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MUTATIONAL ANALYSIS OF THE TOBACCO ETCH POTYVIRUS CYLINDRICAL INCLUSION PROTEIN

by

MARY EVELYN BROWNING-KELLEY

DISSERTATION

Submitted to the Graduate School of

Wayne State University, Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

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MAJOR: BIOLOGICAL SCIENCES

Approved by:

Advisor Date

DEDICATION

To my parents who taught me that curiosity was a good thing and that an education was invaluable;

To my brother and sister who told me I could do it;

To the friends and family who remembered who I was even though they never got to see me;

And most importantly to my beloved husband Scott, who has loved, encouraged and supported me above and beyond the call of marriage while I did it.

ACKNOWLEDGEMENTS

I would like to thank God Almighty for the opportunity to pursue this degree and the friends He has placed in my path on this journey.

I would like to thank Dr. V. Hari for his endless faith in and support of me while I was at Wayne State University. I could not have done this without his mentorship and friendship through the tough and easy times.

I would like to thank my former and current labmates: David Baunoch, Pritam Das, Shiowwen Chen (who is really more a sister than a friend), Murali Krishnan, Paula Turner, Chitra Sridar and Jeremy Johnston for their help technically and their friendship.

I would like to thank my committee members Dr. R. Arking, Dr. T. Holland and Dr. A. Nicholson, for their invaluable input both technically and academically over the years and their evaluation of this manuscript.

A very special thanks to Ms. Linda Van Thiel for first her friendship and second her help with all things computer.

To the many friends who have been of help technically and otherwise, mostly the members of the Nicholson Lab, the Lilien Lab, the Greenberg Lab and the Smith Lab.

And to the many faces who have in small but important ways contributed to the completion of this program, especially Ms. Jill Pruitt for always knowing what piece of paper needed to be filled out when and where to send it.

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Abbreviations List

BCIP	5-bromo-4-chloro-indoylyphosphate
DMF	N,N,-dimethylformamide
DTT	dithiothreitol
EDTA	ethelyenediamine tetraacetic acid
HEPES-KOH	N-2-hydroxyethylpoperazine-N'-2-ethanesulfonic acid
IPTG	isopropyl-1-thio-β-D-galactopyranoside
NBT	nitrobluetetrazolium
PAS staining	periodic acid-Schiff's staining
PEI-cellulose	polyethyleneimine cellulose
PBS	phosphate buffered saline
PMSF	phenylmethyl-sulphonyl-fluoride
TEV	tobacco etch virus
NBT	nitrobluetetrazolium

Introduction.

The potyviridae are the largest group of 34 families of plant viruses. They are divided into 3 accepted subfamilies based on their method of transmission and sequence homologies; the potyviruses, which are aphid transmitted; the bymoviruses, which are transmitted by fungi; the rymoviruses, which are transmitted by mites, and one proposed family, the ipomoviruses, which are transmitted by white flies. The potyviruses are named after the type member, potato virus Y (PVY). The potyviruses cause substantial crop and ornamental plant damage every year. This dissertation details my studies of the ATPase activity and related activities of the cylindrical inclusion body protein (CI) of tobacco etch virus (TEV), a member of the potyvirus family.

Tobacco etch virus (TEV) is a positive-sense single-stranded RNA virus. The virion is a 730 nm long filamentous particle, 12 -13 nm wide, and the viral genome is encapsidated by approximately 2000 molecules of a single capsid protein (Dougherty and Carrington, 1988). The single strand of genomic RNA is approximately 9496 bases in length with a genomically linked viral protein (VPg) at the 5' end (Hari *et al.*, 1981; Murphy *et al.*, 1990) and a variable poly (A) tail at the 3' end (Allison *et al.*, 1986; Hari *et al.*, 1979). The RNA contains one open reading frame which is translated into a large polyprotein of 345,943 daltons (Allison *et al.*, 1986) (Figure 1).

The polyprotein is subsequently cleaved into at least 9, and possibly 10, proteins by three virus-encoded proteases (Figure 2) (Dougherty *et al.*, 1980; Carrington *et al.*, 1988; Verchot *et al.*, 1991). Table 1 summarizes the known and suggested gene products of potyviruses and their known or putative functions.

Figure 1. Genome map of the TEV-HAT potyvirus. The 5' end has a genomically linked viral protein (VPg) and the 3' end is polyadenylated. The genome has a single open reading frame which is translated into one large polyprotein that undergoes subsequent proteolytic processing (see Figure 2).

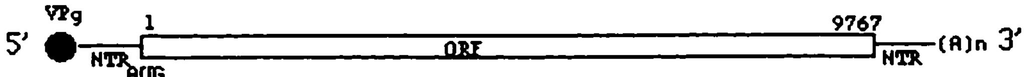

VPg
5'
NTR
AUG
1
ORF
9767
NTR
(A)n
3'

Figure 2. Model for proteolytic processing of the TEV potyviral polyprotein. The polyprotein is processed by three virus-encoded proteases, P1, HC-Pro and NIa. The diagram is divided into autoproteolytic events and *trans* proteolytic events. The cleavage sites are denoted by arrows that originate from the protein responsible for the proteolysis.

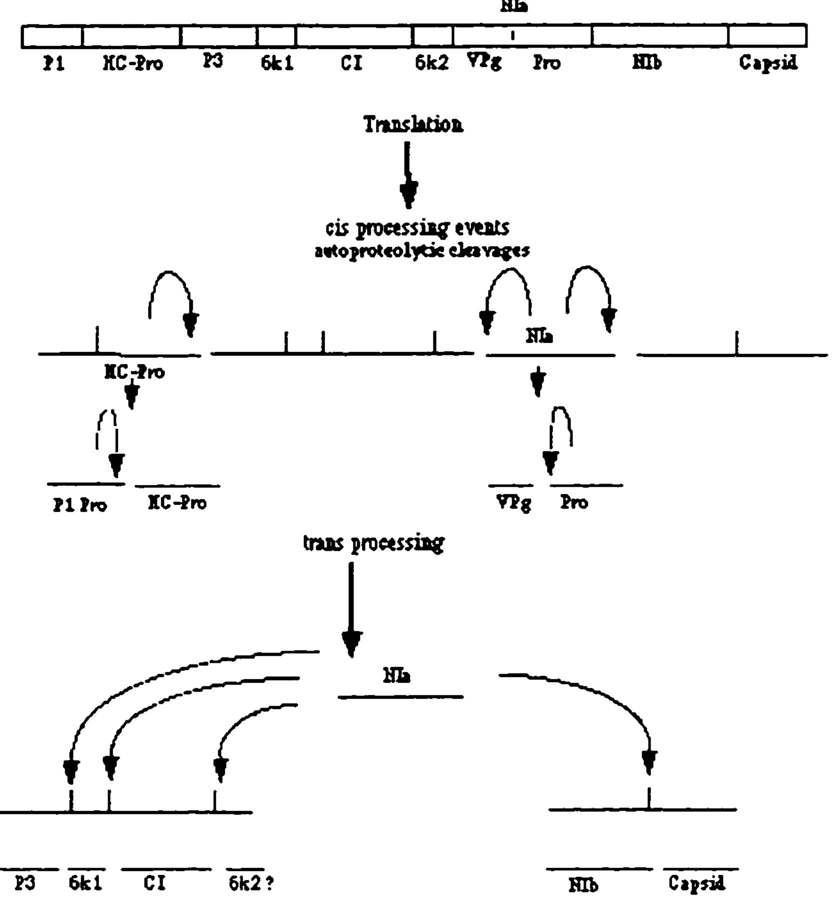

NIa
P1
HC-Pro
P3
6k1
CI
6k2
VPg
Pro
NIb
Capsid
Translation
cis processing events
autoproteolytic cleavages
NIa
HC-Pro
P1 Pro
HC-Pro
VPg
Pro
trans processing
NIa
P3
6k1
CI
6k2 ?
NIb
Capsid

Table 1. Known or predicted functions of the gene products of potyviruses.

Gene Product	Known or Suggested Function
P1	-autocatalytic protease -exhibits non-specific RNA binding -involved in genome replication
Helper Component-Protease (HC-Pro)	-autocatalytic protease -essential for aphid transmission of virus -involved in long distance transport of virus in plant -involved in genome amplification
P3	-unknown function
6K1	-unknown function
cytoplasmic inclusion protein (CI)	-RNA helicase (putative for TEV) -ATPase -RNA binding -putative movement protein -speculated to be involved in genome replication
6K2	-membrane associated protein
nuclear inclusion Ia (NIa)	-N-terminus is VPg -main viral polyprotein protease cleaves most of polyprotein into functional gene products -forms nuclear inclusions late in infection
nuclear inclusion Ib (NIb)	-RNA dependent RNA polymerase -forms nuclear inclusions late in infection
capsid	-30 kD single structural protein of virion -involved in vector transmission

The P1 protein is one of three virus-encoded proteases responsible for the cleavage of the TEV polyprotein. It is a serine-type protease that autoproteolytically cleaves between itself and the N terminus of the HC-Pro. It has been shown to exhibit non-specific RNA binding, and acts in *trans* as a genome amplification factor (Verchot *et al.*, 1992; Verchot *et al.*, 1995, Brantley *et al.*, 1993). The helper component-protease (HC-Pro) is a multifunctional protein. It is a cysteine-like protease that autoproteolytically cleaves between itself and the P3 protein. The N-terminus of the protein is the helper component that is required for transmission of the virus by aphids, and contains sequences that are required for efficient RNA replication, as well as the establishment of a systemic infection (Carrington *et al.*, 1989; Atreya *et al.*, 1992; Cronin *et al.*, 1995; Thornbury *et al.*, 1990). The P3 protein has been detected in tobacco vein mottling potyvirus (TVMV) infected plants, but to date no function has been assigned to it. It has been suggested, based on sequence comparisons, that it could be a transmembrane protein and hence it might play a role in viral replication (Rodriguez-Cerezo and Shaw, 1991). The cytoplasmic inclusion body protein (CI) is an RNA helicase that has RNA stimulated ATPase activity and is also thought to be involved in cell-to-cell spread of the viral RNA (Lee *et al.*, 1997; Chen *et al.*, 1994; Eagles *et al.*, 1994; Baunoch *et al.*, 1991; Lain *et al.*, 1991; Lain *et al.*, 1990; Calder *et al.*, 1990). The nuclear inclusion-a protein (NIa) has two distinct domains; the N-terminus is the genomically linked VPg and the C-terminus is the major virus encoded protease (Murphy *et al.*, 1990; Dougherty *et al.*, 1991). The VPg is suggested to act as a primer for the initiation of RNA synthesis (Shahabuddin *et al.*, 1988, Tobin *et al.*, 1989). The C-terminal protease is a cysteine like protease is a cysteine like protease that is

responsible for cleavage of the C-terminal two-thirds of the potyviral polyprotein yielding the mature viral proteins (Dougherty, 1988). The nuclear inclusion-b (NIb) protein of the potyviruses is the RNA dependent RNA polymerase (RdRp) that replicates the viral RNA (Hong *et al.*, 1996). Together, the NIa and NIb proteins form the nuclear inclusion bodies found in infected plants (Knuhtsen *et al.*, 1973). The NIa contains a bipartite nuclear localization signal, and its movement into the nucleus depends on cleavage of the 6K2 protein from the N-terminus (Resptrepo *et al.*, 1990; Carrington *et al.*, 1991; Restrepo-Hartwig *et al.*, 1992). A nuclear localization signal has been found in the NIb protein and has been found to be dependent on the structural integrity of the protein. Mutations that alter the structural integrity of the protein result in the loss of nuclear transport (Li *et al.*, 1997). The 6K2 protein is adjacent to the N-terminus of the NIa protein. It has been shown to be membrane associated in transgenic plants and found to play a role in RNA amplification. (Restrepo-Hartwig *et al.*, 1994). The presence of a 6K protein adjacent to the N-terminus of the CI protein (6K1) is suggested based on the cleavage maps of tobacco vein mottle potyvirus (TVMV) and plum pox potyvirus (PPV). Its existence has been demonstrated *in vitro* by cloning the P3 and 6K1 region into a bacterial system that also contained the clone for the NIa proteinase. Analysis of the expressed proteins revealed the presence of the expected cleavage products by the NIa protein, which included the free 6K1 polypeptide (Garcia *et al.*, 1992). Although it has been found in the bacterial system, and a region corresponding to the 6K1 is conserved in TEV, the actual cleavage site has not been found, and the 6K1 has not yet been detected in infected plants (Parks *et al.*, 1992). It has been suggested that it may play a role in viral replication due to the presence of hydrophobic stretches of amino acids and their

similarity to the picornaviral 2B and 3A peptides (Reichmann *et al.*, 1992). The capsid protein (CP) is the single coat protein that encapsidates the genomic viral RNA (Shukla, 1989). The CP is involved in aphid transmission of the virus and is required for cell-to-cell and long distance movement of the virus in the infected plants (Atreya *et al.*, 1992; Dolja *et al.*, 1994; Dolja *et al.*, 1995).

To be classified as a member of the Potyviridae, a virus must exhibit the formation of cytoplasmic cylindrical inclusion bodies (Borges, 1958; Edwardson, 1974). These cylindrical inclusion bodies are formed by the aggregation of the virus encoded cytoplasmic inclusion protein (CI) and are the most important phenotypic criterion for the identification of potyviruses (Milne, 1988; Shukla *et al.*, 1989; Ward *et al.*, 1991). All potyviral CI proteins have a similar molecular weight between 65 to 75 kD. The CI of TEV is approximately 70 kD (Brakke *et al.*, 1987; Dougherty *et al.*, 1980).

Electron microscopic and immunogold labeling studies to date have shown that the CI protein is found in five cytoplasmic locations: (1) in early infection, the CI proteins are closely associated with the plasma membrane near the plasmodesmata and aggregate in the morphological form of spikes (Baunoch *et al.*, 1988); (2) in association with the endoplasmic reticulum (Langenberg, 1986); (3) in free form associated with thread-like filaments in the cytoplasm (Edwardson, 1974); (4) in cylindrical inclusion bodies of various morphologies (Edwardson, 1974); and (5) in association with virus infection-induced vesicles (Calder *et al.*, 1990; Chen *et al.*, 1994).

The spike like protrusions seen in early infection eventually elongate and dissociate from the membrane as the infection progresses (Lawson *et al.*, 1971; Christie *et al.*, 1977; Baunoch *et al.*, 1991). The CI are later found free in the cytoplasm, where

they will aggregate to form pinwheel-shaped or scroll-like inclusion bodies. The actual morphology of these inclusion bodies is virus-specific. Initially, researchers tried to utilize this feature to group particular potyviruses into four subdivisions and for diagnosis of which particular potyvirus (or type of) may be in an infected plant (Edwardson, 1974; Edwardson *et al.*, 1984). These subgroupings are no longer used, however, since the four morphological inclusion subgroupings proposed by Edwardson and co-workers does not correlate with either the four known transmission mechanisms of potyviruses, or with the major subgroupings that are based on genome sequence comparison. Ward *et al.*, (1992) have since suggested that CI morphology should not be used as a criterion for determining subgroups while establishing divisions within the potyviruses.

Sequence analysis of the CI protein led to the discovery of 7 conserved domains that the CI protein shares in common with known and putative RNA and DNA helicases. These evolutionarily conserved sequences occur in proteins involved in: (1) replication, transcription, translation, recombination and repair; (2) DNA packaging, (3) dNTP generation, and (4) possibly mRNA splicing (Gorbalenya *et al.*, 1989; Wassarman *et al.*, 1991; Koonin *et al.*, 1993; Schaeffer *et al.*, 1993; Selby *et al.*, 1993). Gorbalenya and Koonin developed a set of three superfamilies that comprised all of the known proteins that contained these domains or variations of them (Gorbalenya and Koonin, 1989).

Superfamily II consists of proteins from the Poty-, Flavi-, Pesti-, Herpes-, and Poxviridae, as well as cellular proteins such as eIF-4a and p68, *Saccharomyces cerevisiae* RAD3 and Tif (translation initiation factor), and *E. coli* recQ and uvrB proteins. The members of superfamily II which are of considerable interest for the purposes of this dissertation include the DEAD/DEAH family which includes the CI protein of

potyviruses and the putative/known RNA helicases of the flavi- and pestiviruses (Hodgman, 1988; Gorbalenya *et al.*, 1989b; Koonin, 1991; Dolja *et al.*, 1992). A second classification of the viral proteins is suggested by Habili and Symons (Habili and Symons, 1989). Utilizing the subdivision of positive sense RNA plant viruses into two large supergroups, Sindbusvirus-like (supergroup A) and the Picornavirus-like (supergroup C), they developed a series of subgroupings based on the evolutionary relationship of their known and putative RNA polymerases and helicases of these viruses, with the addition of a new supergroup, the Luteovirus-like (supergroup B) (Goldbach *et al.*, 1988).

Of the seven conserved domains in the CI protein, functions are known for four of them. Each domain has within it consensus sequences that are invariant within each superfamily. Domains I and II (or the A and B sites) together comprise the purine NTP-binding motif (NTBM). Domain I (the 'A' site) has the general motif sequence, <hydrophobic stretch>(G/A)xx(G)xGKS/T, where the hydrophobic stretches contain at least 2 hydrophobic residues out of 5 (Gorbalenya *et al.*, 1989). Domain I has been implicated directly in the binding of the pyrophosphate (Pi) moiety of the NTP (Rozen *et al.*, 1989). Domain II (the 'B' site) (Walker, 1982) is proposed to be involved in the interaction with the magnesium cation (Mg^{++}) complexed with the same phosphate group and has the sequence motif, <hydrophobic stretch>D(E/D) (Moller *et al.*, 1985; Jurnak, 1985; Bradley *et al.*, 1987; De Vos *et al.*, 1988; Hodgman, 1988). More recently, domain II has also been implicated in intracellular virus movement (Lee *et al.*, 1997; Mushegian and Koonin, 1993). Domains Ia and VI are involved in pH dependent non-specific single-stranded RNA binding (Fernandez *et al.*, 1995, 1996). To date no

functions are known for domains III, IV, and V.

In all RNA viruses with a genome size of at least 6.0 kb, the conserved sequences for NTPase activity and helicase activity are present, with the only exception being the human astrovirus with a genome of 7.2 kb (Gorbalenya and Koonin, 1989; Gorbalenya *et al.*, 1989b; Jiang *et al.*, 1993). All known RNA helicases have been shown to possess an intrinsic NTPase activity, where the hydrolysis of the nucleotide is thought to provide the energy necessary to unwind the dsRNA.

