

DNA species associated
CHARACTERISATION OF SUBGENOMIC RNAs FROM PLANT TISSUE
~~INFECTED~~ WITH TOMATO BUSHY STUNT VIRUS

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ABSTRACT

Tomato bushy stunt virus (TBSV) is the type member of the tombusvirus group of plant viruses, which comprises over ten different serologically related viruses responsible for causing a number of serious viral disease problems. Although TBSV was the first icosahedral plant virus to be crystallized, and is one of the best studied of all viruses from a structural perspective, little work has been done on the genomic organization.

The genome of single-stranded RNA (ssRNA) was isolated from TBSV particles purified from infected tobacco plants. By electrophoresis through denaturing gels the RNA was shown to be approximately 4800 nucleotides in length. Also encapsidated in the virus particles were a number of lower molecular weight ssRNA species. By Northern blotting two of these (2000 and 900 nucleotides) were shown to be related to the viral genome. The other lower molecular weight RNAs hybridised to RNA from healthy plants, showing them to be encapsidated host RNA.

Radioactive cDNA probes were prepared from the purified genomic RNA and used to probe Northern blots of infected plant RNA. However, most of the numerous RNA species detected were later shown to be artefacts. Purification of the ssRNA from infected plants away from the ribosomal RNA removed the artefacts and resulted in the detection of only two lower molecular weight ssRNA species; these co-migrated with denatured double-stranded RNA from infected tissue and with the two encapsidated TBSV specific RNAs.

Using random primers and reverse transcriptase a number of overlapping clones of the TBSV genomic RNA were obtained. These were restriction mapped and located with respect to the ends of the genomic RNA. The clones were used to hybrid select TBSV specific RNAs from infected

tissue. The genomic RNA and both lower molecular weight species were then translated in rabbit reticulocyte lysates. The 2000 nucleotide species produced a 40 kilodalton polypeptide, which was identified as the coat protein by immunoprecipitation. The 900 nucleotide RNA species produced a 22 kilodalton polypeptide, and the genomic RNA produced a 37 kilodalton polypeptide. However, when the translation of the genomic RNA was repeated in the presence of high levels of tRNA, a higher molecular weight polypeptide of 90 kilodaltons was also produced. These results indicate that the expression of the TBSV genome is achieved by using both subgenomic RNAs and readthrough of at least one stop codon on the genomic RNA.

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CHAPTER 1

INTRODUCTION

1.1 AIMS OF THE THESIS

At the beginning of this project, both the particle structure and cytopathology of tomato bushy stunt virus (TBSV) had been studied extensively. However, little work had been done on the genome organization and replication of TBSV, or any other tombusvirus. Therefore, the work in this thesis attempts to establish the genomic organization and expression of TBSV, and proposes a genetic map for the virus.

The thesis is divided into a number of parts, and includes an introduction and a discussion. To provide a background for the project and the discussion, the introduction contains much of the relevant information known about TBSV and the other tombusviruses prior to the beginning of this work, and a general review of the genome organization, replication and expression of a number of plant RNA viruses. During the course of the work, a number of reports were published regarding the genome of both TBSV and another tombusvirus, cymbidium ringspot (CyRSV). These findings, and a comparison to the results presented in this thesis, are included in the discussion.

1.2 THE TOMBUSVIRUS GROUP

The tombusvirus group consists of tomato bushy stunt virus and several related viruses. They are readily transmitted by mechanical inoculation. No insect or mite vector has been found, and the majority of experiments with fungus vectors have proved inconclusive; cucumber necrosis virus is, however, transmitted by zoospores of

Olpidium species (Rochon & Tremaine, 1988). The viruses, while present in the pollen, are not transmitted through seed. However, the particles are very stable, are released in large amounts from infected plants (Smith et al., 1969) and have been found in high concentrations in both stream water and soil (Tomlinson et al., 1982). The virus may therefore be transmitted mechanically in soil (Hiruki & Teakle, 1987).

In 1982 the International Committee on Taxonomy of Viruses (ICTV) recognized seven viruses as definitive members of the tombusvirus group: TBSV, artichoke mottled crinkle (AMCV), carnation Italian ringspot (CIRV), CyRSV, eggplant mottled crinkle (EMCV), pelargonium leaf curl (PLCV), petunia asteroid mosaic (PAMV) viruses, and two viruses as tentative members: saguaro cactus (SCV) and turnip crinkle (TCV) viruses (Matthews, 1982). However, recent work has shown that three additional viruses should also be considered definitive members; a virus originally reported from Morocco as the pepper strain of TBSV (Fisher & Lockhart, 1974) was found to be sufficiently serologically different from TBSV to warrant a name of its own, i.e. Moroccan pepper virus (MPV) (Makkouk et al., 1981; Vetten & Koenig, 1983), and Tombusvirus Neckar (TVN) (Koenig & Lesemann, 1985) and grapevine Algerian latent virus (GALV) appear to be additional tombusvirus members because of their biological, physicochemical, ultra-structural and serological properties (Koenig & Lesemann, 1985; Koenig & Gibbs, 1986).

The expanded group of ten members has recently been recognised by the ICTV (unpublished Minutes of the 17th Meeting of the Executive Committee of the ICTV, 1987). Because of significant differences with the definitive tombusviruses in biological behaviour (i.e. host range) and strategy of replication (Henrique & Morris, 1979), it has been suggested that TCV and SCV are unlikely to be

members of the group (Martelli, 1981). Indeed, the recent report from the ICTV (unpublished Minutes of the 17th Meeting of the Executive Committee of the ICTV, 1987) places these viruses in a newly formed carmovirus group together with carnation mottle virus (CarMV). Based upon double-stranded RNA profiles and genome size, the same report now considers cucumber leaf spot (CLSV), cucumber necrosis (CNV) and cucumber soilborne (CSBV) viruses as possible members of the tombusvirus group (Rochon & Tremaine, 1987; Martelli et al., 1988). However, CLSV and CSBV are serologically unrelated to all the tombusviruses tested, do not show detectable sequence homology with TBSV RNA (Martelli et al., 1988) and have a cytopathology different from that of tombusviruses (Di Franco & Martelli, 1987).

By comparing the properties of the definitive tombusvirus members (Table 1.1), it can be seen that they are very similar in both their physicochemical and cytopathological properties, and are all related to TBSV as determined by both serological and nucleic acid hybridization techniques. The serological relationships of the group were studied most recently by Koenig and Gibbs (1986) using over 220 antisera and 2000 titrations. The relatedness of each pair of viruses, simplified and summarized in Table 1.1, were given as a serological differentiation index (SDI), which is the mean number of two-fold dilution steps separating the homologous and heterologous titres of the antisera prepared against one of the viruses (Van Regenmortel 1975, Koenig 1976). The most distant relationship that can be measured accurately has an SDI of 9, the closest has an SDI of 1. A value of >9 indicates that cross reaction occurs with only some of the antisera.

TABLE 1.1: SOME PROPERTIES OF TOMBUSVIRUSES
(adapted from Martelli et al. 1988)

Virus	S _{20w}	Bouyant density in CsCl (g/cm ³)	Size of genomic RNA (kb)	Mr of coat protein (kD)	Estimated percent sequence homology with TBSV-type	Serological relationship with TBSV-type	Presence of multivesicular bodies in peroxisomes of infected cells
TBSV	130	1.35	4.7	42	100	++++	Yes
AMCV	132	1.35	4.7	43	7	+++	Yes
CIRV	135	1.36	4.7	37	11	+	No*
CyRSV	127	1.36	4.7	43	10	+/-	Yes
EMCV	132	1.34	4.7	41	7	++	Yes
GALV	128	1.34	4.7	37	15	-	Yes
MPV	140	1.36	4.7	43	10	++	Yes
PAMV	134	1.35	4.7	41	11	+++	Yes
PLCV	136	1.34	4.7	41	24	+++	Yes
TVN	133	1.36	4.7	45	15	+	Yes

Notes for Table 1.1

- = Not determined.
- ++++ = Serological differentiation index of 1
- +++ = Serological differentiation index of 2-3
- ++ = Serological differentiation index of 4-5
- +
- + = Serological differentiation index of 6-7
- +/- = Serological differentiation index of >9
- = No cross reaction with any antisera tested.
- * = Multivesicular bodies found in mitochondria.

