SEQUENCES INVOLVED IN THE CONTROL OF TRANSLATION IN POTATO VIRUS S

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I dedicate this thesis to my mammy and daddy
Specific cis-acting sequences within the carlavirus potato virus S (PVS) genomic RNA molecule appear to control gene expression at the translational level. Two sequences have been investigated, the untranslated sequence upstream from the initiation codon of the viral coat protein gene, designated VTE and the 5’ untranslated leader sequence from the genomic RNA molecule (PVS 5’). In vitro and in vivo, either of these sequences enhance the translation of a downstream open reading frame when provided as the untranslated leader in a transcript molecule. Translational enhancement was also detected at the transgenic plant level.

Both PVS sequences were deleted in an attempt to identify the core regulatory element responsible for this translational enhancement phenomenon. Results indicate that in vitro and in vivo, the functional motif is contained within the 5’ proximal portion of both sequences. When the sequences of these important regions were compared, a homologous block of nucleotides was identified, a block which is also highly conserved within the 5’ untranslated leader of another carlavirus, blueberry scorch virus (BBScV).

In addition to the function of translational enhancement, the VTE sequence, and a parallel sequence from another carlavirus, Helianthus virus S (HelVS), direct internal ribosome entry and initiation of translation in vivo in a prokaryotic system and in vitro in a eukaryotic system, respectively.

Results have also indicated that the mechanism of expression associated with the VTE and PVS 5’ leader may be at least partially cap-independent in nature. This may represent an adaption by the virus to achieve a maximum rate of translation and multiplication when cellular translational machinery is depleted or altered upon viral infection.

Preliminary investigation has also assigned additional functions to both sequences. The VTE sequence may contain the recognition site for the viral polymerase in the production of the PVS 1.3 kb subgenomic RNA molecule. The PVS 5’ leader may contain the specific sequence or structure that is recognised by the viral coat protein in the initiation of genomic RNA encapsidation.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full form</th>
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<tr>
<td>AMPS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumen</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside-5'-triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Dideoxynucleoside-5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ds</td>
<td>Double-stranded</td>
</tr>
<tr>
<td>E.coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N-(2-ethane sulphonic acid)</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamp</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholine)ethanesulphonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microitre</td>
</tr>
<tr>
<td>OLB</td>
<td>Oligo labelling buffer</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDW</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N',tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight/weight</td>
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Introduction

1.1 General introduction

Like their counterparts in the animal kingdom, plant viruses have developed ingenious and complex mechanisms to maintain and enhance their existence in a particular host. Many have evolved additional genomic functions which reflect the special needs dictated by a plant environment. The ecological and economical impact of plant viruses is enormous, especially in warmer continents such as Africa or South America where the rate of virus spread may be dramatic. The tremendous loss incurred in valuable crop plants has led to a growing demand for the development of new viral control measures and for this to be possible a detailed knowledge of mechanisms involved in viral replication and gene expression is essential. Viruses, both plant and animal, are extremely versatile. The genomes of some viruses encode as few as four gene products so the economy of the coding capacity is quite startling. Being invaders of an eukaryotic environment they must employ strategies, some of them quite ingenious, to maximise virus entry, multiplication and spread throughout the host.

The subject of this thesis will be the carlaviruses, potato virus S (PVS), and the mode of translation associated with it. The general and molecular characteristics of this virus will be discussed in this introductory chapter. The mechanism of cap-independent translational initiation will also be discussed, a process that has been proposed to explain an alternate mode of genome expression exploited by many animal viruses and which may also play a role in plant virus gene expression. Some of the consequences of viral invasion of the host plant will also be briefly outlined.

1.2 Carlaviruses

1.2.1 General carlavirus characteristics

Generally carlaviruses induce latent symptoms in host plants with the exception of potato virus M (PVM) and those carlaviruses infecting legumes. As a result of this many of the group members were discovered by chance, detected as complex infections with other viruses. The group was originally called the potato virus S group (Brandes and Wetter, 1963) but this was changed by Harrison and co workers in 1971 to the carlavirus group after, at that time, the more intensely studied member, carnation latent virus (CLV). Symptoms, although generally mild, vary quite considerably in different host plants and
with different group members. One example of a carlavirus induced disease is that caused by lily symptomless virus (LSV) (Allen, 1972). On its own this virus does not cause severe symptoms but as a complex with cucumber mosaic virus it causes a necrotic fleck in Easter lily. A more detailed account of carlavirus induced diseases is provided by Wetter and Milne, (1981). Although the above mentioned symptom appears to be quite severe it does represent an extreme situation and most carlaviruses are generally symptomless in most plants with effects varying from being indetectable to a mild chlorosis.

Carlaviruses have a flexous, filamentous rod shaped structure which often appears curved to one side under electron microscopy. Virions have a particle length of 610-700 nanometres and diameter of 12 nanometres (Brands and Wetter, 1959). The viral genome consists of a single-stranded, positive-sense RNA molecule about 7.5 kilobases in size (Mr 2.3-2.6 x 10^6) which is encapsidated in 1600-2000 identical coat protein subunits of 30-34 kilodaltons (Varma et al, 1968). Spectrophotometrically an RNA content of 5-6% has been determined (Gibbs and Harrison, 1976). Genomic carlavirus RNA molecules have a 5' proximal cap structure (Foster and Mills, 1990a) and a 3' terminal poly A tract (Mackenzie et al., 1989).

Because of the little degree of interest in this group of plant viruses, until recently, the main criteria for classification has been particle length (Brands and Wetter, 1959) which has resulted in the misrepresentation of some members. Molecular methods have now emerged which allow a more sophisticated classification, for example, on the basis of serological relationship or direct sequence homology.

The entire viral sequence for potato virus M (PVM) (Morozov et al., 1990) and for blueberry scorch virus (BBScV) (Cavileer et al., 1994) have been determined. Partial nucleotide sequences of many other group members such as potato virus S ordinary and Andean (PVS^OA) (Mackenzie et al., 1989; Foster and Mills, 1991a), carnation latent virus (CLV) (Meehan and Mills, 1991), *Helianthus* virus S (HelVS) (Foster et al., 1990a), and lily symptomless virus (LSV) (Memelink et al., 1990), among others, have also been documented. This sequence analysis has revealed that the carlaviruses genome encodes six major open reading frames. The larger 5' proximal gene encodes the viral replicase protein which ranges in size from 190-220 kDa. The second, third and fourth cistrons encode products of 25 kDa, 12 kDa and 7 kDa, respectively, and these are collectively called the triple gene block proteins (TGB). The fifth ORF encodes the viral coat protein and the 3' terminal gene encodes an 11 kDa product. The function of this protein and the TGB proteins is at present still largely unclear. The PVS genome organisation is represented diagrammatically in Fig 1.1.

### 1.2.2 The function of carlavirus proteins

The 5' proximal carlavirus gene product, the putative viral replicase, ranging in size
from 190-220 kDa, has had that function assigned due to close sequence homology and the identification of specific replicase associated domains with replicase ORFs from other plant and animal viruses. Cavileer et al., (1994), has reported a 44% sequence identity between the replicase components of PVM and BBScV.

In addition to all the general mechanisms for virus classification, the elucidation of full length sequences for individual viruses has resulted in a classification from an evolutionary point of view, on the basis of nucleotide sequence homology, which reinforces the interesting phenomenon of common ancestry between plant and counterpart animal viruses. On the basis of homology in the viral replicase gene two “supergroups” of plant viruses have been identified (Goldbach, 1986).

Members of the first of these groups are the “picornavirus like” viruses that possess a virus encoded protein (VPg) at the 5’ end of the genome and a poly A tail at the 3’ end. Members of this group express their genome as a large polyprotein which is subsequently autocatalytically cleaved to individual viral constituents (Dougherty et al., 1989; 1990). Members of this supergroup include the comov-, nepov- and potyviruses. The second group is the “Sindbis like” viruses which exhibit quite a wide variation in genome structure and translational strategy but share certain characteristics such as sequence homology in particular proteins, especially in the large non-structural proteins. Members of this group include the bromov-, cucumov-, tombus- and tobamoviruses. These striking similarities to animal viruses bring about quite important implications about common ancestry and evolution among both virus types.

Sequence analysis has placed the carlavir in the “Sindbis like” supergroup of positive-stranded RNA viruses on the basis of the replicase protein. The N- and C-terminal regions appear to contain the major conserved domains between different groups of positive-sense, single-stranded RNA viruses, for example PVM and BBScV with potex- and tymoviruses, (Morozov et al., 1990; Cavileer et al., 1994), the latter two groups with which sequence homology with the large proteins of the animal alphaviruses and the “Sindbis like” viruses has been previously reported (Rozanov et al., 1989; Morozov et al., 1989a; 1989b).

Three conserved putative replicase domains have been identified, a methyltransferase domain, an NTPase-helicase domain and an RNA polymerisation domain. The C-terminal region contains a conserved RNA polymerisation domain that is not only conserved within the group but which is common to all RNA-dependent RNA polymerases of plant and animal viruses (Morozov et al., 1989a; 1989b). A conserved asparagine dipeptide and several surrounding conserved residues is a common feature of this domain (Poch et al., 1989). Also in the C-terminal region is an NPT-binding domain which contains the common motif GLY-LYS-SER/THR (GKS/T) which constitutes part of the nucleotide binding pocket of ATP- and GTP-binding proteins (Zarić et al., 1991). This region has structural similarity to regions in bacterial proteins that are involved in unwinding which indicates some helicase function (Gorbalenya and Koonin, 1989). The N-terminal region contains an homologous domain that is conserved between potex-, tymo- and carlaviruses. It is interesting to observe that the lack of sequence conservation in this area with the Sindbis virus is caused by a mutation in the latter which results in
enhanced methyltransferase activity (Scheidel et al., 1989). This domain has consequently been associated with this activity.

The second, third and fourth cistrons are the triple gene block or TGB proteins and they encode proteins 25 kDa, 12 kDa and 7 kDa in size, respectively. Of the TGB proteins the 25 kDa ORF displays the greatest degree of sequence homology throughout the carlaviruses. A conserved G-GKSS/T motif has been identified as being common to various ATP-, GTP-binding proteins (Zimmern, 1987; Gorbalenya et al., 1988). The 12 kDa protein is quite homologous among carlaviruses. A conserved domain within this protein, common to many membrane associated proteins has been identified. Homology in this domain has also been associated with the 13 kDa product of beet necrotic yellow vein virus (BNYVV) (Bouzoubaa et al., 1987). The least homologous of all the TGB products is the 7 kDa protein. Although sequence homology is low, 29% among carlaviruses, 23% between carlaviruses and potexviruses (Foster and Mills, 1990e), blocks of hydrophobic amino acids have been identified which are characteristic of membrane spanning domains and the incorporation of the 7 kDa proteins of PVS and PVM into membranes has actually been demonstrated in vitro (Morozov et al., 1991).

All these observations have lead to the tentative suggestion that one or more of these TGB proteins may be involved in cell-to-cell movement of viral particles or viral RNA. Plant viruses pass from cell-to-cell through plasmodesmata, pores in plant cell membranes that connect one living cell with its neighbour. It has been estimated that the plasmodesmatal exclusion ratio is as little as 0.73 nm so viruses must extensively modify these pores to allow their passage (Terry and Robards, 1987). So called "movement proteins" have been widely documented for plant viruses with some examples including the P3 protein of alfalfa mosaic virus (AlMV) (Baker et al., 1983; Stussi-Garaud et al., 1987), the 3A protein of cucumber mosaic virus (CMV) (Mackenzie and Tremaine, 1988) and the 30 kDa protein of tobacco mosaic virus (TMV) (Tomenius et al., 1987). With TMV the movement function of virus particles containing mutated forms of the 30 kDa protein has been complemented in trans in transgenic plants expressing the wild type TMV movement protein (Deom et al., 1987; Meshi et al., 1987). This movement function appears to be a general effect as the transport function of one virus may also promote movement of another apparently unrelated virus, for example, Tm-2 resistance to TMV in tomato operates at the level of the movement protein but this resistance can be overcome by co-inoculation with the potexvirus potato virus X (PVX) (Taliansky et al., 1982). The membrane association properties, a characteristic of these TGB proteins indicates that one or more of these gene products, maybe together with other host factors, may potentate the phenomenon of short distance transport. No direct evidence has been proposed to support this theory.

The viral coat protein gene is one of the 3' terminal genes and it is thought to be translated from the smaller subgenomic RNA molecule which is produced in planta. This gene product is about 30-36 kDa in size and these coat protein units encapsidate the viral genomic RNA molecule, being arranged with helical symmetry. Serological relationship
was one of the primary molecular methods by which individual viruses could be classified into groups before the development of more sophisticated techniques such as direct sequence comparison. The coat proteins of members of the carlavirus group are highly conserved, as high as 70% identity at the amino acid level for some members (Foster et al., 1991d). Homology appears to be highest in a central block which is also conserved among the potexviruses, this homology is quite low at the N- and C-termini. Cross reactivity between antisera of different members is variable with some of the group viruses being more closely related to each other serologically than to others. Foster and Mills, (1990f) have reported cross reactivity of antisera raised against impatiens latent virus (ILV) with HelVS, AHLV, and PVS whereas PMV antisera does not react with any of these viruses but does react with PVM.

Genetic strategies to induce viral resistance in plants such as cross protection and coat protein-mediated protection have been demonstrated among members of the carlavirus group. Transgenic Nicotiana debneyi expressing PVS coat protein have been shown to be resistant to the subsequent challenge inoculation by PVS viral particles or PVS RNA (Mackenzie and Tremaine, 1990). Simultaneous resistance to the closely related potexvirus, PVX, and the potyvirus potato virus Y, PVY, has also been reported in transgenic potato plants expressing both nucleocapsid proteins (Lawson et al., 1990).

Another observation that emphasises the possible importance of the carlavirus coat protein relates to virus movement. White clover mosaic virus (WCIMV) belongs to the potexvirus group of plant viruses (Forster et al., 1988) which, as has been stated above, is closely related to the carlaviruses in genome organisation, mode of gene expression and sequence homology, especially in the coat protein ORF. Forster et al., (1992) has demonstrated that coat protein mutants of this virus were able to replicate in protoplasts but were unable to establish an infection in planta. The cell-to-cell transport function was therefore abolished by mutations in the coat protein ORF. The coat protein gene of this particular potexvirus could be interacting in some way with a viral encoded movement protein to promote transport. It is possible that the same phenomenon may also hold true for the carlaviruses.

The 3' terminal ORF encodes a gene product approximately 11 kDa in size. It is the presence of this protein that distinguishes carlaviruses from the closely related potexviruses which do not possess this terminal cistron. The 11 kDa proteins of members of the carlavirus group are highly homologous and the central region of the protein contains a cystine-rich zinc finger motif domain which is found in many nucleic acid binding proteins (Berg, 1986; Klug and Rhodes, 1987). Gramstat et al., (1990), has demonstrated nucleic acid binding to both single- and double-stranded nucleic acid, with the 12 kDa protein of PVM. As a result of this observation it has been suggested that the 11 kDa protein may be involved in viral replication or host transcription. It has also been suggested that this protein may function as a “helper component” for non-persistent aphid transmissibility since this protein is absent from potexviruses which are transmitted mechanically and do not have an associated viral vector. Several plant virus helper components have already been identified such as the 56 kDa product of PVY.
(Robaglia et al., 1989) or the helper component of TEV (Allison et al., 1986) but there appears to be no definite sequence conservation between these different proteins except for the presence of a zinc finger motif. Evidence presented here about the function of the 11 kDa protein, as with that presented for the TGB proteins, is purely speculative and the actual role of these proteins in vivo in the plant still remains unclear.

1.3 Potato virus S (PVS)

1.3.1 General PVS characteristics

The work presented in this thesis will concentrate on the carlaviruses potato virus S (PVS). As mentioned above, like many of the other carlaviruses, PVS was discovered in field plants by accident (de Bruyn Ouboter, 1952). PVS is one of the most common viruses infecting potatoes and, although symptoms in this host vary from undetectable to mosaic bronzing to a fine necrotic spotting effect on lower leaves (Rozendaal and Brust, 1955), it has been estimated that this virus may reduce tuber yield by as much as 15-20% (Travantzi, 1983). PVS is one of the most important filamentous viruses in Europe (Lecoq et al., 1988). The host range for the virus includes most potato cultivars, Nicotiana debneyii (Bagnall et al., 1956) and Chenopodium quinoa. C. quinoa is often used as an indicator plant for PVS infection as it gives a characteristic local lesion symptomology (de Bolcx, 1970; Hiruli, 1975; Kaczmarek and de Bolcx, 1977).

Andean strains of PVS (PVS\(^\text{A}\)), so called because symptoms induced by these strains of PVS are similar to symptoms induced by an isolate from a Peruvian potato variety (Gibbs et al., 1965), induce more severe symptoms in potato, such as chlorosis and necrosis, and a systemic response in C. quinoa as opposed to the local lesion response induced by the ordinary strain (PVS\(^\text{o}\)) (Hinostroza-Orihuela, 1973). These strains appear to be more active and tend to reach a higher concentration in the plant than the more usual varieties. PVS can be transmitted mechanically but more usually in a nonpersistent manner by aphids (Rose, 1983; Dolby and Jones, 1987) and it has also been observed that the Andean strain is transmitted more readily than the ordinary strain (Rose, 1983; Slack, 1983). From a molecular point of view a comparison of the two strains have revealed little differences in the coat protein with regards to cross reactivity with antisera and size but sequence comparison has revealed quite different results (Foster and Mills, 1991d). Although the sequences of the coat proteins are highly homologous (93%) a region of variability exists at the N-terminal region. Within this variable region is a block of 11 consecutive non-homologous amino acids. Sequence comparison of the 11 kDa 3' terminal gene has also revealed a block of 8 non-homologous consecutive amino acids. It has been suggested that these differences may reflect the differences in transmissibility and symptomology that has been observed between the two strains.
Fig 1.1 Diagrammatic representation of the genome structure of the carlavirus PVS (Foster, 1990d; Foster and Mills, 1990a; 1992a; 1992b). RNA molecules are represented as single lines and gene products are represented as shaded boxes. Sizes are indicated in kilobases (kb) and kilodaltons (kDa), respectively. 5' and 3' terminal structures are also indicated.
Fig 1.1

7.5 kb genomic RNA

190 kDa Replicase

2.5 kb subgenomic RNA

25 kDa

7 kDa

12 kDa

Triple Gene Block Proteins

1.3 kb subgenomic RNA

32 kDa

Coat Protein

11 kDa
1.3.2 The PVS translational strategy

Viruses, both plant and animal, employ a diverse range of ingenious translational strategies to attain expression of their genome in the eukaryotic host environment. Strategies include segmentation of the viral genome in such a way that essential ORFs are placed at the 5' proximal end of a mRNA molecule, a strategy employed by brome mosaic virus (BMV) (Ahlquist et al., 1984). A similar mechanism to this is the production of subgenomic RNA molecules. Subgenomic RNAs are identical in sequence to the genomic molecule but they are 3' co-terminal and their production effectively places internal genes in a position where they will be expressed by the eukaryotic ribosome which will only recognise the first gene on a polycistronic RNA molecule. Examples of viruses that produce subgenomics are, BMV (Ahlquist et al., 1981) and tobacco mosaic virus (TMV) (Joshi et al., 1983; Hunter et al., 1976). TMV, among other viruses, exhibits the phenomenon of translational readthrough. At a frequency of about 10% an amber termination codon at the end of the 126 kDa protein open reading frame (ORF) is ignored resulting in the production of a larger 183 kDa readthrough product (Pelham, 1978). Other plant viruses, such as members of the como- or potyvirus group, express their entire genome in the form of a large polyprotein which is subsequently autocatalytically cleaved to give constituent viral components (Franssen et al., 1984). The potential for differential translational regulation among the polyprotein producing viruses does not exist as all products are produced in equimolar quantities, with segmented genome viruses or those producing subgenomic molecules the potential for regulation is much greater. It has been estimated that each TMV particle requires about 2150 subunits for complete encapsidation whereas only one RNA polymerase molecule may be required. The potential for regulation at the stage of mRNA production is possible and also smaller coat protein subgenomics appear to be able to compete more efficiently for ribosomes than larger subgenomics. Coat protein is synthesised in vivo at much larger amounts than would be expected of the 5:1 ratio of encapsidated subgenomic to genomic molecule (Davis and Hull, 1982).

The major strategy employed by PVS, and carlaviruses in general, is to express their internal gene products via the production of subgenomic RNA molecules. For PVS two subgenomics have been detected in infected tissue. These subgenomic RNA molecules, 2.5 kb and 1.1 kb in size, were detected in addition to the full length 7.4 kb molecule when poly-A(+)RNA extracted from infected potato tissue was probed with a PVS coat protein clone (Foster and Mills, 1991a). These molecules appear to be polyadenylated as both were shown to bind well to oligo(dT)-cellulose. Subgenomic molecules have also been detected with the ordinary strain of PVS (Foster and Mills, 1990a). It has been demonstrated that the translation of capped mRNA molecules is sensitive to the addition of cap analogue. Bearing this in mind evidence suggests that PVS subgenomic molecules do not possess a cap structure at the 5' end as the expression of subgenomic encoded products do not appear to be affected by the addition of exogenous cap analogue to an in
In vitro translation system as opposed to the sensitivity exhibited by the genomic RNA encoded proteins (Foster and Mills, 1990a). About a 78% decrease in incorporation of radioactive label was observed in the presence of cap analogue but there appeared to be little or no effect on the production of viral coat protein. The analysis of HelVS particles under electron microscopy has revealed that in addition to the full length viral genomic particle, two smaller particles can also be detected within the smear of fragmentation of about 320 nm and 180 nm. Northern analysis on purified RNA isolated from HelVS viral particles using a 3' terminal clone has identified the presence of small subgenomic molecules, 3.3 and 1.5 kb in size, in addition to the full length genomic molecule (Foster and Mills, 1990b). For many members of the group subgenomic RNA species have as yet not been detected, and for those viruses with which they have been identified these RNA species are only present in trace amounts.

In vitro translation of RNA extracted from PVM, PVS, HelVS and CLV (Szybiak and Legocki, 1981; Foster and Mills, 1990a; 1992b; Meehan and Mills, 1991) results in the production of a broad range of peptides of about 190 kDa to 31 kDa in size. Time-course translation studies have revealed that, in vitro, coat protein is one of the primary products of translation and one of the most abundant, being produced after only a 10 minute incubation. It is not clear how a protein, that is evident in such large quantities, is produced from a subgenomic RNA molecule that is present at almost undetectable amounts.

In addition to the generally accepted carlavirus translational strategy, that is the production of subgenomic RNA molecules, some evidence has also suggested that proteolytic processing of viral products may occur. In vitro, one of the primary products of PVS genomic RNA translation is a 190 kDa peptide, a protein which is presumed to be the viral replicase. Time-course translation experiments have revealed a decrease in intensity of this band on a polyacrylamide gel, over a 60 minute period, with the simultaneous accumulation of a 150 kDa peptide. When amino-acid analogues were incorporated into the original translation reaction this phenomenon was not observed (Foster and Mills, 1992b). The incorporation of these analogues into viral products has previously been shown to block proteolytic cleavage (Pelham, 1979; 1980; Vance and Beachy, 1984).

It has been suggested that the protease activity responsible for this processing could be a function of a subgenomic encoded product because fractionated PVS viral RNA, from which the larger genomic band was selected in preference to the subgenomic molecules, when translated in vitro, displayed a reduced degree of proteolysis when compared to the level when the full complement of viral RNA was translated (Foster and Mills, 1992b). This protein processing has also been observed with the HelVS replicase protein and the 36 kDa coat protein of AHLV which is produced from a 38 kDa precursor product (Foster and Mills, 1991c). Protease activity could be a viral encoded property or a specific host encoded protease could be activated either directly or indirectly by the virus. It is interesting to note that the C-terminal portion of the PVM coat protein, does in fact show
Fig 1.2 Eukaryotic cap-dependent initiation of translation (Hershey, 1991). Ribosomal small (40S) and large (60S) subunits are represented as semi-circles. Translational initiation factors are represented as circles, and the initiating transfer RNA molecule as an L-shaped structure. The 7-methyl-guanylic acid "cap" at the 5' end of the RNA molecule is represented as a solid circle. The direction of the scheme of events is indicated by arrows. This process is explained in greater detail in Section 1.4.
Peptidyl chain elongation
homology in sequence and position to the functional catalytic domain that has been recognised in a number of serine proteases (Strauss et al., 1987). Instead of the characteristic -His-Asp-Ser- (HRS) catalytic triad, PVM has a -His-Asp-Thr- (HRT) triad at the same position. Similar homologies have also been detected within the C-terminal portions of the PVS, CLV and LSV coat proteins (Foster and Mills, 1992b).

1.4 Initiation of translation in eukaryotes

The major subject of this thesis is a study of the translational strategy exploited by plant viruses, consequently, a brief account will be presented regarding the general features involved in this mechanism, in particular those involved in the stage of translational initiation.

Eighteen years ago Marilyn Kozak proposed a model for eukaryotic translational initiation which today is still generally accepted (Kozak, 1978). The "scanning model" for translational initiation dictates that the 40S small ribosomal subunit initially binds to the 5' end of a mRNA molecule and migrates along the untranslated leader to the first AUG codon in a favourable context for initiation of translation. Since the initial proposal of this theory extensive research is beginning to reveal the mechanism involved and the protein-protein and protein-RNA interactions that may occur. The more that becomes known the more complex the mechanism appears to be.

A whole array of eukaryotic initiation factors (eIF) have now been identified and the function of some have been deduced. Most are involved in the formation of a complex between the small ribosome subunit, the mRNA molecule and the initiating tRNA molecule (tRNA\textsuperscript{i^\*}). The initiation factors dissociate from the complex before or during 40S and 60S ribosomal subunit association when another set of factors join to promote peptidyl chain elongation. A great number of covalent and non-covalent associations between protein factors and RNA have been identified in this system with only hydrolysis of pyrophosphate bonds in ATP and GTP occurring. At least 10 initiation factors have been partially characterised; more may also be involved that are as yet unknown. Most evidence regarding the actual mechanism has been derived from in vitro studies but a proposal regarding the scheme of events that are thought to occur during translation initiation has been developed. Cap-dependent translational initiation has been reviewed extensively (Kozak, 1983; 1989; Hershey, 1991), so major steps involved are only outlined briefly below though they are essential for an explanation of the mechanism of cap-independent translation that will be discussed in the following section (Section 1.5). A diagrammatic representation of the scheme of events involved is provided in Fig 1.2.

The cap-dependent translation initiation pathway can be divided into four major steps:

1. Ribosome dissociation into 40S and 60S subunits.
2. At physiological conditions 80S ribosomes exist in an active equilibrium with
constituent subunits. Two initiation factors, elF-1A (eukaryotic initiation factor-1A) and 
elF-3 shift the equilibrium to dissociated subunits by binding to the 40S subunit and 
preventing its association with the 60S subunit. It is thought that these two factors 
remain associated with the 40S subunit throughout translational initiation.

(2) Met-tRNA_J binding to the 40S ribosomal subunit to form a preinitiation complex.

eIF-2 in a binary complex with GTP binds with the initiating methionine transfer RNA 
(Met-tRNA_J) to form a ternary complex. Another initiation factor eIF-2C stabilises this 
ternary complex and stimulates the formation of the 40S preinitiation complex whereby 
the ternary complex binds to the 40S ribosomal subunit.

(3) mRNA binding to the 40S preinitiation complex to form a 40S initiation complex.

An initiating 40S ribosomal subunit interacts with the mRNA molecule in association 
with the 7-methyl-guanylic acid “cap” structure at the 5’ terminus. There is also evidence 
of internal ribosome binding that will be discussed later. eIF-4F (the cap binding 
complex) recognises and then binds to the cap through its α subunit (eIF-4E component 
of the 4F complex). Two additional components eIF-4A and eIF-4B then bind and their 
associated ATP dependent RNA helicase activity melts the secondary structure in the 5’ 
region of the mRNA leader. eIF-4A is an RNA dependent ATPase. Once secondary 
structure has been melted the 40S preinitiation complex can scan the mRNA leader in 
search of the initiation codon.

(4) Joining of the 40S initiation complex with the 60S subunit to form an 80S initiation 
complex.

The 60S ribosomal subunit binds to the 40S subunit carrying the mRNA molecule and 
Met-tRNA positioned at the initiation codon. This ribosome association is stimulated by 
eIF-5. The GTP molecule bound to eIF-2 is hydrolysed in this reaction which results in 
the ejection of the eIF-2.GDP complex and the other bound initiation factors. The 
association of the 60S subunit may be rapid as the 40S ribosomal subunit has low 
stability in the absence of bound eIF-2.

eIF-2.GDP must be recycled to catalyse another round of initiation. This marks one of 
the major control steps in the process of translation. This recycling is catalysed by eIF-2B, 
otherwise known as the GEF factor (guanine nucleotide exchange factor) which 
promotes GTP:GDP exchange. eIF-5A stimulates the formation of the first peptide bond 
and chain elongation occurs.

Several criteria must be met for a mRNA molecule to be efficiently translated. One of 
the major factors appears to be the context of the sequence surrounding the AUG start 
codon. Mutagenesis studies with monkey cells originally identified a - 
GCC^A/GCCCAUGG- sequence as the optimal context for translational competence. 
Although the other residues augment translation the important bases appear to be a purine 
at position -3 and a G residue at position +4 when the A of the AUG is designated 
residue +1. This has also been confirmed in vitro and in transgenic plants (Kozak, 1986; 
1987). An analysis of plant mRNAs has identified an expected purine at position -3 in 
93% of the molecules studied and a G at position +4 in 74% of those studied (Kozak,
Another factor is the presence of a 7-methyl-guanylic acid “cap” structure at the 5’ end of a mRNA molecule. The importance of the cap structure on the efficiency of mRNA translation in vitro was first reported by Shatkin, (1976). The general rule is that the translation of most cellular mRNAs is stringently dependent on the presence of a cap structure in vivo and uncapped transcripts are translated inefficiently. The cap recognition initiation factor associates with the methylated cap during translational initiation, but this recognition may be dispensable for the translation of mRNAs with relatively long unstructured leaders, a situation that will be discussed later.

The translatability of a cellular mRNA molecule is also determined by the nature of the 5’ untranslabeled leader. The length of the untranslated leader sequence affects the efficiency of translational initiation. Cellular mRNA leaders are generally 40-80 ribonucleotides in length. It appears that a leader length of at least about 20 residues is essential for initiation codon recognition as it has been demonstrated with synthetic leaders that the first AUG in a favorable context for translation was ignored at a level of about 50% when it was positioned 12 nucleotides from the cap structure (Kozak, 1991a; 1991c). Presumably this is due to the lack of sufficient sequence for ribosome assembly upstream from the translational start site. Kozak has also proposed that translational efficiency in vitro is proportional to leader length, using synthetic leaders in the range 17-80 nucleotides, i.e., translational efficiency increases with increasing leader length (Kozak 1991b). Some cellular leaders and the well documented leaders from viral RNAs have extremely long 5’ untranslated regions but there is no general evidence that this may be detrimental to translation unless they contain extensive secondary structure, indeed quite the opposite appears to be true. As was mentioned above the scanning model for translational initiation dictates that ribosomes scan the untranslated leader until they reach the first AUG codon in a favorable context for translational initiation. Presumably, in line with this theory, the longer the leader the larger the assembly pad for ribosome binding and the greater the pool of associated ribosomes for translational initiation.

Another important consideration for translational efficiency concerns the degree of secondary structure contained within the 5’ leader. The majority of cellular mRNAs have untranslated regions with a high GC content (50-70%). Some have been identified with GC contents as high as 90%, this is a characteristic of mRNAs of proteins that are usually present in the cell at a low abundance, such as oncoproteins or growth factors (Kozak, 1987b; 1991d; 1992). The corresponding high degree of associated secondary structure will act as a barrier to ribosome scanning and it has been postulated that RNA helicases may be involved in the melting of extreme secondary structure to facilitate ribosome migration. Helicase activity has been associated with the eIF-4A initiation factor (Grifo et al., 1984; Linder et al., 1989). This high degree of secondary structure in cellular leaders brings forward implications regarding translational control. The translational efficiency of high GC cellular mRNA leaders may be detrimentally affected under conditions of stress such as during viral infection and this may play a role in the host translational shutdown observed under such conditions. An interesting observation from an evolutionary point of view is that lower eukaryotes such as slime moulds and
yeasts have leaders which are as much as 90% AU rich (Cigan and Donahue, 1987), a characteristic also displayed by viral 5' untranslated leaders. This unstructured leader conformation which facilitates ribosome migration and scanning may partly explain why viral RNAs are translated more efficiently than host molecules. Whether this factor alone explains the high efficiency of viral translation will be discussed later.

The regulation of gene expression at the translational level has been studied in detail recently and it appears that the 5' leader may play a vital role in this form of control. It appears that secondary structure or the presence of upstream AUG codons can dramatically influence translation. Kozak has recognised the phenomenon of reinitiation or "leaky scanning" whereby ribosomes remain associated with the mRNA molecule and reinitiate at a downstream AUG (Kozak, 1983; 1988). Reinitiation has been observed with cellular mRNAs but only when the upstream cistrons are extremely small.

An example of a closely regulated reinitiation process concerns the translation of the yeast GCN4 gene which encodes a transcription gene which regulates the expression of a series of biosynthetic genes in a response to amino acid starvation. The transcript is constitutively expressed but is only translated in response to cell starvation, therefore regulation is at the translational level.

The 5' leader of the mRNA molecule contains 4 small ORFs upstream from the authentic AUG start codon. The accumulation of uncharged tRNAs that occurs during amino acid starvation results in the activation of the specific kinase that phosphorylates and consequently inactivates the cellular eIF-2 factor α subunit (Dever et al., 1992). As has been stated earlier, eIF-2 is responsible for the escortation of the Met-tRNA$_i$ to the small ribosomal subunit. Its depletion causes an increase in the time required for the 40S subunit to acquire a Met-tRNA and become competent to reinitiate. This reduced competence allows some of the 40S subunits to bypass upstream cistrons and initiate at the authentic AUG of the GCN4 gene (Geballe and Morris, 1994). Eukaryotic reinitiation is, as far as we know, thought to be the exception, especially in the situation described above where it offers such a degree of regulated translation.

1.5 The exception: cap-independent translation

Viruses, of both plants and animals, affect host metabolism in both drastic and quite subtle ways. The subtle interactions of viruses with cellular translational machinery are largely unknown but some interesting observations have been made. Extensive research, from a translational viewpoint, has concentrated on the picornavirus group of animal viruses, perhaps because instead of a methylated cap structure this group of viruses possess a virus encoded VPg structure at the 5' end of the genome (Lee et al., 1977; Flanagan et al., 1977). The scanning model for translational initiation predicts that uncapped transcripts will be translated inefficiently which certainly is not the case with the picornaviruses, consequently this suggests a mode of translation which is not dependent
on the cap-dependent scanning model. This section will discuss the nature of the structural elements of the picornaviral genome that are involved in cap-independent translational initiation. Although these similar regions in different picornaviruses share several characteristics, many differences have also been identified.

1.5.1 Picornaviruses

Members of the picornavirus group have a single-stranded, positive-sense RNA genome of about 7.5 kb in size with one large ORF encoding a polyprotein which is subsequently autocatalytically cleaved to constituent viral components. Instead of a cap structure, picornaviruses have a small polypeptide termed VPg covalently linked to the 5' end of the genome which is removed in the cytoplasm. The 5' untranslated region (UTR) is large, 610-1200 nucleotides in length and several AUG codons have been detected within these picornavirus leaders some even in a favourable context for translational initiation with a characteristic A(-3) and G(+4) configuration (Sonenberg, 1987). Translation products of these small ORFs have not been identified but even if they were produced their size may be too small for detection. It has been argued that ribosomes may scan the leader and initiate translation on these other AUG codons. Once translation of these small ORFs was complete it has been suggested that ribosomes remain associated with the mRNA leader and "reinitiate" translation at another downstream good context AUG (Kozak, 1983; 1988; 1992). It seems unlikely that reinitiation or "leaky scanning" is responsible for picornavirus translation because although leaders of group members share such characteristics as length and secondary structure there is little in the way of sequence conservation. Start codons are present at different positions with different group members in an apparently random manner, consequently, it has been suggested alternately that picornavirus translational initiation occurs by a process of direct internal ribosome binding, totally independent from a mechanism requiring association with the cap structure.

It has been proposed that the UTRs of picornaviruses are highly structured containing several stem loops which have the potential to base pair with cellular factors involved in the process of cap-independent initiation of translation (a structural configuration that would hinder ribosome scanning). The regions of the 5' UTR of picornaviruses involved in internal initiation have been called "internal ribosome entry sites" (IRES) or "ribosome landing pads" (RLP) and much work has been concentrated on an identification of factors that interact with structural components within these regions and how they promote initiation of translation (Sonenberg 1987; Jackson et al., 1990).

Two major types of IRES structures have been identified, the first type is common to members of the entero- and rhinoviruses, the second type is common to cardio- and aphthoviruses. Similarities do exist between leaders of these 2 groups, in particular in the secondary structure configuration. The IRES structures of foot-and-mouth disease virus (FMDV) and encephalomyocarditis virus (ECMV) show about 50% sequence homology whereas the IRES structures of FMDV and poliovirus only display about 35%
homology (Jackson et al., 1990). Cap-independent internal initiation of translation may be a true cellular process with differences between the IRES structures indicating subtle differences in this mechanism.

Dicistronic constructs have been used to demonstrate internal initiation associated with the leaders of picornaviruses. Dicistronic cellular eukaryotic mRNAs have generally not been detected unless the upstream ORF is extremely small but in a prokaryotic environment polycistronic mRNAs are the norm which reflects the differences between the eukaryotic and the prokaryotic ribosome. The downstream cistron in such a construct should be translated very inefficiently with those ribosomes only initiating translation that had scanned through the entire upstream ORF. It has, however, been demonstrated that when many of the picornaviral 5' leaders are placed between two cistrons the second cistron is translated even more efficiently than the first (Pelletier and Sonenberg, 1988).

1.5.1.1 Enteroviruses and Rhinoviruses

Poliovirus has probably been the most extensively studied picornavirus with regard to translational internal initiation. Pelletier and Sonenberg, (1988), has reported, both in vitro (in HeLa cell extract) and in vivo (in COS cells), the efficient expression of the second ORF in a dicistronic construct, chloramphenicol acetyl transferase (CAT), when the poliovirus 5' UTR is placed in the intercistronic region. No CAT translation occurs when the intercistronic region contains a synthetic spacer approximately the same size as the poliovirus UTR. Another interesting observation from this research is that translation of the first ORF, thymidine kinase (TK), is inhibited by about 50 fold when translated under high salt conditions. In this system expression of CAT is slightly stimulated.

The 5' and 3' borders of the poliovirus RLP or IRES has been mapped to between nucleotides 320-630 of the leader (Pelletier and Sonenberg, 1988). Secondary structure predictions within this region have identified a number of stem-loop structures which have the potential to be involved in RNA-RNA and RNA-protein interactions involved in the process of cap-independent initiation of protein synthesis. Haller et al., (1992), has identified a stem loop structure (designated stem loop F) between nucleotides 448-556 which is absolutely essential for virus infectivity and translation. Mutations resulting in the destabilisation of secondary structure in this region dramatically reduce translation. Another absolutely critical cis-acting sequence is a polypyrimidine tract, at nucleotide 558, which is absolutely conserved among picornaviruses. A sequence of UUUCC at the 3' end of this tract appears to be the central functional region associated with internal initiation as point mutations within this sequence totally eradicate translation (Meerovitch et al., 1991; Nicholson et al., 1991). A cryptic AUG at position 586 also appears to be important for function but the stem loop in which this is contained is dispensible.

The polypyrimidine tract sequence was first identified by Beck et al., (1983), in the untranslated leader of FMDV as a sequence showing significant homology to a purine rich sequence at the 3' end of eukaryotic 18S rRNA and it was suggested that it offered a function similar to that of a Shine-Dalgarno sequence for prokaryotic ribosome binding.
This complementarity is evident between the 5' half of this tract in poliovirus and mammalian 18S rRNA and mutations more sensitive to substitution are those in residues that would interact most strongly with the ribosome (Pestova et al., 1991). The fact that this tract is about 200 nucleotides upstream from the authentic AUG codon makes it unlikely that this sequence behaves exactly as a Shine-Dalgarno sequence but it may be important for ribosome association.

It is not as yet clear what secondary ultrastructure that may form within the 5' UTR but individual loop structures and important sequences have been pinpointed by these mutational studies. The actual mechanism of internal initiation also is unclear but it has been proposed that the polypyrimidine tract may mark an integral sequence for ribosome association and the upstream sequence may fulfill a spacer function between this sequence and the authentic AUG start codon downstream.

The IRES of human rhinovirus (HRV) RNA extends to a point between nucleotides 554-562 of the 5' untranslated leader (Borman et al., 1992). Nested deletions of the leader smaller than the 554 nucleotide 5' boundary of the IRES in a dicistronic construct results in the inactivation of translation of the second cistron when translated in vitro in rabbit reticulocyte lysate and in HeLa cell extracts. Deletions larger than the 3' 562 nucleotide boundary results in the translation of the second cistron to the same level as with the full length leader in the same system. Computer secondary structure predictions indicate that this IRES lies in a relatively unstructured region and it appears that it is the specific sequence within the 554-562 boundaries that is important rather than the spacer function that it provides as when this region is replaced with a 15 nucleotide oligonucleotide linker in a dicistronic construct translation of the downstream cistron is again inactivated. Even though the HRV 5' UTR is smaller than the poliovirus UTR there is quite strong sequence homology between the two over the 5' proximal 600 nucleotides and the boundary for the poliovirus IRES has been mapped to a similar region as that of HRV. In both viruses this sequence is quite a distance upstream from the authentic AUG start codon so it has been proposed that ribosomes associate with the RNA at the IRES sequence after which scanning occurs until the authentic AUG codon is reached for initiation of translation.

1.5.1.2 Cardioviruses and Aphthoviruses

The ECMV 5' non-translated leader is 834 nucleotides in length and contains a functional IRES structure for internal ribosome binding within the 3' proximal ~500 nucleotides to the authentic AUG start codon (Jang et al., 1988; 1989). Two features have been identified within this sequence which are thought to be the functional cis-acting elements of the ECMV IRES, a stem-loop structure (stem-loop E) and the polypyrimidine tract that is highly conserved among picornavirus UTRs.

Stem-loop E is absolutely essential for translational function. Mutations that destabilise the secondary structure configuration of this stem greatly decrease translation,
complementary mutations in the opposite side of the stem that restore base pairing results in a wild type level of translation. Stem-loop C at the 5' end of the leader appears to be a duplication of stem-loop E and may augment translation. The widely documented polypyrmidine tract marks the 3' border of the ECMV IRES, any base substitutions in this region strongly reduce the translational efficiency.

The ECMV IRES directs internal initiation of translation in a genetically engineered poliovirus (Molla et al., 1992). This quite ingenious experiment resulted in the construction of a dicistronic virus genome with 573 ribonucleotides of the ECMV IRES inserted between the P1 and P2 ORFs of the poliovirus type 1 RNA. Transcripts translated in HeLa cell-free extracts functioned with comparable efficiencies and resulted in the initiation of a viable infection in vivo.

FMDV is an aphthovirus on which extensive research has been concentrated especially on the elucidation of the actual mechanism of cap-independent translation and the cellular factors that may be involved. The FMDV IRES has been mapped to the 3' terminal 450 nts of the 1300 nucleotide long 5' UTR. As described above, it was Beck et al., (1983), that first identified the conserved polypyrimidine tract as an integral cis-acting sequence in the process of cap-independent initiation of translation. Work regarding the binding of a cellular p57 protein has allowed the beginning of the elucidation of the tertiary leader structure configuration and how this may help to explain the process of internal ribosome initiation (Jang and Wimmer, 1990). p57 binds to 2 distinct regions of the leader which are separated by more than 300 nucleotides. This protein may have two binding domains, or exist in dimeric forms, either way the result will be to bring these regions into close proximity in such a way that a large portion of the leader could be effectively “jumped” by the ribosome. A similar situation is evident with ECMV.

Theilers murine encephalomyelitis virus (TMEV) is another cardiovirus that has been shown to possess a 5' UTR that promotes initiation of protein synthesis in a cap-independent manner. The TMEV UTR is 1064 nucleotides in size and shows sequence and structural homology with the UTRs of other cardioviruses especially in a polypyrmidine sequence 16 nucleotides upstream from the AUG start codon. As has been mentioned for FMDV, this region forms a highly conserved stable stem-loop structure which is thought to be integrally involved in internal translational initiation. Deletions of the leader in both mono- and dicistronic constructs have mapped the TMEV IRES to between nucleotides 500 and nucleotides 1043-1053 (Bandyopadhyay et al., 1992).

1.5.1.3 Other viral IRES types

Another type of IRES structure has been identified within the hepatitis virus A (HAV) 5' UTR. HAV is an enterovirus but it contains an IRES-like structure more closely related to that of ECMV (a cardiovirus) than to members of the same group, such as poliovirus.
The region responsible for internal initiation of protein synthesis has been mapped to the 300 nucleotides proximal to the authentic initiation AUG codon at position 736 (Brown et al., 1991; Glass et al., 1992).

Hepatitis virus C has a genome organisation similar to that of flaviviruses but it possesses a long 5' UTR, containing 3 or 4 AUG codons (depending on the virus strain), which is translated in a cap-independent manner. This has promoted its classification as a separate group. The HCV IRES has been localised to a sequence between nucleotides 101-332 of the UTR which is the smallest IRES as yet identified, at least among the picornaviruses. No real sequence or structural homology is evident with other IRES containing leaders except for the presence of a polypyrimidine tract at position 206 which appears to be the one common feature of picornaviral leaders. A stem-loop structure in the functional IRES sequence may play a role in translational initiation as this structure is conserved between other HCV types (Kohara et al., 1992).

A functional naturally occurring polycistronic mRNA of an eukaryotic virus is the RNA3 molecule of the coronavirus, infectious bronchitis virus (IBV). This molecule appears to be tricistronic in nature, encoding gene products 3a, 3b, and 3c in that order. No subgenomics have been identified to explain the expression of internal gene products. According to Kozak's model for translational initiation it was proposed that translation of the two downstream ORFs occurred as a result of leaky scanning of ribosomes through the upstream initiation codons (Kozak, 1978). The initiator AUGs for 3a and 3b are in fact in poor context for translational initiation but evidence that contradicts this hypothesis is that 3c is the most abundant of the 3 proteins in infected cells and mutations that strengthen the context of the upstream AUG codons appear to have no effect on this expression. This suggests that translation associated with 3c is independent of translation of the two upstream ORFs. To further reinforce this observation Lui and Inglis, (1992), constructed a chimeric tetracistronic construct in which the influenza virus NP protein gene was inserted upstream from the 3a ORF. Translated in vitro the NP protein and the 3c protein were the only products detected. Translation of 3c also appears to be relatively insensitive to the addition of cap analogue, a factor which drastically reduces the translation of the other mRNA products.

All this evidence points towards a cap-independent internal ribosome initiation mechanism for the translation of the 3c cistron of IBV with the internal ribosome landing pad being within the coding region of the 3a or 3b ORF. Structural comparisons of the upstream region from the 3c AUG codon has revealed similarities to that suggested for ECMV. Similarities are most evident in two stem loop structures, one of them stem loop E which contains a polypyrimidine tract which is highly conserved throughout the picornaviruses. Near the beginning of the 3a gene is a 7 nucleotide sequence with base pair complementarity with the 3' end of the eukaryotic 18s rRNA molecule.

