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Theo W. Dreher

Coreplication experiments have revealed that replication of turnip yellow mosaic virus (TYMV) RNA in turnip protoplasts is *cis*-preferential. Genomes encoding mutant p141 or p66, proteins essential for virus replication, were inefficiently rescued by a helper genome. One model for the *cis*-preferential replication of TYMV is that p66 and p141 form a complex that associates with the RNA from which they are translated, limiting their availability *in trans*. Three types of experiments were used in this study in an attempt to obtain physical evidence for the hypothetical interaction between p66 and p141. Immunoprecipitations from *in vitro* translation reactions using antiserum that recognizes p66 (and its progenitor, p206) coprecipitate p141, indicating that the proteins form a complex *in vitro*. The results of coimmunoprecipitations of translation products with in-frame deletions did not lead to definitive information about interaction domains. p66 and the helicase domain of p141 do not detectably interact in the yeast two-hybrid system or in GST fusion interaction assays. Problems with the expression of full length p141 fusions make conclusions about the interaction of other p141 domains with p66 not possible at this time. Since the helicase domain of p141 does not appear to interact with p66, future experiments will focus on obtaining expression of smaller domains of p141, outside the helicase domain, and determining if they interact with p66. Variations to the model that do not necessitate the direct interaction between p66 and p141 are also considered.

Search for Protein-Protein Interactions Underlying the Cis-Preferential Replication of Turnip Yellow Mosaic Virus

By

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1. General Introduction

1.1 Classification of Turnip Yellow Mosaic Virus

The genome of turnip yellow mosaic virus (TYMV) consists of a 6.3 kb positive sense RNA, and is encapsidated by viral-encoded capsid proteins to form a 29 nm icosahedron. TYMV is the type member of the tymoviruses, which are included in the Sindbis-like, or alphavirus-like, supergroup (Morch *et al.*, 1988; Goldbach and Wellink, 1988). Based on similarity of sequences and genome organization, Koonin and Dolja (1993) placed plus strand RNA viruses into three supergroups. Supergroup I contains the picorna-like viruses, supergroup II includes RNA phages and flaviviruses, and supergroup III contains the Sindbis-like viruses. All members of supergroup III produce a subgenomic mRNA, which along with the genomic RNA, has a 5' cap. Polymerase- and helicase-like domains are generally present, as well as a methyltransferase domain that appears to be unique to supergroup III, although some of the smaller members lack this domain (Rozanov *et al.*, 1992). In TYMV and other monopartite members of supergroup III, the order of the conserved domains (N-terminal to C-terminal) is consistently methyltransferase, helicase, polymerase.

1.2 Genome Organization

The three open reading frames encoded by TYMC (Corvallis strain of TYMV) are diagramed in Figure 1.1. ORF-69 initiates at nucleotide 88 and terminates at nucleotide 1971 (Morch *et al.*, 1988; Dreher and Bransom, 1992) and encodes a 69-kDa protein involved in viral cell-to-cell movement (Bozarth *et al.*, 1992). ORF-206 encodes a 206-kDa protein with methyltransferase-, helicase- and polymerase-like domains (Morch *et al.*, 1988; Gorbalenya and Koonin, 1989; Rozanov *et al.*, 1992). The protein also contains a protease domain that functions *in cis* to cleave the 206-kDa protein into 141-kDa and 66-kDa proteins (Bransom and Dreher, 1994; Bransom *et al.*, 1996). ORF-206 initiates at nucleotide 95, terminates at nucleotide 5626 and is in the +1 frame in relation to ORF-69. CP-ORF is in the same reading frame as ORF-69, and encodes a 20-kDa capsid protein expressed from a subgenomic RNA. The CP-ORF extends from nucleotide 5644 to nucleotide 6210 (Morch *et al.*, 1988).

Only 3% of the TYMV genome is non-coding. The 5' untranslated region (UTR) has not been extensively studied, but it does have a m7GpppG cap, as does the the 5' end of the subgenomic RNA (Briand *et al.*, 1978). The 3' UTR contains a tRNA-like structure that can be aminoacylated with valine *in vitro* (Pinck *et al.*, 1970; Dreher *et al.*, 1988) and *in vivo* (Joshi *et al.*, 1982).

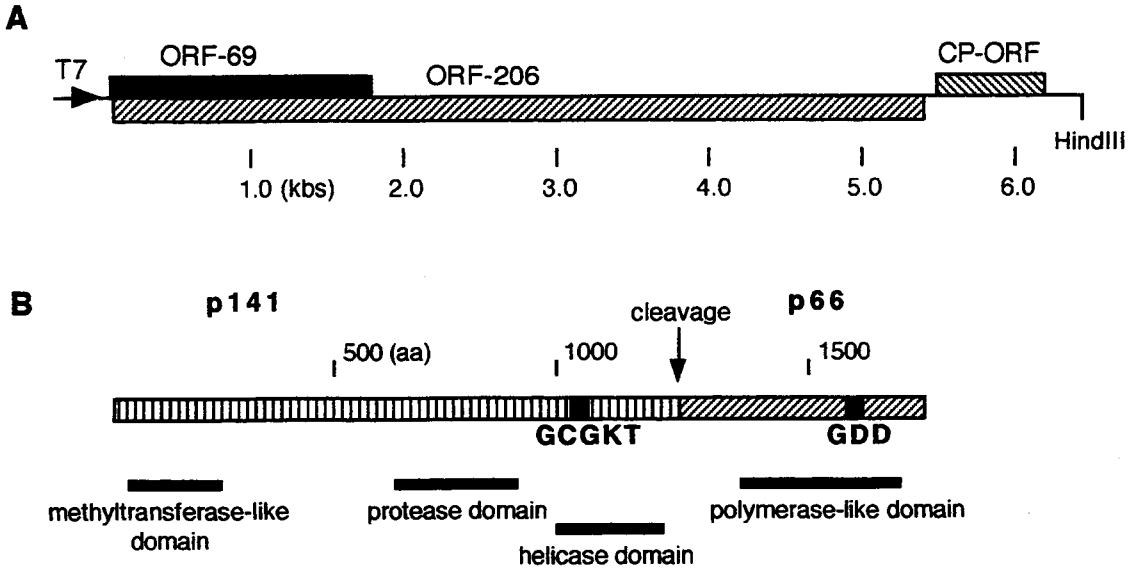


Figure 1.1. Diagram of TYMC. (A) The cDNA insert of pTYMC. Infectious transcripts can be made by using T7 RNA polymerase to transcribe *HindIII* linearized DNA. (B) Diagram of the ORF-206 product. The protein domains with conserved motifs or known function, the core amino acids of the helicase- and polymerase-like domains (GCGKT and GDD respectively), and the protease cleavage site between amino acids 1259 and 1260 are shown.

1.3 Conserved Protein Domains and their Functions

1.3.1 Movement proteins

As was mentioned above, some of the proteins encoded by TYMV share sequence homology with other plant and animal RNA viruses. Movement proteins, like the 69-kDa protein of TYMV, are unique to plant viruses. Although p69 is not necessary for TYMV replication in protoplasts, it is necessary for the spread of infection in whole plants (Bozarth *et al.*, 1992). p69 has a high pI (12.1), suggestive of nucleic acid binding properties. The 69-kDa movement

protein is phosphorylated when expressed in insect cells (Seron *et al.*, 1996) and migrates slower than expected in SDS-PAGE.

The 30-kDa protein of tobacco mosaic tobamovirus (TMV), is a well studied viral movement protein. The phosphorylated protein is a single stranded nucleic acid binding protein that is able to modify plasmodesmata, increasing the molecular exclusion limit from 3.0 nm to 6-12 nm (reviewed in Deom *et al.*, 1992). It is postulated that p30 coats and unfolds TMV RNA, and facilitates cell to cell movement through the plasmodesmata (Citovsky *et al.*, 1990). RNA binding has been observed by the 26-kDa movement protein of foxtail mosaic potexvirus (Rouleau *et al.*, 1994) and by the gene I product of cauliflower mosaic caulimovirus (Citovsky *et al.*, 1991).

1.3.2 Capping domains

The amino terminal region of p206/p141 of TYMV contains a methyltransferase-like domain. This domain is conserved and specific to members of the Sindbis-like supergroup, and is thought to be responsible for the methylated 5' cap structure found on the genomes of these viruses. In contrast, picorna-like viruses have a VPg protein associated with their 5' genome ends (Rozanov *et al.*, 1992; Goldbach and Wellink, 1988).

The methyltransferase domain consists of 5 motifs and contains four strictly conserved amino acids: H in motif I, NXXR in motif II and Y in motif IV (Rozanov *et al.*, 1992). A methytransferase activity was detected by methylation of guanylylimido-diphosphate by the nsP1 protein of Sindbis virus (Mi and Stollar, 1991). It was determined that changing any of the four conserved methyltransferase amino acids to A resulted in a protein with no methyltransferase activity and an RNA that was not infectious (Wang *et al.*, 1996). The methyltransferase domain of TYMV is required for replication (Weiland and Dreher, 1993), but it has not been extensively studied.

Guanylyltransferase activity has been detected in the nsP1 proteins of both Semliki Forest virus and Sindbis virus. This activity is likely responsible for the addition of GMP to the 5' end of the genome, creating a cap 0 structure (m7GpppG). It was found that this reaction was dependent on the presence of S-adenosylmethionine (AdoMet), indicating that the methyl group is added to GTP before GMP is covalently added to the 5' end of the genome (Ahola and Kaariainen, 1995). The mRNA capping mechanism found in most mammalian and viral systems involves first the addition of GMP followed by its methylation. A GTP binding activity has been detected in tobacco mosaic virus, and unlike that of Sindbis virus, it was not dependent on the presence of AdoMet (Dunigan and Zaitlin, 1990). Guanylyltransferase activity has not been studied in association with TYMV, but it is likely that the activity is viral-encoded since viral replication occurs in the cytoplasm.

1.3.3 Protease domains

Proteolytic processing of viral-encoded proteins is a common feature in both plant and animal viruses (Dougherty and Semler, 1993). The proteases responsible for the processing are typically virally encoded. The protease domain of p206/p141 is responsible for the specific cleavage of p206 between A^{1259} and T^{1260}, to form the N-terminal product, p141 and the C-terminal product, p66 (Chapter 2; Bransom *et al.*, 1996; Kadare *et al.*, 1995). Cleavage of p206 is essential for viral replication; viruses with mutations that inactivate the protease or that alter the cleavage site cannot replicate in protoplasts. *In vitro*, proteolysis occurs *in cis*, as is indicated by the lack of a dilution effect; increasing the reaction volume did not result in a measurable decrease in proteolysis (Bransom *et al.*, 1994). It was found that cotranslations in rabbit reticulocytes of a TYMV RNA encoding a defective cleavage site, and

of a TYMV RNA encoding a defective protease, did not result in any proteolysis products, indicating that the protease does not function *in trans*. However, it is possible in this system that the functional protease was bound to the defective cleavage site and unavailable to work *in trans*. C^{783} and H^{869} were found to be essential residues and help classify the TYMV protease as a papain-like protease (Bransom *et al.*, 1994).

1.3.4 Helicase domains

NTP-binding motifs, as part of helicase domains, are common among viruses. Negative strand RNA viruses and retroid viruses seem to be the only groups that consistently lack this domain (Gorbalenya and Koonin, 1989). Helicase activity has been detected in the cytoplasmic inclusion (CI) proteins of plum pox potyvirus (Lain *et al.*, 1990), and tomarillo mosaic potyvirus (Eagles *et al.*, 1994). In an ATP-dependent manner, these proteins were able to separate the strands of circa 20 base pair duplex RNA molecules with 3' and 5' overhangs

Properties consistent with helicase activity in TYMV have recently been described for amino acids 916 to 1259 of p206/p141. This domain was expressed in *E. coli* and was found to have ATPase, GTPase and sequence-independent RNA binding activities. The ATPase and GTPase activities were stimulated two-fold by the addition of RNA fragments (Kadare *et al.*, 1996). Changing the conserved nucleotide binding motif GxGKT to GxGST had been previously shown to prevent replication of TYMV RNA in protoplasts (Weiland and Dreher, 1993). The same alteration has now been shown to decrease ATPase and GTPase activity to 15-20% of wild type (Kadare *et al.*, 1996). In TYMV, the helicase domain could be responsible for unwinding the 3' tRNA-like structure to allow negative strand synthesis, and for separating double-stranded replication intermediates.

1.3.5 RNA-dependent RNA polymerases

RNA-dependent RNA polymerases are essential for the replication of positive stranded RNA viruses. The conserved sequence motif characteristic of these polymerases, GDD (Argos *et al.*, 1988), is found in p206/p66, and mutations in this domain prevent viral replication (Weiland and Dreher, 1993). A number of viral and host proteins has been found to be necessary for the function of the polymerases of other viruses, and will be discussed later.

1.3.6 Capsid protein

As stated earlier, the 20-kDa capsid protein is expressed from a subgenomic RNA. The assembled capsid consists of 180 subunits with T=3 symmetry (Matthews, 1991). It is thought that the loss of 5-9 capsid subunits creates a small hole that allows the release of the RNA and the initiation of infection (Adrian *et al.*, 1992). It has been observed that deletions of the RNA that encodes the coat protein prevent systemic symptoms in turnip plants. The coat protein is therefore important in the establishment of a systemic infection, perhaps by facilitating long distance viral movement (Weiland and Dreher, 1993; Bransom *et al.*, 1995).

1.4 Noncoding Regions and other *Cis* Elements

The 5' untranslated region (UTR) of TYMV does not share sequence homology with TMV, brome mosaic bromovirus (BMV), or alfalfa mosaic virus (AlMV) (Briand *et al.*, 1978). In TMV, the 5' UTR contains a so-called Ω sequence that enhances translation. This

sequence works in conjunction with a sequence just 5' of the 3' TLS of TMV, called the upstream pseudoknot domain (UPD). These sequences are recognized by proteins in wheat germ and carrot extracts, which presumably facilitates translation (Leathers *et al.*, 1993). TYMV does not contain an Ω-like sequence, but it does contain a putative pseudoknot just 5' of the TLS. Even with the pseudoknot present, the 3' UTR of TYMV was not able to significantly increase the expression of a reporter gene (Gallie and Kobayashi, 1994). Another tymovirus, wild cucumber mosaic virus, has two pseudoknot structures just upstream of the TLS that closely resemble the UPD of TMV (Hellendoorn *et al.*, 1996).

The TYMV pseudoknot mentioned above may also be involved in virus replication. It was found that a mutation that would destabilize the pseudoknot structure (U-96⇨G), resulted in a virus that replicated poorly and could not cause systemic infection in plants. Viruses that had regained their ability to spread systemically contained reversions or a second site suppressor mutation that restored the pseudoknot structure (Tsai and Dreher, 1992).

tRNA like structures (TLS) are found on the 3' ends of many plant RNA viruses and most can be aminoacylated. The TLS of TYMV can be charged with valine, and mutational analysis of the anticodon loop indicated that the middle nucleotide of the anticodon is the most important factor for efficient charging (Dreher *et al.*, 1992). Identity element changes from valine to methionine result in a TLS that aminoacylates specifically and efficiently with methionine, and an RNA that is infectious (Dreher *et al.*, 1996).

The tymobox is a highly conserved sequence found in all tymoviruses. Although it is found in coding regions, the nucleotide sequence is conserved, not just the amino acids they encode (Ding *et al.*, 1990). Subgenomic RNA synthesis is initiated on full length minus-strand template (Gargouri *et al.*, 1989) in a similar manner as with BMV and AlMV. The tymobox is postulated to be involved in the recognition or binding of the replicase and an initiation box is

thought to dictate the subgenomic RNA transcription start site (Ding *et al.*, 1990).

The tymobox is an example of how *cis* elements are not always limited to noncoding regions of the genome. There may be *cis* elements in TYMV RNA that have not been identified or characterized, and this needs to be considered when designing and analyzing mutants. Deletions, insertions and point mutations in the coding region of replication proteins may also interfere with RNA secondary structure and result in a defective template. In the RNA bacteriophage Qβ, *cis* elements (RNA elements required for replication) have been found in the replicase coding region (Mills *et al.*, 1990). Plasmid-encoded Qβ replicase proteins were provided *in trans*, and the ability of mutant RNAs to be replicated and produce virus particles was measured. Many deletions and insertions in the RNA, some of which were in the replicase coding region, resulted in low or no virus production. Similar experiments with the Qβ readthrough region, which adds a 200 amino acid extension to the capsid protein, have led to the conclusion that stable secondary RNA structure in this region is required for viral propagation (Arora *et al.*, 1996).

1.5 Replication Complexes

No replication complexes induced by eukaryotic positive-strand RNA viruses have been characterized sufficiently to determine subunit composition. However, the viral replication complexes are thought to be composed of both viral and host proteins; they are associated with membranes and RNA templates. In the case of poliovirus, electron microscopy and immunocytochemistry have been used to deduce how the replication complexes are formed, and how they are associated with membranes. It is proposed that soon after the P2 replication proteins are synthesized, they associate with rough endoplasmic

reticulum (rER) membranes. The 2BC protein is thought to induce the formation of smooth vesicles, which eventually detach from the rER (Bienz *et al.*, 1987). The 2C protein or a 2C containing precursor, facilitates the attachment of the viral RNA to the membrane and directs proper replication complex assembly (Bienz *et al.*, 1990).

Plant viral replication complexes are also associated with membranes. Viral RNA synthesis for TYMV has been shown to occur at invaginations in the chloroplast membrane (Garnier *et al.*, 1986).

The viral proteins associated with the replication complex of TYMV are probably p206 and/or its cleavage products p141 and p66. For BMV, proteins 1a and 2a are known to be part of the replication complex and have been shown to interact directly with each other both *in vitro* (Kao *et al.*,1992) and in the yeast two-hybrid system (O'Reilly *et al.*, 1995).

Proteins 1a and 2a of BMV are expressed from separate RNAs (RNA1 and RNA2) and have helicase- and polymerase-like domains, respectively. In a specially designed BMV replication system in yeast, assembly of functional replication complexes containing proteins 1a and 2a was dependent on the presence of RNA3. The role or RNA3 is unclear, but it appears to be involved in either replication complex assembly, activity or stability (Quadt *et al.*, 1995). Since RNA3 is not required for BMV replication in protoplasts, it is thought that RNA1 and RNA2 can also facilitate replication complex formation.

There is to date relatively little information on host proteins associated with viral replication complexes. In BMV, a replication complex-associated barley protein is immunologically cross-reactive with the p41 subunit of eIF-3 from wheat germ. It was found that adding eIF-3 to the replication complex stimulated negative-strand RNA synthesis (Quadt *et al.*, 1993). The replication complex of the Qβ bacteriophage contains the ribosomal protein S1 and the elongation factors Tu and Ts (Blumenthal *et al.*, 1979). HF-I is a heat stable host protein involved in minus-strand RNA synthesis of Qβ, but of unknown function in *E. coli* (Kajitani and Ishihama, 1991). It

is interesting that translation factors seem to play a role in the replication complexes of both these viruses.

It has been proposed that different forms of replication complexes may be required for negative-strand versus positive-strand synthesis. In Sindbis virus, the replication proteins P1-4 are translated as a single polypeptide. More P123 is synthesized than P1234, which is made by readthrough of a termination codon. Cleavage of P123 by the protease domain of P2 occurs *in trans* and results in the cleavage products P1, P2, and P3, but the cleavage site between P3 and P4 can be cleaved *in cis* and is preferred This preferential cleavage results in the initial accumulation of P123 and P4. P4 contains the polymerase-like domain and is involved in both positive and negative-strand synthesis. P123 is associated with the complex responsible for negative strand synthesis but this complex initiates positive strand synthesis inefficiently. Cleavage of P123 to produce P1, P2 and P3, results in a replicase complex with a preference for positive strand synthesis (Lemm *et al.*, 1994).