References: 1, Gallitelli & Russo (1986, 1988); 2, Mayo & Jones (1973); 3, Gallitelli & Hull (1985); 4, Koenig & Gibbs (1986); 5, Appiano et al. (1978); 6, Quacquarelli et al. (1966); 7, Martelli & Russo (1973); 8, Hollings et al. (1970); 9, Martelli & Russo (1972a); 10, Hollings et al. (1977); 11, Martelli & Russo (1981); 12, Makkouk et al. (1981); 13, Ambrosino et al. (1967); 14, Hollings & Stone (1965); 15, Martelli & Russo (1972b).

The degree of homology between the genome of TBSV and the other tombusviruses was studied by Gallitelli et al. (1985b) using nucleic acid hybridization techniques. As shown in Table 1.1, there was found to be a moderate sequence homology between the definitive members, with TBSV and PLCV most closely matched with 24% of the TBSV sequence found in PLCV. In contrast only 7% of the sequences of TBSV and AMCV were found to be common.

Comparison of the serological and sequence relatedness shows that there is little correlation between the two (Table 1.1). For example, CyRSV is serologically the most distantly related tombusvirus. Koenig and Gibbs (1986) found cross reactions with TBSV with only a few antisera, while Hollings et al. (1977) found no cross reaction at all. However, nucleic acid hybridization tests did not indicate an especially distant relationship between CyRSV and TBSV. Likewise, TBSV and AMCV are fairly close serologically, but their genome homology was only 7%. TBSV and PLCV are approximately as close serologically as TBSV and AMCV, but hybridization studies indicate that 24% of the genomes are common.

Koenig and Gibbs (1986) suggested that the lack of correlation between SDI values and estimates of genome homology may be due to different parts of the tombusvirus genome being conserved to different extents during evolution; with TBSV and AMCV the sequence encoding for the antigenic determinants may have been conserved during evolution while other areas of the genome have changed considerably. In CyRSV, however, the areas encoding for the antigenic determinants have been conserved to a much smaller degree than other areas. Gallitelli et al. (1985) also concluded that regions of RNA homology may be different for different tombusviruses. Hybridization between the definitive members of the tombusviruses using

different areas of the cloned RNA genome would allow these suggestions to be tested.

Although TBSV is considered in Table 1.1 to be a single virus, a number of isolates exist which can be distinguished from each other serologically and electrophoretically, but which cause similar diseases in tomato (Lycopersicon esculentum). Hollings and Stone (1975) studied the serological relationships of a number of TBSV strains, namely TBSV-Type, TBSV-Tulip, TBSV-Cherry and TBSV-GCRI strains. During serological cross-reactions in double diffusion tests, both the type and the tulip strains formed spurs with each other and with TBSV-Cherry, TBSV-GCRI, PLCV, AMCV, PAMV and CIRV, showing that TBSV-Tulip and TBSV-Type are only distantly related to these other tombusviruses. However, the cherry and GCRI strains of TBSV formed continuous precipitin lines with PAMV, showing a close relationship. Because of this serological relationship, the TBSV-Cherry strain has been described as the cherry strain of PAMV by Koenig and Gibbs (1986).

Another well studied strain of TBSV is TBSV-BS3. This strain was originally derived by passaging the type strain several times through differential hosts (Steere, 1953). Even though the two strains migrate in different directions when electrophoresed through agarose gels (Koenig & Gibbs, 1986), they are very closely related serologically (Koenig & Gibbs, 1986). Interestingly, in recent years no natural outbreaks of TBSV-Type have been recorded; all new records have been attributed to TBSV-BS3 (Koenig & Avgelis, 1983).

1.3 VIRUS STRUCTURE AND COMPOSITION

The structure of TBSV has been studied extensively by both X-ray defraction (Harrison et al., 1978) and electron microscopy (Crowther & Amos, 1971). The particles are of isometric shape 30 nm in diameter (Matthews, 1982), or of 34 nm in diameter according to recent estimates (Franki et al., 1985). Protein analysis has established that each particle consists of 180 polypeptides with a Mr of about 41 kD (380 amino acid residues). Minor polypeptides with Mr of 28 kD and 87 kD have also been found in TBSV coat protein preparations (Butler, 1970; Ziegler et al., 1974). The smaller polypeptide may be a cleavage product of the major coat protein (Ziegler et al., 1974), as it is often not detected when freshly made preparations of the virus are analysed (McCarthy, 1983).

It has been calculated that there is enough of the larger polypeptide (87 kD) for each virus particle to contain only one molecule. Its location and function remain unknown, but Butler (1970) suggested that it may be a polymerase, possibly attached to the viral RNA. However, more recently it has been shown that the viral RNA alone is infectious when applied to leaves of an appropriate host, even after treatment of the RNA with proteinase K or pronase (Mayo et al., 1982), which would argue against such a suggestion. It has now been ascertained that the 87 kD protein is a stable covalently linked dimer of two protein subunits (Stockley et al., 1986).

1.4 CYTOPATHOLOGY OF TBSV INFECTION

Definitive and tentative members of the tombusvirus group have been isolated in nature from at least twenty diverse plant species, including annual crops, woody perennials, vines, ornamentals and cacti, in the Mediterranean basin and in different countries of the

American and European continent (Martelli, 1981). The type strain of TBSV induces stunting and bushy growth of tomato (Lycopersicon esculentum Mill.) plants accompanied by severe deformation of young leaves and, sometimes, by yellowing and purpling of lower leaves. Chlorotic blotching, ring and line patterns show on the ripe fruits, thus rendering the crop unmarketable (Martelli, 1981). In tobacco species (eg. Nicotiana clevelandii) the young leaves become severely deformed, and the lower leaves develop necrotic lesions. Often the plant dies due to withering of the stem within two to three weeks.

The ultrastructure of localised and systemic infections of TBSV has been extensively studied (Russo & Martelli, 1972; Martelli & Russo, 1977; Martelli et al., 1977; Martelli et al., 1988). The intracellular sites of virus replication, coat protein synthesis and particle assembly are still unknown, but a number of structures specific to infection have been described.

Perhaps the most characteristic feature of TBSV infection is the existence of multivesicular bodies (MVB) about the size of a large mitochondrion located in the cytoplasm in both local and systemic infections. Cytochemical studies showing that the MVB contain glycolate oxidase (Martelli et al., 1984) provide evidence that these structures derive from peroxisomes. In infected cells, the peroxisomes undergo a progressive vesiculation of the bounding membrane through the possible addition of membranous material from the endoplasmic reticulum. Modified peroxisomes may become very plastic, engulfing portions of the ground cytoplasm through the invagination of the limiting membrane (Russo et al., 1983, Koenig & Lesemann, 1985). These modifications lead to a novel structure made up of three major components: (1) a peripheral, irregularly thickened membrane, sometimes obviously connected with the cytoplasmic reticulum

strands; (2) an electron dense finely granular or crystalline matrix; and (3) many globose to ovoid vesicles measuring 80-150 nm in diameter which contain finely fibrillar material. With CIRV, the MVB originate from the mitochondria rather than from peroxisomes (Di Franco et al., 1984). As with peroxisomes, mitochondria undergo a progressive peripheral vesiculation leading to a transformation of the organelles into disorderly aggregates of vesicles intermingled with the remnants of stroma.