RNA-stimulated NTPase activity has been found in the NS3 proteins of West Nile and yellow fever flaviviruses, the p80 protein of bovine viral diarrhea (BDV) pestivirus (Wengler *et al.*, 1991; Tamura *et al.*, 1993; Warrener *et al.*, 1993) and the CI proteins of PPV, TEV and tamarillo mosaic virus (TaMV) potyviruses (Lain *et al.*, 1991; Chen *et al.*, 1994; Eagles *et al.*, 1994). RNA helicase activity has been demonstrated for the CI proteins of PPV and TaMV potyviruses, the NS3 protein of the Hepatitis C (HCV) flavivirus, and the p80 protein of BDV (Lain *et al.*, 1990; Eagles *et al.*, 1994; Kim, *et al.*, 1995; Warrener *et al.*, 1995).

The *in vitro* requirements for NTPase activity are an NTP and a divalent cation cofactor, generally magnesium, but it has been reported that the manganese cation yields a higher activity level for the TEV CI (Chen *et al.*, 1994) and that it is necessary for the activity of the BVDV p80 protein (Warrener *et al.*, 1993). The NTP requirement varies by protein and in general all of the studied proteins seem to hydrolyze all of the NTPs, as well as some or all of the dNTPs, although to different degrees of efficiency. The *in vitro* assay results for helicase activity have shown that both the PPV and TaMV CI protein are able to unwind only dsRNA substrates with 3' single-strand overhangs, indicating that it

functions in the 3' to 5' direction (Lain *et al.*, 1993; Eagles *et al.*, 1994). The RNA helicase activity of PPV CI protein was the first report of helicase activity associated with a protein encoded by an RNA virus. The molecular mechanism of RNA unwinding is unknown for any RNA helicase.

It has been suggested that these RNA helicase proteins may have roles in several different viral processes. These include viral RNA replication, vesicular trafficking of replication complexes, RNA encapsidation, and cell to cell movement (Gorbalenya *et al.*, 1989; Peters *et al.*, 1994; Rodriguez *et al.*, 1993; Mirazayan *et al.*, 1994; Rouleau *et al.*, 1994)

Potyviral replication is poorly understood. To date five of the virus encoded proteins have been shown to be involved in virus amplification *in vivo*, however the exact mechanism or cellular location has yet to be elucidated. The five proteins that are involved in virus amplification are the P1, CI, NIa, NIb, and the 6K2. The P1 protein has been shown to be necessary for genomic amplification. Studies where the P1 was removed from the viral genome showed a marked decrease in genome amplification in plants (Verchot *et al.*, 1992 and 1995). Further evaluation determined that it is possible that the P1 plays a role in enhancing translation due to its RNA binding abilities (Brantley *et al.*, 1993). Mutational analysis of the CI of TVMV showed that functional CI was necessary for genome amplification, however, exactly how the mutations in the CI affected the viral RNA replication is not known (Klein *et al.*, 1994). Data for several viruses indicate that the helicase-like protein is needed for the continued accumulation of all classes of viral RNA. For brome mosaic virus (BMV) and cucumber mosaic virus (CMV), in the absence of the Ia protein, which is the putative RNA helicase, only

negative-stranded RNA molecules were synthesized (Kroner *et al.*, 1990; Hayes *et al.*, 1990). For the poliovirus 2C protein, a putative superfamily III RNA helicase, the essential importance of the purine NTP-binding motif for viral reproduction was confirmed by site-directed mutagenesis (Mirzayan *et al.*, 1992; Teterina *et al.*, 1992). It seems that the 2C protein is involved in the release of newly synthesized positive-strand RNA from the replication complex (Bienz *et al.*, 1987; Li *et al.*, 1988). In the case of PPV, it has been shown that a crude membrane fraction containing capsid protein, CI protein, NIa and NIb has RNA replication activity (Martin *et al.*, 1991). The NIa protein contains the VPg at its N-terminus. This protein is thought to be essential for priming the RNA synthesis steps during viral replication (Shahabuddin, 1988). A more recent study by Li *et al.*, has lead to a proposed model for the initiation of the (+)RNA strand synthesis in the potyviruses (Li *et al.*, 1997). The model proposes that the VPg/NIa and NIb come together to form a priming complex (Figure 3). The 6K2 protein has been found to be membrane associated. Transgenic plant studies by Restrepo-Hartwig and Carrington, 1994, have shown that mutations in the hydrophobic stretches within this protein lead to a decrease in the amplification of the virus (Restrepo-Hartwig *et al.*, 1994). This would suggest that viral replication (or at least some of the steps involved) takes place in association with the cytoplasmic membrane, or other membranous structures, such as chloroplasts or virally induced cytoplasmic vesicles (Gadh *et al.*, 1986; Gunasinghe *et al.*, 1991; Chen *et al.*, 1994). The actual location of viral replication, and the form of viral replication whether through a replicative form (RF) or replicative intermediate (RI) is not known.

It has been suggested that the potyviral CI protein is involved in cell-to-cell

movement of the virus because of its association with both infection induced vesicles and the plasmodesmata in early infection (Calder *et al.*, 1990; Langenberg, 1986; Baunoch *et al.*, 1991; Chen *et al.*, 1994).

Viral movement proteins are responsible for the spread of the viral infection into surrounding cells and ultimately the entire plant. These proteins are thought to somehow modify the intercellular connections of the plant cells (the plasmodesmata) and allow the transport of either entire virion particles, as for cow pea mosaic virus (CPMV) (van Lent *et al.*, 1990) or nucleoprotein complexes as for tobacco mosaic virus (TMV) (Wolf *et al.*, 1989) into adjacent cells.

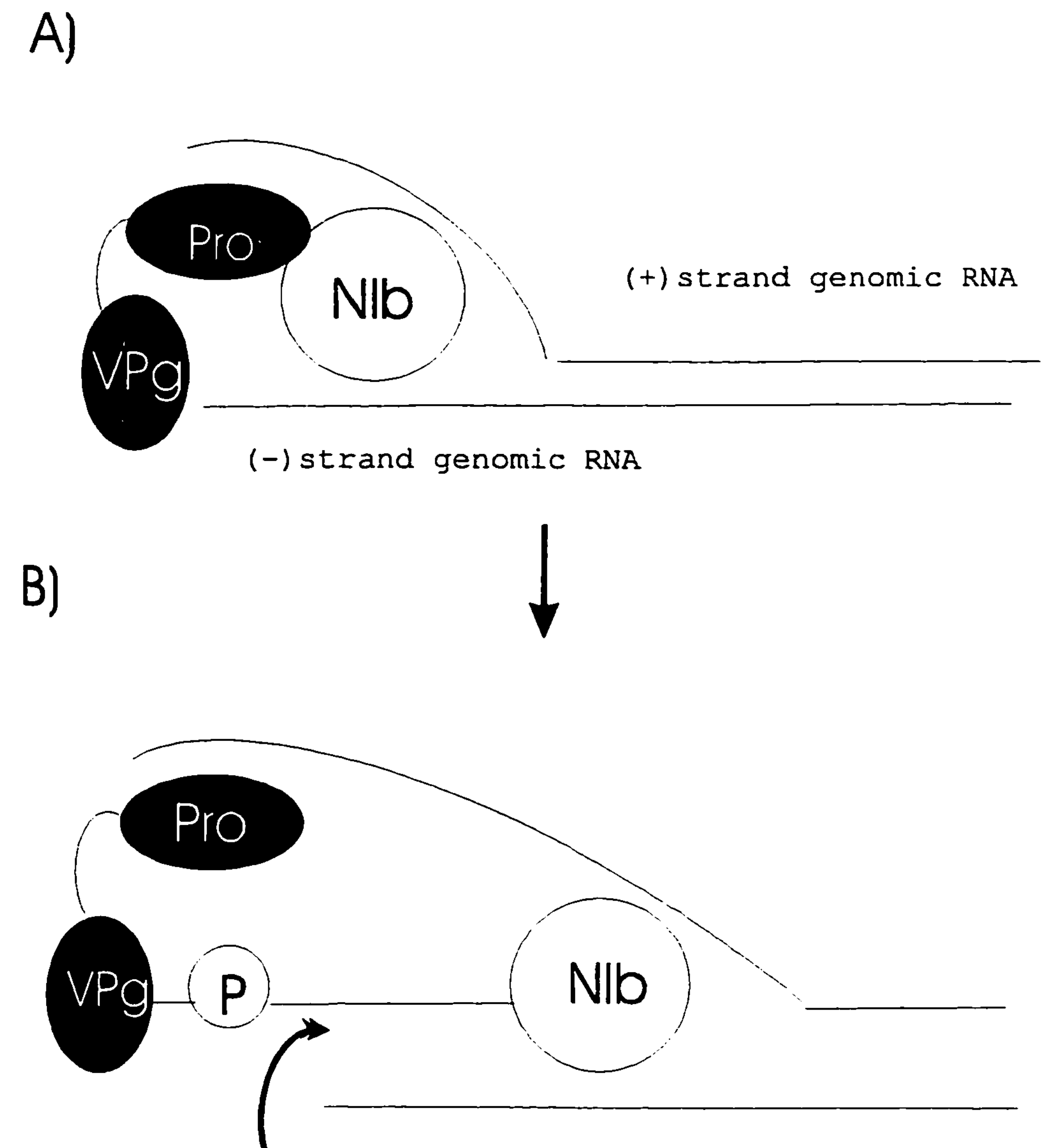
To further characterize the role of the CI protein in the virus life cycle, studies were initiated where mutants of the CI protein were constructed and analyzed for ATPase activity (Chen, 1994). Internal deletion mutants were constructed in the plasmid pET 11a, a bacterial protein expression system which is under the control of an inducible T7 *lac* promoter. The mutants generated, Δ151-633, Δ347–444, and Δ438–633 are missing the amino acids indicated by the numbers in the name of each mutant. Subsequent analysis showed that the Δ151-633 and Δ347–444 mutant proteins did not have ATPase activity, whereas the Δ438–633 mutant protein did have ATPase activity.

As a continuation of this study several other mutants were constructed in the pET 15b protein expression system. This system has the same inducible promoter as the pET 11a plasmid system, however, it also contains a N-terminal his-tag that is fused to the expressed protein to allow for affinity column purification.

This dissertation deals with (1) construction of bacterially expressed mutants of the CI protein in pET 15b, (2) characterization of the ATPase activity of these pET 15b

mutants, (3) the ATP binding ability of CI protein mutants constructed in pET 11a and pET 15b mutants deficient in ATPase activity, (4) the RNA binding ability of all the pET 11a and pET 15b mutants, and (5) analysis of the apparent size difference between the native plant CI and the bacterially expressed full-length constructs of the CI protein.

Figure 3. A model of the suggested interactions of the NIa and NIb proteins during priming of potyviral RNA synthesis as proposed by Li *et al.*, 1997. **A)** The VPg portion of the NIa protein associates with the genomic RNA while the Pro portion of the NIa interacts with the NIb (the RNA-dependent RNA polymerase) to form a priming complex **B)** After initiation of synthesis, the VPg portion of the NIa protein is covalently linked through a tyrosine residue to the new strand of viral RNA at the 5'-phosphate group.


A)
Pro
NIb
VPg
(+)strand genomic RNA
(-)strand genomic RNA
B)
Pro
VPg
P
NIb
Newly synthesized (+)strand genomic RNA with VPg attached

Materials and Methods.

Virus, cDNA Clone and Antisera.

The highly aphid-transmissible strain of TEV (TEV-HAT) was obtained from Dr. C. D. Atreya of the University of Kentucky, KY. The virus was propagated in *Nicotiana tabacum* L var *Xanthi* n.c. plants grown in university greenhouse facilities. The full-length CI gene clone in pET 11a, pCI, was previously generated in our laboratory (Chen, S., Ph.D. Dissertation, 1994). The antisera to TEV-CIP used in this study was prepared in our laboratory (Baunoch, D. Ph.D. Dissertation, 1991, Wayne State University).

TEV cylindrical inclusion protein preparation and purification.

Tobacco leaves (*Nicotiana tabacum* L var *Xanthi* n.c.) systemically infected with TEV-HAT were used as the source for TEV cytoplasmic inclusion (CI) bodies. The CI purification was performed according to the method described by Heibert (Heibert *et al.*, 1984) as modified by Lain (Lain *et al.*, 1990).

Gel electrophoresis and Western Blot analysis of proteins.

All protein SDS-PAGE (Laemmli, 1970) and western blots (Towbin *et al.*, 1979) were carried out according to standard protocols for the BIO-RAD Mini Protean II units (Bio Rad Labs, Hercules, CA).

Recombinant DNA techniques.

Recombinant DNA and cloning procedures were carried out by standard methods (Sambrook *et al.*, 1989) or according to supplier's protocols. All plasmids were maintained in the DH5α strain of *E. coli* for DNA purification and manipulations. Three plasmids were used for the work described here, pET 15b, pET 11a, (Novagen Inc., Madison, WI), pBS SK(+/-) (Stratagene, Inc., La Jolla, CA) and the pCI plasmid which

contains the full-length CI gene in pET 11a (Chen, 1994).

Generation of the full-length CI clone and N-terminal deletions.

The plasmid pET 15b (Novagen, Inc., Madison, WI) contains an N-terminal histidine tag that allows for affinity purification of the fusion protein on a Ni^{2+} column. By incorporating the cleavage site for the NIa TEV protease (available commercially from Gibco-BRL) it is possible to remove the histidine tag from the fusion, leaving only the native CI amino acid residues. All constructs described in this thesis that have the TEV protease site and are cloned into pET 15b are designated by the prefix pro-.

All polymerase chain reactions (PCR) were carried out with the Pwo DNA polymerase (Boehringer-Mannheim, Indianapolis, IN) and the reaction conditions varied for each of the PCRs carried out. For engineering the expression of the TEV-HAT CI protein in pET 15b, specific primers were used to amplify the CI gene from the pCI plasmid by polymerase chain reaction (PCR). The fragment encompasses the complete 633-amino acid coding region of the CI from plasmid pCI and the TEV protease site. The sequence of the 5' primer used for generation of the full-length CI and the two internal deletion mutants is:

5'-GCGCGGATCC*GAGAACCTCTACTTTCAG***AGTTTGGATGA**-3'

It incorporates the cleavage sequence for the TEV protease (Carrington and Dougherty, 1988). The 3' primer was the same for all of the constructs described, its sequence is: 5'-GCGCGGATCCTCA**TTGGAGATAGATAGTTTC**-3'. It incorporates a translation termination codon immediately following the codon of the last amino acid (residue 633) of the mature CI protein.

The sequences of the three specific 5' primers for the N-terminal deletions are:

ΔN40: 5'-GCGGATCC*GAGAACCTCTACTTTCAGGGA***CAAATCAGCCGAGGC**-3'

ΔN80: 5'-GCGGATCC*GAGAACCTCTACTTTCAGGGA***GTGAGAGGTGCT**-3'

ΔN95: 5'-GCGCGGATCC**TACCATTTATCAAAGAGAGGGAGAGTG**-3'

The ΔN40 mutant removes the first N-terminal 40 amino acids of the CI protein. The ΔN80 mutant removes the first 80 N-terminal amino acids of the CI protein and the ΔN95 mutant deletes the first 95 N-terminal amino acids of the CI protein.

For all primers, the nucleotides in **bold** indicate the TEV-HAT positive- or complementary-strand sequences, *italicized* bases represent the TEV protease cleavage sequence, and underlined sequences are the BamHI restriction site that allow for cloning of the PCR fragments into the BamHI site of the pET 15b bacterial expression vector.

PCR generation of the internal deletion mutants.

Two internal deletion mutants were generated by recombinant PCR (Higuchi, 1990) to delete the conserved domains I and IA of the CI protein. Two sets of primers were required to do each recombinant PCR. The internal primers were designated IA upstream (the internal 3' primer) and IA downstream (the internal 5' primer). The external primers used were the same ones used to generate the full length CI protein. The PCR proceeded in two steps. The first was the generation of the two fragments that lack the IA domain. The second used the upstream and downstream fragments of the first reaction and the external 5' and 3' primers to regenerate the in-frame, full-length clone minus the spliced out deleted sequence (Figure 4).

The three primer sets used were:

Set 1. Generates the 5' and 3' ends of the deletion mutants, which are identical to the full-length CI protein. This set is used for both of the internal deletion mutants.

5' primer is: 5'-GCGCGGATCC*GAGAACCTCTACTTTCAG***AGTTTGGATGA**-3'

3' primer is: 5'GCGCGGATCCTCA**TTGGAGATAGATAGTTTC**-3'

Set 2. Domain I deletion internal mutants. These were used to generate, in conjuction with Set 1, the two PCR fragments that had the domain I sequences deleted from them.

Upstream 3' primer: 5'-CCCGCAAGAGATTTTCTTTACCATTTATCAAAGGAG-3'

Downstream 5' primer:5'-TCTCTTTGATAAATGGTAAAGAAAATCTCTTGCGGG-3'

Set 3. Domain IA internal deletion mutants. This set was used in conjuction with Set 1 to generate the two PCR fragments that had the domain IA sequences deleted from them.

Upstream 3' primer: 5'-CTTGTGCATGTTATCCACTCTCCCTCACTT-3'

Downstream 5' primer: 5'-AAGAGAGGGAGAGGGGATAACATGCACAAG-3'

Cloning of the full-length and mutant genes into pET 15b.

Cloning of the fragments was done by utilizing the Bam HI site of pET 15b. Since the fragments have a Bam HI site at each end, this resulted in a non-directional cloning event. This necessitates a two-step screening process to verify the presence of insert and its orientation. The transformants were initially digested with Bam HI to verify the presence of the inserted fragment. They were then analyzed by restriction mapping for the orientation of the 5'end of the insert with respect to the pET 15b RNA polymerase promoter. Orientation was determined by the size of the restriction fragments generated. The full-length CI (proCI) and mutants ΔN40, ΔDom I, and ΔDom IA were screened for orientation using Eco RV. The mutants ΔN80 and ΔN95 were screened using either Sph I or Ssp I.

Sequence verification of the mutants.

After verification of the orientation of the inserts, the constructs were sequenced across the ligation junctions and the site (region) of the mutations. All sequencing was

carried out on an ALF Automated Sequencing System (Pharmacia Biotech, Inc., Piscataway, NJ) using fluorescein labeled primers. The primers were obtained from the Biological Sciences Molecular Core Facility (Wayne State University, Detroit, MI). The pET T7 promoter primer (Novagen, Inc.) was used to sequence across the 5' Bam HI insertion site of both the pET 15b and pET 11a constructs. This primer was also used to verify the deletions for the ΔN40, ΔN80, and ΔN95 mutants. Internal primers were designed to sequence across the deletions of the ΔDom I and ΔDom IA mutants. The pET terminator primer (Novagen, Inc.) was used to sequence the 3' Bam HI site of all the pET 15b and 11a constructs.