Recently, a replicase preparation from TYMV has been characterized. This replicase preparation can copy TYMV, TMV and BMV RNAs to relative levels of 100, 18 and 6%, respectively, but it does not copy poly U, poly A or alfalfa mosaic virus (AlMV) RNA. The viral and host protein components of this replication complex have not yet been identified (Ravindra Singh, personal communication).

1.6 *Cis*-Preferential Action

Cis-preferential proteins are those that work predominantly *in cis*. They are usually discovered when complementation experiments show that the proteins do not function, or function poorly, *in trans*. *Cis*-preferential action is a common method to restrict transposition of bacterial transposons, as transposases generally function much more efficiently *in cis* than they do *in*

trans. There are a number of mechanisms that explain the *cis*-preferential action of transposases, some of which will be discussed below.

A model for the *cis*-action of the transposase (Tnp) of Tn*5* is based on the formation of nonfunctional multimers. Tnp is known to dimerize but this form of Tnp is unable to bind DNA. It is thought that one monomer of Tnp binds one end of the Tn*5* sequence and another Tnp monomer binds the other end. The two Tnp monomers are thought to then form a productive dimer that initiates transposition. This reaction occurs *in trans* infrequently because Tnp is likely to form a nonproductive multimer with another Tnp (or inhibitor) molecule before it reaches a distal Tn*5* sequence (Weinreich *et al.*, 1994).

The Tnp of the Tn*10* transposon also functions poorly *in trans*. The *cis*-preferential action of this Tnp is thought to be due to either slow transcript release or poor mRNA stability; it was found that changes in the mode of transposase gene expression at steps subsequent to transcription initiation could affect the degree of its *cis* action (Jain and Kleckner, 1993). In the case of Tn*903*, the lack of *trans* action by the transposase seems to be due to the protein's instability. Using a *lon* deficient strain of *E. coli* resulted in *trans*-action of the transposase (Derbyshire *et al.*, 1990). Inefficient translation initiation also appears to limit the levels of transposase produced by Tn*903* (Derbyshire and Grindley, 1996).

1.7 *Cis*-Preferential Replication

As with the bacterial transposons, some viruses also encode proteins that exhibit *cis*-preferential action. Often they are non-capsid proteins involved in replication, and show a preference for the template from which they were synthesized. In these cases where translation and replication are coupled, replication is termed *cis*-preferential.

Since RNA polymerases have relatively high error rates, it is to the advantage of the RNA virus to have some mechanism to prevent the unnecessary replication of defective RNAs. It was noted that defective interfering (DI) RNAs in polioviruses were very rare, and the ones that did occur still produced functional replication proteins. Deletions and insertions were found only in the structural genes and they all maintained the reading frame of the replication protein genes. *In vitro*-constructed poliovirus DI genomes were active replicons only if the insertions maintained the reading frame (Hagino-Yamagishi and Nomoto, 1989). On further investigation, it was determined that a number of poliovirus non-capsid proteins cannot be provided *in trans,* including 2A (Novak and Kirkegaard, 1994), 2B (Johnson and Sarnow, 1991), 3A and 3B (Giachetti *et al.*, 1992).

Clover yellow mosaic potexvirus (CYMV), beet necrotic yellow vein virus (BNYVV) and alfalfa mosaic virus (AlMV) are plant viruses that exhibit *cis*-action that affect replication. CYMV is the only potexvirus that has had associated defective RNAs identified. All of the naturally occurring defective RNAs examined maintained the reading frame of both ORF1 (encodes the putative polymerase) and ORF5 (encodes the coat protein). It was suggested that translation of these two reading frames is required to either facilitate replication of the RNA or to ensure its encapsidation (White *et al.*, 1992). The cysteine-rich p14 of BNYVV is involved in cell-to-cell movement of the virus, and is required *in cis* for the replication of RNA2 (Hehn *et al.*, 1995). Complementation experiments were used to determine that the replication of RNAs 1 and 2 of AlMV is coupled to the translation of the encoded proteins (van Rossum *et al.*, 1996).

Complementation experiments with TYMV revealed that its replication is also *cis*-preferential (Weiland and Dreher, 1993). Replication was detected in turnip protoplasts by Northern analysis. The constructs used and the relative accumulation levels of viral RNA in various complementation experiments are shown in Figure 1.2 (adapted from Weiland and Dreher, 1993). The helper genome,

ΔCP, has a deletion in the CP-ORF and its replication, as determined by the accumulation of positive-sense genomic RNA, is only 10% of that of wild type transcripts. This is thought to be due to the high degradation rate of unencapsidated RNA, not to lower replication rates, as negative-sense RNA levels were similar to that of wild type. The possibility exists, however, that the coat protein has a role in the stimulation of positive strand synthesis. The helper genome poorly complemented RNAs with in-frame deletions or substitutions in ORF-206 (Fig. 1.2). In addition, only a low level of complementation was observed in experiments with inocula consisting of a genome (GCGST) containing a substitution in the conserved helicase motif and a genome (RDD) with a substitution in the conserved polymerase motif. By contrast, accumulation levels of 49% of wild type resulted from complementation experiments between a constructs with a large deletion in the p66 coding region (ΔSP-G) and the GCGST genome, indicating that *cis*-preference can be overcome (Weiland and Dreher, 1993).

Some of the mutant genomes have deletions that affect more than just protein size and conformation. Δ4085-6062, which has most of the p66 and coat protein coding regions deleted, also has the *cis*-acting tymobox and the subgenomic initiation region deleted. This deletion prevents subgenomic RNA synthesis, so coat protein levels will be low, and the RNA more readily degraded, possibly explaining the low levels of positive-sense RNA accumulation when inoculated together with the GCGST genome (Weiland and Dreher, 1993). This deletion may also remove *cis*-elements important for the replication of genomic RNA.

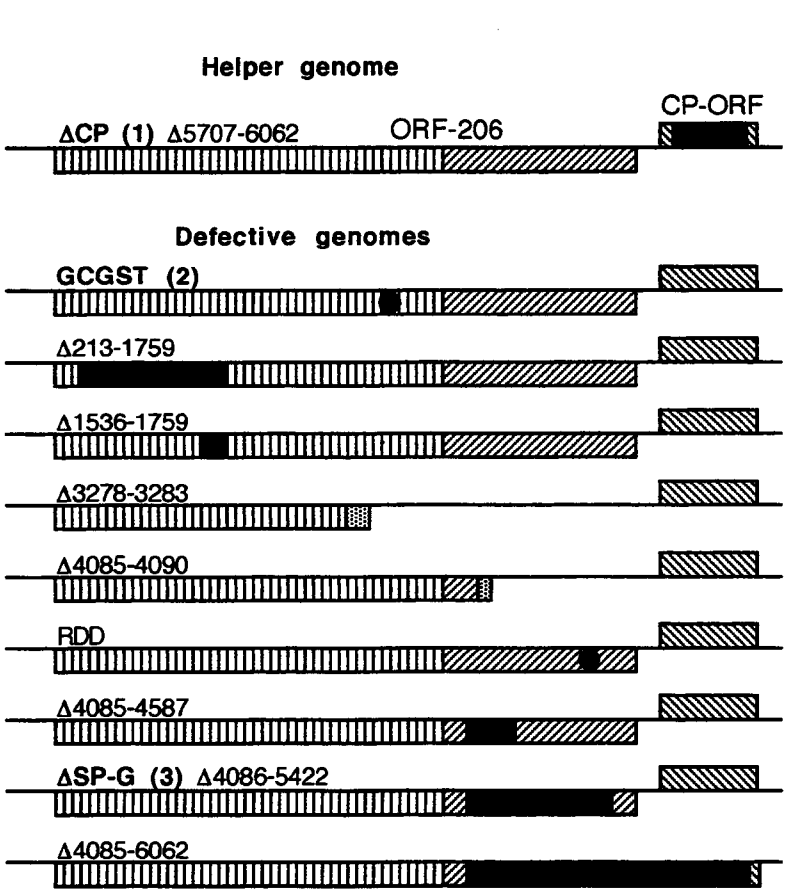


Figure 1.2. Diagram of ORF-206 and CP-ORF for the mutant genomes studied by Weiland and Dreher (1993). Deleted sequences are represented by solid bars, dotted bars represent out of frame sequences prior to stop codons (frame-shift mutants), and black circles represent point mutations that change the amino acids of the conserved helicase and polymerase motifs. The names of genomes used in common coinoculation experiments (ΔCP, GCGST and ΔSP-G) are in bold and are numbered 1-3. The table to the right of the genome maps shows the accumulation of genomic RNA, relative to wild-type levels, in coreplication experiments with genomes 1-3. The ΔSP-G genome was refered to as TYMC-Δ4085-5406 by Weiland and Dreher (1993). This genome was sequenced and it was determined that the nucleotides joined were 4086 and 5422, as reflected for ΔSP-G in this figure.

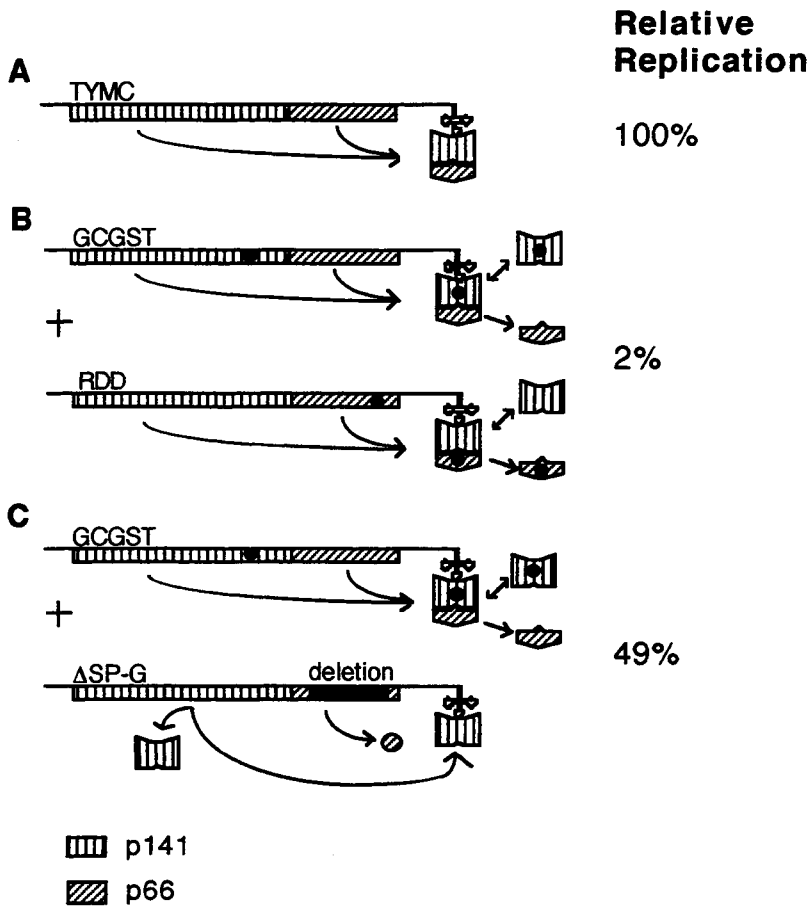


Figure 1.3. Model proposed by Weiland and Dreher (1993) that explains the *cis*-preferential replication of TYMV. Three inoculum combinations and their relative replication compared to wild type are given. The proposed interactions between p141, p66 and the template RNA are indicated. In (A), wild type, active replication complexes are formed that are strongly associated with the template. The complexes formed in (B) are inactive but still strongly associated with the template. Active complex formation in (C) is postulated to be due to the availability of wild type p66 provided *in trans,* translated from the GCGST genome, and the assembly of active replication complexes on the ΔSP-G template. The large deletion in the p66 coding region of the ΔSP-G genome prevents the interaction between the encoded p66 and p141, so that wild type p66 provided *in trans* can be incorporated into active replication complexes. p141 must likewise be provided in *trans.*

Genomes Δ3278-3283 and Δ4085-4090 both cause frameshifts that prevent the expression of all or most of p66, respectively. These frameshifts could affect the suitability of mutant genomes as replication templates by decreasing RNA stability. Also, since these frameshifts cause early termination of translation, replication can be affected if translation of these regions is required to provide a functional template.

A number of mechanisms can explain the *cis*-preferential replication of RNA viruses (discussed in Novak and Kirkegaard, 1994). One possibility is that only newly synthesized proteins are functional and able to associate with the template. Tight regulation of translation could assure that only limited amounts of replication proteins are made, and they quickly become associated with the template and form replication complexes. Active translation could be required so that ribosomes could remove or induce RNA secondary structure in the template or deliver host proteins needed in replication. It is also possible that protein diffusion is somehow restricted, either by a membrane or by the protein's association into a complex with other proteins.

A model was proposed that explained the *cis*-preferential replication of TYMV based on a theoretical interaction between p141 and p66. The finding in TYMV that genomes with deletions in the p66 coding region could be complemented by a helper virus, led to the hypothesis that the deletions might correspond to loss of an interaction domain. If the interaction of p66 was with p141, analogous to the interaction identified between the helicase and polymerase-like proteins of BMV (Kao *et al.*, 1992), the replication proteins could be sequestered and unavailable *in trans*. The p141 and p66 proteins were postulated to be channeled to the template, possibly to bind the negative-strand promoter, and assemble into replication complexes (Weiland and Dreher, 1993). Deletion of an interaction domain in one of the interacting proteins could disrupt the protein-protein interaction, allowing the proteins to diffuse from the template, and for complementation to occur. This model, diagramed in Figure 1.3, explains the *cis*-preferential replication

data for TYMV; note that this model was suggested in the absence of evidence for or against a direct protein-protein interaction between p141 and p66. The purpose of this study was to determine if an interaction between the two proteins does indeed occur.

1.8 Well Characterized Protein-Protein Interaction Domains

A few proteins involved in protein-protein interactions have been well studied and characterized. The interaction between two Gcn4 (a yeast regulatory protein) molecules is an example of a leucine zipper. This type of interaction domain is found in α helices where every seventh amino acid is a leucine. The leucine side chains can project into an α helix of another protein with a similar cyclical arrangement of leucines and a stable coiled-coil is formed (O'Shea *et al.*, 1991).

The SH2 domain of the Src protein, a tyrosine kinase, has been well studied and has been crystallized in both free and complexed forms. Whereas the leucine zipper motif can comprise a relatively large interaction domain, the SH2 domain is restricted to an interaction region of 5 or 6 residues. The domain consists of anitiparallel sheets surrounded by two α-helices and has been likened to a two-holed socket formed by hydrophobic pockets. The interacting peptide contains phosphotyrosine and has been likened to a two-pronged plug (Waksman *et al.*, 1993).

The SH3 domain, also of the Src protein, is composed of antiparallel sheets and a C-terminal 3_{10} helix. The domain contains a hydrophobic groove, lined with the side chains of aromatic amino acids (Yu *et al.*, 1992) that can interact specifically with proline-rich peptides. Proline-rich peptides that form left-handed type II polyproline helices fit into the groove of the SH3 domain (Yu *et al.*, 1994).

1.9 Methods to Detect Protein-Protein Interactions

There are numerous methods used to detect protein-protein interactions. Each method has its benefits, obstacles, and drawbacks. With *in vitro* techniques, some interactions that are detected are not necessarily biologically important, and could be just an artifact of the method. For this reason, it is important to obtain confirmation from a number of detection methods, and to verify that the two proteins can occur in the same part of a cell at the same time.

In this study, three techniques were used: coimmunoprecipitations, yeast two-hybrid studies, and glutathione S-transferase (GST) fusion interaction assays. Immunoprecipitations in nondenaturing conditions are a common way of detecting protein complexes. If a protein coprecipitates with the immunoprecipitation of another protein, the two proteins likely interact, either directly or indirectly. Immunoprecipitations can be used in *in vivo* experiments with cell lysates or with *in vitro* translated proteins. An advantages of *in vivo* experiments include that the protein complexes are pre-assembled, and as long as cell lysis does not disrupt these complexes, they should be in their native state during the immunoprecipitations (Phizicky and Fields, 1995). Sometimes *in vivo* experiments are difficult or not possible due to low concentrations of the proteins of interest.

Immunoprecipitations of *in vitro* translated proteins are also commonly performed and they were used in this study. In both *in vivo* and *in vitro* immunoprecipitations, it is important to use well characterized antisera. The antibodies must be specific for the proteins they were made against, and must not cross react with the coprecipitated proteins. Pre-immune sera should be used to assure that other antibodies in the sera do not recognize the proteins, and that the proteins do not nonspecifically adhere to the beads or Protein A. In *in vivo* and *in vitro* immunoprecipitations, coprecipitating proteins do not necessarily indicate a direct protein-

protein interaction, as the interaction could be mediated by another protein (Phizicky and Fields, 1995).

The yeast two-hybrid system developed by Fields and Song (1989), has become a very popular method to detect protein-protein interactions. In this system, hybrid proteins are made with the proteins of interest and either the yeast Gal4 DNA binding domain or the Gal4 activation domain. If the two proteins of interest interact, then transcription of a reporter gene is activated. This method has become popular because it is an *in vivo* genetic system that allows for library screening using the bait protein fused to the Gal4 DNA binding domain. A similar system that replaces the Gal4 DNA binding domain with the *E. coli* LexA protein is also commonly used.

False positives can occur with the yeast two-hybrid system if one protein of interest activates transcription on its own. False negatives can also occur for reasons including low fusion expression, poor fusion stability, improper protein folding that makes the interaction domain inaccessible, and failure of the fusion to locate to the nucleus. Designing clones that express small domains can avoid some of these problems (Bartel *et al.*, 1993).

GST fusion interaction assays involve the construction of GST fusions with the proteins of interest using a pGEX vector or derivative. This system uses the protein purification system developed by Smith and Johnson (1988), where GST fusion proteins produced in *E. coli* can be purified in nondenaturing conditions taking advantage of the affinity of GST for glutathione. GST fusion interaction experiments use the partially purified GST fusion proteins bound to glutathione-Sepharose or agarose, and add a test protein, either in a cell lysate or from an *in vitro* translation. If the two proteins interact, the test protein will be copurified. One advantage of this method is that it is possible to produce large quantities of the fusions in *E. coli*, which increases the likelihood of detecting weak interactions. Disadvantages include the difficulties of expressing some fusions and the possible absence of essential post translational modifications in *E. coli*.

2. Identification of the Cleavage Site Recognized by the Turnip Yellow Mosaic Virus Protease

2.1 Introduction

The viral proteins required for the replication of TYMV are encoded by ORF-206 (Fig. 1.1). The 206 kDa protein encoded by ORF-206 contains methyltransferase-, helicase- and polymerase-like domains, and is autocatalytically processed into p141 and p66 (previously known as p150 and p70) by its own protease domain (Fig. 1.1). In order to better characterize the ORF-206 proteins and their role in *cis*-preferential replication, it was important to identify the peptide bond that is cleaved by the protease to form p141 and p66. Knowledge of the cleavage site has been used in the designing of clones used to investigate protein-protein interactions (Chapters 4 and 5).

Previous work has identified the probable active site residues of the protease as C^{783} and H^{869}, helping to classify the protease as a papain-like protease (Bransom and Dreher, 1994). Experiments using triple amino acid mutations across the sequence 1253-GPKLNGATP-1261 of p206 have suggested that cleavage occurred within this sequence (Bransom *et al.*, 1991), but the exact cleavage site is unknown.