Appiano et al. (1981) showed that MVB appear in inoculated tissue before progeny virus particles. This led to the suggestion that MVB were the site of viral RNA replication, a notion supported by the finding that double-stranded RNA, supposed to be of viral origin, was detected in the vesicles of MVB of cells infected with CyRSV and CIRV (Russo et al., 1983; Di Franco et al., 1984), and that MVB of TBSV-BS3 infected cells incorporate (³H)uridine (Appiano et al., 1983).

Plants naturally infected with TBSV are systemically infected. However, in some experimental hosts, such as Gomphrena globosa (Appiano & D'Agostino, 1980), the infection remains localised due to the hypersensitive response. In these cases the virus remains confined within necrotic lesions developed upon inoculation. The centre of the lesions consist of necrotic cells that contain large numbers of virus particles. The cells of the inner part of the boundary which separates the healthy and infected tissue contain virus particles at a lower concentration, and have cell walls heavily thickened by lignin and suberin. Their chloroplasts are deprived of starch but have sac-like evaginations connected to multivesicular bodies (Appiano et al., 1977). Cells on the outer part of

the boundary are metabolically active with thickened cell walls. No virus particles are present, and the chloroplasts contain very large amounts of starch (Appiano et al., 1977).

Although the cell walls of the necrotic cells and those at the boundary have thickened cell walls, this is in itself not likely to act as a barrier to virus movement, as the thickening often happens after the appearance of multivesicular bodies and the first virus particles. It has been postulated that the resistance is due to physiological changes within the cells similar to ageing (Pennazio et al., 1978), and that cell wall thickening is a consequence of necrosis rather than a cause of localization (Appiano et al., 1981).

1.5 GENOME STRUCTURE OF POSITIVE-STRANDED RNA PLANT VIRUSES

For a plant virus to be successful, it must be able to replicate and spread systemically throughout the host. This involves the expression of a number of open reading frames (ORFs) to produce viral proteins. It would be expected that both the timing of the production of the viral proteins during the replication cycle and their concentrations should be regulated. This may be achieved by altering the affinity of both the genomic and subgenomic RNAs for the ribosomes, and altering the levels of subgenomic RNAs via the RNA-dependent RNA polymerase. Therefore, the sequences around the ribosome binding sites and the polymerase binding sites are of considerable importance in plant RNA virus infections.

TABLE 1.2: CHARACTERISTICS OF SOME PLANT RNA VIRUS GROUPS

VIRUS GROUP	TYPE MEMBER	STRUCTURE		ADDITIONAL ENCAPSIDATED RNA	TRANSLATION STRATEGY ¹
		5'	3'		
Carmo-	CarMV	cap	-OH	SG, sat	RT, SG
Luteo-	BYDV	VPg	-OH	?	?
Potex-	PVX	cap	poly(A)	-	(RT)
Poty-	PVY	VPg	poly(A)	-	PTc
Sobemo-	SBMV	VPg	-OH	SG	SG
Tobacco necrosis	TNV	ppA	-OH	(SG), sat ²	(SG)
Tobamo-	TMV	cap	tRNA	SG, sat	RT, SG
Tombus-	TBSV	(cap)	(-OH)	SG, sat	RT, SG
Tymo-	TYMV	cap	tRNA	SG	SG, (RT), (PTc)
Diantho-	RCNMV	(cap)	(-OH)	(SG)	(RT), (SG)
Como-	CPMV	VPg	poly(A)	-	PTc
Furo ³ -	BNYVV	cap	poly(A)	SG	RT, SG
Nepo-	TBRV	VPg	poly(A)	-	PTc
Pea enation mosaic	PEMV	VPg	-OH	-	(PTc)
Tobra-	TRV	cap	-OH	SG	RT, SG
Cucumo-	CMV	cap	tRNA	SG	SG
Bromo-	BMV	cap	tRNA	SG	SG
Ilar-	TSV	cap	-OH	SG	SG
Alfalfa mosaic	AlMV	cap	-OH	SG	SG
Hordei-	BSMV	cap	poly(A)	SG	SG

Notes on Table 1.2

Only those groups containing viruses that have been studied in any detail are included. The information contained within the table is from The Plant Viruses Volume 1, edited by R.I.B. Franki (1985), The Plant Viruses Volume 2, edited by M.H.V. Van Regenmortel and H. Fraenkel-Conrat (1986), The Plant Viruses Volume 3, edited by R. Koenig (1988) and The Molecular Biology of Positive Strand RNA Viruses, edited by D.J. Rowlands, M.A. Mayo and B.W.J. Mahy (1987).

() = to be confirmed.

? = unknown.

1 = Strategies of expression include post-translational cleavage (PTc), readthrough (RT) and subgenomic RNA (SG).

2 = The satellite is encapsidated in separate capsids composed of its own coat protein molecules.

3 = Although BNYVV has been described as the type member of the Furovirus group, the terminal structures of this virus differs greatly from other members. For example, soil-borne wheat mosaic (SBWMV) has neither a 5'-cap or VPg, and is not polyadenylated.

The 5'-structure

Three different types of structures have been found at the 5'-end of plant virus RNAs: "cap", genome-linked protein or (p)ppX (Table 1.2):

(1) Cap. A cap structure, m⁷GpppX (where X is any nucleotide) is present at the 5'-end of all eukaryotic nonorganelle cytoplasmic mRNAs and most eukaryotic viral RNAs, picornaviral and some plant viral RNAs being notable exceptions (Shatkin, 1976, 1985). Numerous studies have shown that the cap structure facilitates stable complex formation between 40S ribosomal subunits and mRNA during translation initiation (Sonenberg, 1987). The mRNA cap has been shown to bind to a cellular 24 kD protein. The 24 kD protein in turn associates with two other proteins of 220 kD and 50 kD (the 50 kD protein was shown to be an altered form of the elongation factor eIF-4A) to form a cap binding protein (CBP) complex. In the presence of ATP both eIF-4A and eIF-4B bind to the CBP, and there are several lines of evidence that show the association of proteins results in the melting of the 5'-end secondary structure in an ATP-mediated process (Sonenberg, 1987). Presumably this facilitates subsequent ribosome binding and/or migration of the ribosomes.

(2) VPg: The 5'-end of the RNAs from several como- and nepoviruses and also of southern bean mosaic virus (SBMV) have been shown to be linked to a protein (Table 1.2). The precise role of the VPg is not known, but it is thought to be involved in viral RNA replication and encapsidation. Removal of the VPg of cowpea mosaic virus (CPMV) by digestion with proteinase K does not influence the infectivity of the CPMV RNAs (Stanley et al., 1978; Reisman & De Zoeten, 1982). However, removal of VPg from

the RNA of nepoviruses leads to a significant, often drastic decrease of infectivity (Harrison & Barker, 1978; Mayo et al., 1982)

(3) (p)ppX. To date only tobacco necrosis virus (TNV) and its satellite (STNV) have been found to be neither capped nor protein coupled at their 5'-ends (Table 1.2), similar to prokaryotic mRNAs. The 5'-terminal 42 nucleotides of STNV can be folded into a hairpin loop which may play the role of a signal for recognition of the RNA by the 40S ribosomal subunit.

The 3'-structure

Three different types of structures have been found at the 3'-termini of plant viral RNAs: poly(A) tail, tRNA-like or 3'-OH (Table 1.2):

(1) Poly(A) tail. In contrast to most eukaryotic mRNAs, only a few plant virus RNAs have been shown to carry a poly(A) tail. The poly(A) tails of CPMV have been shown to have a mean length of 167 residues for M-RNA and 87 residues for B-RNA (Ahlquist & Kaesberg, 1979). The function of poly(A) tails may be to reduce the rate of degradation of mRNAs (Brewer & Ross, 1988). Unlike cellular mRNAs, the poly(A) tails are not synthesised by a poly(A) polymerase. The polyadenylation signal (AAUAAA) is missing from the 3'-ends of the CPMV RNAs (Davies et al., 1979), and poly(U) stretches have been identified in the minus-strands of RF molecules (Lomonossoff et al., 1985), indicating that the poly(A) tail is included in the regular replication process.