Combining all of this evidence allows a picture of the mechanism that may be occurring in association with these sequences to emerge. Richard Jackson, (1990), has proposed...
that ribosomes actually bind to the 3' end of the leader in very close proximity to the authentic AUG initiation codon. The model favoured by Sonenberg, (1991), proposes that the site of ribosome initiation is internal in the IRES structure with subsequent ribosome scanning until the AUG start codon is reached. It is thought that with cardio- and aphthoviruses the leader ultrastructure brings the ribosome entry site into the region surrounding the authentic AUG as the conserved IRES structure extends right up to this codon. The situation with entero- and rhinoviruses is more complex with sequence conservation extending not as far as with the other group leaving a variable sequence region upstream from the AUG. Evidence points to the ribosome entry site being just downstream from a conserved polypyrimidine tract after which a small amount of scanning occurs until the authentic AUG codon is reached. The defined structures identified and the cellular factors that bind to these associate in a specific manner to potentate internal ribosome association. Although it may share many characteristics, initiation of translation among the different picornaviral groups do show some differences. Poliovirus translation is very inefficient and inaccurate in rabbit reticulocyte lysate or in Xenopus oocytes whereas translation of FMDV or cardioviruses is extremely efficient under these conditions. Poliovirus translation, in these systems, requires the supplementation of factors present in HeLa cells for efficient expression. This suggests that different cellular factors are required for the different mechanisms of translation. Cap-independent translational factors will be discussed in more detail in Sections 1.5.3 and 1.5.4.

1.5.2 Cellular mRNAs

Organisms as primitive as viruses are not unique in their ability to circumvent the constraints of normal eukaryotic translation initiation. It is quite plausible to suggest that many cellular mRNAs may also have evolved to employ a cap-independent translational mechanism. It may be of advantage to some mRNAs to be translated during developmental stages in the organism during which cap-dependent translation is inhibited, for example, a dramatic decrease in host translation under high salt or stress conditions, during viral infection or during mitosis. With the latter it is thought that underphosphorylation of the eIF-4E cap binding protein during this cellular developmental stage is responsible for the decrease (Bonneau and Sonenberg, 1987).

One example of such a cellular molecule is the glucose-regulated protein 78/immunoglobulin heavy chain-binding protein (GRP78/BiP) which is upregulated on poliovirus infection, during which there is a global down regulation of host cellular translation. Translation of dicistronic constructs with the GRP78/BiP untranslated leader between two reporter genes results in an efficient expression of the second cistron (Macejak et al., 1991). Dicistronic chloramphenicol (CAT) - luciferase (LUC) constructs containing intercistronic leaders, synthetic, poliovirus UTR and BiP 5' leader, transfected into HeLa cells express CAT at the same level but LUC is translated 20-30 times more
efficiently with the constructs containing the poliovirus and BiP leaders than in the construct containing the synthetic leader.

The 5' leader of the homeotic gene Antennapedia (Antp) mRNA of Drosophila contains an IRES-like structure (Oh et al., 1992). Transcription of this mRNA molecule is driven by two promoters, P1 and P2, giving RNA transcripts that differ in their 5' non-coding regions (NCR). The long NCR of the mRNA molecule generated from the P2 promoter contains 15 AUG codons all in a favourable context for initiation of translation and the sequence contains quite strong secondary structure which would hinder the process of ribosome scanning. Using a CAT-LUC dicistronic construct, expression of the downstream LUC cistron was shown to be 240 times higher when the Antp leader was placed in the intercistronic region, in cultured SL2 (Schneider line 2) cells. The cis-acting region involved in initiation has been mapped to a 252 nucleotide exon sequence in the leader which is thought to act as an IRES analogous to the situation with the picornaviruses. This sequence is also present in the leader of the mRNA molecule generated from the P1 promoter and it appears to exhibit quite high homology throughout Drosophila species which indicates perhaps some conserved function. Up to 20% of Drosophila mRNA leaders have long 5' NCRs containing multiple AUG codons which indicates that initiation of translation by this mechanism may be quite frequent in this species (Cavener et al., 1991).

Translation of many other cellular transcripts may be regulated in this way and a study of these mRNA molecules may help to explain changes in the translational machinery that occur during varying stages of cellular development. Several examples of developmentally regulated genes, or genes that respond to external stimuli, have been reported and translational regulation has been implicated as the controlling factor. The 5' untranslated region of Xenopus c-myc 1 mRNA is responsible for the selective expression of the gene product during oogenesis and early embryogenesis (Lazarus, 1992). The conditions that select this mRNA for translation at this stage in development are unclear. Other examples include the response to light that controls translational initiation of the ribulose-1,5-bisphosphate carboxylase gene in Amaranth cotyledons (Berry et al., 1990), or the light response that triggers the differentiation of cells in Volvox synchronous cultures (Kirk et al., 1985).

1.5.3 The mechanism of cap-independent translation (evidence from picornaviruses)

Little is known about the mechanisms of cap-independent translation and the transacting factors that may be involved. Meerovitch and co-workers, (1989), have identified a 52 kDa protein (p52) from HeLa cell extract which forms a specific protein-RNA complex between nucleotides 559-624 of the 5' untranslated poliovirus leader. More recent data has identified p52 as the La autoantigen which is a nuclear protein involved in the
termination of RNA polymerase III transcripts (Meerovitch et al., 1993). This protein appears to be relocated to the cytoplasm during poliovirus infection. Several proposals have been made regarding the nature of the p52/La protein. One suggestion is that La plays a role in secondary structure unwinding in association with the translational initiation factors eIF-4A and eIF-4B or in the recruitment of translational factors or ribosomes to the specific region of the leader for internal initiation. Another view is that it acts as an initiating correction factor (ICF). Translation of picornaviral RNA is very inefficient in rabbit reticulocyte lysate with upstream AUGs being selected for translational initiation as opposed to the authentic start site, but this is enhanced by the addition of a 54 kDa ICF isolated from Krebs-2 cells which corrects the selection of the AUG (Svitkin et al., 1988).

A transacting factor has also been identified which specifically interacts with the ECMV 5' leader. This 57 kDa (p57) protein appears to bind to a stem loop structure, predicted as being an integral part of the internal ribosome entry site (IRES) of the untranslated leader for this virus. The same factor is thought to be involved in FMDV translation (Jang and Wimmer, 1990). Mutations that alter secondary structure in this region totally abolish p57 binding. Immunoprecipitation has identified this protein as the nuclear pyrimidine tract-binding protein (PTB) which is involved in the splicing of RNA polymerase II transcripts but it also appears to be associated with eukaryotic ribosomes in the cytoplasm (Hellen et al., 1993; Patton et al., 1991). The IRES function of ECMV can actually be decreased by immunodepletion of the PTB protein (Ghetti et al., 1992). Again, as with La protein suggestions of functions for this protein are purely speculative. p57/PTB appears to be a ribosome bound protein and so may be involved in the recruitment of ribosomes for translation. Both proteins appear to have dual function, nuclear and cytoplasmic.

The addition of extract from fresh mouse liver to rabbit reticulocyte lysate stimulates the IRES function of Hepatitis virus A by 12 fold (Glass and Summers, 1993). This enhancement was not evident when extract from any other part of the mouse was added suggesting the presence of liver specific factor was responsible for this increase. This brings forward the theory of a tissue specific or developmental regulation of translational initiation.

1.5.4 The involvement of cap-dependent translational initiation factors

An integral component of eukaryotic translational initiation is the eIF-2 factor. This factor marks a control point in the eukaryotic translation mechanism, its activity being controlled at the level of phosphorylation. As was mentioned briefly above, eIF-2 is involved in the initial complex formation with GTP and the initiating methionine tRNA (Met-tRNA Met), eIF-2 effectively escorts the Met-tRNA Met to the 40S ribosomal subunit with which a 43S initiation complex is formed. This complex binds to the 5' end of a mRNA molecule and migrates along the untranslated leader. eIF-5 joins the complex
and this triggers the hydrolysis of GTP which in turn induces a conformational change in the eIF-2 protein which results in a reduced affinity for 40S binding. The eIF-2.GDP complex dissociates leaving the initiator tRNA and the 40S ribosomal subunit free to associate with the 60S ribosomal subunit giving a functional ribosome. The α subunit of eIF-2 may be phosphorylated by two specific kinases, namely the p68 kinase and the HRI factor (Kozak, 1979).

The partial phosphorylation of eIF-2, as a result of viral infection, has been demonstrated with many animal virus infections such as those of adenovirus and poliovirus (O'Malley et al., 1989; Black et al., 1989). This inactivation of a cellular translational initiation factor may reflect the dispensability of this cap-dependent initiation factor for cap-independent translation. In addition to the degradation of an integral component of the cap-binding complex (p220) on infection with poliovirus, the degradation of other cellular factors such as eIF-3 and the p68 kinase has also been reported. In contrast to the above situation this indicates, perhaps, a requirement for the eIF-2 translational factor due to the degradation of one of the specific kinases responsible for its phosphorylation and consequent inactivation. If this kinase degradation or inactivation is not just a random event this eIF-2 factor may be an essential component of the process of cap-independent translation.

Eukaryotic initiation factors 4F and 4B have also been implicated as having some direct involvement in the process of cap-independent translation (Schepet et al., 1992). eIF-4F is the first factor to bind to the 5' end of the mRNA molecule in association with the cap structure during normal cap-dependent translation. eIF-4B is the next factor to bind so both factors are involved in the very initial stages of translational initiation. Anthony et al. (1991), have translated dicistronic constructs containing the poliovirus UTR in vitro in the presence or absence of additional initiation factors. Their findings indicate that eIF-4F and eIF-4B both facilitate translation of the downstream ORF in a capped dicistronic while having no effect on the translation of the first ORF. The translational stimulation associated with these factors appears to be cap-independent in nature as both factors stimulate translation of ORF2 in the presence of cap analogue, i.e., under conditions where cap-dependent translation and therefore the translation of ORF1 is inhibited. The stimulation is in the order of 3-4 fold. The question remains as to why factors that mark integral points in the cap-dependent translational initiation pathway effect cap-independent translation in a stimulatory manner. It has been proposed that cap-dependent translation is more competitive such that initiation factors will be used primarily for this process. However under conditions where cap-dependent translation is inhibited, for example, during virus infection or during mitosis, factors become more available for cap-independent translation. The degradation of the p220 α-subunit of eIF-4F during poliovirus infection may modify the initiation factor making it more likely to participate in the cap-independent translational pathway. It is quite plausible to assume that an overlap will occur between the two mechanisms especially if we assume that cap-independent translation has evolved as a mechanism to overcome the constraints of normal eukaryotic translation.

The majority of the research on this subject of viral translation in a eukaryotic
Fig 1.3
Fig 1.3 Diagrammatic representation of the structure and genome organisation of the viruses discussed in Section 1.6. Information concerning the tymovirus, turnip yellow mosaic virus (TYMV) (Richards et al., 1977); the bromovirus, brome mosaic virus (BMV) (Ahlquist et al., 1984); alfalfa mosaic virus (AIMV) (Koper-Zwarthoff et al., 1977); the tobamovirus, tobacco mosaic virus (TMV) (Goelet et al., 1982); the potexvirus, potato virus X (PVX) (Huisman et al., 1988); and the potyvirus, tobacco etch virus (TEV) (Allison et al., 1986), is provided. RNA molecules are represented as single lines and gene products are represented as shaded boxes, sizes are provided in number of nucleotides (ntd) and kilodaltons (K), respectively. 5' and 3' terminal structures are also indicated.
environment has been with animal viruses. The following section will provide some information regarding this situation with plant viruses and how they overcome the constraints of the eukaryotic ribosome.

1.6 Plant viral translational enhancers, potential exploitation in biotechnology for increased gene expression.

The regulation of gene expression is extremely important for all organisms not least for plant viruses that require a maximum rate of production of viral proteins to allow rapid multiplication and spread. An ever growing number of plant viral translational enhancers have been documented in recent years, that is, specific nucleotide sequences whose presence results in the elevation of the expression of vital gene products to required high levels. These enhancers constitute the 5' untranslated nucleotide regions of genomic or subgenomic RNA molecules. The presence of these sequences at the 5' proximal end of a mRNA molecule results in an increase in expression of a downstream ORF at the translational level. Reported candidates include one of the best known and most intensely studied virus, tobacco mosaic virus (TMV), members of the potyvirus group, and even a small satellite RNA of tobacco necrosis virus. Enhancement values range from 2-100 fold with different viruses, different reporter genes, and in different systems. With the emergence of new viral leaders the mechanism by which these sequences operate becomes even more confused. Similarities in sequence do exist between some leaders, for example, the TMV Ω leader and the potato virus X (PVX) 5' UTR, but there appears to be no general consensus sequence for translational enhancement except for an absence of secondary structure, but whether this factor alone can explain the phenomenon is debatable. Whatever the mechanism, the presence of these sequences upstream from an ORF results in an elevated level of protein production and may feature as an important tool in biotechnology in the future. The variation in sequence between leaders suggests possibly more than one mechanism by which enhancement occurs.

The viruses discussed and the positions in the genome from which these enhancer sequences are derived are outlined in Fig 1.3. The properties displayed by these leaders are summarised in Fig 1.6.

1.6.1 Capped plant translational enhancers

The first reported example of a plant viral leader acting as a translational enhancer was that of the 37 nucleotide 5' untranslated region from the RNA 4 molecule of alfalfa mosaic virus (AIMV) reported by Jobling et al., (1987) (Fig 1.4). In wheat germ and rabbit reticulocyte lysate in vitro systems the presence of this leader confers as much as a 35 fold enhancement of the expression of the foreign barley α-amylase message, and a 6-7 fold enhancement of the human interleukin 1β gene. The AIMV leader also enhances the expression of the β-glucuronidase (GUS) reporter by 6 fold in an in vivo E.coli.
prokaryotic system using a recombinant trp promoter plasmid. Capped GUS transcripts containing the AIMV UTR expressed the reporter gene at a level of 8 times the level expressed from control transcripts fold enhancement of translation in tobacco protoplasts (Gallie et al., 1989a).

Probably the most widely documented enhancer is the 5' non-coding leader of the TMV genomic RNA molecule. This leader is 68 nucleotides in length, contains no internal guanosine residues and has a cap structure at the 5' end (Fig 1.4). The TMV leader is commonly referred to as omega (Ω) which represents the entire sequence, including the first AUG of the TMV genome, but omits the 5' terminal G residue associated with the cap structure. Omega prime (Ω') does not include this first initiation codon. Reports of translational enhancement properties have been derived from both in vitro and in vivo studies (Gallie et al., 1987a; 1987b; 1989; 1992; Sleat et al., 1987; 1988). Enhancements of between 2 and 10 fold of chloramphenicol acetyl transferase (CAT) and neomycin phospho transferase II (NPT II) have been reported in wheat germ, rabbit reticulocyte, and an E. coli in vivo system. In vivo both with capped and uncapped transcripts of these Ω' reporter constructs the presence of the leader confers a 5-6 fold enhancement of reporter expression in tobacco protoplasts and 3-4 fold enhancements in Xenopus oocytes. Using the GUS reporter gene up to 80 fold enhancements have been observed, again in tobacco protoplasts and Ω is also capable of enhancing luciferase (LUC) translation in carrot protoplasts by more than 90 fold (Gallie et al., 1992). Recent data reports a 16 fold enhancement in the expression of the 10KZ maize protein storage gene in transgenic tobacco plants when the Ω leader is present as opposed to non leader control transformants (Kang et al., 1994).

Ω has a highly organised sequence with distinct regions of conservation between different strains of TMV. Central features appear to be a (CAA)n residue region and three 8 base pair direct repeat sequences (ACAAUUAC). Extensive mutation and deletion of the leader has identified one direct repeat sequence and the (CAA)n region as the "core regulatory element", ie, the functional motif region responsible for TMV leader-mediated translational enhancement (Gallie et al., 1988; 1992).

The 5' untranslated leader sequence of the PVX genomic RNA molecule has been extensively studied to the point that a pentanucleotide sequence has been identified as the region responsible for translational enhancement activity (Fig 1.4). Initial results associating this region with translational enhancement were derived from in vitro studies. Smimyagina et al., (1991), reported a 6-60 fold enhancement in translation of the NPT II in rabbit reticulocyte lysate. In vivo the presence of this PVX leader results in a 4 fold enhancement of translation of this reporter gene in Nicotiana tabacum protoplasts (Zelenina et al., 1992), and this enhancement has also been demonstrated with GUS (Pooggin et al., 1992).

More recent data has involved a molecular dissection of the leader and subsequent identification of the active regions associated with translational enhancement. The PVX sequence of 83 nucleotides has been divided into two regions, the α region consists of
Fig 1.4 Sequences of capped plant translational enhancers discussed in Section 1.6.1. The first and last nucleotide of each sequence is indicated. The AUG translational initiation codon is underlined. For the omega TMV leader the ACAAUUAC 8 base pair direct repeat and the (CAA)n sequence, discussed in Section 1.6.1 are indicated by an overline and a dotted underline, respectively. For PVX the CCACC pentanucleotide sequence with base pair complementarity to the 5' end of 18S rRNA is indicated by being overlined in bold.
Fig 1.4

TMV (omega) 5' untranslated leader

m7Gppp UAUUUUAACAAUAUACACACACAAACACCAAGCAUGAUG

PVX (αβ) 5' untranslated leader

m7Gppp GAAACUAAACCAACACCAACCAACCAACCAACCAACCAACCAUGAUG

BMV RNA3 untranslated leader

m7Gppp GUAAAUUACACCAUUUGCUUGCGAUGAUG

TYMV 5' untranslated leader

m7Gppp GUAAUCACACACAAUCCACACAGCUUGAUGAUG

AIMV RNA4 untranslated leader

m7Gppp GUUUUUUAUUUUUAUUUUUAUACCCCAUGCCAUAAUGAUG
the first 41 nucleotides from the 5' proximal end and the \( \beta \) region which consists of the remaining 43 nucleotides down to the first AUG. The entire \( \alpha \) region contains no G residues, has a distinct unpaired conformation, a characteristic also of the \( \Omega \) leader, within the 5' terminal 30 residues and, most importantly, this sequence contains a CCACC pentanucleotide motif which has base pair complementarity with the 5' terminal structure of the 18S rRNA molecule. Deletions of the leader have revealed, at least \textit{in vitro}, that the entire \( \beta \) region is dispensable with regard to translational enhancement. Also, surprisingly, deletion of the 30 nucleotide unstructured 5' region causes a stimulation of translation. It appears that the CCACC pentanucleotide is the central region associated with translational activity as any manipulations of this region result in a dramatic decrease in translation (Tomasherskaya \textit{et al.}, 1993).

Some evidence has suggested that the brome mosaic virus (BMV) RNA3 5' UTR acts as a translational enhancer in some systems (Fig 1.4). Capped GUS transcripts containing this leader stimulates translation by 8 fold in tobacco protoplasts and a 3 fold stimulation is also evident with this reporter gene in a prokaryotic \textit{E.coli in vivo} system. The 5' UTR from turnip yellow mosaic virus stimulates GUS expression by a factor of 5 in \textit{E. coli} under the influence of a \textit{trp} promoter (Fig 1.3) (Gallie \textit{et al.}, 1987a).

\subsection{1.6.2 Picornavirus-like plant translational enhancers, potyviruses}

Translational enhancement has been widely documented among the potyvirus group of plant viruses. Members of this group express their genome as a single polyprotein which is subsequently autocatalytically cleaved to individual viral components. This polyprotein production also occurs within the picornavirus group of animal viruses.

The tobacco etch virus (TEV) 5' UTR is 144 nucleotides in length, naturally uncapped and has a virus encoded protein (VPg) covalently linked to the 5' terminus instead of a cap structure (Fig 1.5). \textit{In vitro} the presence of this leader at the 5' end of a mRNA molecule results in the enhancement of GUS expression in both wheat germ and rabbit reticulocyte lysate systems with capped and non-capped leader GUS messages showing 11 fold and 4 fold enhancements respectively over non-leader control transcripts. Electroporation of both DNA and RNA into tobacco protoplasts has demonstrated that this enhancement is also evident \textit{in vivo}. Transgenic plants expressing TEV leader GUS mRNA have a mean value of expression of the GUS reporter gene 5 times higher than that of GUS control plants. Carrington and co-workers, (1990) have suggested that the functional region of the leader is contained within the 3' terminal 63 nucleotides as this region, when supplied in \textit{trans} to an \textit{in vitro} translation system, can effectively compete out the translational enhancement properties of the leader.

Several strains of plum pox potyvirus (PPV) have been identified (Lain \textit{et al.}, 1989; Teycheney \textit{et al.}, 1989). All possess a conserved 147 nucleotide long 5' UTR to the first
AUG in a favourable context for translational initiation (Fig 1.5). An AUG at position 36 has been ruled out as the authentic AUG start codon. As with the non-translated leaders of other potyviruses, or for that reason other viruses, the PPV 5' UTR is devoid of secondary structure and is rich in A and U residues. Deletion of the leader between residues 19-101 (with the first nucleotide of the leader being designated residue 1) does not cause any noticeable decrease in translational enhancement or internal ribosome initiation (Reichmann et al., 1991). This indicates that the region responsible for this phenomenon, at least in this virus and maybe potyviruses in general, may be extremely small.

Another characterised potyvirus translational enhancer is the 143 nucleotide 5' UTR of pea seedborne mosaic virus (PShMV) (Fig 1.4). This enhancer has been shown to be effective in vivo in both pea and tobacco protoplasts with enhancement levels of about 20 fold (Nicolaisen et al., 1992). One interesting observation with this leader is that a deletion which leaves only the first 83 nucleotides of the authentic enhancer results in a doubling of the level of translational enhancement. Computer predictions have indicated that this deletion may decrease secondary structure, a characteristic which would presumably facilitate ribosome scanning along the leader.

Recently the 5' UTR, 185 nucleotide leader of potato virus Y (PVY) has been associated with an enhancement at the translational level (Fig 1.5). The presence of this sequence as mRNA leader results in a stimulation of translation in both rabbit reticulocyte and wheat germ lysate, in vitro (Levis et al., 1992; 1993). Interestingly, the addition of 91 extra nucleotides to the 5' proximal end of the leader results in a further 6 fold enhancement in this translation level. It is thought that this addition alters the secondary structure configuration of the leader. This mirrors the situation above with PShMV and indicates that the secondary base pair configuration of the leader may be important. In an attempt to identify the region(s) involved in enhancement a hybrid arrest translation mechanism has been used. This has involved the annealing of oligonucleotides complementary to specific PVY UTR regions to transcripts containing this leader and monitoring the effect these have on translation of the downstream ORF. These studies have indicated that one of the central functional region of the leader is contained within the first 16 residues of the genomic RNA molecule as the addition of an oligonucleotide complementary to this region almost totally eradicates translational enhancement properties.

Data has also implicated that the 5' UTR of turnip mosaic potyvirus acts as a translational enhancer. When fused to GUS this 130 nucleotide region increased translation in vivo by about 7 fold when compared to a synthetic GUS construct when bombarded into tobacco suspension culture cells (Dallaire et al., 1993). More recent documentation has resulted in the proposal of the existence of a picornavirus-like IRES structure for internal ribosome binding and initiation of translation. Basso et al., (1994) inserted a stable hairpin structure, either before or after the 5' UTR linked to the GUS gene. GUS activity was only detected in vivo when the structure was placed upstream.
Fig 1.5 Sequence of potyvirus plant virus translational enhancers discussed in Section 1.6.2. The first and last nucleotides of each sequence is numbered. The AUG translational initiation codons are underlined.
Fig 1.5

**TEV 5' untranslated leader**

```
NAAAUAACAAUUUCAACACAACAUUACAAAAACAAACGAAUCUCAAGCAJ
CAAGCAUUUCUACUUUCUAUGCAGCAAUUUAAAUCAUUCUUUUAAAGCAA
GCAAUUUCUGAAAAUUUUCACCAUUACUAAACGAUAUGCA AUG
```

▲

144

**PSbMV 5' untranslated leader**

```
AAAAAUAACACUCACAAACAAAAACGAUAUCGAAAUUGCUUUAAAACUCAUGJ
UGCAUUAUGUUCACAGCUUUGAUUAUACUGUUAUCAUUUUACACACU
UUUGCAACUUUUCACUUAAAGUACAAGCUCAUCA A AUG
```

▲

145

**PPV 5' untranslated leader**

```
AAAAUAUAAAAACUCACACAAACAAUACAAAAUUUUAUGCAUUCAAUCUAAU
CUCAAGCUUACAAAUUUUCAAUCACUCUUUGAAAAGAUCAAAAAAAUCAACA
AAGAAAAUCUUUAAAUUUCACCAAAAUACUGCAAGUCA AUG
```

▲

146

**PVY 5' untranslated leader**

```
AAUAAAAACACUCAAUACACAAAGAAAAACACACGAAAAAAACACUCUAAU
AACGCUCAUUCUACAAAGCUCUUGCAGUUUCAGUUUUAAAUCAUUUC
CUUGCAUUUCUACAGAACAUAUUGGAAAACAUUUCACUCAACAAGCAAU
UUCAUCACUUACACAAUUUCAGAUCUCA AUG
```

▲

184
Fig 1.6 Summary of the translational properties of the plant viral enhancer sequences discussed in Section 1.6. A + indicates that the sequence enhances translation in a particular system. A - indicates that the sequence does not enhance translation in a particular system. An N indicates that the sequence has not been tested for a translational enhancement property in that particular system.
### Fig 1.6

<table>
<thead>
<tr>
<th>TRANSLATIONAL LEADER</th>
<th>RABBIT RETICULOCYTE</th>
<th>WHEAT GERM</th>
<th>E.COLI trp promoter</th>
<th>PROTOPLANTS OR SUSPENSION CULTURE</th>
<th>XENOPUS OOCYTES</th>
<th>TRANSGENIC PLANTS</th>
<th>INSENSITIVITY TO CAP ANALOGUE</th>
<th>INTERNAL INITIATION BICISTRONIC</th>
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<td>+</td>
<td>+</td>
<td>TOBACCO</td>
<td>+</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>TOBACCO, CARROT</td>
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<td>+</td>
<td>+</td>
<td>N</td>
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<td>+</td>
<td>+</td>
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<td>N</td>
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<td>N</td>
<td>+</td>
<td>TOBACCO</td>
<td>-</td>
<td>N</td>
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</tr>
<tr>
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<td>N</td>
<td>+</td>
<td>-</td>
<td>N</td>
<td>N</td>
<td>N</td>
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<td></td>
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<td>+</td>
<td>N</td>
<td>TOBACCO</td>
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<td>N</td>
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</tr>
</tbody>
</table>
from the leader which indicates that some internal ribosome association may be occurring. The form that a potyvirus IRES structure would take is unclear. Presumably, due to the relatively small size and lack of secondary structure, it would differ from that of picornaviruses.

One small RNA that complicates the story even further is the RNA satellite of tobacco necrosis virus (STNV). This 1239 nucleotide viral RNA molecule possesses a 29 residue 5' UTR and a larger 619 nucleotide 3' UTR. This virus does not possess either a 5' proximal cap structure like TMV or PVX or a VPg structure like the potyviruses. Experimental evidence has suggested that the 5' UTR does not enhance translation in the absence of the 3' UTR. Most mutational analysis has concentrated on the disruption of computer predicted stem loop structures in both UTRs, with alterations of a potential 18S rRNA binding region, or mutation of potential base pair regions between the 5' and 3' UTRs. It appears that a specific interaction between the both UTRs is responsible for this enhancement (Timmer et al., 1993). This contradicts the lack of secondary structure theory by suggesting, alternately, that it is the presence of this specific secondary structure that allows enhancement to occur, at least with this RNA.

Some new evidence presented by Tanguay and Gallie, (1994), has also suggested that the enhancement associated with the Ω leader is dramatically enhanced even further when the 52 nucleotide TMV 3' UTR is present. The presence of this 3' UTR stimulates expression of the luciferase reporter by 22 fold over expression with the Ω leader only. This observation brings about important implications about translational enhancement and whether an association of the 5' and 3' UTRs may occur with TMV and other translational enhancers and perhaps play a role in the natural virus system.

1.6.3 The mechanism of plant viral translational enhancement

Translational enhancement could occur by several mechanisms. For AlMV it has been proposed that the phenomenon of translational enhancement is achieved by the facilitated scanning of ribosomes along the leader to the authentic AUG codon due to an absence of secondary structure (Jobling et al., 1987). It is thought that this unstructured leader conformation will promote the efficient scanning and subsequent initiation of translation according to the generally accepted model proposed by Kozak (1978). An AU rich content and absence of secondary structure do appear to be a general characteristic of these leaders but whether this property alone can explain translational enhancement is debatable. Exceptions to this rule do exist. Wilson and co-workers have observed that the BMV RNA3 leader enhances the expression of a downstream ORF in vitro despite the presence of stable secondary structure (Gallie et al., 1987a; Alquist et al., 1984). They have also demonstrated that synthetic leaders of the same length as Ω which are also devoid of secondary structure do not enhance translation (Gallie et al., 1987b). This general absence of secondary structure makes it seem unlikely that enhancement is a result of mRNA stability on the basis of protection from nuclease digestion as messenger
molecules containing these enhancer sequences if anything would be less stable and hence more susceptible than molecules with more structured leaders.

With Ω and the PVX leaders in particular, extensive research has resulted in the identification of sequence motifs or "core regulatory elements" which appear to be responsible for translational enhancement properties (Gallie et al., 1988; 1992; Tomasherskaya et al., 1993). Experiments with the potyvirus group indicate that enhancement may be associated with a very small region of the leader even though this has not directly been identified and may be different with different group members (Reichmann et al., 1991; Levis and Astier-Manifacier, 1993). Sequence conservation between enhancers is, on the whole, nonexistent which indicates that there may be more than one mechanism by which enhancement can occur.

Some experimental evidence has implicated the involvement of some cap-independent or internal ribosome initiation mechanism. Cap-independent translation has been demonstrated with the long 5' UTRs of members of the picornavirus group (Jackson et al., 1990; Pelletier and Sonenberg, 1988) and even for cellular mRNA leaders (Macejak and Sarnow, 1991; Oh et al., 1992). As has been discussed above, poliovirus actually encodes a specific protease that degrades the p220 component of the cap binding complex effectively playing a major role in the shut down of normal cap-dependent host translation and recruitment of ribosomes for viral translation at the expense of the cell (Etchison et al., 1982). Normal cap-dependent translation dictates that ribosomes bind, in association with the 5' cap structure, to a mRNA molecule and then scan the untranslated leader until the authentic AUG codon is reached for initiation of translation (Kozak, 1978). The presence of free cap or cap analogue in any translation system results in the competition for factors involved in normal cap-dependent translation leaving less available for actual host mRNA expression. Enhancement associated with the TEV and PVY leaders appears to be relatively insensitive to the addition of cap analogue to an in vitro translation system. The insensitivity to cap displayed by these leaders suggests possibly that some cap-independent mechanism may be employed (Carrington et al., 1990; Levis et al., 1993; 1994).

Observations with TMV have demonstrated direct binding of two eukaryotic ribosomes within the Ω sequence in the presence of sparsomycin (Konarska et al., 1981). This so-called disome formation has also been demonstrated with TYMV (Filipowicz and Haenni, 1979). It has been proposed that this 80S binding capacity provides an extra site for ribosome association upstream from the AUG start codon. This lends foundation to the idea that some form of internal ribosome association and subsequent initiation of translation may be occurring in association with these leader sequences. Internally placed leader dicistronic constructs, constructs that have been widely used to demonstrate internal ribosome initiation of translation among the animal picornaviruses, containing the PVY and PPV UTRs promote the expression of the downstream cistron. Ribosomes may bind internally in association with elements within these leader regions to initiate translation of the second gene. This downstream ORF appears to be independently translated from the upstream cistron by a mechanism of internal ribosome binding as opposed to reinitiation (Reichmann et al., 1991; Levis and Astier-Manifacier, 1993).
observation that the turnip mosaic virus leader contains a secondary structure configuration analogous to the picornavirus IRES structure also lends evidence to this theory (Basso et al., 1994).

In conclusion we can say that these observations all suggest that facilitated ribosome scanning due to an unstructured leader conformation may not fully explain this enhancement phenomenon, although it may play a contributory factor. A specific secondary configuration, analogous to the situation with the IRES structures of animal picornaviruses, may also be operating to some degree with these plant enhancers. Evidence has pointed towards the involvement of specific motifs, perhaps binding domains for plant host translation factors or ribosomes as well as the secondary leader configuration. A specific interaction between 5' and 3' UTRs has also been implicated as having some involvement, at least with some viruses. Some of these plant viral leaders have displayed a cap-independent mode of translation, a phenomenon again discussed in detail for picornaviruses, but whether this is a general characteristic of translational enhancer sequences is not yet clear. This enhanced expression of viral gene products at the translational level may therefore be a contradiction of the universally accepted scanning model for translational initiation, and is yet another example of how these versatile organisms have adapted to maximise gene expression and subsequent multiplication and spread.

The application of these leaders in biotechnology could be quite dramatic. The use of the Q leader to enhance the expression of a foreign gene in transgenic tobacco plants has been discussed above (Kang et al., 1994). In the animal virus world, the reovirus untranslated mRNA leader of the s4 gene, when fused to the coding region of the s1 mRNA, elevates the expression of this gene over the endogenous expression level by four fold (Roner et al., 1989). Most analysis of these plant viral leaders has involved reporter genes and this has shown that these plant translational enhancers operate efficiently and independently from their related coding sequence and they could easily be exploited to enhance the expression of specific targeted genes in a transgenic system. The use of viral translational enhancers to elevate the expression of foreign genes in plants is an additional expressional control level to transcription that may be exploited to maximise protein production which may often be a drawback in transgenic plants.

Viral infection, to a lesser or greater degree, has a dramatic effect on the cellular metabolism of the host plant. Consequently, one of the processes often affected is the process of translational initiation, the integral control step in the gene expression of single-stranded, positive-sense RNA viruses. The examples described above indicate mechanisms whereby both plant and animal viruses may exploit the host translational machinery for their own aims. The following section will discuss some of the effects of viral infection on the host plant.
1.7.1 The response of the host plant to viral infection.

The interaction between the virus and its host is very specific. Fraser, (1986) has suggested that resistance to single-stranded RNA viruses is predominantly monogenic and these resistance genes appear to be either constitutive or very rapidly induced. The use of plant resistance genes to engineer resistance in other plant species may be a futile operation because viral genomes mutate so rapidly in nature that these resistance genes may be quite quickly overcome. Viral genomes are so small and the coding capacity is so limited that it is unlikely that the virus could encode a factor whose sole function is virulence. Determinants of virulence are contained within genes encoding other essential functions such as viral replication or spread. All plants are not resistant to viral infection, nor is the opposite situation true. The host response to viral attack may be displayed in four general ways depending upon the specific interaction with the virus.

At one end of the scale we have “total immunity”. This is a non-host interaction between a non-host and a non-pathogenic virus. This immunity may be positive, with the plant producing some factor(s) which inhibits viral multiplication or pathogenesis, or the immunity may be negative with the inhibition of multiplication or pathogenesis being due to the absence of a factor(s) required by the virus in the host.

“Subliminal infections” allow viral replication in initially infected cells and the few surrounding this region but do not allow spread throughout the plant. It is thought that this form of immunity operates at the stage of cell-to-cell movement, with perhaps an absence of factors required to interact with viral encoded movement proteins in the host cell or, alternately, the production of some factor(s) by the host which interacts in a negative manner with the movement protein, therefore inhibiting cell-to-cell spread.

The virulence determinant of the Tm-2 resistance gene to TMV in tomato has been mapped to the 30 kDa movement protein. Evidence supporting this include the demonstration that co-inoculation with PVX allows the resistance conferred by the Tm-2 gene product to be overcome as this second virus complements TMV movement function (Talinasky et al., 1982). Also the use of coat protein mutants of TMV have allowed the localisation of single amino acid substitutions in the movement protein of strains overcoming Tm-2 resistance (Meshi et al., 1989). It appears likely that the the Tm-2 gene product interacts with the TMV movement protein in a negative manner thereby confining viral infection to the subliminal level and inducing the hypersensitive response.

The “hypersensitive response” (HR) or local lesion response is a specific plant response to pathogen attack and it usually results in a necrotic or chlorotic lesion a few mm in diameter from the initial site of virus entry in which viral particles are confined. With some TMV host interactions a systemic necrotic response of this nature, rather than protecting the host from pathogen attack, may be so severe that widespread necrosis occurs leading to plant death. The genetic trigger of the HR response may be quite simple. In Nicotiana glutinosa and N. sylvestris the N and N’ genes respectively have been identified as the dominant resistance determinants to TMV infection (Ponz and Bruening, 1986). The target of the N’ factor has been localised to the TMV coat protein.
gene (Saito et al., 1987). The initial signal, whatever form this assumes, triggers a complex cascade of signalling pathways inducing the production and activating all kinds of defence related proteins. The involvement of cytokinins and the process of lignification have also been implicated in the initial trigger reaction. One much studied aspect of the response is the rapid production of pathogenesis related (PR) proteins of which about 10 have been identified. The PR-1 group has been most extensively studied and contains 3 members with 90% sequence homology all which contain a transit peptide sequence for excretion into the extracellular space. The HR response also appears to induce a “systemic acquired immunity” to super-infection by another pathogen. The nature of the induction of the hypersensitive response is not clear but once activated a cascade of reactions are triggered resulting in the characteristic local lesion response.

The phenomenon of “total susceptibility” whereby most of the plant cells become infected by virus is in fact quite a rare event. Most plants are resistant to some extent to most plant viruses but total susceptibility when it does occur usually results in devastating symptom development and even plant death. Viral accumulation in susceptible hosts of TMV, brome mosaic virus (BMV), cucumber mosaic virus (CMV) and alfalfa mosaic virus (AMV) reaches maximum concentration after about 10-14 days. After this stage the concentration of TMV remains fairly constant whereas the concentration of BMV and AMV tends to begin to decrease. CMV displays a cyclic pattern of virus accumulation with particle concentration decreasing after this initial peak had been reached only to increase again after about 7 days. The reason for this cyclic accumulation could possibly be due to a depletion of the amino acid pool or to the action of host nucleases on viral genetic material. The demands of viral accumulation, especially of those viruses that multiply to high levels in the host, drastically effect the metabolism of the plants that they invade. Symptoms in the infected susceptible host range in severity from indetctable to chlorosis or even to necrosis and plant death. Many aspects of cellular metabolism may be interfered with such as the processes of photosynthesis and growth.

1.7.2 Tobacco mosaic virus infection of a susceptible host

Tobacco mosaic virus (TMV) belongs to the tobamovirus group of plant viruses. Members of this group have a rigid rod-shaped morphology with particles sizes of about 300 x 18 nm and a positive-stranded, single-sense RNA genome of about 6400 nucleotides. The genome encodes at least four proteins (Goel et al., 1982). The two larger 5' genomic products 126- and 183- kDa in size, are both required for efficient viral replication. The larger product is produced as a result of translational readthrough of an amber termination codon (Pelham, 1978). A diagrammatic representation of the TMV genomic organisation is provided in Fig 1.3. The 3' terminal genomic products are a protein approximately 30 kDa in size that is involved in cell-to-cell transport (Meshi et al., 1987) and the 17.5 kDa structural coat protein. The two larger products (126 kDa
and 183 kDa) are translated from the genomic RNA molecule and the smaller products (30 kDa and 17.5 kDa) are expressed from subgenomic RNA molecules (Hunter et al., 1976).

As mentioned above, total susceptibility to viral invasion is rare but it often manifests itself with such devastating consequences that it has received much attention due to the ecological and economical losses incurred. Resistance to TMV infection is widespread with several dominant loci encoding resistance in a variety of plant species. Examples include the Tm-2 gene, which results in the elimination of TMV cell-to-cell spread in tomato (Pelham, 1966) or the N gene that results in the induction of a HR response against TMV in Nicotiana glutinosa (Holmes, 1938). Natural virus resistance, although an extremely important issue, is not a subject that will be considered further here. Concentration will be placed on the effect of TMV infection in susceptible hosts, especially in relation to the effect this virus has on host translation.

Host species, lacking any encoded resistance to TMV, display quite devastating symptoms on infection. The most typical TMV induced symptom is a dark green and light green mosaic pattern in developing leaves caused by alterations in chloroplast development in viral infected cells. The development of mosaic patterns depends upon the stage of leaf development at the point of initial infection and this symptom will only be displayed in leaves that are less than 1.5 cm in length at that initial stage and those subsequently developing. Mosaic tissue contains normal dark green cells that contains no virus and light cells in which the viral concentration is extremely high. These dark green islands appear to be resistant to viral infection. Leaves that are at quite advanced stages of development, on infection typically remain symptomless (Culver et al., 1991). Some strains of TMV, however, do induce chlorosis in these developed leaves due to alterations in the structural configuration of mature chloroplasts rather than affecting chloroplast development.

Most evidence suggests that viral translation is carried out by cytoplasmic ribosomes. In infected TMV tissue after an initial stimulation, the concentration of cytoplasmic rRNA is reduced to about 50% of the level in healthy tissue, probably due to the demand for precursors of viral RNA synthesis (Fraser, 1973). The effect on chloroplastic rRNA appears to be even more severe with TMV infection causing both an almost total inhibition of synthesis (Fraser, 1969; Hirai and Wildman, 1969) and an accelerated degradation of chloroplastic ribosomes (Schuch, 1974). This difference in response of the two types of rRNA may reflect the dispensability of chloroplastic and the requirement for cytoplasmic ribosomes for viral translation.

Despite this theory that TMV multiplies in the cytoplasm, viral particles, viral RNA and viral coat protein have also been detected within the chloroplast (Schoelz and Zaitlin, 1989). TMV RNA and coat protein has been isolated from Percoll-purified chloroplasts from TMV infected cells (Reinero and Beachy, 1986; Schoelz and Zaitlin, 1989). Reinero and Beachy, (1989), demonstrated that TMV coat protein has a membrane associated accumulation and it also appears to have an inhibitory effect on photosystem
II. In addition to TMV many other viruses have been detected within the chloroplast. TYMV (Chalcroft and Matthews, 1966) and barley streak virus (Carroll, 1970) have been detected in invaginations in the chloroplast periphery.

It still remains unclear whether or not TMV coat protein is within the chloroplast due to transportation or due to translation by chloroplastic ribosomes. It has been demonstrated that 70S prokaryotic E.coli ribosomes are capable of producing TMV coat protein from a viral RNA template in vitro (Glover and Wilson, 1982). Camerino et al., (1982), have actually demonstrated the production of TMV coat protein on chloroplasts purified from spinach cells. If these 70S ribosomes do play a role in viral translation, coat protein could be synthesised from a full length genomic viral molecule, thereby making the production of subgenomic RNA molecules redundant. In most TMV strains the nucleotide region upstream from the coat protein ORF contains a putative Shine-Dalgarno sequence for prokaryotic ribosome binding. This conserved sequence could be functional in the host environment.

Not surprisingly, when the effects induced by viral infection are considered, TMV infection also appears to affect the process of photosynthesis, presumably due to the decreased chlorophyll content of infected leaves. In one experiment the incorporation of 14C into photosynthate was monitored. This revealed an initial stimulation in photosynthesis a day after initial viral infection which subsequently decreased quite rapidly during the later stages of virus accumulation. The magnitude and rapidity of this response may be a subtle interaction between the virus and the host rather than a general response to accumulating virus concentration (Fraser, 1987).

It is thought that plant growth reduction on infection with TMV is primarily of a "competitive" nature with the sheer physical presence of high viral concentrations acting as a sink for cellular resources such as amino acids, ribonucleosides and the plant protein synthesis apparatus. This is true especially for susceptible tobacco species in which the viral concentration may exceed 4.2 mg/g fresh weight (Fraser and Gerwitz, 1980) with the viral coat protein becoming the most predominant protein in the leaf.

TMV infection does not appear to have any deleterious effects on host poly(A) mRNA concentration but up to a 75% decrease in host protein synthesis has been observed during the period of most active viral accumulation (Fraser and Gerwitz, 1980). It appears, therefore, that inhibition of protein synthesis during TMV infection operates at the translational level. This dramatic and rapid decrease in host translation has been demonstrated in various ways. Fraser, (1972) monitored 3H-histidine incorporation into protein in both infected and uninfected tobacco leaf tissue. The use of the amino acid histidine allowed host protein translation only to be monitored, effectively ignoring viral translation as histidine is not a constituent of the TMV coat protein (Hennig and Wittman, 1972). As has been mentioned above a 50-75% decrease in translation in infected tissue as opposed to healthy tissue was observed, only to be recovered once viral accumulation.
had ceased.

How this devastating effect on host protein synthesis at the translational level occurs is not clear, nor is the advantage that this decrease makes to the virus. It is possible that inhibition of host translation may not be a deliberate action of viral invasion, rather an incidental consequence of it. Many animal viruses such as the paroviruses or the retroviruses replicate in their host without affecting host protein synthesis in any way whatsoever. One contributory aspect of the phenomenon could be the enhanced competitive nature of the TMV coat protein subgenomic RNA molecule and even the genomic molecule for cellular ribosome binding and subsequent initiation of translation. The effect could be a more subtle direct effect on host translational machinery.

The rate of incorporation of $^{35}$S-sulphate into individual host proteins including the chlorophyll a binding protein and the large and small subunits of ribulose,1-5, diphosphate carboxylase, are also dramatically effected, in a negative manner, by viral infection (Doke and Hirai, 1970). The encapsidation of chloroplastic DNA or RNA, often of that encoding rubisco, by TMV viral coat protein resulting in the formation of "pseudovirion" particles has also been demonstrated (Rochno and Siegel, 1984). This could be a contributory factor to the process of host translational down regulation observed on infection with TMV. Host transcripts could be encapsidated, effectively masking them from the cellular translational apparatus. The evolutionary and regulatory implications of these observations are unclear.

1.8 Project aims

The aim of this thesis is to investigate some of the molecular characteristics of the PVS genome. The major aspect explored will be viral translation and how the virus manages to be efficiently expressed in a eukaryotic cellular system. Concentration will be placed on the process of translation. The analysis will involve the characterisation of two sections of the viral genome. The first sequence is the 101 bp region upstream from the ATG of the viral coat protein ORF (VTE). A detailed molecular characterisation of this sequence will hopefully help to explain the production of large quantities of viral coat protein despite the absence of large quantities of RNA template. Another sequence that will be analysed is the 5' untranslated leader of the PVS genomic RNA molecule. The characterisation of this sequence will hopefully provide an insight into the efficiency with which this virus multiplies in a host environment and manages to compete for factors involved in endogenous host translation. These investigations will result in a detailed examination of the process of viral translation as a whole, and the effect that viral translation may have on a host plant.
Chapter 2

Materials and Methods

2.1 Manipulation, Modification and Cloning of DNA

2.1.1 Restriction digestion of DNA

Restriction digests were carried out according to the manufacturer’s directions for particular enzymes (Gibco-BRL). Generally one microgram (1 μg) of DNA was digested in a 20 μl volume with 2 μl of the appropriate 10 x restriction enzyme buffer. Approximately 0.5 μl (1-5 units) of restriction enzyme was added and digests were incubated at 37 °C for at least an hour.