Attempts at N-terminal sequencing *in vitro* translated p66 labeled with [^{3}H]proline have been unsuccessful. In this study, we hoped that [^{35}S]methionine, with its high specific activity, would provide a better label for sequencing. Because of the lack of methionine residues in the N-terminal region of p66, oligonucleotide-directed mutagenesis was used to change the underlined residues to methionines in the wild-type p206 sequence 1257-NGATPSASPTHR-1268 (performed by Kay Bransom). Since it had been noted that small deletions in p66 resulted in higher yields of *in vitro* translated p66 (Kay Bransom, unpublished observation),

an in-frame deletion of codons 1331-1468 was also introduced to create pTYMC-MetΔ66. Δ66 protein labeled with [^{35}S]methionine was subjected to sequential Edman degradation and the released amino acids from each cycle were collected and analyzed for radioactivity (insufficient material was present for mass detection). The peak fractions corresponded to the two methionine residues, and were used to deduce the TYMV protease cleavage site.

2.2 Methods

2.2.1 Preparation of transcripts

300 ng of linear plasmid DNA (pTYMC or derivative) was used to make RNA transcripts using T7 RNA polymerase. The 30 μl reactions contained 80 mM Hepes-KOH, pH 7.5, 12 mM magnesium chloride, 2 mM spermidine, 2.9 mM NTPs, 9 mM dithiothreitol, and 50 units T7 RNA polymerase. The samples were incubated at 37°C for 30 minutes, then 50 more units of T7 RNA polymerase were added, and the incubation continued another 30 min. After treatment with *E. coli* deoxyribonuclease I, the transcripts were phenol extracted, ethanol precipitated and dissolved in 50 μl water.

2.2.2 *In vitro* translation

Transcript RNA (30 ng/μl) was translated at 30°C for 90 minutes in a rabbit reticulocyte lysate (Promega), in the presence of 0.33-0.66 μCi [^{35}S]methionine/μl, 25 mM Hepes-KOH, pH 7.2, 40 mM potassium chloride, 0.1 M potassium acetate, 0.5 mM magnesium acetate, 0.1 mM spermidine, 57 μg/ml bovine liver tRNA, and 50 μM each amino acid except methionine. Reaction volumes were 60 μl.

2.2.3 Protein sequencing

The proteins of *in vitro* translations of mutant transcripts were separated by 0.1% SDS/10% PAGE and transferred to polyvinylidene difluoride membrane (PVDF, DuPont) using a semi-dry electrophoretic transfer apparatus (Semi-phor, Hoefer Scientific Instruments) with a minimal amount of transfer buffer containing 20 mM Tris-base, 150 mM glycine and 20% methanol. The band corresponding to the Δ66 protein (51 kDa) was cut out of the PVDF membrane and was subjected to N-terminal sequencing cycles in an Applied Biosystems gas-phase analyzer (performed by Barbara Robbins, Central Services Laboratory, the Center for Gene Research and Biotechnology). The fractions were collected and counted in a liquid scintillation counter (Packard Tri-Carb).

2.3 Results and Discussion

Previous to protein sequencing, it was determined that the methionine substitutions introduced in the N-terminal region of p66 did not interfere with viral replication or infectivity (Bransom *et al.*, 1996). RNA transcripts of the methionine mutant genome (TYMC-Met) and of wild type TYMC were inoculated onto turnip plants as previously described (Weiland and Dreher, 1989) and both produced typical signs of infection by 5-6 days. The yield of virions from methionine-mutant infected leaves was similar to that of TYMC-infected leaves. RT-PCR was used to amplify mutant genomes purified from the infected leaves, and the product was sequenced to confirm the stability of the two methionine mutations.

Once the methionine substitutions had been shown to be stable and not to affect the proteolytic processing of p206 (as determined by analysis of *in vitro* translation products), a large

scale (60 µl) *in vitro* translation of the pTYMC-MetΔ66 genome was performed. The Δ66 protein (51 kDa) was subjected to sequential Edman degradation, and the fractions were analyzed for radioactivity (Fig. 2.1). Analysis of two independently prepared samples indicated the release of [^{35}S]methionine in fractions 6 and 8. From this data, the cleavage site was deduced to be between amino acids A^{1259} and T^{1260} and the molecular weights of the two released proteins were calculated at 140,618 and 66,036 Da. The cleavage site of the TYMV protease has been confirmed by the work of Kadare *et al.* (1995).

The sequence around the cleavage site is as follows (with cleavage occurring between P1 and P1':

P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'
G	P	K	L	N	G	A	T	P	S

With the cleavage site identified, the results of previous studies where amino acids were mutated to glycine or alanine in triplets (Bransom *et al.*, 1991), now indicate that changes in the amino acids at and immediately upstream of the cleavage site are the changes that will most likely reduce or prevent cleavage. Substitution of LNG (P4 to P2) with AAA resulted in no detectable cleavage by the protease, and the substitution of ATP (P1 to P2') with GAA resulted in a partial loss of cleavage. The cleavage site preferences of other papain-like viral proteases also indicate the strongest sequence requirements are at P1 and P2, although substitutions by related amino acids can be permissible (Carrington and Herndon, 1992; Strauss and Strauss, 1994).

TYMV is the first tymovirus for which the exact protease cleavage site is identified and it was hoped that sequence comparisons would allow predictions for cleavage sites in other tymoviruses. However, the sequences of tymoviruses are divergent around the cleavage site and do not allow for clear predictions at

this time. Direct cleavage site determinations will be required to learn how broad a range of sites is recognized by tymoviral proteases.

The TYMV protease cleavage site was considered in the design of pAS1-66, pACTII-66 (Chapter 4), and pGEX-66 (Chapter 5).

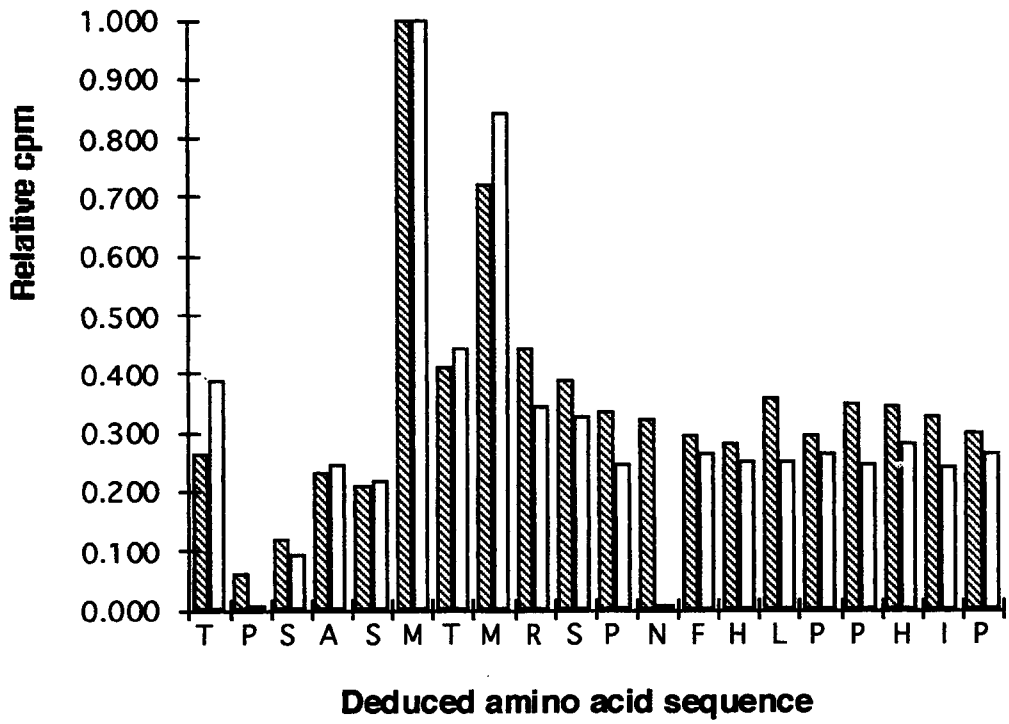


Figure 2.1. Analysis of N-terminal sequence of the internally deleted 66 kDa protein. The graph shows the relative levels of [^{35}S]methionine (relative to cycle 6, taken as 1.0) recovered from sequential cycles of Edman degradation completed by an automated protein sequencer on two independent samples. The recovery of [^{35}S]methionine in two peaks separated by one cycle identifies the methionine residues introduced into p206 at positions 1265 and 1267 and permits identification of the N-terminal residue of the deletion derivative of the 66 kDa protein. The deduced amino acid sequence for cycles 1-20 is indicated on the bottom.

3. TYMV Protein Interactions: Coimmunoprecipitation Results

3.1 Introduction

As described in Chapter 1, complementation experiments with TYMV revealed that its replication is *cis*-preferential. Most non-independently replicating genomes could not be efficiently rescued by a helper genome, as determined by the accumulation of genomic RNA in turnip protoplasts (Weiland and Dreher, 1993). There were some mutant TYMV genomes, however, that were able to accumulate to high levels when coinoculated with a genome containing a point mutation in the helicase active site (GCGST). These mutant genomes included ΔSP-G which could accumulate to 49% and Δ4085-4587 which could accumulate to 21% compared to wild type (Fig. 1.2). These two genomes contained large deletions in the p66 coding region. It was postulated that the ability of these deleted genomes to replicate *in trans* related to the absence of the usual *cis*-interactions proposed between viral proteins. According to a proposed model, the newly synthesized replication proteins, p141 and p66, would associate with one another and with their template (Fig. 1.3). The deletions in p66 disrupt the protein-protein interaction, and the proteins would then be available *in trans* (Weiland and Dreher, 1993). The goal of this study was to obtain physical evidence for the hypothetical interaction between p66 and p141.

The approach used was to perform immunoprecipitations on *in vitro* translated TYMV RNAs. If p66 and p141 form a complex, immunoprecipitations using antibodies against one protein would be expected to coprecipitate the second protein. The objectives of these experiments were to first determine if p141 and p66 form a complex *in vitro*, and then to attempt to map the interaction domain in p66 using nested in-frame deletion mutants. This approach was

successful in detecting an interaction between the two BMV replication proteins, and in mapping the interaction domain (Kao *et al.*, 1992a and b).

The IgG fractions of three antisera directed against synthetic peptides were available for use. N-206 is directed against the sequence VPEGHEAGSYNOPSDA at the N-terminal region (methyltransferase domain) of p206, C-141 is directed against the sequence ANGLVALYTSRSGV at the C-terminal region of p141, and C-206 is directed against the sequence AESELLHYVQ at the C-terminus of p206 (Fig. 3.1). It was hoped that immunoprecipitations using each of these antibodies would coprecipitate other protein(s) involved in the proposed complex.

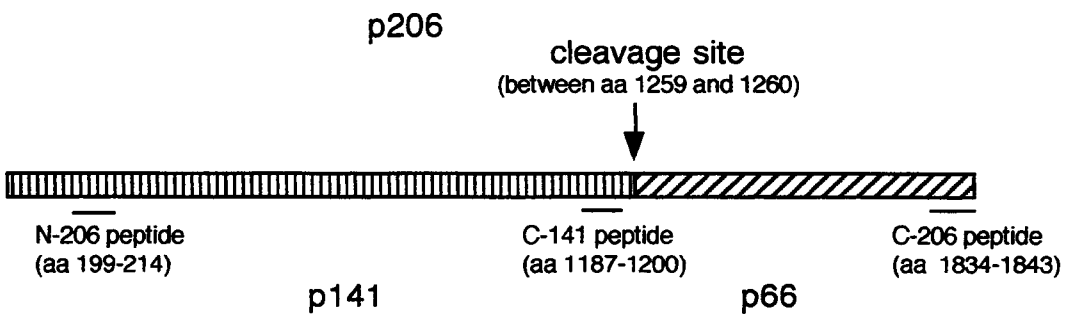


Figure 3.1. Epitopes recognized by ORF-206 specific antiserum. This diagram of p206 shows the protease cleavage site and the locations of the amino acid sequences corresponding to the synthetic peptides used in antibody production.

3.2 Methods

3.2.1 Preparation of transcripts

300 ng of linear plasmid DNA (pTYMC or derivative) was used to make RNA transcripts using T7 RNA polymerase. The 30 µl

reactions contained 80 mM Hepes-KOH, pH 7.5, 12 mM magnesium chloride, 2 mM spermidine, 2.9 mM NTPs, 9 mM dithiothreitol, and 50 units T7 RNA polymerase. The samples were incubated at 37°C for 30 minutes, then 50 more units of T7 RNA polymerase were added, and the incubation continued another 30 min. After treatment with *E. coli* deoxyribonuclease I, the transcripts were phenol extracted, ethanol precipitated and dissolved in 50 μl water.

3.2.2 *In vitro* translation

Transcript RNA (30 ng/μl) was translated at 30°C for 90 minutes in a rabbit reticulocyte lysate (Promega), in the presence of 0.33-0.66 μCi [^{35}S]methionine/μl, 25 mM Hepes-KOH, pH 7.2, 40 mM potassium chloride, 0.1 M potassium acetate, 0.5 mM magnesium acetate, 0.1 mM spermidine, 57 μg/ml bovine liver tRNA, and 50 μM each amino acid (except methionine). Reaction volumes were typically 15 to 60 μl.

3.2.3 Assay for label incorporated into protein

The amount of [^{35}S]methionine incorporated into protein during *in vitro* translations was determined by trichloroacitic acid (TCA) precipitation. Aliquots (1.5 μl) of the *in vitro* translation reactions were spotted on filter paper and allowed to dry. The filter paper was then boiled in 10% TCA for ten min, rinsed twice with 5% TCA, twice with 95% ethanol, and once with acetone. The filter paper was then air dried, added to scintillation fluid, and counted in a liquid scintillation counter (Packard Tri-Carb). Typical readings were between 20,000 and 100,000 cpm/μl translation reaction.

3.2.4 IgG purification

The antipeptide antisera were first de-salted by adding 1 ml serum volumes to NAP 10 columns (Pharmacia Biotech). The serum was eluted with 1.5 ml application buffer (20 mM Tris-HCl, pH 8.0, and 28 mM sodium chloride). The desalted serum was added to a DEAE Affi-blue gel column (Bio-Rad), and eluted in 20 ml application buffer, collecting 6 fractions of approximately equal volumes. The concentration of IgG in each fraction was determined by reading the absorbance at 280 nm and converting to IgG concentration using the relationship $A_{280} = 1.35$ for 1 mg/ml IgG (Harlow and Lane, 1988). The three most concentrated fractions were pooled and used in the subsequent experiments. The pooled IgG fractions represent 1:6 dilutions of the original sera.

3.2.5 Enzyme immunoassay

A 20 μg/ml peptide solution in PBS (1.8 mM potassium phosphate, 10 mM sodium phosphate, pH 7.2, 140 mM sodium chloride, 2.7 mM potassium chloride) was used to coat the wells of a polystyrene microtiter plate. Fifty μl of the solution was added to each well, and the plate was covered and incubated overnight at 4° C. The plate was washed twice with wash buffer (PBS containing 0.05% Tween 20), and then the wells were filled completely with PBS containing 2% BSA. The plate was incubated at room temperature for two hours, and then washed twice with wash buffer (Fuller *et al.*, 1988).

The antiserum to be tested was diluted 1/100, 1/400, 1/1600 and 1/6400 in diluting buffer (PBS containing 0.25% BSA, 0.05% Tween 20, and 0.02% sodium azide). Fifty μl of diluted serum was added to each well, and the plate was incubated at room temperature for 2 hours, followed by four washes with wash buffer (Fuller *et al.*, 1988).

Fifty µl of a 1/1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antiserum in diluting buffer was added to each well. The plate was again incubated at room temperature for 2 hours, followed by 4 washes with wash buffer, and two with substrate buffer (10 mM diethanolamine, pH 9.5, 0.5 mM magnesium chloride). Fifty µl of a 0.1% solution of p-nitrophenyl phosphate in substrate buffer was added to each well, and the plate was incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 50 µl of 0.1 M EDTA to each well (Fuller *et al.*, 1988) and the plate was read at 405 nm.

3.2.6 Immunoprecipitations

Between 2 and 5 µl (100,000-150,000 incorporated cpm) of the *in vitro* translations were diluted to 80 µl with cold L buffer (50 mM Tris-HCl, pH 7.4, 50 mM potassium chloride, 15 mM magnesium chloride, 1 mM dithiothreitol and 10% glycerol) in 1.7 ml tubes. Twenty-five µl of a 50% slurry of freshly washed Protein A-Sepharose (Sigma) in L buffer was added to each, and the tubes were rotated at 4°C for 10 min. The tubes were then centrifuged for 90 seconds at 2040 x g. After this pre-adsorption step, the supernatants were transferred to new tubes containing 1 µl of antiserum or 5 µl of a purified IgG fraction, and rotated 2-5 hours at 4°C. The tubes were then spun briefly, 40 µl of the Protein A-Sepharose slurry was added to each, and the rotation at 4°C was continued overnight.

The Sepharose beads were washed twice with 500 µl cold L buffer, once with 500 µl cold 500 mM lithium chloride, 20 mM Tris-HCl, pH 7.4, and twice again with 500 µl cold L buffer (Kao *et al.*, 1992a). Ten µl 2X Laemmli buffer (31 mM Tris, pH 6.8, 4% SDS, 20% sucrose, 0.01% bromphenol blue, 10% β-mercaptoethanol) was added to each tube and the tubes were boiled for 5 min. The

dissociated proteins were analyzed by 0.1% SDS/10% PAGE (Laemmli, 1970).

Except where noted, the L buffer included 0.05% SDS and 0.5% Triton X-100.

3.2.7 Immunoprecipitation analysis

A phosphorimager and integration software (Image Quant, Molecular Dynamics) was used to determine the relative amounts of the ORF-206 proteins present in the immunoprecipitations. Some of the protein bands were close together on the SDS-PAGE, but chromatograms of each lane made it possible to determine the peaks corresponding to each band. The ratio of p141/p66 corrected for the number of methionines in each protein was determined. The values for the ratios were similar whether peak height or peak area were used in the calculations.

3.3 Results and Discussion

3.3.1 Coimmunoprecipitations with different antisera

Ideally, the immunoprecipitations would have been performed in nondenaturing conditions so that protein complexes would only be minimally disturbed. It was determined, however, that SDS and Triton X-100 were required in the immunoprecipitation buffer in order to prevent nonspecific binding of the proteins to the Protein A-Sepharose. As shown in Figure 3.2 lanes 2-4, immunoprecipitations of *in vitro* translations of TYMV transcripts using preimmune sera precipitated both p141 and p66 when the buffer did not contain both SDS and Triton X-100. Note that these antibodies are specific when detecting SDS-denatured

proteins (Connie Bozarth, unpublished). Other conditions tried in order to reduce non-specific binding included adding 2% BSA in the immunoprecipitation buffer, extending the wash times to 10 min each, and using Protein A-acrylic beads (BioRad) instead of Sepharose. None of these variations reduced the non-specific binding (data not shown). The buffer used in all subsequent immunoprecipitations contained 0.05% SDS and 0.5% Triton X-100. While these conditions are not ideal for the study of protein-protein interactions, they were necessary in this case and in other studies where immunoprecipitation were successfully used to identify protein-protein interactions (Hartley *el al.*, 1995; Horisberger, 1992).

Immunoprecipitations using the C-206 antiserum precipitated p206 and p66, which contain the epitope recognized by the C-206 antibodies, and to a lesser degree, p141 which does not (Fig. 3.2, lane 7). Since the C-206 antiserum does not immunoprecipitate p141 from *in vitro* translations of transcripts missing the coding region for the C-206 epitope (ΔSP-W, Fig. 3.2, lane 14), it was concluded that the p141 in Fig. 3.2, lane 7 was coprecipitated through its interaction with p66 and/or p206.

