stussi-Garaud, C. (1992). Nucleic acid binding properties of the alfalfa mosaic virus movement protein produced in yeast. Virology 188:896-899.

Shepherd, R.J., and Fulton, R.W. (1962). Identity of a seed borne virus of cowpea. Phytopathology 52:389-493.

Shih, D.-S., Dasgupta, R., and Kaesberg, P. (1977). 7-Methyl guanosine and efficiency of RNA translation. J. Virol. 19:637-642.

Sonenberg, N. (1990). Poliovirus translation. Curr. Top. Microbiol. Immunol. 161:23-47.

St Johnston, D. (1995). The intracellular localization of messenger RNAs. Cell 81:161-170.

Storms, M.M.H., Kormelink, R., Peters, D., Van Lent, J.W.M., and Goldbach, R.W. (1995). The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. Virology 214:485-493.

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N., and Takanami, T. (1991). Functional analysis of deletion mutants of cucumber mosaic virus RNA 3 using an *in vitro* transcription system. Virology 183:106-113.

Tarun, S.Z., and Sachs, A.B. (1995). A common function for mRNA 5' and 3' ends in translation initiation in yeast. Genes Dev. 9:2997-3007.

Tarun, S.Z., and Sachs, A.B. (1996). Association of the yeast poly(A) tail binding protein with translation initiation factor eIF4G. EMBO J. 15:7168-7177.

Traynor, P., Young, B.M., and Ahlquist, P. (1991). Deletion analysis of brome mosaic virus 2a protein: Effects on RNA replication and systemic spread. J. Virol. 65:2807-2815.

Tremaine, R.I., and Hamilton, R.I. (1983). CMI/AAB Descriptions of Plant Viruses; Commonwealth Agricultural Bureaux/Association of Applied Biologists. Southern Bean Mosaic Virus. Vol. 274.

Vaewhongs, A.A., and Lommel, S.A. (1995). Virion formation is required for long-distance movement of red clover necrotic mosaic virus in movement protein transgenic plants. Virology 212:607-613.

Vale, R.D. (1987). Intracellular transport using microtubule-based motors. Annu. Rev. Cell Biol. 3:347-378.

van Lent, J., Wellink, J., and Goldbach, R. (1990). Evidence for the involvement of the 58K and 48K proteins in the intercellular movement of cowpea mosaic virus. J. Gen. Virol. 71:219-223.

Van Lent, J., Storms, M., van der Meer, F., Wellink, J., and Goldbach, R. (1991). Tubular structures involved in movement of cowpea mosaic virus are also formed in infected cowpea protoplasts. J. Gen. Virol. 72:2615-2623.

von Arnim, A.G., and Deng, X.W. (1994). Light inactivation of arabidopsis photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. Cell 79:1035-1045.

Wang, S., and Miller, W.A. (1995). A sequence located 4.5 to 5 kilobases from the 5' end of the barley yellow dwarf virus (PAV) genome strongly simulates translation of uncapped mRNA. J. Biol. Chem. 270:13446-13452.

Wang, S., Browning, K.S., and Miller, A.W. (1997). A viral sequence in the 3'-untranslated region mimics a 5' cap in facilitating translation of uncapped mRNA. EMBO J. 16:4107-4116.

Weiland, J.J., and Edwards, M.C. (1996). A single nucleotide substitution in the aa gene confers oat pathogenicity to barley stripe mosaic virus strain ND18. Mol. Plant-Microbe Interact. 9:62-67.

Wellink, J., and van Kammen, A. (1989). Cell-to-cell transport of cowpea mosaic virus requires both the 58K/48K proteins and the capsid proteins. J. Gen. Virol. 70:2279-2286.

Wellink, J., van Lent, J.W.M., Verver, J., Sijen, T., and Goldbach, R.W. (1993). The cowpea mosaic virus M RNA-encoded 48-kilodalton protein is responsible for induction of tubular structures in protoplasts. J. Virol. 67:3660-3664.

Wieczorek, A., and Sanfaçon, H. (1993). Characterization and subcellular location of tomato ringspot nepovirus putative movement protein. Virology 194:734-743.

Wittmann, S., Chatel, H., Fortin, M.G., and Laliberte, J. (1997). Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic

initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two-hybrid system. Virology 234:84-92.

Wolf, S., Deom, C.M., Beachy, R.N., and Lucas, W.J. (1989). Movement protein of tobacco mosaic virus modifies plasmodesmata size exclusion limit. Science 246:377-379.

Wu, S., Rinehart, C., and Kaesberg, P. (1987). Sequence and organization of southern bean mosaic virus genome RNA. Virology 161:73-80.

Yerkes, W.D., and Patino, G. (1960). The severe bean mosaic virus, a new bean virus from Mexico. Phytopath. 50: 334-338.

Yogo, Y., and Wimmer, E. (1972). Polyadenylic acid at the 3'-terminus of poliovirus RNA. Proc. Natl. Acad. Sci. USA. 69:1877-1882.

APPENDIX

ACRONYMS FOR SELECTED VIRUSES

AMV	Alfalfa mosaic AMV group
BBSSV	Blueberry shoestring sobemovirus
BMV	Brome mosaic bromovirus
BYDV	Barley yellow dwarf luteovirus
CfMV	Cocksfoot mottle sobemovirus
CMV	Cucumber mosaic cucumovirus
CPMV	Cowpea mosaic comovirus
LTSV	Lucerne transient streak sobemovirus
PV	Poliovirus
RCNMV	Red clover necrotic mosaic dianthovirus
RYMV	Rice yellow mottle sobemovirus
SBMV-B	Southern bean mosaic sobemovirus – bean strain
SBMV-C	Southern bean mosaic sobemovirus – cowpea strain
SBMV-G	Southern bean mosaic sobemovirus – Ghana strain
SBMV-M	Southern bean mosaic sobemovirus – Mexican strain
SCMV	Subterranean clover mottle sobemovirus
SNMV	Solanum nodiflorum mottle sobemovirus
SoMV	Sowbane mosaic sobemovirus
TEV	Tobacco etch tobamovirus
TMV	Tobacco mosaic tobamovirus
TRV	Turnip rosette sobemovirus
TuMV	Turnip mosaic potyvirus
VTMV	Velvet tobacco mottle sobemovirus

VITA

Kailayapillai Sivakumaran was born in Mirusuvil, SriLanka, and graduated from Trinity College, Kandy, SriLanka. He received his Bachelor of Science degree (1985), and a Master of Science degree (1987) from the Madras University, Madras, India. He worked for the Madras Science Foundation from 1987-89 as a technical assistant. He then enrolled in a Master's Program at Montana State University, and graduated with a Master of Science in Agronomy (1992). He is presently working on his Ph.D. in Microbiology at the University of Tennessee at Knoxville, and will receive his degree in the spring of 1998.