2.1.2 Electroelution of DNA fragments

Electroelution was used to isolate small DNA fragments from agarose gels that fell below the threshold size recommended for gene clean (Section 2.1.3). An electroelution gel tank was filled with 1 x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) up to the level of the wells and wells were flushed out with a syringe to expell any air bubbles. 200 μl of electroelution high salt binding buffer (3 M NaOAc (pH 7.9), 0.01% bromophenol blue) was added to the central tube of the v-shaped well to be used. The gel slice, isolated from an ethidium bromide stained agarose gel, was placed in the well. Electroelution was carried out at 100 volts for 1 hour. After this time the buffer was removed from the well using a syringe and the solution was transferred into a 1.5 ml Eppendorf tube. The DNA migrates from the gel slice, enters the well and is contained in the high salt buffer. DNA was precipitated by adding 2.5 volumes of cold ethanol and collected by centrifugation in a mini-centrifuge. The DNA pellet was resuspended in 20 μl of SDW after being dried under vacuum. Fragments were either used for ligation or as template for the production of a radioactive probe.

2.1.3 Gene clean isolation of DNA fragments

All reagents and protocols were supplied by BIO 101 Incorporated. The gel slice containing the required DNA band was cut out of an ethidium bromide stained agarose gel and was placed in a 1.5 ml Eppendorf tube. To this three volumes of 6 M NaOAc (pH 7.9), 0.01% bromophenol blue) was added to the central tube of the v-shaped well to be used. The gel slice, isolated from an ethidium bromide stained agarose gel, was placed in the well. Electroelution was carried out at 100 volts for 1 hour. After this time the buffer was removed from the well using a syringe and the solution was transferred into a 1.5 ml Eppendorf tube. The DNA migrates from the gel slice, enters the well and is contained in the high salt buffer. DNA was precipitated by adding 2.5 volumes of cold ethanol and collected by centrifugation in a mini-centrifuge. The DNA pellet was resuspended in 20 μl of SDW after being dried under vacuum. Fragments were either used for ligation or as template for the production of a radioactive probe.
For each wash, the glassmilk pellet was resuspended in 600 µl of New Wash, vortexed to suspend the glassmilk, and recentrifuged briefly for 10 seconds before the supernatant was discarded. After the last wash all remaining traces of New Wash were removed and the pellet was resuspended in 20 µl of SDW. This was incubated at 60 °C for a few minutes to remove the DNA from the glass beads, followed by recentrifugation. The supernatant containing the suspended DNA was transferred to a fresh tube and the pellet was discarded. One tenth of the supernatant was electrophoresed on an agarose gel to visually estimate the DNA concentration.

2.1.4 Dephosphorylation of DNA

Dephosphorylation of restricted vector terminal sequences, digested with only one enzyme were carried out to prevent recircularisation during ligation. About 1 µg of vector DNA was digested with the appropriate restriction enzyme. The DNA was extracted twice with an equal volume of phenol/chloroform and precipitated with 1/10 volume of 3 M NaOAc (pH 7.0) and 2.5 volumes of cold ethanol. DNA was collected by centrifugation, the supernatant discarded, the pellet dried under vacuum, and resuspended in 18 µl of SDW. 2 µl of CIP buffer (100 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 10 mM ZnCl₂), and 1 unit of Calf Intestinal Alkaline Phosphatase (CIP) was added and the reaction incubated at 37 °C for 15-30 minutes. Dephosphorylated DNA was then extracted twice with phenol/chloroform and precipitated with ethanol. After centrifugation the pellet was dried and resuspended in 20 µl SDW. Dephosphorylated vector DNA could now be used in ligation reactions.

2.1.5 Amplification of DNA by Polymerase Chain Reaction (PCR)

Reactions varied slightly in volume or in the annealing temperature used, but the general procedure for DNA amplification is outlined below. The following components were mixed in a 0.5 ml Eppendorf tube:

- 4 µl of diluted DNA template (5-100 ng)
- 2 µl of primer 1 (100 ng/µl)
- 2 µl of primer 2 (100 ng/µl)
- 1.8 µl of (x11) PCR buffer : (67 mM Tris-HCl, 16.6 mM NH₄SO₄, 6.7 mM MgCl₂, 10mM β-mercaptoethanol, 6.7 mM EDTA (pH 8.0), 1.5 mM of each dNTP, 170 µg/ml BSA. This was made up to a final concentration of 676 µl and stored at -20 °C)
- 2.5 units of Taq DNA polymerase (Promega)
- SDW to a final volume of 20 µl.

Approximately 30 µl of mineral oil was layered over the reaction to prevent evaporation and tubes were placed in a Perkin-Elmer Cetus Thermocycler. A denaturing temperature of 95 °C for one minute, an annealing temperature between 50-55 °C for one minute, and an extension temperature of 72 °C for one to three minutes were used for 25-30 cycles. PCR products were visualised by electrophoresis on an ethidium bromide stained agarose gel.
2.1.6 Ligation of DNA

Generally ligation of molecules with compatible cohesive ends was carried out. 100-500 ng of gel purified or dephosphorylated vector was mixed with 100-500 ng of gel purified insert fragment. Reactions were made up to a final volume of 25 µl before the addition of 5 µl of 5 x ligation buffer (BRL, supplied with DNA ligase). 1-10 Weiss units of DNA ligase was added and reactions were incubated at room temperature overnight. DNA fragments derived from polymerase chain reaction amplification were also cloned directly into a PCR cloning vector which has T overhangs to facilitate the cloning of PCR products. Ligation into the pCR11 (Invitrogen) and pGEM-t (Promega) cloning vectors was carried out according to the manufacturer’s instructions. Information regarding these plasmids are provided in Section 2.16.

2.1.7 Production of competent *E.coli* cells for transformation.

Taken from the method by Cohen *et al.*, (1972).

100 mls of L-broth (Section 2.17.1) was inoculated with 1 ml of an overnight culture of either JM109, TG2 or XL1-blue *E.coli* cells (Section 2.15). This was incubated in a shaking incubator at 37 °C until an OD600 of 0.5 was reached. Cells were collected by centrifugation at 4 °C at 5000g for 5 minutes. The spent media was discarded and the bacterial pellet was resuspended in a half volume (50 mls) of sterile ice-cold CaCl₂. Cells were resuspended by gentle pipetting and were incubated on ice for 1 hour. After this incubation cells were again pelleted by centrifugation at 4°C at 5000g for 5 minutes. The supernatant was discarded and cells were resuspended in 6 mls of cold CaCl₂ if they were to be used immediately or in ice-cold CaCl₂ containing 20% glycerol if they were to be frozen. Cells to be frozen were divided into 200 µl aliquots in Eppendorf tubes and were flash frozen in liquid nitrogen and stored at -80 °C until required.

2.1.8 Transformation of competent *E.coli* cells.

Plasmid DNA was mixed with a 200 µl aliquot of competent cells and the mixture incubated on ice for 20 minutes. After this incubation, tubes were heat shocked at 42 °C for 2 minutes and placed on ice for 5 minutes. 1 ml of L-broth was added to tubes and they were incubated at 37 °C for 1 hour to allow expression of the antibiotic resistance gene encoded by the plasmid. Cells were collected by centrifugation and, after the removal of the spent media, the pellets were resuspended in 200 µl of L-broth. 190 µl and 10 µl volumes of resuspended cells were spread on L-agar (see section 2.17.1) plates containing the appropriate antibiotic at the correct concentration. Plates were allowed to dry, inverted, and incubated at 37 °C overnight.
2.1.9 Glycerol colony stocks

Bacteria can be stored almost indefinitely in frozen stocks containing glycerol. 5 mls of L-broth containing the appropriate antibiotic at the correct concentration was inoculated with a single bacterial colony and incubated overnight with shaking at 37 °C. The following day 850 μl of culture was transferred into a screw top 1.5 ml tube. 150 μl of sterile glycerol was added and the tube vortexed to mix contents thoroughly. Cells were flash frozen in liquid nitrogen and stored at -70 °C. When required, tubes containing the frozen cell stock, were removed and transferred immediately to dry ice. A scraping from the frozen glycerol stock was used as inoculum for a fresh culture.

2.1.10 Small-scale preparation of plasmid DNA

Taken from the method by Birnboim and Doly, (1979).

Solutions:

Solution 1 - 50 mM glucose
- 25 mM Tris-Cl (pH 8.0)
- 10 mM EDTA (pH 8.0)

Solution 2 - 0.2 M NaOH
- 1% SDS

Solution 3 - 60 ml potassium acetate (5 M)
- 11.5 ml glacial acetic acid
- 28.5 ml H₂O

1.5 mls of an overnight culture grown up at 37 °C in L-broth was pelleted in a mini-centrifuge for 5 minutes. The supernatant was removed and the bacterial pellet resuspended in 100 μl of solution 1, then mixed with 200 μl of solution 2, and finally 150 μl of solution 3, in that order with mixing between each addition. Tubes were briefly vortexed followed by a 10 minute centrifugation to remove the white genomic DNA precipitate. The supernatant was transferred to a fresh tube and DNA precipitated by the addition of an equal volume of isopropanol. The DNA was pelleted by centrifugation, all of the supernatant discarded, and the pellet resuspended in 400 μl of SDW. 200 μl of both phenol and chloroform were added to the tube to remove protein contaminants. After vortexing and centrifugation, the supernatant was transferred to a fresh tube. Care was taken to avoid the cloudy material at the phenol/chloroform interface. The phenol/chloroform extraction was repeated once more. DNA was finally precipitated by adding 1/10 volume of 3 M NaOAc (pH 7.0) and 2.5 volumes of cold ethanol. After a 10 minute centrifugation the ethanol was discarded and the pellet washed in 70% ethanol to remove remaining traces of salt. The pellet was dried under
vacuum and resuspended in 50 μl SDW. About 5 μg of plasmid DNA was obtained from the original 1.5 ml of bacterial culture. 10 μl was used in a restriction digest.

2.1.11 Midi-scale preparations of plasmid DNA

A 100-200 ml culture was grown in L-broth at 37 °C overnight. Cells were harvested by centrifugation for 10 minutes in 50 ml disposable plastic tubes at 5000g in a Beckman GPR bench top centrifuge. The broth was poured off and the bacterial pellet was resuspended in 4 ml of solution 1, 8 ml of solution 2, and 6 ml of solution 3, in that order with shaking between each addition. Tubes were centrifugation at 5000g for 10 minutes and the supernatant was transferred to a fresh tube, to remove genomic DNA. Polyallomer wool was be used to filter the supernatant to ensure that there was no contamination from the white genomic DNA precipitate. An equal volume of isopropanol was added to precipitate plasmid DNA and this was collected by centrifugation, again at 5000g. The isopropanol was removed and the DNA pellet resuspended in 300 μl of SDW. At this stage the resuspended DNA could be transferred into a 1.5 ml Eppendorf tube. 300 μl of 10 M LiCl2 was added to precipitate RNA, the tube vortexed vigorously, and centrifuged in a mini-centrifuge for 10 minutes. The supernatant was transferred to a fresh tube and an equal volume of PEG solution added (1.6 M NaCl, 13 % (w/v) polyethylene glycol 6000) to precipitate the DNA. After a 10 minute incubation on ice the tube was centrifuged for 10 minutes. The supernatant was discarded and the clear glassy pellet retained and resuspended in 100 μl SDW. 5 μl of RNase (15 μg/μl) was added and the tube incubated at 37 °C for 30 minutes.

After this incubation 50 μl of each phenol/ chloroform were added, the tube vortexed, centrifuged for 5 minutes and the supernatant transferred to a fresh tube. This phenol/chloroform extraction was repeated twice more or until the supernatant layer was clear. Plasmid DNA was precipitated by the addition of 1/10 volume of 3 M NaOAc (pH 7.0) and 2.5 volumes of cold ethanol. Tubes were centrifuged for 10 minutes to collect the DNA pellet, the ethanol discarded and the pellet washed in 70% ethanol. The pellet was dried under vacuum and resuspended in 100 μl of SDW. DNA concentration was determined spectrophotometrically or visually on an ethidium bromide stained agarose gel.

2.1.12 Caesium chloride preparation of plasmid DNA

500 ml of L-broth was inoculated with 10 ml of an overnight culture and this was incubated at 37 °C in a conical flask overnight with shaking. Cells were harvested by centrifugation at 30,000g in a Sorval RC-5B centrifuge for 5 minutes in GSA polypropylene bottles. The supernatant was discarded and the bacterial pellet resuspended in 10 ml of solution 1 (Section 2.1.10). To this 20 ml of freshly prepared solution 2 was added and the tube inverted to mix the contents. 15 ml of ice cold solution 3 was finally added and the tube contents again mixed. The white genomic DNA precipitate was isolated by centrifugation at 30,000g for 5 minutes and the clear supernatant filtered using polyallomer wool into a fresh tube. An equal volume of isopropanol was added and nucleic acids
collected by centrifugation at 30,000 g for 5 minutes. To remove RNA an equal volume of 10 M LiCl₂ was added, tube contents mixed by inverting and RNA collected by centrifugation at 30,000 g for 10 minutes. The supernatant was transferred into a fresh tube, DNA precipitated by adding an equal volume of isopropanol, and collected by centrifugation at 10,000 rpm. The supernatant was poured off and the bacterial pellet was air dried before being resuspended in 12 ml of SDW. To this 12 g of caesium chloride was added and mixed to dissolve. Finally 0.8 ml (10 µg/ml) of ethidium bromide was added, tube contents were carefully mixed and transferred to caesium chloride prep plastic Beckman tubes through the hole in the top using a syringe. Tube weights were equalised on a balance and sealed before centrifugation in a Sorval at 110,000 g for 12 hours.

The next day tubes were removed and viewed under UV-illumination. Two bands should be obvious in the tube, an upper genomic DNA band and a lower plasmid DNA band. The upper tube region was pierced with a needle to release the pressure in the tube. A second needle and 2 ml syringe were used to pierce the tube just below the second plasmid DNA band and draw the band into the syringe. Ethidium bromide was removed by extracting with H₂O saturated caesium chloride until the solution became clear. The solution was transferred into siliconised corex tubes and 3 volumes of SDW followed by an equal volume of isopropanol added. Tubes were centrifuged at 30,000 g for 15 minutes at 4 °C to collect precipitated DNA, the pellet washed with 70% ethanol, dried under vacuum and resuspended in 500 µl of SDW.

2.2 Agarose gel electrophoresis

Usually a 1% agarose (Sigma) gel was used but lower percentage gels were used to separate smaller DNA or RNA fragments. Gels were made with and electrophoresed in 1 x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Ethidium bromide was added to both the gel and the running buffer to a final concentration of 5 x 10⁻⁴ µg/ml. One fifth of a volume of DNA or RNA loading buffer (2 x TAE, 20% glycerol, 0.25% bromophenol blue) was added to samples. Samples were loaded and electrophoresed at 100 volts to separate nucleic acid fragments. Marker DNA (1 kb ladder, Promega) or RNA (brome mosaic virus RNA, Promega) containing fragments of known molecular size were electrophoresed at the same time to allow the size of bands to be determined. Bands were visualised on a UV-transilluminator and were photographed using a video camera processor.
2.3 Sequencing of DNA

2.3.1 Preparation of single-stranded template

5 ml of L-broth with ampicillin (100 µg/ml) was inoculated with 1 ml of an overnight culture of bacterial cells containing the plasmid to be sequenced. The culture was grown to an OD600 of 0.5-0.8 at 37 °C with shaking. 2 ml of this culture was transferred to a fresh universal bottle and infected with 50 µl of M13KO7 helper phage (10 plaques) which carries the kanamycin selectable marker. The culture was incubated at 37 °C for 1 hour. After this incubation 400 µl of infected cells were transferred into 10 ml of L-broth containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml). The culture was incubated overnight in a 37 °C shaker.

The following day 1.5 ml of culture was transferred to a 1.5 ml Eppendorf tube. After centrifugation 1 ml of supernatant was removed and transferred to a fresh Eppendorf tube. 250 µl of 20% PEG/2.5 M NaCl solution was added and the tube incubated at room temperature for 15 minutes. After this incubation the tube was centrifuged for 10 minutes to collect the phage. At this stage a visible phage pellet should be visible. The supernatant was removed using a vacuum line and the pellet was resuspended in 400 µl of TE containing 0.2% β-mercaptoethanol. The resuspended phage suspension was extracted twice with an equal volume of phenol/chloroform and single-stranded DNA was precipitated by adding 1/10 volume of 3 M NaOAc (pH 7.0) and 2.5 volumes of cold ethanol. DNA was collected by centrifugation and supernatant was discarded. The tube was dried under vacuum and the DNA pellet resuspended in 20 µl of SDW and stored at -20 °C until needed. A T7 DNA sequencing kit (Promega) was used for all subsequent reactions.

2.3.2 Annealing of primer to single-stranded template

10 µl of single-stranded DNA template, prepared according to the method described in the previous section, was placed in a 1.5 ml Eppendorf tube. To this 2 µl of sequencing primer (3 ng/µl) and 2 µl of annealing buffer (1 M Tris-HCl (pH 7.5), 100 mM MgCl₂, 160 mM DTT) were added. Contents were mixed gently and the tube incubated in a water bath at 60-65 °C for 10 minutes followed by a 10 minute incubation at room temperature. Primer annealing now complete, sequencing reactions were carried out immediately or primer annealed DNA was stored at -20 °C until needed.

2.3.3 Annealing of primer to double-stranded template

DNA from midi-scale plasmid preparation was used for double-stranded sequencing reactions. The concentration of template was adjusted so that 32 µl contained 1.5-2 µg of plasmid DNA. Double-stranded template was denatured by adding 8 µl of freshly prepared 2 M NaOH. This was incubated at room temperature for 10 minutes before the addition of 7 µl
of 3 M NaOAc (pH 7.0), 4 μl of distilled water and 120 μl of ethanol. Contents were mixed
and were placed on dry ice for 15 minutes before precipitated DNA was collected by
centrifugation. The pellet was washed in ice cold 70% ethanol, dried under vacuum and
resuspended in 10 μl of SDW. Denatured DNA was resuspended in 10 μl of SDW and
primer was annealed in the same way as for annealing to single-stranded template (Section
2.3.2). Primer annealed template was used immediately in sequencing reactions or was
stored at -20 °C until needed.

2.3.4 Sequencing reactions

Sequencing reactions were carried out according to the method by Sanger et al., (1977). A
T7 DNA sequencing kit (Pharmacia) was used in all cases with sequencing reactions being
carried out according to the manufacturer’s instructions. Labelled [α-35S]dATP (370
mBq/ml, specific activity 110 TBq/mmol, Amersham) was used as a radioactive label.
Reactions were stored at -20 °C until needed and were heated to 75-80 °C for 2 minutes
before loading 3 μl of each sample onto a sequencing gel, preheated to 55 °C.

2.3.5 DNA sequencing gels

A 6 % (w/v) acrylamide gel solution was made by mixing 34.11 g of DNA sequencing
grade acrylamide, 1.8 g of N,N’-methylenebisacrylamide, 252 g of urea, 60 ml of x10 TBE
buffer (108 g Tris base, 5.8 g EDTA, 55 g boric acid/l) and water to to a final volume of 600
ml. The solution was denised by mixing with amberlite which was removed by filtration.
A BioRad sequencing gel kit was used throughout the project (20 cm x 40 cm). The top plate
was siliconised and the apparatus assembled according to the manufacturer’s instructions.
The base was sealed in the casting tray supplied with the kit by pouring in 20 ml of 6% (w/v)
gel solution with 480 μl of ammonium persulphate (AMPS) and 64 μl of N,N,N,N’,
tetramethyl-ethylenediamine (TEMED) being added just before pouring. This was allowed to
set before the gel was poured. 480 μl of AMPS and 64 μl of TEMED was added to 50-60
ml of 6 % gel solution in a beaker. This was immediately transferred to a 50 ml syringe and
was carefully inserted between the two glass plates of the gel apparatus. A shark tooth comb
was placed in the top of the gel, straight side downwards. The gel was allowed to
polymerise for about 1 hour. When the gel had set the buffer tank was filled with 1 x TBE
buffer and the gel was heated to approximately 50 °C before inversion of the shark tooth comb
and loading of sequencing reactions. The samples were electrophoresed at 2500 volts.
When electrophoresis had been completed the gel apparatus was dismantled and the gel was
transferred onto 3 MM Whatman paper. This was covered with cling film and dried on a
BioRad gel dryer under vacuum for 2 hours. Autoradiography was usually carried out for 1-
3 days depending on the radioactive counts detectable on the gel.
2.3.6 Analysis of DNA sequence

Sequence was read manually and was analysed using a Biosoft sequence analysis program or the DNASIS sequence analysis program from Pharmacia.

2.4 Nucleic acid extraction from plant tissue

2.4.1 RNA extraction

RNA was extracted according to the method by Wadsworth et al., (1988). As with all RNA work care was taken to keep all solutions and apparatus nuclease free. 250-500 mg of plant tissue was ground to a fine powder in liquid nitrogen in a pestle and mortar and 500 μl of buffer A was added.

Buffer A:
- 25 mM sodium citrate (pH 7.0)
- 4M guanidium isothiocyanate
- 1.5% (w/v) sodium lauryl sarcosine
- 100 mM β-mercaptoethanol.

The homogenate was transferred to a 1.5 ml Eppendorf tube and vortexed for 15 seconds. 250 μl of both phenol and chloroform were added and the tube vortexed for a further 15 seconds before centrifugation for 5 minutes. 500 μl of the aqueous phase was transferred to a fresh Eppendorf tube and the phenol/chloroform extraction was repeated two more times. After the extractions were completed 400 μl of the aqueous phase was again transferred to a fresh Eppendorf tube and to this 400 μl of 6 M LiCl₂ was added. The tube was incubated on ice for 1 hour to precipitate the RNA and was then centrifuged at 4 °C for 10 minutes. The supernatant was removed leaving a large pellet. The pellet was disrupted with a glass rod and 1 ml of 3 M LiCl₂ was added. The tube was vortexed to evenly resuspend the pellet, centrifuged for 5 minutes to collect the RNA, and the supernatant removed. This wash was repeated two more times. After the final spin all traces of supernatant was removed, the pellet was resuspended in 400 μl potassium acetate and heated to 55 °C for 10 minutes to dissolve the RNA. The tube was centrifuged for a further 5 minutes to remove insoluble matter and the supernatant transferred to a fresh Eppendorf tube. RNA was precipitated by adding 1 ml of ethanol and incubating at -80 °C for 15 minutes. The RNA was pelleted by centrifugation for 15 minutes at 4 °C, dried under vacuum, and dissolved in 50 μl of nuclease free water. Samples were stored at -80 °C.
2.4.2 DNA extraction for PCR analysis

An Eppendorf lid was used to pinch off a piece of plant leaf material and this was frozen in an Eppendorf tube on dry ice. The tissue was ground thoroughly for at least 15 seconds using a micro-homoginiser.

400 µl of PCR extraction buffer (200 mM Tris-Cl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and the tube vortexed for 30 seconds to ensure all the plant extract was in suspension. To remove insoluble plant debris tubes were centrifuged for 5 minutes and 300 µl of the supernatant was transferred to a fresh Eppendorf tube. Nucleic acids were precipitated by adding an equal volume of isopropanol and incubating at room temperature for 2 minutes. After a 10 minute centrifugation the supernatant was removed and the pellet dried. The pellet, which usually appeared orange or pink in colour, was resuspended in 50-100 µl of SDW and 5 µl of this was used in a 50 µl PCR reaction.

2.5 Autoradiography

2.5.1 Labelling of DNA probes

Probes were labelled according to the method by Feinberg and Vogelstein, (1984). The DNA template for radioactive labelling was either purified from an agarose gel or was generated by PCR. Oligo labelling buffer (OLB) was made by mixing solutions A,B and C in the ratio 2 : 5 : 3.

Solution A :
2 M Tris-Cl (pH 8.0) 625 µl
5 M MgCl₂ 225 µl
SDW 350 µl
β-mercaptoethanol 18 µl
0.1 M of each dATP, dTTP, dGTP 5 µl of each

Solution B :
2 M HEPES (titrate with NaOH to pH 6.0)

Solution C:
Hexadeoxyribonucleotides at 90 OD₂₆₀ units/ml in 3 mM Tris-Cl, 0.2 mM EDTA (pH 7.0)

A minimum of 10-20 ng of DNA was used for each labelling reaction. The DNA in a 10 µl volume was boiled in an Eppendorf tube for 1 minute. The tube was transferred to a 37 °C water bath for a further 5 minutes and centrifuged briefly to collect the denatured DNA in the bottom of the tube.
The following were mixed:

3 µl of OLB
0.6 µl of DNase free BSA
0.5 µl of [α-32P]dCTP (370 mBq/ml, specific activity 110 TBq/mol, Amersham)
0.6 µl of DNA polymerase 1 Klenow fragment

Reagents were mixed by pipetting and incubated in a 37 °C water bath for 1 hour. After this incubation the percentage incorporation of label into the probe was determined. Probes were again boiled before being added to pre-hybridising filters.

2.5.2 Determination of percentage incorporation of label into a radioactive probe

85 µl of SDW was added to the radioactive probe, prepared according to method described in Section 2.5.1. 1 µl of this was removed and added to 500 µl of herring sperm DNA (500 µg/ml). 125 µl of 50% TCA (trichloroacetic acid) was then added to precipitate the DNA and the solution filtered under vacuum using a GF-C glass filter. The filter was washed 2 times in 10% TCA before a final rinse with industrial methylated spirits (IMS). To offer a means whereby the total number of counts in the probe could be determined, 1 µl from the unfiltered probe (probe with 85 µl of SDW) was spotted onto a separate filter. Both were placed in separate scintillation vials with 1-3 ml scintillation fluid. The % incorporation of radioactivity into the probe could therefore be determined as the value obtained for the filtered sample divided by the value for the untreated sample multiplied by 100.

2.5.3 Southern and Northern blotting

Blotting of nucleic acids was carried out according to the method by Southern, (1975).

DNA or RNA samples were first separated on an ethidium bromide stained agarose gel and photographed under a UV-transilluminator with a ruler alongside. The gel was placed in depurinating solution (0.25 M HCl) for 10 minutes, transferred to denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes and then to neutralising solution (3 M NaCl, 0.5 M Tris-Cl (pH 7.4)) for 30 minutes.

RNA gels required no depurinating, denaturing or neutralising so they could be blotted immediately after photography. To prevent RNA degradation due to the action of RNases all apparatus was rinsed in a 0.2% NaOH solution or were treated with detergent.

To blot the gel a rectangular shaped sponge was placed in a tray filled with 20 x SSC and this was allowed to soak up the SSC solution. A piece of 3 MM paper (Whatman) cut to the size of the sponge was placed on top and the gel was placed on top of this. Cling film was
carefully placed around the outside of the gel to prevent the 20 x SSC from passing through the edges as opposed to through the gel. A piece of Hybond-N (Amersham) cut to the exact size of the gel was soaked in sterile water and placed on top of the gel. A glass rod was used to press out any air bubbles between the gel and the filter. Two pieces of gel sized 3 MM paper soaked in 2 x SSC were placed on top of the Hybond-N and two pieces of dry 3 MM paper were placed on top of these. A stack of paper towels were placed on top with a weight of approximately 400 grams on top of this. DNA or RNA was allowed to transfer to the membrane by capillary action overnight.

2.5.4 Fixing of nucleic acid blots

Nucleic acids were fixed onto Hybond-N membranes by exposing them to UV-transillumination in a UV Stratalinker 2400 (Stratagene)

2.5.5 Hybridisation of filters

Annealing of radioactive probes to filters was carried out at 42 °C in the presence of formamide. The formamide helps to keep the probe in a single-stranded form during the experiment. Hybridisation solution was made as follows:

5 mls Formamide
2.5 mls 20 x SSC
1 ml NaH₂PO₄ (0.5 M)
0.5 ml 100 x Denhards (2% BSA, 2% Ficoll, 2% PVP)
1 ml Herring Sperm DNA (1 mg/ml) (boiled for 10 minutes and incubated on ice for 10 minutes before use)

The filter was pre-hybridised at 42 °C for at least an hour during which time the radioactive probe was made and the incorporation of radioactivity into the probe checked. The probe was boiled for 2 minutes before being added.

2.5.6 Washing filters

Low stringency washes were carried out at 42 °C in wash A (3 x SSC, 0.1 % SDS). Wash was changed and discarded periodically until no counts could be detected in the wash solution using a Geiger counter. Higher stringency washes were carried out at 42 °C in wash B (0.5 x SSC, 0.1 % SDS) in the same way. Blots were allowed to air dry (blots were not allowed to dry completely in case further washing or stripping was required), were wrapped in Saran wrap and placed on X-ray film in a cassette at -70 °C for several hours to 1 week depending upon the radioactive counts detected.
2.5.7 Membrane stripping

Occasionally probe needed to be stripped from filters for screening with another probe. For the successful removal of probe, membranes were never be allowed to dry completely after hybridisation and washing. A 0.1% (w/v) solution of SDS was heated to boiling temperature, poured over the membrane to be stripped, and allowed to cool to room temperature before being discarded. The method used was the same for both DNA and RNA blots.

2.5.8 Dot blots

A strip of Hybond-N membrane was divided into squares. The membrane was then soaked in 2% SSC and allowed to air dry on a piece of 3 MM paper. When the membrane was nearly dry either RNA or leaf sap samples were directly dotted onto individual squares. DNA was denatured by boiling before being dotted on. Filters were allowed to air dry before fixing and hybridisation.

2.5.9 Colony hybridisation

Colonies to be screened were streaked onto duplicate plates containing the appropriate antibiotic at the correct concentration. One of these plates contained a piece of Hybond-N membrane, this plate provided a filter for radioactive screening, the other plate provided a duplicate copy of screened colonies. Both were incubated at 37 °C overnight. The following day the filter was removed from the first plate and placed, colony side down, on a piece of 3 MM paper soaked in a 2 x SSC solution containing 0.5% SDS, for 2-3 minutes. The membrane was then removed and baked in a microwave on full power for 2 minutes before fixing the DNA onto the filter in a UV-Stratalinker (Stratagene). The filter was screened radioactively according to the protocol given in (Section 2.5.5). Positive colonies could be recovered from the duplicate master plate.

2.6 Western blotting

2.6.1 Transfer of protein to millipore membrane

Solutions:

Anode buffer 1 - 0.3 M Tris (pH 10.4), 10% methanol
Anode buffer 2 - 25 mM Tris (pH 10.4), 10% methanol
Cathode buffer - 25 mM Tris, 40 mM aminohexanuric acid, 20% methanol (pH 9.4)
A (Sartorius) gel blotter was used for protein transfer. The transfer was arranged from the cathode to the anode in the following order. Two pieces of 3 MM paper cut to the size of the gel were soaked in Anode buffer 1 and were placed in the centre of the cathode electrode of the gel blotter apparatus. Placed on top of this was one piece of 3 MM paper soaked in Anode buffer 2. The polyacrylamide SDS gel was placed on top of this with a gel sized piece of Nitrocellulose C membrane (Sartorius) soaked in water on top. A glass rod was used to smooth out air bubbles between the gel and the membrane. On top of this was placed three pieces of 3 MM paper soaked in Cathode buffer. The anode lid of the gel apparatus was screwed on top and transfer was carried out at 75 mA for 1 hour for 1 gel and at 150 mA for 1 hour for 2 gels.

2.6.2 Probing with antibody

Solutions:

Tris-buffered saline (TBS) (pH 7.6)

<table>
<thead>
<tr>
<th>Component</th>
<th>/1000 ml</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>2.42 g</td>
<td>20 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.00 g</td>
<td>137 mM</td>
</tr>
<tr>
<td>HCl</td>
<td>3.80 ml</td>
<td>1 M</td>
</tr>
<tr>
<td>H2O</td>
<td>to 1000 ml final volume (check pH 7.6)</td>
<td></td>
</tr>
</tbody>
</table>

TBS-Tween - 0.1 % Tween 20

Nitro-blue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were made up in dimethyl formamide (DMF).

BCIP buffer - 0.1M Tris HCl (pH 9.5), 1 mM MgCl₂

Protein binding sites on the membrane were blocked by incubation in TBS containing 5% marvel (milk proteins) for a minimum of 1 hour at room temperature on a shaker. Blocking can also be achieved overnight at 4 °C. All subsequent steps were carried out at room temperature on a shaker. After blocking the membrane was washed in 1 x TBS-Tween for 5 minutes. The primary antibody was diluted to the appropriate concentration in TBS containing 3% marvel. This can be reused and sodium azide may be added at a concentration of 0.05% to prevent bacterial growth. The membrane was incubated in primary antibody for at least an hour after which time the antibody solution was poured off and the membrane washed three times for 5 minutes in TBS-Tween. The secondary antibody, usually anti-rabbit immunoglobin linked to alkaline phosphatase, was diluted 1/1000 fold in TBS-3%
marvel and the membrane incubated in this for at least an hour. After this time the secondary antibody was poured off (this may be saved as well) and the membrane again washed three times for 5 minutes in TBS-Tween and finally rinsed in BCIP buffer. 5 mls of developing solution was made, BCIP buffer which contained 50 μl of 30 mg/ml NPT and 50 μl of 50 mg/ml BCIP. This was poured onto the membrane and left until the satisfactory development of purple bands. The developer was then rinsed off with water. The membrane was photographed when wet.

2.6.3 Protein extraction from plant tissue

A mortar and pestle were cooled on dry ice to 4 °C. Plant tissue was then ground with the addition of liquid nitrogen and the fine powder obtained transferred to a 1.5 ml Eppendorf tube. To this 200-400 μl of 4 x cracking buffer was added (2 % (w/v) NaDodSO4, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.01% bromophenol blue, all in 0.125 M Tris-HCl (pH 6.8)) and the tube boiled for 2-3 minutes before centrifugation at full speed for 5 minutes. The supernatant was transferred to a fresh tube and stored at -20 °C until needed. Samples were again boiled for 2 minutes before loading onto a SDS polyacrylamide gel.

2.7 In vitro transcription and translation reactions

2.7.1 In vitro transcription of plasmid DNA

Template plasmid DNA was linearised using a restriction enzyme site at the 3’ terminal end of the DNA plasmid insert. This digest was extracted twice with an equal volume of phenol/chloroform and linearised DNA was precipitated using 1/10 volume of 3 M NaOAc (pH 7.0) and 2.5 volumes of cold ethanol. After a 10 minute centrifugation at full speed the pellet was dried under vacuum and resuspended in 20 μl of sterile distilled water. 2 μl of digest was electrophoresed on an agarose gel and the DNA concentration was estimated visually under UV-transillumination. In vitro transcription reactions were carried out using a kit supplied by Stratagene. Reaction components were as follows:

- 1-2 μg of linear DNA template
- 5 μl RNA transcription buffer (200 mM Tris-HCl (pH 8.0), 40 mM MgCl₂, 10 mM spermidine, 250 mM NaCl)
- 1 μl of each rATP, rTTP, rCTP and rGTP (10 mM)
- 1 μl Dithiothreitol (DTT) (0.75 M)
- 1 μl RNase inhibitor (40 units/μl) (Promega)
- Diethylpyrocarbonate (DPEC) treated SDW up to a final volume of 25 μl
- 0.5 μl T3/T7 RNA polymerase (5 units)

The tube was centrifuged briefly to mix components and incubated at 37 °C for 30 minutes.
To remove DNA from transcription reactions 1 µl of RNase free DNase (10 U/µl) was added after 25 minutes and incubated at 37 °C for a further 5 minutes. RNA transcript was purified by phenol/chloroform extraction and precipitated using ethanol. The pellet was dried under vacuum and resuspended in 20 µl of SDW. 5 µl was electrophoresed on an agarose gel to estimate the concentration of RNA template visually under UV-transillumination. Capped transcripts were generated by adding 2.5 µl of cap (m^7G(5')ppp(5')G)(5mM) to transcription reaction.

2.7.2 In vitro translation of transcript RNA

In vitro translation reactions were carried out in both wheat germ and rabbit reticulocyte lysate (Promega).

For wheat germ in vitro translations the following were added in a typical reaction: 25 µl wheat germ extract (Promega); 1-3 µl potassium acetate (1 M); 4 µl amino acid mixture minus methionine (1mM); 1 µl RNase inhibitor (40 units/µl); 1-2 µg RNA transcript; nuclease free water to a final volume of 50 µl; 1 mCi/ml L-[35S] methionine (800 Ci/mM, Amersham). For non-radioactive translations 4 µl of amino acid mixture containing the full complement of amino acids (1 mM) was used.

For rabbit reticulocyte lysate in vitro translations the following were added in a typical reaction: 35 µl rabbit reticulocyte lysate (Promega); 1 µl amino acid mixture minus methionine (1 mM); 1 µl RNase inhibitor (40 units/µl); 1-2 µg RNA transcript; Nuclease free water to a final volume of 50 µl; 1 mCi/ml L-[35S] methionine (800 Ci/mM, Amersham).

As discussed previously for non-radioactive translations, 1 µl of amino acid mixture containing the full complement of amino acids (1 mM) was used. Before the addition of protein loading buffer samples were removed for radioactivity incorporation or luciferase assays. Translation reactions were stopped by adding an equal volume of protein cracking buffer (2% SDS (w/v), 5% β-mercaptoethanol (v/v) and 10% glycerol (v/v), 0.01% bromophenol blue (w/v) all in 0.125 M Tris-HCl (pH 6.8)). Samples were boiled for 2 minutes before separation on a SDS polyacrylamide gel.

2.7.3 Determination of radioactive methionine incorporation into protein

2 µl of the completed translation reaction was mixed with 98 µl of 1 M NaOH/2%H2O2 in a 1.5 ml Eppendorf tube and incubated at 37 °C for 10 minutes. 900 µl of ice cold 25% TCA/2% casamino acids was then added and the tube incubated on ice for 30 minutes to precipitate translation products. 250 µl of this TCA mixture was collected by vacuum filtering using a Whatman GF/A glass filter and rinsed 3 times with 1-3 mls of ice cold 5%
TCA. After a final rinse with acetone the filter was allowed to air dry at room temperature. To determine the total counts present in the reaction a 5 μl aliquot from the remaining TCA mixture was placed directly onto a separate glass filter and allowed to dry at room temperature. Both filters were placed separately in scintillation vials with 1-3 ml of scintillation fluid (Opti-fluor O, Packard). 

$^{35}$S methionine incorporation was determined therefore as the count per million (cpm) value of the washed filter divided by 50 times the cpm value of the washed filter multiplied by 100.

2.8 SDS polyacrylamide gel electrophoresis

2.8.1 SDS polyacrylamide gels

12.5% SDS polyacrylamide gels were used in all cases, that is for both western blots and for in vitro translation gels, and proteins were electrophoresed using a BioRad mini gel kit. The proportions of constituents in a 12.5% SDS polyacrylamide gel are given below:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>6.75 ml</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH8.8)</td>
<td>5.00 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris (pH 6.8)</td>
<td>-</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>10% w/v SDS</td>
<td>0.20 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>8.00 ml</td>
<td>0.65 ml</td>
</tr>
</tbody>
</table>

The constituents of the separating gel were mixed in a glass beaker. A 30% acrylamide stock was used (29.2 g acrylamide; 0.8 g bis-acrylamide per 100 ml volume). 50 μl of 10% AMPS and 5 μl of TEMED were added and the gel solution was immediately poured between the two mini gel plates of the apparatus. A fine film of water was placed over the top to ensure a even interface. Polymerisation was allowed to proceed for 30 minutes. After the gel had formed, the water overlay was removed and the constituents of the stacking gel were mixed in a glass beaker. 25 μl of 10% AMPS and 2.5 μl of TEMED were added and the stacking gel poured on top of the separating gel with the gel comb in place. The stacking gel was allowed to polymerise for 30 minutes.

Protein samples were electrophoresed in glycine running buffer (0.3% Tris base, 1.44% glycine, 0.1% SDS (pH8.3)) at voltages between 100 and 200 until the dye front had disappeared into the running buffer. Standard protein markers were electrophoresed with protein samples to determine the size of protein bands.
2.8.2 Staining and fixing of polyacrylamide gels

Proteins were stained with Coomassie stain (H\textsubscript{2}O, methanol and acetic acid at a ratio of 5:5:1 with 0.2% Coomassie brilliant blue dye) for 30 minutes on a shaker. To destain the gel and visualise protein bands the gel was first rinsed in H\textsubscript{2}O and placed in destain solution (H\textsubscript{2}O, methanol and acetic acid at a 5:5:1 ratio) for 30 minutes to 1 hour with shaking. The destain was changed periodically and was either recycled, or discarded if the gel was radioactive. After staining and destaining non-radioactive gels were photographed and \textit{in vitro} translation gels were dried on a BioRad gel dryer under vacuum for 1 hour. Autoradiography was carried out for 1-3 days depending upon the radioactivity detected using a Geiger counter.

2.9 Transient expression assays

2.9.1 Sucrose gradient purification of tobacco protoplasts

A modification of the methods by Guerineau \textit{et al.}, (1991), and Chapeau \textit{et al.}, (1974).

Solutions:

\textbf{Protoplast enzyme solution} (pH 5.6, filter sterilised and stored frozen)

- Macerozyme R-10 (Yakult Honsha) 0.2 g/l
- Cellulase onozuka (Yakult Honsha) 1.0 g/l
- Mannitol 80.0 g/l
- Sucrose 20.0 g/l
- MS salts (Murashige and Skoog, 1962) 2.35 g/l

\textbf{Protoplast wash solution} (pH 5.6, filter sterilise and store frozen)

- Mannitol 80.0 g/l
- MS salts 2.35 g/l

Protoplasts were prepared from SR1 tobacco leaves (\textit{Nicotiana tabacum}, cv Petit Havana) that were almost fully expanded. 30 ml of protoplast enzyme solution was defrosted and poured into a 9 cm Petri dish. Using fine forceps the lower epidermis of the leaf was carefully peeled off and underlying sections of leaf tissue were cut out with a sharp scalpel and were floated, peeled side down, on the enzyme solution. Cefotaxime (300 mg/l, Sigma) was added to prevent bacterial growth. The plate was sealed with nesofilm and the and leaf sections were incubated at room temperature (15°C) overnight.

The following day the plate was agitated gently to release any protoplasts still attached to the
leaf into the solution and left for a further 30 minutes. The protoplast solution was then poured through a 64 µm nitex sieve into a fresh Petri dish to remove any leaf tissue and gently pipetted into a 50 ml disposable plastic tube. Protoplasts were collected by centrifugation at 1000g in a Beckman GPR benchtop centrifuge for 5 minutes, the supernatant pipetted off and discarded, and protoplasts resuspended very gently in an equal volume of protoplast wash solution. Protoplasts were again collected by centrifugation at 1000g, the supernatant discarded, and protoplasts resuspended in a third volume of protoplast wash solution (approximately 10 ml). 2.5 ml of 21% sucrose solution was placed in 10 ml screw top tubes. 5 ml of resuspended protoplasts were gently laid over the sucrose with care being taken not to disturb the boundary. Tubes were centrifuged for 5 minutes at 1000g with slow acceleration and deceleration. At this stage a distinct layer of protoplasts could be observed floating at the interface of the sucrose and wash solution. Burst protoplasts and leaf debris appeared as a pellet in the bottom of the tube. Protoplasts were carefully removed and transferred into a fresh 10 ml tube. Protoplasts were counted on a haemocytometer slide, the number of protoplasts per ml being equal to the sum of the number present in 5 large squares multiplied by 1000. Protoplasts were recentrifuged at 1000g for 5 minutes to resuspend in the appropriate volume of protoplast wash solution to give 5 x 10^5 - 1 x 10^6 protoplasts per 200 µl volume.

2.9.2 Transfection of tobacco protoplasts

Solutions:

**PEG solution** (pH 6.0, filter sterilise and store frozen in small aliquots)
- Polyethylene-glycol (6000) 250 g/l
- Ca(NO₃)₂ x 4H₂O 23.6 g/l
- Mannitol 82 g/l
- MES 3.9 g/l

**0.275 M Calcium nitrate solution** (pH 6.0, autoclave)
- Ca(NO₃)₂ x 4H₂O 65 g/l
- MES 2 g/l

**Protoplast recovery media** (pH 5.6, filter sterilise and store frozen)
- Mannitol 80 g/l
- Sucrose 20 g/l
- MS salts 2.35 g/l

20-40 µg of supercoiled DNA was mixed with 200 µl of PEG solution in 10 ml plastic tubes. 200 µl of sucrose gradient purified protoplasts (approximately 10⁶ protoplasts) were immediately added and tubes were agitated gently to mix. After a 10 minute incubation at
room temperature, 1 ml of 0.275 M calcium nitrate solution was slowly added dropwise to tubes. A further 4 ml of 0.275 M calcium nitrate was then added and reactions incubated for a further 10 minutes at room temperature (approximately 15 °C). Tubes were centrifuged at 1000g for 5 minutes, supernatant media removed and protoplasts gently resuspended in 5 ml of protoplast recovery media. Resuspended protoplasts were carefully poured into 3 cm Petri dishes which were sealed with nescofilm. Reactions were incubated at room temperature for 5-8 hours after which time they were transferred into 10 ml tubes and centrifuged at 1000g for 5 minutes. The supernatant was removed and protoplasts were resuspended in 500 µl of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT, and ground in a pestle and mortar for 1 minute. 50 µl of protoplast extract was used for luciferase assays, 100 µl of protoplast extract was used for GUS assays.

2.9.3 DNA microprojectile bombardments

Tungsten M-10 particles (DuPont) were suspended at a concentration of 50 mg/ml in ethanol in an Eppendorf tube and stored at -20 °C in this form until needed. Just before use the particles were washed three times in SDW and resuspended after the final wash in 500 µl in SDW.

DNA bombardment transient expression assays were carried out using a ballistic microprojectile gun. 7 µg of supercoiled test plasmid and 3 µg of supercoiled reference plasmid were mixed with 25 µl of pre-prepared tungsten microprojectile particles (1.25 mg). DNA was precipitated onto the tungsten particles by adding 25 µl of 1 M CaCl2 and then 10 µl of 0.1 M spermidine (free base) with mixing after each addition. Tubes were incubated at room temperature for 15 minutes after which time the tungsten settled out at the bottom of the tube. 25 µl of the clear upper layer was removed and was discarded. Tubes were then sonicated to evenly resuspend the tungsten particles and 2-3 µl of these were immediately pipetted onto a macro-projectile. Macro-projectiles were now ready for firing according to the method described by Twell et al., (1989).

Sterile tissue culture grown Samsun tobacco leaves, greenhouse grown potato leaves, greenhouse grown Nicotiana benthamiana and Samsun tobacco pollen, were the tissue types used for bombardment experiments. With leaf bombardments roughly equally sized leaves were placed in the centre of Petri dishes containing MSO agar. For pollen bombardments pollen was spread on a nylon Hybond membrane on solidified pollen germination medium (300 mM sucrose, 1.6 mM HBO3, 3 mM Ca(NO3)2.4H2O, 0.8 mM MgSO4.7H2O, 1 mM KNO3, 25 mM MES-KOH (pH 5.9), 0.8 % agar (Tuppy et al., 1991). Plates, with lids removed, were placed in the firing chamber in the central slot position. A stopping plate was placed in the upper region of the chamber with the little hole side facing upwards. The macro-projectile on which the tungsten particles had been placed was inverted and placed in the firing hole with care being taken not to loose the tungsten spot. A blank charge was placed on top and the lid was screwed on. The chamber was evacuated to 25 imperial inches of Mercury and the charge was fired by hitting the blank with a firing pin and hammer. The vacuum was released and the sample removed. Petri dishes were sealed with nescofilm and
were incubated at 25 °C for 24 hours for leaf tissue or for 16 hours for pollen tissue. After this time luciferase and/or GUS assays were carried out.