The pET 11a constructs pCI (the full-length CI), pCI Δ151-633, pCI Δ347-444, and pCI Δ435-633, were also sequenced. The pET T7 promoter primer (Novagen, Inc.) was used to sequence the 5' Bam HI site and the pET terminator primer (Novagen, Inc.) across the internal deletions. The internal primers for the sequencing of the pET 11a constructs were:

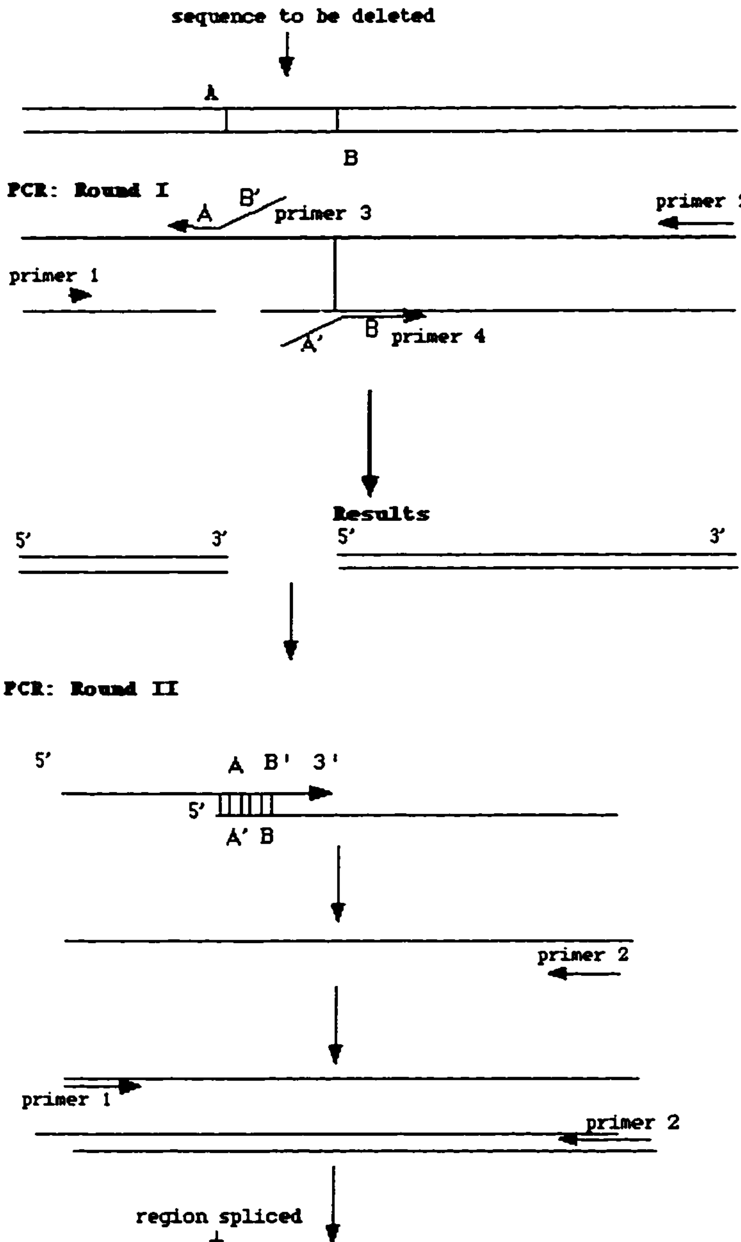
pCI Δ151-633: 5'-TGTTAATGCTTGAGCCTAC-3'

pCI Δ347-444: 5'-ATTTGCCAGTGACAACACC-3'

pCI Δ435-633: 5'-TGAAGAGTGGAGGAACTG-3'

Expression and purification of the full-length CI and mutants in HMS174(DE3). Log-phase cultures of transformed *E. coli* strain HMS174(DE3) were adjusted to 1.0 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) to induce expression, and then grown for an additional 3 hours at 37 °C with vigorous shaking at 250 rpm. The cells were harvested by centrifugation and stored at -70°C. The cells were thawed and resuspended

Figure 4. Schematic representation of the recombinant PCR method for generating internal deletion mutants. In the first round of PCR, two fragments were generated, using a set of internal and external primers, that lack the region to be deleted. Each fragment, designated upstream and downstream with respect to the deleted region, contained complementary sequences to the other fragment. During the second round of PCR, the fragments prime off one another to give back the gene in frame minus the region to be deleted.


sequence to be deleted
A
B
PCR: Round I
A
B'
primer 3
primer 2
primer 1
A'
B
primer 4
Results
5'
3'
5'
3'
PCR: Round II
5'
A B' 3'
5'
A' B
primer 2
primer 1
primer 2
region spliced

in 20 ml of buffer A (50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 2.0 mM EDTA, and 100 mg/ml phenylmethyl-sulphonyl-fluoride (PMSF) and 0.2 mg/ml lysozyme) and incubated on ice for 20 min. Triton X-100 was added to a final concentration of 1.0% and the cell suspensions were incubated for an additional 10 min on ice before extensive sonication. The lysates were centrifugated at 11,000*g* for 20 min. The resulting pellets were washed three times with buffer W (1% Triton X-100, 25% sucrose, 5.0 mM EDTA, and 100 mg/ml PMSF in phosphate buffered saline (PBS) (Sambrook *et al.*, 1989)). This was done by resuspending the pellets in buffer W and incubating on ice for 10 min. Additional sonication was required at times to resuspend the pellets completely. The protein was collected by centrifugation at 25,000*g* for 10 min. The final pellets were resuspended in buffer A and loaded onto a step sucrose gradient. The gradient consisted of 3 steps of 50%, 60%, and 80% sucrose in 25 mM Tris-Cl, pH 7.5.

The sucrose gradients were ultracentrifuged at 21,000*g* for 1 hour at 4°C in a Beckman SW 28.1 swinging bucket rotor. After centrifugation 1.0 ml fractions were collected using a Buchler fractionator (Fort Lee, NJ). Each fraction was analyzed for protein content by BIO-RAD protein microassay and ATPase activity by the ATPase assay. Those fractions containing protein and ATPase activity were further analyzed by SDS-PAGE and western blot immunoassay.

ATPase assay.

The ATPase assay used is based on a modification of the procedures described by Lain and coworkers (Lain *et al.*, 1990). Approximately 50ng of protein was mixed with 25 μl of ATPase buffer (15 mM HEPES-KOH, pH 7.5, (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 2.5 mM magnesium chloride and 1.0 mM dithiothreitol (DTT)) and 0.5 μCi of [α-^{32}P]ATP (3000 Ci/mmol) (Amersham Life Sciences, Arlington Ht., IL). The reactions were incubated at room temperature for 15 min. and stopped by the

addition of EDTA to a final concentration of 0.2 M.

The ATPase reaction for the sucrose fractions was performed using 6μl of each fraction in 30 μl of the ATPase buffer described above. Larger sample volumes could not be used due to the presence of high concentrations of sucrose in the last fractions from the 60 and 80% gradient regions. The PEI-cellulose chromatography was carried out as described below.

After completion of the reaction, 1.0 μl of the reaction mixture was spotted onto polyethyleneimine (PEI) cellulose chromatography plates (Sigma Chemicals, St. Louis, MO) and ascending chromatography was done using 0.15 M formic acid-0.15 M LiCl as the mobile phase solvent. The dried PEI-cellulose plates were then autoradiographed and quantitative analysis was performed with an Ambis Image Acquisition and Analysis System (Ambis, Inc., San Diego, CA).

RNA binding by UV cross-linking and Northwestern Assay.

^{32}P-labeled RNA transcripts were prepared by transcribing pBluescript SK(+/-) phagemid (Stratagene, Inc., La Jolla, CA) with either T7 RNA polymerase or with T3 RNA polymerase (both polymerases were purchased from Gibco-BRL, Gaithersburg, MD) in the presence of [α-^{32}P]UTP or [α-^{32}P]ATP (Amersham Life Sciences, Arlington Ht., IL) according to the protocol provided by the company. The T7 transcripts were generated using pBluescript that was linearized with either SmaI or BamHI and the T3 transcripts were generated with pBluescript that had been linearized with either Eco RV or Hind III (all restriction enzymes are from Promega, Madison, WI). Cross-linking by UV light was carried out by incubating approximately 1.0 μCi of [α-^{32}P]UTP labeled RNA with 250ng of protein in 25 μl of cross-linking buffer (20 mM HEPES-KOH, pH 7.5, 2.5 mM magnesium chloride and 1.0 mM dithiothreitol (DTT)). Samples were UV

irradiated in open 0.6 ml microfuge tubes in a Stratagene Stratalinker UV cross-linker (Stratagene Inc., La Jolla, California) at 1.8 J of energy (254 nm wavelength) for 20 min (Lain *et al.*, 1991).

Unprotected RNA was digested with 5.0 μg of RNase A at 37 °C for 30 min. The cross-linked RNA-protein complexes were then subjected to 8% SDS-PAGE electrophoresis (Laemmli, 1970). The gels were stained, dried and autoradiographed.

RNA binding was also studied by Northwestern Assay. The proteins were run on a 10% SDS-PAGE and then transferred to a nitrocellulose membrane by western blotting. The proteins were subsequently renatured in buffer R (10mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 50 mM NaCl, 0.1% Triton X-100, and 1X Denhardt's reagent) for 1 hour at room temperature. This was repeated 4 more times. The membrane was incubated in a sufficient volume to allow free movement during gentle agitation. The membrane was then incubated in 2.0 ml of buffer R containing approximately 5 x 10^5 cpm of labeled RNA for a minimum of 1 hour at room temperature without agitation. After 3 washes (15 min each) in buffer R, the membrane was air dried and stained with Ponceau S (Schneppenheim *et al.*, 1991) to visualize the proteins. The blot was photographed, air dried and autoradiographed.

ATP binding by cross-linking studies.

The ATP binding cross-linking assays were performed using 10μCi of [α-^{32}P]ATP (3000 Ci/mmol) with approximately 250 ng of protein in the buffer detailed above. The samples were cross-linked in open 0.6 ml microfuge tubes in a Stratalinker at 1.8 J of energy for 20 min (Lain *et al.*, 1991). The cross-linked ATP-protein complexes were then subjected to 8% SDS-PAGE electrophoresis. The gels were stained, dried and autoradiographed.

Sub-sequence Analysis of the CI protein.

Sub-sequence analysis of the CI protein to determine the presence of post-translational modifications signal sequences was carried out using the MacVector 5.0 computer analysis program. The CI sequence was entered into the program (amino acids 1-633) according to the sequence determined for the CI protein by Allison *et al.*, and the sequence was analyzed for protein sub-sequences, short protein motifs and long protein motifs.

Periodic acid-Schiff's (PAS) staining for the detection of glycosylated proteins.

CI protein purified from plants, bacterially expressed proCI protein, and proteins known to be glycosylated, were subjected to 10% SDS-PAGE and stained for glycosylation (Fukuda and Kobata, 1993). The gel was fixed for 1 hour in fix solution (10% acetic acid, 40% methyl alcohol, 50% distilled water). The gel was then soaked in freshly prepared periodate solution (0.7 gm periodic acid in 100 ml 5% acetic acid/distilled water) for 1 hour. The gel was then washed briefly with distilled water and soaked in 50 ml of freshly prepared *meta*-bisulfite solution (0.2 gm *meta*-bisulfite in 100 ml of 5% acetic acid/distilled water) until the entire gel turned yellow. The gel was transferred to fresh *meta*-bisulfite solution and incubated for 10-15 minutes or until the gel had just decolorized. The gel was then incubated in Schiff's Reagent (Fukuda and Kobata, 1993) until pink or red bands appear. After 1 hour no increase in band intensity was detected. The gel was then photographed and stained with Coomassie Blue to detect the non-glycosylated proteins.

Immunoblot Detection of Proteins that have Tyrosine Phosphorylation.

This method is based on the known affinity of the commercially available PY-20 biotinylated polyclonal antibody for proteins that have phosphorylated tyrosine residues (Transductions Laboratories, Lexington, KY). CI protein isolated from plants, bacterially expressed proCI protein, and tyrosine phosphorylated control proteins, were subjected to

10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 15 min at room temperature with 1% non-fat dry milk powder in TTBS (Tris buffered saline, 20mM Tris (pH 7.5), 500mM NaCl, 0.1% Tween 20). The blot was then washed 3x 5 min each wash in TTBS.

The blot was incubated overnight with PY-20 biotinylated primary polyclonal antibody. The PY-20 antibody was used at a 1:200 titer in TTBS with gentle agitation at room temperature. The blot was then washed 3 x 5 min. each wash in TTBS and incubated with ExtrAvidin-AP (streptavidin conjugated to alkaline phosphatase activity) (Sigma Biochemicals) at a 1:10,000 titer in TTBS for 1 hour at room temperature. The blot was then washed 3 x 5 min each wash in TTBS. The blot was developed using 15 mg NBT (nitroblue tetrazolium, Sigma Biochemicals) in 1.0 ml of 70% N,N,-dimethylformamide (DMF) and 7.5 mg BCIP (5-bromo-4-chloro-indoylyphosphate, Fisher Scientific) in 1.0 ml of 100% DMF. The NBT and BCIP were added to 50 ml of carbonate buffer (0.1M $NaHCO_3$, pH 9.8, 1.0 mM $MgCl_2$) and then added to the blot. Development was allowed to occur in the dark without agitation for 5 min. to several hours until the bands appear. The blot was then photographed, air dried and stored.

Results.

Expression of the CI protein and mutants in bacteria.

The CI gene was cloned into the *E. coli* expression vector pET 15b under the control of the T7 *lac* promoter as described in the materials and methods. This was done in order to introduce mutations into the gene and analyze the biochemical activities of the mutant proteins generated with regard to ATPase activity, ATP- and RNA- binding abilities.

Five mutants were constructed along with the full-length CI protein. ProCI is the full-length CI clone that contains a His tag and the TEV NIa protease cleavage sequence at the N-terminus. The ΔN40, ΔN80, and ΔN95 mutants are N-terminal deletions that delete the first 40, 80 and 95 amino acids respectively from the N-terminus of the full-length CI protein. The ΔDom I and ΔDom IA are internal deletion mutants that delete domain I, which contains amino acids 81-94, and domain IA, which contains amino acids 105-114.

In all six constructs, the protein was fused to the His tag of the pET 15b vector and incorporated the peptide cleavage signal for the TEV NIa protease. This results in a combined 23 amino acid tag that is N-terminally fused. A termination codon was introduced after the last amino acid encoded for by the CI protein. This results in recombinant proteins that are all fusion proteins. Figure 5 outlines all of the constructs discussed in this text and the location of the seven conserved domains in the CI protein. The pET 15b and pET 11a constructs are shown here, including the full-length CI protein constructs and all mutants.

Figure 5. A schematic representation of all the pET 11a and pET 15b constructs.

Tag: Amino acid residues that are fused to the N-terminus of the proteins.
His: there is a His tag with the TEV protease signal built in after the His tag fused to the N-terminus of the pET 15b constructs
14aa: there are 13 amino acids from the gene 10 protein of the bacteriophage T7 and an AUG fused to the N-terminus of the pET 11a constructs.

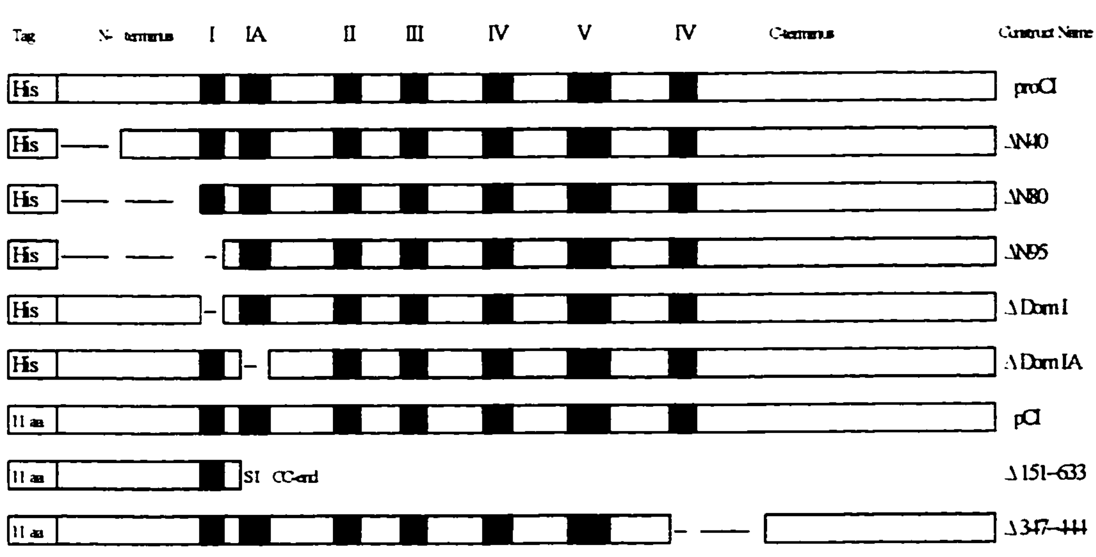
N-terminus: is here referred to as the first 80 amino acids of the native CI sequence up to domain I.

Conserved domains: are shown in relative scale and are represented by the shaded areas and numbered with Roman numerals.

C-terminus: is here referred to as all amino acids downstream of the last residue of domain VI.

Unboxed, hypenated areas represent where regions of the protein sequence have been deleted. The numerals in the names of the constructs indicate which amino acids or domains have been deleted from the protein.

All of the plasmid constructs shown here were sequenced for verification.


Tag
N- terminus
I
IA
II
III
IV
V
IV
C-terminus
Construct Name
His
proCI
ΔN40
ΔN80
ΔN95
Δ Dom I
Δ Dom IA
11 aa
pCI
S1 CC-end
Δ 151-633
Δ 347-444

The plasmids with each construct were transformed into the HMS174(DE3) expression strain of *E. coli.* After induction with IPTG, all plasmid containing strains showed the presence of induction specific proteins of the new expected sizes. The proteins were all expressed at different levels, with the full-length proCI showing the smallest amount of induction with the ΔDom I and ΔDom IA mutants showing the next lowest yields (observation, data not shown). All the proteins were present in an insoluble aggregate form when isolated from the bacteria. No proteins of any size from IPTG induced pET 15b/HMS174(DE3) reacted with the anti-TEV CI antiserum (figure 13).