(2) tRNA-like structure. The 3'-terminus of some virus RNAs can be aminoacylated eg. turnip yellow mosaic virus (TYMV) can be charged with valine (Pinck et al., 1970).

The RNAs of the viruses of a given taxonomic group usually accept the same amino acid.

The viral RNAs were found to interact with a number of tRNA specific enzymes in vitro, namely the tRNA nucleotidyl-transferase, the aminoacyl-tRNA synthetase, the peptidyl-tRNA hydrolase, the peptide elongation factor EF-1a, RNase P and a tRNA-specific cytosine methylase. Although TYMV and brome mosaic virus (BMV) can be esterified in vivo, and the tRNA -like structure of BMV RNA has promoter activity (Hall et al., 1987), the role of 3'-aminoacylation, if any, in the replication cycle of plant viruses remains to be elucidated.

(3) 3'-OH. It has been shown that the RNAs from several viruses which do not terminate with a poly(A) tail cannot be aminoacylated. For alfalfa mosaic virus (AlMV) this was rather surprising since this virus has a lot in common with BMV. However, sequence analysis of the 3'-end showed that the 3'-terminal sequences could not be folded into a tRNA-like structure and did not contain a 3'-terminal CCA characteristic of tRNAs (Koper-Zwarthoff & Bol, 1980; Gunn & Symons, 1980). The RNAs of AlMV and several ilarviruses have a high degree of affinity for the coat protein subunits (van Vloting-Doting & Jaspers, 1972; van Vloting-Doting, 1975; Houwing & Jaspers, 1978). It has been proposed that the binding of the coat protein to the 3'-terminus of these RNAs is required for proper recognition of the genome by the viral replicase (Houwing & Jaspers, 1978).

5'- and 3' sequences of genomic RNA

Expression of the ORFs requires the interaction of the 5'-terminal non-coding sequences of the genomic and subgenomic RNAs with ribosomes. Protein synthesis in eukaryotes is usually initiated at the AUG codon closest

to the 5'-end of the mRNA and does not reinitiate internally. The 5'-noncoding regions in eukaryotic mRNAs tend to be low in guanosine, and purines are preferred at position -3 and +4 surrounding the initiator codon, the canonical sequence being A/G---AUGG (Kozak, 1981, 1984). In tobacco mosaic virus (TMV) and other multicistronic plant viral RNAs only the first AUG codon is active. The initiation codons of both the genomic and subgenomic RNAs follow U/A rich non-coding sequences and have purines at the preferred positions (Goelet et al., 1982). These U/A rich sequences are not common in eukaryotic mRNA in general, but appear to be regular features of the 5' non-coding regions of plant viruses.

Interestingly, although the sequence around the initiation codon might be suboptimal, in some cases this does not appear to have an adverse effect on the level of translation. An example is the CarMV coat protein subgenomic RNA which, although only representing less than 10% of the encapsidated RNA, has a messenger activity in vitro far exceeding that of the genomic RNA (Guilley et al., 1985; Carrington & Morris, 1985). This might be due to the primary or secondary structural characteristics of the 5'-untranslated sequence, or perhaps the initial coding sequences. It is noteworthy that the untranslated region of the subgenomic RNA is also U/A rich and deficient in guanosine residues.

Divided genome viruses should be especially useful in defining the signals for RNA encapsidation, RNA replication and other functions crucial for viral infectivity, because these signals should exist on each of the genomic RNAs with a degree of similarity of primary and secondary structure required for effective interaction with the relevant proteins. Both the 5'- and 3'-termini of M- and B-RNAs of CPMV (Van Wezenbeck et al., 1983; Lomonossoff & Shanks, 1983) contain regions with high

sequence homology. The first 44 nucleotides in the two leader sequences show 86% homology, while at the 3'-terminus, the last 65 nucleotides preceeding the poly(A) tail show 83% homology. Particularly noteworthy is a stretch of 11 nucleotides found in the leader of both RNAs (nucleotides 19-29, ie UUUUGAUAAAA) which is complementary (allowing one G-U base pairing) to the first 4 A's of the poly A tail and the 7 nucleotides proceeding this tail in each RNA. This implies, furthermore, that both the plus and minus-strand of either genome segment share a similar sequences at their 3'-termini. This sequence may therefore well be the recognition signal for the viral replicase.

Examination of the 3'-terminal sequences of RNA-1, RNA-2, RNA-3 and RNA-4 from each of the bromoviruses and CMV (cucumber mosaic virus) shows that there is considerable inter-viral and intra-viral homology (Ahlquist et al., 1981). In BMV RNAs, intra-viral homology extends for at least 230 residues from the 3'-termini, while in CMV the terminal 138 nucleotides of RNA-1 and RNA-2 are identical to those of RNA-3 and RNA-4 except for one residue in RNA-1 and three residues in RNA-2 (Symons, 1979). The inter-viral homology is more obvious when the secondary structures are compared; as described above, the 3'end 190 or so bases of BMV and CMV can be folded to form tRNA-like structures with very similar structures (Ahlquist et al., 1981). Although the 3'-termini of AlMV cannot be folded into a tRNA-like structure, the 3'-terminal 142 residues of all four RNAs are also substantially conserved, after which there is no significant homology (Cornelissen et al., 1983a, 1983b; Barker et al., 1983).

1.6 STRATEGIES OF EXPRESSION

As mentioned above, eukaryotic ribosomes typically start translation at the first AUG initiation codon from the 5'-end of the mRNA (Kozak 1981, 1983). For the translation of plant viral RNAs this rule implies that only the 5'-ORF in the genomic RNAs are directly available for translation while downstream ORFs normally remain closed. Plant RNA viruses may employ one (or more usually a combination) of the following three translation strategies to overcome the limitation of their host's ribosomes (Table 1.2).

- 1) The viral RNA may contain a leaky termination codon which some of the ribosomes will read-through to translate the second gene. Two proteins will be produced which are identical over the total length of the shorter protein, e.g. TMV, CarMV.
- 2) The virus may produce subgenomic RNAs to express downstream genes, e.g. TMV, CarMV, SBMV.
- 3) The viral RNA may be translated (as a monocistronic messenger) into a polyprotein which is proteolytically processed to give several mature proteins with distinct functions, e.g. potato virus Y (PVY), TYMV, CPMV

These strategies not only allow the virus to synthesize more than one protein from a unique RNA, but also permit the virus to modulate the relative abundance of the viral proteins. In the case of read-through the relative amount of the two proteins depends on the efficiency with which the termination signal is overcome. For example, if only a small amount of the larger protein was required, the termination codon could be used for which there was only a relatively low concentration of suppressor tRNA in the host plant.