2.10 Tobacco transformation

2.10.1 Conjugation of recombinant plasmid into Agrobacterium

The kanamycin resistant binary vectors, pBI121 or pBin19 (Section 2.16), were used to make constructs for tobacco transformation. The *Agrobacterium* tumefaciens strain LBA4404 (Hoekema *et al.*, 1983) and the helper plasmid pRK2013 (Ditta *et al.*, 1980) were used. The helper plasmid provides the mobilising (mob) and transfer (tra) factors for conjugative transfer of the binary vector from *E.coli* to *A. tumefaciens*.

Triparental mating was carried out as follows. 100 μl of an overnight culture of *E. coli* containing the binary vector, *A. tumefaciens* LBA4404 and pRK2013 were spread on a nutrient agar plate containing no antibiotics. The plate was incubated at 28 °C for 2 days. After this incubation a wire loop was used to streak a loopful of the bacterial lawn that had formed onto a nutrient agar plate containing the antibiotics rifampicin (100 mg/ml) and kanamycin (50 mg/ml) to select for *Agrobacterium* cells containing the binary vector. This was incubated for 2 days at 28 °C. Single colonies were selected and were grown in N-broth with rifampicin (100 mg/ml) and kanamycin (50 mg/ml) for 2 days at 28 °C. Cultures were used for total nucleic acid extraction and Southern blot analysis or for plant transformation.

2.10.2 Total nucleic acid extraction from Agrobacterium tumefaciens

1.5 mls of *Agrobacterium* culture derived from a single colony was centrifuged in an Eppendorf for 5 minutes. The supernatant was removed and the bacterial pellet resuspended in 300 μl of SDW. 100 μl of 5% Sarkosyl and 150 μl of pronase E (15 mg/ml) were added, tube contents were mixed and incubated at 37 °C for 1 hour. The solution was extracted three times with an equal volume of phenol/chloroform. During the extraction the sticky mass present in the bacterial lysate was sheered by gentle sucking up and down. 1/10 volume of 3 M sodium acetate (pH 7.0) and 2.5 volumes of cold ethanol were added to the supernatant from the final phenol/chloroform extraction. The nucleic acid pellet was collected by centrifugation for 10 minutes, washed in 70% ethanol and dried under vacuum. The pellet was resuspended in 50 μl of SDW and 20 μl was used for restriction digest analysis. Southern blots were carried out on restricted *Agrobacterium* DNA using appropriate diagnostic probes to identify specific foreign DNA sequences.

2.10.3 Tobacco leaf disc transformation

*Nicotiana tabacum* SR1 plants supplied from the Leicester University botanical gardens were used for tobacco leaf disc transformation. Almost fully expanded leaves were surface
sterilised in a sterile casserole dish in a 20% bleach solution for 15 minutes. Leaves were
washed three times for 15 minute periods to remove any traces of bleach and were cut into 1
cm squares on a sterile ceramic tile. The mid rib of the leaf was avoided. Discs were placed
on MSD4 x 2 (Section 2.17.2) plates for 2 days to allow swelling. After swelling discs were
floated on 50 or 100 times dilution of Agrobacterium, containing the intended DNA for
transfer, for 10 minutes. Discs were transferred again to MSD4 x 2 plates, plates were sealed
with clingfilm and placed in a tissue culture growth room for about 2 days (until
Agrobacterium growth was just visible). Leaf discs were then transferred again to fresh
MSD4 x 2 plates but this time containing the antibiotics, augmentin (400 mg/ml) to select
against the Agrobacterium and kanamycin (100 mg/ml) to select for transformants containing
transferred DNA. After 2-3 weeks shoots began to appear from the sides of the leaf discs.
These were removed, with care being taken to avoid any callus material, and placed in sterile
powder round jars containing MSO agar (Section 2.17.2) containing augmentin and
kanamycin at the above concentrations. After root development these transformants were
either subcultured to maintain in tissue culture, or potted into soil and placed in transgenic
growth rooms.

2.11 Infection of plants with tobacco mosaic virus (TMV)

Leaves to be infected were dusted with carborundum powder which when rubbed gently
over the leaf causes mild abrasion which results in breakages in the leaf epidermis, it is this
allows virus to enter. Virus, extracted from infected plant leaf tissue, was gently rubbed onto
the leaf to be inoculated. Mock inoculations were carried out with water being used instead
of virus. Plants were grown in growth cabinets at 22 °C with 8 hours of darkness and 16
hours of light. Uninfected and infected plants were kept in different cabinets and, in the case
of TMV, mosaic symptoms became evident after 10-15 days. Samples were taken from
inoculated or systemic leaves of both infected and uninfected plants at day time-points post-
inoculation for either fluorometric GUS assays or determination of the presence of viral
RNA.

2.12 Assays for reporter gene expression

2.12.1 Luciferase assays

1 µl from an in vitro translation reaction in a 10 µl volume with SDW was directly assayed
for luciferase activity. Plant tissue was first ground in a pestle and mortar with 300 µl of
potassium phosphate buffer (100 mM, pH 7.5) containing 1 mM DTT, (0.1 M potassium
phosphate buffer was made by mixing 8.4 mls of 1M K₂HPO₄ and 1.6 mls of 1M KH₂PO₄
per litre volume). Extract was transferred to 1.5 ml Eppendorf tube and centrifuged for 5
minutes. The supernatant was transferred to a fresh tube and 50 µl of this extract was
assayed for luciferase activity.

Samples were placed in a bioluminescence photometer reaction cuvette and were assayed in a luminometer in the presence of 100 μl of ATP buffer (500 mM HEPES (pH 7.8), 1M MgCl₂, 20 mM ATP in 100 mM KPO₄ (pH 7.5)) and 100 μl of D-luciferin (0.5 mM in 100 mM KPO₄ (pH 7.5), Sigma). The light emitted was measured by the machine and the luciferase unit value was printed out directly.

2.12.2 Fluorometric β-glucuronidase (GUS) assays

GUS extraction buffer:

- 50 mM Na₂PO₄ (pH 7.0)
- 10 mM EDTA
- 0.1% Triton X-100
- 0.1% Sarcosyl
- 10 mM β-mercaptoethanol

(0.1 M Na₂PO₄ pH 7.0 stock solution was made by mixing 5.77 mls of 1M Na₂HPO₄ and 42.3 mls of 1M NaH₂PO₄ per litre volume)

Plant material was ground in an Eppendorf with a micro-homogeniser with 500 μl of GUS extraction buffer. Tubes were centrifuged for 5 minutes to remove plant debris and the supernatant transferred to a fresh tube. 100 μl of extract was transferred to a tube containing 400 μl of GUS extraction buffer containing 1 mM methyl umbelliferyl glucuronide (MUG). Tubes were incubated at 37 °C with 100 μl volumes being removed at time points of 0, 30, 60 and 90 minutes and added to microtitre wells which contained 100 μl of GUS reaction stop solution (200 mM Na₂CO₃). Fluorescence was measured directly from the microtitre plate in a Perkin-Elmer fluorometer with an excitation wavelength of 365 nm and an emission wavelength of 455 nm. The rate of GUS activity, taken as the fluorometer measurement of the conversion of MUG to methyl umbelliferrone (MU), was expressed as fluorometric units per hour (FU/h).

2.13 Protein concentration determination

Protein concentration was determined using Bradford assays (Bradford, 1976). 20 μl of protein samples were placed in wells in a microtitre plate and 180 μl of Bradfords reagent was added (600 mg/l coomassie brilliant blue G-250, 2% (v/v) perchloric acid, allowed to settle overnight and filtered (OD464 1.3-1.5)). Protein standards of BSA ranging in concentration from 0 to 1 mg/ml were prepared and 20 μl volumes of each of these were also placed in microtitre plate wells with Bradfords reagent. The plate was read by a Dynatech MR5000 microtitre plate reader. The programme used provided a direct calculation of the protein concentration of the samples in mg/ml.
2.14 Determination of nucleic acid concentration

DNA and RNA concentration was usually estimated both visually on an ethidium bromide stained agarose gel, or by spectrophotometry. DNA and RNA concentrations were measured at an OD<sub>260</sub> of 260 and 280, respectively. A scan between OD<sub>260</sub> 200 and 300 provided some indication of purity of a nucleic acid extract.

2.15 Bacterial Strains

E.coli JM109 (Yanisch-Perron et al., 1985) : rec A<sub>1</sub>, sup E<sub>44</sub>, end A<sub>1</sub>, hsd R<sub>17</sub>, gyr A<sub>96</sub>, rel A<sub>1</sub>, thi Δ (lac-pro AB). This strain is a recombination deficient mutant which will modify but not restrict DNA.

E.coli XLl-blues (Bullock et al., 1984) : rec A<sub>1</sub>, end A<sub>1</sub>, gyr A<sub>96</sub>, thi-1, hsd R<sub>17</sub>, sup E<sub>44</sub>, rel A<sub>1</sub>, lac, [F<sup>+</sup> pro AB, lacI <sup>q</sup> Z<sup>_M15</sup>, T<sub>n</sub>10(tet<sup>+</sup>)].

E.coli TG2 (Biggin et al., personal communication) : sup E, Δ(lac-pro AB), hsdAS, Δ(srl-rec A)306::T<sub>n</sub>10(tet<sup>+</sup>) F<sup>+</sup> D<sub>36</sub>, pro AB<sup>+</sup>, lacI<sup>1</sup>, lac Z<sup>_M15</sup>. This strain is a recombination deficient strain of TG1 (TG1, (Gibson et al., 1984) EcoK<sup>+</sup> derivative of JM101, does not modify or restrict DNA)

2.16 Plasmids

pBluescript (SK+/-) :
(Short et al., (1988), Stratagene) This vector has a ColE1 origin of replication and confers ampicillin resistance in bacteria. It exhibits blue/white colour selection, possesses a T3 and T7 promoter for in vitro transcription, can be used for single-stranded rescue of DNA or for double-stranded sequencing and has numerous unique restriction enzyme sites in the multiple cloning site.

pSL301 :
(Invitrogen) This plasmid contains a ColE1 origin of replication, confers resistance to the antibiotic ampicillin in bacteria and has blue/white colour selection. It also has the SL2 superlinker for cloning.

pGEM5Zf(+/-) :
(Promega) This plasmid has an F1 origin of replication and confers ampicillin resistance in
bacteria. It has blue/white colour selection.

pGEM-t :
(Promega) This plasmid is a derivative of pGEMSZf. It was constructed by EcoRI restriction of this plasmid and the addition of thymine residues onto each cut end to facilitate the cloning of PCR products.

pCR11 :
(Invitrogen) This plasmid possesses T overhangs at the cloning site to facilitate the cloning of PCR products. It contains a ColE1 origin of replication and confers both ampicillin and kanamycin resistance in bacteria.

pBin19 :
(Bevan, 1984) This plasmid has a wide host range origin of replication (pRK252). It confers resistance to the antibiotic kanamycin in bacteria and plants and has associated blue/white colour selection. pBin19 has several unique enzyme restriction sites and is used for Agrobacterium-mediated transfer of DNA into plants.

pBI121 :
(Jefferson, (1987), Clonetech) This plasmid is a derivative of the pBI101 plasmid with the only difference being the addition of an 800 base pair fragment containing the cauliflower mosaic virus 35S promoter. pBI101 is a derivative of pBin19 but contains the GUS ORF. It contains a RK2 origin of replication and confers kanamycin resistance in both bacteria and plants. It is used for Agrobacterium-mediated transfer of DNA into plants.

2.17 Media

2.17.1 Bacterial Media

L-broth (Luria-Bertani Medium)
The following were added to 950 ml of deionised water:

10 g of bacto-tryptone  
5 g of bacto-yeast extract  
10 g of NaCl

The solutes were allowed to dissolve and the pH was adjusted to 7.0 with 5 M NaOH. The volume was made up to 1 litre with deionised water. For L-agar 15 g of bacto-agar was added per litre of L-broth just before autoclaving.
2.17.2 Plant Media

MSO media (a media based on MS (Murashige/Skoog) salts)
(The pH was adjusted to 5.6 with 0.1 M HCl)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂.2H₂O</td>
<td>440.000</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>160.000</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900.000</td>
</tr>
<tr>
<td>KI</td>
<td>0.830</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170.000</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.200</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>0.250</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>370.000</td>
</tr>
<tr>
<td>MnSO₄.4H₂O</td>
<td>22.300</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>ZnSO₄.4H₂O</td>
<td>8.600</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>27.850</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>37.250</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.000</td>
</tr>
<tr>
<td>Inositol</td>
<td>100.000</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.500</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.500</td>
</tr>
<tr>
<td>Thiamine HCL</td>
<td>0.100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30,000.000</td>
</tr>
</tbody>
</table>

(The pH was adjusted to 5.6 with 0.1 M HCl)

MSD4 x 2

The same as MSO with the following being added in addition:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration mg/l</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>6-BAP</td>
<td>1.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30,000.0</td>
</tr>
</tbody>
</table>

(pH 5.8)

All media was sterilised by autoclaving for 20 minutes at 15 lb/sq.in with slow cooling.
Most methods outlined in this chapter were derived from Draper et al., (1988) and Sambrook et al., (1989). Most chemicals were obtained from Sigma unless otherwise stated.
Chapter 3

Translation of carlaviruses mRNAs in a prokaryotic system

3.1 Introduction

The mechanism of carlavirus gene expression involves the production of two subgenomic RNA molecules that promote the translation of internal open reading frames (Foster and Mills, 1990a; 1990b; 1991a). The larger of these is thought to act as template for translation of the triple gene block proteins, the smaller for viral coat protein production. With the carlavirus, Helenium virus S (HelVS), the genome takes the form of a single-stranded, positive-sense RNA molecule, 7.4 kb in size, which is encapsidated in single Mr 31 kDa coat protein subunits (Kuschki et al., 1978). In addition to full length genomic 650 nm encapsidated viral particles, smaller viral particles, 320 nm and 180 nm in size have been detected in purified virus preparations (Foster and Mills, 1990b). These smaller molecules are present only in trace amounts when compared to the concentration of full length genomic particles, and, on the basis of their size, it is thought that they are encapsidated subgenomes, the larger containing a 3.3 kb RNA and the smaller containing a 1.5 kb RNA molecule (Foster and Mills, 1990b). The HelVS 1.5 kb RNA molecule is an efficient message for coat protein synthesis in vitro (Foster et al., 1992c). PVS also has two smaller RNA species, 2.5 kb and 1.1 kb in size, in addition to the full length genomic RNA molecule which have been detected when RNA from infected plant tissue was probed with a 3' terminal PVS clone from the Andean strain of the virus (Foster and Mills, 1991a). Similar to the situation with HelVS, these RNA species are present only in trace amounts when compared to the concentration of genomic RNA.

Time-course experiments on in vitro translation of many carlaviruses has revealed that coat protein is one of the primary proteins produced eventhough it is one of the 3' terminal genes, reaching a detectable level only 10 minutes into the reaction, and it is produced in large amounts to cope with the demands of viral encapsidation (Mackenzie et al., 1989; Foster and Mills, 1990a; 1991c; 1992b). This is true also for the type member of the group carnation latent virus (CLV) for which subgenomics have yet to be identified (Meehan and Mills, 1991).

Encapsulation by coat protein is absolutely essential for the protection of viral genetic material from the degradative nature of the environment of which they are invaders. Carlaviruses require large quantities of individual coat protein subunits to completely encapsidate the large genomic RNA molecule with the formation of a flexous filamentous particle. This figure has been estimated at between 1600-2000 individual subunits per viral
particle (Varma et al., 1968). This does not take into account the coat protein required for the encapsidation of subgenomic RNAs.

A detailed comparison of the sequences of the upstream untranslated regions from the carlavirus 25 kDa and the coat protein ORFs, the putative 5' termini of the carlavirus subgenomic RNA molecules, has revealed the presence of sequences with homology to Shine-Dalgarno sites for prokaryotic ribosome binding which are essential for efficient translation of prokaryotic and chloroplastic mRNAs (Shine and Dalgarno, 1975; Foster and Mills, 1991b). These Shine-Dalgarno sequences exhibit base pair complementarity to the pyrimidine rich sequence at the 3' end of the prokaryotic 16S rRNA molecule. Two such regions have been identified upstream from the second carlavirus ORF, the 25 kDa gene of the triple gene block. These have been designated Ribosome Binding Sites 1 and 2 (RBS1/2) and are situated about 40 and 20 nucleotides upstream from AUG initiation codon, respectively. A third site, RBS3, is situated about 40 nucleotides upstream from the AUG of the viral coat protein ORF. These sequences are highly conserved throughout the carlavirus group which indicates some functional importance and these conserved blocks may help to explain mechanism of carlavirus translation. These sequences and their position are indicated in Fig 3.1.

It appears that viral coat protein and perhaps the triple gene block proteins are produced from subgenomic RNA molecules that are present in such low quantities as to make them almost indetectable. Subgenomic RNA molecules may therefore be extremely efficient, a theory that is explored in Chapters 4 and 5, and/or viral coat protein could be produced as a result of internal ribosome binding and initiation of translation on the full length genomic RNA molecule or on the larger 2.5 kb subgenomic. The aim of this chapter is to investigate whether or not these carlavirus sequences could actually operate in a prokaryotic system, a phenomenon that has been suggested by the identification of these prokaryotic ribosome binding sequences. A positive result would bring forward important evolutionary implications and raise questions as to why these sequences have been conserved despite their apparent redundancy.

### 3.2 Expression of HelVS coat protein in E.coli.

#### 3.2.1 Construct

Two clones, pHEL19 and pHEL23, were provided by Dr. G. Foster (Botany Department, University of Leicester). Both were constructed following double-stranded cDNA synthesis from HelVS genomic RNA, the first strand being primed using oligo-dT, and ligation into Smal digested vector pUC13 vector. The two clones were selected by immunoscreening using HelVS polyclonal antisera for their ability to express the HelVS coat protein, presumably fused to the alpha peptide of the lacZ gene after induction of the lacI promoter using IPTG. Restriction enzyme analysis revealed insert sizes of 1.4 kb and 1.6 kb for
Fig 3.1 Diagrammatic representation of the 3' terminal carlaviruses genomic organisation indicating the position of conserved sequences with homology to prokaryotic Shine-Dalgarno sequences. Ribosome Binding Sites 1, 2 and 3 (RBS) are indicated as are the 3' terminal genome products. The sizes of these proteins, in kilodaltons (K), are also indicated. The actual nucleotide sequences of these regions for various carlaviruses is provided to demonstrate the degree of sequence conservation among the group. Gaps (-) have been introduced for maximum alignment. Taken from Foster and Mills, (1991b).
Fig 3.1

AA
LSV AGGAACCTTAGAT
PVS AGGAGGTTTGAT
PVM AGGGTGCTTTAA

PVM — CCTTTAGGT-CAT
PVS -ACCTTTAGGTTTCAC
CLV AACC-TTAGGTTTCAC
LSV -ACCTTTAGGTTTCAC
HeLVs --CTTTTACCTTAC

RBS1 RBS2 RBS3

LSV AGCTTAGGGTTAT
PVS AGCTTAGGTAAT
PVM AGCTTAGGTTATT

25 K 12 K 7 K

AAA

33 K 11 K
Fig 3.2 The nucleotide sequence corresponding to the 7 kDa gene and upstream partial 12 kDa gene of HelVS (Turner et al., 1993). The DNA sequence is shown as the equivalent of the viral plus strand and nucleotide residues are numbered from the first nucleotide at the 5' end of the sequence obtained. The amino acid sequence is presented below the nucleotide sequence. Termination codons are indicated by a (*). The ATG start codon of the HelVS 7 kDa gene is underlined with a bold line. Nucleotides proposed as having some involvement in ribosome binding are underlined with a dotted line.
Fig 3.2

CCAAGGAAATTAACACGATTTGAGGGTFACTGCAACTACGGGTGGCTTACGTG
PRKLNSEGNCRKXYHPWAYV

ATGGCAARTCCTCCTTTCTATTTGACCTTCTACCTGCTAATTTGACCTTCTACCTG
ISLVLISLWDSRRVCSC

GTTGCGCTTTACATTAAAGTTGACAGACTTTTTTCTTATTAGTATTACAGATATCT
GRRH*HEELLIIALLAILGS

ATTGTCTGGCTATTAACTAATCAACAAGAGCAGTGCGTAGTTCTTATCACAGGGTAATCGGG
IVWLLLTNTQQEQCVVLYLTCESV

CGATAGTGGCTTTGCAAGTTTACCCCTGGAATTGATTAGTTCTTTATTACAGAGGSAECCNC
RIVSCCKFTPEIEYAYAKALKPA

AACTGTTTGGCCCTTTTACGATTCAAGCAGRAGYUTNFACAGAAGAAGFPACN
NSC*--------------

AAAAAXG
PHEL19 and PHEL23, respectively. It was determined that the difference in nucleotide length was at the 5’ end due to the detection of a poly (A) tail in both clones (Foster and Mills, 1992c). The PHEL19 clone was sequenced and was shown to contain the entire HelVS coat protein open reading frame with a downstream 12.6 kDa protein gene (Foster et al., 1990c), but one aspect of this clone was of interest with respect to its expression after induction with IPTG. Between the initiating methionine of the viral coat protein and the initiating methionine of the lacZ of the vector two stop codons were detected, indeed there were stop codons in all reading frames between the two methionines thus raising the question as to how the coat protein ORF was being efficiently expressed in E. coli as it was clearly not using the Shine-Dalgarno sequence and initiating methionine of the lacZ protein. It was therefore suggested that ribosomes could possibly be binding internally in association with a sequence between these stop codons to initiate translation (Foster et al., 1992c). Investigation of the PHEL23 clone would perhaps provide stronger evidence for internal binding as it appeared that this clone contained more sequence extending 5’ from the ATG of the coat protein gene.

3.2.2 The sequence of the upstream region from the ATG of the coat protein ORF in the PHEL23 clone

In order to obtain the nucleotide sequence to the 5’ upstream region of the coat protein ORF, single-stranded DNA rescued via helper phage from the PHEL23 clone was sequenced using the dideoxynucleotide termination method of Sanger et al., (1977), and compiled using the computer program DNASIS (Pharmacia). Sequence data obtained extended about 380 nucleotides upstream from the ATG of the coat protein gene. Within this region one complete ORF was identified to the 5’ region of the coat protein gene, consisting of 183 nucleotides, with the deduced peptide containing 61 amino acids, equivalent to a molecular mass of 6723.64 daltons. The sequence obtained is provided in Fig 3.2.

3.2.3 Amino acid conservation of the HelVS 7 kDa protein

The entire 183 nucleotide ORF contained within the 381 nucleotides sequenced is presumably, based on size and genomic position, the 7 kDa protein of the HelVS triple gene block (TGB). The predicted amino acid sequence of this 7 kDa peptide was compared with equivalent peptides of other carlaviruses and potexviruses (Fig 3.3). Homologies of 30.8% and 31.8% were evident at the amino acid level with the 7 kDa peptides of the other carlaviruses PVSA (the Andean strain of potato virus S) and PVM (potato virus M), respectively (Mackenzie et al., 1989; Rupasov et al., 1989). A slightly lower though significant homology of 28.6% was detected with the equivalent peptide of the potexvirus, PVX (Huisman et al., 1988). The central region of the sequence exhibited the greatest degree of homology with a degree of sequence variability at the N-proximal and C-terminal ends of the protein. A region of very strong conservation was a central 11 amino acid -
Alignment of the 7 kDa protein amino acid sequence of HelVS with the equivalent ORFs from PVS, PVM and PVX (Turner et al., 1993). Homologous amino acids are boxed. Termination codons are represented as *.
Fig 3.4 Alignment of a portion of the amino acid sequence of the ORF upstream from the HelVS 7 kDa protein with the C-terminal amino acid portion of the 12 kDa proteins of PVS, PVM and PVX (Turner et al., 1993). Gaps (-) have been introduced for maximum alignment. Termination codons are represented as "*".
Fig 3.4

HelVS 12K  
PRKLNSESIE-GCRCYHKQPW

PVS 12K  
PTKLNSEVEPGNYWTQPW

PVM 12K  
PGRLNSteamAIPGPW

PVX 12K  
PN-LGRSVSLHNQKN-AAF

AYVIGSLLVLI-LSLWD

LLVI---LLVALICLSGRH

AVV--LLLVLWIASHKL

SRRVCCS--GRRRH*

AQCCPRC---NRVHSA*

GRPRCAGS-HT*

ISQRNHTCAGNHHSSH*
Fig 3.5 Autoradiograph produced after SDS-polyacrylamide gel electrophoresis of an equal volume of \textit{in vitro} translation reaction containing transcript generated from pHEL19 and pHEL23 pBluescript clones in rabbit reticulocyte and wheat germ lysate. Lane 1, pHEL19 in rabbit reticulocyte lysate; lane 2, pHEL23 in rabbit reticulocyte lysate; lane 3, pHEL19 in wheat germ extract; lane 4, pHEL23 in wheat germ extract. The position of the 31 kDa HelVS coat protein band is indicated.
CVVLITGESVR- block which was extremely homologous among all the viruses compared. Amino acid homologies are presented in Fig 3.3.

Sequence data obtained extended beyond the start codon of the 7 kDa ORF into what was presumed to be 12 kDa protein gene of the triple gene block. This sequence extended 141 nucleotides upstream from the 7 kDa gene ATG start codon with a deduced partial peptide containing 47 amino acids, presumably corresponding to the C-terminal region of the HelVS 12 kDa protein. This sequence was compared with the predicted C-terminal regions of equivalent peptides from other carla- and potexviruses. Significant amino acid similarity of 46.3% and 41.6% homology was evident at the amino acid level with the equivalent regions of the 12 kDa proteins of PVS and PVM respectively with 25.4% similarity detected with the equivalent region of PVX. Amino acid homologies are provided in Fig 3.4.

3.2.4 Expression of pHEL19 and pHEL23 in a eukaryotic system *in vitro*

Both insert fragments from pHEL19 and pHEL23 were subcloned from pUC13 asScaI/HindIII digested fragments into pBluescript SK(+) digested with the same enzymes. Using the T3 RNA polymerase promoter positive-sense mRNA transcripts were generated and equal amounts of RNA and translated *in vitro*, both in rabbit reticulocyte lysate and wheat germ extract. On translation of both clones a 31 kDa protein band was detected which corresponds to the size of the HelVS viral coat protein. This agrees with previous western blot results when the pHEL19 and pHEL23 clones were induced with IPTG in a prokaryotic system (Section 3.2.3). Results are presented in Fig 3.5. Approximately equal amounts of coat protein was produced from both clones which indicates that both pHEL19 and pHEL23 derived transcripts act as efficient template for HelVS coat protein. pHEL23 is as efficient as pHEL19 in an eukaryotic system despite the presence of additional information upstream.

3.3 Internal initiation of translation with potato virus S

3.3.1 Constructs

Experiments designed to further investigate the expression of the carlavirus coat protein in a eukaryotic and prokaryotic system were carried out with potato virus S (PVS). This virus was chosen because constructs were available for the analysis. PVS constructs were generated which would allow the analysis of the untranslated sequence upstream from the ATG of the coat protein gene. A 3' terminal PVS construct, p7CP, was provided by Dr. G. Foster (Botany Department, University of Leicester). This clone contained, in addition to the PVS coat protein ORF and downstream 11 kDa protein gene, 101 nucleotides of viral sequence upstream from the initiating methionine of this gene (Foster and Mills, 1992a). This untranslated region has been designated, VTE or viral translational enhancer, and it includes all of the sequence between the termination codon of the 7 kDa protein gene of the triple gene block and the ATG of the coat protein ORF. It therefore contains the conserved nucleotide block with base pair complementarity to the pyrimidine rich sequence at the 3' end.
**Fig 3.6 A** Diagrammatic representation of constructs used for the analysis of the PVS VTE sequence with regard to internal initiation of translation. The VTE, 101 nucleotides between the termination codon of the 7 kDa ORF and the start codon of the PVS coat protein is represented as a black block. The synthetic sequence used as a control leader in experiments is represented as a dotted block. The position of the RNA polymerase promoter (T3 or T7) used to generate *in vitro* transcripts is indicated by an arrow. GUS β-glucuronidase reporter gene : 7CP PVS 3' terminal coding sequence containing the coat protein and 11 kDa ORFs with upstream VTE sequence : 35S promoter cauliflower mosaic virus 35S transcriptional promoter : 35S term cauliflower mosaic virus 35S transcriptional terminator : 7CP gene products.

**Fig 3.6 B** VTE and SYN sequences in constructs represented in A. Leader sequences from pVTE-LUC and pSYN-LUC are provided in Fig 4.1 B and 4.5 B, respectively. ATG start codons are underlined.
VTE sequence, 101 nucleotides between the termination codon of the 7 kDa ORF of the TGB and the ATG of the PVS coat protein gene

B

VTE leader sequence in pBI121, pGUS-7CP and pGUS-VTE-LUC constructs

SYN leader sequence in pGUS-SYN-LUC construct
of the 16S rRNA molecule that has been discussed in Section 3.1 (Foster and Mills, 1991b). The entire p7CP insert was isolated as a SacI/EcoRI fragment from the pBluescript-KS(+) vector in which it was contained, and was cloned into SacI/EcoRI-digested pBI121 (Clontech, Section 2.16) binary vector. This cloning event resulted in the p7CP insert being placed downstream from the GUS open reading frame. In this clone, designated pBI121-7CP, the VTE sequence acted as the intercistronic spacer sequence between the GUS and the PVS coat protein ORFs. This GUS-PVS coat protein dicistronic fragment was isolated as an XbaI/EcoRI fragment from pBI121-7CP and was cloned into pBluescript SK(+)-digested with the same enzymes. This construct was designated pGUS-7CP. Diagrammatic representations of the constructs pBI121-7CP and pGUS-7CP are provided in Fig 3.6.

The upstream 101 nucleotide region from the PVS coat protein gene, or VTE, was isolated by PCR amplification and was linked to the 5' end of the luciferase reporter gene. A detailed description of the construction of this plasmid, pVTE-LUC, and a control plasmid used in the experiment containing a synthetic sequence linked to the luciferase coding gene, pSYNLUCb, is provided in Sections 4.2 and 4.6, respectively. To produce dicistronic constructs, derivatives of these two plasmids in which the leader sequences would be placed at an internal location between two open reading frames, the GUS reporter gene was cloned into the region upstream from both leader sequences. The GUS gene was isolated as a SacI fragment from a pSYN-GUS plasmid (Section 4.2) and was cloned into SacI-digested and dephosphorylated pVTE-LUC and pSYN-LUCb vectors. The orientation of the GUS coding sequence was determined by PCR amplification using a primer within the GUS gene and a primer in the pBluescript vector. Clones were selected which contained the GUS gene in the correct orientation, with the ATG start codon at the 5' end of the construct. Diagrammatic representation of the constructs pGUS-VTE-LUC and pGUS-SYN-LUC are provided in Fig 3.6.

3.3.2 Preliminary investigations into internal ribosome binding with PVS in a eukaryotic environment.

Preliminary experiments with pGUS-7CP constructs, the GUS reporter gene linked to the PVS coat protein ORF and upstream VTE sequence, both in vitro and in vivo, were largely unsuccessful. In vitro translation of the pGUS-7CP clone in rabbit reticulocyte lysate did result in the production of a band corresponding to the size of the coat protein gene but a similarly sized band was also observed when a clone containing the GUS ORF was translated alone, presumably produced due to premature termination of translation. Although this band was not of the same intensity as the band produced with the GUS-coat protein VTE dicistronic clone, pGUS-7CP, it could not conclusively say that this band was viral coat protein (results not provided).

Transgenic plants were also generated by Agrobacterium-mediated leaf disc transformation with the pBI121-GUS-7CP binary construct such that GUS-7CP RNA would be produced in vivo in a natural eukaryotic environment. Primary transformants expressed GUS at high levels but coat protein was not detected when protein extracts were probed with PVS coat.
Table 3.1

Luciferase expression in *E. coli*

<table>
<thead>
<tr>
<th>Construct</th>
<th>Luciferase activity after IPTG induction</th>
<th>Fold enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSYN-LUC</td>
<td>40620</td>
<td></td>
</tr>
<tr>
<td>pVTE-LUC</td>
<td>11199150</td>
<td>X 275.7</td>
</tr>
<tr>
<td>pSYN-LUC</td>
<td>2376390</td>
<td></td>
</tr>
<tr>
<td>pVTE-LUC</td>
<td>313628400</td>
<td>X 131.9</td>
</tr>
</tbody>
</table>
Table 3.2

<table>
<thead>
<tr>
<th>Construct</th>
<th>Luciferase activity after IPTG induction</th>
<th>Fold enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGUS-SYN-LUC</td>
<td>8980</td>
<td></td>
</tr>
<tr>
<td>pGUS-VTE-LUC</td>
<td>351250</td>
<td>X 39.1</td>
</tr>
<tr>
<td>pGUS-SYN-LUC</td>
<td>107554</td>
<td></td>
</tr>
<tr>
<td>pGUS-VTE-LUC</td>
<td>3071090</td>
<td>X 28.6</td>
</tr>
</tbody>
</table>
protein polyclonal antisera. The quality of the antisera used was, however, questionable because when extract from PVS infected control potato plants was probed only a very faint band could be detected (results not presented). It was decided to overcome this detection problem by replacing the coat protein ORF with the reporter gene luciferase as this would provide a more sensitive assay system.

3.3.3 The activity of the VTE sequence in a prokaryotic system in vivo.

The lac promoters of pVTE-LUC and pSYN-LUC constructs (monocistronic constructs, Sections 4.2 and 4.6) were induced using IPTG in E.coli. Care was taken to ensure that the AUG of the luciferase gene was out of frame with the AUG of the lacZ protein so that luciferase was not expressed as fusion protein with the alpha peptide of this protein, using the Shine-Dalgarno sequence for prokaryotic ribosome binding of the lacZ. Any detectable luciferase expression should therefore be through an interaction of ribosomes within the sequence between these two initiation codons.

Expression of luciferase from the construct which contained the VTE sequence, i.e. the PVS sequence upstream from the AUG of the viral coat protein with the carlaviruses conserved canonical Shine-Dalgarno sequence, was a mean value of 204 times higher than the level expressed from the construct that contained a randomly selected synthetic sequence. These experiments were carried out in triplicate and results are presented in Table 3.1.

In the same in vivo prokaryotic system a similar induction experiment was carried out with pGUS-VTE-LUC and pGUS-SYN-LUC, that is the two dicistronic constructs with either the VTE sequence or a synthetic sequence in the intercistronic region (Fig 3.6). Theoretically, expression of the second ORF should only occur if ribosomes bind internally in association with the intercistronic sequence to initiate translation. After induction, about 30 times the level of luciferase was expressed from bacterial cells containing the pGUS-VTE-LUC construct when compared to the level expressed from cells containing the pGUS-SYN-LUC construct. The experiment was carried out in duplicate and results are presented in Table 3.2.

3.4 Discussion

A purine-rich sequence preceding the initiator codon within a mRNA leader and showing some degree of complementarity to the pyrimidine rich sequence near the 3' end of the 16S rRNA molecule, is thought to play an essential role in mRNA-ribosome recognition and initiation of translation in a prokaryotic system (Shine and Dalgarno, 1975). The common "Shine-Dalgarno" sequence 5'-AGGAGGT-3' is thought to be essential for prokaryotic and chloroplastic ribosome recognition but many mRNA molecules are efficiently expressed even though they possess only 3 or 4 nucleotides in common with the sequence at the 3' end of the 16S rRNA molecule. The positions of Shine-Dalgarno sequences may range from 3-28 nucleotides from the AUG start codon which indicates a considerable degree of flexibility (Bahramian, 1980). This is particularly true for chloroplastic ribosomes that appear to be able to withstand quite large distances between recognition sites and initiating AUG codons.
It has previously been reported, by Foster and Mills (1991b), that the sequences upstream from carlavirus internal ORFs, upstream from the 25 kDa gene product of the TGB and upstream from the coat protein cistron, that is the proposed termini of the carlavirus subgenomics, contain blocks of nucleotide sequence that could potentially act as prokaryotic ribosome recognition site on the carlavirus genomic or subgenomic RNA molecule, due to sequence homology with these Shine-Dalgarno sequences. A nucleotide alignment of these ORF upstream regions has determined that these sequences are extremely highly conserved between group members which indicates that they may have some functional importance. The positions and sequences of these conserved Ribosome Binding Sites (RBS) are provided in Fig 3.1.

Results presented indicate that two HelVS clones, pHEL19 and pHEL23, express HelVS coat protein in vivo in a prokaryotic system despite the presence of at least two stop codons in pHEL19, and the presence of a complete open reading frame, corresponding to a peptide approximately 7 kDa in size, upstream from the ATG of the coat protein gene, with pHEL23 (Foster et al., 1992c; Turner et al., 1993). This prompted the proposal that the canonical Shine-Dalgarno sequence present upstream from the HelVS coat protein ORF could possibly be functioning as a ribosome recognition sequence promoting ribosomal translational initiation in this prokaryotic system. The HelVS sequence with Shine-Dalgarno homology is underlined in Fig 3.2. These observations are supported by experiments carried out with a corresponding region of the PVS genome. In a prokaryotic in vivo system a luciferase construct containing the 101 nucleotide VTE sequence, that is the upstream PVS coat protein sequence which contains the canonical Shine-Dalgarno sequence mentioned above, linked to the luciferase, expresses the reporter gene at a mean value of about 200 times the level expressed from a construct that contains a randomly selected synthetic sequence in place of the VTE. This enhanced expression cannot be attributed to production of luciferase as a fusion with the a peptide of the lacZ protein as the AUG start codon is out of frame with the initiation codon of the of the lacZ ORF.

Dicistronic GUS-LUC constructs with either the VTE or a synthetic sequence in the intercistronic region, pGUS-VTE-LUC and pGUS-SYN-LUC, respectively, were used in an attempt to demonstrate internal ribosome binding and subsequent initiation of translation in association with the VTE sequence. Dicistronic constructs have been widely used to demonstrate internal initiation with the animal picornaviruses (Pelletier and Sonenberg, 1988) and theoretically the second cistron should only be translated, in a prokaryotic system, if it possesses a functional Shine-Dalgarno sequence for ribosome recognition. Factors such as leaky ribosome scanning or reinitiation result in a low level of expression of the second cistron in the absence of such a sequence. In experiments, expression of the downstream ORF from pGUS-VTE-LUC was at least 30 times higher than the level of expression from pGUS-SYN-LUC. The presence of the VTE leader in the intercistronic region therefore resulted in at least a 30 times increase in the level of luciferase expression when compared to that observed when a SYN leader is present. These results suggest that the canonical Shine-Dalgarno sequences upstream from the coat protein ORFs in HelVS and PVS are functionally

(Ruf and Kossel, 1988).
Fig 3.7 Potential secondary structure configuration of the upstream regions from the ATG start codons of the PVS 25 kDa gene of the TGB and the coat protein gene. ATG initiation codons are underlined. Potential binding sites for prokaryotic ribosome binding are indicated either in bold type, or are underlined by a double hatched line.
Fig 3.7

GTGTATGAGGAGGTTTGAT TAGTAGTATTGAATATATATG

---

GTTGAAAC AAAAGCTCGAAATATACAGTCTCACAGCAAGAATG
active in a prokaryotic environment. Why prokaryotic or chloroplastic ribosome recognition sites have been conserved in an organism that exists in a eukaryotic environment will be discussed later in detail.

The predicted secondary structure configuration of the 5' leaders of prokaryotic and chloroplastic mRNAs place the Shine-Dalgarno sequence on the exposed loop of a “hairpin” structure, the hairpin being maintained by the RNA-RNA associations around this region. On the basis of the interaction of ribosomes with this structure, two groups of hairpin types have been identified. In the first group, the 3' end of the 16S rRNA can base pair to the 5' shoulder of the hairpin or at an additional site present on the non-hydrogen bonded loop, for example the hairpin upstream from the \textit{E. coli} galE gene (Musso \textit{et al}., 1974). The second group consists of a single Shine-Dalgarno sequence situated in the non-hydrogen bonded loop of the hairpin, for example with the bacteriophage Qφ A2 gene (Steitz, 1975). The effect of secondary structure on prokaryotic translational initiation has been studied widely and generally an increase in secondary structure decreases the rate of translational initiation (Kozak, 1991a), but with the r11B messenger of the T4 phage the strengthening of the hairpin loop stem structure surrounding the Shine-Dalgarno site appears to have no detrimental effect on translation (Shinedling \textit{et al}., 1987). This has led to the development of the “trough” theory whereby the Shine-Dalgarno sequence exposed at the loop associates with the 30S ribosomal subunit in a cleft like structure. In line with this theory it is plausible to suggest that many cryptic AUG codons may be prevented from operating because they are buried in double stranded regions (Steitz and Jakes, 1975). An experiment by Platt \textit{et al}., (1976), that lends support to this proposal is that a mild denaturing of bacteriophage f2 RNA with formaldehyde greatly increases the rate of chain initiation of two of the three normally recognised f2 proteins and in addition to this ribosomes also recognise and initiate translation at three additional usually silent initiation codons.

With regard to the experiments described in this chapter, it has been observed that the RBS sequences upstream from the 25 kDa protein gene of the TGB (RBS1) and upstream from the coat protein gene (RBS3) in PVS form stable hairpin structures with their canonical Shine-Dalgarno sequences exposed on a non-hydrogen bonded loop at the 5' shoulder of the hairpin and on the non-hydrogen bonded loop of the hairpin, respectively, Fig 3.8. These conform well to the hairpin types identified for prokaryotic and chloroplastic mRNAs. The Shine-Dalgarno like sequence upstream from the PVS 25 kDa gene is 44 nucleotides from the initiating methionine and can be folded into a secondary structure conformation with a 4 base pair stem and a 9 nucleotide loop. The sequence resides on a non-hydrogen bonded loop on the 5' shoulder of the hairpin. The similar RBS site upstream from the coat protein ORF is located 43 nucleotides from the initiation codon and is displayed on the non-hydrogen bonded 10 nucleotide loop of a hairpin structure with a 4 base pair stem. These structures are located quite a distance upstream from the initiation codon but as stated earlier in natural prokaryotic mRNA leaders this distance appears to be quite flexible so it is not unreasonable to propose that these structures could be operating as ribosome recognition signals in PVS. This could be a general carlavirus characteristic. The potential structural conformations which may be formed with PVS are provided in Fig 3.7.
At present it is unclear why a sequence with homology to sequences present within the region 5' to the initiation codon in prokaryotic and chloroplastic mRNAs should be present upstream from the AUG of a viral RNA from a eukaryotic host. *In vitro* translation of pHEL.19 and pHEL.23 in rabbit reticulocyte lysate and wheat germ extract has indicated that this mechanism, whatever it may be, of coat protein production, despite the presence of upstream stop codons or open reading frames, is functional both in a prokaryotic and eukaryotic environment. Results with the PVS clones also strongly support this theory. A *cis*-acting sequence or structural feature of the leader may provide the recognition signal necessary for ribosome binding and subsequent initiation of translation. Therefore the PVS and HeLV coat protein sequences, and perhaps similar regions from all carlaviruses, appear to be functionally equivalent to a prokaryotic Shine-Dalgarno sequence for prokaryotic ribosome binding, a phenomenon that has been proposed for the TMV 5' untranslaced leader sequence (Gallie and Kado, 1989).

One proposal which may explain this phenomenon is that these subgenomics may have the potential to be translated on prokaryotic like chloroplastic ribosomes as opposed to cellular eukaryotic ribosomes in the plant (Foster and Mills, 1991b). Evidence supporting this theory is by no means conclusive but homology has been identified between sequences upstream from the initiation codons of carlavirus coat protein ORFs and 25 kDa protein genes with prokaryotic Shine-Dalgarno sites. The presence of viral particles in chloroplasts has been demonstrated for TYMV (Hatta and Matthews, 1976), TMV (Reinero and Beachy, 1986), and barley stripe virus (Carroll, 1970), and a symptom of viral infection very often is an almost immediate shut down of host photosynthetic machinery (Hinari and Wildman, 1969). Camerino et al., (1982), have actually demonstrated the expression of heterologous mRNAs, such as TMV RNA, in an *in vitro* translation system derived from spinach chloroplasts. It has been previously been stated in Chapter 1 that chloroplastic ribosomes are able to withstand greater distances between their recognition signals and initiating AUG than prokaryotic ribosomes. This would be an important consideration if PVS subgenomics are being translated on chloroplastic ribosomes because the sequence with homology to a Shine-Dalgarno site is 43 nucleotides upstream from the initiating methionine of the PVS coat protein ORF (Foster and Mills, 1991b).

Results obtained with HeLV, and preliminary results with PVS, suggest also that internal ribosome entry and initiation of translation is occurring in a eukaryotic *in vitro* environment, despite the absence of the eukaryotic 5'-CCTCC-3' ribosome recognition sequence. The exact nature of the sequences or structures that may be responsible for either prokaryotic or eukaryotic internal ribosome entry in carlaviruses are unknown. Speculation has only been made regarding the existence of sequence and conformational homology with prokaryotic ribosome recognition sequences. It is possible that this phenomenon in prokaryotes and eukaryotes occurs as a result of recognition of different *cis*-acting sequences. Although it is unlikely that the same sequences are recognised in both systems it may be that it is the actual hairpin secondary structure in the region surrounding the carlavirus canonical Shine-Dalgarno sequence is responsible for ribosome recognition, prokaryotic and eukaryotic, and
subsequent initiation of translation, rather than the specific sequence. This ribosome secondary structure recognition has been widely reported among the animal picornaviruses that contain stem loop hairpin IRES (Internal Ribosome Entry Sites) structures within their long UTRs which direct internal ribosome association and initiation of translation in a cap-independent manner (Pelletier and Sonenberg, 1988; Jang et al., 1988; 1989; Borman and Jackson, 1992). The positioning of the carlavirus RBS sequences, just upstream from the AUGs of the 25 kDa ORF and coat protein gene, indicates that carlavirus subgenomics could be expressed in a similar cap-independent manner. In addition, these gene products could be expressed as a result of internal ribosome entry on the full length genomic molecule. Constraints on prokaryotic and eukaryotic translation may not therefore be as defined as has been previously proposed.
Chapter 4

In vitro analysis of a translational enhancer upstream from the coat protein open reading frame of potato virus S

4.1 Introduction

Potato virus S (PVS) is a member of the carlavirus group of plant viruses and as such it exhibits a characteristic carlavirus genomic organisation and translational strategy. The regulation of protein synthesis at the translational level is the only real control strategy available to positive-sense, single-stranded RNA viruses to attain differential or temporal gene expression. One example of this regulation is the expression of the PVS coat protein gene, which is required in large quantities to meet the demands of viral encapsidation but is expressed from a subgenomic RNA molecule that is present at almost indetectable amounts (Foster and Mills, 1990a; 1990b; 1991a; 1991c; 1992b; Mackenzie et al., 1989). This is discussed in more detail in Chapter 3.

One possible explanation for the above stated phenomenon is that the PVS coat protein subgenomic RNA molecule, and carlavirus coat protein subgenomics in general, may be very efficiently translated. In light of what is known about factors that influence the expression of a transcript molecule in a eukaryotic expression system a possible candidate influencing the efficiency of translation is the 5' non-coding leader sequence of the subgenomic RNA molecule.

Another factor which suggests that this leader sequence could possibly have some involvement in translational regulation is the identification of a sequence homologous to a Shine-Dalgarno site for prokaryotic ribosome binding in the immediate upstream sequence from the coat protein ORF of all carlaviruses (Foster and Mills, 1991b). With PVS this sequence is contained between the termination codon of the 7 kDa cistron and the ATG of the coat protein gene which itself is contained within a larger 42 kDa ORF which includes the coat protein cistron and extends upstream to include the sequence to the 5' side of this ORF (Foster and Mills, 1992a). Whether this 42 kDa cistron is translated is not known. With other carlaviruses for which the sequence in this region is known the sequence between the 7 kDa and coat protein ORFs is non-coding (Foster and Mills, 1990c; Rupasov et al., 1989). The sequence with homology to a Shine-Dalgarno site, -AGGTCAC-, contained within the PVS coat protein upstream region, is conserved throughout the viral group which suggests that it may have some translation function in the plant eukaryotic environment. The existence of these conserved sequences have been discussed in detail in Chapter 3 and their positions in
the PVS genome are indicated in Fig 3.1.