Purification of bacterially expressed proteins.

Extracts from all induced cell lines were processed and fractionated as described in Materials and Methods. Proteins from the gradient fractions were subjected to 10% SDS-PAGE. In all cases, proteins of the predicted sizes were detected, as well as several bands of smaller sizes. Due to their reactivity with anti-TEV CI serum and their absence in the pET 15b/HMS174(DE3) samples, these smaller proteins were considered to be truncations or degradation products of the CI mutants. Initially, affinity purification was attempted using the His tag on all the pET 15b constructs, however the proteins are highly insoluble even in 6M guanidinium and 8M urea, and hence no or little binding to the columns was obtained (data not shown). An alternative method of purification based on sucrose density gradient purification, described in the materials and methods, was chosen based on the large size of the protein inclusions.

ATPase activity was detected in fractions 10-12 for the proCI and ΔN40 proteins (figures 7C and 8C) in fractions 10 and 11 for the ΔN80 and ΔDom IA proteins (figures 9C and 12C). ATPase activity above background levels was not detected in any fraction for the ΔN95 or ΔDom I mutants (figures 10C and 11C). ATPase activity was not

detected in the HMS174(DE3)/pET 15b sample fractions at a level higher than that of the background (figure 6C). The bulk of the ΔN95 protein consistently isolates to the last and pellet fraction of the gradient. The reason for this behavior in the case of ΔN95 is not known, and it is speculated that the mutation may have caused structural changes that cause the formation of super-aggregates. The appropriate fractions for each sample were pooled and used for future experiments. Fraction 14 was collected for the ΔN95 mutant and used for all experiments. The pooled fractions were dialyzed to remove the sucrose and subjected to SDS-PAGE to determine the purity of the protein fractions. Figure 13 shows all the dialyzed fractions along with pET15b/HMS174(DE3) controls.

Characterization of ATPase activity.

ATPase assays were carried out as described previously in the material and methods. Proteins from the pooled fractions were analyzed and roughly 250ng of protein was assayed. The full-length proCI protein and the ΔN40, ΔN80 and ΔDom IA mutants all possess ATPase activity. The ΔDom I and ΔN95 mutants lack ATPase activity

Quantitative analysis shows the relative levels of activity for each of the mutants compared to the native plant isolated CI protein and the bacterially expressed full-length proCI protein (figure 14). Table 2 summarizes the results of the ATPase activity assay.

Table 2. Summary of ATPase activity of all full-length CI proteins and mutants.

Protein and Result of ATPase Assay									
proCI	ΔN40	ΔN80	ΔN95	ΔDom I	ΔDomIA	PCI	Δ151-633	Δ347-444	Δ438-633
(+)	(+)	(+)	(-)	(-)	(+)	(+)*	(-)*	(-)*	(+)*

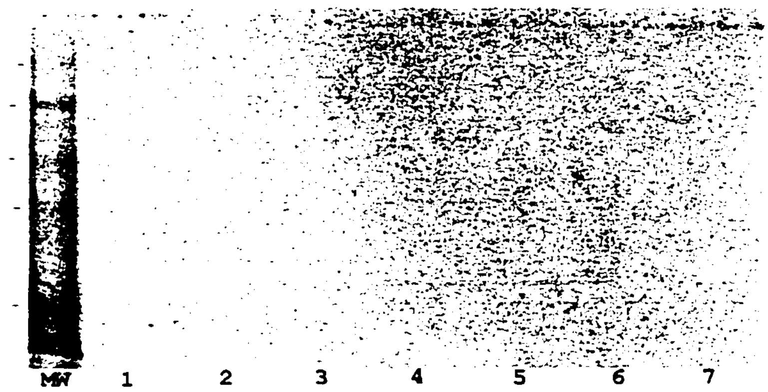
* These results were obtained from S. Chen. Ph.D. Dissertation 1994.
(+) indicates the presence of ATPase activity
(-) indicates the absence of ATPase activity

ATP Binding Analysis.

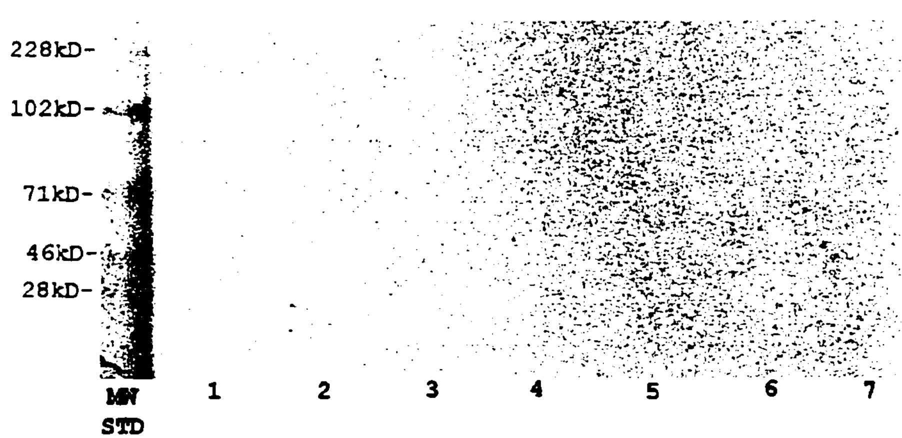
UV crosslinking studies were carried out as previously described. Roughly 250ng of each protein was analyzed for ATP binding ability. The full-length pCI protein and ATPase deficient mutants, ΔN95, ΔDom I, Δ151-633 and Δ347-444, and the ATPase

Figure 6. Analysis of the pET 15b/HMS174(DE3) sucrose fractions. Each set of fractions was analyzed by SDS-PAGE, Western blot and ATPase assay with quantitation by Ambis Image Analysis. **A)** SDS-PAGE was done on 30 μl of every other sucrose fraction and stained with coomassie blue. Lanes 1-7 are fractions 2, 4, 6, 8, 10, 12 and 14 respectively in both the SDS-PAGE and Western blots. **B)** Western blot analysis of every other fraction as described in A. **C)** The autoradiograph of the ATPase assay performed on every fraction collected as described in the materials and methods. All fractions here are spotted in duplicate, B is the blank control and contains no protein, set 1 is CI protein from plants used as a positive control, sets 2-15 are fractions 1-14 respectively. **D)** Ambis quantitation of the ATPase autoradiograph graphed to determine where the ATPase activity is localized to in the sucrose gradient. Note the lack of proteins comparable to the CI protein in A, the lack of CI reactive proteins in B, and the lack of ATPase activity in C and D as compared with figure 7.

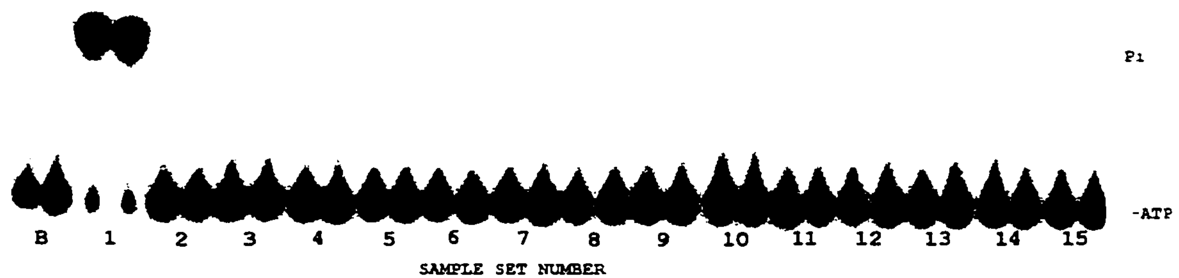
A) pET 15b/HMS174(DE3) SDS-PAGE.



B) pET 15b/HMS174(DE3) Western Blot.



C) Autoradiograph of ATPase assay of pET 15b/HMS174(DE3) sucrose fractions.



D) Ambis quantitation of pET 15b/HMS174(DE3) sucrose fraction ATPase assay.

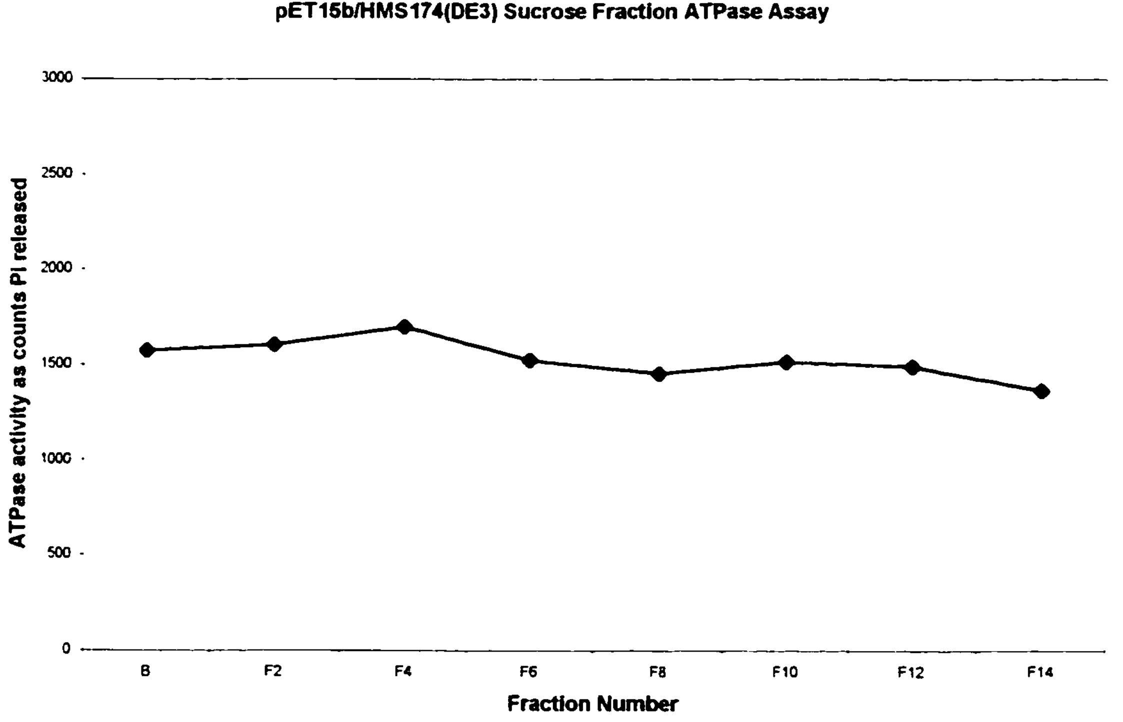
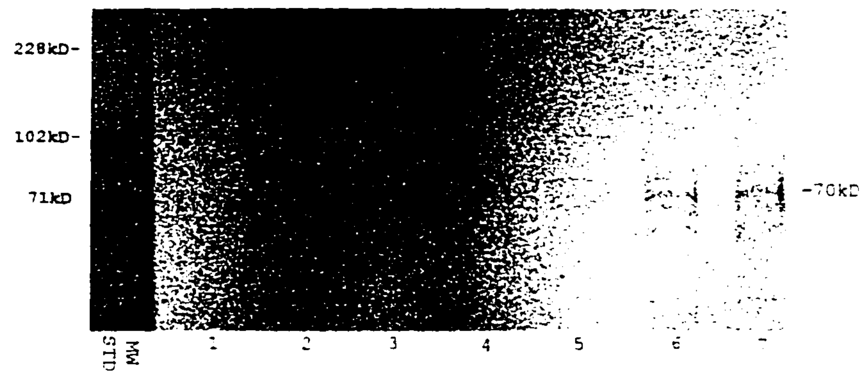


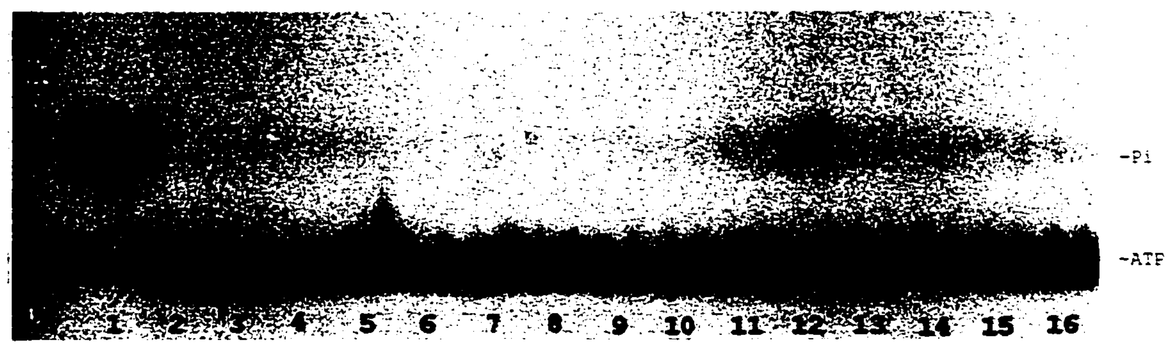
Figure 7. Analysis of the proCI sucrose fractions. Each set of fractions was analyzed by SDS-PAGE, Western blot and ATPase assay with quantitation by Ambis Image Analysis. **A)** SDS-PAGE was done on 30 μl of every other sucrose fraction and stained with coomassie blue. Lanes 1-7 are fractions 2, 4, 6, 8, 10, 12 and 14 respectively in both the SDS-PAGE and Western blots. **B)** Western blot analysis of every second sucrose fraction as described in A. **C)** The autoradiograph of the ATPase assay performed on every fraction (in duplicate) as described in the materials and methods, B is the blank control and contains no protein, set 1 is CI protein from plants used as a positive control, sets 2-15 are sucrose fractions 1-14 respectively. **D)** Ambis quantitation of the ATPase autoradiograph graphed to determine where the ATPase activity is localized to in the sucrose gradient. Note the presence of a 70 kD protein in A, and that these proteins are CI reactive in B. Note also that the ATPase activity observed isolates to those fractions that contain the CI reactive proteins.

A) proCI SDS-PAGE.



B) proCI Western Blot.

C) proCI autoradiograph of ATPase assay of sucrose fractions.



D) Ambis quantitation of proCI sucrose fraction ATPase assay.

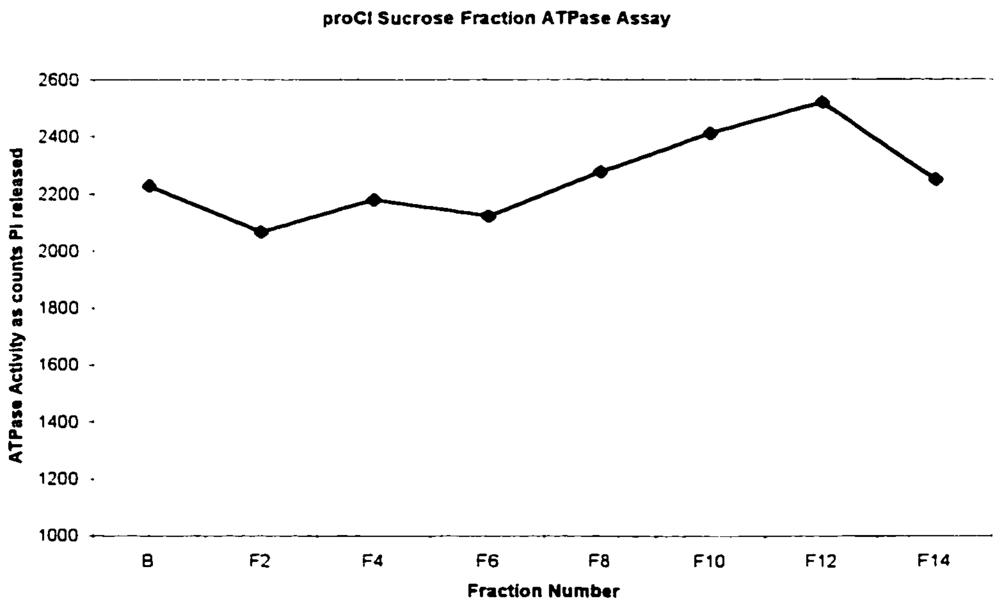
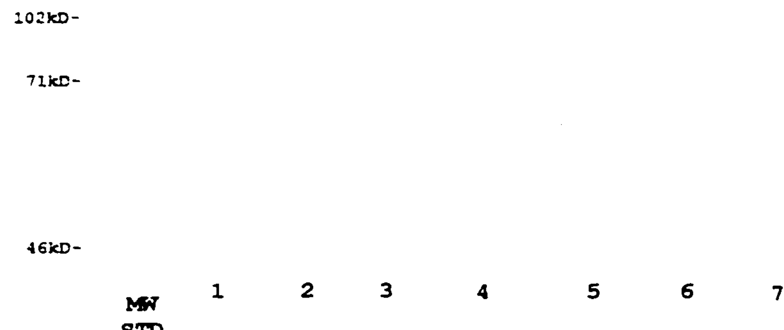
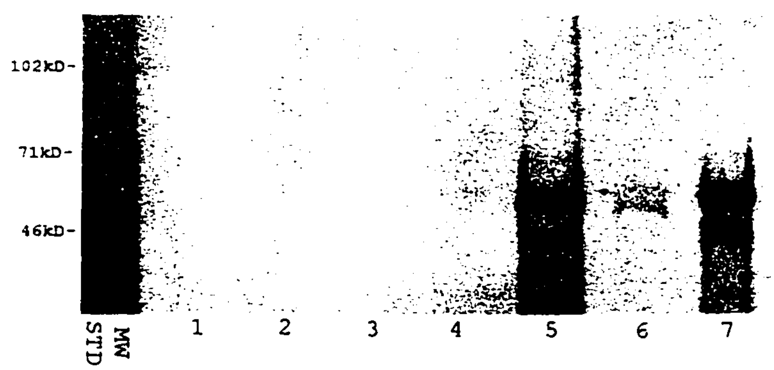


Figure 8. Analysis of the ΔN40 sucrose fractions. Each set of sucrose fractions was analyzed by SDS-PAGE, Western blot and ATPase assay with quantitation by Ambis Image Analysis. **A)** SDS-PAGE was done on 30 μl of every other sucrose fraction and stained with coomassie blue. Lanes 1-7 are sucrose fractions 2, 4, 6, 8, 10, 12 and 14 respectively in both the SDS-PAGE and Western blots. **B)** Western blot analysis of every second sucrose fraction as described in A. **C)** The autoradiograph of the ATPase assay performed on every sucrose fraction (done in duplicate) as described in the materials and methods, B: is the blank control and contains no protein, set 1 is the CI protein from plants used as a positive control, sets 2-15 are sucrose sucrose fractions 1-14 respectively. **D)** Ambis quantitation of the ATPase autoradiograph graphed to determine where the ATPase activity is localized to in the sucrose gradient. Figure 8A shows the presence of a protein that corresponds to the predicted size of the ΔN40 mutant and figure 8B shows that this protein is reactive against the CI antiserum. Figures 8C and 8D demonstrate that the ATPase activity detected is present in those sucrose fractions that contain the 67.7 kD CI reactive protein.