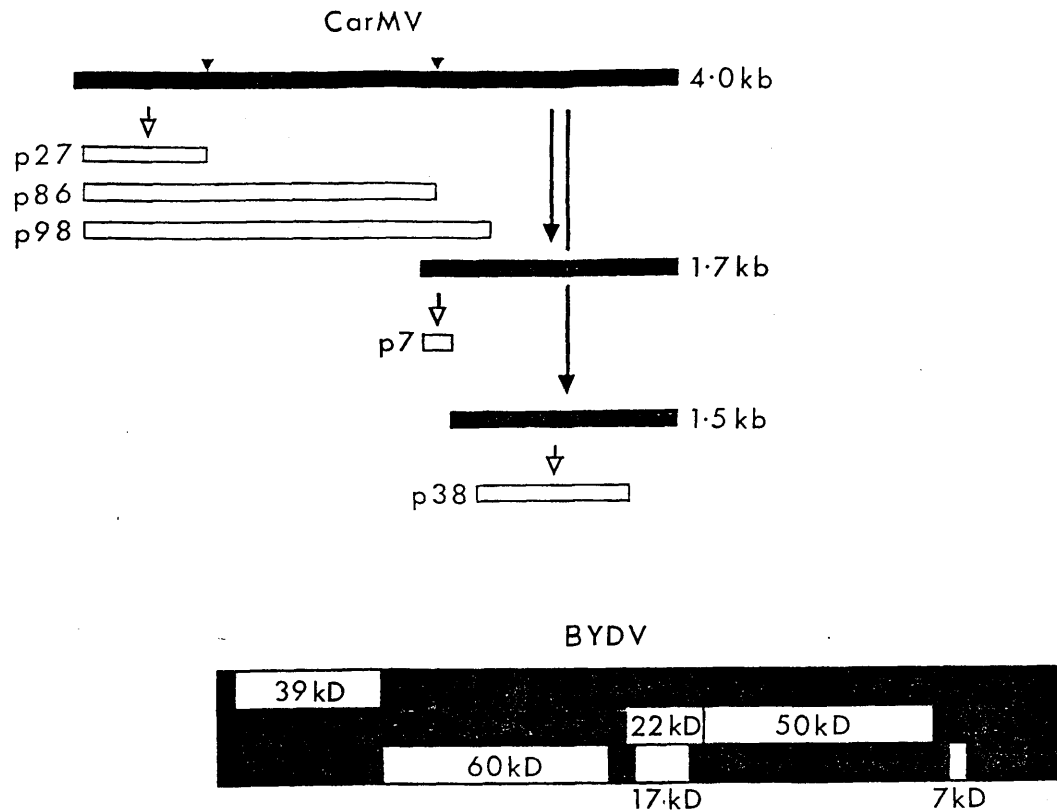
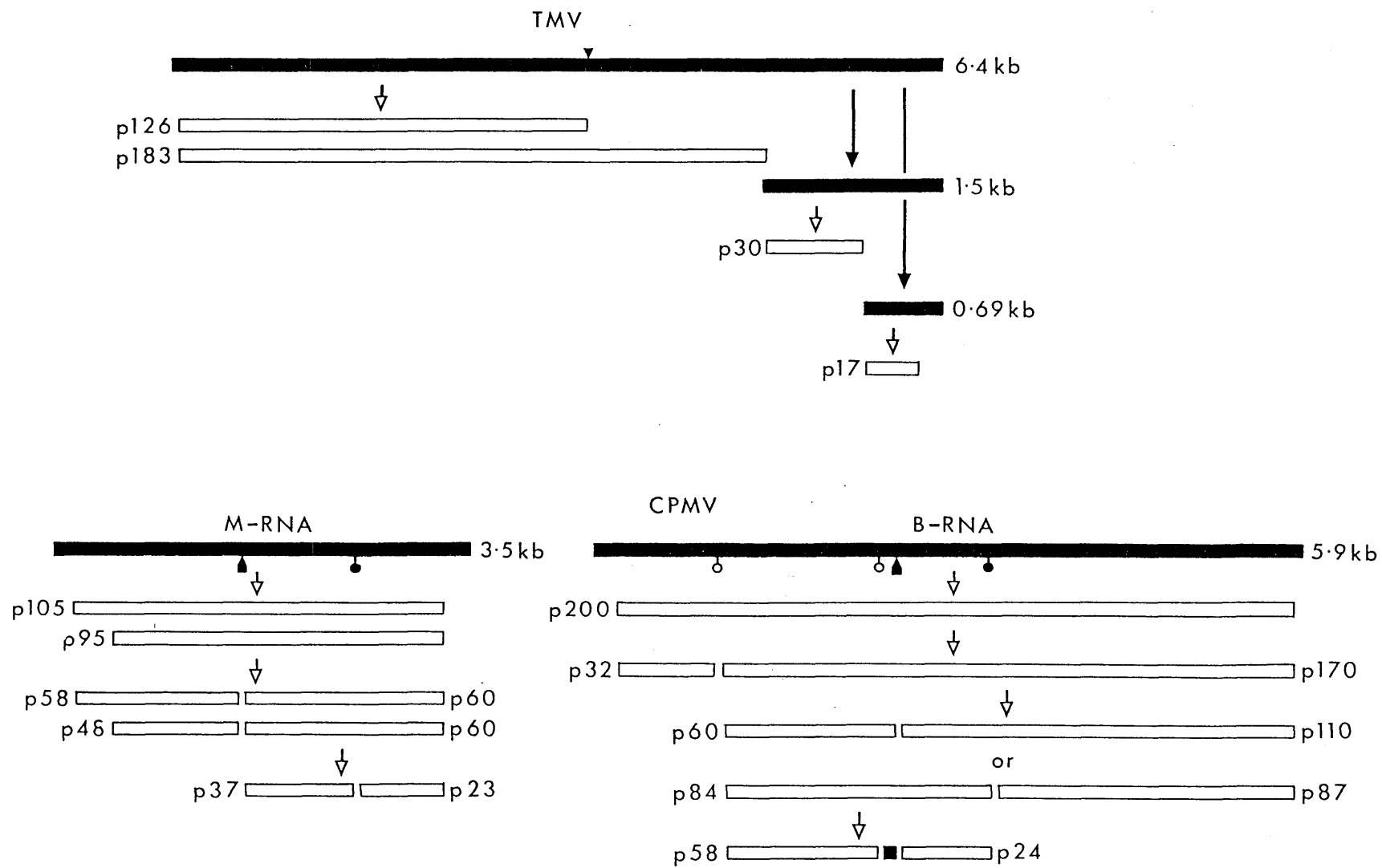


Fig.1.1. Strategies of genome expression of three plant RNA viruses, and the open reading frames of BYDV genomic RNA. The approximate size of the genomic and subgenomic RNAs of CPMV, TMV and CarMV are given in kilobases.

For CPMV, TMV and CarMV: ■ = RNA molecule; □ = protein molecule; p = protein in kilodaltons; ▲ = glutamine-methionine cleavage site; ● = glutamine-glycine cleavage site; ○ = glutamine-serine cleavage site; □ = CPMV coat protein. indicates presumed derivation of subgenomic RNAs and of protein fragments.

For BYDV: the open reading frames are shown in white, and the sizes of the potentially encoded polypeptides are given in kilodaltons.



The levels of expression of internal ORFs can be regulated by the relative abundance of subgenomic RNAs and the efficiency of their translation. The 5' non-coding regions, the sequence around initiation codon and the subgenomic promoter strength may all play a role in this level of regulation.

Readthrough

The genetic map of TMV is shown in Fig.1.1. When TMV RNA is translated in vitro, two major proteins of 126 kD and 183 kD are produced (Pelham & Jackson, 1976). By two dimensional peptide mapping Pelham (1978) deduced that the two proteins are initiated from the same AUG and have overlapping amino acid sequences. The 126 kD protein gene is terminated by a UAG codon (Goelet et al., 1982). The leakiness of this codon leads to the synthesis in small amounts of the read-through protein of 183 kD. The synthesis of the larger protein is increased significantly when yeast UAG suppressor tRNA is added to the in vitro translation system.

In TMV infected cells the 126 kD and 183 kD proteins are readily detectable (Paterson & Knight, 1975; Beier et al., 1980). It was suggested that tobacco plants contains natural UAG suppressor tRNA, a suggestion which proved to be correct when such a molecule was isolated from both tobacco and wheat leaves and sequenced (Beier et al., 1984a, 1984b).