This chapter will present evidence that the 101 nucleotides upstream from the PVS coat protein ORF, when provided as a mRNA leader, enhances the translation of downstream reporter genes over a basal level of translation conferred by a random synthetic leader in vitro. Evidence will also be presented which suggests that the enhancement properties associated with this region are mediated through cis-acting sequences within the 101 nucleotide region and a possible association with trans-acting factors. This sequence has been designated VTE or viral translational enhancer due to the effect of the presence of this leader has on the levels of translation of a downstream ORF.

4.2 Investigation of translational enhancement properties associated with the VTE

4.2.1 Constructs

The source of the region upstream from the coat protein ORF in PVS was a clone designated p7CP (Foster and Mills, 1992a). This clone was shown to contain the 3' terminal 11 kDa protein gene common to all carlaviruses and the upstream coat protein ORF. It also contains 101 nucleotides of viral sequence upstream from the initiating ATG of the coat protein cistron which consists of all the sequence between the termination codon of the 7 kDa ORF of the carlavirus triple gene block and the initiating methionine of the coat protein gene. This region contains the block of nucleotides with sequence homology to a Shine-Dalgarno site for prokaryotic ribosome binding that is conserved throughout the carlavirus group (Foster and Mills, 1991b).

Initially this 101 nucleotide sequence was isolated by polymerase chain reaction amplification (PCR) using the primers, 5'-GCTCTAGAGCTCACAAGAGAT-3' for the 5' primer, and 5'-CGGATCCATGGTCTCTGTGGGACAGT-3' for the 3' primer, the latter of which covers the ATG start codon of the coat protein ORF. Restriction enzyme sites were incorporated into primer sequences to facilitate cloning with the 5' primer including an XbaI site and the 3' primer including a BamHI site. The viral sequence in and around the ATG coat protein start codon was also changed to incorporate an Ncol site which represents a change in viral sequence from AA to CC at positions 1 and 2 downstream from this initiation codon.

After PCR amplification using these primers the product was digested with XbaI and BamHI and was cloned into XbaI/BamHI digested pBluescript-SK(+) (Stratagene, Section 2.16). Single-stranded DNA rescued via helper phage, from clones containing the appropriately sized inserts, was sequenced using the deoxynucleotide chain termination method of Sanger et al., (1977), to ensure that there were no PCR generated mutations in the sequence. The resulting plasmid, containing the 101 nucleotides upstream from the PVS coat protein gene, was designated pVTE, where VTE represents viral translational enhancer.
Fig 4.1 A. Diagrammatic representation of the constructs used to generate RNA transcripts for *in vitro* translation analysis. An arrow indicates the position of the RNA polymerase promoter (T3) within the plasmids. SYN - Synthetic leader; VTE - viral translational enhancer, PVS 101 nucleotide coat protein upstream sequence; APG - plant mRNA leader; GUS - β-glucuronidase; NOS - nopaline synthetase terminator with the GUS constructs; 35S - the cauliflower mosaic virus 35S terminator with the luciferase constructs.

Fig 4.1 B. Nucleotide sequences of the leaders generated from the constructs shown in A. Viral sequences are shown in bold type and synthetic sequences which are derived from plasmids are indicated in italics. Positions of the initiation codons (ATG) are underlined.
A

► VTE   GUS   NOS
► SYN   GUS   NOS
► VTE   LUCIFERASE   35S
► SYN   LUCIFERASE   35S
► APG   LUCIFERASE   35S

B

VTE-GUS:
GGGAACAAAAGCTGGAGCTCCACCACCGGCCGCTCTAGCTGCTGCTCAGGTAAGAGTTCAGGAAGGACAGGCTGGAGCCATG

SYN-GUS:
GGGAACAAAAGCTGGAGCTCCACCGGCCGCTCTAGCTGCTGCTCAGGTAAGAGTTCAGGAAGGACAGGCTGGAGCCATG

VTE-LUC:
GGGAACAAAAGCTGGAGCTCCACCGGCCGCTCTAGCTGCTGCTCAGGTAAGAGTTCAGGAAGGACAGGCTGGAGCCATG

SYN-LUC:
GGGAACAAAAGCTGGAGCTCCACCGGCCGCTCTAGCTGCTGCTCAGGTAAGAGTTCAGGAAGGACAGGCTGGAGCCATG

APG-LUC:
GGGAACAAAAGCTGGAGCTCCACCGGCCGCTCTAGCTGCTGCTCAGGTAAGAGTTCAGGAAGGACAGGCTGGAGCCATG
In order to monitor the translational regulatory properties associated with the VTE, reporter genes were inserted downstream from the 101 nucleotide cloned sequence. The 13-glucuronidase (GUS) gene was isolated as a BamHI/EcoRI fragment from pBI101.2 and was cloned into pVTE, digested with the same enzymes. This GUS fragment contained the nopaline synthetase (Nos) transcriptional terminator at the 3' end of the coding sequence. Transcripts derived from this construct will have the VTE sequence as a 5' untranslated leader. A diagrammatic representation of the construct and the leader sequence of mRNA transcripts generated from the resultant plasmid, pVTE-GUS, are provided in Fig 4.1.

This BamHI/EcoRI GUS fragment was also cloned into pBluescript-SK(+) vector digested with the same enzymes to give a control construct which contained the reporter gene but not the authentic viral leader sequence. This plasmid was designated pSYN-GUS where SYN represents the synthetic pBluescript polylinker region between the transcriptional start site and the BamHI restriction enzyme site. This sequence is 74 nucleotides in length which is approximately comparable to the length of test leaders used in experiments. It is this synthetic sequence that acts as a mRNA leader in transcripts generated from this construct and the actual sequence of this leader and a diagrammatic representation of the clone is provided in Fig 4.1.

Constructs containing the VTE and SYN leader sequences were also generated which contained the luciferase (LUC) reporter gene instead of GUS. This provided an additional extremely sensitive reporter system that could be used to analyse the properties of the VTE. The plasmid, designated pVTE-LUC, was constructed following the insertion of an NcoI/HindIII digested LUC fragment into NcoI/HindIII digested pVTE-GUS. This LUC restriction fragment was isolated from a plasmid designated pRTL2-LUC (Section 5.2) and it contains the cauliflower mosaic virus 35S transcriptional terminator at the 3' end of the coding sequence. This cloning event effectively replaced GUS with LUC taking advantage of the unique NcoI site engineered into the region surrounding the LUC start codon (Fig 4.1).

Two synthetic leader-LUC plasmids were generated, the first was constructed by insertion of NcoI/HindIII digested LUC into pSL301 (Invitrogen, Section 2.16) digested with the same enzymes. pSL301 contains the large SL superlinker sequence which contains the rare NcoI enzyme site. The mRNA leader sequence in transcripts generated from this plasmid was therefore the sequence between the transcriptional start site in the plasmid and the NcoI site of the polylinker. A diagrammatic representation of the construct, designated pSYN-LUC, and the actual sequence of the leader in transcripts generated from this construct are provided in Fig 4.1.

A second synthetic-LUC plasmid was also constructed whereby a NcoI/HindIII LUC fragment from a plasmid designated pRTS2-LUC (Section 5.2) was cloned into NcoI/HindIII digested pBluescript-SK(+) vector. This LUC restriction fragment contained an additional portion of synthetic sequence, namely the sequence between the Ncol and NcoI sites of the pGEM-5Zf (Promega, Section 2.16) plasmid polylinker. A diagrammatic representation of this
construct, designated pSYN-LUCb, and the sequence of the leader in transcripts generated from this construct are provided in Fig 4.5.

With preliminary experiments analysing the activity of the VTE (Sections 4.2.2, 4.3, 4.4 and 4.5) pSYN-LUC has been used. pSYN-LUCb has been used in experiments which analysed the effect of deletion of the VTE sequence (Section 4.6.2).

A randomly selected authentic plant leader was used from a selection available within our laboratory. This sequence, being an authentic plant sequence, was used to ensure that the synthetic leaders in pSYN-LUC and pSYN-LUCb were not having an inhibitory effect on translation. This plant leader corresponded to 74 nucleotides upstream from the ATG of the APG gene which was originally isolated from Arabidopsis thaliana (Roberts et al., 1993). A plasmid designated pAPG-LUC was provided by Neil Bate (University of Leicester). The leader had originally been isolated by PCR amplification using the primers 5'-GTCGACCAAAGCAGATATTGAC-3' as the 5' primer and 5'-CAACCATGGTTTTACAACTTTACG-3' as the 3' primer. The PCR generated fragment was digested using SaI and NcoI, restriction enzyme sites engineered into primer sequences, and was ligated to Ncol/HindIII generated LUC from pSYN-LUC. The resulting ligation product was ligated into pBluescript-KS(+) which generated the construct, pAPG-LUC. Again a diagrammatic representation of this construct and the sequence of the untranslated leader in mRNA transcripts generated from this construct are provided in Fig 4.1.

According to Kozak's scanning model for translation (Kozak, 1978) the context of the initiating AUG codon in a mRNA molecule is important for translational competence. An AUG codon with a poor surrounding context will be recognised inefficiently by ribosomes resulting in a low level of translation of the associated downstream cistron. All the plasmids constructed for this analysis have initiation codons in the optimal -ACCATGG- context so this factor should not be variable in the analysis.

Constructs were therefore available which would enable the generation of transcript molecules for an in vitro characterisation of translational properties associated with the 101 nucleotides upstream from the PVS coat protein gene, or VTE. This could be easily monitored using the GUS and LUC reporter genes with the translational properties associated with the VTE being compared to the level of expression of these two reporter genes in its absence.

4.2.2 The presence of the VTE enhances translation in vitro

The insert orientation of all clones detailed in Section 4.2.1 was such that the T3 RNA polymerase promoter was used for the generation of sense RNA in vitro transcripts. These transcripts contained the entire sequence from the transcriptional start site of the vector to the terminus of the cloned insert at the 3' restriction enzyme site at which the plasmid DNA had been linearised. RNA in vitro transcripts were generated for plasmids, pVTE-GUS, pSYN-GUS, pVTE-LUC, pSYN-LUC and pAPG-LUC. The amount of RNA generated for the
### Table 4.1

#### Relative Percentage Incorporation of Radioactivity

<table>
<thead>
<tr>
<th>WheatGerm Extract</th>
<th>Fold Enhancement</th>
<th>Rabbit Reticulocyte Lysate</th>
<th>Fold Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construct</td>
<td>Construct</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSYN-GUS</td>
<td>1</td>
<td>pSYN-GUS</td>
<td>1</td>
</tr>
<tr>
<td>pVTE-GUS</td>
<td>2.9</td>
<td>pVTE-GUS</td>
<td>2.8</td>
</tr>
<tr>
<td>pSYN-LUC</td>
<td>1</td>
<td>pSYN-LUC</td>
<td>1</td>
</tr>
<tr>
<td>pVTE-LUC</td>
<td>1.7</td>
<td>pVTE-LUC</td>
<td>2.1</td>
</tr>
</tbody>
</table>

#### Relative Luciferase Activity

<table>
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<th>Rabbit Reticulocyte Lysate</th>
<th>Fold Enhancement</th>
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<tbody>
<tr>
<td>Construct</td>
<td>Construct</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSYN-LUC</td>
<td>1</td>
<td>pSYN-LUC</td>
<td>1</td>
</tr>
<tr>
<td>pAPG-LUC</td>
<td>0.85</td>
<td>pAPG-LUC</td>
<td>0.45</td>
</tr>
<tr>
<td>pVTE-LUC</td>
<td>2.37</td>
<td>pVTE-LUC</td>
<td>4.9</td>
</tr>
</tbody>
</table>

#### Relative % Incorporation

<table>
<thead>
<tr>
<th>WheatGerm Extract</th>
<th>Fold Enhancement</th>
<th>Rabbit Reticulocyte Lysate</th>
<th>Fold Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construct</td>
<td>Construct</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capped pSYN-LUC</td>
<td>-</td>
<td>Capped pSYN-LUC</td>
<td>1</td>
</tr>
<tr>
<td>Capped pVTE-LUC</td>
<td>-</td>
<td>Capped pVTE-LUC</td>
<td>2.08</td>
</tr>
</tbody>
</table>
Fig 4.2 A. In vitro translation analysis of GUS linked to synthetic (SYN) or viral (VTE) leaders. Lane 1 SYN-GUS RNA, wheat germ extract; lane 2 VTE-GUS RNA, wheat germ extract. Position of the GUS product is indicated by an arrow. Bands of lower molecular weight are due to premature termination. B. In vitro translation analysis of luciferase linked to synthetic (SYN) or viral (VTE) leaders. Lane 1 SYN-LUC RNA, rabbit reticulocyte lysate; lane 2 VTE-LUC, rabbit reticulocyte lysate; lane 3 SYN-LUC, wheat germ extract; lane 4 VTE-LUC RNA, wheat germ extract. The position of the luciferase band is indicated by an arrow. Bands of lower molecular weight are due to premature terminations. Equal quantities of RNA were translated in vitro, after 1 hour incubations, equal volumes were removed from the translation mixes and were analysed by gel electrophoresis and autoradiography.
Fig 4.2
pVTE-GUS and pSYN-GUS, and for the pVTE-LUC, pSYN-LUC and pAPG-LUC was equalised visually on an ethidium bromide stained agarose gel before being translated either in rabbit reticulocyte lysate or in wheat germ extract. Both capped and uncapped transcripts were generated and used in translation reactions. In vitro translation experiments were performed in triplicate and results were represented as the value of the mean enhancement conferred by the presence of the VTE sequence above the level conferred by the SYN or APG leaders.

When uncapped RNA transcripts of pVTE-GUS and pSYN-GUS were translated in vitro in rabbit reticulocyte lysate and wheat germ extract and the levels of translation compared, the presence of the VTE conferred higher levels of reporter gene translation than levels conferred by the synthetic leader. Translation was expressed as the percentage of $^{35}$S-methionine incorporated into protein (that is the percentage incorporated into the TCA-insoluble fraction). VTE-GUS transcript expressed the GUS ORF at mean value of 2.8 times the level that was expressed from SYN-GUS transcript in rabbit reticulocyte lysate. The enhancement conferred by the VTE was 1.9 fold higher than the level conferred by the SYN leader in wheat germ extract. These results are presented in Table 1 and are visually represented in the autoradiograph produced after SDS-polyacrylamide gel electrophoresis of equal volumes of in vitro translation reactions containing VTE-GUS and SYN-GUS transcript (Fig 4.2a).

Similar experiments were carried out with the LUC constructs and a similar effect was evident. Assaying for the stimulation of $^{35}$S-methionine incorporation into the TCA-insoluble fraction of the translation reactions containing transcripts generated from constructs pVTE-LUC and pSYN-LUC in vitro, revealed that the presence of the VTE enhanced the level of translation in rabbit reticulocyte lysate by a factor of 2.1 fold and in wheat germ extract by a factor of 1.7 fold. Results are presented in Table 1 and are visually represented in Fig 4.2b where equal volumes of translation reactions containing VTE-LUC and SYN-LUC mRNA were analysed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Luciferase, being a very sensitive reporter, offered the added advantage of being able to quantify the levels of biologically active peptide produced from added transcript within the in vitro translation system. This allowed the contribution played by premature termination of translation to be ignored, a factor that is not taken into account when the level of translation is expressed as the percentage incorporation of radioactivity into protein. When direct luciferase assays were performed on equal volumes of in vitro translation reaction the translational enhancement conferred by the presence of the VTE was evident. In rabbit reticulocyte lysate, translation of the VTE-LUC transcript resulted in 4.9 times the level of expression of biologically active luciferase than the level produced from the transcript containing the synthetic leader. This stimulation was also evident in wheat germ extract with a 2.37 fold increase in luciferase production being conferred by the presence of the VTE leader.

The level of translation from luciferase transcripts containing the VTE as an untranslated
Table 4.2

<table>
<thead>
<tr>
<th>Construct</th>
<th>Time after initiation of translation reaction (mins)</th>
<th>% Incorporation</th>
<th>Fold enhancement of VTE over SYN leader</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVTE-LUC</td>
<td>20</td>
<td>6.78%</td>
<td>x 2.25</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>8.25%</td>
<td>x 2.56</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>10.29%</td>
<td>x 1.98</td>
</tr>
<tr>
<td>pSYN-LUC</td>
<td>20</td>
<td>3.01%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.2%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.2%</td>
<td>-</td>
</tr>
</tbody>
</table>
leader was also compared to the level of translation from transcripts containing the APG as a leader. Enhancements of 11.6 fold in rabbit reticulocyte lysate and 2.8 fold in wheat germ extract were conferred by the presence of the viral leader. These approximately correspond with the fold enhancement values when reporter gene expression from transcripts containing the VTE were compared with the level of reporter expression from transcripts containing the synthetic leader. These results are presented in Table 1.

Capped transcripts gave the same levels of enhancement as those discussed above for uncapped transcripts. Translation of capped VTE-LUC mRNA in rabbit reticulocyte lysate resulted in the expression of the luciferase reporter at a level of 2.08 times that expressed from the capped SYN-LUC transcript. Again these results are presented in Table 1.

4.3 Enhancement is not due to mRNA stability

A possible explanation for the translational enhancement conferred by the presence of the VTE is that mRNA molecules containing this sequence at the 5' end are more stable and less susceptible to exonuclease degradation. To confirm that it was not stability, but alternately an intrinsic property of the VTE sequence, that was responsible for the enhancement, a time-course translation assay was carried out. Equal amounts of uncapped VTE-LUC and SYN-LUC transcript were translated in rabbit reticulocyte lysate. At time points during the hour incubation period, samples of both reactions were removed and the percentage of radioactivity incorporated into protein was determined (35S-methionine incorporation into the TCA-insoluble fraction). At all times during the reaction the enhancement ratio remained approximately the same, that is a 2-2.5 stimulation in translation was observed when the VTE leader was present throughout the in vitro translation. This indicates that the enhancement observed was not due to the more rapid degradation of the SYN-LUC transcript during the course of the reaction. Results are presented in Table 2.

4.4 The mode of translation associated with the VTE is at least partially cap-independent

From the literature it has become evident that the mode of translation associated with many translational enhancers, both from plants and animals, displays some degree of independence from the normally accepted scanning model for translation proposed by Kozak, (1978). The 7-methyl guanidine acid "cap" structure present at the 5' terminus of the majority of eukaryotic mRNA molecules is thought to be vital for the process of mRNA recognition and subsequent initiation of translation. Many viruses, including the animal picornaviruses and the plant potyviruses, do not possess such a structure so it has been proposed that these viruses employ an alternative translation mechanism that does not depend upon this cap recognition, a cap-independent translational initiation mechanism. Evidence has also suggested that carlavirus subgenomics are naturally uncapped which points towards the
Levels of translation of capped transcripts generated from pSYN-LUC and pVTE-LUC in the presence of cap analogue. Translation reactions with capped transcripts from pSYN-LUC and pVTE-LUC were carried out for 1 hour in the presence of 0, 0.24 mM, 0.36 mM and 0.48 mM cap analogue (m\(^7\)G\(^5\)pppG) in rabbit reticulocyte lysate (RRL). Levels of translation were calculated from radioactive incorporation assays. Results are expressed as a percentage of the level of translation of the transcripts in the absence of cap analogue which was taken as 100%.
Fig 4.3

![Graph showing relative percentage translation in RRL at different mM concentrations of cap analogue. The x-axis represents mM concentration of cap analogue: 0.24, 0.36, and 0.48. The y-axis represents relative percentage translation in RRL. Open bars represent SYN-LUC mRNA, and closed bars represent VTE-LUC mRNA.]
possible use of this similar alternative translation mechanism (Foster and Mills, 1990a).

It has been determined that the process of cap-dependent translation is extremely sensitive to the addition of cap analogue to an in vitro translation system. Cap-independent translation appears to be less affected by this addition, rather, transcripts translated in this manner appear to be translated even more efficiently under conditions where cap-dependent translation is limiting in vivo.

Capped transcripts from both pVTE-LUC and pSYN-LUC were generated and RNA was equalised visually on an ethidium bromide stained agarose gel. Transcripts were translated in rabbit reticulocyte lysate in the presence of increasing concentrations of cap analogue and the effect of these additions on translation was monitored. The efficiency of translation was determined as the percentage $^{35}$S-methionine incorporation into protein (TCA-soluble fraction) for each system and the effect of cap analogue was expressed as the percentage decrease in the level of translation under the inhibitory conditions produced as compared to the level without the addition of the inhibitor. Results are presented in Fig 4.3.

Cap analogue had a dramatic effect on translation of the capped SYN-LUC transcript with percentage decreases in the level of translation of 46.5% and 80.2% at cap analogue concentrations of 0.24 mM and 0.35 mM, respectively, when compared to translation levels in the absence of cap analogue. The effect of the same concentrations of cap analogue on translation of the capped VTE-LUC transcript was not so pronounced with reductions of only 5.4% at the 0.24 mM cap concentration and 51.8% at the 0.35 mM concentration, respectively. Luciferase production from both transcripts, that is the transcript containing the VTE leader and the transcript containing the SYN leader, was severely inhibited under high concentrations conditions of cap analogue.

The response of some other leader sequences, in addition to the VTE and SYN leaders, to the inhibitory translation conditions produced by the addition of cap was also analysed. Three additional sequences were used. The first was the APG leader, the authentic plant leader sequence which has been shown to promote a similar level of translation as that conferred by the SYN leader (Section 4.2.2). The second was the TEV leader which is the 5' untranslated leader from the tobacco etch virus (TEV) genomic RNA molecule. It has been previously demonstrated that this sequence appears to promote an enhanced level of translation both in vitro and in vivo in a manner that appears to be at least partially cap-independent (Carrington et al., 1990). Instead of a cap structure, the TEV genomic RNA molecule possesses a VPg (virus encoded protein) at the 5' end, a fact which reinforces the proposal of the operation of this alternate translational initiation mechanism. The final sequence is the LAT52 leader which is the 5' untranslated leader of the pollen specific LAT52 (Late Anther Tomato) gene of tomato (Twell et al., 1989).

The level of translation associated with transcripts containing the APG leader was inhibited to nearly the same degree as that demonstrated earlier with transcripts containing the SYN leader. This inhibition was as much as 28.3% at the lowest cap analogue concentration used (0.24 mM), a concentration where transcripts containing the VTE leader were only inhibited
by 5.4%. Transcripts containing the TEV and the LAT52 leaders appeared to be relatively insensitive to the addition of cap analogue. The lowest cap concentration (0.24 mM) caused an almost negligible decrease in translation with no decrease at all with transcripts containing the TEV leader and a decrease of 7.9% with transcripts containing the LAT52 leader. These results agree with those previously published by Carrington et al., (1990) for TEV. At the higher 0.35 mM cap analogue concentration translation of transcripts containing the APG leader was inhibited by 70.2%, translation of transcripts with the TEV leader was inhibited by 66% and translation of transcripts containing the LAT52 leader were inhibited by 49.8%. At very high cap concentrations translation of all transcripts was severely inhibited. In conclusion, the translation of transcripts containing the SYN and APG leaders is very sensitive to cap analogue but the translation of transcripts containing the VTE, TEV and LAT52 leaders is not inhibited by cap to the same degree.

4.5 Enhancement properties associated with the VTE may be mediated by a trans-acting factor

The results from the cap-inhibition experiment indicated that the mode of translational initiation associated with the VTE leader may be at least partially cap-independent. An integral component of this cap-independent translational initiation mechanism in picornaviruses involves the association of as yet largely unknown translational factors or ribosomes with cis-acting sequences. Similar factors or ribosomes could be associating with a sequence within the VTE to promote translational enhancement. It was this phenomenon that was investigated in the following experiment.

Small transcripts, of either the VTE or a synthetic leader sequences, were generated from the plasmids, pHVE (Section 4.2.1) and pBluescript SK (Section 2.16), respectively. These were added individually to rabbit reticulocyte lysate in vitro translation reactions containing VTE-LUC mRNA transcript. If factors or ribosomes are in fact binding to the VTE leader sequence in a cap-independent manner the addition of excess uncapped VTE leader may result in competition for these binding factors, limiting the amount available to partake in VTE-LUC mRNA expression. We could therefore predict that the addition of excess VTE leader would result in a decrease in the level of luciferase translation. In this model the addition of excess uncapped synthetic leader should not have a deleterious effect as it presumably would not contain the specific cis-sequence responsible for association of the cap-independent translation factor.

A transcript corresponding to the 101 nucleotides of VTE sequence was generated in vitro using the pHVE plasmid as template linearised with NcoI. A control synthetic transcript was also generated using KpnI digested pBluescript-SK as template. The DNA templates for both of these in vitro generated leader transcripts were removed using RNase free DNase and the concentrations were equalised both visually on an ethidium bromide stained agarose gel and spectrophotometrically. Increasing concentrations of leader transcripts were added to a rabbit reticulocyte lysate translation system containing a final concentration 0.5 µg of uncapped VTE-LUC mRNA.
**Fig 4.4** Trans-inhibitory effect of the VTE and SYN leaders on the levels of translation of VTE-LUC transcripts *in vitro*. Translation reactions were carried out using uncapped VTE-LUC transcripts in the presence of either the VTE or SYN leader. Leaders were added at concentrations of 0.15 μg, 0.25 μg, 0.5 μg and 2.25 μg. Reactions were carried out for 1 hour in rabbit reticulocyte lysate.
Fig 4.4

Leader concentration (ug)

Percentage translation in RRL

- □ VTEleader
- ● SYNleader
The addition of both leaders resulted in a decrease in luciferase translation from the VTE-LUC transcript but the decrease on addition of the uncapped VTE leader transcript was more pronounced than with the addition of the uncapped synthetic leader mRNA. At a synthetic leader concentration of 2.5 µg a 46% decrease in translation occurred but at this same concentration the VTE leader inhibited translation dramatically with a 75% decrease. Results are presented in Fig 4.4.

4.6 Deletion analysis of the VTE

In an attempt to isolate the actual region of the VTE responsible for translational enhancement a series of constructs were generated which contained deletions of the 101 nucleotide viral sequence. The effects of these deletions on the level translation was determined.

4.5.1 Constructs

The VTE was deleted from 101 nucleotides in length to only 20 nucleotides by PCR amplification using p7CP (Section 4.2.1) as template and the primers, 5'-GAAACTGTCCCACAGACCATGTC-3' to the viral sequence, and a primer within the pBluescript polylinker. This generated a PCR fragment representing the entire coat protein gene and down stream 11 kDa ORF but this fragment only contained a portion of the VTE sequence, only the 20 nucleotides of viral sequence immediately upstream from the ATG initiation codon of the coat protein cistron. This PCR product was first cloned into pCR11 (Section 2.16, Invitrogen) and was then subcloned as a KpnI/Ncol fragment into pBluscript-SK + digested with the same enzymes. The sequence of the PCR generated clone was confirmed by single-stranded DNA sequencing using the dideoxynucleotide termination method of Sanger et al., (1977).

From this clone, designated pSL-7CP, the coat protein ORF was replaced by the luciferase reporter gene. The luciferase fragment was isolated from pSYN-LUC (Section 4.2.1) as an Ncol/HindIII fragment and was cloned into Ncol/HindIII digested pSL-7CP. This takes advantage of the Ncol site engineered into the sequence surrounding the ATG start codon. The resulting plasmid was designated pSLVTE-LUC. Diagrammatic representations of the constructs, pSL-7CP and pSLVTE-LUC, and the sequence of the leaders of transcripts generated from these plasmids are provided in Fig 4.5.

Two additional deletions were constructed VTEW and VTEM. Both of these deleted sequences contained the conserved carlaviruses block of nucleotides which has been discussed in detail in Section 3.2 and the viral sequence downstream from this sequence to the ATG of the luciferase ORF but they were deficient in the sequence upstream from this block. As indicated by the name, VTEW was derived from wild type (W) viral sequence whereas, VTEM contained a 2 base mutation (M) which results in a change in the viral sequence from CC to GG within the conserved block. Both clones were generated by PCR amplification.
Fig 4.5 A. Diagrammatic representation of the constructs used to generate RNA transcripts for *in vitro* translation analysis of VTE deletions. An arrow indicates the position of the RNA polymerase promoter (T3) within the plasmids; SYNb - Synthetic leader derived from the poly linker of the pGEMSZf plasmid; VTE - viral translational enhancer, PVS 101 nucleotide coat protein upstream sequence; SL - VTE 20 nucleotide deletion; VTEW - VTE wildtype deletion; VTEM - VTE mutated deletion; 35S - the cauliflower mosaic virus 35S terminator with the luciferase constructs; CP - PVS coat protein.

Fig 4.5 B. Nucleotide sequences of the leaders generated from the constructs shown in A. Viral sequences are shown in bold type and synthetic sequences which are derived from plasmids are indicated in italics. Positions of the initiation codons (ATG) are underlined.
A

VTE-CP

GGGAACACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTA GAGCTCAC AAGAGAGTTTGGTGAGCCAAGCGTACAGCAACATTGGGGCGTGGAAGCAGC CTTTAGGTTCACAGGTAAAGAGTTGCTGAAAGAAGACTGTGCCCACAGAGAC CATG

SYNb-LUC

GGGAACACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTATGATATCCCGCGGCCCTATG

VTEW-LUC

GGGAACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTA GAAGCACC TTTAGGTTCACAGGTAAAGAGTTGCTGAAAGAAGACTGTGCCCACAGGACCATG

VTEM-LUC

GGGAACACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTAGAAGCACC TTTACCTTCACAGGTAAAGAGTTGCTGAAAGAAGACTGTGCCCACAGGACCATG

SLVTE-CP/LUC

GGGAACACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTA GAAGCACC TTTACCTTCACAGGTAAAGAGTTGCTGAAAGAAGACTGTGCCCACAGGACCATG

B

SYNb-LUC

GGGAACACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTATGATATCCCGCGGCCCTATG

VTEW-LUC

GGGAACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTA GAAGCACC TTTAGGTTCACAGGTAAAGAGTTGCTGAAAGAAGACTGTGCCCACAGGACCATG

VTEM-LUC

GGGAACACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTAGAAGCACC TTTACCTTCACAGGTAAAGAGTTGCTGAAAGAAGACTGTGCCCACAGGACCATG

SLVTE-CP/LUC

GGGAACACAAAGCTGGAGCTCCACCACCGCGGTGCCGGCGCCGCTCTA GAAGCACC TTTACCTTCACAGGTAAAGAGTTGCTGAAAGAAGACTGTGCCCACAGGACCATG
**Fig 4.6** Nucleotide sequence of the VTE (101 nucleotides upstream from the ATG of the PVS coat protein gene). The positions of leader deletions are indicated by arrows. The GG-CC mutation in VTEM is also indicated. The conserved carlaviruses block of nucleotides with base pair complementarity to the prokaryotic 16S ribosomal RNA molecule is underlined by a dotted line. The ATG start codon of the coat protein gene is also underlined.
Fig 4.6

GAGCTCACAAGAGATTTGGTGGAAGCCGTAGCAACATTGG

VTEW AND VTEM DELETIONS  CC  MUTATION IN VTEM

GGCCGTTGAAGCACCTTTAGGTCACAGGTAAGAGCTCGA

CONSERVED CARLAVIRUS BLOCK

AGAAACTGTCCCCACAGAGACCATG

VTESL DELETION
using pVTE-LUC (Section 4.2.1) as template. Specifically designed 5' primers, 5'GAAGCACCTTTAGGTTCAC-3' for VTEW and, 5'-GAAGCACCTTTACTTCAC-3' for VTEM, were used in conjunction with a primer to the sequence in the vector at the 3' terminal end of the luciferase coding sequence, to generate amplified fragments of the VTE deletions linked to the luciferase gene. Both PCR products were digested with XbaI and EcoRI and the resulting fragments, about 500 base pairs in size, were cloned into XbaI/EcoRI digested pBluescript-SK-I. XbaI was used because this site was engineered into both 5' primers and EcoRI was used because this site was within the luciferase coding sequence. At this stage both constructs, pVTEW and pVTEM, were sequenced from the 5' end of each clone to ensure that there were no PCR generated mutations in the leader sequences (Sanger et al., 1977). NcoI/HindIII digested luciferase from pSYN-LUC was cloned into both pVTEW and pVTEM plasmids digested with the same enzymes to give constructs designated pVTEW-LUC and pVTEM-LUC respectively. This cloning ensured that a wild type luciferase gene was contained within both clones without any PCR generated mutations. Diagrammatic representations of the constructs discussed and the sequence of the leaders of transcripts generated from these plasmids are presented in Fig 4.5. The actual positions of these deletions relative to the full VTE sequence are highlighted in Fig 4.6.

4.5.2 Translational properties of VTE deletion transcripts \textit{in vitro}

RNA transcripts derived from the VTE deletion clones detailed in Fig 4.5 were translated \textit{in vitro} in rabbit reticulocyte lysate and wheat germ extract. As with the original clones described in Section 4.2.1 the orientation of cloned inserts was such that the T3 RNA polymerase promoter was used to generate \textit{in vitro} transcripts. RNA transcripts were equalised visually on an ethidium bromide stained agarose gel and luciferase assays were carried out on equal volumes of translation reaction. Results regarding the levels of translation of VTE deletions, were expressed as the percentage decrease in translation of luciferase compared to the level of translation from transcript containing the full VTE sequence which is given a value of 100%. All experiments were performed in triplicate.

The deletion which reduced the VTE leader sequence length to only the 3’ terminal 20 nucleotides upstream from the PVS coat protein gene, the SLVTE deletion, had a dramatic effect on the translation of the PVS coat protein ORF. When expression was represented as the percentage incorporation of labelled methionine into coat protein this deletion resulted in a 78.43% decrease in the level of translation when compared to the level of translation from transcripts containing the full VTE leader. These results are presented in Table 3 and are represented visually, after SDS polyacrylamide gel electrophoresis and autoradiography of equal volumes of translation reaction of VTE-CP and SLVTE-CP transcript in Fig 4.7.

The level of translation associated with the SLVTE leader deletion when linked to luciferase did not show such a dramatic decrease. The presence of the full VTE sequence in transcripts resulted in 1.4 times the level of luciferase expression in rabbit lysate than the level expressed by transcripts containing the SLVTE deletion. In wheat germ extract the level of enhancement conferred by the VTE over that conferred by the SLVTE was 2.03 fold. These values represent a 29.13% decrease in translation in rabbit lysate and a 49.2% decrease in wheat
Fig 4.7 Translation products of VTE-CP and SLVTE-CP in rabbit reticulocyte lysate. Equal quantities of RNA were translated *in vitro*. After 1 hour incubations, equal volumes were removed from the translation mixes and analysed by gel electrophoresis and autoradiography. Lane 1 VTE-CP, the translation product of the transcript containing the 101 nucleotide VTE sequence linked to the coat protein gene; lane 2 SLVTE-CP, translation product of the transcript containing 20 nucleotides of the VTE sequence linked to the coat protein gene. The position of the 34 kDa coat protein *in vitro* translation product is indicated by an arrow.
Table 4.3

<table>
<thead>
<tr>
<th>Construct</th>
<th>Mean Fold Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVTE-CP</td>
<td>1</td>
</tr>
<tr>
<td>pSLVTE-CP</td>
<td>4.53 +/- 0.39</td>
</tr>
</tbody>
</table>

% Incorporation of radioactivity in rabbit reticulocyte lysate
Fig 4.8.A Graph representing the translational efficiencies of VTE deletion mRNA molecules in rabbit reticulocyte lysate (RRL). Translation is expressed as a percentage of the level of "wild type" translation when the full length VTE leader is present. Solid bars represent the mean expression value from three repeat experiments for each construct and error bars represent the standard error from this mean. Translation associated with the SYN leader, that is SYNb in this case, is also represented in this way.

Fig 4.8.B Graph representing the translational efficiencies of VTE deletion mRNA molecules in wheat germ extract (WG). Translation is expressed as a percentage of the level of "wild type" translation when the full length VTE leader is present. Solid bars represent the mean expression value from three repeat experiments for each construct and error bars represent the standard error from this mean. Translation associated with the SYN leader, that is SYNb in this case, is also represented in this way.
germ as a consequence of this deletion. Results are represented graphically in Fig 4.8. The reason why this deletion does not inhibit translation to the same extent when linked to luciferase than when linked to the PVS coat protein is unclear. It could be that the downstream luciferase coding sequence is interacting with the VTE to mediate this effect.

Both the VTEW and VTEM deletions had a drastic effect on the level of translation with luciferase expression in most cases being not even as efficient as the level associated with the basal translation level associated with transcripts containing the synthetic leader. No statistical significance was evident in the levels of translation when both deletions were compared, so it appeared that the mutation within the conserved carlavira block did not unduly effect translation. In rabbit lysate, mRNA molecules containing the VTEW and VTEM sequences as leaders, expressed luciferase at only about a quarter of the efficiency of the level of luciferase expression from a mRNA molecules containing the full VTE as a leader (77.6% and 77.7% decreases in expression with VTEW and VTEM, respectively). Expression values of only 0.314 and 0.294 were observed when the levels of luciferase expression from transcripts containing the VTEW and VTEM leaders, respectively, were compared to the level of expression from transcripts with the full length VTE sequence. In wheat germ expression values were 0.38 for VTEW and 0.32 for VTEM, respectively, when compared to the level of translation with transcripts containing the VTE. These values represent a 62.49% decrease in wheat germ with VTEW and a 74.4% decrease with VTEM form the level of luciferase expression with the full VTE sequence. Results are represented graphically in Fig 4.8.

The inclusion of transcripts with synthetic leaders in the experiment offered a type of control, in that the level of translational enhancement conferred by the VTE over the SYN leader was already known. The presence of the VTE resulted in an enhancement of translation by 5.473 fold in rabbit reticulocyte lysate and by 2.64 fold in wheat germ extract, over the level of translation associated with the synthetic leader, a different synthetic leader in this case that was used in original assays (pSYN-LUCb). This level is very similar to the previously stated translation enhancement results of, 4.9 fold in rabbit reticulocyte lysate and, 2.37 fold in wheat germ. Results are indicated graphically in Fig 4.8.

4.7 Hybrid arrest translation studies

The effect of the addition of oligonucleotides, complementary in sequences to regions within the VTE leader, on the translational competence of transcripts derived from pVTE-LUC and pSYN-LUC in rabbit reticulocyte lysate was also analysed. Two oligonucleotides were used, the first VTEA with sequence 5'-GTCGGATCCATGGTTCGAACTCTTAC-3', and the second VTEB with sequence 5'-GACGGATCCATGGCTTCAACGGCCCC-3'. In relation to the secondary structure prediction model for the VTE sequence presented in Fig 3.8, the VTEA sequence is situated in the region downstream from the internally situated stem loop structure nearest the AUG initiation codon, VTEB is situated upstream from this structure.

In this experiment a 50 fold molar excess of oligonucleotide, either VTEA or VTEB, was
added to transcript and the mixture was incubated at 60 °C and then allowed to cool to room temperature. This results in the annealing of oligonucleotides to complementary regions of transcript. It was proposed that the binding of a complementary sequence to a region of the VTE leader could possibly have an influence on the level of translation of a downstream cistron. The binding of this oligonucleotide sequence could possibly block the association of a cis-acting sequence with a trans-acting factor, involved in the mediation of the translational enhancement phenomenon.

Results indicated that the VTEB oligonucleotide, the sequence complementary to the region of the VTE upstream from the stem-loop structure, inhibited translation of the VTE-LUC transcript in rabbit reticulocyte lysate, to a greater degree than the inhibition observed with the VTEA oligonucleotide. Three individual inhibition experiments were carried out. With the VTEB oligonucleotide inhibited translation of the VTE-LUC transcript by a mean value of 53% whereas the VTEA oligonucleotide inhibited expression by a mean value of only 15.6%. The effect of the addition of these oligonucleotides to translation reactions containing SYN-LUC transcript was negligible.

4.8 Discussion

An enhanced level of translation conferred by the presence of a 5' untranslated leader in a mRNA molecule, contradicts the normally accepted model for translational initiation proposed by Kozak, (1978). Recently a large number of plant translational enhancers have been documented (Chapter 1; Gallie, 1993; Turner and Foster, 1995) and the results presented in this chapter suggest that the VTE PVS sequence may be a candidate to join this list. The 101 nucleotides upstream from the coat protein ORF of PVS enhances the expression of downstream reporter genes at the translational level, in vitro. This enhancement was compared with the level of translation associated with two different random synthetic leaders, of comparable length and degree of secondary structure, and that associated with an authentic plant leader. Results also indicated that enhancement was not a result of mRNA stability which has often been argued.

The actual mechanism through which this enhancement is potentiated is at present still largely unclear, especially since very little is known about the whole process of cellular cap-dependent translation. Generally, it appears that, enhancers are not constrained in their expression under conditions where normal cap-dependent translation is repressed. Such situations include viral infection, high salt conditions and certain stages in cellular development such as during mitosis (Bonneau and Sonenberg, 1987). Such an inhibitory situation can be effectively copied, in vitro, by the addition of cap analogue to a translation system. A cap (m^7G^'pppG) structure at the 5' terminal end of a eukaryotic mRNA molecule is generally essential for its efficient translation. Translational initiation factors recruit ribosomes to the sequence associated with this cap structure, after which the ribosome scans the leader until it reaches the authentic AUG start codon. Exogenous free cap in the translation system will also associate with eukaryotic initiation factors (eIF) factors and
ribosomes reducing their availability for actual expression.

Results from translation experiments with capped transcript molecules containing a synthetic leader supports this theory, with expression being dramatically repressed even under conditions of quite low concentrations of cap. Translation of capped mRNA molecules containing the VTE sequence as a 5’ leader were not inhibited to the same extent under similar conditions. Foster and Mills have previously reported that when PVS viral RNA, extracted from plant tissue, was translated in the presence of cap analogue, the expression of the subgenomic encoded products was not inhibited to the same extent as the genomic encoded products (Foster and Mills, 1990a). This has lead to the suggestion that PVS subgenomics may be uncapped. This apparent cap-independency has been demonstrated with other plant viral leaders, for example the 5’ untranslated leader of the TEV genome (Carrington et al., 1990) and this suggests that plant viral translational enhancement could occur by an alternative mechanism to the generally accepted process of normal cellular translation. This process could share some similarities to the cap-independent translational initiation process identified for the animal picornaviruses (Pelletier and Sonenberg, 1988; Jackson et al., 1990).

In the animal system cellular transcripts have been identified that are efficiently translated under conditions where the expression of the majority of endogenous transcripts is repressed (Macejak et al., 1991; Oh et al., 1992). Our results also showed that a 5’ leader sequence derived from a pollen specific gene, the LAT52 gene of tomato, behaved as a translational enhancer in vitro and strongly in vivo in pollen (Twell et al., unpublished results). This leader also exhibits this insensitivity to cap analogue with regard to its translation. This observation will not be discussed further except to say that the LAT52 transcript may be translated efficiently during stages of pollen development during which cap-dependent translation may be inhibited.

Results from competition assays also point towards some form of cap-independent translation and indicate that this enhancement could be through the intermediacy of cis-acting sequences within the VTE and associating trans-acting factors of some description. The actual mechanism of cap-independent translation has largely been deduced from studies on the animal picornaviruses. Specific sequences within the characteristic long UTRs of picornaviruses or distinct regions of secondary structure have been identified as the cis-acting elements involved in translation or internal ribosome entry (IRES structures). Cap-independent cellular factors have also been identified and their association with these cis-sequences has been demonstrated (Meerovitch et al., 1989; Jang and Wimmer, 1990). It is thought that eukaryotic cap-independent translation is mediated through the association of these factors with structures within the leader IRES in conjunction with already identified cap-dependent initiation factors. If enhancement is operating in this manner it can be proposed that translation of a mRNA molecule that uses this mechanism would be effected by the addition of excess 5’ leader to an in vitro translation system. This will result in the decrease in efficiency of cap-independent translation as factors will associate with leader sequences and less will be available to partake in translation. Although cellular factors have not been identified for plant enhancers, the addition of VTE leader to an in vitro translation
system resulted in a dramatic decrease in VTE-LUC translation. This decrease was not observed when a synthetic leader was added. Further investigation will be required to identify and characterise cap-independent plant translational initiation factors.

The molecular dissection of the VTE sequence has resulted in the identification of a core element within the leader responsible for functional translational enhancement. The removal of 80% of the leader sequence has a dramatic effect on the translational competence of mRNA molecules when this leader is linked to the PVS coat protein gene. This deletion removes the conserved carlavirus nucleotide block which may be important in a prokaryotic system and the entire sequence to the 5' side of this. This sequence with homology to a Shine-Dalgarno site is discussed in detail in Chapter 3. Subsequent deletion results suggest that this sequence is not important with regard to the process of translational enhancement and it may be the sequence upstream of this, to the 5' proximal end of the leader, that may be important. One confusing observation, however, was that when the deleted SLVTE leader was linked to luciferase the translation of these transcripts was not effected to the extent of the situation with the coat protein gene. This could be perhaps to some interaction of the leader with the luciferase coding region.

The VTEW wild type leader deletion that contained this potential Shine-Dalgarno sequence and the rest of the 3' sequence of the VTE translated the luciferase ORF at a very poor efficiency, even lower than that displayed by a transcript with a synthetic leader. The introduction of mutations in this Shine-Dalgarno like sequence did not appear to further reduce the translation level significantly. This evidence identifies the sequence upstream from the Shine-Dalgarno site as the important region involved in the translational enhancement phenomenon. The actual sequence(s) involved and how they mediate their effects is at present unclear. It could perhaps be through some recognition of the secondary structure configuration of the leader or a portion of the leader in this region by an, as yet unidentified, cellular factor. These deletion results are also supported by results from hybrid arrest translation experiments, where the region upstream from the Shine-Dalgarno sequence appeared to be more important with regard to the process of translational enhancement.

In conclusion, the 101 PVS VTE sequence is a translational enhancer in vitro. Enhancement is likely to operate, at least partially, by a cap-independent translational initiation mechanism which may involve the association of specific cellular trans-acting factors with cis-acting sequences or structures within the sequence upstream from a conserved carlavirus nucleotide block with homology to a prokaryotic ribosome binding site. The nature and exact positions of these cis-acting sequences are at present unclear and will need further characterisation. Results have shown that translational enhancement is an intrinsic property of the leader sequence and not a result of enhanced mRNA stability, a proposal that has until recently been used to explain the phenomenon.
Chapter 5

In vivo characterisation of the translational enhancer upstream from the coat protein ORF of PVS

5.1 Introduction

Chapter 4 has reported the translational enhancement properties associated with the 101 nucleotide upstream region from the PVS coat protein ORF in vitro. The use of an in vitro system is a convenient tool to attain preliminary information or ideas regarding the characteristics of a biological system but it is effectively an artificially manufactured environment and there is the danger that results derived from such a system may not truly reflect the situation in vivo. For this reason the results presented in this chapter will concern the translational enhancement properties of the VTE in two in vivo transient systems, namely microprojectile bombardment into tobacco plant tissue and transfection into tobacco protoplasts. It will also present data regarding the activity of the VTE at the whole plant level in transgenic tobacco plants. The generation of transgenic plants, expressing either GUS RNA transcript with a random synthetic leader or GUS RNA transcript containing the VTE sequence as an untranslated leader, has also enabled us to further investigate the mode of translation associated with the VTE in support of previous predictions from in vitro results.