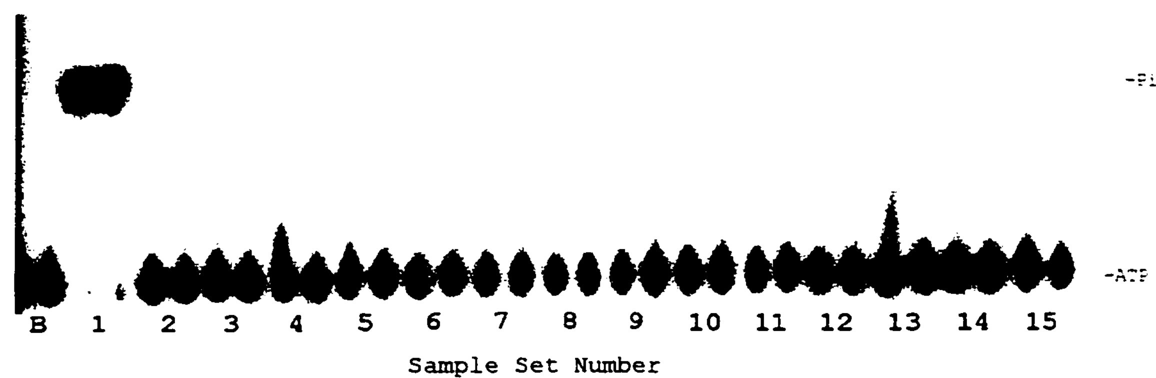
A) ΔN40 SDS-PAGE



B) ΔN40 Western Blot.



C) ΔN40 autoradiograph of ATPase assay of sucrose fractions.



D) Ambis quantitation of ΔN40 sucrose fraction ATPase assay.

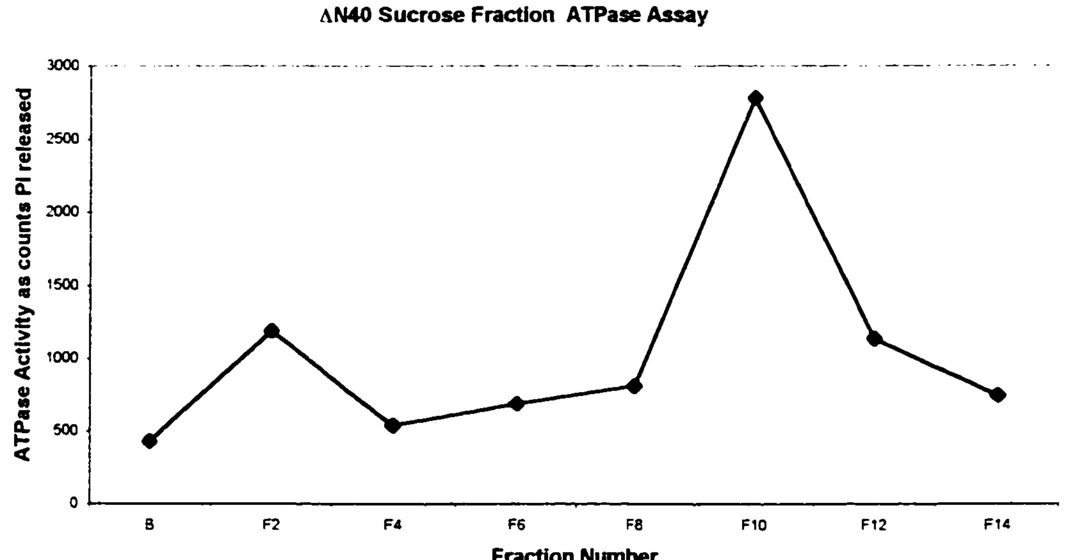
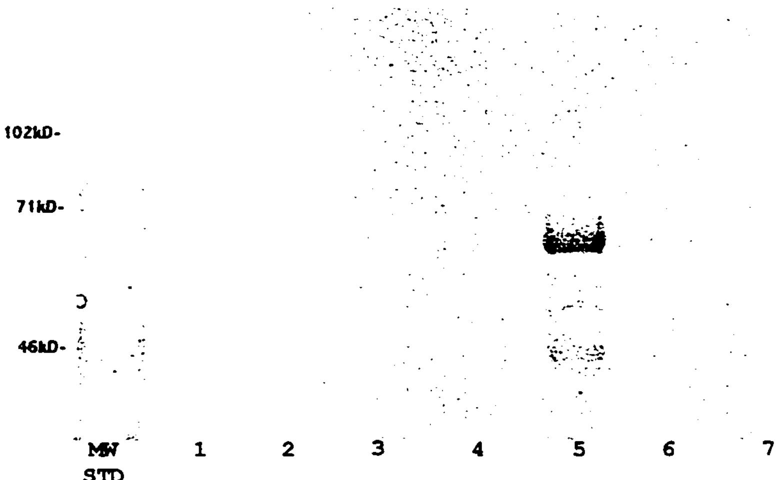
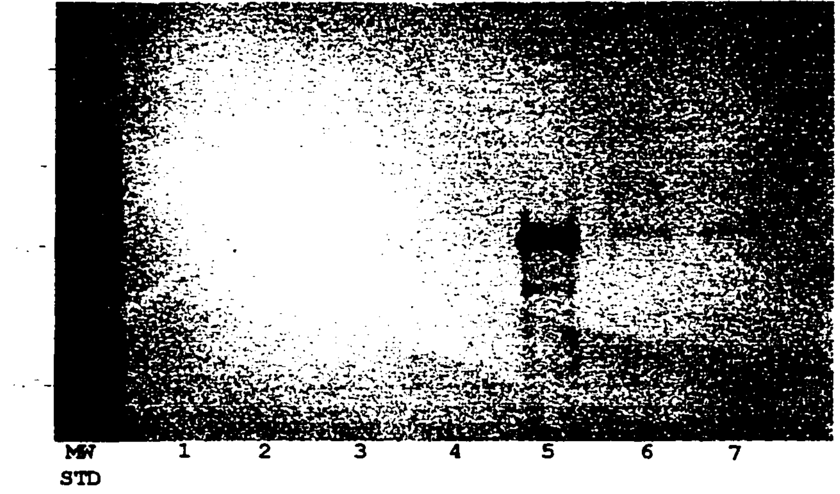


Figure 9. Analysis of the sucrose fractions for ΔN80. Each set of sucrose fractions was analyzed by SDS-PAGE, Western blot and ATPase assay with quantitation by Ambis Image Analysis. **A)** SDS-PAGE was done on 30 μl of every other sucrose fraction and stained with coomassie blue. Lanes 1-7 are sucrose fractions 2, 4, 6, 8, 10, 12 and 14 respectively in both the SDS-PAGE and Western blots. **B)** Western blot analysis of every other sucrsoe fraction as described in A. **C)** The autoradiograph of the ATPase assay performed on every sucrose fraction (done in duplicate) as described in the materials and methods, B: is the blank control and contains no protein, set 1 is CI protein from plants used as a positive control, set 2-15 are sucrose fractions 1-14 respectively. **D)** Ambis quantitation of the ATPase autoradiograph graphed to determine where the ATPase activity is localized to in the sucrose gradient. Figure 9A shows the presence of a protein that corresponds to the predicted size of the ΔN80 mutant protein. Figure 9B shows that this protein in reactive to the CI antiserum. Figures 9C and 9D show that the ATPase activity seen is localized to the same sucrose fractions that contain the 63 kD CI reactive protein.

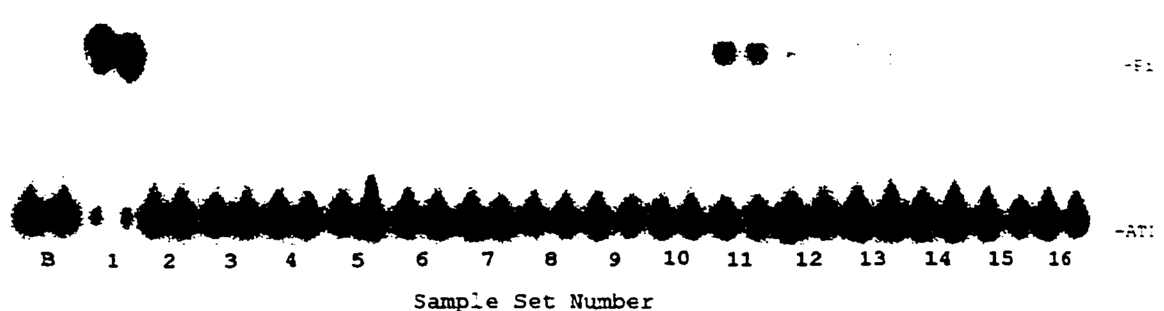
A) ΔN80 SDS-PAGE.



B) ΔN80 Western Blot.



C) ΔN80 autoradiograph of ATPase assay of sucrose fractions.



D) Ambis quantitation of ΔN80 sucrose fraction ATPase assay.

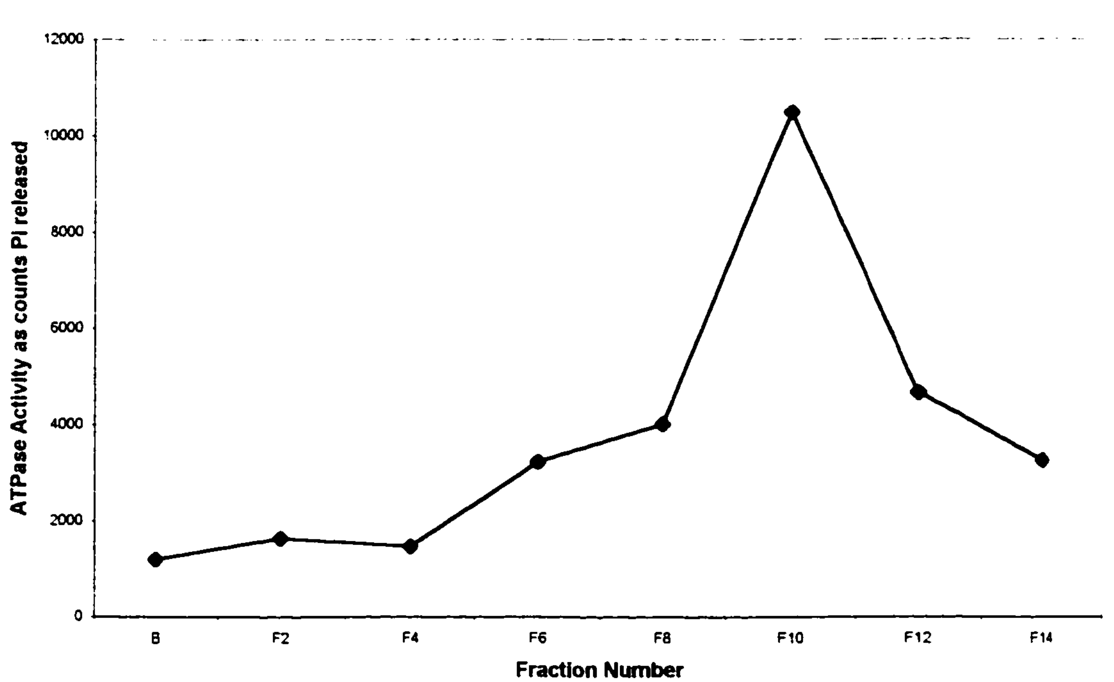
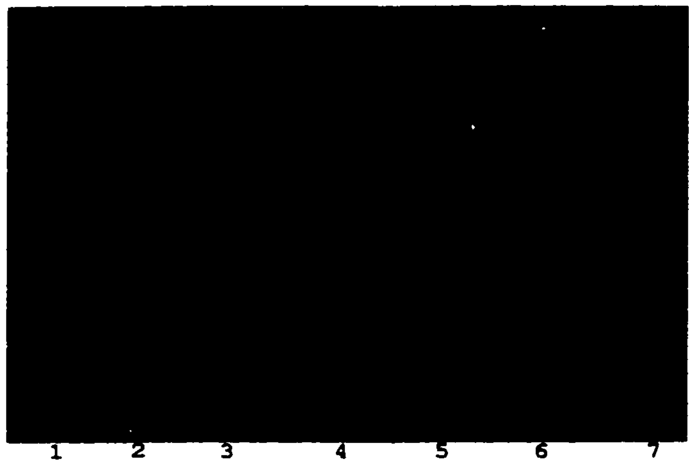
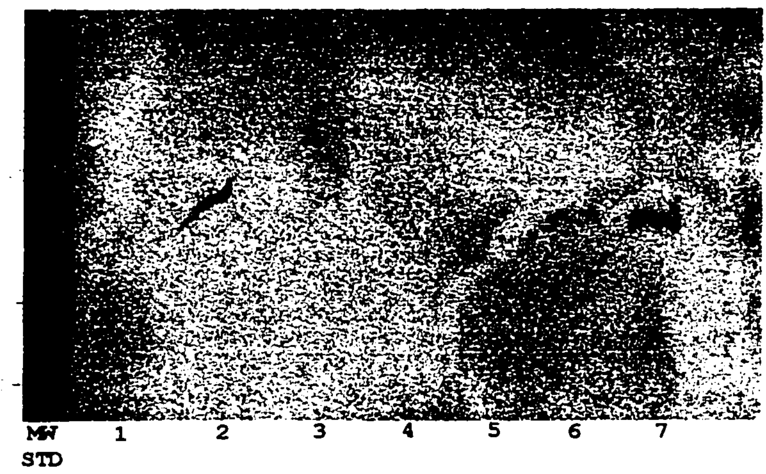


Figure 10. Analysis of the ΔN95 sucrose fractions. Each set of sucrose fractions was analyzed by SDS-PAGE, Western blot and ATPase assay with quantitation by Ambis Image Analysis. **A)** SDS-PAGE was done on 30 μl of every other sucrose fraction and stained with coomassie blue. Lanes 1-7 are sucrose fractions 2, 4, 6, 8, 10, 12 and 14 respectively in both the SDS-PAGE and Western blots. **B)** Western blot analysis of every second sucrose fraction as descrbied in A. **C)** The autoradiograph of the ATPase assay performed on every sucrose fraction (done in duplicate) as described in the materials and methods, B: is the blank control, and it contains no protein, sets 1-15 are sucrose fractions 1-15 respectively, (+) control is CI protein isolated from plants used as a positive control. **D)** Ambis quantitation of the ATPase autoradiograph graphed to determine where the ATPase activity is localized to in the sucrose gradient. Figures 10A and 10B show the presence of a 61.7 kD protein (the predicted size for the ΔN95 mutant protein) that is reactive to the CI antiserum. Figures 10C and 10D show that there is no detectable ATPase activity above the background level for any of the sucrose fractions assayed.

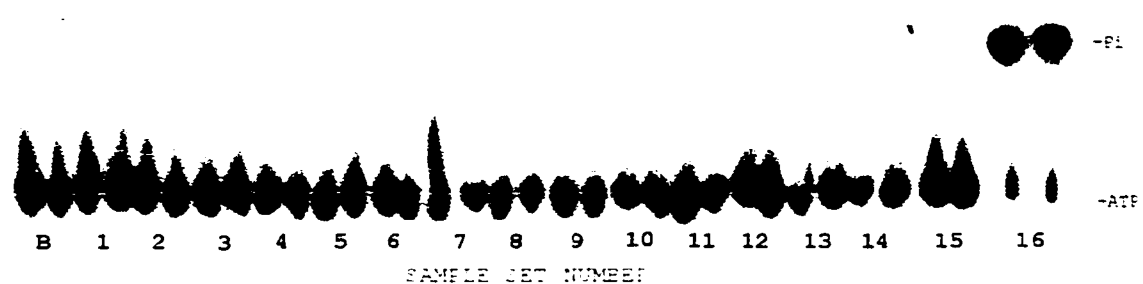
A) ΔN95 SDS-PAGE.



B) ΔN95 Western Blot.



C) ΔN95 autoradiograph of ATPase assay of sucrose fractions.



D) Ambis quantitation of ΔN95 sucrose fraction ATPase assay.