Immunoprecipitations using the N-206 or C-141 antisera precipitated p141, which contains both of the recognized epitopes, but precipitated p206 very poorly (Fig. 3.2, lanes 8-13). Both of these antisera had been shown in an immunoassay to contain antibodies that recognized the synthetic peptides they were directed against (data not shown) so it was surprising that neither could efficiently precipitate p206. Possibly both epitopes are inaccessible in the full length protein. No p66 was detected in immunoprecipitation using either of the antisera (Fig. 3.2, lanes 10 and 13), but the amounts of p141 precipitated were fairly low. Even if every p141 molecule precipitated was associated with a p66 molecule, the amount of p66 coprecipitated would likely be undetectable because of its smaller molecular weight and lower methionine content compared to p141. It is also possible that if p66

associates with p206, it was not coprecipitated with either the N-206 or C-141 antisera because of the lack of precipitated p206.

Another potential problem is that some or all of the epitopes are inaccessible in a protein complex. If this is the case, then the use of polyclonal antibodies directed against full length or domains of the proteins would be more likely to precipitate the complexes. Such antisera are unavailable at present.

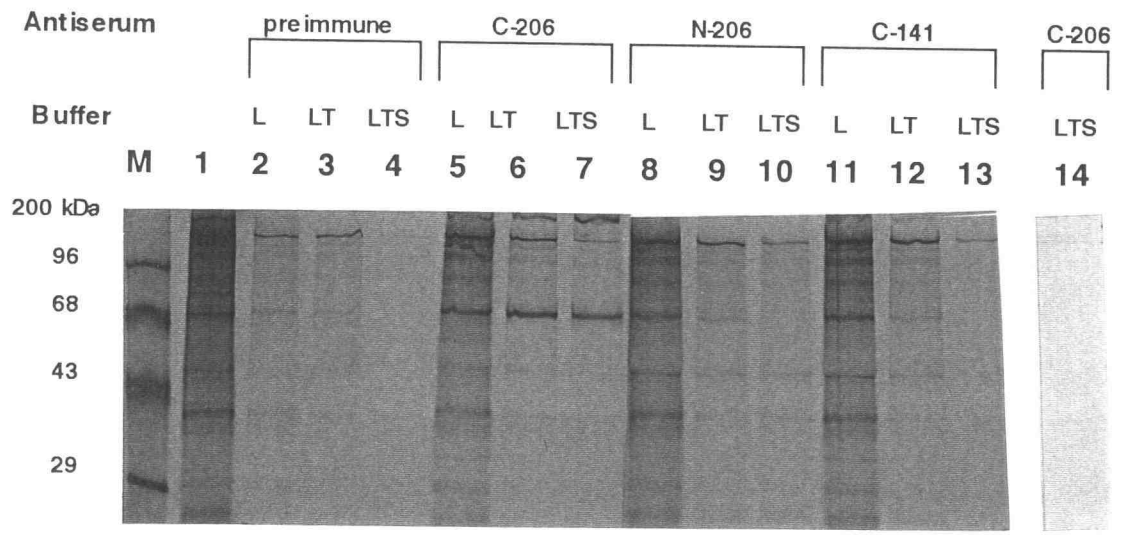


Figure 3.2. Immunoprecipitations of TYMC *in vitro* translations. Lane 1 represents 1 μl of the [^{35}S]methionine-labeled *in vitro* translation products of TYMC transcripts. Lanes 2-13 are immunoprecipitations of 5 μl of translation products directed by TYMC transcript RNA, using either preimmune serum (lanes 2-4), C-206 antiserum (lanes 5-7), N-206 antiserum (lanes 8-10) or C-141 antiserum(lanes 11-13). L buffer (L) was used in the immunoprecipitations in lanes 2, 5, 8 and 11, L buffer with 0.5% Triton-X 100 (LT) was used in lanes 3, 6, 9 and 12, and L buffer with 0.5% Triton-X 100 and 0.05% SDS (LTS) was used for the immunoprecipitations in lanes 4, 7, 10 and 13. Lane 14 is an immunoprecipitation of 5 μl of a ΔSP-W translation using C-206 antiserum and in the presence of LTS buffer.

3.3.2 Is the 3' end of the TYMC genome required for the proposed protein complex formation?

The model for *cis*-preferential replication envisions the involvement of the 3' untranslated region of the genome in either protein complex formation or stability. For BMV, it has been found that the 3' and intercistronic noncoding regions of RNA3 are required, along with the replication proteins, for the formation or the stability of active replication complexes in yeast (Quadt *el al.*, 1995). Since pTYMC/*Hind* III produces TYMC transcripts with an extra few bases on the 3' end (ACGU) not found in the genomic RNA, it was important to determine whether these extra bases would interfere with protein complex formation detected as a change in the p141/p66 ratio of the immunoprecipitations. This could occur if complex formation required an aminoacylated 3' end. As can be seen in Fig. 3.4, the immunoprecipitations of the translation products of TYMC and TYMC virion RNA are very similar, and the p141/p66 ratios are 0.28 and 0.30 respectively. So even though the additional bases on the TYMC transcript interfere with aminoacylation of the 3' end, this does not appear to affect protein complex formation in this assay.

The Δ3' construct, linearized at *Sma* I[6061] and therefore missing 257 nucleotides from the 3' end of the genome, was also used in immunoprecipitation experiments to determine the importance of the 3' end of the TYMV genome in the formation or stability of the proposed protein complex. The transcripts made from this construct lack the 3' untranslated region of the genome, which includes the tRNA-like structure. The p141/p66 ratio for the Δ3' immunoprecipitation was 0.25, similar to 0.28, the ratio for TYMC (Table 3.1). This result indicates that the 3' end of the TYMV genome, which includes the tRNA-like structure, does not affect the p141/p66 ratio and therefore does not appear to be involved in the formation of the putative p141/p66 or p141/p206 complex *in vitro*.

3.3.3 Mapping the interaction domain in p66

Since the immunoprecipitations using the C-206 antiserum provided some evidence for an interaction between p141 and p66/p206, this antiserum was used in subsequent experiments in an attempt to map an interaction domain in p66. Figure 3.3 diagrams ORF-206 for TYMC and the mutant genomes used in this study. The clones from which these RNAs were transcribed were constructed by Kay Bransom and David Gilmer. The ΔSP-G construct, containing a deletion between nucleotides 4086 and 5422 in the p66 coding region, allowed for efficient *trans* replication in turnip protoplasts (Weiland and Dreher, 1993). Since this deletion was proposed to disrupt the postulated interaction between p141 and p66, the other mutant genomes used in this study contain smaller in-frame deletions between nucleotides 4086 and 5422.

The objective was to perform immunoprecipitations on the *in vitro* translations of mutant transcripts, and determine if any of the deletions resulted in a loss or decrease in the amount of coprecipitated p141. Figure 3.4A shows the *in vitro* translation products of mutant and wild type transcripts and Figure 3.4B shows the results of their immunoprecipitations with the C-206 antisera using buffer containing SDS and Triton X-100.

The p141/p66 ratios for the immunoprecipitations were determined as described in the Methods section. The p141/p66 ratio for TYMC was calculated at 0.28, which was similar to that of many of the deletion mutants. The ratios for the deletion mutants , ΔSP-G, Pst 4276 and ΔMP 4708 were estimated to be 0.087, 0.089 and 0.12 , respectively, about three times smaller than the ratio for TYMC. The lower ratios indicate that less p141 was coprecipitated with these constructs. These results suggest that the common domain missing in the three mutants, in the C-terminal half of p66, could be involved in the proposed protein-protein interaction.

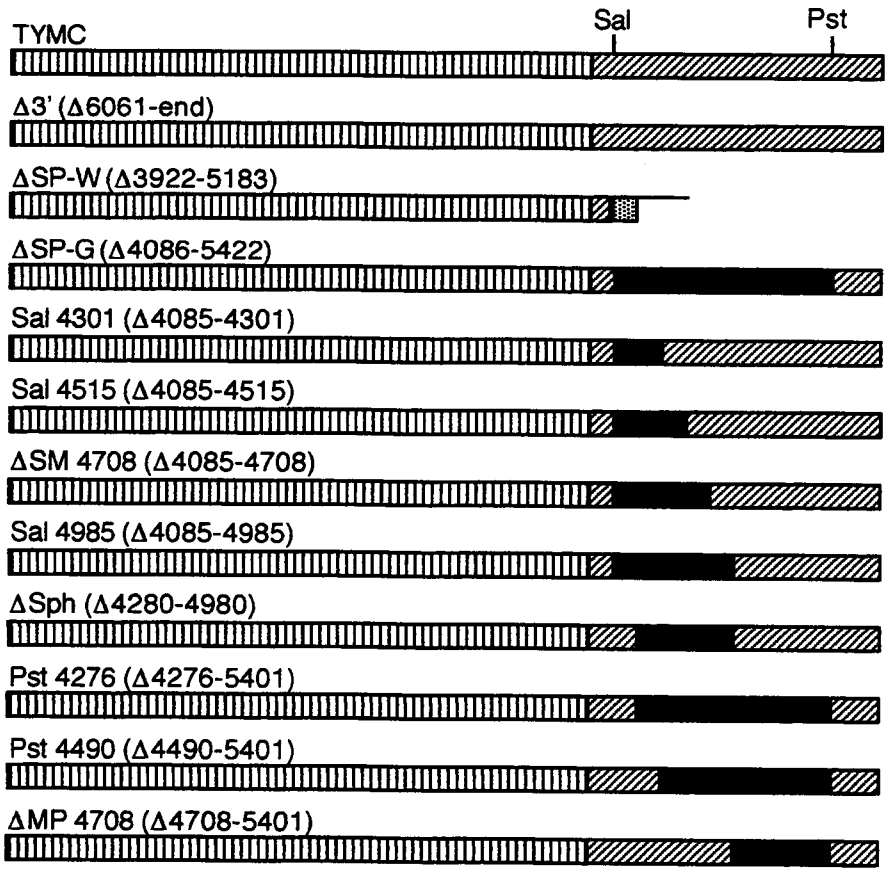


Figure 3.3. ORF-206 of TYMC and the modified genomes used in this study. Δ3' contains a wild type ORF-206 but the genome is linearized at *Sma* I[6061] and lacks the tRNA-like structure on the 3' end. ΔSP-W contains a frameshift mutation and translation of this genome produces a truncated version of p206 that is missing the C-206 epitope. ΔSP-G has the region between nucleotides 4086 and 5422 deleted. The other deletion mutants are nested in-frame deletions within nucleotides 4086 and 5422.

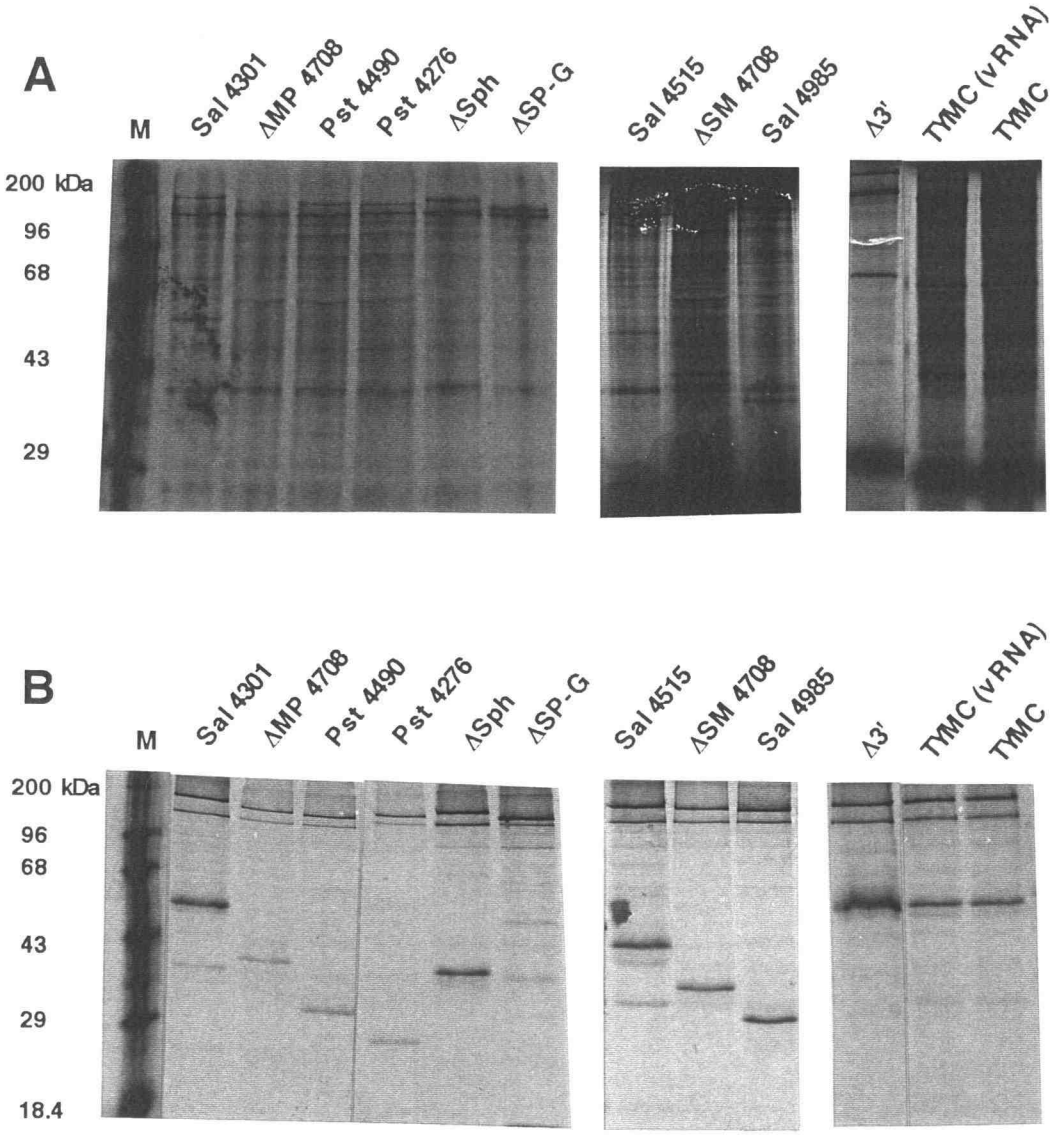


Figure 3.4. Autoradiograph of deletion mutant translations and immunoprecipitations. Transcripts of the constructs in Fig. 3.3 were translated *in vitro* as described in the Methods section. The translation products are shown in panel A, and the immunoprecipitations are shown in panel B. The L buffer used for the immunoprecipitations contained both 0.5 % Triton-X 100 and 0.05 % SDS. TYMC vRNA indicates that virion RNA was used in the translation. Proteins were separated by 0.1% SDS/10% PAGE.

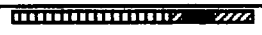
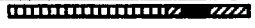
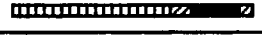
Construct	Deletion size in amino acids	# of met in p141	# of met in p66	p141/met p66/met (average +/- 1 SD)	p141/p66 ratio relative to that of TYMC	*relative co-replication in protoplasts
TYMC 	0	19	7	0.28 +/- 0.11	1.0	1.0
TYMC (virion) 	0	19	7	0.30 +/- 0.016	1.1	1.0
Δ3' 	0	19	7	0.25	0.89	NA
ΔSP-G 	445	19	2	0.087 +/- 0.017	0.31	0.54
Sal 4301 	72	19	7	0.29 +/- 0.013	1.0	0.12
Sal 4515 	143	19	7	0.36 +/- 0.14	1.3	0.37
ΔSM 4708 	208	19	6	0.33 +/- 0.074	1.2	0.37
Sal 4985 	300	19	4	0.19 +/- 0.014	0.68	0.44
ΔSph 	233	19	5	0.27 +/- 0.092	0.96	0.16
Pst 4276	375	19	2	0.089 +/- 0.026	0.32	0.43
Pst 4490 	304	19	2	0.12 +/- 0.044	0.43	0.24
ΔMP 4708 	208	19	3	0.18 +/- 0.052	0.64	0.49

Table 3.1. The number of methionines in p66 and p141, the size of the deletion and the ratio of p141/p66 in the immunoprecipitation are recorded for each construct. The immunoprecipitations were repeated at least twice for all of the constructs except Δ3' which was only performed once. The calculated ratios were determined using peak heights as described in the Methods section. The relative replication as compared to wild type was recorded for each construct. Coreplication was measured by Northern analysis of turnip protoplasts coinoculated with the mutant transcripts indicated and transcripts of a complementation partner (GCGST) containing a point mutation in the helicase active site. *Data of Kay Bransom.

Two of the deletion mutants, Sal 4985 and ΔMP 4708, have p141/p66 ratios (0.19 and 0.18 respectively) that are lower than that of TYMC, but not as low as the ratios for Pst 4276, Pst 4490 and ΔSP-G. All the other deletion mutants (Sal 4301, Sal 4515, ΔSM 4708. and ΔSph), have ratios similar to that of TYMC. The result of ΔMP 4708, with its lower than wild type p141/p66 ratio, is in agreement with the findings from the ΔSP-G, Pst 4276 and ΔMP 4708 genomes that indicated the C-terminal half of p66 contains a possible interaction domain. The deletion in Sal 4985 (Δ4085-4985) is very similar to the deletion in ΔSph (Δ4280-4980) but their ratios are different (0.19 and 0.27 respectively). The 195 nucleotides that are present in ΔSph but missing from Sal 4985, are also missing in a number of the other mutants (Sal 4301, Sal 4515, and ΔSM 4708) that have ratios similar to TYMC, so it is unlikely that the nucleotides correspond to an interaction domain. Lower recovery of p141 could be due to overall structural changes in p66 caused by the Δ4085-4985 deletion.

A factor that complicates the deletion mapping is that some of the p141 in the immunoprecipitations could be present due to its non-specific binding to the Protein A-Sepharose and not necessarily due to its specific interaction with p66/p206. Although the non-specific binding of p141 to the Protein A-Sepharose was greatly reduced by the addition of Triton X-100 and SDS to the immunoprecipitation buffer, some p141 did continue to bind non-specifically (Fig. 3.2, lane 4). The small amount of p141 that binds non-specifically to the Protein A-Sepharose would be expected to be similar in all of the immunoprecipitations of mutant genomes. However, since the amount of p141 coprecipitated was relatively small, especially with some of the deletion mutants, any variations in the amount of p141 in the immunoprecipitations due to non-specific binding could skew the p141/p66 ratios.

It is also possible that since none of the deletions resulted in a complete loss of the coprecipitation of p141, only a lowering of p141/p66 ratio compared to wild type, p66 regions outside the

deletion between nucleotides 4086 and 5422 could be involved in the protein-protein interaction.

Table 3.1 also contains the results of coreplication experiments performed by Kay Bransom. These complementation experiments in turnip protoplasts used the mutant transcripts together with a complementing transcript that contains a point mutation in the helicase active site (GCGST) as inocula. It was hoped that these complementation experiments would help map a domain whose absence promotes trans-replication, and that this phenomenon could be correlated to the postulated protein-protein interaction domain of p66. Genomes with deletions that corresponded to an interaction domain would be expected to be efficiently rescued by the GCGST genome. It was hoped that the results of these experiments would lend support to the immunoprecipitation results that suggest the involvement of the C-terminal region of p66 in an interaction with p141/p206. However, the coreplication results show that any deletion in the p66 coding region resulted in an increase in the accumulation of genomic RNA as detected by Northern analysis, indicating complementation with the GCGST genome. As described in Chapter 1, coreplication experiments with GCGST transcripts and transcripts containing a polymerase point mutation (RDD), resulted in replication levels of 2% compared to wild type (or 0.02 relative replication). The data summarized in Table 3.1 show that the smallest deletion in p66 (Sal 4301) resulted in an increase in the relative replication from 0.02 to 0.12. ΔSP-G, which contains the largest deletion, could accumulate to levels up to 0.54 relative to wild type.