5.2 Constructs

A plasmid, pRTL2-LUC, from which all the following plasmids were derived, was provided by Neil Bate (Botany Department, Leicester University). This construct contained the TEV 5' untranslated leader (Carrington et al., 1990) linked to the luciferase coding sequence, downstream from a double 35S cauliflower mosaic virus promoter. The promoter drives the expression of a sense luciferase transcript in vivo with the TEV leader sequence at the 5' end. A synthetic leader luciferase construct, pRTS2-LUC, in which a double 35S cauliflower mosaic virus promoter drives the expression of a sense luciferase transcript, in vivo, with a randomly selected synthetic sequence as the 5' untranslated leader, was constructed following PCR amplification of the pGEM5Zf (Promega) polylinker and insertion into pRTL2-LUC as a Sall, NcoI fragment. This cloning event effectively replaced the TEV UTR with a synthetic sequence. An authentic plant leader from an Arabidopsis pollen gene (APG) (Roberts et al., 1993), was inserted into pRTL2-LUC as an XhoI, NcoI fragment which replaced the TEV leader with the APG leader with the resultant plasmid
Figure 5.1 A Diagrammatic representation of constructs used to characterise translational efficiencies of leaders and deletions of leaders in vivo. VTE - viral translational enhancer, potato virus S subgenomic leader of 101 nucleotides; SYNb - pGEM5Zf polylinker synthetic leader; SYN121 - pBluescript synthetic leader; APG - plant mRNA leader; 35S CaMV promoter - single promoter from the cauliflower mosaic virus genome; 35SX2 CaMV promoter - double promoter from the cauliflower mosaic virus genome; 35S 3' term - terminator from the cauliflower mosaic virus genome; NOS term - terminator from the nopaline synthetase gene.

Figure 5.1 B Nucleotide sequences of the leaders generated in vivo from the constructs shown in A. Viral sequences are shown in bold type, plant leader (APG) in italics, and sequences derived from plasmids are indicated in plain text. Initiation codons are underlined.
A

<table>
<thead>
<tr>
<th>Construct</th>
<th>Promoter</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRTV2-LUC</td>
<td>35SX2 CaMV</td>
<td>VTE Lucifase (LUC)</td>
</tr>
<tr>
<td>pRTS2-LUC</td>
<td>35SX2 CaMV</td>
<td>SYNb Lucifase (LUC)</td>
</tr>
<tr>
<td>pRTA2-LUC</td>
<td>35SX2 CaMV</td>
<td>APG Lucifase (LUC)</td>
</tr>
<tr>
<td>pRTSL2-LUC</td>
<td>35SX2 CaMV</td>
<td>VTESL Lucifase (LUC)</td>
</tr>
<tr>
<td>pRTW2-LUC</td>
<td>35SX2 CaMV</td>
<td>VTEW Lucifase (LUC)</td>
</tr>
<tr>
<td>pBI121</td>
<td>35SCaMV</td>
<td>NDS B-Glucuronidase (GUS)</td>
</tr>
<tr>
<td>pBI121-VTE</td>
<td>35SCaMV</td>
<td>VTE B-Glucuronidase (GUS)</td>
</tr>
</tbody>
</table>

B

**SYNb-LUC:**
CTCGACCTGCAGGCGGCACACTAGTGATATCCCGCGGCCATG

**VTE-1-LUC:**
ACCTCGAGCTCAACAAGAGTTGGTTGGAGCCGTAGCAACATTGGGGCCGTTGAAAGCACCTTGAGTTGCACACAGGTAAGAGTTGGGCGCCGTTGAAAGCACTTGAATTAACGTTTGGTACTACTTTCTGTAGTAATACCTGGAT

**APG-LUC:**
CTCGACCAAGACAGATATTGACATGACAGTGCGAAGACACTTGGCCAT

**VTESL-LUC:**
CTCGACCTGCAGGCGGCACACTGCTACTAGTGATATCCCGCGGCCATG

**VTEW-LUC:**
CTCGACCTGCAGGCGGCACACTAGTGATATCCCGCGGCCATG

**SYN(121)-GUS:**
TCTAGAGGATCCCGCGGGTGGTCAGTCGCCCTATG

**VTE-GUS:**
TCTAGAGGCTCACAAGAGATTGCTGAGCCGTAAGCAACAATTGGGCGGTTGAAAGCACCTTCTAGTTGCACACAGGTAAGAGTTGGGCGCCGTTGAAAGCACTTGAATTAACGTTTGGTACTACTTTCTGTAGTAATACCTGGAT
designated pRTA2-LUC. The APG leader was included in the experiment because being an authentic plant leader it should perhaps give a more representative basal translation level. The VTE-LUC fragment was isolated from the pBluescript vector that was used for in vitro studies (Section 4.2), as a SacI, BamHI fragment and was cloned into pSL301 digested with the same enzymes. This allowed the subsequent insertion into pRTL2-LUC as an XhoI, BamHI fragment thereby replacing the TEV-LUC fragment with VTE-LUC with the resultant plasmid being designated pRTV2-LUC. These constructs were used in transient in vivo expression assays with the initiation ATG codon being in the most favourable context for translational initiation of a plant gene (Kozak, 1989). A diagrammatic representation of the constructs pRTS2-LUC, pRTA2-LUC and pRTV2-LUC and the actual sequence of transcript leaders generated in vivo are presented in Fig. 5.1.

Two of the VTE deletions, analysed in vitro (Section 4.6), were also analysed in vivo. A VTESL-LUC double 35S cauliflower mosaic virus promoter plasmid was constructed following isolation of the VTESL-LUC fragment from the pBluescript vector used for in vitro experiments as a NotI, BamHI fragment and was cloned into pRTS2-LUC digested with the same enzymes. A VTEW-LUC plasmid was constructed in the same way. These cloning event effectively replaced SYN-LUC with either VTESL-LUC or VTEW-LUC, giving plasmids designated pRTSL2-LUC and pRTW2-LUC, respectively. As with the other constructs the initiating ATG codon was in a favourable context for translational initiation. A diagrammatic representation of these two constructs and the sequence of leaders generated in vivo are provided in Fig. 5.1.

Plasmids were also constructed that would allow the integration of VTE-GUS and SYN-GUS DNA into the tobacco genome, under the influence of a transcriptional promoter using Agrobacterium-mediated plant transformation. A pBI121 (Clonetech) plant transformation vector was used as the pSYN121-GUS control plasmid. This construct drives GUS expression using a single 35S cauliflower mosaic virus transcriptional promoter. The leader sequence in this construct is the synthetic polylinker of the construct, the sequence between the transcriptional start sequence and the ATG of the GUS gene. To construct the pBI121-VTE-GUS binary plasmid, the VTE-GUS fragment from the pBluescript vector used for the in vitro analysis (Section 4.1), was isolated as an XhoI, EcoR1 fragment and was cloned into pBI121 digested with the same enzymes. This construct could drive VTE-GUS transcript RNA production in vivo under the influence of the single 35S cauliflower mosaic virus promoter. A diagrammatic representation of the constructs pBI121-GUS and pBI121-VTE-GUS and the sequences of transcript leaders generated in vivo are presented in Fig. 5.1.

5.3 Microprojectile bombardments into leaf and pollen tissue

DNA bombardment transient expression assays were carried out using a ballistic microprojectile gun as described by Twell et al., (1989). Equal amounts of supercoiled DNA
Fig 5.2  DNA microprojectile bombardments into leaf and pollen tissue.  

A  Expression of pRTV2-LUC (VTE-LUC), pRTA2-LUC (APG-LUC) and pRTS2-LUC (SYNb-LUC) constructs bombarded into Samsun tobacco leaves using a microprojectile gun. Luciferase units are expressed as the LUC value obtained on assaying one tenth of the total bombarded leaf extract divided by the rate of GUS activity for the same bombarded tissue. Solid bars represent the mean expression values of three repeat experiments and error bands represent the standard deviation from those mean values.

B  Expression of the constructs bombarded in A fired into pollen tissue using a microprojectile gun. Again, LUC units are represented as the LUC value on assaying one tenth of the total bombarded pollen extract divided by the rate of GUS activity for the same bombarded tissue. The mean values of three repeat experiments are represented by solid bars and error bars represent the standard deviation from those mean values.
Fig 5.2

A

Luciferase units/rate of GUS activity

B

Luciferase units/rate of GUS activity
Fig 5.3 Expression of pRTV2-LUC (VTE-LUC) and pRTS2-LUC (SYNb-LUC) constructs on transfection into sucrose purified tobacco protoplasts. $10^6$ protoplasts were used for each transfection reaction and luciferase values were expressed as the LUC activity of one tenth of the protoplast extract adjusted by corresponding GUS values to equalise transfections. Solid bars represent the mean expression values of three repeat experiments and error bars represent the standard deviation from each of these means.
Fig 5.3

![Graph showing luciferase units per 100,000 protoplasts for VTE-LUC and SYN-LUC.]

- VTE-LUC: 3x10^4 units
- SYN-LUC: 1x10^4 units
of pRTS2-LUC, pRTA2-LUC and pRTV2-LUC, were used in individual firing experiments into tissue culture grown tobacco leaf and pollen tissue. A control pRTL2-GUS construct was co-bombarded with the various test plasmids. pRTL2-GUS is effectively the same as pRTL2-LUC (Section 5.2) except for the presence of the GUS reporter gene instead of luciferase. It contains the TEV 5' UTR linked to the β-glucuronidase coding sequence with an upstream double 35S cauliflower mosaic virus transcriptional promoter. The inclusion of this plasmid in the assay provided a second reporter system to give some indication as to the efficiency of individual bombardment events. The rate of GUS activity associated with pRTL2-GUS, expressed as fluorometric units per hour (FU/h), for individual reactions was divided into corresponding luciferase values to obtain a more accurate representation of actual translation and minimise any discrepancies caused by variability in bombardment efficiency.

The pRTV2-LUC construct, that is the construct that drives the production of a luciferase transcript with the VTE sequence as an untranslated leader in vivo, resulted in a higher level of luciferase expression, 2.1 and 2.9 times higher in tobacco leaf and pollen tissue, respectively, over the level of luciferase expression from the pRTS2-LUC construct, that is the construct that results in the production of a luciferase transcript with a synthetic leader. pRTA2-LUC gave levels of reporter gene expression of 1.2 times in leaf and 0.8 times in pollen as compared to the level of expression from pRTS2-LUC. Results are represented graphically in Figs 5.2 A and B with individual bars representing the mean value of three individual bombardment experiments.

5.4 Transfection of tobacco protoplasts

Transient expression assays were also carried out with sucrose purified tobacco (SR1) protoplasts. Equal amounts of supercoiled pRTS2-LUC or pRTV2-LUC DNA were transfected in individual reactions. As with the bombardments, the pRTL2-GUS construct was co-transfected in an attempt to equalise the efficiency of each individual transfection reaction. The rate of GUS activity, expressed as fluorometric units per hour (FU/h) was divided into the corresponding luciferase value. In tobacco protoplasts the pRTV2-LUC construct expressed the luciferase reporter at 2.9 times the level expressed by the pRTS2-LUC construct. Experiments were carried out three times with separate batches of protoplasts and results are expressed graphically in Fig 5.3.

5.5 The expression of VTE deletions in tobacco protoplasts

Sucrose purified tobacco protoplasts were transfected with equal amounts of supercoiled DNA of pRTV2-LUC, pRTS2-LUC, pRTSL2-LUC and pRTW2-LUC. pRTL2-GUS was co-transfected to equalise the efficiency of individual reactions and the rate of GUS activity in fluorometric units per hour (FU/h) was divided into corresponding luciferase values. Translation associated with the full VTE sequence was represented as 100% and the reporter expression of the deletions of this sequence was expressed as percentages of this value.
Fig 5.4 Luciferase expression from constructs containing deletions of the VTE in vivo.

Equal amounts of DNA of pRTV2-LUC (VTE-LUC), pRTSL2-LUC (VTESL-LUC), pRTW2-LUC (VTEW-LUC) and pRTS2-LUC (SYNb-LUC), were transfected into sucrose purified tobacco protoplasts. Luciferase values are provided as the value obtained on assaying one tenth of the protoplast reaction divided by the rate of GUS activity for that particular reaction. Luciferase expression with the full VTE sequence is designated 100% and expression from other constructs are represented as percentages of this value. Error bars represent the standard error of the mean for each construct. Experiments were carried out in triplicate.
Fig 5.4

![Bar chart showing luciferase activity for different constructs: pRTV2-LUC, pRTSL2-LUC, pRTW2-LUC, pRTS2-LUC. The bars decrease in height from left to right, indicating a decrease in luciferase activity.](image-url)
Fig 5.5 The effect of the VTE on GUS expression in transgenic plants. Transgenic plants were generated by Agrobacterium-mediated leaf disc transformation using the binary constructs, pBI121-GUS and pBI121-VTE-GUS, which would produce a GUS RNA message with a synthetic leader (121SYN) or a viral leader (VTE), respectively. GUS assays were carried out on 11 independently transformed plants for both pBI121-GUS and pBI121-VTE-GUS (represented by asterisks). The mean GUS values for each group (GUS units/mg soluble protein/min) are indicated by solid columns. Error bars represent the standard error for each mean.
Fig 5.5
The VTESL deletion which contains only the 20 nucleotides of the VTE upstream from the ATG of the PVS coat protein gene, expressed luciferase at 44.5% the level expressed by the construct that contained the entire VTE sequence. The VTEW deletion, that is the 3' sequence of the VTE that does contain the region with homology to a Shine-Dalgarno site for prokaryotic ribosome binding that has been discussed extensively previously, expressed luciferase at only 34.7% of the level expressed form a construct with the full VTE sequence. As a type of control the pRTS2-LUC construct was also transfected into tobacco protoplasts at the same time as the various deletion constructs as the degree of enhancement conferred by a construct containing the VTE leader over that containing the SYN leader had already been determined in vivo from previous protoplast experiments (Section 5.4). pRTS2-LUC resulted in luciferase expression at 26.7% the level expressed by pRTV2-LUC. This indicates an enhancement of 3.7 fold with the construct containing the VTE over the construct containing the synthetic leader which closely reflects previous protoplast results. Results are represented graphically in Fig 5.4.

5.6 Transgenic plants

Transgenic SR1 tobacco plants were generated, either expressing SYN-GUS or VTE-GUS RNA driven by a single 35S CaMV transcriptional promoter, using Agrobacterium-mediated leaf disc transformation. Transgenic shoots were selected for their ability to form roots in a medium containing kanamycin as the pBI121 vector contains the neomycin phosphotransferase antibiotic selectable marker gene. The incorporation of the target DNA into transgenic plants was also confirmed by PCR amplification analysis. Plants containing either SYN-GUS or VTE-GUS DNA gave a band 900 base pair in size after PCR using specific primers designed to amplify a sequence within the GUS coding sequence (Results not presented). 11 transgenic plants for each SYN-GUS (pBI121-GUS) and VTE-GUS (pBI121-VTE-GUS) were selected by both methods discussed above. The levels of activity of GUS-positive plants are provided in Fig 5.5. GUS values were expressed as pmol of enzyme required to produce one mg of MU in one minute. The mean GUS activity value for pBI121-VTE-GUS transformed plants was observed to be 3.8 fold higher than the mean value measured for pBI121-GUS control transformants.

5.6 Infection of transgenic plants with tobacco mosaic virus (TMV)

5.6.1 Preliminary data: Translational competence of the VTE in infected cells

Initially, randomly selected primary transformants, two producing SYN-GUS RNA and two producing VTE-GUS RNA, maintained and cloned in tissue culture, were inoculated with TMV and GUS assays were carried out on samples from the inoculated leaf over a 10
Fig 5.6 Graph representing the effect of TMV infection of primary transgenic plants on GUS expression. Each line represents the percentage of GUS expression, in the inoculated leaf, at time points post-inoculation from individual values at day 0, for which GUS expression is designated 100%. Different plants are represented by a different line as indicated by the legend.
Fig 5.6

Days post inoculation

% GUS expression

- VTE-GUS #3
- VTE-GUS #6
- GUS #7
- GUS #13
Fig 5.7 Graphical representation of GUS expression in the inoculated leaf of two second generation transgenic tobacco plants infected with TMV. Results are given for two plants: A VTE-GUS#2B/2 and B GUS#17/2. Reporter gene expression is represented as a percentage of the level of expression at day 0 (which is designated 100%), at subsequent days post-inoculation.
day period. GUS activity was expressed GUS units/mg soluble protein/minute and was represented as a percentage change from the level of reporter gene activity at time 0 (t=0) which was designated 100%. All plants showed a decrease in GUS expression over the duration of the experiment but this decrease appeared to be more pronounced with the SYN-GUS plants.

With plants VTE-GUS#3 and VTE-GUS#6 GUS expression decreased by 59% and 55% of expression at t=0 after 5 days, and to 59.8% and 39.9% of expression at t=0 after 10 days, respectively. With plants GUS#7 and GUS#13, GUS expression decreased dramatically, by 86% and 79% of the expression at t=0 after 5 days and by 83% and 78.3% of the expression at t=0 after 10 days, respectively. Results are represented graphically in Fig 5.6.

A similar experiment was carried out with second generation transgenic plants (second generation transformants are represented as /2), one expressing GUS RNA and the other expressing VTE-GUS RNA, GUS#17/2 and VTE-GUS#2B/2, respectively. GUS activity, GUS units/mg protein/min, in the inoculated leaf, was measured 0, 4, 7 and 12 days post inoculation with TMV. GUS activity at time 0 (t=0) was given a value of 100% and GUS values at time points after this were expressed as a % of this value. With the GUS#17/2 plant, decreases in GUS activity by 93.5% at t=4, 92.5% at t=7 and 76.7% at t=12, from the t=0 100% value, were observed. With VTE-GUS#2B/2, decreases of only 32% at t=4, 30.8% at t=7 and 21.4% at t=12, from the t=0 100% value, were observed. Results are expressed graphically in Fig 5.7 A and B.

These two experiments indicated that the expression of GUS RNA transcript produced in transgenic plants containing the VTE sequence as the 5' untranslated leader, may not be as sensitive to the inhibitory conditions placed on translation caused by virus infection as GUS RNA from transgenic plants containing a synthetic leader sequence. One of the criticisms of this experiment was that the stage of the viral infection over the course of the experiment had not been monitored. It was decided to carry out a more detailed examination of this phenomenon which would correlate virus concentration to GUS expression and take into account any variability caused by plant senescence over the time of the experiment.

5.6.2 Translational competence of the VTE in cells containing detectable concentration of TMV RNA

Four second generation transgenic plants were analysed, GUS#12/2, GUS#8/2, VTE-GUS#1/2 and VTE-GUS#2B/2. Samples from the inoculated leaf were taken at time points of 1, 3, 7 and 14 days post-inoculation with TMV virus particles. GUS assays were carried out on samples, with GUS activity being expressed as GUS units/mg protein/min, and sap samples were also probed with TMV RNA to detect the presence of viral RNA. Over the course of the experiment samples were also taken from duplicate uninfected transgenic plants (one for each of the four experimental transgenics) to rule out any contribution of plant senescence to variability in GUS production over the 14 days.
Fig 5.8 Expression of GUS in the inoculated leaf of transgenic second generation plants on infection with TMV. Results are presented for 4 plants: A GUS#12/2, B GUS#8/2, C VTE-GUS#1/2 and D VTE-GUS#2B/2. Reporter expression is presented by solid bars as a percentage increase or decrease from the level of GUS expression at day 1 for individual plants, at subsequent days post-inoculation. GUS expression in leaves displaying TMV mosaic type symptoms for each plant is represented by striped bars. Dot blots, indicating viral RNA accumulation, are provided above graphs for the corresponding plant, with the autoradiograph for each sample being placed below the corresponding graph bar.
Fig 5.8

A  GUS#12/2

B  GUS#8/2

C  VTE-GUS#1/2

D  VTE-GUS#2B/2

Virus accumulation

Virus accumulation
A general trend appeared to emerge. With VTE-GUS plants, where virus concentration was high, as indicated by dot blots, GUS activity appeared to be enhanced. This phenomenon was not observed in SYN-GUS plants.

With the SYN-GUS plants, GUS expression either decreased either slightly or considerably over the 14 days. With GUS#12/2, after an initial increase in expression by just over 30% at day 7, expression decreased, at day 14, to just over 13% of the level of GUS activity at t=1. In addition to this t=14 sample from the inoculated leaf a sample was also taken at the same time from a leaf displaying obvious TMV mosaic-like symptom, which obviously represented a situation where virus concentration was high. GUS expression in this leaf was determined to be only 7.3% of reporter expression in the inoculated leaf at day 1. Dot blots revealed that TMV RNA concentration was detectable after 14 days in the inoculated leaf and in the leaf displaying mosaic-type symptoms. A parallel leaf in a duplicate GUS#12/2 plant was mock-inoculated and samples were also taken from this leaf over the course of the experiment and these did not vary to any great extent from each other with expression values of 7.8, 6.9 and 7.4 pmol/mg/min. Compared to the variabilities displayed in the infected plant these values represent a fairly linear relationship.

The other SYN-GUS plant, GUS#8/2, displayed a quite similar pattern, although perhaps not as pronounced. GUS expression decreased from 100% at day 1 to 75.5%, 84.6% and 80% at days 3, 7 and 14, respectively, in the inoculated leaf. In the leaf displaying mosaic symptoms at t=14, GUS expression was reduced to only 34% of the value in the inoculated leaf at day 1. Dot blots allowed the detection of TMV RNA most strongly at day 14 and in the inoculated leaf and in the leaf displaying characteristic viral symptoms, but a low signal was also detected in the inoculated leaf as early as day 7. Again, as with the previous plant, in the parallel mock-inoculated leaf of the duplicate transgenic, GUS values varied little over the course of the experiment with values of 134, 148.7 and 141 pmol GUS/mg/min.

The pattern with the VTE-GUS plants was very different. VTE-GUS#1/2 displayed an initial stimulation in GUS expression, 42.8% higher than expression in the inoculated leaf at day 1, at day 3 and at day 7 a slight decrease in expression was observed with expression only 42.9% of the value at t=1. The most dramatic effect was shown to occur at day 14 with a 3.43 fold enhancement in GUS expression over the day 1 value, in the inoculated leaf, and this increased expression was also evident in the symptomatic leaf at the same day point with expression an order of 3.1 fold higher. TMV RNA concentration was observed to be highest at day 14 in the inoculated leaf and in the leaf displaying symptoms. Trace amounts were also evident in the inoculated leaf at day 7. As with all the other plants the variability in GUS expression over the time of the experiment in the mock-inoculated leaf of the duplicate transgenic plant was slight with expressions of 36.3, 32 and 44.5 pmol GUS/mg/min. When compared to the variability observed with the infected leaf these values almost represent a linear relationship.

The final VTE-GUS transgenic displayed a similar pattern to that exhibited by VTE-GUS#1/2. VTE-GUS#2B/2 exhibited an initial stimulation in GUS expression at day 3 with expression increasing by 173% over the level of enzyme activity at day 1. This initial
stimulation was followed by a decrease at t=7 from this day 3 value to a similar value to the
day 1 expression value (11.5% higher than expression at t=1). At day 14 there was a
dramatic increase in reporter expression, in the inoculated leaf, by an order of magnitude
3.65 fold higher than the day 1 value and this fold enhancement was 3.81 in the leaf with
mosaic-type symptoms, at the same time point. Dot blots revealed the presence of TMV
RNA at day 14 in the inoculated leaf and in the symptomatic leaf. GUS values in the mock-
inoculated leaf of the duplicate transgenic plant did not vary to any significant degree, with
values of GUS expression of 59.4, 53 and 52.2 pmol/mg/min.
Results are represented graphically in Fig 5.8.

5.7 Discussion

Results presented in this chapter indicate that the VTE, 101 nucleotide sequence upstream
from the PVS coat protein gene ATG codon, when provided as an untranslated leader
sequence, enhances the translation of a downstream ORF in vivo, both in transient
expression assays when bombarded into leaf or pollen tissue and in tobacco protoplasts, and
in transgenic plants. The latter system is probably not the best way to quantitatively
determine the degree of enhancement associated with the VTE due to the variability in gene
transcription between individual transformants but, alternately, it does demonstrate how this
sequence will operate at the whole plant level in an in vivo environment and it is this factor
that will be important from a biotechnological point of view. It has previously been
demonstrated in Chapter 4, that the VTE sequence enhances translation of a downstream
ORF in vitro also. Deletions of the sequence has indicated that the cis-acting region
responsible for enhancement properties is at the 5' end, and a sequence with homology to a
Shine-Dalgarno site for prokaryotic ribosome binding does not appear to be involved in this
function.

As has been mentioned in the main introduction, the incorporation of translational enhancers
into expressional cassettes may help to increase the expression of foreign genes in transgenic
plants, a situation that is often a problem in reality. For example, Kang et al., 1994, has
recently reported a 16 fold enhancement in expression of the 10kZ maize protein storage gene
in transgenic tobacco plants when the TMV Ω leader was incorporated as the 5' untranslated
leader. The VTE and other translational enhancers could be used in future to increase the
expression of biochemically important introduced foreign genes in agronomically important
crops.

Results from the in vivo activity of deletions of the VTE reflect previous in vitro results
indicating, as has been proposed in Chapter 4, that the functional sequence of the leader, with
relation to translational enhancement, may be contained within the 5' proximal region of this
sequence.

Controls on cellular translation are tightly regulated to allow temporal and differential
expression of host cellular proteins. Post-transcriptional regulation has been shown to
operate under various conditions of environmental or developmental stress. This response may be specific for a particular gene, for example the light-mediated control of translation of ribulose-1,5-bisphosphate carboxylase RNA in *Amaranth* seedlings (Berry *et al.*, 1990) or the response of mRNAs involved in ferritin biosynthesis when exposed to iron (Hentze *et al.*, 1987). Alternately, the response may be more general causing a gross dramatic effect on host cellular protein synthesis. Some environmental conditions stimulate a mass "shut-down" of host translation, examples include high salt concentrations or certain cellular developmental situations such as during mitosis (Bonneau and Sonenberg, 1987).

Perhaps the most widely documented down-regulation of cellular metabolism is that which occurs on viral infection. The viruses only aim is to attain a maximum rate of multiplication and spread through the host so many viruses establish conditions within the cell that favour translation of the viral species at the expense of the cellular species. This shut down effect is mediated at the post-transcriptional level and as cellular translation is a tightly regulated process the availability of translation factors will not be sufficient to meet the demands of a virus that multiplies to a high concentration in its host. The shut down of endogenous cellular metabolism is a quite ingenious mechanism, employed by the virus, to overcome this problem and recruit cellular translation factors and ribosomes for its own use. It should be mentioned here that all viruses do not affect protein synthesis, paroviruses and retroviruses often replicate in their host without any detriment to cellular metabolism in any way whatsoever.

Over the past few years attention has concentrated on the situation during picornavirus infection and several theories have been proposed to explain how host translational shut down is mediated. Infection with poliovirus results in the complete shut off of host translation only two hours after initial infection. With poliovirus, it was originally proposed that host translational shut down was entirely a result of degradation of the p220 component of the cap-binding cellular translational initiation factor, eIF-4F (Echison *et al.*, 1982). In viral infected cells two peptides, 110 kDa and 300 kDa in size, were detected that reacted with p220 antisera. Neither the 3C or 2A poliovirus encoded protease peptides can directly degrade the p220 protein *in vitro* (Lee *et al.*, 1985; Lloyd *et al.*, 1986), so it seems likely that inactivation of p220 is mediated through the intermediacy of an endogenous cellular protease that is activated in some way by viral infection. It has also been reported this inactivation of p220, requires the presence of eukaryotic initiation factor-3 (eIF-3) (Wyckoff *et al.*, 1990) and it has been proposed that this initiation factor could possibly contain the catalytic site for p220 cleavage. Presumably, this degradation would inhibit cellular cap-dependent translation but would allow viral translation that would operate by an alternative translational mechanism, independent of this cap-binding function, but whether this is the sole mechanism whereby poliovirus inhibits cellular translation is debatable. Adenovirus infection also results in the inactivation of p220 (Huang and Schneider, 1991). Picornaviral infection affects other proteins, examples include the p68-kinase that is involved in the regulation of eIF-2α (Black *et al.*, 1989), and one of the subunits of eIF-3 (Echison and Smith, 1990) so the host translational shut down observed on infection with this particular virus could be through any one or a combination of these effects.

Another cellular translational factor that is affected by viral infection is the α subunit of eIF-
2. This factor is partially inactivated by phosphorylation in cells infected with vesicular stomatitis virus (Centrella and Lucas-Lenard, 1982), reoviruses (Dratwka-Kos et al., 1984) and adenoviruses (Samuel et al., 1984). It could be that viral translation still requires this factor but the depletion of an active form from the endogenous supply may decrease the amount present for cellular translation without having severe effects on viral protein synthesis which may compete more efficiently. There is also considerable evidence regarding the existence of virus infection specific proteins such as the p52 peptide in poliovirus infected cells (Meerovitch et al., 1989) or the p57 peptide in ECMV and FMDV infected cells (Jang and Wimmer, 1990; Luz and Beck, 1991). These have been discussed at large in the main introduction.

In addition to the degradation of factors involved in the translation pathway, more general factors have been suggested which may be responsible, or at least play a contributing factor, for host translational shut down. The ability of viral mRNAs to out compete cellular species has been reported in many cases, for example, mengovirus mRNA competes 35 times more efficiently in cell free translation extracts than does globin mRNA (Rosen et al., 1982), this is, however not true for poliovirus RNA that is translated less efficiently than its cellular competitors. The intracellular cation concentration is another factor that can affect translation, presumably by affecting secondary configuration (Carrasco, 1977). This concentration does increase in poliovirus infected cells and poliovirus RNA is translated more efficiently under these conditions but this increase does not occur until several hours after initial infection, that is several hours after the inhibition of cellular protein synthesis so it is unlikely that this factor alone is responsible for the inhibition. With ECMV however, there is little or no decrease in host protein synthesis until relatively late in the infection cycle which correlates well with the timing of increased intracellular cation concentration (Lacal and Carrasco, 1982). With the latter infection this characteristic may be more important. There is also some evidence that cellular mRNA molecules are degraded more rapidly in both poxvirus (Rice and Roberts, 1983) and herpesvirus (Fenwick and McMenamin, 1984). It therefore seems likely that a combination of these effects may be responsible for host translational shut down and the contributing factors appears to be different with different viruses.

This overall phenomenon has also been observed on infection with plant viruses and although very little is known about the biochemical changes that induce it, it seems plausible that they are similar to the situation with the animal picornaviruses. This effect is mediated at the translational level by some interaction with the host cellular translational machinery to the detriment of plant mRNA molecules and to the advantage of viral molecules. As has been stated in Chapter 1, TMV infection may cause a 75% decrease in host protein synthesis during the period of most active virus accumulation (Fraser, 1987) and it has been suggested that this inhibition may be, at least in part, of a competitive nature with the sheer physical presence of the virus, acting as a sink for cellular resources such as amino acids and ribonucleotides. This type of inhibition, by sheer abundance, has also been detected with VSV (Lodish and Porter, 1981) where cellular and viral polysomes are the same size but most are programmed with VSV RNA due to the predominant concentration in the infected cell. Other factors may also contribute, such as the ability of the TMV RNA molecule to out
compete host molecules for translational components, or, it is also possible that TMV infection could induce specific changes in the cellular translational apparatus to its expressional advantage.

The cellular glucose-regulated protein 78/immunoglobin heavy chain-binding protein (GRP78/BiP) was discovered for its ability to circumvent the translational shut down that occurs during poliovirus infection (Macejak and Sarnow, 1991). It has been proposed that this mRNA molecule is translated by an alternative cap-independent translational mechanism, a mechanism which has been proposed poliovirus itself, due to the ability of this virus to express its genes efficiently under conditions where normal cap-dependent translation is repressed. It was basically this phenomenon of efficient expression in an environment where cap-dependent translation was inhibited that has been investigated in experiments with transgenic plants. Infecting these plants with TMV creates, in whatever way, the environment in which TMV viral molecules are preferentially translated.

Results from this Chapter and Chapter 4 have indicated that the VTE PVS viral sequence is a translational enhancer and there is also evidence, at least in vitro, that the mode of translation associated with it may be at least partially cap-independent. By infecting VTE-GUS transgenic plants with virus we were, hopefully, artificially creating the type of conditions that mRNA molecules containing the VTE sequence as a 5' leader would be selectively translated in. Results do indeed indicate that VTE-mediated expression appears to be higher when viral concentration is high, quite late in the infection. Initial infection studies indicated that translation of transcripts containing the synthetic leader was inhibited more than transcripts containing the VTE leader but this could not be conclusively stated because the presence of virus had not been definitely associated with this effect. In the final transgenic infection experiment, enhancements in GUS activity of 3.43 and 3.65 fold were observed with VTE-GUS plants #1/2 and #2B/2, respectively, when the viral concentration was high. In SYN-GUS plants, #12/2 and #8/2, decreases in GUS activity of 7.4 and 1.25 fold, respectively, were observed, again at high virus concentrations. In leaves with characteristic TMV symptoms, GUS activity was dramatically increased within VTE-GUS plants but decreased in SYN-GUS plants. Direct comparisons cannot be made between this leaf and the inoculated leaves of the same plants because both are at different developmental stages and consequently exhibiting different rates of protein synthesis but the fact that this pattern reflects that exhibited in the inoculated leaf reinforces these results. This could indicate that the RNA molecules containing the VTE sequence are preferentially translated compared with cellular molecules, perhaps by an ability to out-compete endogenous transcripts, brought about by the altered cellular conditions generated by TMV infection. As this increased VTE-associated expression occurs late in the infection this phenomenon could have something to do with an increased intracellular cation concentration, a situation that has been proposed as a contributing factor to host translational shut off in ECMV infected cells.

In summary, the VTE, 101 nucleotide sequence upstream from the ATG of the PVS viral coat protein gene, enhances the expression of a downstream reporter gene in vivo. Transcripts produced in transgenic plants containing the VTE sequence as a 5' leader appear to be selectively expressed over transcripts produced in transgenic plants containing a
synthetic leader in an environment that favours the expression of TMV RNA. The conditions induced by PVS infection may therefore reflect those associated with TMV infection.
Chapter 6

The 5’ untranslated leader sequence from the PVS genomic RNA acts as a translational enhancer

6.1 Introduction

Viruses have employed the use of quite ingenious mechanisms to achieve a maximum rate of multiplication and spread through the host which they have invaded. The regulation of translation offers a major area for potential control of protein synthesis. The more efficient the translation the greater the rate of protein production and the more raw materials available for viral multiplication.

One important factor affecting translational competence is the nature of the 5’ untranslated leader (UTR) sequence of the mRNA molecule. Characteristics such as leader length and secondary structure configuration all contribute to the efficiency with which the downstream ORF will be translated (Kozak, 1978; 1989; 1991b; 1991c). Plant viral 5’ sequences have characteristically long untranslated leaders which have a high AU base composition, a characteristic of a relatively unstructured configuration. As a result of this translational enhancement, the properties associated with these leaders have often been attributed to the facilitated scanning along the leader by ribosomes (Jobling et al., 1987). The variability in enhancement properties displayed by leaders with comparable length and secondary structure configuration contradicts this theory. Core regulatory cis-acting elements have been identified within the UTRs of TMV and PVX (Gallie et al., 1992; Smirnyagina et al., 1991) which indicate that enhancement could be a result of specific protein-RNA interactions.

Translational enhancement conferred by the 5’ UTR of a positive-sense, single-stranded RNA genomic molecule, as has been discussed at length in Chapter 1, has been documented for members of many plant viral groups. Examples include the potyviruses, TEV (Carrington et al., 1990) and PSbMV (Nicolaissen et al., 1992), the tobamovirus, TMV (Gallie et al., 1987a; 1987b), and even the satellite RNA of tobacco necrosis virus (TNV) (Timmer et al., 1993) among others, but until now the nature of translation associated with the 5’ UTR of carlaviruses has been unknown.

Two observations indicated that translational enhancement could possibly be a property associated with the PVS genomic RNA leader. The first was the documentation that the untranslated genomic leader from PVX, a member of the closely related potexvirus group of plant viruses, was a strong translational enhancer both in vitro and in vivo (Zelenina et al., 1992; Pooggin et al., 1992), and the second was the identification of the VTE translational enhancer upstream from the PVS coat protein gene (Turner et al., 1994a; 1994b). It was these factors that prompted an analysis of the translational properties of
the PVS genomic UTR. The translational enhancement properties of the VTE, in vitro and in vivo, have been discussed in detail in Chapters 4 and 5, respectively.

Another property which may be associated with the PVS genomic 5' UTR is that of initiation of coat protein binding resulting in viral encapsidation. Encapsidation initiation requires a specific cis-acting recognition site on the viral RNA molecule. With TMV this sequence has been localised to an internal region of the genomic RNA molecule (Zimmern and Butler, 1977), but with the potexvirus, papaya mosaic virus (PMV) the origin of coat protein assembly (OAS) has been mapped to within the 5' UTR leader sequence (Sit et al., 1994). As PVS is so similar to PVX in other characteristics such as genomic organisation, translational strategy and even sequence homology, it is also possible that a sequence within the PVS UTR could be responsible for this function also.

The most compelling piece of evidence that supports this theory that the PVS OAS may also be contained within the 5' UTR comes from experiments with coat protein-mediated protection in transgenic plants. The first reported demonstration of this phenomenon, that is engineered resistance in a transgenic plant to a virus due to the expression of the viral coat protein, was by Powell-Abel et al., (1986). Findings indicated that transgenic plants expressing the TMV coat protein displayed a delay in symptom development on inoculation by the virus. Nelson et al., (1987) reported that this resistance was effective against challenge with TMV viral particles but was overcome by inoculation with TMV viral RNA. As has been stated above, the TMV OAS is located at an internal position within the viral genome (Zimmern and Butler, 1977; Zimmern and Wilson, 1976). It is thought that when transgenic plants, expressing the TMV coat protein, are inoculated with viral particles the presence of exogenous coat protein inhibits the process of disassembly thereby preventing expression of the viral genome. The situation is different on challenge inoculation with viral RNA. When naked RNA enters the cell encapsidation initiates at the OAS but because this site is internal within the viral genome translation can initiate at the 5' end and once initiation has been achieved ribosomes can effectively strip coat protein from the viral RNA. The position of the TMV OAS can therefore explain the effectiveness of coat protein-mediated protection against TMV viral particles but not against TMV viral RNA.

The situation with the potexvirus, potato virus X (PVX), is different to that with TMV. Coat protein-mediated protection has also been demonstrated for this virus (Lawson et al., 1990) but this resistance is effective against challenge with both PVX viral particles and PVX viral RNA. The OAS of another potexvirus, papaya mosaic virus (PMV), has been located to the 5' proximal end of the viral genomic RNA molecule. In this case the effectiveness of coat protein-mediated protection against challenge with viral particles may be explained as above, that is the inhibition of disassembly due to the presence of exogenous coat protein. It has been proposed, however, that the effectiveness of this process against viral RNA is due to at least a partial recoating of the potexvirus RNA which would result in the prevention of translation of the replicase gene (Hemenway et al., 1988).

Transgenic Nicotiana debneyi expressing PVS viral coat protein are resistant to challenge inoculation with both PVS viral particles and PVS viral RNA (Mackenzie and
Fig 6.1 Nucleotide sequence alignment of the untranslated leaders of blueberry scorch virus (BBScV)(top) and potato virus S (PVS)(bottom). Homologous nucleotides are indicated by small circles. The positions of the two conserved blocks (Block 1 and 2) are indicated by thick solid lines. The position and sequence of leader deletions are also provided. PVS 5'A, leader deletion removing the 3' terminal viral leader sequence up to the first conserved sequence block (Block 1); PVS 5'B, leader deletion removing the 3' viral leader sequence up to the second conserved sequence block (Block 2); PVS 5'Amut, the mutated version of PVS 5'A which has an extra T residue in a run of three T residues within the sequence. The sequences of deletions are indicated.
Fig 6.1

Blueberry scorch virus (top)
potato virus S (bottom)

Block 1

NTAACACCTCCCGAATATACCGAGATATAAACAACCGACGTTGATATACATAG

Block 2

TAACACCTCCGAAATAGTTGACTAAACACCGAGTTGATATACATAG

PVS 5′ Deletion A

TAATTTGACTAAACACGCGACTTTCAAGCAAATTACTTAACATG

PVS 5′ Deletion Amut

TTCAAGACAATTACTTAACATG

PVS 5′ Deletion B
Tremaine, 1990). This response, in addition to the close similarity between potexviruses and carlaviruses in properties such as genome structure and expression, suggests that the PVS OAS could possibly be contained within the genomic 5' UTR. This property was also investigated in this chapter.

The following results will provide evidence that the PVS 5' UTR enhances the translation of a downstream cistron \textit{in vitro} and \textit{in vivo} in both a eukaryotic and prokaryotic environment when provided at the 5' proximal end of a mRNA molecule as the untranslated leader. Some preliminary data will also suggest that the function of origin of coat protein assembly could also be contained within this leader sequence. The possible exploitation of this sequence to achieve viral resistance, and even regulated gene expression, will be discussed.

6.2 The PVS 5' untranslated leader is a translational enhancer \textit{in vitro}.

6.2.1 Conservation between carlavirus UTRs

A clone, pE12, which contained the entire PVS 5' UTR sequence and the N-terminal coding region of the PVS replicase gene was provided by Eithne McShane (Queen's University, Belfast). This clone was constructed following cDNA synthesis using a primer complementary to a sequence near the 5' end of the PVS replicase ORF. When the sequence of the PVS untranslated leader was compared to the published sequence for another member of the group, blueberry scorch virus (BBScV)(Cavileer et al, 1994), it was evident that the two sequences were closely related. Two areas of high sequence conservation were evident, the first of these regions encompassed the 5' terminal 14 residues of the leader and the second a nucleotide block consisting of 13 residues downstream. This first sequence has been designated Block 1 and the second region has been designated Block 2. The positions of these nucleotide sequences in relation to the 5' UTR sequence are provided in Fig 6.1. This indicated that these regions could possibly have some functional importance. A degree of sequence homology has also been detected between the PVS 5' UTR and the PVM 5' UTR, particularly at the 5' proximal end of the sequence, but the similarity is not as pronounced as that observed with BBScV (Cavileer \textit{et al.}, 1994). No obvious sequence homology was detected between the UTR sequences of PVS and the potexvirus PVX except for a general AC rich composition.

6.2.2 Constructs

The 5' untranslated leader of the genomic PVS RNA molecule was initially isolated by PCR amplification from the pE12 clone detailed above. The 63 nucleotide sequence was amplified using a specially designed 3' primer 5'-GCGGATCCATGGAAGTAATT-TGCTTTGACAG-3' and a primer complementary to a sequence within the Bluescript
Fig 6.2 A  Diagrammatic representation of constructs used to generate *in vitro* mRNA transcripts for translation analysis. A closed arrow indicates the position of the RNA polymerase promoter (T3) within the plasmids. PVS 5' UTR, potato virus S 5' untranslated leader region; PVS 5'A, potato virus S leader deletion A; PVS 5'A mut, potato virus S leader deletion A with an extra T residue generated during PCR amplification; PVS 5' B, potato virus S leader deletion B; 35S term, cauliflower mosaic virus 35S transcriptional terminator.

Fig 6.2 B  Nucleotide sequence of the transcript leaders generated from the constructs shown in A. Viral sequences are shown in bold type and synthetic sequences that are derived from plasmids are indicated in italics. The position of the extra T nucleotide in PVS 5'A mut is indicated by a * and positions of the initiation codons (ATG) are underlined.
A

- **PVS 5'**
  - **LUCIFERASE**
  - 35S term

- **PVS 5'A**
  - **LUCIFERASE**
  - 35S term

- **PVS 5'Amut**
  - **LUCIFERASE**
  - 35S term

- **PVS 5'B**
  - **LUCIFERASE**
  - 35S term

B

**PVS 5'-LUC**

```
GGGAACAAAAAGCTGGAAGCTC TAAACACTCCGAAAATAATTGACTTAAA
CAACGCGACTGTCTTCAAGCAAATTACTTACCATG
```

**PVS 5' A-LUC**

```
GGGAACAAAAAGCTGGAAGCTC TAATTGACTTTAACAACGCGACTGTCTTCA
GCAAATTACTTACCATG
```

**PVS 5' AMut-LUC**

```
GGGAACAAAAAGCTGGAAGCTC TAATTGACTTTAAACACGCGACTGTCTCA
AGCAAATTACTTACCATG
```

**PVS 5' B-LUC**

```
GGGAACAAAAAGCTGGAAGCTC GTTCAAGCAAATTACTTACCATG
```
vector of the initial clone, pE12, as the 5' primer. The PCR product was digested with SacI, an enzyme site in the vector upstream from the 5' UTR, and BamHI, a site incorporated into the specifically designed primer at the 3' end of the PCR product. The use of the 3' primer also change the sequence surrounding the ATG of the polymerase gene to generate a NcoI site which represents a change in viral sequence from GG to CC. This SacI/BamHI PCR fragment was cloned into SacI/BamHI digested pBluescript-SK+.

Clones with inserts of the correct size were sequenced by the dideoxynucleotide method of Sanger, (1977), using a double-stranded template. All were shown to contain the authentic PVS 5' UTR sequence except for a single base mutation which resulted in a change in the viral sequence from A to T, at a position 21 nucleotides upstream from the ATG initiation codon. This mutation was present in all clones sequenced but instead of reamplification it was decided to continue with the analysis of these clones as this mutation was not contained within a region of conservation when this sequence was compared to that of another carlaviruses, BBScV (Fig 6.1). This clone was designated pPV SS' UTR.

The luciferase reporter gene was cloned downstream from this sequence to allow an analysis of translational properties of the leader. The LUC fragment was isolated from pSYN-LUC (Section 4.2) as a NcoI/HindIII fragment and was cloned into NcoI/HindIII digested pPVS 5' UTR. The LUC fragment contains the cauliflower mosaic virus 35S transcriptional terminator at the 3' end of the luciferase coding sequence. This clone was designated pPVS 5'-LUC and is represented diagrammatically in Fig 6.2A. The leader sequence from mRNA transcripts generated from this construct in vitro is provided in Fig 6.2B.

When strong translational enhancement properties were found to be associated with this PVS leader two deletions were also constructed. These were specifically designed on the basis of sequence comparison with another member of the carlaviruses group, BBScV (Cavileer et al., 1994; Lawrence and Hillman, 1994) which revealed two blocks of nucleotide homology (Fig 6.1). Deletions of this leader were specifically designed in an attempt to identify which of these blocks, if any, were involved in translational enhancement. The first deletion resulted in the removal of the 3' portion of the leader which effectively removed the terminal region immediately downstream from the second of the carlavirus blocks of conservation (Block 2). The second deletion removed conserved Block 2 and all the sequence downstream. These deletions were constructed by PCR amplification using specifically designed 5' primers and a primer at the 3' terminal end of the luciferase gene in the Bluescript vector, using the pPVS 5'-LUC clone as template.

For the first deletion, designated PVS 5' A, the primer sequence of the 5' primer was 5'-CCGAGCTCTAATTTGACTTAAACAAC-3' and for the second deletion, designated PVS 5' B the sequence of the 5' primer was 5'-CCGAGCTCGTTCAAGCAAATTA-CTTAC-3'. In the construction of both clones the PCR amplified fragments, which approximately corresponded to the size of the luciferase ORF, were digested with SacI, the restriction enzyme site engineered into both 5' primers, and EcoRI, an internal restriction site in the luciferase gene. The resultant fragment, about 500 base pairs in
size, was cloned into SacI/EcoRI digested pBluescript-SK(+) fragment and digested with the same enzymes. Clones were designated pPVS 5' A-LUC and pPVS 5' B-LUC, respectively, and the sequence of the deleted leaders was confirmed by dideoxynucleotide sequencing according to the method devised by Sanger, (1977), using a double-stranded template. A diagrammatic representation of these constructs is provided in Fig 6.2A and the leader sequences from transcripts generated from these constructs in vitro are provided in Fig 6.2B.