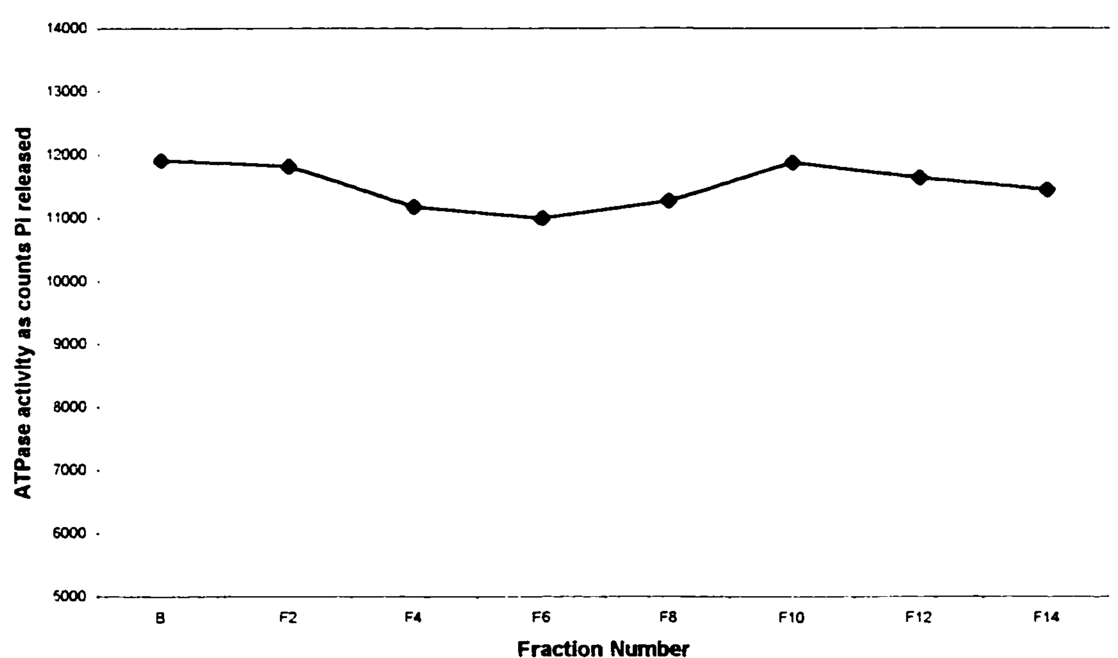
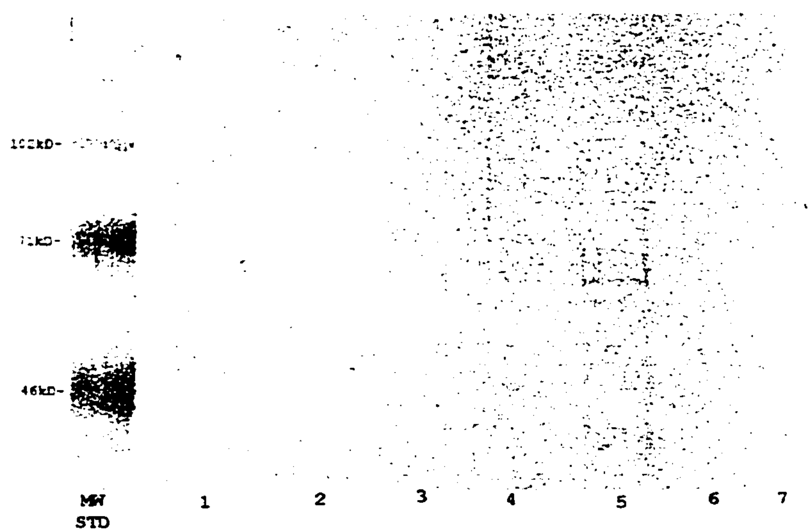
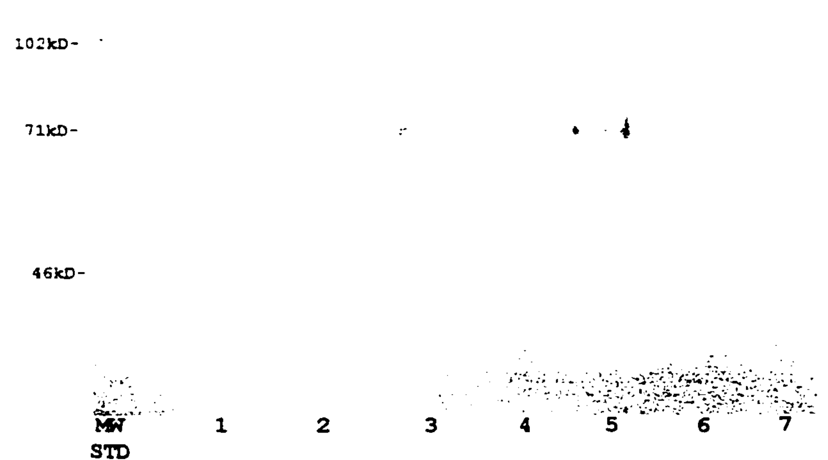


Figure 11. Analysis of the ΔDom I sucrose fractions. Each set of sucrose fractions was analyzed by SDS-PAGE, Western blot and ATPase assay with quantitation by Ambis Image Analysis. **A)** SDS-PAGE was done on 30 μl of every other sucrose fraction and stained with coomassie blue. Lanes 1-7 are sucrose fractions 2, 4, 6, 8, 10, 12 and 14 respectively in both the SDS-PAGE and Western blots. **B)** Western blot analysis of every second sucrose fraction as described in A. **C)** The autoradiograph of the ATPase assay performed on every sucrose fraction (done in duplicate) as described in the materials and methods, B: is the blank control and contains no protein, sets 1-15 are sucrose fractions 1-15 respectively, set 16 is CI protein isolated from plants used as a positive control. **D)** Ambis quantitation of the ATPase autoradiograph graphed to determine where the ATPase activity is localized to in the sucrose gradient. Figures 11A and 11B show the presence of a 70.8 kD protein that is reactive to the CI protein antiserum. The predicted size of the ΔDom I mutant protein is approximately 70.8 kD. Figures 11C and 11D demonstrate that there is no ATPase activity present in any of the sucrose fractions above the level of the background control.

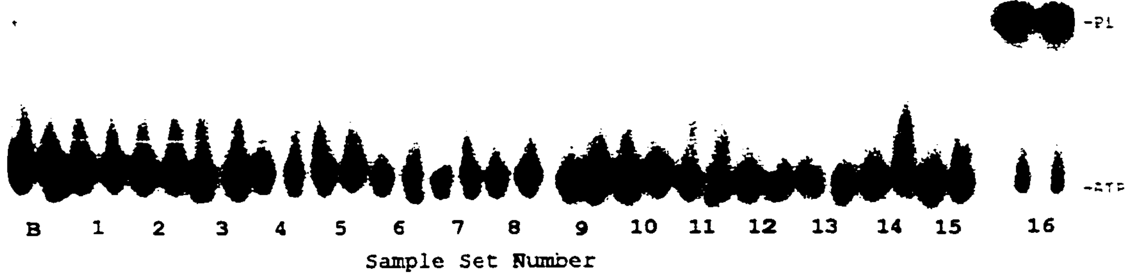
A) ΔDom I SDS-PAGE



B) ΔDom I Western Blot



C) ΔDom I autoradiograph of ATPase assay of sucrose fractions.



D) Ambis quantitation of ΔDom I sucrose fraction ATPase assay.

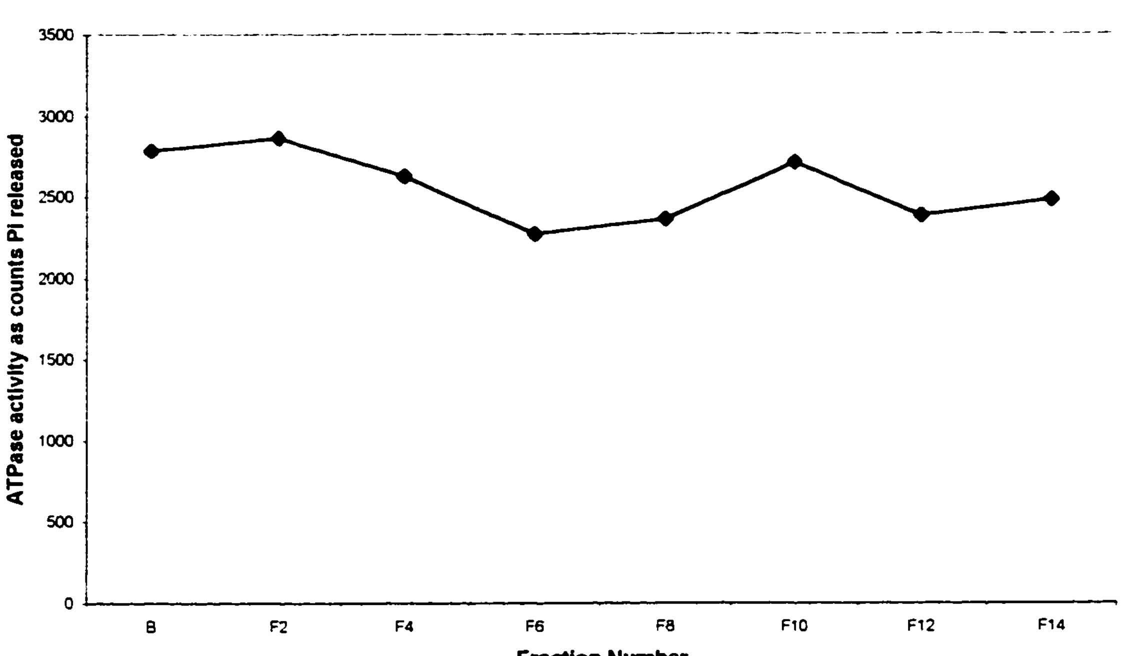
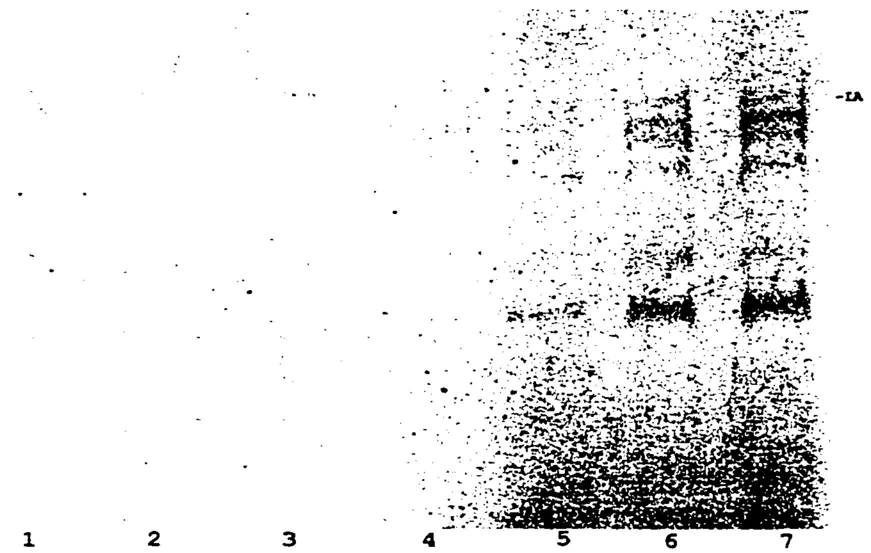


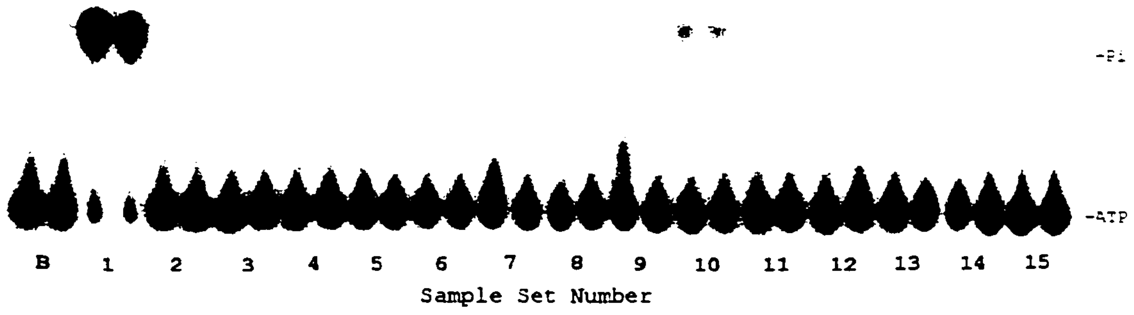
Figure 12. Analysis of the Dom IA sucrose fractions. Each set of sucrose fractions was analyzed by SDS-PAGE, Western blot and ATPase assay with quantitation by Ambis Image Analysis. **A)** SDS-PAGE was done on 30 μl of every other sucrose fraction and stained with coomassie blue. Lanes 1-7 are sucrose fractions 2, 4, 6, 8, 10, 12 and 14 respectively in both the SDS-PAGE and Western blots. **B)** Western blot analysis of every second sucrose fraction as described in A. **C)** The autoradiograph of the ATPase assay performed on every sucrose fraction (done in duplicate) as described in the materials and methods, B: is the blank control and contains no protein, set 1 is CI protein isolated from plants used as a positive control, sets 2-15 are sucrose fractions 1-14 respectively. **D)** Ambis quantitation of the ATPase autoradiograph graphed to determine where the ATPase activity is localized to in the sucrose gradient. Figures 12A and 12B show the presence of 70.8 kD protein that is reactive to the CI antiserum. The predicted size of the ΔDom IA mutant protein is approximately 70.8 kD. Figures 12C and 12D show that the ATPase activity detected in the sucrose fractions localizes to the same sucrose fractions that contain the CI reactive proteins.

A) ΔDom IA SDS-PAGE.



B) ΔDom IA Western Blot.

C) ΔDom IA autoradiograph of ATPase assay of sucrose fractions.



D) Ambis quantitation of ΔDom IA sucrose fraction ATPase assay.

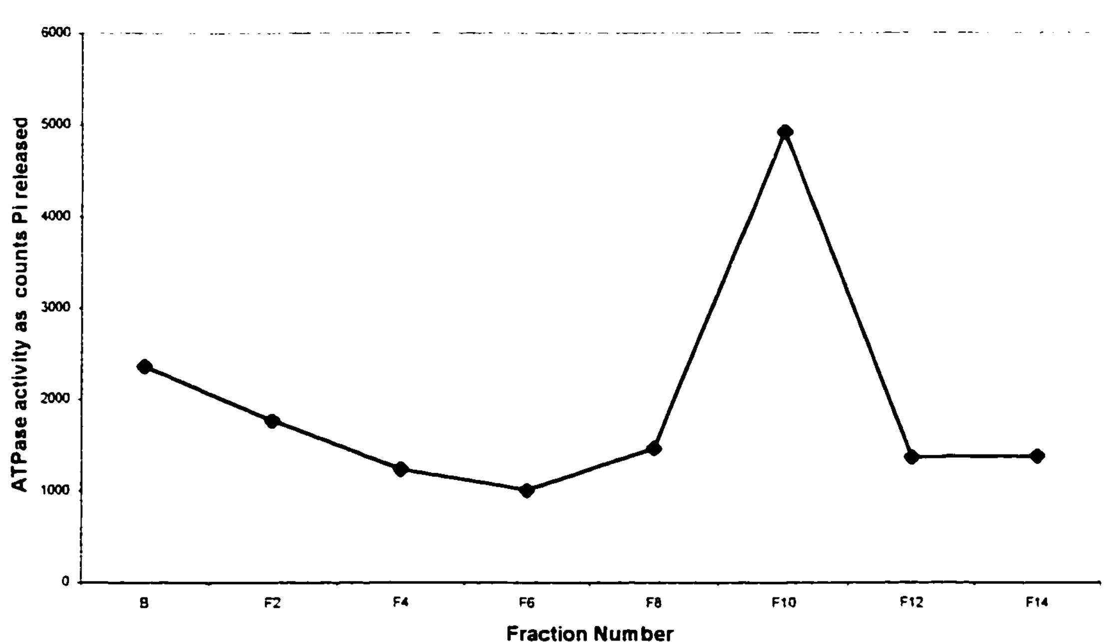
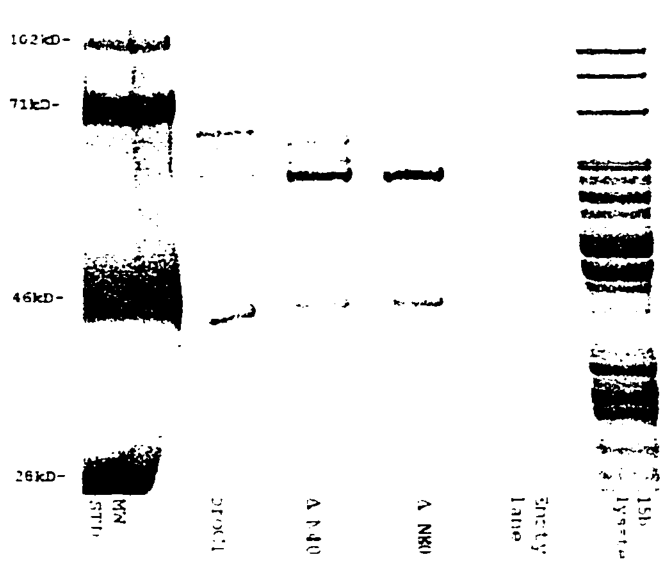


Figure 13. SDS-PAGE and Western Blot analysis of purified protein fractions. SDS-PAGE and western blot analysis was performed on the pooled, dialyzed sucrose fractions for each protein. A) The samples are the bacterially expressed proCI, ΔN40, ΔN80, and pET 15b/HMS174(DE3) lysate. B) The samples are the same as listed for the SDS-PAGE in A. The blot was incubated with anti-CI antiserum and then developed according to the materials and methods. The proCI, ΔN40 and ΔN80 all react to the serum, while there is a lack of reactivity with the bacterial control lysate. This clearly demonstrates that the isolated proteins are CI specific products and that there are no cross-reactive proteins found in the bacteria.

A) SDS-PAGE of purified protein fractions.



B) Western blot of analysis of purified protein fractions.