It is possible that many of the deletions in p66 result in improper folding of the protein that distorts the interaction domain and hinders the ability of p66 to interact with p141. This could explain the coreplication experiments where all the deletion mutants were more efficiently rescued than was the RDD mutant. Although most of the immunoprecipitation data does not support the idea that the deletions disrupt proper protein folding (many of the deletion mutations had p141/p66 ratios similar to TYMC), the

difference in the ratios between the Sal 4985 and ΔSph genomes, even that the deletions are very similar, could be explained by potential differences in the folding of the mutant p66 proteins.

Another explanation for the coreplication results is that the proposed interaction domain of p66 is formed by the tertiary structure of the protein, such as with the SH2 and SH3 domains of Src (Yu *et al.*, 1992) where a hydrophobic pocket is formed on the surface of the protein. If this was the case, the interaction domain would not map as one domain detected in the protein's primary and secondary structure. Any deletion could potentially affect the protein's conformation and weaken its interaction with p141. It is also possible that there are multiple interaction domains in p66.

3.3.4 Conclusions

The immunoprecipitation data gave some indication that p141 and p66/p206 could form a complex *in vitro*. The attempt to map the interaction domain with immunoprecipitations of the translation products of deletion mutants led to the conclusion that the C-terminal half of p66 may be involved in the protein-protein interaction. The immunoprecipitation results could be misleading, however, due to variations in the small amounts of p141 that bind nonspecifically to the Protein A-Sepharose, and to potential problems the deletions cause in protein folding. The coreplication experiments where all deletions resulted in increased *trans* replication, do not lend support to the involvement of any particular domain in an interaction. As discussed in Chapter 1, it is important to use more than one method when investigating protein-protein interactions. Since the immunoprecipitation data gave some indication of an interaction between *in vitro* translated p141 and p66/p206, a yeast two-hybrid system was used next to verify the interaction.

4. TYMV Protein Interactions: Yeast Two-Hybrid Results

4.1 Introduction

In the yeast two-hybrid system (Fields and Song, 1989), the ability of two hybrid (chimeric or fusion) proteins to interact correlates with the transcriptional activation of a reporter gene. This system is commonly used to search a library of fusion proteins for ones that interact with a bait protein. In this study it was used to determine if two known proteins interact. In contrast to the *in vitro* immunoprecipitations described in Chapter 3, the yeast two hybrid system is an *in vivo* genetic method. It was hoped that the study would provide data in support of the immunoprecipitation data indicating that p141 and p66/p206 could form a complex *in vitro*.

The *E. coli*/yeast shuttle vectors used in this study are shown in Figure 4.1. pAS1 (Durfee *et al.*, 1993) is a 7.1 kb plasmid with a *TRP1* selectable marker (encodes *N*-(5'-phosphoribosyl)-anthranilate isomerase Plischke *et al.*, 1976 and can restore tryptophan prototrophy in a yeast host) and a 2 micron origin of replication. The plasmid contains an alcohol dehydrogenase 1 (*ADH1*) promoter followed by the coding region for the Gal4 DNA binding domain containing a nuclear localization signal, a hemagglutinin (HA) epitope and a multiple cloning site. These features allow for in-frame cloning into the multiple cloning site and the synthesis of Gal4 DNA binding domain fusion proteins in an appropriate yeast strain.

pACTII (Durfee *et al.*, 1993, modified by Stephen Elledge) is a 7.55 kb plasmid with a *LEU2* selectable marker (encodes 2-isopropylmalate dehydrogenase, Plischke *et al.*, 1976) and can restore leucine prototrophy in a yeast host. An *ADH1* promoter is followed by coding regions for a SV40 large T antigen nuclear localization signal, the Gal4 activation domain, and an HA epitope. Fusion proteins with the Gal4 activation domain can be created by in-frame cloning into the multiple cloning site and using an appropriate yeast strain.

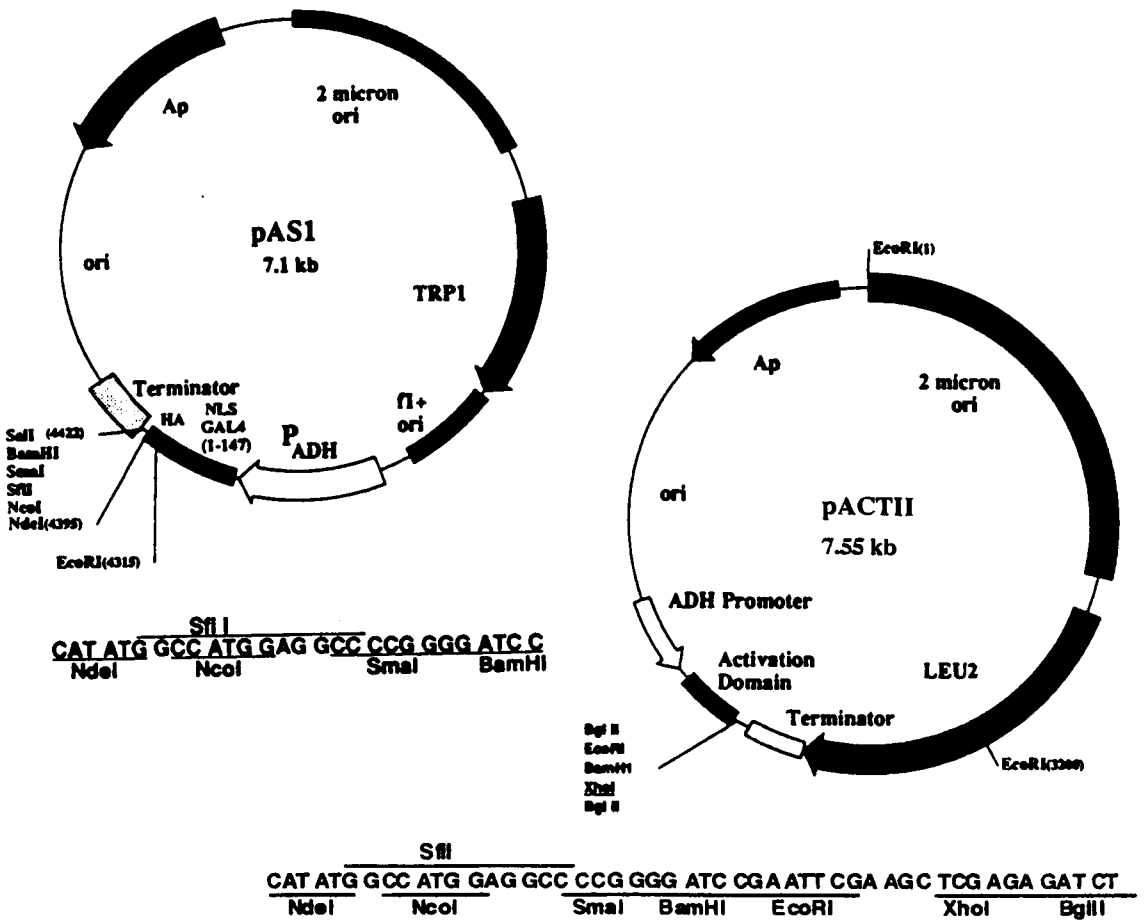


Figure 4.1. Diagrams of pAS1 and pACTII. These diagrams, adapted from Durfee *et al.* (1993), include restriction sites and sequences of the multiple cloning sites.

Both plasmids also contain ampicillin resistance genes and origins of replication that allow for cloning, selection and growth in *E. coli.*

The approach was to use pAS1 and pACTII to make fusion proteins with the ORF-206 proteins of TYMV and to test their ability to interact in the *Saccharomyces cerevisiae* strain Y166. If the fusion proteins interact, then the Gal4 DNA binding domain and the Gal4 activation domain would activate transcription of the chromosomal *lac* Z reporter gene of Y166.

4.2 Methods

4.2.1 Construction of plasmids used in the yeast two-hybrid study

Oligonucleotides were designed that, with pTYMC (or one of its derivatives) as template, allowed for the PCR synthesis of the inserts used in the construction of the plasmids employed in this study. The p66 insert was created by using the pTYMC-derived template pTYC H3-Nco (linearized), which contains an *Nco* I site at nucleotide 3864, the 5' wild type sequence oligonucleotide TYS+3825 (GTTCCCGCAACTCAAGCT) and the 3' oligonucleotide Xho-5610(GGGCTGATTGCCTCGAGACGTAGTGAAGCA) that creates an *Xho* I site at nucleotide 5627. The TYMV insert containing the conserved helicase domain was created by using the 5' oligonucleotide 2768+Nco(ATCAGAGCCATGGAGTCTTTCAAAGT) which creates an *Nco* I site at 2775, and the 3' oligonucleotide Xho-3857 (GCGCTGGGGCTCGAGCCGCCGTTGAGTTT) which creates an Xho I site at 3872. In the p141 insert, the wild type *Nco* I site at nucleotide 209 was eliminated and the *Nco* I site at nucleotide 93 was created by the synthesis of a mega primer (Sarkar and Sommer, 1990) using

the 5' oligonucleotide 84+Nco (GCAAATGAGCCATGGCCTTCCAATTAG) and the 3' oligonucleotide 199-NcoX (CTTCGGAATGGACCAGGGGTAGGTCTGT) that removed the wild type *Nco* I site without changing the amino acid sequence of the translated protein (the wild type codon CCA is changed to CCC; both code for proline). The resulting 110 base pair mega primer was used along with the 3' oligonucleotide Xho-3857 to create the full length p141 insert. The bases that are underlined in the above sequences indicate changes from the wild type sequence. The inserts were digested with *Nco* I and *Xho* I and ligated to pACTII cut with *Nco* I and *Xho* I, and to pAS1 cut with *Nco* I and *Sal* I (Fig. 4-2).

Both the helicase and the p141 inserts have a wild type *Xho* I site at nucleotide 3278. Complete digestion with *Nco* I, and partial digestion with *Xho* I followed by gel purification provided the full helicase insert. Complete digestion of the p141 insert with *Xho* I created the p141-XF insert. The nucleotide 3278-3872 *Xho* I fragment was ligated to pACTII-141-XF cut with *Xho* I to create pACTII-141. The p141 insert used to create pAS1-141 was provided by digestion of pACTII-141 with *Nco* I followed by a partial digest with *Xho* I (Fig. 4.2). The above ligation reactions were used to transform *E. coli* strain DH5α using standard molecular biology techniques (Maniatis *et al.*, 1988). The transformants were then screened for the presence of vectors containing the above inserts as determined by diagnostic restriction digests of the plasmid DNA.

4.2.2 Polymerase chain reaction (PCR)

PCR was used to synthesize the inserts used in the yeast two-hybrid constructs. Ten pmoles of each oligonucleotide and 100 ng of template were combined in 10 µl reactions containing 50 mM Tris, pH 8.5, 2 mM magnesium chloride, 500 µg/ml BSA, 0.2 mM dNTPs

and 0.5 units of Taq DNA polymerase (Promega). The samples were transferred to capillary tubes and placed in an Idaho Technologies air thermo-cycler. Typical temperature conditions were: Denaturation at 94°C for two minutes before cycling 30 times between 50-60°C for one second, 72°C for 20-150 seconds and 94°C for one second. Taq was the polymerase used for the p66 and helicase inserts. Because of the enzyme's proofreading capabilities, rTth DNA polymerase (Perkin-Elmer), was used at 1 unit per 10 µl reaction, and using an elongation temperature of 68° instead of 72°C, for the p141 3.8 kb insert synthesis

4.2.3 Yeast transformations

Strain Y166 is a *S. cerevisiae* strain with the genotype *MATa, gal4, gal80, his3, trp1-901 ade2-101, ura3-52, leu2-3, leu2-112, rnr3::GAL1--->URA3 [GAL1-lacZ].* Y166 was grown in 2-10 ml YEPD (2% peptone, 1% yeast extract, 2% glucose) overnight at 30°C. The culture (1.5 ml) was transferred to a microfuge tube, and centrifuged 16,000 x g for 20 seconds to pellet the cells. The supernatant was discarded and the pellet was resuspended in the residual medium. Between 1 and 5 µg of plasmid DNA, 50 µg single stranded carrier DNA and 500 µl of polyethyleneglycol solution (10 mM Tris-HCl, pH 7.5, 40% PEG 8000, 1 mM EDTA, 0.1 M lithium acetate) was added to the cells. The cells were incubated at 30°C for 15 min on a shaker, followed by incubation in a 42°C water bath for 10 min. Ethanol was added to 10% and the cells were incubated another 5 min at 42°C (Lauermann, 1991). The cells were then pelleted, resuspended in 200 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), plated on the appropriate dropout medium (medium lacking an essential amino acid) and incubated at 30°C for 3-5 days. The dropout media were composed of 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, and 0.15% of an amino acid mix that excludes leucine, tryptophan, histidine and uracil. A filter-sterilized

solution of uracil was added to a concentration of 50 mg/l, and leucine, histidine and tryptophan were added where appropriate to concentrations of 100 mg/l, 200 mg/l, and 50 mg/l respectively.

Transformations for the double transformants were done sequentially; ie. the yeast strain was transformed with one plasmid, and a single yeast colony was picked to liquid medium and grown overnight at 30°C before being transformed with the second plasmid. It was found unnecessary to grow the single transformants in selective liquid medium before performing the second transformations.

4.2.4 Recovery of plasmids from yeast

Yeast double transformants were grown overnight at 30°C in 2 ml of -leu,-trp dropout medium. The cultures were transferred to microfuge tubes and centrifuged 16,000 x g for 20 seconds to pellet the cells. The pellets were resuspended in the residual medium and then vortexed for 2 min with 200 µl lysis solution (10 mM Tris, pH 8.0, 2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA), 200 µl phenol/chloroform, and 1 scoop (approx. 0.3 g) 425-600 µm glass beads. The tubes were then centrifuged at 16,000 x g for 5 min. The supernatants were transferred to new tubes, and the DNA was precipitated with ethanol and then resuspended in 30 µl of TE with ribonuclease (Bartel *et al.*, 1993). Between 1 and 10 µl of the DNA was used to transform calcium chloride competent *E. coli strain* DH5α (Maniatis, 1988).

4.2.5 β-galactosidase assays

Yeast colonies were initially screened for β-galactosidase activity by a filter paper assay. A sterile 7 cm-diameter Whatman 1MM filter paper was placed on top of 4 day-old yeast colonies. A sterile needle was used to make three orienting holes in the paper and agar. The filter was then placed in liquid nitrogen, colony side up, for 5 seconds. The filter paper was then transferred to a 8 X 8 cm Whatman 3MM paper soaked in Z buffer (100 mM sodium phosphate, pH 7.0, 1 mM magnesium sulfate, 10 mM potassium chloride) containing 0.27% β-mercaptoethanol and 0.03% (v/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and incubated overnight at room temperature in a humid environment. Colonies producing β-galactosidase turn blue within this time (Bartel *et al.*, 1993).

β-galactosidase activity was quantified using the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG). Yeast transformants were incubated overnight at 30°C in the appropriate dropout medium. The optical density of the cultures at 600 nm was then measured, and 100 µl of each was transferred to 1.7 ml microfuge tubes. Seven hundred µl of Z buffer/β-mercaptoethanol, 50 µl chloroform, and 50 µl 0.1% SDS was added to each tube, and the tubes were vortexed for 30 seconds. One hundred and sixty µl of an ONPG solution (0.4% in Z buffer) was added, the tubes were mixed, and then incubated at 30°C for one hour. The reactions were stopped by the addition of 400 µl of 1 M sodium carbonate, and the tubes were centrifuged at 16,000 x g for 10 min. The OD_{420} was measured for the supernatants, and the β-galactosidase activity was calculated using the following equation from Bartel *et al.*, (1993):

$$\beta\text{-galactosidase units} = 1000 \times \frac{OD_{420}}{60 \text{ min} \times 0.1 \text{ ml} \times OD_{600}}$$

4.2.6 Analysis of fusion protein expression in yeast

Overnight cultures of Y166 single transformants were used to inoculate 5 ml of the appropriate dropout medium to OD_{600}≅0.15. These tubes were incubated with shaking at 30°C until the OD_{600}≅0.5 (about 6 hours). A total of 3 ml of each culture was centrifuged at 16,000 x g for 1 minute, and the pellets were resuspended in 50 µl of 2X Laemmli sample buffer (31 mM Tris, pH 6.8, 4% SDS, 20% sucrose, 0.01% bromphenol blue, 10% β-mercaptoethanol). The samples were frozen at -80°C, boiled for 5 min and then centrifuged briefly (Golemis *et al.*, 1996). Twenty µl of each sample were subjected to 0.1% SDS/10% PAGE (Laemmli, 1970) followed by semi-dry electrophoretic transfer (Semi-phor, Hoefer Scientific Instruments) to polyvinylidene difluoride membrane (PVDF, DuPont) using a minimal amount of transfer buffer containing 20 mM Tris-base, 150 mM glycine and 20% methanol.

The PVDF membrane was blocked for a minimum of 30 min at room temperature in MTBS which contains 20 mM Tris-HCl, pH 7.5, 0.5 M sodium chloride (TBS) and 5% Carnation instant non-fat milk. The blocking was followed by an incubation in a 1:1000 dilution of anti-HA monoclonal antibodies (Berkeley Antibodies) in MTTBS (MTBS with 0.5% Tween-20) for 2 hours. The membrane was then washed twice with TTBS (TBS with 0.5% Tween-20) followed by a 1 hour incubation in a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (BioRad) in MTTBS. The membrane was washed twice in TTBS and twice in TBS before detection of the peroxidase with ECL (Amersham) chemiluminescent reagents.

4.2.7 His3 assay

S. cerevisiae strain CG-1945 has the genotype *MATa ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542,*

gal80-538, cyh^{r}2, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3::GAL4$_{17\text{-}mers(x3)}$-CyC1$_{TATA}$-lacZ (Clontech, 1996) and was used for the screening of the ability of transformants to activate transcription of the *HIS3* gene. In order to determine the amount of 3-aminotriazole (3AT) required to counteract the low level of constitutive His3 expression, media lacking leucine, tryptophan, and histidine, and containing varying amounts of 3AT were used. The double transformant pAS1/pACTII was used as the negative control and pAS1-LEF5/pACTII-LEF5 was used as the positive control. LEF5 of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is a protein known to dimerize (Steve Harwood, personal communication). A colony of each control was suspended in separate tubes containing 50 µl of sterile water. A sterile loop was used to streak these suspensions on media containing varying amounts of 3AT; the plates were incubated at 30°C for three days and then analyzed for growth. It was determined that 3 mM was the minimal amount of 3AT that prevented the growth of the negative control. Media containing 3 mM 3AT were used to screen for the expression of His3 in selected double transformant combinations.

The His3 assay was performed on double transformants in a similar manner as above. Suspensions of yeast colonies were made in sterile water. Four suspensions of each double transformant, along with one positive and one negative control, were streaked onto the assay medium. The plates were analyzed for growth after incubation at 30°C for three days.

4.3 Results and Discussion

4.3.1 β-galactosidase assays

The fusion proteins made and screened in this study are shown in Figure 4.2. All single transformants and all possible

combinations of double transformants of *S. cerevisiae* strain Y166 were analyzed for β-galactosidase expression using a filter paper assay that employs X-gal as a substrate. The positive control used in these assays was pAS1-IE1; IE1 of the baculovirus AcMNPV fused to the GAL4 DNA binding domain can activate β-galactosidase expression in Y166 independently of the expression of the GAL4 activation domain (Steve Harwood, personal communication). In all cases except the positive control and the transformant pAS1/pACTII-141, the colony lifts remained white or pale pink. The lifts from the positive control began to turn blue within 30 minutes, and the lifts from the transformant pAS1/pACTII-141 turned blue by the following morning. The transformant pAS1/pACTII-141 was negative for β-galactosidase expression in the liquid assay using ONPG as substrate. Since X-gal is more sensitive than ONPG for the detection of β-galactosidase, the filter paper assay often detects weak positives that the ONPG assay cannot detect or quantify (Clontech, 1996).