Subgenomic RNAs

In addition to the 126 kD and 183 kD proteins, a 30 kD protein is also produced when TMV RNA is translated in vitro. This smaller protein has no peptides in common with the higher molecular weight proteins, and was found to be

translated from the separately encapsidated sgRNA (I2) (Bruening et al., 1976; Higgins et al., 1976; Beachy & Zaitlin, 1977). The 30 kD protein is found in both tobacco leaves and protoplasts where it appears early in the onset of infection, before the synthesis of detectable levels of the higher molecular weight proteins (Joshi et al., 1983; Watanabe et al., 1984)

Translation of vulgare strain of TMV RNA in vitro does not yield the coat protein, even though the protein is produced in very high levels in vivo (Hunter et al., 1976; Siegal et al., 1976). Hunter et al. (1976) isolated a short RNA species from infected plant RNA that translated to produce the coat protein. Higgins et al. (1976) and Bruenig et al. (1976) showed that when the genomic RNA from the cowpea strain of TMV was translated in vitro significant amounts of the coat protein were produced. When TMV virions are studied by electron microscopy 300 nm rods are usually seen. Higgins et al. (1976) and Bruenig et al. (1976) showed that virion preparations of the cowpea strain also contained 40 nm rods not found in the vulgare strain infection, which when purified free from the 300 nm rods yielded mRNA that coded exclusively for the coat protein. This sgRNA is 3'-coterminial with the genomic and I2-RNA. Bruenig et al. (1976) also showed an intermediate size of rod exists; the RNA from this size of rod (I2) directed the synthesis of a 30 kD protein.

The reason for the encapsidation of the coat protein sgRNA (cpsgRNA) in the cowpea strain was later shown to be due to the relative location of the origin of assembly (Goelet et al., 1982; Meshi et al., 1981, 1982). The assembly origin for the vulgare strain is contained within the 30 kD protein gene which results in the encapsidation of the genomic and I2-RNA, but not the cpsgRNA. In the cowpea strain the position of the assembly origin is shifted to the coat protein gene.

The entire nucleotide sequence of the genomic RNA of the vulgaris TMV strain (Goelet et al., 1982) and the TMV-I strain (Ohno et al., 1984), and large parts of the cowpea strain (Meshi et al., 1981, 1982) have been established. These sequences have established the presence of the 126, kD, 183 kD, 30 kD and coat protein genes, the leaky termination codons and the origins of assembly. The end of the reading frame for the 183 kD protein overlaps by five codons with the ORF, in a second phase, for the 30 kD protein.

The 30 kD ORF terminates with a UAA codon two nucleotides before the initiator codon for the coat protein ORF:

Thus the 30 kD protein is read in a different frame from the one used for the 126/183 kD proteins and the coat protein. In the cowpea strain the 30 kD protein is 15 amino acids longer than the vulgare and TMV-L strains and overlaps in its 5' and 3' regions with the 183 kD and coat protein genes by eight and twenty six nucleotides respectively.

Re-investigation of the number of subgenomic RNA molecules of TMV by Sulzinski et al. (1985) concluded that there is a third such molecule, termed I1, of Mr 1.1×10^6 . This RNA species had been identified before (Siegal et al., 1976; Beachy & Zaitlin, 1977; Goelet & Karn, 1982; Dorokhov et al., 1983) but its role as a mRNA had not been established. Northern blotting and S1 mapping using ^{32}P labelled clones to known regions of the TMV genome established the I1-RNA to have a 5' terminus at guanosine residue 3405, with the first 5'-proximal AUG codon at residues 3495. If this codon is the functional initiator, then the I1-RNA coding sequence is preceded by a 90 base noncoding leader sequence. Of the ten bases immediately 5' proximal to this AUG codon, eight of those residues are uridine or adenosine, a characteristic of the other sgRNAs.

If the AUG at position 3495 is indeed the codon used for initiation, then the I1 ORF is in frame with and co-terminates with the ORF that encodes the 183 kD protein. A polypeptide product of 54 kD would be the expected product of the I1 ORF. Such a polypeptide is produced when gradient fractions of total encapsidated TMV RNA enriched for I1 are translated in vitro in the rabbit reticulocyte lysate system. Asselin et al. (1985) detected a prominent 50 kD product following the in vitro translation of TMV RNA in a E.coli based translation system. This data suggests that the 50 kD product is the product of an internal gene, since in this system internal initiation of translation is not restricted.

Sulzinski et al. (1985) acknowledge that such a protein had not been reported in either protoplasts or infected leaf material, but suggested that the 54 kD protein would be masked by host proteins of a similar size, and suggest that the protein might be revealed using western blotting and the correct antibodies. However, when antibodies

raised using a 183 kD fusion protein were used in a western blot of TMV infected protoplasts (Saito et al., 1986), the 54 kD protein was not detected. It was therefore concluded by Saito et al. (1986) that the 54 kD protein does not play a role in the replication of TMV, at least in tobacco protoplasts.

There are three models for the production of subgenomic RNA: (1) internal initiation by the replicase on the minus-strand of the genomic RNA (Kennedy, 1980; Nassuth et al., 1981); (2) premature termination during minus-strand synthesis, followed by independent replication of the subgenomic RNA (Goelet & Karn, 1982); and (3) processing by nuclease cleavage of genome length RNA (Gonda & Symons, 1979). Using the BMV replicase, Miller et al. (1985) showed that the BMV subgenomic RNA-4 was synthesised from minus-strand templates of BMV RNA-3; dscDNA of RNA-3 was cloned behind a SP6 promotor and transcribed to produce minus-strand RNA-3. Positive-strand subgenomic RNA-4 was then produced upon incubating this transcribed RNA with the BMV replicase. The minus-strand, and hence the double-stranded form seen occasionally in tissues, probably results from the replicase binding to the 3'-end of the RNA-4; as the subgenomic is 3' co-terminal it has the same replicase binding site as the genomic RNA. The newly transcribed RNA-4 minus-strand cannot, however, serve as a template for plus-strand synthesis because the BMV replicase binds upstream of the RNA-4 initiation site on RNA-3.

Marsh et al. (1988) and French and Ahlquist (1988) have located the the subgenomic promotor of RNA-4 in BMV RNA-3. Truncated and mutant minus-strand RNA templates were produced by in vitro transcription of altered RNA-3 cDNA clones and used as templates by the RNA-dependent RNA polymerase as described by Miller et al. (1985). While the control RNA-3 clone yielded plus-strand sgrNA-4 of the

expected length, the mutant RNAs gave rise to either altered lengths and/or reduced yields of RNA-4. While sequences no more than 20 bases upstream of the initiation site suffice to direct specific initiation at a low level, sequences at least as far as 74 bases upstream are required for full activity (French & Ahlquist, 1988). The location of the mutations within the template by Marsh et al. (1988) revealed a promotor of approximately 62 bases grouped into functional domains:

$$\text{-}\underline{\text{UUAUUAUU}}\text{-(A)}_{20}\text{-}\overline{\text{AAGAUCUAUGUCCUAAUUCAGC}}^*\text{GUAUUAUAUAUGUCGAC-}$$

(The asterisk proceeds the initiation codon, and the A₂₀ represents the poly A tract. Other features are described in the text). However, French and Ahlquist (1988) noted that the consensus sequence AUCUAUGUU (the sequence closest to the initiation codon and within the 62 bases defined by Marsh et al., 1988 is overlined) occurs three times in the upstream region, each with a single mismatch, and that removal of each of the upstream elements in turn results in a successive loss of promotor activity. French and Ahlquist (1988) have suggested that the consensus elements might constitute binding sites for trans-acting factor(s) involved in initiation of sgRNA synthesis.

As mentioned above, the core sequence consists of about twenty bases immediately upstream of, and including, the initiation G nucleotide. When these 20 bases are compared with 19 bases present upstream of the subgenomic initiation site of several alphaviruses a strong homology (bold letters in sequence) can be seen. Three regions (totalling 16 bases) have complete homology with the alphavirus consensus promotor (Marsh et al., 1988).