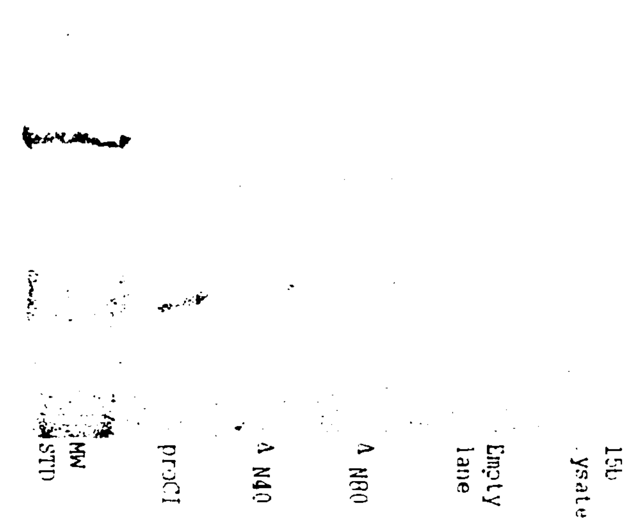
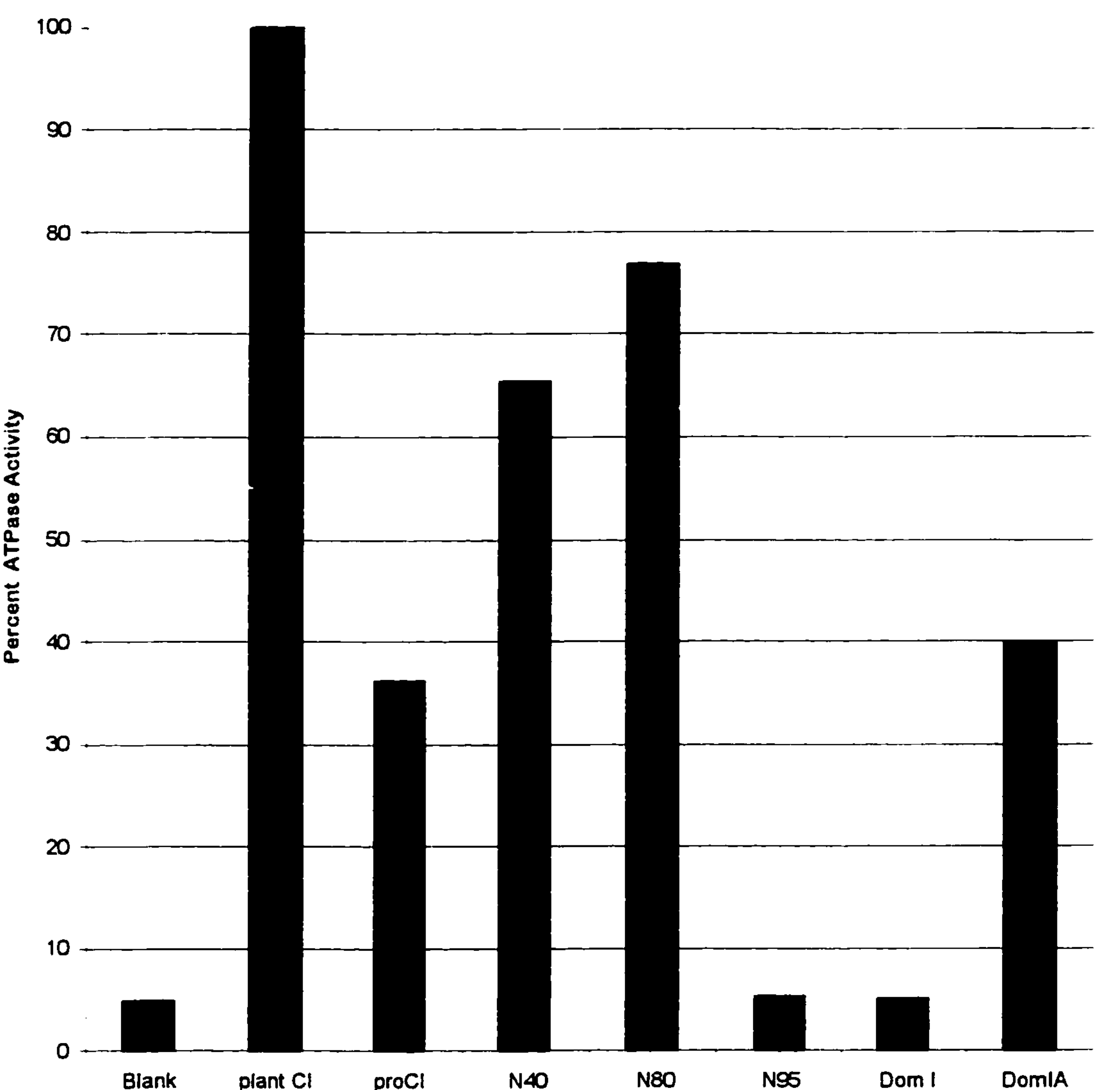


Figure 14. Quantitative analysis of ATPase activity. Approximately 50ng of each protein was analyzed for ATPase activity, with CI protein isolated from plants being the standard control and its level of activity taken to be 100%. The result is expressed as the amount of Pi released by the bacterially expressed protein divided by the Pi released by the plant isolated CI protein. The Pi counts were obtained by an Ambis Image Analysis System. A negative control which contained no protein was also subjected to the ATPase assay and used as a background control (blank). The experiment was done in triplicate.


Comparison of ATPase Activity as % of plant CI activity
Percent ATPase Activity
100
90
80
70
60
50
40
30
20
10
0
Blank
plant CI
proCI
N40
N80
N95
Dom I
DomIA

active mutant Δ438-633 were analyzed. The full-length pCI and Δ438-633 mutant proteins are positive controls and exhibit ATP binding The ΔDom I and ΔN95 deletion mutants lack ATP binding capabilities (figure 15). The Δ151-633 and Δ347-444 mutants (pET 11a constructs) also showed ATP binding. Table 3 summarizes the results of the ATP binding assay.

Table 3. Summary of ATP binding for pCI and ATPase activity deficient mutants.

Protein and Result of ATP Binding Assay					
pCI	ΔDomI	ΔN95	Δ151-633	Δ347-444	Δ438-633
(+)	(-)	(-)	(+)	(+)	(+)

(+) indicates ATP binding (-) indicates no ATP binding

RNA Binding Analysis.

RNA binding studies were carried out both by UV crosslinking and by Northwestern methods. The full-length proCI and the ΔN40, ΔN80, ΔN95, ΔDom I, and ΔDom IA mutants all displayed RNA binding under both the Northwestern and UV cross-linking reaction conditions.

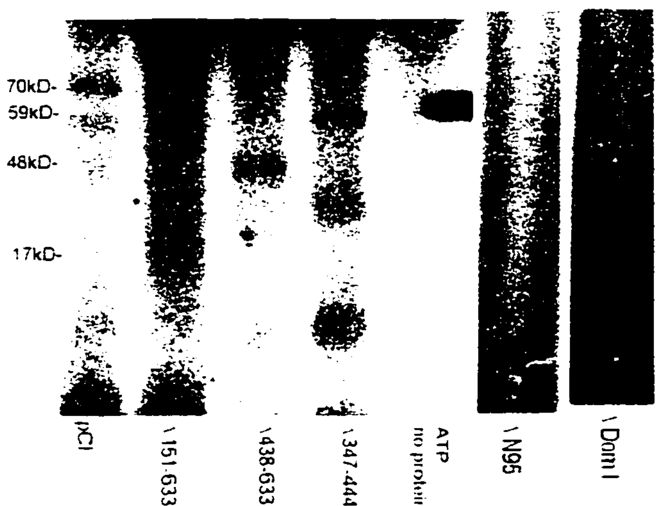
The pET 11a constructs, the full-length pCI, and the internal deletion mutants Δ348-444 and Δ438-633 showed RNA binding by UV crosslinking studies (figure 16B). The Δ151-633 mutant does not demonstrate any RNA binding. Table 4 summarizes the results of the RNA binding assay.

Table 4. Summary of RNA binding of both full-length CI proteins and mutants.

Protein and Result of RNA Binding Assay									
proCI	ΔN40	ΔN80	ΔN95	ΔDom I	ΔDomIA	pCI	Δ151633	Δ347-444	Δ438-633
(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)

(+) indicates RNA binding. (-) indicates no RNA binding

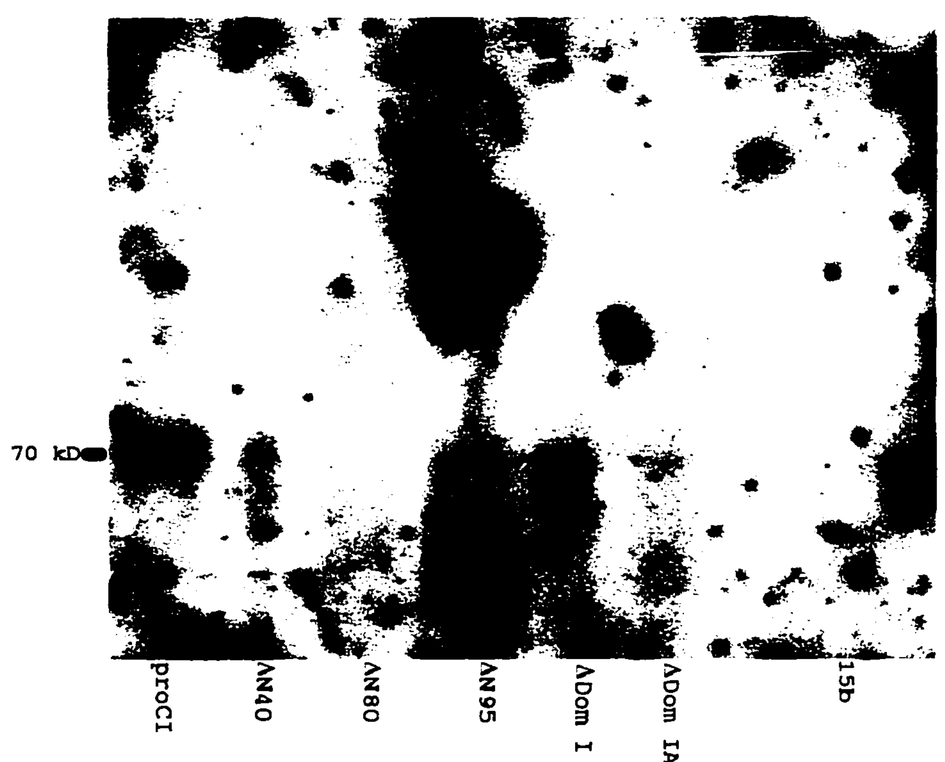
Figure 15. ATP binding assay. Mutant proteins that lacked ATPase activity, the ΔN95, ΔDom I, Δ151-633 and Δ347-444 mutants, were assayed for their ability to bind ATP. The pCI and Δ438-633 proteins were used as positive controls since they are positive for ATPase activity. Approximately 250ng of each protein was reacted with [α-^{32}P]ATP, UV irradiated and subjected to SDS-PAGE. Lanes 1-6 are pCI, Δ151-633, Δ438-633, Δ347-444, [α^{32}P]ATP with no protein, ΔN95 and ΔDom I respectively. The molecular weights of the proteins are: 70 kD, 48 kD, 59 kD, 17 kD, 62 kD, and 71 kD respectively (Chen, 1994). The protein locations are indicated by a line with the respective molecular weight next to them. The pCI, Δ438-633, Δ347–633 and Δ151–633, show ATP binding, while the ΔN95 and ΔDom I proteins show no evidence of ATP binding. The [α^{32}P]ATP with no protein shows the absence of any radioactive species, indicating the [α^{32}P]ATP will be present in the gel if bound to a protein species.



70kD-
59kD-
48kD-
17kD-
pCI
151-633
438-633
347-444
ATP
N95
Dom I

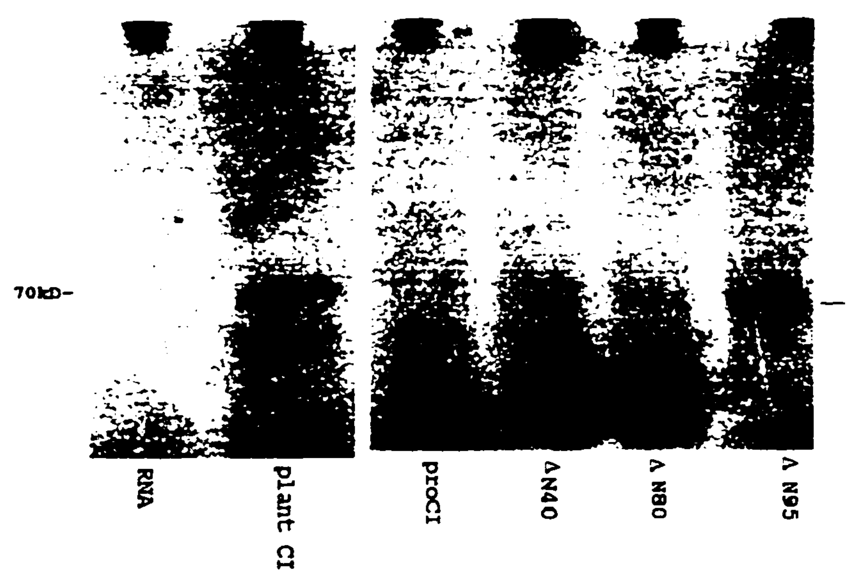
Figure 16. RNA binding assay. All of the protein constructs described in figure 5 were assayed for their ability to bind RNA. **A)** RNA binding by Northwestern assay. The Northwestern was performed as described in the materials and methods. Lanes 1-7 are proCI, ΔN40, ΔN80, ΔN95 ΔDom I, ΔDom IA, and pET 15b/HMS174(DE3) respectively. RNA binding is observed in the proCI, ΔN40, ΔN80, ΔN95 ΔDom I and in ΔDom IA lanes. No binding is observed for the pET 15b/HMS174(DE3) control sample. **B)** RNA binding by UV cross-linking. The UV cross-linking was performed as described in the materials and methods. Gel 1 contains: RNA alone with no protein, CI isolated from infected plants, proCI, ΔN40, ΔN80 and ΔN95, respectively. Gel 2 contains: Δ151-633 (Δ151), Δ347-444 (Δ347), Δ438-633 (Δ438), pET 11a/HMS174(DE3) and pET 15b/HMS174(DE3) respectively. Note the absence of any bands in the RNA alone lane in gel 1. RNA binding is evident in the plant isolated CI protein as well as in the proCI, ΔN40, ΔN80 and ΔN95 lanes, although to a lesser degree than in the plant isolated CI lane. In gel 2, RNA binding can be seen for the Δ347-444 and Δ438-633 samples, but not for the Δ151-633, pET 11a/HMS174(DE3) or pET 15b/HMS174(DE3), which are the bacterial controls samples.

A) Northwestern Binding Assay.

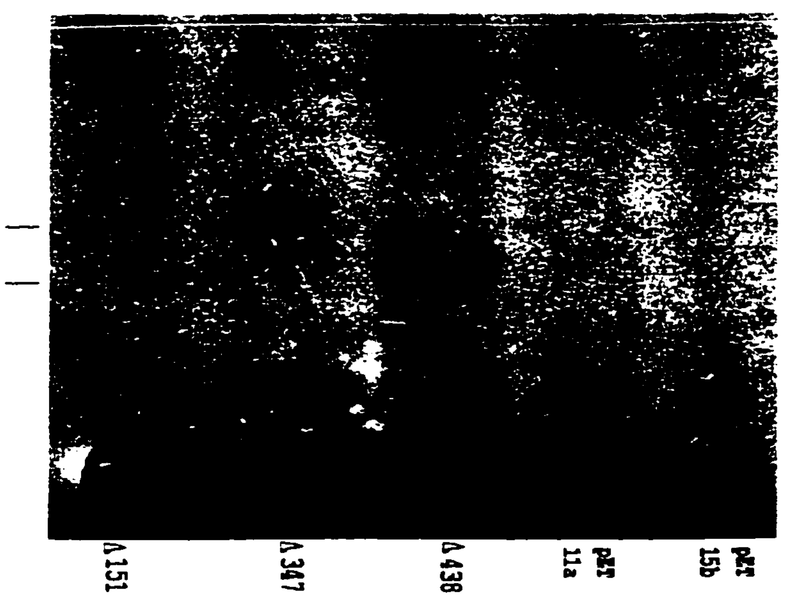


B)

RNA UV cross-linking assay gel 1.



RNA UV cross-linking assay gel 2.



Summary.

Table 5 below summarizes the ATPase, ATP- and RNA- binding activities of the full-length CI proteins, pCI and proCI, as well as all of the mutants described in this text and outlined in Figure 6.

Table 5. Composite summary of the ATPase, ATP- and RNA-binding activities of both full-length CI proteins and all pET 11a and pET 15b mutants.

Protein[1]	ATPase Activity	ATP Binding	RNA Binding
proCI	Yes	ND	Yes
ΔN40	Yes	ND	Yes
ΔN80	Yes	ND	Yes
ΔN95	No	No	Yes
ΔDom I	No	No	Yes
ΔDom IA	Yes	ND	Yes
pCI	Yes*	Yes	Yes
Δ151-633	No*	Yes	No
Δ347-444	No*	Yes	Yes
Δ438-633	Yes*	Yes	Yes
pET 15b/HMS174(DE3)	No	No	No

* These results were obtained from S. Chen, Ph.D. Dissertation 1994.
1 See Figure 5 on page 33 for a summary of the protein constructs.
ND: These proteins were not assayed.

N-Glycosylation and Phosphorylation Studies on CI protein isolated from plants.

It is well known that glycosylated proteins run at varying mobilities when subjected to SDS-PAGE. Sequence analysis using the MacVector 5.0 sequence analysis program revealed the presence of several N-glycosylation sites (Kornfeld *et al.*, 1985) in the CI protein (figure 17). PAS staining, which detects N-glycosylated proteins, was carried out as described in the materials and methods to see if this modification could be responsible for the difference in band mobility between the plant isolated CI protein and the full-length bacterial proteins (figure 18).

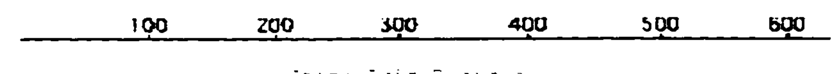
The results reveal that the CI protein isolated from plants is weakly N-glycosylated. The CI protein isolated from bacteria does not stain under these conditions

indicating that it is not glycosylated (Figure 10). Further studies will be necessary to determine the extent of the glycosylation and the nature of the carbohydrate groups attached to the CI protein.

Phosphorylation is another type of post-translational modification that occurs in eukaryotic and some prokayotic proteins. Sequence analysis using the MacVector 5.0 sequence analysis program revealed the presence of a complete signal for tyrosine phosphorylation in the CI protein (Cooper *et al*, 1984) (figure 17).

Western blot analysis was done using the specific antibody PY-20 described in the materials and methods. The blot shows that the plant isolated CI protein is tyrosine phosphorylated and that the bacterially expressed full-length CI proteins are not tyrosine phosphorylated (Figure 19).

Figure 17. Map showing the N-glycosylation and tyrosine phosphorylation sites on the CI protein. The known amino acid sequence of the TEV-HAT CI protein was entered into the MacVector 5.0 sequence analysis program and the sequence was scanned for known protein motifs that are in this program. Using the sequences for N-glycosylation (Kornfeld *et al.* 1985) and tyrosine phosphorylation (Cooper *et al.*, 1984) the program determined there were 5 potential N-glycosylation sites and 1 potential tyrosine phosphorylation site.


100
200
300
400
500
600

N-GLYCOSYL
N-GLYCOSYL
N-GLYCOSYL
N-GLYCOSYL
N-GLYCOSYL

Figure 18. PAS stained SDS-PAGE of plant isolated CI protein for detection of N-glycosylation. The proteins were run according to standard SDS-PAGE protocols and stained by the PAS method (materials and methods) to detect any glycosylated proteins. Lanes 1-7 are: molecular weight standards, plant isolated CI protein, bacterially expressed proCI, ovalbumin, bovine serum albumin, tobacco mosaic virus capsid protein, and chicken retina lysate respectively. The ovalbumin, bovine serum albumin, and chicken retina lysate all are glycosylated or contain glycosylated proteins and therefore are used as positive controls here. Tobacco mosaic virus capsid protein is not glycosylated and is the negative control. Note that the bacterially expressed proCI protein is not stained here showing that it is not N-glycosylated, while the CI protein isolated from plants is stained in the gel indicating that it is N-glycosylated. The molecular weight standards are prestained with coomassie blue, therefore staining here does not indicate glycosylation of the proteins.