In addition to the two deletions discussed, sequencing identified a mutated leader sequence generated by PCR amplification in the construction of the larger PVS 5' A deleted leader. This mutated sequence contained an extra T residue in a wild type sequence run of three T nucleotides. A construct containing this sequence linked to luciferase was also included in the analysis. A diagrammatic representation of this construct, designated pPVS 5' Amut-LUC is provided in Fig 6.2A and the actual leader sequence of transcripts generated from this construct is indicated in Fig 6.2B. The positions of these deletions in relation to the full length PVS leader sequence are indicated in Fig 6.1.

6.2.3 Translational enhancement properties of the PVS 5' UTR in vitro

As with all the constructs mentioned in the previous chapters, transcripts from pPVS 5'-LUC were generated using the T3 RNA polymerase promoter. The translational properties of transcripts containing the PVS 5' UTR as an untranslated leader were compared to properties exhibited by transcripts with a synthetic leader, this construct, pSYN-LUCb, has been detailed in Chapter 4 (Section 4.2.1).

Transcripts generated from constructs pSYN-LUCb and pPVS 5'-LUC were equalised visually on an ethidium bromide stained agarose gel and equivalent amounts of transcript were used in individual in vitro translation reactions both in rabbit reticulocyte lysate and wheat germ extract.

When PVS 5'-LUC and SYN-LUCb transcripts were translated in vitro, and the level of luciferase expression compared, the enhancement properties associated with the untranslated leader of the PVS genomic RNA molecule was evident. In rabbit reticulocyte lysate the presence of this viral leader conferred a mean enhancement value of approximately 30 fold on the expression of luciferase over the level of translation from transcripts containing the synthetic leader. This mean enhancement value over SYN-LUCb transcripts in wheat germ extract was nearly 15 fold. These values are represented graphically in Fig 6.3.

Transcripts derived from pVTE-LUC were also included in the analysis. This allowed a comparison of the PVS 5' UTR with a leader that was known to enhance translation over a basal level exhibited by the synthetic leader used in the investigation. The inclusion of the VTE leader also acted as a type of control in that the level of enhancement conferred by this leader over the synthetic leader was already known from previous
**Fig 6.3 A** Graphical representation of results obtained as to the relative luciferase activity on translation of PVS 5'-LUC, VTE-LUC and SYNb-LUC transcripts *in vitro* in rabbit reticulocyte lysate. Expression is represented as the fold increase in luciferase translation with the PVS 5'-LUC and the VTE-LUC transcripts as compared to the SYNb-LUC transcript. Error bars indicate the value for the standard error of the mean enhancement with each construct. Experiments were carried out in triplicate. PVS 5', potato virus S 5' untranslated leader; VTE, potato virus S 101 nucleotides upstream from the coat protein gene; SYNb, synthetic leader.

**Fig 6.3 B** Graphical representation of results obtained for the relative level of luciferase activity on translation of PVS 5'-LUC, VTE-LUC and SYNb-LUC transcripts *in vitro* in wheat germ extract. Expression is represented as the fold increase in luciferase translation with the PVS 5'-LUC and the VTE-LUC transcripts when compared to the SYNb-LUC transcript. Error bars indicate the value for the standard error of the mean enhancement for each construct. Experiments were carried out in triplicate. PVS 5', potato virus S 5' untranslated leader; VTE, potato virus S 101 nucleotides upstream from the coat protein gene; SYNb, synthetic leader.
Fig 6.4 Graph demonstrating the effect of the addition of cap analogue on the translation of PVS 5'-LUC and SYNb-LUC transcripts in vitro in rabbit reticulocyte lysate. Transcripts were translated in the absence and in the presence of increasing millimolar (mM) concentrations of cap analogue. Luciferase translation is expressed as a percentage decrease from the level of translation without the inhibitor for each transcript.
Fig 6.4

- SYN-LUCb
- PVS 5'-LUC

Percentage luciferase expression

Cap analogue concentration (mM)
experiments. In rabbit reticulocyte lysate PVS 5'-LUC transcripts expressed luciferase at a mean value of 12 times the level of that expressed by transcripts containing the VTE leader. In wheat germ the presence of the PVS 5' UTR conferred a mean fold enhancement of 5 fold in reporter gene expression over the level expressed from transcripts containing the VTE leader. Fold enhancement conferred by the presence of the VTE as an untranslated leader on the level of luciferase expression over the level of expression exhibited by transcripts containing the SYNb leader gave similar results to those previously reported in Chapter 4 (Table 4.1). In rabbit reticulocyte lysate VTE-LUC transcripts expressed luciferase at 4.9 times the level expressed by SYN-LUCb transcripts. In wheat germ extract enhancement conferred by the VTE leader was 3.9 times that exhibited by the SYNb leader (Fig 6.3). All experiments were carried out at least in triplicate.

An experiment was also carried out to investigate the mechanism by which this translational enhancement associated with the PVS 5' UTR occurred. In a similar experiment to that previously reported for the VTE (Fig 4.3), capped luciferase transcripts containing the PVS 5' UTR as an untranslated leader, were translated in vitro under conditions of increasing concentration of cap analogue, 0, 0.1, 0.2 and 0.3 mM. Translation of capped PVS 5' leader luciferase transcripts was observed to be only inhibited by 16% at the highest cap concentration used in the experiment (0.3 mM). At this same concentration of inhibitor translation of transcripts containing the synthetic leader (SYNb) was inhibited by 48%. This mirrors the situation with the VTE leader. The translation of capped transcripts containing the VTE as a leader sequence was observed to be less sensitive to the presence of cap analogue than capped transcripts containing a synthetic leader. PVS 5' UTR results are represented graphically in Fig 6.4, VTE results are provided in Fig 4.3.

6.2.4 Deletions of the PVS 5' UTR

All transcripts were generated using the T3 RNA polymerase promoter. Transcripts generated from constructs, pSYN-LUCb, pPVS 5'-LUC, pPVS 5' A-LUC, pPVS 5' B-LUC and pPVS 5' Amut-LUC were equalised visually on an ethidium bromide stained agarose gel and equal amounts of transcript were used in in vitro translation reactions in rabbit reticulocyte lysate and wheat germ extract and the level of luciferase expression was compared.

The removal of the terminal leader sequence downstream from conserved Block 2 had little or no effect on translation. Transcripts generated from this pPVS 5' A-LUC deletion construct expressed luciferase at small decreased values of 12.95% and 12.78% in rabbit reticulocyte lysate and wheat germ lysate, respectively, when translation was compared to the level expressed from transcripts with the full PVS 5' leader.

The removal of both Block 2 and the sequence downstream had a more severe effect on the level of translation. Transcripts generated from this larger pPVS 5' B-LUC deletion clone, expressed luciferase at decreased efficiencies of 32.69% in rabbit reticulocyte lysate and 52.7% in wheat germ extract, when translation was compared to the level of
Fig 6.5 A  Graphical representation of results obtained from translation of PVS 5' leader deletions \textit{in vitro} in rabbit reticulocyte lysate. Luciferase translation from PVS 5' leader deletion transcripts is expressed as a percentage of the wild type translation level from transcripts with the full PVS 5' UTR leader. Error bars represent the value for the standard error of the mean expression with each construct. Experiments were carried out in triplicate. PVS 5', potato virus S 5' untranslated leader; PVS 5'A, PVS leader deletion removing the conserved Block 1; PVS 5'B, PVS leader deletion removing Block 1 and a portion of Block 2. These conserved blocks are indicated in Fig 6.1.

Fig 6.5 B  Graphical representation of results obtained from translation of PVS 5' leader deletions \textit{in vitro} in wheat germ extract. Luciferase translation from PVS 5' leader deletion transcripts is expressed as a percentage of wild type translation level with transcripts containing the full PVS 5' UTR leader. Error bars represent the value for the standard error of the mean expression with each construct. Experiments were carried out in triplicate. PVS 5', potato virus S 5' untranslated leader; PVS 5'A, PVS leader deletion removing conserved Block 1; PVS 5'B, PVS leader deletion removing Block 1 and a portion of Block 2. These conserved blocks are indicated in Fig 6.1.
Fig 6.5 A

![Bar chart for Fig 6.5 A showing percentage luciferase expression for constructs PVS 5', PVS 5'A, PVS 5'B, and SYN.]

Fig 6.5 B

![Bar chart for Fig 6.5 B showing percentage luciferase expression for constructs PVS 5', PVS 5'A, PVS 5'B, and SYN.]

---

**Construct**: PVS 5' | PVS 5'A | PVS 5'B | SYN
---|---|---|---
**Percentage Luciferase Expression**: 120 | 100 | 80 | 0
Fig 6.6 A Diagrammatic representation of the construct, pRTS'2-LUC generated for an in vivo analysis of the translational properties associated with the PVS 5' untranslated leader. Components of the construct and restriction sites are indicated. The fragment cloned into pBin19 is also indicated.

Fig 6.6 B Leader sequence of transcripts generated in vivo from the constructs detailed in A. The position of the ATG start codon is underlined. Viral sequence is represented in bold type, plasmid sequence is represented in italics.
A

pRT5'2-LUC

CaMV 35S Promoter  PV5.5' UTR  LUCIFERASE  CaMV 35S Term

DNA Fragment Cloned into pBIN19 to make pBin195'2-LUC

B

pRT5'2-LUC/pBin19-5'2-LUC leader

CTCGAGCTC TAAACACTCCGAAAATAATTTGACTTAACAAACGCGACTGTTCACAAGCAATTACCTACCATG
luciferase expression from transcripts containing the full PVS 5' leader. The level of translation from transcripts containing the mutated version of the PVS 5' A leader, PVS 5' Amut, was the same as the level associated with the wild type deleted sequence. Results are represented graphically in Fig 6.5. All experiments were carried out in triplicate.

6.3 The PVS 5' UTR can function as a translational enhancer in vivo

6.3.1 Constructs

Transient in vivo expression assays were carried out in tobacco protoplasts. For this analysis a construct was needed which contained a transcriptional promoter to drive mRNA synthesis in vivo. A double 35S promoter from cauliflower mosaic virus was used, placed immediately upstream from the PVS 5' UTR-LUC DNA fragment. This plasmid was constructed following the insertion of the SacI/BamHI digested PVS 5' UTR-LUC fragment from pPVS 5' UTR-LUC into SacI/BamHI digested pRTV2-LUC (This plasmid has been discussed in detail in Chapter 5 and contains a VTE-LUC DNA fragment downstream from a double 35S promoter in such an orientation that the luciferase transcript with the VTE sequence as an untranslated leader will be produced in vivo). This cloning event effectively replaced the VTE-LUC fragment with the PVS 5' UTR-LUC fragment. A diagrammatic representation of this construct, designated pRT5'-2-LUC, and the actual leader sequence of the transcript generated in vivo are provided in Fig 6.6. The DNA fragment containing the 35S cauliflower mosaic virus promoter linked to PVS 5' UTR-LUC was isolated as a HindIII fragment from pRT5'-2-LUC and was cloned into HindIII digested and dephosphorylated pBin19 binary vector. This plasmid was constructed so that the translational enhancement properties of the PVS 5' UTR could be investigated in transgenic plants. A diagrammatic representation of this construct designated pBin195'-2-LUC and the sequence of the transcript leader generated in vivo in transgenic plants are provided in Fig 6.6. A pBin19 construct containing a synthetic leader instead of the PVS leader sequence was kindly provided by Neil Bate (University of Leicester) to act as a control in this experiment.

6.3.2 Translational enhancement properties of the PVS 5' UTR in vivo

Equal amounts of supercoiled plasmid DNA (30 μg) of pRTS2-LUC (this plasmid has been discussed in detail in Chapter 5 and contains a double 35S cauliflower mosaic virus promoter linked to a SYNb-LUC fragment in such an orientation that a luciferase transcript with a synthetic untranslated leader will be generated in vivo), and pRT5'2-LUC were transfected into sucrose gradient purified tobacco protoplasts. A control
**Fig 6.7** Graph representing the relative level of luciferase expression from transcripts derived from the constructs pRTS2-LUC and pRTS'2-LUC *in vivo* in tobacco protoplasts. Luciferase activity is expressed as the mean fold enhancement value from pRTS'2-LUC transcripts over the mean fold enhancement value associated with pRTS2-LUC transcripts. Experiments were carried out in triplicate. The rate of GUS activity for individual transfections was divided into corresponding luciferase values to equalise reactions. Error bars represent the standard error of the mean enhancement with each construct.
Fig 6.7

Relative luciferase activity of GUS activity

Construct

pRTS2-LUC  pRTS2'-LUC
<table>
<thead>
<tr>
<th>Construct</th>
<th>Experiment number</th>
<th>Luciferase value/rate of GUS activity</th>
<th>Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRTS2-LUC</td>
<td>1</td>
<td>195593.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3763.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>41160.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>22297.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>16675.5</td>
<td></td>
</tr>
<tr>
<td>pRT5'2-LUC</td>
<td>1</td>
<td>4017063.3</td>
<td>x 20.54</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>58467.6</td>
<td>x 15.54</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>527740.3</td>
<td>x 12.82</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>216240.3</td>
<td>x 9.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>193967.3</td>
<td>x 10.63</td>
</tr>
</tbody>
</table>

Mean enhancement value = 14.1
plasmid, pRTL2-GUS, was also co-transfected with test plasmids in an attempt to equalise transfection reactions, as, if the efficiency of transfection was the same in all reactions the expression of GUS from this plasmid should also be the same. The use of a completely different reporter system for this analysis meant no interference with the reporter system used for the test plasmids. pRTL2-GUS contains the double 35S cauliflower mosaic virus promoter with downstream TEV UTR linked to the GUS reporter gene. Equalising reactions was achieved by dividing the value of the rate of GUS activity per minute for a particular reaction into the corresponding luciferase value obtained in that same reaction.

In vivo in tobacco protoplasts, a DNA construct which drives expression of a mRNA transcript with the PVS 5' UTR sequence as an untranslated leader, expresses luciferase at a mean value 14 times higher than the level expressed from a DNA construct which drives the expression of a luciferase transcript with a synthetic leader as the untranslated leader. The only difference between these two constructs is the nature of the untranslated leader sequence. Results are provided in Table 6.1 and are represented graphically in Fig 6.7. Protoplast experiments were carried out at least in triplicate. An analysis of the translational properties of the PVS 5' UTR in transgenic plants is being carried out as part of another project. Preliminary results have been obtained. Three transgenic tobacco plants have been generated which express LUC RNA with the PVS 5' leader sequence at the 5' proximal end of the transcript molecule. When LUC assays were carried out on these transgenics and the level of expression compared to the level of LUC expression from transgenics expressing the LUC transcript with a synthetic sequence as the UTR, a 13.5 fold increase was found to be conferred by the presence of the viral leader.

6.3.3 The PVS 5' UTR enhances translation in a prokaryotic system in vivo

The translational properties associated with the PVS 5' UTR were also analysed in a prokaryotic system in vivo. Two constructs were used in this experiment, pSYN-LUCb and pPVS 5'-LUC. pSYN-LUCb has been discussed in detail in Chapter 4 (Section 4.2.1) and consists of a synthetic luciferase fragment in a pBluescript vector. pPVS 5'-LUC has been detailed in this chapter (Section 6.2.2) and consists of a PVS 5' UTR luciferase fragment in a pBluescript vector.

Analysis of the sequence of both leaders revealed that with both constructs the ATG initiation codon of the luciferase reporter gene was out of frame with the ATG of the initiation codon of the lacZ protein of the vector. The lacZ promoter in E.coli bacterial cells containing both constructs, was induced using IPTG. Any luciferase produced would presumably be in the form of a fusion protein with the α peptide of the lacZ operon but as the luciferase coding sequence is out of frame in both clones a functional peptide would not be produced. Results from induction assays are provided in Table 6.2. The level of luciferase expressed from the induced bacterial culture containing the pPVS 5'-LUC construct was much higher than the level produced from the bacterial culture containing the pSYN-LUCb construct. With three individual experiments, fold
Table 6.2

Luciferase expression in *E. coli*

<table>
<thead>
<tr>
<th>Construct</th>
<th>Luciferase activity</th>
<th>Fold enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYN-LUCb</td>
<td>40620</td>
<td>X 878.6</td>
</tr>
<tr>
<td>PVS 5'-LUC</td>
<td>35690600</td>
<td></td>
</tr>
<tr>
<td>SYN-LUCb</td>
<td>97030</td>
<td>X 322.5</td>
</tr>
<tr>
<td>PVS 5'-LUC</td>
<td>31302510</td>
<td></td>
</tr>
<tr>
<td>SYN-LUC</td>
<td>271040</td>
<td>X 18462.2</td>
</tr>
<tr>
<td>PVS 5'-LUC</td>
<td>5003995200</td>
<td></td>
</tr>
</tbody>
</table>
enhancements in luciferase activity of 332.5, 878.6 and 18462.2 were observed with constructs containing the PVS 5' UTR over constructs containing the synthetic leader.

6.4 PVS coat protein may bind to a sequence within the 5' untranslated leader of the genomic molecule

The second part of this chapter involved the investigation of leader properties in relation to coat protein binding. One consequence of coat protein binding to the leader could possibly result in the initiation of viral encapsidation. An experiment was carried out to investigate whether or not the PVS 5' leader molecule could direct the encapsidation of a downstream ORF in tobacco protoplasts. If this leader sequence could direct the initiation of RNA encapsidation it is possible that the presence of high levels of coat protein in the cell would result in the down regulation of reporter gene synthesis as a result of transcript molecules being effectively hidden from the cellular translational machinery.

DNA constructs, pRT5'-2-LUC and pRTS2-LUC, were co-transfected into SR1 tobacco protoplasts with clones containing the entire PVS coat protein ORF downstream from a double 35S cauliflower mosaic virus transcriptional promoter. In vivo these clones, designated pWP33 and pWP, produce a functional PVS coat protein, the only difference in them being that the pWP clone will also produce the PVS 3' terminal 11 kDa gene product. These clones were constructed to allow the expression of PVS coat protein in plants and they were provided by Jonathan Weston (Queen's University, Belfast). In vivo transcription and translation of both the LUC ORF and the PVS coat protein ORF will occur but if the PVS 5' UTR contains the OAS for encapsidation we would expect the PVS 5'-LUC transcript to be encapsidated a proportion of the time leaving less transcript available to be translated.

pRTS2-LUC and pRT5'-2-LUC (~15 μg) were transfected separately in individual reactions. The same amount of DNA for each of these two plasmids was also cotransfected with about 30 μg of one of the coat protein plasmids (pWP33 or pWP). pRTL2-GUS was cotransfected into all reactions to equalise transfection efficiencies. The rate of GUS activity per minute was calculated for each reaction and this value was divided into corresponding luciferase values. This experiment was carried out 3 times.

The presence of the PVS coat protein appears to have a dramatic and specific effect on the expression of the clone containing the PVS UTR. A mean decrease of 73.8% was observed in luciferase expression from the clone containing the PVS leader when PVS coat protein was present in tobacco protoplasts. This decrease was not evident with the clone containing the synthetic leader with a corresponding mean increase in luciferase expression of 17.7% when the coat protein was present. Results are provided in Table 6.3.
Table 6.3

<table>
<thead>
<tr>
<th>Construct(s)</th>
<th>Luciferase value/rate of GUS activity</th>
<th>Effect of coat protein on LUC expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
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<tr>
<td>pRT5'2-LUC</td>
<td>1946.70</td>
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</tr>
<tr>
<td>pRT5'2-LUC</td>
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<td></td>
</tr>
<tr>
<td>+ WP33</td>
<td>637.02</td>
<td>67.27% decrease $\downarrow$</td>
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<tr>
<td>pRTS2-LUC</td>
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<tr>
<td>pRTS2-LUC</td>
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<tr>
<td>+ WP33</td>
<td>161.07</td>
<td>28.42% increase $\uparrow$</td>
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<tr>
<td>Experiment 2</td>
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<tr>
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<td>798.87</td>
<td>58.96% decrease $\downarrow$</td>
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<td>pRTS2-LUC</td>
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<tr>
<td>+ WP</td>
<td>153.35</td>
<td>22.3% increase $\uparrow$</td>
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<tr>
<td>Experiment 3</td>
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<td>pRT5'2-LUC</td>
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<td>pRT5'2-LUC</td>
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<td>+ WP33</td>
<td>9675.6</td>
<td>94.9% decrease $\downarrow$</td>
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<td>pRTS2-LUC</td>
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<td>pRTS2-LUC</td>
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</tr>
<tr>
<td>+ WP33</td>
<td>17181.8</td>
<td>3.04% increase $\uparrow$</td>
</tr>
</tbody>
</table>
6.5 Discussion

Plant viral translational enhancers have been discussed in detail in Chapter 1. Several have been identified in recent years since the documentation of the first such leader, the 5' UTR of the AIMV RNA4 subgenomic molecule (Jobling et al., 1987). Whatever the mechanism promoting this translational competence, the presence of these leaders at the 5' end of a mRNA molecule results in an enhanced translation of the transcript. The situation with the picornavirus-like potyviruses, with regard to how these leaders may operate, is perhaps easiest to understand and make conclusions about. Potyviruses possess long 5' UTRs and instead of a 5' proximal cap structure they have a virus encoded protein (VPg) covalently linked to the proximal end of the UTR. The absence of this cap structure means that translation associated with these leaders must occur by an alternative mechanism to the generally accepted "cap-dependent scanning model" for translational initiation proposed by Kozak, (1978). The study of cap-independent translation in plant systems has not really been investigated to any degree but suggestions towards the mechanism that could be involved have come from the animal world. In a eukaryotic system, cap-independent translation appears to operate most efficiently under conditions where cap-dependent translation is inhibited, such as during specific stages of cellular development such as mitosis (Bonneau and Sonnenberg, 1987) and it appears to involve a combination of novel host factors (Meerovitch et al., 1989; Jang et al., 1990; Pestova et al., 1991) with cap-dependent eIF factors (Anthony and Merrick, 1991; Scheper et al., 1992) in association with structural elements within the leader RNA. The situation with the naturally capped enhancers is more difficult to explain. Perhaps by possessing a 5' proximal cap structure these leaders can take advantage of both cap-dependent and cap-independent translational mechanisms. By using both mechanisms viral proliferation could occur under conditions of limited cellular cap-dependent translation. Experiments with the PVS UTR has indicated that translation associated with this leader is less sensitive to the presence of cap analogue than that associated with a synthetic leader in an *in vitro* translation system. This apparent cap-independency suggests that the above situation may be true.

When sequences of the PVS 5' UTR and that of the carlaviruse blueberry scorch virus were aligned two regions of conservation were detected, the 5' most proximal of which was designated Block 1 and the second of which was designated Block 2. This homology indicated that these blocks could possibly be involved in some conserved function, translational enhancement could possibly be associated with one of these blocks. It was on the basis of these conserved sequences that deletions of the 5' UTR were constructed. Results from deletions have revealed that removal of the quite conserved 5' terminal region of the leader appears to have little or no effect on the level of translation. It appears that enhancement properties are largely attributed to conserved Block 2, or at least a portion of it, and the sequence upstream with translation values being decreased by about 50% when it is removed. Even the smallest deletion constructed which contained only Block 1 still enhanced translation considerably over the
level conferred by a synthetic leader so further deletions or mutations in this region will be required to determine the exact sequence responsible for this enhancing activity. In PVX this function has been assigned to a sequence as small as pentanucleotide motif -CCACC- (Tomashevskaya *et al.*, 1993). This exact sequence is not contained within the PVS UTR but a sequence as small as this could be the *cis*-acting domain responsible for translational enhancement.

Experiments have also determined that the PVS 5' UTR enhances translation *in vivo* both in a eukaryotic (Section 6.3.2) and a prokaryotic (Section 6.3.3) environment. These properties have also been reported for the VTE subgenomic enhancer (Chapters 5 and 3) and they suggest that this sequence may be involved in enhanced viral translation and consequently multiplication and spread in a natural environment. The reason why this sequence acts as an enhancer in a prokaryotic environment is difficult to explain. If the PVS 5' UTR contained some sequence which was at one stage essential for prokaryotic translation this would not be expected to be conserved through the evolution from a viral prokaryotic to eukaryotic invader. These sequences could however be involved in chloroplastic translation which utilises prokaryotic like ribosomes, a situation that has been discussed in detail in Chapter 3.

One requirement that all viruses share, both animal and plant, is a need to protect their genetic information. In the susceptible host, either eukaryotic or prokaryotic, viral genomic DNA and especially RNA would be quickly degraded, so to promote genome longevity the viral genetic material is completely wrapped up or “encapsidated” in viral encoded coat protein subunits which effectively protect the nucleic acid from host nuclease attack. Encapsidation may be quite complicated with the involvement of several different coat protein units in the generation of a complex icosahedral capsid molecule, as is the case with the animal picornaviruses. Alternately encapsidation may be quite simple as is the case with TMV whereby single viral encoded coat protein subunits wrap the single-stranded RNA genome with helical symmetry. The mechanism of encapsidation has been extensively studied for many viruses and, rather than being a random event, initiation of coat protein assembly appears to be a specific protein-RNA interaction, of the coat protein unit with a *cis*-sequence or structure on the viral nucleic acid molecule.

Tobacco mosaic virus (TMV) was the first virus for which the actual structure was deduced and since then research has moved at a dramatic rate. TMV is a 6.4 kb RNA molecule coated in helical virus proteins, about 2100 copies of a single 17.4 kDa protein per virus particle. The origin of coat protein assembly initiation (OAS) has been localised to within a portion of the viral genome 900-1300 nucleotides from the 3' end of the molecule (Zimmern and Butler, 1977; Zimmern and Wilson, 1976). This region is quite highly structured particularly in the sequence involved in the formation of a marginally stable hairpin loop structure, designated stem loop 1. It is thought that it is this structural *cis*-acting element that is involved in coat protein recognition and two nearby hairpins appear to be dispensible for this function. Mutational studies have determined that the secondary structure configuration of the loop, rather than the primary RNA sequence, is important for subunit recognition and the function of the loop appears to be in the display of a specific *cis*-acting “signal sequence”, namely -AAGAAGUCG-, in a form that can be
recognised by the coat protein. Oligoribonucleotides derived from this sequence have been shown to bind virus subunits and cause their aggregation into imperfect helices resembling virus particles. Site directed mutagenesis has identified the importance of G residues in this sequence. When the G residues are changed to C nucleotides, protein recognition is severely effected (Turner et al., 1988). Many chloroplastic mRNA molecules contain similar OAS sequences in their 5' leaders which results in the encapsidation of chloroplastic transcripts in TMV coat protein to form "pseudovirion" particles. One such mRNA is the transcript coding for the most abundant chloroplastic protein, ribulose-1,5-bisphosphate carboxylase. It is thought that the formation of these pseudovirion particles may have some function in symptom development (Rochon and Siegel, 1984). Perhaps, during TMV infection, the wrapping up of host transcripts in coat protein decreases the pool available for translation and results in a decrease in protein synthesis, it is this situation that we have artificially mirrored in our encapsidation experiment.

Another example is the encapsidation of the plant virus, turnip crinkle virus (TCV). TCV is a small icosahedral virus with a single-stranded positive-sense RNA genome of about 4051 ribonucleotides in size. The OAS in this virus is a stem loop structure surrounding a UAG termination codon in the viral polymerase gene. Two coat protein binding sites have been identified on this loop and synthetic oligoribonucleotides derived from these sequences bind coat protein in vitro (Wei and Morris, 1992). Any mutations that disrupt the secondary configuration of the stem decrease encapsidation initiation.

The site of encapsidation initiation has been identified for many other viruses and the general rule appears to be a recognition by the coat protein of a structural element on the viral nucleic acid molecule. This situation is the same with the double-stranded RNA spleen necrosis retrovirus (SNV) avian animal virus. With this virus a double hairpin has been identified as recognition sequence for assembly (Konings et al., 1992). Linker scanning mutagenesis has revealed that the stem region of the hairpin, and not the major loop structure, contains conserved 4 ribonucleotide -GAGG- loops, and at least one of these is required for function (Yang et al., 1994). The order of the hairpins appears to be unimportant but both are required for efficient encapsidation.

An experiment with this virus has highlighted the lack of recognition stringency associated with these encapsidation signals. Replacement of the SNV encapsidation signals with the parallel sequence from the closely related virus, Moloney murine sarcoma virus (mMSV) results in the generation of a recombinant virus that replicates well in its host (Yang et al., 1994). This lends evidence to the theory that encapsidation sequences are not strictly virus specific rather recognition operates at the level of secondary or tertiary structure.

The tobaviruses have often been documented for their ability to form pseudorecombinant virus populations with the RNA1 molecule of one virus isolate and the RNA2 molecule of another (Angenent et al., 1989; Robinson et al., 1987). Recently McFarlane et al., (1994), has demonstrated a functional pea early browning virus encapsidated in tobacco rattle virus coat protein. The OAS of both viruses may share a common secondary configuration if not primary sequence.

Two proposals have been suggested to explain the phenomenon of coat protein-mediated
protection. These are that coat protein, or mutated coat protein, could either, interfere with RNA repackaging (de Zoeten and Fulton, 1975), or, inhibit the disassembly of the invading virus (Dodds et al., 1985). Another possible explanation is that exogenous coat protein in the cell could associate at the viral OAS and initiate repackaging before translation or replication can occur.

The region of assembly initiation in carlaviruses is unknown. An indication that this sequence could be contained within the 5' UTR came from some recent evidence that the OAS of the closely related potexvirus, papaya mosaic virus (PMV), was contained within the untranslated leader of the genomic RNA molecule (Sit et al., 1994). In vitro, coat protein assembly with this virus requires a sequence within the 5' proximal 38 ribonucleotides of the genomic UTR. AC residues, in particular the presence of adenine nucleotides, has been implicated as the important residues involved in encapsidation initiation, whether or not any structural elements are involved is at present unknown. Carlaviruses and potexviruses are closely related, with regard to genome structure, translational strategy, protein functions and particle morphology. The only major difference appears to be the presence of an 11 kDa 3' terminal gene in carlaviruses that is not present in potexviruses. The potato virus X (PVX) 5' UTR has been demonstrated to enhance translation of a downstream cistron both in vitro and in vivo (Zelenina et al., 1992, Pooggin et al., 1992) and this phenomenon has now been demonstrated with the PVS 5' UTR. It was therefore plausible to suggest that the PVS OAS could possibly be contained within the 5' untranslated leader of the PVS genomic RNA molecule.

Results have tentatively indicated that the presence of PVS coat protein results in the decrease of expression of transcripts containing the PVS 5' UTR as a 5' untranslated leader in vivo. The expression of cistrons associated with a synthetic leader are not effected by coat protein, in fact a slight stimulation in luciferase translation occurs with the synthetic construct when PVS coat protein is provided. This effect is likely to operate at the translational level as the only difference between the two clones investigated, pRT5'2-LUC (this construct contains the PVS 5' leader) and pRTS2-LUC (this construct contains a synthetic leader), is the nature of the untranslated leader present at the 5' proximal end of the transcript molecule generated from these constructs in vivo. Although no direct evidence has been offered, down regulation of luciferase in association with the PVS 5'-LUC transcript could contain a sequence or structure which promotes initiation of coat protein assembly. Encapsidation of transcript molecules in this in vivo protoplast system could possibly prevent ribosome entry and initiation of translation, hence a decrease in luciferase expression. Research being carried out as part of another project will continue to investigate this phenomenon in vivo and using the deletion constructs discussed above will attempt to isolate the region of the leader responsible for this phenomenon.

As was discussed above, two blocks of conservation have been observed within the carlavirus UTR sequence. Deletions constructed in an attempt to identify the functional region involved in translational enhancement have indicated the involvement of a portion of the second of these conserved regions (Block 2). We could therefore tentatively suggest that the PVS OAS could possibly be contained within the first of these regions of
conservation (Block 1) as this sequence is dispensible with regard to translational enhancement.

In conclusion we can say that the untranslated leader of the genomic RNA PVS molecule enhances translation of a downstream open reading frame \textit{in vitro} and \textit{in vivo}. This enhancement property appears to be a function of all or a sequence contained within the 3' terminal 36 nucleotides of the leader. The mode of translation associated appears to be cap-independent. The function of origin of coat protein assembly may also be a function of this leader sequence.
Chapter 7

Promoter activity for the production of a subgenomic RNA molecule may be associated with the VTE

7.1 Introduction

The VTE sequence consists of the 101 nucleotides upstream from the ATG of the PVS coat protein ORF. When provided as the untranslated leader sequence in a transcript molecule the VTE enhances the expression of a downstream ORF both \textit{in vitro} (Chapter 4; Turner \textit{et al.}, 1994a) and \textit{in vivo} (Chapter 5; Turner \textit{et al.}, 1994b). Eventhough these translational enhancement properties have been associated with the VTE it is not clear where the exact start of the PVS coat protein subgenomic is located. It may be that only a portion of the VTE constitutes the untranslated leader of the smaller PVS subgenomic. The use of conventional methods to map the exact termini, for example by Northern analysis and PCR amplification, have been unsuccessful, largely due to the almost indetectable concentration of the carlavirus subgenomics and due to contamination from fragmented genomic RNA. An alternative approach was to attempt to create an artificial subgenomic molecule which was present at a greater concentration or was easier to detect than the authentic coat protein subgenomic molecule. This approach depends on a \textit{cis}-acting sequence, the subgenomic promoter sequence, being present within the VTE that is recognised by the PVS viral replicase in the production of positive-sense subgenomic molecules from a negative-sense genomic molecule.

This chapter deals largely with experimental attempts to assign the function of subgenomic promoter to the VTE in addition to the function of translational enhancement. The PVS triple gene block products, and the 3' terminal coat protein and 11 kDa protein, are produced from subgenomic RNA molecules 2.5 kb and 1.3 kb in size, respectively (Foster and Mills, 1991a). Subgenomic RNAs are produced by the association of the viral encoded replicase protein, in conjunction with additional host factors, with a specific recognition sequence on the negative-sense genomic molecule. The position of the VTE, just upstream from the coat protein ORF, suggests that this sequence, or an area within it, could possibly be such a region.

One observation that suggested that a replication function could possibly be associated with the VTE came from experiments with the potexvirus, clover yellow mosaic virus (CYMV). The identification of a 1.2 kb defective interfering (DI) particle on polyribosomes from the CYMV infected cells has allowed the analysis of the replication strategy of potexviral genomic RNA (White and Mackie, 1990). It has been determined that this small RNA
molecule is composed of 757 nucleotides of the 5' and 415 nucleotides of the 3' termini of the CYMV genomic RNA molecule, therefore the molecule contains the viral replication signals at both the 5' and 3' ends of the genome. This RNA species can accumulate in virus infected cells. A conserved hexanucleotide sequence, -ACUUAA-, has been identified within these terminal regions and mutations in and around this motif results in the irradiication of DI accumulation. This sequence is not only strongly conserved throughout the potexvirus group but more interestingly, from the point of view of this chapter, the carlavirus group. An identical motif has also been located upstream from the ATG codon of both potex- and carlavirus coat protein ORFs and 25 kDa protein genes of the triple gene block on the negative-sense genomic molecule. It has been proposed that these sequences may act as the promoter recognition sequences for coat protein subgenomic production (White et al., 1992).

Very little is known about the method of subgenomic RNA production. It is thought that the viral replication complex recognises a specific internal sequence on the negative-sense genomic RNA molecule and mediates the production of the complementary positive-sense subgenomic molecule. These proposed subgenomic promoter sequences are located between 8-63 nucleotides upstream from the ATG of the coat protein ORF and between 14-40 nucleotides upstream from the ATG of the 25 kDa ORF. In PVS this hexanucleotide conserved sequence is located 39 nucleotides upstream from the coat protein ATG, within the VTE sequence.

An additional observation that lends support to this theory is the sequence homology that exists between the VTE and the untranslated sequence upstream from the 25 kDa ORF (Foster and Mills, 1991c). This homology has been discussed extensively in Chapter 3. The position of these homologous regions correspond approximately to the 5' terminal sequences of the PVS subgenomic RNA molecules on the basis of size comparisons so it is possible that these upstream regions may contain the cis-acting sequence that is recognised by the viral replicase. In attempting to assign such a function to the VTE the process of positive-sense, single-stranded RNA virus replication has been analysed in detail. An account of some of the aspects of the process will be presented in this section. The potential for the development of engineered viral resistance based on the type of experiments discussed will also be considered.

Molecular strategies to engineer virus resistance in plants usually involves that targeting of one particular feature of the viral life cycle, such as virus cell-to-cell spread or transmission, and the form of resistance obtained, if successful, is usually effective only against the virus to which it has been devised. 75% of plant viruses possess a positive-sense, single-stranded RNA genome, and, as can be imagined, these types of viruses play the major contribution to losses incurred, as a result of virus infection, from an economical point of view. All aspects of the viral life cycle mark potential targets that can be exploited for engineered resistance, none more, perhaps, than the process of viral replication.

An understanding of the process of viral replication is necessary before the type of study described in this chapter can be understood. It is generally accepted that when a positive-sense, single-stranded RNA virus enters a cell, the 5' genomic encoded products are expressed first and these are usually components of the replication complex. This complex,
which is composed of both viral and host encoded products, mediates the conversion of the viral genomic positive-strand to a negative-sense complementary strand. This negative-strand then acts as template for progeny positive-strand synthesis. These molecules are either translated or are incorporated into viral particles. Specific cis-acting sequences at the 3' end of the positive and negative-strands are the target sequences for replicase recognition.

Three models have been proposed to explain the production of subgenomic RNA molecules. The first two, premature termination during negative strand synthesis followed by replication of the negative-sense subgenomic produced (Goelet and Carn, 1982), or nuclease processing of the genomic length RNA (Gonda and Symons, 1978), have largely been discounted. The most favoured theory to explain the production of these RNA species is a process of internal initiation by the viral replicase on the negative-strand genomic RNA (Nassuth et al., 1981).

The nature of the replicase complex and the interaction of this complex with specific sequences on the viral RNA molecule has been the topic of extensive research over recent years. Much is known especially about the tricornavirus, brome mosaic virus (BMV). With this virus a 134 nucleotide long tRNA like structure has been identified at the 3' end of the viral genomic positive-strand as the cis-sequence involved in replicase association in the production of the negative-strand template (Miller et al., 1986). Unlike BMV, alfalfa mosaic virus (AMV) does not possess a tRNA like structure at the 3' end, but the cis-sequence responsible for negative-strand synthesis has been located within the 3' terminal 210 nucleotides, and the region responsible for progeny strand synthesis from negative-strand template has been located within the 5' terminal 169 nucleotides (Van der Kuyl et al., 1991a). Viral coat protein appears to play a regulatory role in synthesis ratio of positive and negative molecules (Van der Kuyl et al., 1991b).

Miller et al., (1985), has also demonstrated the production of the BMV RNA4 subgenomic molecule by a process of internal initiation from the negative-sense RNA 3 species. The functional region responsible for this internal initiation process is at the 3' end of the negative-strand and consists of about 62 bases grouped into four functional domains. Sequences with homology to one of these domains, a -UUUAUUUU- box, have been identified within sequences upstream from the subgenomic initiation sites of other plant viruses (Marsh et al., 1988). Homology within this region is also evident with the parallel sequence of the animal alphaviruses (Ou et al., 1982) which indicates that this box could have some functional importance.

The RNA-dependent RNA polymerase complex from several viral infected cells has been isolated and its activity demonstrated in vitro. The soluble replicase complex isolated from cucumber mosaic virus (CMV) infected Nicotiana tabacum plants is capable of replicating CMV RNA (Hayes and Buck, 1990). The replicase complexes from turnip yellow mosaic virus (TYMV) and BMV are capable of replicating TYMV and BMV RNA, respectively (Mouches et al., 1975; Miller and Hall, 1983). The involvement of two BMV encoded non-structural proteins, P1 and P2, in the replication complex has been demonstrated (Quadt et al., 1988) but much less is known about the nature of the host component, or components.

As was stated earlier, the major aim of this chapter was to investigate the activity of the VTE
Fig 7.1 A Positions of the putative subgenomic RNA promoters on the PVS genomic RNA molecule. The genomic viral RNA, positive and negative-strands, is represented as a solid line. The subgenomic RNA promoter sequence is represented as a solid box. Subgenomic RNA molecules, produced via the activity of the viral replicase, are presented as dotted lines.

Fig 7.1 B A diagrammatic representation of the activity of a negative-sense transcript encoding the luciferase gene in an antisense orientation with a subgenomic RNA promoter sequence at the terminal 3' end of the molecule, in both healthy and infected leaves. This negative-sense transcript is represented as a dotted line. The positive-sense transcript, produced in infected tissue due to the activity of the viral replicase, which acts as template for luciferase expression, is represented as a solid line.
Positive-Sense Viral Genomic RNA

\[ \text{5'} \rightarrow \text{SGP} \rightarrow 25K \rightarrow 12K \rightarrow 7K \rightarrow \text{SGP} \rightarrow \text{Coat Protein 33K} \rightarrow \text{poly A} \rightarrow \text{3'} \]

Negative Sense

\[ \text{3'} \rightarrow \text{SGP} \rightarrow \text{SGP} \rightarrow \text{5'} \rightarrow \text{poly A} \rightarrow \text{3'} \]

Viral Replication Complex

Positive-Sense Subgenomic RNA Molecules

\[ \text{5'} \rightarrow \text{poly A} \rightarrow \text{3'} \]

B

\[ \text{35S} \ | \ \text{LUC} \ | \ \text{SGP} \]

Healthy Leaf

\[ \text{5'} \rightarrow \text{Negatiive-Sense} \rightarrow \text{3'} \]

Infected Leaf

\[ \text{5'} \rightarrow \text{Negatiive-Sense} \rightarrow \text{3'} \]

NO Luciferase Activity

\[ \text{3'} \rightarrow \text{Positive Sense} \rightarrow \text{5'} \]

Viral Replicase

\[ \text{HIGH Luciferase Activity} \]
sequence in relation to viral replication. The VTE, being positioned upstream from the PVS coat protein gene, in a region that corresponds approximately to the proposed terminal 5' end of the smaller 1.3 kb subgenomic RNA molecule, could possibly behave as a cis-acting subgenomic promoter sequence for replicase recognition. To analyse this theory constructs were made which would generate an antisense VTE-LUC transcript, in vivo. In this transcript the VTE sequence exists in a negative orientation at the 3' terminal end of the molecule, a form in which it should, theoretically, be recognised by the PVS replication complex, according to the favoured proposal for subgenomic RNA production offered by Nassuth et al., (1981).

In healthy plant tissue this negative-sense transcript will be produced but the reporter gene encoded by the transcript will not be expressed. In infected tissue, or in the presence of the viral replicase complex, if the VTE is a subgenomic promoter sequence, the complex will bind to the VTE or a cis-acting sequence within it to mediate the conversion of this negative-strand to a positive-sense transcript. The reporter gene encoded by the transcript will subsequently be expressed. A diagrammatic representation of this process has been presented in Fig 7.1.

In addition to the VTE sequence another viral sequence was also included in experiments. This was the 400 base pair region upstream from the ATG of the 12 kDa protein of the pea early browning virus (PEBV) RNA1 molecule. This region of PEBV was chosen because infectious clones were available for both RNA molecules. Other reasons for the choice were that the 12 kDa PEBV subgenomic molecule has only been detected in trace amounts, a situation paralleled in carlaviruses, and more importantly that the PEBV replicase can operate efficiently in trans, that is the replicase protein encoded on RNA1 can replicate the RNA2 molecule. This latter consideration would be important if a mechanism as described above was to be successful. This region is thought to contain the cis-sequence required for the production of a subgenomic RNA molecule to allow the expression of this protein (MacFarlane et al., 1989). Constructs were made with this sequence similar to those described above with the VTE.

The tobavravirus group of plant viruses has only three known members, PEBV, tobacco rattle virus (TRV) and pepper ringspot virus (PRV) (Robinson and Harrison, 1985a; 1985b; Robinson, 1983). Tobavraviruses forms a specific interactions with several species of soil inhabiting plant parasitic nematodes, which act as vectors for transmission. The virus does not appear to replicate in the vector but can be retained in a transmissible form for months or even years (van Hoof, 1970). Virus particles take the form of rigid rods with a particle diameter of 21-23 nm and two predominant particle lengths, designated long and short, of 50-115 nm and 210 nm, respectively (Harrison and Robinson, 1978). Particles contain two distinct single-stranded, positive-sense RNA molecules.

The complete nucleotide sequence of both PEBV RNA species, RNA1 and RNA2, have been determined. It has been observed that RNA1 can replicate independently from RNA2 but infections with RNA1 alone do not result in the production of characteristic viral particles, this suggests that RNA1 encodes the replicative function and RNA2 encodes the viral coat protein that is responsible for viral RNA encapsidation. RNA1 is 7073 nucleotides in length and contains four ORFs (MacFarlane et al., 1989), encoding a 5' proximal 141 kDa
peptide, a 201 kDa product produced by translational readthrough of the termination codon of the 141 kDa product (Hughes et al., 1976), a 30 kDa protein and a 12 kDa product. It is the sequence upstream from this protein that has been used in constructs to analyse the activity of subgenomic promoters. The two larger products are the putative replicase proteins and the 30 kDa product is the cell-to-cell spread protein. The function of the 12 kDa product is still a matter of speculation. RNA2 is 3374 nucleotides in length and contains three ORFs (Goulden et al., 1990). Encoded proteins are the viral coat protein, a 29.6 kDa product and a 23 kDa protein.

Results presented concern the activity of two viral sequences, the PVS VTE sequence and the PEBV sequence, in relation to the process of replication. In light of these results, implications regarding the potential for engineered viral resistance will be discussed.

### 7.2 Constructs

Constructs were built that would promote the expression of antisense reporter gene RNA transcripts with a viral subgenomic promoter sequence at the 3' terminal end of the molecule, *in vivo*. Expression of the reporter gene encoded by the transcript should only occur if the viral replicase complex binds to this promoter region and converts this antisense transcript to a positive-sense form (Fig 7.1). Two antisense reporter constructs were generated. Both contained the luciferase reporter gene in an antisense orientation. At the 3' end of this antisense reporter gene coding sequence one construct contained the PVS VTE sequence and the other contained the subgenomic promoter sequence upstream from the 12 kDa protein gene of the PEBV RNA1 molecule. A diagrammatic representation of these constructs is presented in Fig 7.2.

A construct designated pAnti-VTE-LUC, was generated by the insertion of a double 35S cauliflower mosaic virus transcriptional promoter into the plasmid pVTE-LUCb. This plasmid is basically the same as pVTE-LUC (Section 4.2) except for the absence of a 35S transcriptional terminator at the 3' end of the luciferase coding sequence. The 35S cauliflower mosaic virus transcriptional promoter was isolated as a KpnI, HindIII fragment from a plasmid, pWP83 (Wyatt Paul, Leicester University), and was cloned into pVTE-LUCb digested with the same enzymes. The orientation of the promoter was such that an antisense transcript, containing the luciferase coding sequence with the VTE at the 3' end, would be produced *in vivo*. Diagrammatic representations of the construct pAnti-VTE-LUC is provided in Fig 7.2.