Both false positives and false negatives can occur with the yeast two-hybrid system (Bartel et al., 1993). False positives can occur if one fusion protein can activate transcription of *lac* Z (or other reporter gene) on its own. The p141-Gal4 activation domain fusion seems to be able to interact with the Gal4 DNA binding domain, as the transformant pAS1/pACTII-141 turned blue over time in the filter paper assay. However, the p141-Gal4 activation domain fusion was not able to interact with the Gal4 DNA binding domain-ORF-206 fusions. False negatives are also possible and can be due to the failure of the inserts to be maintained in the yeast, poor fusion protein expression or stability, improper fusion protein folding, and the failure of the fusions to locate to the nucleus (Bartel *et al.*, 1993). Another possible reason for a negative result is that the proteins do not interact strongly enough to be detected with this system.

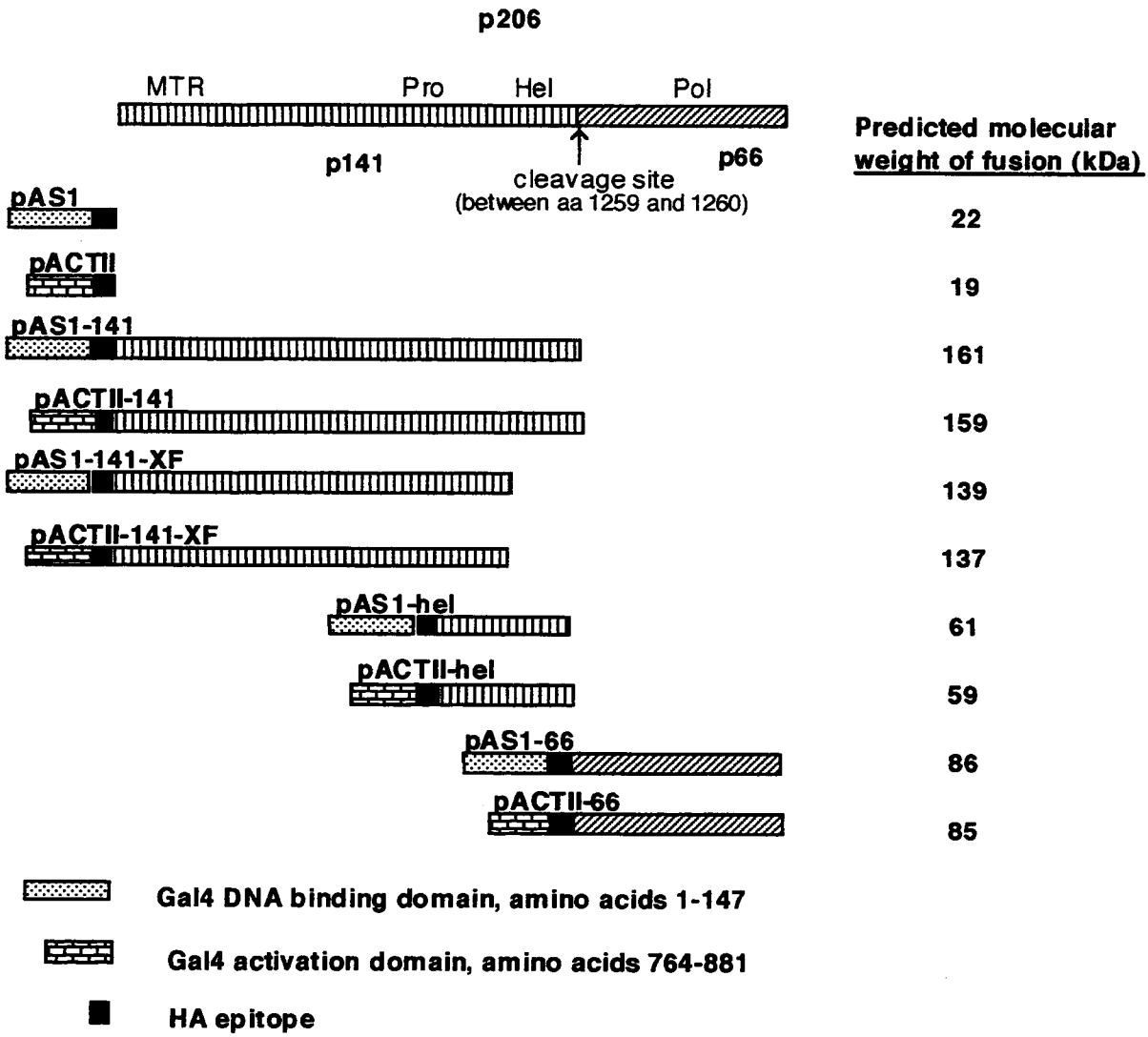


Figure 4.2. Diagrams and predicted molecular weights of the fusion proteins produced by pAS1, pACTII and the constructs with TYMV inserts used in the yeast two-hybrid analysis. The fusion proteins encoded by the pAS1-66 and pACTII-66 constructs include amino acids 1257 to 1844 of p206 (the entire p66). The fusions encoded by pAS1-hel and pACTII-hel include amino acids 894 to 1259 of p206. The helicase-like domain consists of amino acids 960-1198 of p206. The 141-XF constructs encode fusions that include amino acids 1-1061 of p206, and the 141 constructs, amino acids 1-1259 of p206, the entire p141. All of these constructs create fusion proteins comprising the Gal4 DNA binding domain or activation domain, an HA epitope and the insert protein.

4.3.2 Insert stability and expression

In order to determine whether the inserts were maintained in the pAS1 and pACTII vectors throughout their analysis in Y166, the vectors were shuttled back into *E. coli*, and digested with restriction enzymes to confirm the presence and size of the inserts. The plasmids were purified from three Y166 colonies of each of the following four double transformants: pAS1-66/pACTII-141, pAS1-hel/pACTII-141-XF, pAS1-141-XF/pACTII-hel, and pAS1-141/pACTII-66. The total of twelve Y166 plasmid preparations were used to transform *E. coli*. The plasmids were repurified from *E. coli* and digested with restriction enzymes to confirm the presence of inserts of the correct size. In all cases, the plasmids were found to have maintained the full-size inserts (Fig. 4.3).

Toxicity of the fusion proteins in either *E. coli* or in yeast did not seem to be a problem as there was not a noticable difference between the growth of cultures transformed with pAS1 and pACTII with or without TYMV inserts.

Both the pAS1 and pACTII vectors produce fusion proteins that contain HA epitopes (Fig. 4.2). Monoclonal anti-HA antibodies were used to detect the fusion proteins by western analysis so as to determine if some fusions were not being expressed, or were unstable. Both vectors alone produced proteins of the expected size, as did pACTII-66, pACTII-hel, and pAS1-hel (Fig. 4.4). No fusion protein expression was detected for pAS1-66, pAS1-141, pAS1-141-XF, pACTII-141 and pACTII-141-XF (Fig. 4.4). If the fusions were expressed, they did not accumulate to high enough levels to be detectable with these methods. It is unknown whether the synthesis of the fusions was extremely low, or whether the proteins were degraded. Multiple bands were detected in the pACTII-66, pACTII-hel, and pAS1-hel extracts (Fig. 4.4) which could be due to degradation. The use of C-206, N-206 and C-141 antisera (Fig. 3.1) were not useful in the detection of ORF-206 fusion proteins produced in yeast because the antisera also react with many yeast proteins.

Figure 4.3. Insert stability in pAS1 and pACTII. These ethidium bromide-stained 1% agarose gels represent some of the digests performed on the plasmids shuttled back to *E. coli* from the yeast strain Y166. The plasmids were identified by the fragments produced by the digestions shown in this figure, and by a second digestion not shown. Lane 1 in each panel is a digestion of the corresponding plasmid before being transformed into Y166 and is included as a reference. Lanes 2-4 are digested plasmids rescued from three separate yeast colonies. pAS1 contains a *Bgl*II site (not shown in Fig. 4.1) on the ADH promoter side of the multiple cloning site. Since all the ORF-206 inserts contained *Bgl* II sites near the 3' ends, *Bgl* II proved to be a useful screening enzyme. pAS1 also contains a Pst I site on the terminator side of the multiple cloning site, not shown in Fig. 4.1. pACTII contains *Bgl* II sites on either side of the multiple cloning site and it was used for the screening of pACTII-66 which contains an internal *Bgl* II site. *Hin*d III recognizes two sites in pACT II, one on either side of the multiple cloning site. The plasmids in panel (A) were identified as the pAS1 derived plasmids and those in panel (B) were identified as the pACTII derived plasmids. M is a 1 kb ladder (Gibco-BRL).

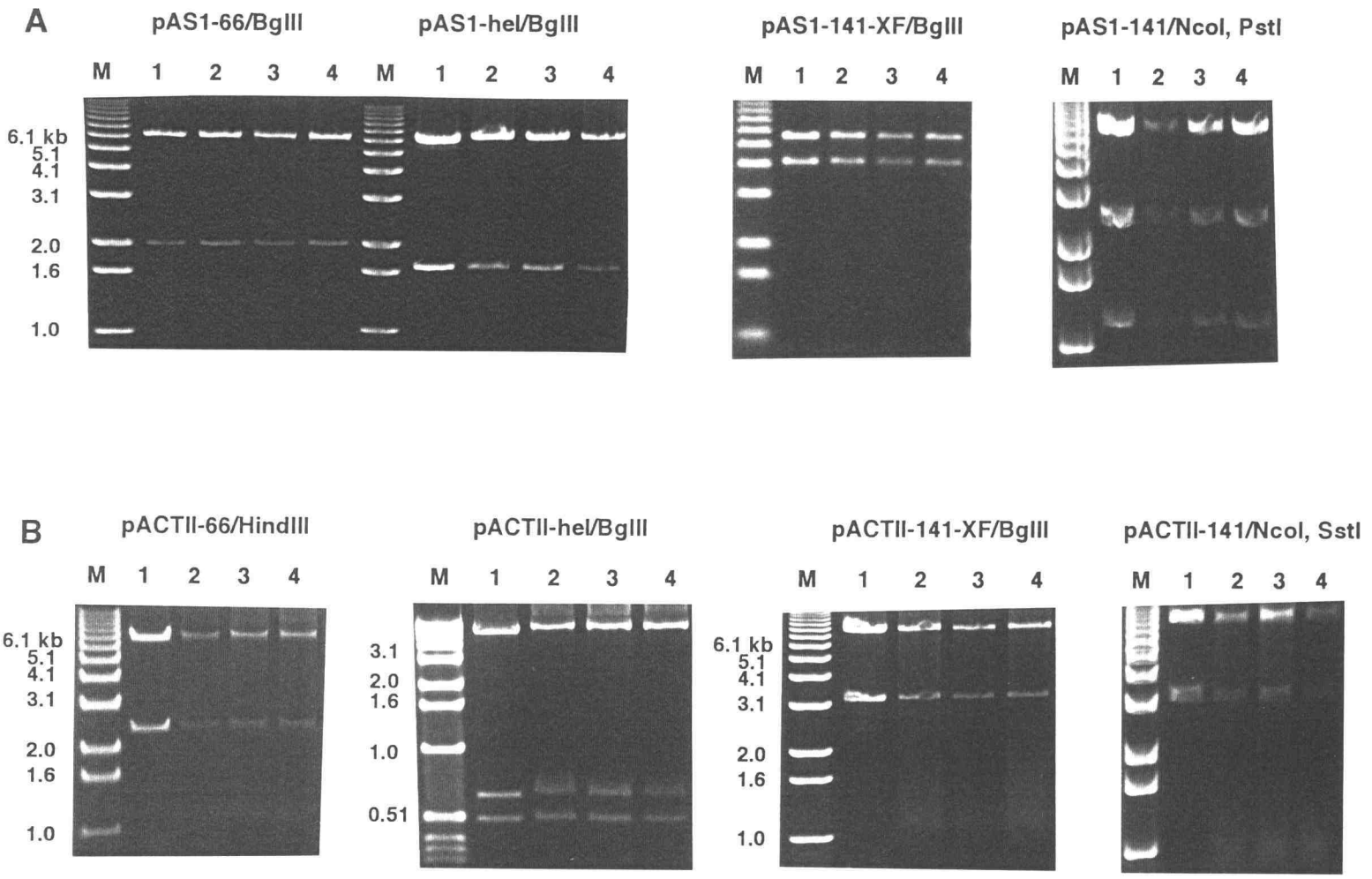

A
pAS1-66/BglII
pAS1-hel/BglII
pAS1-141-XF/BglII
pAS1-141/NcoI, PstI
M 1 2 3 4 M 1 2 3 4
M 1 2 3 4
M 1 2 3 4
6.1 kb
5.1
4.1
3.1
2.0
1.6
1.0
B
pACTII-66/HindIII
pACTII-hel/BglII
pACTII-141-XF/BglII
pACTII-141/NcoI, SstI
M 1 2 3 4
M 1 2 3 4
M 1 2 3 4
M 1 2 3 4
6.1 kb
5.1
4.1
3.1
2.0
1.6
1.0
3.1
2.0
1.6
1.0
0.51
6.1 kb
5.1
4.1
3.1
2.0
1.6
1.0

Figure 4.3

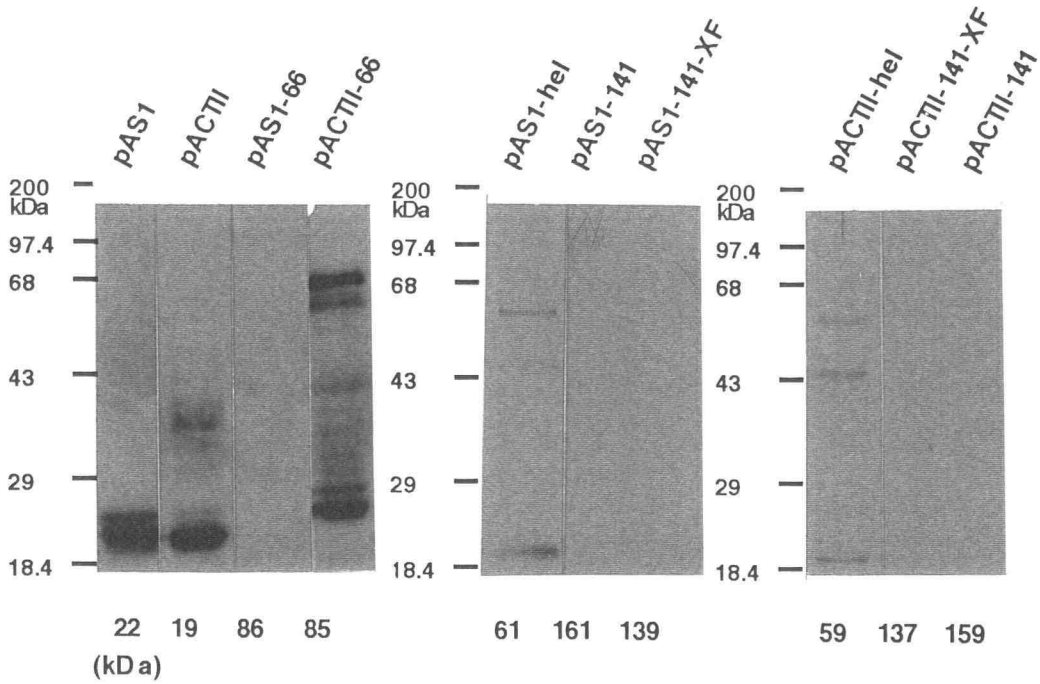


Figure 4.4. Fusion protein expression in yeast. Extracts of Y166 transformed with pAS1, pACTII, or one of their derivatives, were obtained and immunoblotted with anti-HA antibodies as described in the Methods section. The expected protein sizes are given at the bottom of each lane in kDa.

There is the possibility that the fusion proteins are unable to reach the nucleus, and therefore cannot activate transcription of the reporter genes. The fusions encoded by pAS1 and pACTII both contain nuclear localization sequences, but it is unknown how well the fusions used in this study were able to reach the nucleus. Since TYMV replicates in the cytoplasm of plants, its replication proteins may be somehow unsuitable to enter the yeast nucleus. The ORF-206 fusion proteins may interact with yeast cytoplasmic proteins which in turn prevents them from entering the nucleus. It is also possible that the Gal4 DNA binding domain does not bind the *GAL* upstream activation sequence (UAS_G) as well when expressed as a fusion as when it is expressed on its own. It has been determined that most LexA (a DNA binding protein that is used in the place of the Gal4 DNA binding domain in yeast two-hybrid studies) fusions expressed in yeast do not bind their operator as well as does native LexA (Golemis and Brent, 1992).

4.3.3 His3 assays

In order to examine the possibility that the fusions interact only very weakly, another *S. cerevisiae* strain, CG-1945, that is more sensitive to weakly interacting proteins (Craig Giroux, personal communication) was used. With this strain, the *HIS3* gene was used as the reporter. Because the transcription of this gene, under the control of a *GAL1* promoter, is somewhat leaky, 3-aminotriazole (3AT) was added to the yeast media to counteract any constitutive expression of His3. 3AT is an inhibitor of imidazole glycerol phosphate dehydratase, an enzyme involved in histidine synthesis and encoded by *HIS3* (Plischke *et al.*, 1975). It was found that 3 mM was the minimal amount of 3AT that prevented the growth of a negative control (pAS1/pACTII) and allowed the growth of a positive control (pAS1-LEF5/pACTII-LEF5) on minimal medium plates lacking leucine, tryptophan, and histidine. These minimal

medium plates containing 3 mM 3AT were used to screen the double transformants in Table 4-1. The transformant combinations include the pAS1-66 and pACTII-66 constructs with each of the other ORF-206 containing constructs. None of the transformants were able to grow on the histidine dropout plates, indicating that if the fusions were expressed and were stable, they did not detectably interact.

Double transformant	Growth on -leu, trp dropout media	Growth on -leu, trp, his dropout media with 3 mM 3AT
pAS1-LEF5/pACTII-LEF5	yes	yes
pAS1-66/**pACTII-hel**	yes	no
pAS1-66/pACTII-141-XF	yes	no
pAS1-66/pACTII-141	yes	no
pAS1-hel/pACTII	yes	no
pAS1-hel/pACTII-66	yes	no
pAS1-hel/pACTII-hel	yes	no
pAS1-141-XF/**pACTII-66**	yes	no
pAS1-141/**pACTII-66**	yes	no

Table 4.1. His3 assay results. This table lists the double transformants of *S. cerevisiae* strain CG-1945 that were assayed for His3 expression by their ability to grow on media lacking histidine. The transformants in bold indicate that fusion proteins were produced at detectable levels in *S. cerevisiae* strain Y166.

4.3.4 Conclusions and future plans

Since the helicase- and polymerase-like proteins of BMV have been shown to interact (Kao *et al.*, 1992, O'Reilly *et al.*, 1995), these two domains of TYMV seemed the most likely candidates for a proposed protein-protein interaction. In the yeast two-hybrid system, the double transformant pAS1-hel/pACTII-66 was the most hopeful to activate transcription of the reporter gene because the

fusions contained the helicase and polymerase domains, and their expression was high enough to be detected by western analysis (Fig. 4.4). No interaction was detected, however, with this double transformant in either of the yeast strains. Although there are reasons why this result could be a false negative, it is an indication that the helicase domain of p141 is not involved in an interaction with p66.