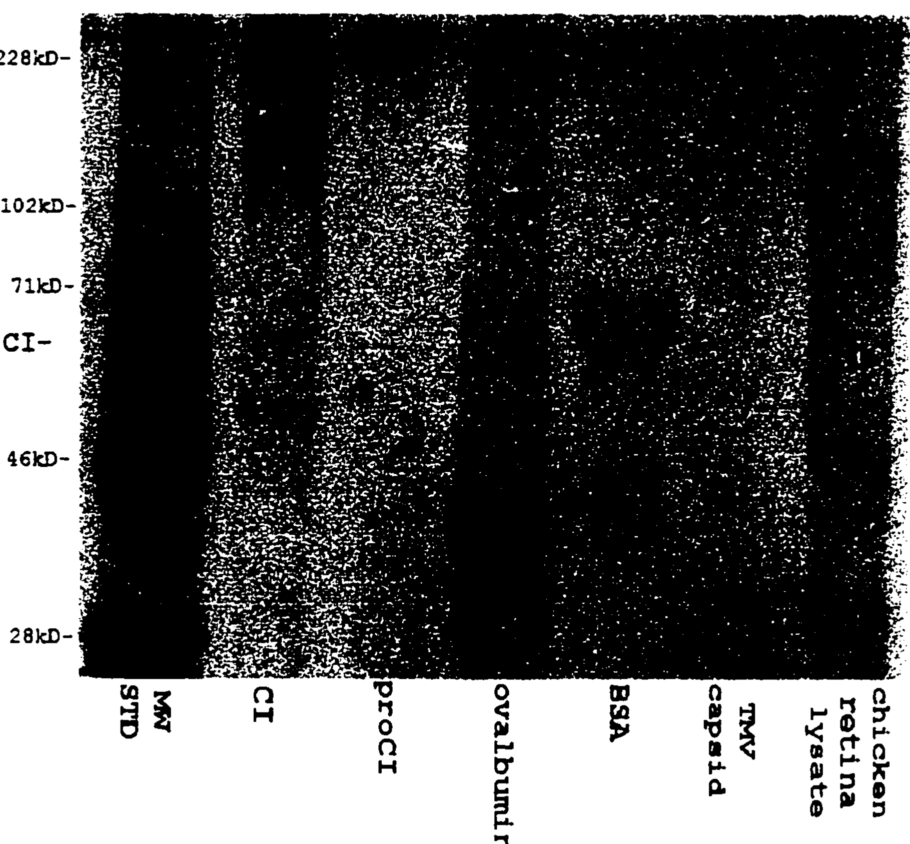
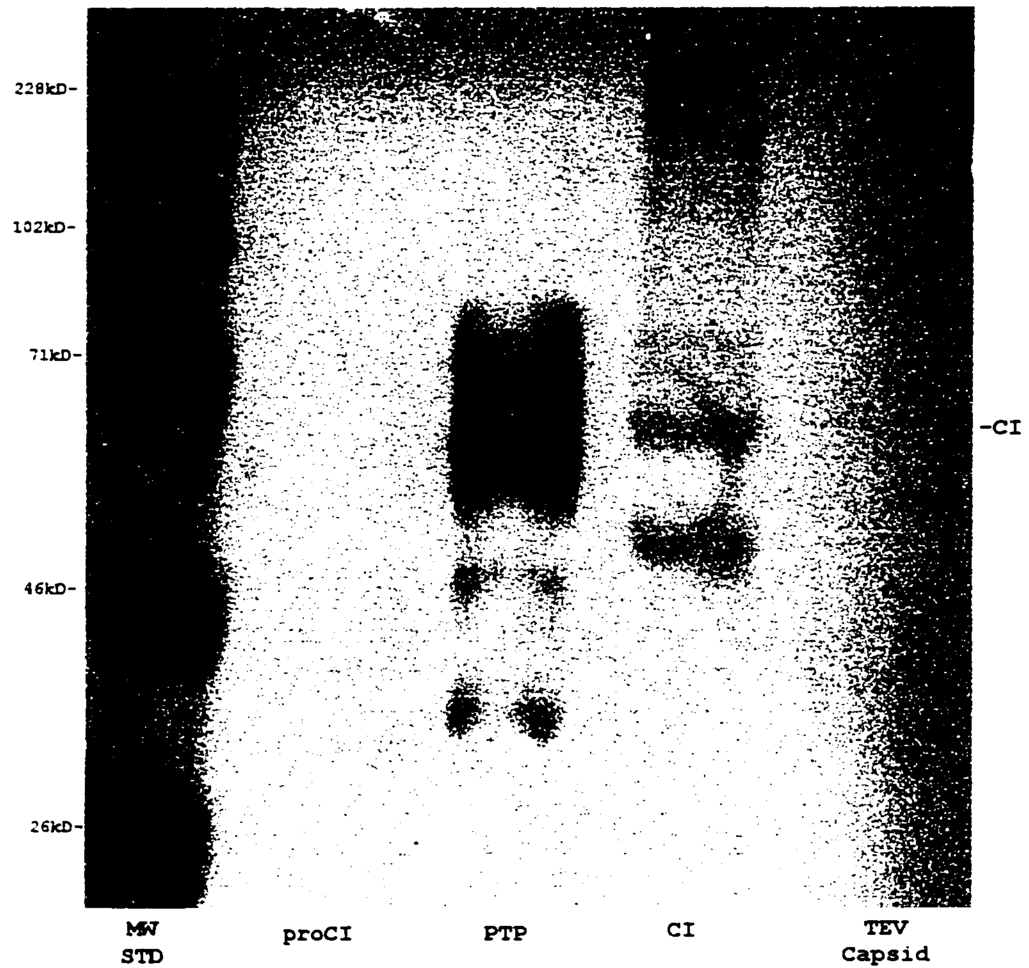

228kD-
102kD-
71kD-
CI-
46kD-
28kD-
MW STD
CI
proCI
ovalbumin
BSA
TMV capsid
chicken retina lysate

Figure 19. Immunoblot detection of tyrosine phosphorylation of CI protein isolated from plants. The proteins were subjected to a standard SDS-PAGE and electroblotted to nitrocellulose membrane for the western blot. The proteins were incubated overnight with the PY-20 antiserum that is specific for phosphorylated tyrosine residues (materials and methods). Lanes 1-5 are: molecular weight standards, proCI, protein tyrosine phosphatase (PTP), CI protein isolated from plants and tobacco etch virus capsid protein, respectively. The PTP protein is known to be tyrosine phosphorylated and is the positive control. The tobacco etch virus capsid protein is not known to be tyrosine phosphorylated and is the negative control for the experiment. The CI protein isolated from plants (lane 4) is tyrosine phosphorylated and the bacterially expressed proCI protein is not tyrosine phosphorylated 9 (lane 5). The molecular weight standards are prestained and are not reactive with the CI antiserum.


228kD-
102kD-
71kD-
46kD-
26kD-
-CI
MW
STD
proCI
PTP
CI
TEV
Capsid

Discussion.

1. ATPase activity of bacterially expressed proCI and mutants.

The TEV-HAT gene was cloned into the *E. coli* expression vector pET 15b under the control of the T7 *lac* promoter in order to characterize, by mutation, those regions of the protein that are required for ATPase activity. The fusion protein, proCI, which contains the full-length CI gene has ATPase activity. This activity is considerably lower than that of the native plant isolated CI protein (Figure 7). A previous study, Chen 1994, also found that the full-length pCI protein was also less active with respect to ATPase activity when compared to the activity of the CI protein purified from infected plants. The reason for this difference in enzymatic activity is not clear. However the finding that the CI protein isolated from plants is N-glycosylated and tyrosine phosphorylated suggests, that since the bacterially expressed proteins are not glycosylated or phosphorylated, that these post-translational modifications may affect the enzymatic activity of the CI protein.

Previous work done in our laboratory (Chen, S. Ph.D. Dissertation, 1994, Wayne State University) has shown that all of the C-terminal residues downstream of domain VI (amino acids 436-633) can be removed without loss of ATPase activity. However, no experiments had been performed on the ATPase activity of mutants lacking N-terminal sequences upsteam of the first conserved domain, domain I. In view of this, two N-terminal deletion mutants were constructed to determine the extent to which the residues upstream of the conserved domains are involved in ATPase activity. The two N-terminal deletion mutants, ΔN40 and ΔN80 exhibit ATPase activity (Figure 7). Since the N-terminal deletions remove all the amino acids upstream of the first conserved domain,

domain I, it seems that these amino acids are not required for ATPase activity.

Removal of domain IA (ΔDom IA) does not affect ATPase activity either and it is interesting to note that in the picornavirus-like superfamily to which the potyviral CI proteins belong, this is the least conserved domain both in sequence and presence in the proteins. The function of domain IA has not been characterized completely and has only recently been shown to be involved in RNA binding (Fernandez, *et al.*, 1996). This study indicates that it does not play a role in ATPase activity.

Domain I contains the sequences of the nucleotide binding motif (NTBM) of all known ATPases and is subsequently thought to be the ATP binding site in the CI protein. Two mutants were constructed to determine if domain I alone is responsible for ATP binding. Domain I is absent in both the ΔDom I and ΔN95 mutants. In the ΔDom I mutant it is spliced out and the rest of the protein is present and in frame, and in the ΔN95 mutant the first 95 amino acids of the N-terminus, which includes all of the domain I sequences, of the CI protein are removed. The ΔDom I and ΔN95 deletion mutants both lack ATPase activity. This further supports the notion that the presence of domain I is absolutely required for ATPase activity .

2. ATP binding ability of mutants that lack ATPase activity.

For mutants that had no ATPase activity an ATP binding study was carried out. This was done to determine if the loss of ATPase activity was due to loss of binding of ATP or loss of the catalytic activity. In other words, are the binding and cleavage activities located in the same region (domain I) of the protein, or is more than one region (or domain) involved in the ATPase activity.

ATP binding assays were carried out on the ΔDom I and ΔN95 mutants, as well as mutants that had been previously generated in our lab, namely the Δ151-633 and Δ347-

444 mutants. All of these mutants lack ATPase activity. Neither the ΔDom I or ΔN95 mutants displayed any ATP binding, while both the Δ151-633 and Δ347-444, which contain domain I, bound ATP. This gives direct evidence that domain I is essential for ATP binding since both the ΔDom I or ΔN95 mutants lack domain I and both the Δ151-633 and Δ347-444 mutants contain domain I.

It is interesting to note that while the Δ151-633 binds ATP, it has no ATPase activity. This protein lacks all of the regions downstream from domain IA. Domain II is the Mg^{++} binding domain. It is known that the ATPase activity of the CI protein requires a divalent cation cofactor (Mg^{++}, Mn^{++}, etc.). Since domain II is the binding location of this moiety, its absence would cause a loss of activity. The binding of ATP by this mutant indicates that none of the downstream domains play a role in ATP binding.

The Δ347-444 mutant binds ATP but lacks ATPase activity. This mutant contains all of the amino acids except for domain VI and the C-terminus, which is known to be dispensable for ATPase activity. If domain I and domain II alone are sufficient for ATPase activity, then this mutant should be active, since it contains both. The fact that it is not active and that domain VI is the only other domain missing suggests that other domains may play a role in ATPase activity either by structural contributions or direct involvement in the enzymatic activity. Further studies are necessary however to determine which of these scenarios is correct.

3. RNA binding of the bacterially expressed proCI and its mutants and pCI and its mutants.

All of the pET 11a and pET 15b were assayed for RNA binding. All of the N-terminal deletion mutants, the ΔN40, ΔN80 and ΔN95 mutants, exhibited RNA binding and all of these mutants contain both domains IA and VI. Domain VI has long been

thought to be the only RNA binding domain because it contains many positively charged residues. Recent work by Fernandez, *et al.*, (1996), has shown that domain IA is also an RNA binding domain. The binding by these proteins indicates that the N-terminal residues upstream of and including domain I are not necessary for RNA binding.

The two pET 15b internal deletions, ΔDom I and ΔDom IA also have RNA binding capabilities. The ΔDom I protein contains both domains IA and VI, so it may be expected to bind RNA. The ΔDom IA protein lacks domain IA, but contains domain VI and was still able to bind RNA. This indicates that the presence of domain VI is enough for RNA binding.

Of the pET 11a constructs, the pCI, the Δ347-444 and Δ435-633 mutants show RNA binding. The Δ151-633 mutant does not display any RNA binding. The Δ347-444 mutant shows RNA binding and contains domain IA and lacks domain VI. In the case of Δ151-633, it only has domains I and IA and all of the upstream N-terminal residues, since it does not bind RNA this indicate that even though domain IA has been shown to bind RNA, for this mutant it is not sufficient. This could be due to the lack of other downstream regions of the protein whose role in RNA binding has yet to be elucidated or that under the experimental conditions described here binding is not obtained. The Δ435-633 mutant has RNA binding and contains both domains IA and VI.

4. Analysis of the apparent size differences between the native plant isolated CI protein and the full-length bacterially expressed pCI and proCI.

It was consistently observed that the full-length CI protein isolated from the bacteria ran at a slightly faster rate in SDS gels than the CI protein isolated from infected plants (data not shown). It was speculated that the possible causes for this difference in mobility between the two proteins: 1) The bacterially expressed proteins were being

degraded by bacterial proteases, resulting in slightly smaller products, or 2) The CI protein expressed in infected plants has post-translational modifications, such as N-glycosylation or phosphorylation whereas the bacterially expressed counterparts do not contain these modifications.

There is a significant amount of proteolysis of the bacterially expressed proteins. Many different methods of protein purification were tried to reduce the amount of proteolysis, such as adding increased levels of protease inhibitors, using different protease deficient bacterial strains such as BLR(DE3) (Novagen, Inc.) etc. None of these attempts affected the levels of proteolysis significantly (data not shown). An attempt was made to determine if the proteins were being degraded from the N-terminus. An anti-His tag antibody was obtained to see if the His tag was still attached to the proteins. Although this antibody is supposedly specific for the His tag it was found that it cross reacted with the plant isolated CI protein therefore this approach could not be used to determine if there was any N-terminal degradation. C-terminal degradation could not be determined since there is no antibody available that is specific for the C-terminus of the CI protein and microsequencing was not attempted.

Sequence analysis of the CI protein revealed that there are sites for both N-glycosylation and tyrosine phosphorylation (Figure 17). Experiments were performed to determine if these modifications were present in the CI protein.

PAS staining revealed that the CI protein is weakly N-glycosylated (Figure 18). Ovalbumin is a weakly N-glycosylated protein and was used as a positive control for visual comparison of the degree of N-glycosylation. The bacterially expressed CI proteins do not stain under these conditions, indicating that they are not glycosylated.

Glycosylated proteins are generally membrane associated proteins. This finding

is interesting in that the CI protein early in infection is found associated with various membranous structures before it forms its characteristic cytoplasmic inclusion bodies. The CI protein has been implicated to have a role in spread of the viral infection. Its proximity to the plasmodesmata and glycosylation lend more evidence to this idea.

The CI protein contains a signal for tyrosine phosphorylation. The signal is composed of three parts, (R/K)XXX(D/E)XXXY (Cooper, et al., 1984), where X is any residue, and is located in domain II of the CI protein which is the divalent cation binding site. In view of the low level of ATPase activity observed for the both of the full-length bacterially expressed proteins (pCI and proCI), it is interesting to speculate that since the bacterially expressed proteins are not tyrosine phosphorylated, they are not as active as the plant isolated CI protein, which even at very low concentrations is very active. An even more interesting notion is that the native CI in the plant is not active until it is phosphorylated. Further studies are necessary to determine the validity of these speculations.

Summary.

The experiments discussed here show: **(1)** that non-domain N-terminal sequences and domain IA do not have a role in ATPase activity or ATP binding (mutants ΔN40, ΔN80, ΔDom IA), **(2)** removal of domain I by either N-terminal deletion or splicing (mutants ΔN95 and ΔDom I) abolishes both ATPase activity and ATP binding, but does not affect RNA binding, **(3)** the presence of domain IA alone in the Δ151-633 mutant is not sufficient for RNA binding, suggesting that there are other regions of the protein needed for binding in the absence of domain VI as indicated by the results of the Δ347-444 mutant, which lacks domain VI and all regions downstream of it, but contains all the upstream sequences; **(4)** the CI protein isolated from TEV-infected plants is weakly N-glycosylated and is tyrosine phosphorylated.

determine if these modifications were present in the CI protein.

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Summary.

The experiments discussed here show: **(1)** that non-domain N-terminal sequences and domain IA do not have a role in ATPase activity or ATP binding (mutants ΔN40,

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ABSTRACT

MUTATIONAL ANALYSIS OF THE TOBACCO ETCH POTYVIRUS CYLINDRICAL INCLUSTION PROTEIN

by

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May 1998

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The CI protein of tobacco etch potyvirus is known to be an NTPase as well as a putative RNA helicase. In a previous study, the CI gene and three mutants were constructed and expressed in the *E. coli* expression vector pET 11a.

In this study the TEV CI gene was cloned into *E. coli* expression vector pET 15b and five other mutants were constructed. These contructs in pET 15b, along with the pET 11a constructs, were sequenced to verify the mutations made and the reading fram of the protein. The pET 15b constructs were expressed and assayed for ATPase, ATP binding (only those proteins that were ATPase activity deficient) and RNA binding activities. The pET 11a constructs were expressed and assayed for ATP binding (only those proteins that were ATPase activity deficient) and RNA binding activities. Experiments on the bacterially-expressed CI protein and its mutants showed that (1) the non-domain N-terminal regions of the CI protein (sequences up to domain I) are dispensible for both ATPase activity and RNA binding, (2) that domain I is necessary for ATP binding and ATPase activity, but it alone is not sufficient for ATPase activity, (3)

that domains IA and VI are both RNA binding domains and that the presence of one or the other is sufficient for RNA binding.

In addition this study showed that the CI protein expressed in virus-infected plants is glycosylated and tyrosine-phosphorylated. This observation is important since it leads to speculations that the CI protein may have roles in signal transduction, membrane transport and other biological functions.

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PRESENTATIONS:

Browning-Kelley, M.E., Chen, S., Sridar, C. and Hari, V. (1997). Mutational analysis of the potyviral ATPase gene. Poster, Wayne State University and Parke-Davis Pharmaceutical Research. Detroit, MI

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