Parts of the promotor sequence (underlined) show homology with a number of other putative subgenomic promoters of plant viruses such as TRV, TMV and AlMV, although these sequence blocks are often spaced differently. BSMV, however, does not have the AAGA sequence, but does have the UCUA sequence instead. Both the upstream UUAUUAUU block and the poly(A) tract apparently have a specific function in promotor activity because deletions in this area effect the levels of sgRNA-4 produced, but not the correct initiation of sgRNA-4.

In addition to the core sequence and other blocks of homology, a A-U rich domain overlapping the 5'-untranslated end of RNA-4 apparently determines correct initiation. Deletions in this region result in a large proportion of sgRNA-4 initiated at the incorrect nucleotide (Marsh et al., 1988).

The other main domain immediately upstream of the promotor core consists of the internal poly(A) tract of RNA3. This probably serves as an non base-paired spacer facilitating access of the replicase to the promotor within the generally high secondary structured RNA-3.

Polyprotein

As shown in Table 1.2, a number of viral RNAs are translated into polyproteins which are then proteolytically processed. Probably the most studied example is CPMV. As shown on Fig.1.1, the B-RNA is translated to produce a 200 kD polypeptide which is processed to a number of smaller polypeptides (Franssen et al., 1984a). The 170 kD polypeptide is processed by alternative cleavages to give either 110 kD + 60 kD polypeptides or 87 kD + 84 kD polypeptides (Goldbach & Rezelman, 1983). A similar situation is seen in the proteolytic processing of poliovirus where the polyprotein

3CD can be cleaved into the proteins 3C (20 kD) and 3D (52 kD) or into 3C' (36.4 kD) and 3D' (35.6 kD). The two amino acids on either side of the cleavage site are different for the two sites, and it is thought that different proteases are used. Cleavage at the 3C'-3D' site may be fortuitous (other sites in the polyprotein are cleaved by this protease to yield essential proteins) and of no biological significance other than lowering the yield of 3C and 3D. When the 3C'-3D' cleavage site was mutated so that cleavage could no longer occur at this site, the virus replication cycle was found to be unaffected (Wimmer et al., 1987).

Linear scanning, internal initiation and translational frameshift

As mentioned above, eukaryotic ribosomes typically start translation at the first AUG initiation codon from the 5'-end of the mRNA (Kozak 1981, 1983). Although the majority of plant viruses use one or more of the strategies described above to express internal ORFs, there are a few instances where more novel methods are employed to overcome the limitations of the host ribosomes.

Translation of CPMV M-RNA in vitro yields 105 kD and 95 kD polypeptides (Fig.1.1) as primary translation products in both reticulocyte lysates and wheat germ extracts. Although the production of both of these polyproteins in vivo has yet to be confirmed, the M-RNAs of all comoviruses tested produce two large proteins in vitro, despite extreme sequence divergence. The proteins have different N-termini (Franssen et al., 1982) and result from translation commencing at initiation codons at positions 161 and 512 (Vos et al., 1984). This might be explained by the sequences around these codons. The linear scanning ribosome model of Kozak (1983, 1986, 1987), shown

to apply to a number of animal RNA viruses (Kozak, 1986), postulates that the 40S ribosomal subunit binds initially at the 5'-end of mRNA and migrates linearly until it reaches the first AUG. If the first AUG codon lies in an optimal context, which in higher eukaryotes is CCACCAUGG (Kozak, 1986) the subunit migrates no further; it couples with a 60S subunit and protein synthesis initiates uniquely at that site. When the context around the first AUG is less favourable (positions -3 and +4 relative to the A of AUG are especially critical), some subunits nevertheless stop and initiate there, while others bypass the first site and initiate at the next codon downstream. Inspection of the sequences around the two CPMV M-RNA codons reveals that whereas the first site (161) is probably weak, having a A at -3 but a U at +4 (Kozak, 1981, 1984), the second AUG at 512 is strong having G at both -3 and +4.

The AUG codon that initiates the poliovirus polyprotein is preceded by eight other AUG codons in a long 743 nucleotide 5'-untranslated region (Dorner et al., 1982). There is no evidence that the eight upstream AUG codons serve to initiate short viral polypeptides. Originally it was thought that the 40S subunit scanned past these codons, but Pelletier and Sonenberg (1988) have shown that the 40S subunit binds directly to the correct codon (or very close to it). The untranslated region is thought to provide a higher-order structure which causes ribosomes to bind directly to the main initiation codon. CPMV and poliovirus are clearly related evolutionarily, and therefore, while the scanning ribosome model of Kozak (1986) may explain the production of the two CPMV M-RNA polyproteins, the possibility of internal initiation cannot be ruled out.

The RNA genome of barley yellow dwarf virus (BYDV) has five ORFs thought to encode genuine polypeptides (Miller *et al.*, 1988) (Fig.1.1). The 5'-ORF potentially encodes a polypeptide of 39 kD beginning at the first AUG in the genome. The 39 kD ORF overlaps by 13 bases a second ORF which could encode a polypeptide of 60 kD. No subgenomic for this polypeptide has been detected, and as the second ORF is in a different reading frame from the 5'-ORF, the 60 kD polypeptide cannot be expressed as a readthrough product of the 39 kD. It is possible that the 60 kD ORF may be expressed via linear scanning by the ribosomes (Kozak, 1986). However, the 39 kD initiation codon is potentially strong having A at -3 and a U at +4 (Kozak, 1981, 1984), and the 60 kD cistron does not begin with an AUG codon.

Internal initiation (Pelletier & Sonenberg, 1988) is a possible alternative to explain the expression of the 60 kD ORF, but analysis of the sequence around the end of the 39 kD cistron raise the possibility of translational frameshift (Miller *et al.*, 1988). During frameshift the ribosome correctly shifts from one reading frame to the other at a discrete position on the mRNA so as to avoid premature termination of polypeptide synthesis (Craig & Caskey, 1987). Frameshifts in translation of polymerase genes of retroviruses and a coronavirus have been characterised (Craig & Caskey, 1987). These viruses all have either the sequence AAAAAAC or UUUA in the frameshift region, followed by a stem-loop structure which can be quite variable in size and location relative to the stop codon. At the end of the 39 kD cistron, BYDV RNA contains the sequence UUUA

followed by a stem-loop structure. Therefore, it has been proposed (Miller et al., 1988) that the ribosomes bypass the stop codon at the end of the 39 kD cistron and translate the 60 kD cistron, resulting in a 99 kD fusion protein.

1.7 REPLICATION OF RNA VIRUSES

During the replication of RNA viruses at least two types of replicative structures are generated - a replicative form (RF) and a replicative intermediate (RI). RF is considered to comprise fully double-stranded RNA molecules consisting of one full-length genomic (plus-strand) molecule and one full-length complementary (minus-strand) molecule. By contrast, RI is a complex consisting of one full-length RNA molecule (of possibly either plus or minus polarity) and several single-stranded tails of opposite polarity and variable length; it is believed that during replication several polymerases are present on the same template at a given time (Hall et al., 1982).

RF forms have been found in hosts infected with a number of plant RNA viruses. For example, dsRNA corresponding to the three genomic RNAs, the subgenomic RNA and the satellite RNA CARNA-5 have been found in CMV infected tissues (Daiz-Ruiz & Kaper, 1977) and protoplasts (Takanami et al., 1977). Such molecules have probably been derived from functional replicative structures. Double-stranded RNAs corresponding in size to viral RNA-1, RNA-2 and RNA-3 have been found in tissue infected with two other tripartite viruses, BMV and AlMV. However, a dsRNA form of the sgRNA-4 has been reported to be both present in (Pinck & Hirth, 1972; Bol et al., 1975; Bastin & Kaesberg, 1976; Loesch-Fries & Hall, 1980) and absent from (Mohier et al., 1974; Bancroft et al., 1975) cells infected with BMV or AlMV. Other experiments in which RNA-4 did not replicate when

inoculated into protoplasts (Bol & van Vloting-Doting, 1973; Smit & Jaspers, 1982; Huisman et al., 1987; Van Pelt-Heerschap et al., 1987) would seem to support the idea that a replicative form of BMV or AlMV RNA-4 is not normally present in infected tissue.