The proposed p141/p66 protein-protein interaction may be unlike that of BMV, and may involve the polymerase interacting with a region of p141 outside the helicase domain. The helicase domain was the only region of p141 that was expressed to a detectable level in Y166. The failure to detect expression of any of the fusions from the clones containing the large p141 and p141-XF inserts, and the successful expression of the fusions from the clones containing the smaller helicase insert, is an indication that smaller fusions are more likely to be expressed in Y166. With this in mind, more constructs have been designed that will express smaller domains within p141. These include a clone that will express a methyltransferase fusion, a protease fusion, and a third fusion that includes the region between the methyltransferase and protease domains. It is hoped that the clones will allow the expression of p141 domains and will lead to information on the postulated interaction between p141 and p66.

5. TYMV Protein Interactions: GST Fusion Results

5.1 Introduction

Glutathione S-transferase (GST) fusion interaction experiments are a popular way to look at protein-protein interactions. They were used in this study, along with the immunoprecipitations and yeast two-hybrid assays described in the previous Chapters, to examine the postulated interaction between p66 and p141 of TYMV. There are two parts to these experiments. First, one protein is expressed as a GST fusion in a host, generally *E. coli,* although other hosts can be used. Since GST has a high affinity for glutathione, the fusion protein can be readily purified in nondenaturing conditions by adsorption to glutathione-agarose (Smith and Johnson, 1988). The second part of the experiments is to add *in vitro* translated proteins, or alternatively a cell lysate, typically radiolabeled, to the purified GST fusion bound to the glutathione-agarose. It can then be determined if any proteins specifically adhere to the fusion protein.

The objectives of these experiments were to obtain sufficient levels of expression of GST-ORF-206 protein fusions, to partially purify the fusions on glutathione-agarose (Sigma), and to determine if [^{35}S]methionine-labeled, *in vitro*-translated ORF-206 proteins could specifically bind to the fusions.

5.2 Methods

5.2.1 Construction of plasmids used to create GST fusion proteins

The vector used in this study is a modified version of pGEX-CS1 (a gift from Dr. Bill Dougherty, Fig. 5.1). pGEX-CS1 is a derivative

of pGEX-1 (Smith and Johnson, 1988) and has the GST coding sequence from *Schistosoma japonicum*, the coding sequence for the cleavage site of tobacco etch virus (TEV) nuclear inclusion a (NIa) proteinase, and TEV nucleotides 145-200 all under the control of a *tac* promoter (Ptac). This vector also contains a *lac* I^q gene and an ampicillin resistance gene.

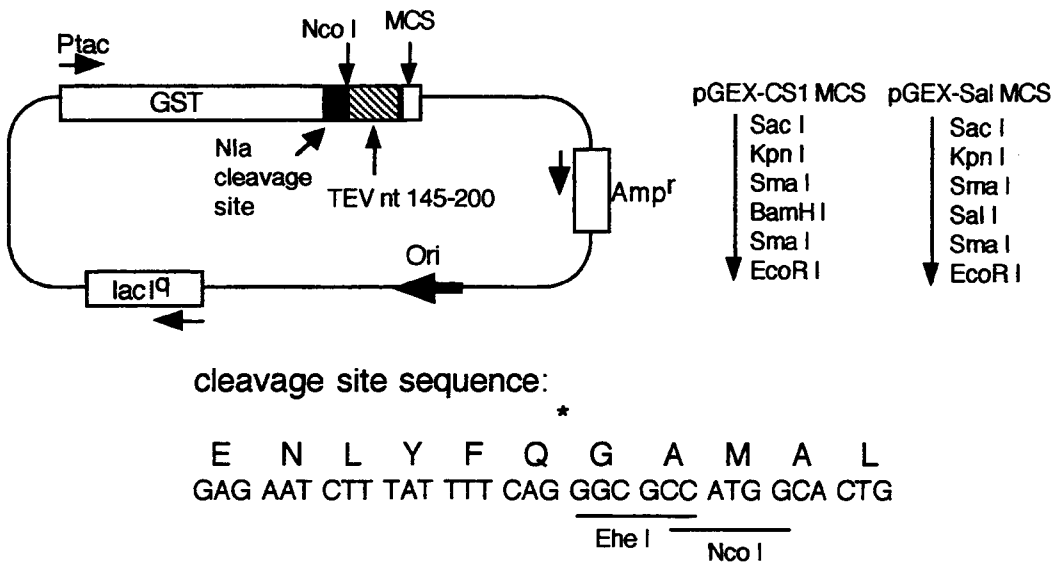


Figure 5.1. Map of pGEX-CS1. This 5 kb vector contains a GST coding region fused to a sequence encoding a NIa proteinase cleavage site. Following the cleavage site is nucleotides 145-200 of TEV cDNA and a multiple cloning site (MCS). The MCS is shown in more detail on the right, where the MCS of pGEX-Sal is also included (the *BamH* I site has been replaced by a *Sal* I site). The cleavage site sequence is shown in detail, and includes the amino acids encoded and two restriction endonuclease sites. The asterisk (*) indicates the peptide bond that is cleaved by TEV NIa proteinase.

A *Sal* I 8mer phosphorylated linker (Promega) was inserted in the multiple cloning site of pGEX-CS1 in order to facilitate further cloning (Fig. 5.1). pGEX-CS1 was first digested with *BamH* I, followed

by treatment with Klenow (USB) and dNTP's to create blunt ends. pGEX-Sal was created by ligating approximately 50 ng of pGEX-CS1 and 25 pmol of the *Sal* I linker. pGEX-Sal allowed for the forced in-frame cloning of the inserts from the yeast two-hybrid study into the *Nco* I and *Sal* I sites of pGEX-Sal, and the deletion of TEV nucleotides 145-200 from the resulting constructs (Fig. 5.2).

5.2.2 Expression of GST fusion proteins and their purification with glutathione-agarose

E. coli strain DH5α transformed with pGEX-Sal or one of the constructs in Figure 5.2, was grown overnight at 30°C in 2 ml of 2YT containing 50 μg/ml ampicillin. The overnight culture was then added to 100 ml of 2YT with ampicillin, and incubated at 30°C until the $OD_{600} \cong 0.5$ (about 6 hours). Isopropyl β-D-thiogalactoside (IPTG) was added to a concentration of 0.1 mM, and the incubation was continued for 8-16 hours. The cells were pelleted, resuspended in 5 ml of cold PBS (10 mM sodium phosphate, 1.8 mM potassium phosphate, pH 7.2, 0.14 M sodium chloride, 2.7 mM potassium chloride), aliquoted 1 ml per tube, and stored at -80°C (Smith and Corcoran, 1990).

On the day an interaction experiment was planned, 1 ml of the above induced culture was thawed on ice. The cells were lysed by sonicating for one minute in 5 second bursts. Lysis of the cells was confirmed by microscopic examination. Triton X-100 was added to 1%, and the tube was mixed gently for 30 min at room temperature. The tube was then centrifuged 16,000 x g for 10 min at 4°C, and the supernatant was transferred to a new tube. One hundred μl of a 50% slurry of glutathione-agarose (Sigma) in PBS was added to the tube of supernatant, and the tube was rotated at room temperature for 0.5-1 hour. The agarose beads were washed three times with 1 ml volumes of PBS, and then resuspended in 50 μl PBS to give a total of 100 μl of slurry (Smith and Corcoran, 1990).

5.2.3 Preparation of competitor proteins

The interaction assays were performed in the presence of competitor proteins to inhibit nonspecific protein binding. *E. coli* strain DH5α was grown overnight at 37°C in 250 ml of 2YT. The cells were pelleted and then resuspended in 2.5 ml of BB buffer (50 mM potassium phosphate, pH 7.5, 150 mM potassium chloride, 10 mM magnesium chloride). The cells were sonicated five times for one min each, alternating with two min rests on ice. The sonicated cells were centrifuged at 48,000 x g for 30 min in a Beckman TL100 ultracentrifuge, and the supernatant was collected, the protein concentration of which was determined by measuring the A_{280} using the following equation that corrects for the presence of nucleic acids: Protein concentration (mg/ml)=(1.55 x A_{280})-(0.76 x A_{260}) (Harlow and Lane, 1988). Glycerol was added to a final concentration of 10%, Triton X-100 was added to 1%, and the extract was diluted with BB buffer so the final protein concentration was 15 mg/ml. This *E. coli* extract was stored until needed at -80°C, at which time it was thawed, diluted to 3 mg/ml with BB buffer containing 10% glycerol and 1% Triton X-100. The extract was centrifuged 175,000 x g for 30 min and the supernatant, containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), was used in the interaction assays.

Turnip leaf extract was made by grinding two turnip leaves with a mortar and pestle in 1 ml of BB buffer. The turnip leaves were then transferred to microfuge tubes and centrifuged at 16,000 x g for 15 min. The supernatant was transferred to a new tube and the protein concentration was determined as described above. Glycerol and Triton X-100 were added to 10% and 1% respectively, the extract was diluted to 7 mg/ml and was stored at -80°C until needed. Prior to use in an interaction assay, the extract was centrifuged at 175,000 x g for 30 min and PMSF was added to a concentration of 0.2 mM.

5.2.4 Interaction assay

Forty µl of the GST fusion glutathione-agarose slurry were added to 200 µl of the above *E. coli* extract and mixed well. *In vitro* translations of selected transcripts were performed as described in the Methods section of Chapter 2. Between 4 and 6 µl of the translation reaction (5,000-20,000 cpm for p70Ω translations and ~150,000 cpm for the TYMC translations) were combined with 200 µl of *E. coli* extract, incubated on ice for 15 min and then centrifuged 16,000 x g for 15 min. The supernatant was combined with the GST-fusion beads-*E. coli* extract mix and then rotated at 4°C for 1-3 hours. The beads were washed three times with BB buffer containing 10% glycerol and 1% Triton X-100 (Swaffield and Johnston, 1996). Ten µl 2X LB (31 mM Tris, pH 6.8, 4% SDS, 20% sucrose, 0.01% bromphenol blue, 10% β-mercaptoethanol) were added, the tubes were boiled for 5 min, and then centrifuged for 1 min at 16,000 x g. The proteins were separated by 0.1% SDS/10% PAGE, and exposed to film for 2-5 days.

5.3 Results and Discussion

5.3.1 Expression of GST fusion proteins

The construct pGEX-hel (Fig. 5.2), directed the production of a GST-TYMV helicase fusion protein that, along with a few degradation products, were bound by the glutathione-agarose (Fig. 5.3, lane 4). The helicase fusion migrated at an apparent molecular weight of about 65 kDa, close to the expected molecular weight of 67 kDa. The fusions were also detectable by immunoblotting with either anti-GST (Sigma) or anti-C141 (Fig. 3.1) antibodies (data not shown).

A similar clone to pGEX-hel has recently been described that directs the synthesis of a GST-TYMV helicase fusion in the expression vector pGEX-2T (Kadare *et al.*, 1996). This fusion protein purified from *E. coli,* has demonstrated ATPase, GTPase and RNA binding activities. The fusion encoded by pGEX-hel differs from the one described by Kadare *et al.* only by the addition of 22 amino acids on the amino terminal side of the helicase domain.

The other three pGEX constructs in Figure 5.2 were capable of directing synthesis of the GST-ORF-206 protein fusions, but not to high enough levels to be of use in GST fusion interaction experiments. Induced DH5α cultures transformed with pGEX-141 or pGEX-141-XF both produced GST-p141 fusions that were detectable by immunoblotting with anti-N-206 (Fig. 3.1) antibodies, indicating that at least the methyltransferase domain of the p141 insert was expressed (data not shown). The protein band pattern produced was very similar for both clones, although no full length fusions were detected, and the induced proteins were not present in Coomassie blue stainable amounts. pGEX-66 was capable of directing the synthesis of full length GST-polymerase fusions (data not shown), but again, the amounts were too low for their use in GST fusion interaction assays.

There are a number of problems that can complicate the overexpression of proteins in *E. coli.* Overexpression of proteins can lead to the formation of inclusion bodies which render the proteins insoluble (Makrides, 1996). Inclusion bodies, which are associated with high levels of protein expression, did not seem to be the obstacle with the three unsuccessful clones in this study since they directed only low levels of fusion synthesis. Toxicity of the proteins encoded by the inserts can also be a problem in the overexpression of proteins in *E. coli* (Makrides, 1996) but this did not seem to be a problem with the TYMV inserts as the growth of the cultures before and after induction with IPTG was not noticeably different from that of pGEX-Sal transformants.

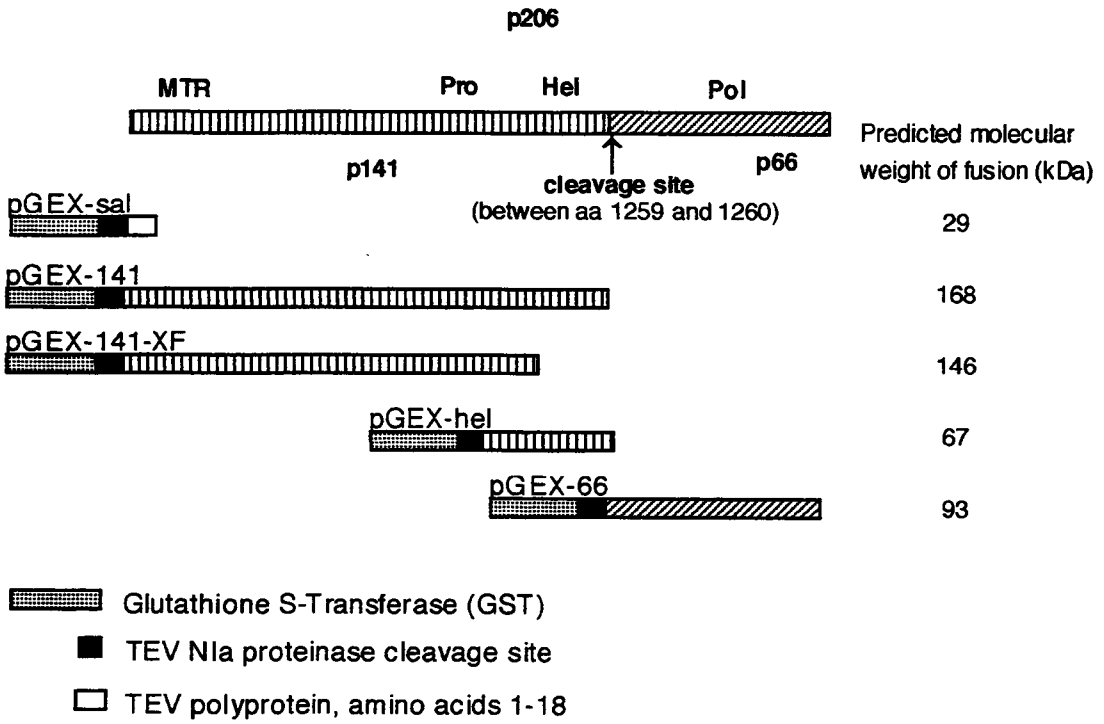


Figure 5.2. Diagrams and predicted molecular weights of the fusion proteins produced by pGEX-Sal and the other constructs used in this study. pGEX-Sal encodes a fusion protein that includes the first 18 amino acids of the TEV polyprotein. The fusion protein encoded by pGEX-66 includes amino acids 1257 to 1844 of p206 (the entire p66). The fusion protein encoded by pGEX-hel includes amino acids 894 to 1259 of p206. The conserved helicase-like domain spans amino acids 960-1198 of p206. The pGEX-141-XF construct encodes a fusion that includes amino acids 1-1061 of p206, and pGEX-141 encodes a fusion protein that includes amino acids 1-1259 of p206, the entire p141. All of these constructs create fusion proteins consisting of GST, a TEV NIa proteinase cleavage site, and the insert protein.

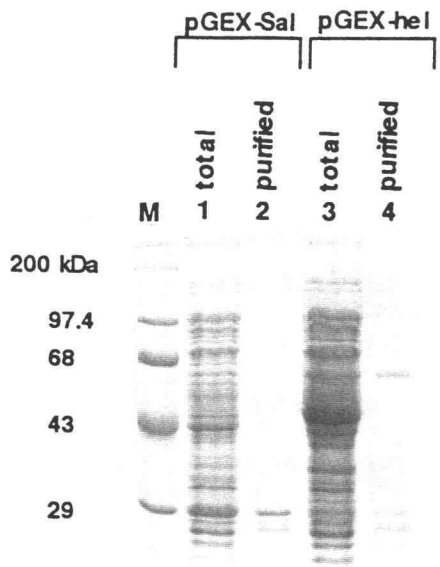


Figure 5-3. Purified GST-fusion proteins. Lanes 1 and 3 of this Coomassie blue-stained 0.1% SDS/10% PAGE represent the total soluble proteins of IPTG-induced cultures of *E. coli* strain DH5α transformed with either pGEX-Sal (lane 1) or pGEX-hel (lane 3). Lanes 2 and 4 represent the proteins purified with 40 μl of a 50% slurry of glutathione-agarose from the pGEX-Sal and pGEX-hel transformed cultures, respectively. The expected molecular weight for the GST-TEV polypeptide fusion is 29 kDa and that for the GST-TYMV helicase fusion is 67 kDa.

The fusion protein band patterns directed by pGEX-66, pGEX-141 and pGEX-141-XF contained numerous bands, most of which corresponded to proteins with smaller molecular weights than those predicted for the fusions (data not shown). It was apparent that fusion protein synthesis was incomplete and/or the fusions were being degraded. Any degradation in this case would not have been caused by sonication, as these cells were added directly to Laemmli buffer and boiled for 5 min. One possible reason for the incomplete and low levels of fusion protein expression is that the TYMV inserts contain codons that are unusual in *E. coli*. The codons AGG, AGA and CCC are rare in *E. coli* (Zhang *et al.*, 1991) but are found throughout

the TYMV genome (Morch *et al.*, 1988; Dreher and Bransom, 1992). The codon CCC is especially common in the TYMV genome, occasionally occurring in pairs and triplets, which can be problematic if the corresponding tRNA is in short supply. It has been found that low levels of the [Arg]-$tRNA_{AGA/AGG}$ (encoded by the *argU* gene) in *E.coli* can hamper the production of certain recombinant proteins (Brinkmann *et al.*, 1989). Supplying the *argU* gene on a high copy number plasmid has been shown to help with the expression of some recombinant proteins. The plasmid pUBS520 (Brinkmann *et al.*, 1989) contains a *argU* gene and a kanamycin resistance gene. The *E.coli* strain DH5α transformed with pUBS520 together with one of the pGEX-TYMV insert clones did not result in higher levels of fusion expression or in the production of longer fusions (data not shown).

Degradation of the fusion protein, either before or after sonication, could also be a problem in the production of the GST fusions. Two protease deficient strains of *E. coli* were transformed with pGEX-66 to see if this could improve the yields of the full length GST-TYMV polymerase fusion. Neither BL21 (Pharmacia), which is *OmpT* minus (*OmpT* encodes an outer membrane-localized protease), or SF110 (Baneyx and Georgiou, 1990), which is *OmpT* minus and *DegP* minus (*DegP* encodes a periplasmic protease), improved the yield of the full length fusion over the yield in DH5α (data not shown).

Similar new clones to those proposed in Chapter 4 that would express smaller regions of p141 (e.g. methyltransferase-like domain, protease domain) as fusions with GST are planned for the future and will hopefully allow for higher level expression of full length fusions. Vectors that direct the synthesis of GST fusions in yeast may also be used.