This entire insert from pAnti-VTE-LUC was isolated as a SacI, KpnI fragment and was cloned into pBin19 digested with the same enzymes. The construct generated was designated pBin-Anti-VTE and is represented diagrammatically in Fig 7.2. This construct was used to transform tobacco plants using *Agrobacterium*-mediated leaf disc transformation.

The PEBV subgenomic promoter sequence upstream from the 12 kDa ORF of the RNA1 genomic molecule was isolated by PCR amplification from a clone, pCAN1, which contained
**Fig 7.2** Diagrammatic representation of the constructs used to determine the activity of antisense transcripts containing subgenomic RNA promoter sequences, *in vivo*. VTE, viral translational enhancer; SGP, subgenomic promoter; PEBV, pea early browning virus. The orientation of the coding sequence of the luciferase ORF is indicated by an arrow, as is the direction of transcription from the 35S cauliflower mosaic virus (CaMV) promoter.
the entire PEBV RNA1 sequence, and which had been shown to be infectious in vivo. This clone was kindly provided by Dr. Stewart MacFarlane at SCRI (MacFarlane et al., 1989). The sequence was isolated using the primers, 5'-GGTCTAGAGGCATGCGACAATAC-TTC-3' as the 5' primer and, 5'-CGGATCCATGGTTTCTTTTTAATGCTCCCAG-3' as the 3' primer. The total sequence isolated extended over a region of about 400 base pairs upstream from the ATG of the PEBV 12 kDa protein coding sequence (MacFarlane et al., 1989). The PCR product was digested with XbaI and BamHI and was cloned into pBluescript-SK(+), digested with the same enzymes. Clones containing appropriately sized inserts were partially sequenced, about 200 nucleotides of sequence was obtained corresponding to the region immediately upstream from this initiation codon, using a double-stranded DNA template and the dideoxynucleotide chain termination method of Sanger, (1977). This precaution ensured that the sequence did not contain any PCR generated errors. The plasmid generated was designated pPEBV.

The luciferase reporter gene was cloned downstream from this PEBV sequence. Luciferase was isolated from pVTE-LUC (Section 4.2) as an NcoI, BamHI fragment and was cloned into pPEBV digested with the same enzymes. This event takes advantage of the Ncol restriction enzyme site engineered into the PEBV 3' primer. The resultant plasmid was designated pPEBV-LUC.

The insert from pPEBV-LUC was isolated as a SacI, BamHI fragment and was cloned into pAnti-VTE-LUC digested with the same enzymes. This cloning event effectively replaced the VTE-LUC fragment in this construct with a PEBV-LUC fragment. This plasmid, designated pAnti-PEBV-LUC, was designed to drive the expression of an antisense PEBV-LUC transcript in vivo, and is represented diagrammatically in Fig 7.2.

The insert from pAnti-PEBV-LUC was isolated as a SacI, KpnI fragment and was cloned into pBin19 digested with the same enzymes. This construct, pBin-Anti-PEBV, is presented diagrammatically in Fig 7.2. This construct was used to transform tobacco leaf discs using Agrobacterium-mediated transformation.

### 7.3 Expression of a VTE antisense reporter construct in vivo

The activity of pAnti-VTE-LUC was analysed, in vivo, by bombardment of DNA into leaf tissue using a ballistic microprojectile gun. Three types of experimental tissue were used in bombardment experiments, healthy tissue cultured Samsun tobacco leaf tissue, healthy greenhouse grown Desiree potato leaf tissue, and PVS infected Desiree potato leaf tissue. Three individual experiments were carried out and the reporter activity of pAnti-VTE-LUC bombarded into PVS infected tissue was compared to the reporter activity on bombardment into healthy tobacco (Table 7.1, Experiments 1), or the activity on bombardment into healthy Desiree potato tissue (Table 7.1, Experiment 3).
pRTS2-LUC (Section 5.2), a plasmid which drives the expression of a sense luciferase transcript with an untranslated leader derived from a synthetic polylinker in vivo, was also bombarded into the various tissues detailed above. The inclusion of this plasmid in experiments allowed the efficiency of translation in the various tissue systems to be compared. A pRTL2-GUS (Section 5.2) was also co-bombarded with test plasmids in an attempt to equalise the efficiency of individual firings. The values obtained for the rate of GUS activity with each firing event were divided into corresponding luciferase values.

Results did indicate that the relative luciferase expression from pAnti-VTE-LUC was higher in PVS infected leaf tissue than in the control uninfected leaf tissues. With the first experiment, the expression of pRTS2-LUC in PVS infected tissue was 7.3 times lower than the expression of this construct in healthy tobacco leaf tissue. The expression of pAnti-VTE-LUC, however, was 1.7 times higher when circular DNA was bombarded, and approximately the same when linear DNA was bombarded, in PVS infected tissue rather than in healthy tobacco tissue. The large decrease in pRTS2-LUC expression in infected tissue may be a result perhaps of host translational down-regulation mediated by the presence of the virus. This same translational down-regulation would also be expected with the expression of the pAnti-VTE-LUC construct, this was not observed. It is possible also that this relative increased expression due to the presence of the VTE sequence is simply a result of the enhanced translational competence of transcripts containing the VTE as the untranslated leader in a viral infected environment, a phenomenon that has been discussed in Chapter 5.

When a more comparable control tissue was used, healthy Desiree potato leaf tissue, this same inhibition in pRTS2-LUC expression in PVS infected tissue was observed. Luciferase expression from this construct in infected leaf was 3 times lower than the level of expression in healthy tissue. The level of luciferase expression from pAnti-VTE-LUC was, however, observed to be the same in both leaf tissue systems.

In conclusion, the luciferase from a reporter construct which drives the expression of an antisense construct with the VTE sequence at the 3' end of this RNA molecule in PVS infected tissue, was observed to be either higher than or comparable to the level of expression in either healthy tobacco or potato tissue. The expression of a construct which drives the expression of a sense luciferase construct in vivo was strongly inhibited in the PVS infected environment. Results are presented in Table 7.1. All values represent the mean value for 3 individual bombardment experiments.

Transgenic SR1 tobacco plants were generated by *Agrobacterium* -mediated leaf disc transformation using the pBin-Anti-VTE construct. These transgenic plants were generated to determine if this attenuation of viral replication observed in firing experiments also occurred at the whole plant level. Primary transformants were generated and the presence of the luciferase coding sequence in the plant genome was confirmed following PCR analysis of a crude total nucleic acid extract, for each plant, using primers designed to amplify a sequence within the luciferase gene. On this basis, 6 positively transformed plants were identified.
Table 7.1

Expression of pAnti-VTE-LUC bombarded into plant tissue *in vivo*

**EXPERIMENT 1**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Luciferase Value / Rate of GUS Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRTS2-LUC</td>
<td>16493.9</td>
</tr>
<tr>
<td>pAnti-VTE-LUC (circular)</td>
<td>487.3</td>
</tr>
<tr>
<td>pAnti-VTE-LUC (linear)</td>
<td>468.2</td>
</tr>
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</table>

**TISSUE TYPE: HEALTHY SR1 TOBACCO LEAF TISSUE**

**TISSUE TYPE: PVS INFECTED POTATO TISSUE**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Luciferase Value / Rate of GUS Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRTS2-LUC</td>
<td>2252.4</td>
</tr>
<tr>
<td>pAnti-VTE-LUC (circular)</td>
<td>836.8</td>
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<tr>
<td>pAnti-VTE-LUC (linear)</td>
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</table>

**EXPERIMENT 2**

**TISSUE TYPE: HEALTHY DESIREE POTATO LEAF TISSUE**

<table>
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<th>Construct</th>
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</thead>
<tbody>
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<td>4861.1</td>
</tr>
<tr>
<td>pAnti-VTE-LUC</td>
<td>64.3</td>
</tr>
</tbody>
</table>

**TISSUE TYPE: PVS INFECTED DESIREE POTATO TISSUE**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Luciferase Value / Rate of GUS Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRTS2-LUC</td>
<td>1604.5</td>
</tr>
<tr>
<td>pAnti-VTE-LUC</td>
<td>74.3</td>
</tr>
</tbody>
</table>
These plants were maintained in tissue culture.

Further research with these transformants is being carried out as part of another project. These plants will be infected with the potexvirus, PVX (a virus that is closely related to PVS, especially displaying sequence homology within the viral replicase gene). This virus will be used because PVS does not infect SR1 tobacco. Transgenic *Nicotiana debneyii* plants expressing the antisense luciferase transcript are at present also being generated. These plants will be infected with PVS, *N. debneyii* being used because it is a host plant for this virus. The effect of viral infection on luciferase expression will be monitored over a period of several days.

### 7.4 The expression of a PEBV antisense construct *in vivo*

Similar to the experiments described above with pAnti-VTE-LUC, the pAnti-PEBV-LUC construct was bombarded into greenhouse grown *Nicotiana benthamiana* leaf tissue. The construct was either bombarded alone or co-bombarded with a pCAN1 construct (MacFarlane *et al.*, 1989), an infectious clone of PEBV RNA1 which produces the PEBV viral replicase *in vivo*. *N.benthamiana* tissue was used because this is a host plant for the PEBV virus.

Again, as a type of control to monitor the efficiency of translation in the presence or absence of the pCAN1 construct, the pRTS2-LUC (Section 5.2) plasmid was used. With all bombardments pRTL2-GUS (Section 5.2) was co-bombarded in an attempt to equalise the efficiency of individual firing events. The rate of GUS activity for each bombardment was divided into the corresponding luciferase value.

Results indicate that the level of luciferase expression *in vivo* in *N.benthamiana* is highest when the pCAN1 plasmid is present. The expression of pRTS2-LUC both in the presence and absence of this infectious clone were comparably equal with the presence of the pCAN1 clone stimulating luciferase expression by as little as 0.14 fold. The level of luciferase expression from pAnti-PEBV-LUC, however, was 2.8 or 2.4 times higher when co-bombarded with pCAN1 than when bombarded on its own (Experiments 1 and 2, respectively).

This suggests that the presence of the viral PEBV subgenomic promoter sequence may result in the elevated expression of luciferase from a clone that produces an antisense reporter gene transcript *in vivo*.

Transgenic SR1 tobacco plants have also been generated by *Agrobacterium*-mediated leaf disc transformation using the pBin-PEBV-Anti construct. The presence of the luciferase gene in a crude DNA extract from resulting plants was checked by PCR amplification using primers specifically designed to amplify the PEBV subgenomic promoter sequence (the sequence of these primers are presented in Section 7.2). Positive plants, those giving an approximately 400 base pair sized band after PCR were maintained and cloned in tissue culture (results not presented).
Table 7.2

Expression of pAnti-PEBV-LUC bombarded into plant tissue *in vivo*

**EXPERIMENT 1**

<table>
<thead>
<tr>
<th>TISSUE TYPE: N. BENTHAMIANA LEAF TISSUE</th>
<th>Construct</th>
<th>Luciferase Value / Rate of GUS Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pAnti-PEBV-LUC</td>
<td>3777</td>
</tr>
<tr>
<td></td>
<td>pAnti-PEBV-LUC + pCAN1</td>
<td>10636.7</td>
</tr>
</tbody>
</table>

**EXPERIMENT 2**

<table>
<thead>
<tr>
<th>TISSUE TYPE: N. BENTHAMIANA LEAF TISSUE</th>
<th>Construct</th>
<th>Luciferase Value / Rate of GUS Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pRTS2-LUC</td>
<td>4671.3</td>
</tr>
<tr>
<td></td>
<td>pRTS2-LUC + pCAN1</td>
<td>5335.4</td>
</tr>
<tr>
<td></td>
<td>pAnti-PEBV-LUC</td>
<td>56.1</td>
</tr>
<tr>
<td></td>
<td>pAnti-PEBV-LUC + pCAN1</td>
<td>137.2</td>
</tr>
</tbody>
</table>
Further research with these plants is being carried out as part of another project. These plants will be infected with TRV, another tobravirus that is closely related to PEBV. pBin-PEBV-Anti is at present also being transformed into *N. benthamiana*, a host for PEBV with transformants being infected with PEBV. With both transformants types the effect of virus infection on the level of luciferase expression over the time of the experiment will be monitored.

### 7.5 Discussion

Although the results presented in this chapter are only preliminary, they do indicate that the VTE may, in addition to possessing a translational enhancement function, also be the *cis*-acting sequence involved in subgenomic RNA production. Only transient data has been presented here, the picture will hopefully be clearer when results from transgenic plants becomes available. The initial reason for producing artificial subgenomics *in vivo* was to map the termini of the authentic carlavirus coat protein subgenomic but unfortunately results have indicated that if these artificial molecules are being produced at all it is in very low concentrations, too low to allow the terminal sequence to be mapped. An alternative strategy will have to be employed to achieve this aim.

The theory behind experiments depends on the rule that antisense RNA cannot be converted into sense RNA in a normal cellular plant environment. Positive-sense, single-stranded RNA viruses do, however, encode a replicase protein which forms a complex with host factors to mediate the conversion of negative (an intermediate of the replication process) to positive sense RNA.

When the VTE sequence was incorporated into a construct that would generate a negative-sense luciferase transcript with this leader at the 3' terminal end, *in vivo*, the level of luciferase expression in infected potato tissue was not significantly higher than the level of expression in healthy potato or tobacco tissue. The expression levels observed were either the same or enhanced only slightly in infected tissue, that is in the presence of the viral replicase. One important observation was, however, that the expression of a sense luciferase transcript was much higher in healthy, either potato or tobacco tissue, by an average factor of 3 and 7.3, respectively. The reason for this difference in expression in healthy and infected tissue may reflect the generally poor state of the plant tissue as a result of the viral infection but, this being the case a similar decrease would be expected with the antisense construct also, this was not observed. It is also possible that in addition to, or instead, of conversion from antisense to sense RNA, the relative increase in expression may be due to an increased translational competence of the VTE-LUC transcript in the viral infected environment. This phenomenon has been discussed at length in Chapter 5.

With PEBV a similar pattern emerges. An infectious clone of RNA1 of PEBV (the molecule that encodes the viral replicase) was co-bombarded with the PEBV-LUC antisense construct. The use of this clone overcomes the problem of different levels of expression in healthy and infected tissue, as observed above with the VTE experiments. The presence of the infectious
clone resulted in about a 3-4 fold increase in the level of luciferase expression.

In conclusion, in a transient *in vivo* system, the presence of both viral sequences, in a negative form in an antisense reporter gene transcript, appears to result in enhanced gene expression in the presence of the viral replicase but not in its absence. The theory to explain this enhancement is that the viral replication complex binds to these viral regions in the conversion from negative to positive transcript, a form that can be translated in the cell. Whether or not this process will operate at the whole plant level is not clear at present but if these experiments prove successful this mechanism could be developed for the engineering of viral resistance.

Many examples of targeted viral resistance at the stage of replication have been documented. Huntley and Hall, (1993), have reported that the co-inoculation of barley protoplasts with RNAs 1 and 2 of BMV with excess antisense RNA to the 3' terminal tRNA structure, results in a reduction in RNA accumulation by 45% and 90%, respectively. This supports earlier results by Ahlquist et *al.*, (1984) which demonstrated the inhibition of BMV negative-strand synthesis, *in vitro*, by the addition of antisense cDNA sequences. Inhibition of TMV replication is only effective when the 3' tRNA like structure is targeted by a hybrid arrest strategy (Powell *et al.*, 1989). It is presumed that these sequences bind to complementary regions of the viral genome and prevent the mediation of the *cis*-acting sequences that are associated with these regions. It is likely that a resistance of this type would be effective in the reduction of virus replication but the resistance would not be absolute, with a degree of multiplication still occurring and it would only be effective when the inoculum concentration is low.

Based on results with the animal Sindbis virus, Olivo *et al.*, (1994), have suggested a mechanism based on replication to facilitate viral detection. A cell line was transformed in such a way that a defective Sindbis virus genome (defective in replication function), which contained the luciferase ORF downstream from the viral subgenomic promoter, was expressed under the influence of the Rous sarcoma virus promoter. This cell line expresses high levels of luciferase when infected with the non-defective form of the virus. It is this type of mechanism that is being employed as a potential strategy for virus resistance in this chapter, that is the use of subgenomic RNA promoter sequences to drive expression of a downstream ORF only when that virus is present in the cell.

The possibility of a broad spectrum resistance against many viruses is appealing. The mechanism detailed here involves the expression of a gene from an antisense transcript that will only be translated into a functional protein, *in vivo*, if the viral replicase to which the resistance has been engineered binds to and copies the antisense transcript into a positive-sense form. The implications of the functional operation of such a process are far reaching.

One possible situation that could be of importance is the use of these replication promoter sequences to allow the expression of a factor that would initiate cell death or have some antiviral activity. At face value the prospect of expressing a cell-cytotoxic gene in a plant would appear to be disadvantageous, but if some form of switch was available which would
promote the expression of the factor only when the virus was in the cell a hypersensitive response could be mimicked with only the initially infected cells and those immediately surrounding being destroyed. This would theoretically have the effect of containing the infection and preventing spread throughout the plant. A switch of this description, based on the preliminary results presented, could be presented by antisense transcripts containing subgenomic promoter sequences. If such a cell-cytotoxic factor was incorporated into an antisense construct like those described above with the luciferase ORF, then its synthesis would only be initiated if the viral replicase protein, and consequently the virus, was present in the cell. The cytotoxic factor would only be produced if conversion from negative to positive message had occurred. The results presented in this chapter with the luciferase reporter gene only tentatively suggest that this form of resistance could be operational in a transient system. Experiments with transgenic plants producing such negative-sense transcripts will determine whether or not this form of resistance could possibly occur at the whole plant level.

It appears that one vital factor contributing towards promoter recognition by the viral replicase complex is the nature of the 5' and 3' terminal sequences. Wu and Kaper, (1994) have demonstrated the inability of the replicase to recognise the 3' promoter sequence of the negative-stranded RNA of cucumber mosaic virus satellite RNA in the absence of a terminal guanosine residue. This residue is not part of the viral sequence but is thought to be incorporated during the synthesis of the negative-strand. This indicates that the sequence required for promoter recognition may be very specific and may depend on secondary structure rather than primary sequence. The clones described in this chapter could possibly be modified to possess the exact subgenomic promoter sequences that exist in nature. Another problem is the high background level of luciferase expression in the absence of the viral replicase. For this system to be successful, that is if a cell cytotoxic gene was being used, the background expression should be absolutely zero to avoid any harm to the plant.

To achieve this novel form of resistance these problems will have to be tackled but if successful it could prove to be extremely important in the engineered resistance to positive-sense, single-stranded RNA viruses and the elevation of their contribution to the economical losses incurred.
Chapter 8
Discussion

The results presented in this thesis provide a further insight into the molecular characteristics of members of the carlavirus group of plant viruses, with concentration being placed on potato virus S (PVS). PVS has a positive-sense, single-stranded RNA genome, 7.5 kb in size. The genomic molecule is encapsidated in individual coat protein subunits (33 kDa), forming a slightly flexuous rod shaped viral particle, 610-700 nm in length (Wetter and Milne, 1981).

The genome organisation and expression of carla- and potexviruses are very similar with the major difference being the presence of a 3' terminal ORF in carlaviruses that is absent from potexviruses. Both possess a 5' proximal gene encoding the replicase protein, a characteristic block of three proteins, the triple gene block, and the viral coat protein gene which is 3' terminal in potexviruses. It is thought that, with both groups, internal gene products which would normally remain silent in a eukaryotic system, are produced through the intermediacy of subgenomic RNA molecules. Coat protein subgenomic RNA molecules have been detected for several members of both groups including the potexviruses white clover mosaic virus (WCMV), clover yellow mosaic virus (CYMV) and narcissus mosaic virus (NMV) (Forster et al., 1988; Bendena et al., 1987; Short and Davis, 1983) and the carlaviruses PVS and HelVS (Foster and Mills, 1991a; 1990b). Larger subgenomic molecules whose 5' proximal end, by size comparison, would approximately correlate to the initiation site of the 25 kDa protein gene of the triple gene block, have also been detected for the potexviruses CYMV and PVX (Bendena et al., 1987; Dolija et al., 1987). With daphne X potexvirus, Guilford and Forster (1986) have detected five subgenomics in infected leaves, perhaps allowing the expression of the two internal triple gene block proteins. It is, however, generally accepted that these proteins are produced by some alternative mechanism such as translational frameshifting or readthrough. Carlavirus subgenomics of corresponding size have also been detected for several members (Foster and Mills 1991a; 1990b). One difference between carla- and potexviruses in relation to their subgenomic RNAs is their concentration. Bendena et al., (1987) and White and Mackie, (1990) have emphasised the relative abundance of the two CYMV subgenomics on the polyribosomal-RNA fraction from CYMV infected tissue. It is this high concentration that has enabled the mapping of the exact terminal residue of the subgenomics of some potexviruses. This is in contrast to the situation with carlaviruses where subgenomics are extremely difficult to detect if this is possible at all.

The majority of the work presented in this thesis will concern the region upstream from the initiation codon of the coat protein ORF in PVS. As with many other carlaviruses, two subgenomic RNA molecules have been detected in infected plant tissue, 2.5 kb and
1.3 kb in size (Foster and Mills, 1991a). PVS subgenomics have been detected only in trace amounts in viral infected tissue, but a subgenomic encoded product, the coat protein, is produced in the large quantities required to meet the demands of viral encapsidation (Foster and Mills, 1990a; 1992b). As was stated above, this is in contrast to the situation with potexviruses where the subgenomics have been detected at high levels (Bendena et al., 1987; White and Mackie, 1990). This observation has led to the proposal that the smaller PVS coat protein subgenomic molecule is translated with such efficiency that only small amounts are required for the production of sufficient quantities of coat protein product. An observation that complicates the story even further is that subgenomic RNA molecules have not, as yet, despite attempts, been detected at all for the carlaviruses CLV but viral coat protein is still one of the primary products of translation in vitro and it is produced at high levels (Meehan and Mills, 1991). In light of this another explanation regarding carlaviruses coat protein expression is that some form of internal initiation of translation occurs on the full length genomic molecule or on the larger 2.5 kb subgenomic molecule.

To analyse the phenomenon of the existence of an efficiently translated carlaviruses coat protein subgenomic, the activity of the untranslated sequence upstream from the ATG of the PVS coat protein ORF was analysed. If efficient translation is occurring, a likely candidate responsible for this enhanced expression activity is the leader sequence of the 1.3 kb subgenomic RNA molecule. Results presented in this thesis have revealed that the 101 nucleotides immediately upstream from the coat protein ORF, or viral translational enhancer (VTE) as it has now been referred to, enhances the translation of a downstream cistron when provided as the untranslated leader in a transcript molecule. This enhancement was evident in vitro in rabbit reticulocyte and wheat germ lysate (Chapter 4; Turner et al., 1994a), and also in vivo when bombarded into leaf and pollen tissue, in tobacco protoplasts and in transgenic plants (Chapter 5; Turner et al., 1994b). From a biotechnology viewpoint the translational enhancement properties conferred by the VTE in transgenic plants could potentially be quite useful. As has been stated in Chapter 1, many plant viral translational enhancers have been documented (Turner and Foster, 1995), but only two of these, the 5' leader sequence from tobacco etch virus (TEV) (Carrington et al., 1990) and the 5' leader sequence from tobacco mosaic virus (TMV) (Kang et al., 1994), have been shown to operate at the transgenic plant level. The VTE sequence could be used to enhance the expression of a foreign gene in a transgenic plant.

The exact terminal residue of the potexviruses CYMV and PVX coat protein subgenomics have been determined (White and Mackie, 1990; Sit et al., 1990). Both terminate with a guanosine residue within the consensus sequence CUCGAA. For PVX this terminal residue is only 5 nucleotides upstream from the coat protein ATG initiation codon. This distance is 10 residues for CYMV. The abundance of potexvirus subgenomics has facilitated the mapping of the termini by standard primer extension methods. Contrary to this, attempts by myself and others to map the exact termini of either carlaviruses subgenomic molecule have proved unsuccessful (Foster, 1990d).
reason for this difficulty is the almost indetectable concentration of these small RNA molecules. Any attempt to map the termini appears to be hindered by contamination from larger fragmented genomic RNA.

Before these terminal residues had been identified, Rupasov et al., (1989) had suggested that, due to sequence comparisons, the potexvirus subgenomic molecules would terminate at this guanosine residue within the consensus described above at the 5’ terminus. In particular, a GAAA tetranucleotide box was identified which is conserved throughout the potexvirus group upstream from the ATG codons of both the coat protein ORF and 25 kDa protein ORF of the TGB. Conservation of this box was also found within the parallel sequences of some carlaviruses. With the carlavirus, PVM such a box has been identified 12 base pairs upstream from the AUG initiation codon of the 34 kDa coat protein gene and a similar GAAU box 8 bases upstream from the AUG of the 25 kDa ORF. This GAAA tetranucleotide sequences have also been identified 20 nucleotides upstream from the PVS coat protein ORF so it may be logical to suggest that this sequence also marks the termini of carlavirus subgenomics. If this is the case the 1.3 kb subgenomic untranslated leader would only consist of the 3’ terminal 20 nucleotides of the VTE sequence.

The presence of the VTE 101 nucleotide sequence upstream from the ATG of the PVS coat protein ORF, when provided as the untranslated leader of a transcript molecule enhances the expression of a downstream gene both in vitro and in vivo (Turner et al., 1994a, 1994b). The enhancement property associated with this leader may possibly explain, at least in part, the efficiency of expression of the PVS coat protein subgenomic and perhaps carlavirus subgenomics in general. When this sequence was deleted to the GAAA tetranucleotide box proposed by Rupasov as being the termini of the PVS coat protein subgenomic, that is only leaving the 3’ terminal 20 nucleotides of the VTE, the expression of the downstream viral coat protein ORF was decreased dramatically, by over 5 fold, as compared to the expression level with the entire VTE sequence, in vitro. This small SLVTE sequence is a very inefficient untranslated leader sequence in vitro with regard to translational initiation. The inefficiency of translation associated with this SLVTE sequence in vitro suggests that it may not act as the coat protein subgenic leader as the PVS subgenomic is expressed at a very high level in planta during a viral infection or in vitro in an artificial system. Additional deletion analysis of the VTE has highlighted the 5’ proximal region of the leader as the sequence responsible for the translational enhancement function. Therefore if Rupasov’s proposals are correct the region of the VTE with translational enhancement properties would not be on the subgenomic RNA molecule. It is possible that the carlavirus subgenomic leader is longer than that of the potexviruses to overcome the problem of low messenger concentration.

Eventhough potex- and carlaviruses are very closely related in terms of the mechanism of gene expression, subtle differences may exist, especially in the expression of the subgenomic encoded products. This is emphasised by the difference in concentration of the subgenomic molecules. Carlavirus subgenomics are present only in trace amounts when compared to the concentration of potexvirus subgenomics therefore carlaviruses may require an extra enhancement property to achieve efficient expression of their
subgenomic encoded products, for example the presence of a longer untranslated leader which confers a high level of expression on a downstream ORF.

Another line of evidence that suggests that the VTE sequence is actually on the 1.3 kb subgenomic molecule and does indeed have a role in translation, is the identification of sequence homology between the VTE sequence and the PVS 5' genomic untranslated leader. The PVS 5' leader sequence of the genomic RNA molecule, as will be discussed later, is a very strong translational enhancer in vitro and in vivo (Chapter 6). This homology is particularly evident in a 21 base region of the VTE immediately upstream from the conserved carlavirus block with homology to a prokaryotic ribosome binding site. It can also be observed that this region of the PVS 5' leader is also highly homologous to a parallel sequence in the BBScV 5' untranslated genomic leader (Fig 6.1). Implications for such sequence conservation are very important. A region of homology has been identified between two sequences which are involved in the function of translational enhancement so it is possible that this sequence may be the cis-acting site responsible for this function. It is unclear why such a conserved region would be maintained if it is not on the subgenomic molecule. Sequence homologies are presented in Fig 8.1.

Results have also suggested that carlavirus coat protein could possibly be produced as a result of internal ribosome initiation on the full length genomic RNA molecule, on the larger 2.5 kb subgenomic or even on smaller subgenomics for the 12 kDa or the 7 kDa proteins of the triple gene block that have as yet not been detected. This factor could possibly fully account for the production of large amounts of coat protein despite the apparent absence of sufficient messenger. It was the identification of prokaryotic ribosome recognition sites, sequences with base pair complementarity to the pyrimidine rich sequence at the 3' end of the 16S prokaryotic rRNA molecule, upstream from the AUG initiation codon of both carlavirus 5' subgenomic encoded products that prompted the initial study of the translational properties associated with these upstream untranslated sequences (Foster and Mills, 1991b). These homologous blocks of nucleotides are conserved throughout the carlavirus group, but have not been identified in closely related potexviruses. This possibly indicates that alternate translational strategies may be exploited by each group, despite their similarity in other characteristics. The reason why these prokaryotic Shine-Dalgarno sequences have been conserved is unclear, but one proposal is that these subgenomic molecules are translated on prokaryotic-like chloroplastic ribosomes. If this is the case this phenomenon would be yet another example of the versatility of viral genome expression and adaptability.

Results presented in Chapter 3 suggest that in vitro translation of a transcript from a Helenium virus S (HelVS) 3' terminal clone results in the production of large quantities of coat protein, and no other product, despite the presence of numerous stop codons and an entire 7 kDa protein ORF upstream (Turner et al., 1993). In vivo expression of the same clone in E.coli also results in the production of coat protein (Foster et al., 1992c) The PVS VTE sequence also directs the synthesis of the downstream reporter gene ORF.
when the VTE is provided as an intercistronic spacer in a GUS-LUC dicistronic construct in a prokaryotic in vivo environment. Internal initiation of translation upstream from carlavirus coat protein ORFs does occur in a eukaryotic environment, at least in vitro, but the extent of contribution this makes to viral gene expression is unclear. This form of translational initiation may operate at a great or lesser extent in conjunction with an efficiently translated subgenomic molecule to achieve the required level of carlavirus coat protein expression.

Eukaryotic internal ribosome initiation has been documented at large among the picornavirus group (Pelletier and Sonenberg, 1988; Molla et al., 1992; Lui and Inglis, 1992) and also to some degree with plant potyviruses (Carrington et al., 1990; Basso et al., 1994). The secondary configuration of the internal ribosome entry site (IRES) within the long untranslated leader is thought to be the factor responsible for recognition, binding and initiation of translation by host ribosomes. Secondary structure recognition also appears to be an important factor in prokaryotic translational initiation (Steitz, 1975; Kozak, 1991a). The VTE sequence does contain a potential stem loop structure based around the conserved prokaryotic ribosome recognition sites discussed in the previous paragraph with the conserved sequence displayed on the non-hydrogen bonded loop of the hairpin. This proposal is in agreement with the "trough" theory whereby the hairpin structure, with the conserved carlavirus sequence exposed on the loop, associates with the ribosome in a cleft like structure in the initiation of translation (Shinedling et al., 1987).

Internal initiation appears to be entirely dependent on recognition of a cis-acting secondary structure on the RNA molecule. The conserved carlavirus "Shine-Dalgarno"-like sequence may direct the entry of ribosomes onto the region of RNA upstream from the ATG of the coat protein ORF. This may be on the smaller coat protein subgenomic molecule, if the leader is longer than that suggested by Rupasov et al., (1989), or on the larger subgenomic or genomic molecules. The process of internal initiation suggests a mode of initiation of translation that is not dependent on the cap structure that is linked to the 5' end of eukaryotic mRNA molecules. Cap-independent translation has been documented extensively among members of the picornavirus group of animal viruses (Jackson et al., 1990; Pelletier and Sonenberg, 1988), with cellular mRNA molecules (Macejak and Sarnow, 1992; Oh et al., 1992) and with some plant viruses (Carrington et al., 1990; Levis et al., 1993; 1994). It appears that leader sequences which direct internal ribosome entry operate efficiently in a host environment which has been depleted of cap-binding component of the elf-4F eukaryotic initiation factor. Foster and Mills, (1990a) have suggested that the PVS 1.3 kb subgenomic molecule is uncapped, due to its insensitivity to the addition of cap analogue in vitro. In contrast to this White and Mackie, (1990) have shown that both subgenomic molecules of the potexvirus CYMV are capped. This is another example of the possible difference in expression of carl- and potexvirus subgenomics.

Experiments described in Chapter 5 with transgenic plant expressing a VTE-GUS transcript RNA, have suggested that the translational competence of the VTE is not detrimentally effected in a tobacco mosaic virus infected environment, an environment in
which the translation of cap-dependent host transcripts is severely impaired. The mode of translation associated with the PVS 1.3 kb subgenomic molecule may be, at least partially, cap-independent in nature. Cap-independently translated transcripts are efficiently expressed under conditions where host translation is impaired. It is advantageous to the virus to decrease the level of host translation to alleviate the demand for cellular translational machinery, leaving more available for viral gene expression. Having an expression mechanism that is not affected by this translational down regulation allows the virus to multiply to high levels at a tremendous rate. If, as has been suggested (Foster and Mills, 1990a), carlaviruses subgenomics are uncapped, the efficient expression of their encoded gene products may be explained by their increased translational competence in the cellular environment created by the presence of the virus.

Very little is known about the mechanism of cap-independent translation but it is thought that specific cap-independent translational initiation factors work in conjunction with cap-dependent factors to mediate the process. \textit{In vitro} competition assays concerning translation associated with the VTE sequence, provided as the untranslated leader sequence in a transcript molecule, has indicated that a protein \textit{trans}-acting factor may bind to a sequence within the VTE, thereby mediating the function of translational enhancement. The addition of exogenous VTE leader RNA to an \textit{in vitro} translation system containing VTE-LUC transcript results in a dramatic decrease in the level of luciferase expression. This decrease is not as pronounced when a synthetic RNA leader is added. It has been concluded that a \textit{trans}-acting factor may bind to the small VTE RNA leaving a limiting amount for association with the VTE leader of the VTE-LUC transcript. A more detailed study of this phenomenon is required but from preliminary results presented it may be postulated that a host cellular factor associates with the VTE sequence resulting in an enhanced expression of the downstream ORF. The nature of such a factor is unclear. It could take the form of a cap-independent recognition protein, that is perhaps a factor that recognises a \textit{cis}-acting secondary structure and mediates internal entry of ribosomes and initiation of translation at the AUG of the downstream ORF.

When translational enhancement properties were found to be associated with the VTE sequence, similar experiments were carried out with the 5' untranslated leader of the PVS genomic RNA molecule. The major reason for these experiments was the homology at the nucleotide level detected between these two viral sequences, perhaps the conserved blocks of nucleotides marked a domain which acted in \textit{cis} to mediate translational enhancement properties. As was stated previously the degree of homology at the nucleotide level is very strong between these two sequences in a region of the VTE just upstream from the Shine Dalgarno-like sequence that has been discussed. It is likely that this conservation is not accidental but may mark a region or structure that is recognised by a \textit{trans}-acting factor to mediate the process of translational enhancement.

Another reason for an interest in the PVS 5' sequence was the documentation of the PVX 5' leader enhancer, that is the UTR from a potexivirus, that is a plant virus group that are very closely related to the carlaviruses in both genome organisation and mode of
Fig 8.1 Sequence homology between the PVS 5' leader sequence (top) and the VTE sequence (bottom). Homologous nucleotides are represented by black dots. The positions of deletions constructed with each sequence are also indicated.
PVS 5'

GAGCTC[AAACACTCCGAAAATA]TTTGACT[AAACAACCGGACGTTCAAGCAATTACTTACCTATGG]

GAGCTCACAAGAGTTTGGTGAGCCGTACAGCAACATGGGGCCGTTGAAGCACCTTTAGGTTCACAGGTA

VTE

AGAGTTCGAAGAAACTGTCACAGCACGAATGG

VTE
gene expression (Smirnyagina et al., 1991; Zelenina et al., 1992; Tomasherskaya et al., 1993). The PVS 5’ UTR was shown to be an extremely strong enhancer both in vitro in rabbit reticulocyte and wheat germ lysate and in vivo in tobacco protoplasts and in transgenic plants, when provided as the leader sequence in a reporter gene transcript (Chapter 6). These enhancements were as great as 30 fold in vitro, and 15 fold in vivo in tobacco protoplasts, over the level of reporter gene expression from a reporter gene transcript with a synthetic leader. The PVS 5’ UTR can therefore join the long list of plant viral leader enhancers that have been documented in recent years, such as the TMV omega leader (Gallie et al., 1987a; 1987b; 1988; 1989; 1992; Sleat et al., 1987; 1988; Kang et al., 1994), the leader sequence from the RNA3 molecule of AIMV (Jobling et al., 1987) and the TEV UTR (Carrington et al., 1990).

With regard to cap-independent translation, similar results were obtained with the PVS 5’ UTR as those described earlier with the VTE. The expression of luciferase transcript molecules containing the PVS 5’ UTR as an untranslated leader was relatively insensitive to the addition of cap analogue in vitro. Previous results do in fact indicate that the PVS 5’ genomic leader is capped as translation of PVS viral RNA in vitro in the presence of cap analogue results in the expression of the subgenomic encoded products in preference to the genomic encoded products (Foster and Mills, 1990a). These results do appear to be contradictory but perhaps the subgenomic molecules are expressed more efficiently in a cap-independent translation system, that is they are recognised in preference to the genomic molecule. In the absence of competition from the subgenomics the PVS 5’ leader may be efficiently recognised and expressed in this system. This brings about important implications regarding the expression of the PVS viral genomic molecule. The entire PVS genome could possibly be expressed by internal entry of ribosomes in a mechanism similar to that documented for picornaviruses. One difference between these two virus types is that the carlaviruses genomic RNA molecule is capped at the 5’ proximal end whereas the picornavirus genomic molecule has a viral encoded protein covalently linked. It is possible that while the picornavirus entirely expresses its genome by a mechanism of cap-independent internal initiation, the carlaviruses uses both mechanisms, that is cap-dependent translation when host translational machinery is plentiful and internal initiation in association with a cis-acting factor within the carlavirus UTR when a host translational factor(s) which is not required for cap-independent translation is limiting.

Preliminary information provided in Chapter 6 has also indicated that the PVS origin of coat protein assembly (OAS) could also be contained within the 5’ untranslated leader. It has recently been documented that the OAS of a potexvirus, papaya mosaic virus (PMV) is located within the 5’ untranslated leader of the genomic RNA molecule and that virus assembly occurs in the 5’ to 3’ direction from the extreme 5’ terminus of the viral RNA (Sit et al., 1994). As potex- and carlaviruses are closely related such factors as genome structure and expression, it is possible that the PVS OAS could also be located within the PVS 5’ untranslated leader.
Another observation supporting this theory was the efficiency of coat protein-mediated protection against PVS in transgenic plants. Transgenic *Nicotiana debneyii* expressing viral coat protein are resistant to infection with both PVS viral particles and PVS RNA (Mackenzie and Tremaine, 1990). As has been discussed extensively in Chapter 6, this different response to two different inocula types suggests an origin of coat protein assembly that is at the 5' proximal end of the genomic RNA molecule.

The TMV OAS has been located to an internal position within the viral genome (Zimmern and Butler, 1977; Zimmern and Wilson, 1976). When viral particles are inoculated onto transgenic plants expressing TMV coat protein, disassembly will be inhibited due to the presence of exogenous amounts of coat protein therefore viral translation will be inhibited. A different situation occurs with TMV RNA. When naked RNA enters the cell encapsidation initiates at the OAS but because this is internal within the viral genome translation can initiate at the 5' end and ribosomes can effectively strip coat protein from the RNA molecule. The effectiveness of coat protein-mediated protection with the potexvirus, potato virus X (PVX) is different (Lawson et al., 1990). Contrary to the situation with TMV, this resistance is effective against challenge with both PVX viral particles and PVX RNA. It has been proposed that coat protein-mediated protection in this case occurs due to at least a partial recoating of the potexvirus RNA which would result in prevention of translation of the replicase gene (Hemenway et al., 1988). Whether or not coat protein-mediated protection is effective against viral RNA may depend largely on the position of the OAS sequence. It appears to be the latter situation that occurs with PVS and perhaps carlaviruses in general.

Preliminary results presented suggest that the presence of exogenous amounts of coat protein results in a dramatic decrease in expression of a downstream open reading frame, the luciferase reporter gene when the PVS 5' sequence is provided as the untranslated leader sequence of the transcript molecule, *in vivo* in tobacco protoplasts. This decrease in expression appears to be specific for the PVS 5' leader sequence because when a synthetic leader sequence is used instead this decrease is not observed. The mean decrease in luciferase expression observed when coat protein was present with the PVS 5' sequence as the leader of the transcript molecule was over 70% of that observed when the coat protein was not present. A 17% fold increase in expression was observed with luciferase transcripts with a synthetic leader when coat protein was present when compared to the level of expression in the absence of coat protein. Further investigation is necessary before we can conclude that this decrease observed is a result of initiation of coat protein assembly and inhibition of ribosome entry to initiate translation but these results do indicate that this could possibly be the case.

Further studies will hopefully assign the function of OAS to within the PVS UTR and identify the specific sequence or structure that is recognised by the coat protein in the initiation of particle assembly. From a biotechnological viewpoint such a sequence or structure could be used, perhaps, to identify the function of a transgene in a transgenic plant. That is, a foreign gene could be introduced into a transgenic plant containing the active OAS sequence from PVS at the 5' proximal end of the coding sequence. This would act as the untranslated leader sequence of the transcript molecule *in vivo*. The
expression of the transgene at the translational level could be switched off by inoculation with the virus. This ability to regulate gene expression with a simple on/off switch would be a novel method of analysing gene function.

If, as in agreement with the proposals of Rupasov et al., (1989), the PVS 1.3 kb subgenomic molecule terminates just 20 nucleotides upstream from the ATG of the coat protein gene, it is likely that the sequence upstream contains a promoter sequence which is recognised by the viral replication complex in the production of the subgenomic RNA molecule. Skryabin et al., (1988) has recognised putative potexvirus subgenomic promoter sequences and this sequence conservation can be extended to the carlavirus group. White and Mackie, (1990) have also detected a degree of sequence homology between the putative subgenomic RNA promoter of the potexvirus CYMV and the known subgenomic promoter of the Venezuelan equine encephalitis virus (VEE). A conserved hexanucleotide sequence, -ACUUAA-, has been identified which is essential for CYMV replication. Mutations in and around this motif result in the eradication of accumulation of a defective interfering particle of the virus. This sequence is not only strongly conserved throughout the potexvirus group but also the carlavirus group. What is of interest with regard to this project is that an identical motif has also been located upstream from the ATG codon of both potex- and carlavirus coat protein ORFs and 25 kDa protein genes of the triple gene block on the negative-sense genomic molecule. It has been proposed that these sequences may act as the promoter recognition sequences for coat protein subgenomic production (White et al., 1992).

Very little is known about the method of subgenomic RNA production. It is thought that the viral replication complex recognises a specific internal sequence on the negative-sense genomic RNA molecule and mediates the production of the complementary positive-sense subgenomic molecule. These proposed subgenomic promoter sequences are located between 8-63 nucleotides upstream from the ATG of the coat protein ORF and between 14-40 nucleotides upstream from the ATG of the 25 kDa ORF. In PVS this hexanucleotide conserved sequence is located 39 nucleotides upstream from the coat protein ATG, within the VTE sequence.

Results have indicated that in vivo, reporter gene expression from antisense luciferase transcripts containing the VTE sequence in a negative form at the 3' end of the molecule, is slightly higher in a viral infected environment as opposed to a healthy environment. The expression of a sense luciferase transcript was, however, observed to be over 7 fold lower in the infected environment. With this antisense transcript, luciferase expression should only occur if the PVS viral replication complex recognises a sequence within the negatively orientated VTE sequence to initiate RNA transcription. These results may be interpreted in two ways. Firstly, that the viral replication complex in the infected environment recognises a subgenomic promoter within the VTE and converts negative template into positive-sense transcript that can be translated by host ribosomes. Secondly, the enhancement properties of the VTE may increase the level of background reporter expression from the antisense construct in the plant infected
environment. Theoretically, luciferase translation should not occur from an antisense transcript but results have indicated that quite a high level has been detected with transient bombardment assays. It is possible that the increased translational competence of the VTE in this viral infected environment may account for the increased expression of the antisense construct in an environment where sense transcript translation is severely impaired. This phenomenon has been discussed in detail in Chapter 5 where the presence of the VTE as a 5' untranslated leader sequence of a sense GUS transcript in transgenic tobacco plants confers an increased expression of the downstream ORF when the plant is infected with TMV, an environment where endogenous translation is usually severely inhibited. It is not known at present whether or not initiation of transcription occurs upstream or downstream from the subgenomic promoter site so if the promoter sequence is within the VTE it is possible that even if the region of the sequence identified as being involved in translational enhancement is upstream it may still be on the subgenomic molecule.

In summary, two untranslated sequences from the carlavirus PVS have been analysed from a molecular viewpoint. Both sequences, the 101 nucleotide VTE sequence upstream from the AUG of the coat protein gene and the PVS 5' leader sequence of the genomic RNA molecule, enhance the expression of a downstream ORF, both in vitro and in vivo, when provided as the untranslated leader sequence in a transcript molecule. Both these sequences have the potential, from a biotechnological viewpoint, to be used to achieve the increased level of expression of a foreign gene in a transgenic plant.

Results obtained bring about implications regarding the mode of expression of the PVS genome and perhaps carlavirus gene expression in general. The apparent cap-independent nature of the mode of translational initiation associated with the PVS 5' untranslated leader, in the absence of subgenomic products, indicates that under conditions of cellular translational down regulation, the PVS genome may be expressed. This will facilitate virus multiplication and proliferation in the host. It appears that the subgenomic encoded carlavirus coat protein ORF may also be expressed in a cap-independent manner, perhaps to an even greater extent than the genomic molecule with the possibility of internal entry of ribosomes on the full length genomic molecule, on the larger carlavirus subgenomic or even on the smaller carlavirus subgenomic itself at a specific sequence or structure upstream from the AUG of the gene. Ribosome scanning may occur from the site of entry to the initiation codon, a situation that has been proposed for picornaviruses. It appears that it is this factor, that is cap-independent internal initiation of translation, combined with the high efficiency of expression of the small carlavirus subgenomic due to the nature of the untranslated leader sequence, that may explain the production of copious quantities of coat protein despite the presence of trace quantities of messenger.

Results have also indicated that the origin of coat protein assembly initiation could be contained within the PVS 5' UTR. This is in agreement with coat protein-mediated protection assays in transgenic plants.

From a biotechnological viewpoint, the use of subgenomic promoter sequences to
promote the expression of genes on infection by a particular virus that would normally remain silent could potentially be very important. Preliminary evidence presented suggests that it may be possible to mimic a type of hypersensitive response in plant cells, that is a type of defence response that is triggered by the presence of the virus. Much more research is necessary in the identification of the replication signals that are required, these may be from a subgenomic promoter, as stated above, or alternately from the 3' end of the negative-sense genomic RNA molecule, that is the promoter sequence responsible for the initiation of transcription of a positive-sense genomic RNA molecule from this negative-sense template. Such a mechanism could prove to be a major breakthrough for engineered resistance against plant viruses.

In conclusion, results indicate that two small, less than 100 nucleotides in length, untranslated carlaviruses may contain at least two biological functions each, that is cap-independent translational initiation and origin of coat protein assembly for the PV5'UTR and translational enhancement, cap-independent internal initiation of translation and subgenomic promoter activity for the VTE. The degree of sequence conservation between these two leaders indicates a shared function. This emphasises the degree of versatility and economy associated with the carlaviruses and reflects the situation in all plant and animal viruses.
Chapter 9

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Publications


### Appendix

#### 4.3.1

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#### 4.4.1

% Decrease in translation of VTE-LUC on addition of leader transcript

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#### 4.7.1

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