5.3.2 Results of interaction assays and conclusions

The relatively high yield of the GST-TYMV helicase fusion from DH5α transformed with pGEX-hel allowed for the use of the fusion in interaction assays. *In vitro* translated proteins labeled with [^{35}S]methionine were added to the agarose-bound fusions, but in no case were the labeled proteins able to specifically bind to the fusions. Translation products from two different transcripts were added to the agarose-bound fusions. In the first case, TYMC full length genomic transcripts were used and it is possible that the p66 and p141 proteins produced associated with one another *in cis* so that the p66 was not available to interact with the agarose-bound helicase fusion. The second transcript used was transcribed from p66Ω (constructed by Kay Bransom), and translation of this transcript results in the synthesis of only p66, the TYMV polymerase. The p66 from the p66Ω translation was unable to interact with the GST-helicase fusion.

A possible reason for the failure of the polymerase and helicase domains of TYMV to interact in these experiments is that the proposed interaction between the polymerase and helicase is mediated by a host protein or TYMV RNA. It has been shown that the polymerase-like and helicase proteins of BMV interact (Kao *et al.*, 1992, O'Reilly *et al.*, 1995) and that template RNA is required for the assembly of BMV replication complexes in yeast (Quadt *et al.*, 1995). However, the use of turnip extract instead of *E. coli* extract for the competitor proteins in the interaction assay, and the addition of TYMV RNA did not result in the retention of p66 on the glutathione-agarose (data not shown).

Although there are reasons why the GST fusion interaction assays may not detect some interactions, the result obtained in this study are in agreement with the yeast two-hybrid studies that indicate the helicase domain of p141 does not interact with p66. Since two different types of experiments could not detect an interaction between p66 and the helicase domain of p141, future plans will focus on regions of p141 outside of the helicase domain

and their potential interaction with p66. It is hoped that the clones planned for the expression of smaller regions of p141 as GST fusions will be capable of directing the synthesis of high levels of the fusions and that interaction assays with the p66Ω translations will help determine where and if p66 and p141 interact.

6. Discussion

6.1 Summary of Results

The purpose of this study was to obtain evidence for the direct interaction between p141 and p66, two proteins encoded by ORF-206 of TYMV RNA. Evidence for a direct protein-protein interaction would support the model, described in Chapter 1 and illustrated in Fig. 1.3, that explains the *cis*-preferential replication of TYMV RNA in turnip protoplasts (Weiland and Dreher, 1993). Three types of experiments were used to study the postulated interaction between p141 and p66: Immunoprecipitation of *in vitro* translated TYMV proteins, yeast two-hybrid experiments using fusions of ORF-206 proteins to Gal4 DNA binding and activation domains, and GST fusion interaction assays where the ability of *in vitro* translated TYMV proteins to interact with GST-ORF-206 fusions was determined.

The immunoprecipitations using C-206 antibodies were able to precipitate all three of the ORF-206 proteins: p206, p141 and p66 (Fig. 3.2). Since p141 does not contain the C-206 epitope (Fig. 3.1), p141 was presumed to be coprecipitated by its interaction with p206 and/or p66, both of which contain the C-206 epitope (Fig. 3.1). The immunoprecipitations using the other two available antisera were unable to show coprecipitation of p66 (Fig. 3.2), and possible reasons for this are discussed in Chapter 3. The use of nested in-frame deletion mutants suggests that a region in the C-terminal half of p66 could be involved in an interaction with p141. However, accurate estimation of p141/p66 ratios was hampered by a background of p141 recovery.

The yeast two-hybrid experiments have afforded a limited study of the proposed interactions, since it was only possible to verify the production of fusion proteins containing the polymerase- and helicase-like domains of p206. Western analysis using

monoclonal HA antibodies revealed that some of the larger fusions did not accumulate to detectable levels (Fig. 4.4), so their involvement in a protein-protein interaction cannot be ruled out. Conclusions can be drawn from the results of the yeast transformant combination of the Gal4 activation domain fusion with the TYMV polymerase-like protein (pACTII-66) and the Gal4 DNA binding domain fusion with the TYMV helicase domain (pAS1-hel, Fig. 4.2) as both of these constructs directed the synthesis of detectable amounts of the full length fusions (Fig. 4.4). Since the brome mosaic virus (BMV) proteins containing helicase-like and polymerase-like domains had been shown to form a complex *in vitro* (Kao *et al*, 1992) and *in vivo* (O'Reilly *et al*, 1995), an interaction between the helicase- and polymerase-like domains of TYMV seemed plausible. However, the pACTII-66/pAS1-hel transformant combination did not activate reporter genes in two different yeast strains, indicating that the helicase- and polymerase-like domains of TYMV do not detectably interact in this system.

The GST fusion interaction assays met with limited success as most of the GST-ORF-206 protein fusions did not accumulate to high enough levels in *E. coli* to permit their use in interaction assays. One construct, pGEX-hel (Fig. 5.2), was capable of directing the synthesis of high amounts of GST-TYMV helicase fusions that were readily purified on glutathione-agarose (Fig. 5.3). *In vitro* translated p66 was added to the agarose-bound helicase fusions but in no case was p66 able to bind to the fusions, even when turnip extract and TYMV RNA were added.

The immunoprecipitation results support the model for *cis*-preferential replication by providing some indication of an interaction between the ORF-206 proteins. However, attempts to verify this interaction using other methods were unsuccessful. The helicase domain of p141 and the polymerase-like protein (p66) do not interact in a manner that is detectable in either the yeast two-hybrid assays or the GST fusion interaction assays described in this study. It is likely that the helicase domain of p141 is not involved

in the proposed protein-protein interaction between p141 and p66. No conclusions can be drawn about the involvement of the other domains of p141, and future experiments will focus on determining their roles in the proposed interaction with p66.

6.2 Possible Modifications to the Model

It is possible that the interaction between the ORF-206 proteins is not direct and that some host protein or host structure is required for the proposed interaction. p66 and p141 are assumed to be associated with the TYMV replication complex, and it is conceivable that p206 is also involved. Analogous proteins to p66 and p141 in BMV (Quadt and Jaspars, 1990), cucumber mosaic virus (Hayes and Buck, 1990) and poliovirus (Bienz *et al.*, 1990) have been shown to be part of the viral replication complexes. It is possible that a deletion in p66 of TYMV could result in the loss of the ability of p66 to be assembled into a replication complex by inhibiting its ability to interact with a host protein or the RNA template, not necessarily by inhibiting a direct interaction with p141. Wild type p66, supplied *in trans*, could then form a functional replication complex with p141. This slight modification to the model could help explain the ability of genomes containing deletions in the p66 coding region, to coreplicate in turnip protoplasts (Weiland and Dreher, 1993) without the requirement for p66 and p141 to interact directly.

Another factor that could affect the coreplication in turnip protoplast of certain mutant genomes is the possible requirement for an active helicase *in cis*. In this variation (Fig. 6.1) of the model, the helicase is required to unwind the secondary RNA structure at the 3' end of the genome either to allow for proper replication complex assembly or in order for the polymerase (p66) to bind the promoter. In the case of the helicase point mutant (GCGST) or a Δhelicase (Δ2995-3705) genome, the helicase is defective or

nonfunctional so the 3' end of the genome is not unwound, replication complexes are not formed or the polymerase cannot bind the promoter, and the polymerase is free to go *in trans* (Fig. 6.1B and 6.1C). It has been shown that the helicase point mutation (GCGST) resulted in a decrease in the ATPase, GTPase and RNA binding activity (Kadare *et al.*, 1996), so it is likely that this mutant would result in reduced template unwinding activity. The Δhelicase genome, which is missing the helicase coding region and would not be expected to have any template unwinding activity, behaves in a similar manner to the GCGST genome in coreplication experiments. In the case of the polymerase point mutant (RDD), the helicase produced is functional and can unwind the 3' end of the genome, allowing replication complexes to form (Fig. 6.1B). If the defective polymerase can still bind the promoter, it would block the binding of wild type p66 provided *in trans*. Whether in a complex or not, the polymerase is defective, so no replication occurs, assuming there is only limited displacement by an active polymerase supplied *in trans*.

The involvement of an active helicase is consistent with the ability of the genome with a large deletion in the p66 coding region (ΔSP-G, Fig. 1.2) to be complemented by the GCGST and Δhelicase genomes if the large deletion in p66 results in a protein incapable of incorporation into a replication complex, or is unable to bind the promoter. In either case, wild type p66 provided *in trans* from the GCGST genome could allow replication of the ΔSP-G genome (Fig. 6.1C). If wild type p141 encoded by the ΔSP-G genome is also available *in trans*, replication of the GCGST genome would also occur.

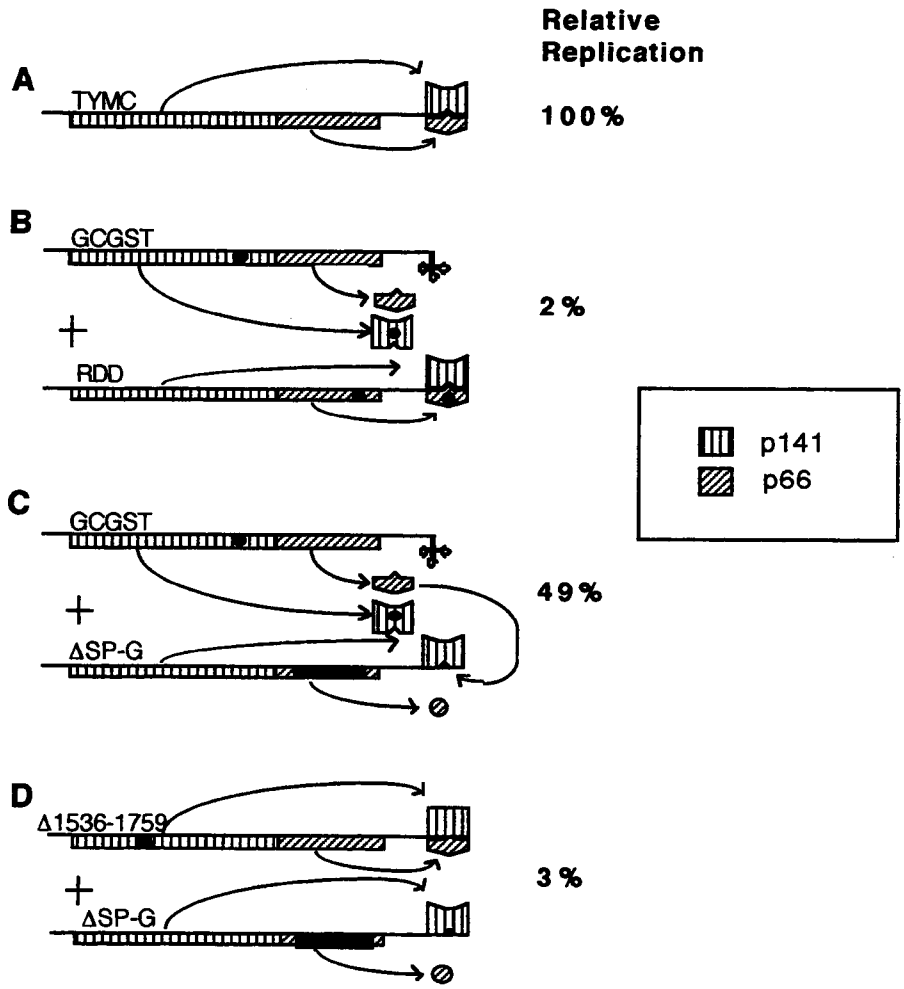


Figure 6.1. Effects of the helicase function on the *cis*-preferential replication of TYMV. (A) Wild type replication complexes are formed and have a high affinity for their template. (B) The helicase produced by the GCGST genome is defective and cannot unwind the 3' end of the genome; the p66 cannot bind its template so it is available *in trans*. The RDD genome directs the assembly of nonfunctional replication complexes, unable to incorporate wild type p66. (C) The p66 encoded by the ΔSP-G genome is not able to be incorporated into replication complexes. The wild type p66 provided *in trans* from the GCGST genome is then able to form active replication complexes with wild type p141 from the ΔSP-G genome. (D) The helicase produced by the Δ1536-1759 genome is functional, the 3' end of the genome is unwound and p66 is able to bind the template. Since the supply of p66 from the Δ1536-1759 genome is limited, there is little complementation with the ΔSP-G genome.

The model that includes the function of the helicase domain can account for the results of some of the coreplication experiments with the defective genomes in Figure 1.2 that could not be explained solely by the proposed protein-protein interaction between p141 and p66. The defective genomes Δ213-1759 and Δ1536-1759 (Fig. 1.2) do not allow for the complementation of ΔSP-G genomes. These defective genomes should still be able to complement the replication of ΔSP-G because, like the GCGST genome, they encode wild type p66. However, the relative coreplication rate for Δ213-1759 and ΔSP-G was <0.1% and for Δ1536-1759 and ΔSP-G was 3% (Fig. 1.2), indicating poor complementation. These defective genomes could be translated *in vitro*, and the deletions were not found to affect proteolytic processing (Weiland and Dreher, 1993).

The lack of complementation between ΔSP-G and the defective genomes Δ213-1759 and Δ1536-1759 can be explained because both Δ213-1759 and Δ1536-1759 encode for wild type helicase domains. According to the model, as long as the helicase is functional, the 3' end of the genome will be unwound, and p66 will be of limited availability *in trans* (Fig. 6.1D). Only genomes that encode for defective helicase domains (GCGST, Δhelicase) will be able to provide p66 efficiently *in trans*. Δ213-1759 has the entire methyltransferase-like domain deleted, and the deletion in Δ1536-1759 corresponds to 75 amino acids in a region between the methyltransferase-like domain and the protease domain, but both genomes encode wild type helicase domains.

The successful complementation of ΔSP-G genomes with ΔCP or wild type genomes (data not shown) indicates that having a nonfunctional helicase is not the only factor that allows for coreplication. Complementation by wild type and ΔCP genomes could occur in two ways: p66 could be provided *in trans* as a free subunit, or p66 could be supplied *in trans* as part of a functional replication complex. If the p66 from the ΔCP and wild type genomes was available *in trans* as a functional replication complex, not as

free p66, these results would still be consistent with the affects of a functional versus nonfunctional helicase. It is possible that only when the genome encodes for a nonfunctional helicase that does not allow complex formation, as with GCGST or the Δhelicase genome, can p66 be provided *in trans* without being part of a complex with p141.

6.3 Unanswered Questions

A number of details about the *cis*-preferential replication of TYMV remain unclear. Future experiments will hopefully help determine whether p141 and p66 interact directly, whether their interaction is mediated by a host factor or RNA element, or whether there is another explanation for the coupling of replication and translation in TYMV. It will be interesting to determine whether the complementation of ΔSP-G genomes by ΔCP and wild type genomes occurs by the *trans* availability of p66 alone or by the availability of p66 as part of a functional replication complex. It is assumed that p141 and p66 are both part of the replication complex, but it is unknown what host proteins may also be associated or whether uncleaved p206 may be involved. Autocatalytic cleavage of p206 to p141 and p66 is never complete *in vitro* (Bransom *et al.*, 1991), but there is no experimental evidence about the role of p206 *in vivo*. It is also unknown how the switch occurs in replication from negative to positive strand synthesis. It is likely that more than one form of TYMV replication complexes exist; one form with a preference for the synthesis of negative strands, one form with a preference for the synthesis of positive strands, and possibly a third for for the synthesis of subgenomic RNA. These different forms may involve variations in the interactions between subunits.

6.4 Future Plans

To answer the question of whether p141 and p66 interact and whether the interaction is direct, new clones have been designed to express the methyltransferase-like domain, the protease domain and the region between the two conserved domains as fusions with either the Gal4 domains or glutathione S-transferase. These constructs will hopefully allow for the expression of domains of p141 that were previously difficult to express as large fusion proteins. The use of these clones in yeast two-hybrid screens and in GST fusion interaction assays will hopefully provide information on the proposed interaction between p141 and p66.

In vivo immunoprecipitations from labeled turnip protoplasts infected with TYMV are also planned. Replication complexes will be pre-formed *in vivo* and as long as the C-206 epitope is not buried in the complex, immunoprecipitations with C-206 antibodies should precipitate the complete replication complexes. These experiments will hopefully show that p141 and p66 form a complex *in vivo*, confirming the results of the *in vitro* immunoprecipitations described in Chapter 3, and indicate if other proteins are also part of the complex. If the labeling of viral proteins is not high enough in the protoplasts to allow for successful immunoprecipitations, immunoprecipitations of a purified and active TYMV replicase preparation may be tried instead. Besides demonstrating that p141 and p66 form a complex, these experiments could eventually help to determine if certain replication complexes have a preference for negative versus positive strand synthesis and what factors affect the preference. It is possible that a host protein is associated with one replication complex form and not in the other, as is the case with Qβ (Blumenthal and Carmichael, 1979), or that an RNA element is required for the assembly of one form, as has been suggested for BMV (Duggal *et al.*, 1994). Proteolytic processing of the replication proteins could presumably allow for conformational changes in the replication complexes. This type of processing has been shown to be

responsible for the switch in the template preference for Sindbis virus (Lemm *et al.*, 1994).

It is hoped that it will eventually be possible to assemble TYMV replication complexes in yeast, as has been done with BMV (Quadt *et al.*, 1995). This will allow for the identification of possible *cis* elements that may be required for complex assembly, as was found with BMV (Quadt *et al.*, 1995), and for the identification of deletions and point mutations in the genome that prevent or inhibit complex assembly. It will be interesting to see whether the helicase point mutation (GCGST) or the Δhelicase construct prevent complex assembly, and support the idea that a defective helicase could allow p66 to be free to diffuse away from its template.

More coreplication experiments in turnip protoplasts are planned to further investigate the effects of the helicase GCGST mutant. The construction of a new mutant genome is planned that would contain the helicase GCGST mutation and the upstream deletion of nucleotides 1536-1759. Coreplication with this new mutant genome and the ΔSP-G genome would be expected to be similar to the rates for ΔSP-G + GCGST genome (49%, see Fig.1.2) if the inactive helicase is what allows for the polymerase to be available *in trans*. If the coreplication rates were lower, as with the Δ1536-1759 + ΔSP-G combination (3%, Fig. 1.2), then it would appear that the Δ1536-1759 mutation has some dominant effect, and the importance of a functional helicase in the coupling of translation and replication of TYMV would not be supported.

Another coreplication experiment that is planned will hopefully determine whether p66, when translated from a ΔCP or wild type genome, is available *in trans* as part of a functional replication complex or is available *in trans* uncomplexed with p141. If the replication of a ΔSP-G genome also containing a GCGST point mutation can be successfully rescued by a ΔCP or wild type genome, it will be clear that both p66 and p141 are provided *in trans* (since p66 and p141 encoded by the ΔSP-G/GCGST genome are both nonfunctional), possibly in a complexed form. If the ΔSP-G/GCGST cannot be complemented by ΔCP or wild type genomes, then it will

appear that in this case p141 cannot be provided *in trans*, and that p66 is available *in trans* in an uncomplexed form.

The use of transgenic plants and cauliflower mosaic virus 35S plasmids were used with alfalfa mosaic virus to show that RNA1 and RNA2 have to be translated, even when the proteins they encode were provided *in trans*, to allow for their replication (van Rossum, *et al.*, 1996). These types of experiments with TYMV will hopefully be possible in the future and will allow the testing of how *cis*-preferential replication is affected if the replication proteins are provided *in trans* and in excess.

6.5 Summary of Conclusions and Recommendations for Future Work

The immunoprecipitation data provided some evidence that p141 forms a complex with p66 and/or p206 *in vitro*. Since yeast two-hybrid and GST fusion interaction assays show that the proposed interaction between p141 and p66 does not involve the helicase domain of p141, future experiments will focus on determining the possible involvement of other regions of p141 in an interaction with p66.

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