As early as 1968 Feix et al. (1968) suggested that RF isolated from the cell could be the result of artefacts formed during RNA isolation steps involving phenol deproteinization. Garnier et al. (1980) showed that in TYMV infected leaves the enzyme-template complex or RI bound to the chloroplast envelope contains no RF, and that the RF detected by polyacrylamide gel electrophoresis is formed during cell disruption and RNA extraction. However, due to the design of the experiments (Garnier et al., 1980), in which nascent RNA was labelled in vivo, any RF which was not actively engaged in replication would not have been detected. Keil and Hofschneider (1973) have provided evidence that RF which is no longer replicating accumulates in E.coli cells infected with bacteriophage M12. Dawson and Dodds (1982) showed that dsRNA molecules have a slow rate of turnover, and Chu and Westaway (1985) showed by using pulse labelling that the RI and RF interconvert in a cyclic manner. However, whatever the origin of RF, the RF isolated from virus infected plants has diagnostic value (Dawson & Dodds, 1982; Dodds & Bar-Joseph, 1983; Gildow et al., 1983; Jordan et al., 1983; Morris et al., 1983) and can be used to aid the classification of plant viruses. For example, cucumber necrosis virus has recently proposed as a tombusvirus by its dsRNA profile on polyacrylamide gels (Rochon & Tremaine, 1988).

RNA-Dependent RNA Polymerases

The replicase of a positive-strand plant RNA virus must be able to make not only the initial complementary RNA copy (the minus-strand) of the RNA template, but also to use the minus-strand to make a new second (typically plus-strand in the sense that it serves as the messenger for protein synthesis) strand that is a replica of the initial RNA template. In healthy plants an enzymatic activity has been found which can: (1) incorporate radioactive ribonucleotide triphosphates into an acid precipitable form, (2) synthesize RNase resistant products, (3) synthesize small, heterodisperse RNA products, (4) synthesize RNA that appears to be complementary to the RNA offered as template, (5) synthesize RNA without any template specificity (Hall et al., 1982). The normal level of these host polymerase activities are enhanced considerably upon viral infection which has led some authors to suggest that such activities are responsible for viral replication. However, much of the data is weak due to the lack of controls and low stimulation of the enzyme activity upon addition of the viral template, and so far no RNA-dependent RNA polymerase that faithfully uses the plus-viral strand to create full-length plus-strands has been found in healthy tissue (see Hall et al., 1982. for a critical review of the data).

A membranous fraction of cell-free extracts of TMV infected tobacco leaves is capable of synthesis of TMV RF and RI (Bradley & Zaitlin, 1971) in a template independent manner, apparently by continuing the synthesis of partially completed replicative structures. The RF is insensitive to RNase A at high salt concentrations, but sensitive to RNase III, showing it to be fully double-stranded, while the RI RNA was partially sensitive to both enzymes, showing it to have both single and double-stranded regions. The RI and RF molecules were labelled by

incorporating radioactive rUTP in the cell-free reaction, and the products denatured and hybridized to single-stranded M13 clones (Young & Zaitlin, 1986). Isolated RI RNA hybridized only to clones of minus polarity (relative to the original plus polarity of the genomic TMV), indicating only plus-strand synthesis. Isolated RF RNA, by contrast, hybridized to M13 clones with inserts of both polarities, with roughly six times greater incorporation into plus-strand than the minus. These experiments indicate that synthesis of plus-strands takes place on replication complexes with one or more progeny strands, while synthesis of minus-strands takes place on complexes with only a single progeny RNA molecule.

Similar results were obtained by Watanabe and Okada (1986) who analysed the in vitro replication products from an extract prepared from TMV infected tobacco protoplasts. The extract produced much more plus-stranded viral RNA than minus-strand RNA. This activity increased in parallel with the appearance of TMV specific RNAs and proteins in vivo (Watanabe et al., 1982, 1984). Moreover, the extract synthesized the sgcpRNA. Neither the RF form or the minus-strand of this sgRNA was detected. Therefore, the sgRNA may be synthesized by internal initiation on the negative strand of the genomic RNA as reported for BMV RNA-4 (Miller et al., 1985).

Dorssers et al. (1983, 1984) analysed the products and protein constituents of a highly purified CPMV RNA replication complex from a particular membrane fraction of infected primary cowpea leaves. The RNA products synthesized in vitro by this fraction revealed the presence of both the host RNA-dependent RNA polymerase, which transcribed both host RNA and viral RNA into small minus-stranded RNA, and a RNA-dependent RNA polymerase which was able to elongate in vitro nascent viral RNA chains initiated in vivo to full-length viral dsRNA.

However, all attempts to obtain a template dependent activity, able to accept and to initiate RNA synthesis on added CPMV failed, even when very crude preparations were used.

The purified CPMV specific RNA-dependent RNA polymerase purified (Dorssers et al., 1984) consisted of polymerase molecules still associated to the endogenous viral RNA template. Only three polypeptides were detected in such preparations - the 110 kD protein encoded by the B-RNA and two host encoded proteins of 68 kD and 57 kD. The 110 kD protein found in the replication complex is possibly the core polymerase of the complex. This protein contains a region of approximately 60 kD with significant sequence homology to the RNA-dependent RNA polymerase of animal poliovirus (see below). Since the conserved domain maps in the 87 kD protein of CPMV as well, it might be expected that the 87 kD also plays a role in replication; infected cells contain such a high level of the 87 kD protein that it is unlikely to be a nonspecific breakdown product. The lack of this protein in significant amounts in the purified RNA-dependent RNA polymerase preparation may be due to its loss during the extraction procedure. The 87 kD protein may be involved in a particular stage of the RNA replication process not performed by the purified preparations analysed so far, such as minus-strand synthesis. The two host proteins (68 kD and 57 kD) may be subunits tightly associated in a multimeric enzyme or, alternatively, one or both may be a host factor analogous to the host factor(s) involved in poliovirus RNA and Q β replication (Wimmer et al., 1987; Blumenthal & Carmichael, 1979).

Miller and co-workers have isolated an enzyme capable of synthesizing the RNAs of BMV in vitro (Bujarski et al., 1982; Miller & Hall, 1983; Miller et al., 1986). The enzyme has an absolute requirement for BMV RNA and it will

accept templates of either plus or minus polarity, but in either case only the specific RNA complementary to the template is produced i.e. the replication cycle, in which these complementary RNAs are then used as templates for the synthesis of RNA of the original polarity, is not completed. However, as initiation of de novo synthesis on the supplied template has been shown (Miller et al. 1986), the BMV replicase probably represents the best purification of a plant RNA-dependent RNA polymerase yet obtained.

The RNA genome of BMV consists of RNA-1 (3234 b), RNA-2 (2865 b) and RNA-3 (2117 b). A subgenomic component (RNA-4, 876 b), from which the coat protein is expressed, has a sequence co-linear with the 3' end of RNA-3 (Ahlquist et al., 1981, 1984b). When protoplasts are inoculated with only RNA-1 plus RNA-2, replication of the introduced RNAs occurs (Kiberstis et al., 1981; French et al., 1986). Inoculation with each RNA separately does not lead to replication. Therefore, the proteins of the two RNAs (RNA-1 codes for protein 1a of 110 kD and RNA-2 codes for 2a of 105 kD) are required for replication of BMV. As expected, protein 1a was detected in purified BMV RNA-dependent RNA polymerase. Protein 2a, however was not detected. It is probable that the 2a protein is lost in the purification process, and similarly to CPMV, the loss of the protein might explain why the purified RNA-dependent RNA polymerase does not perform all the functions expected (Miller & Hall, 1983).

Using the purified BMV replicase, several replication experiments were conducted on 3'-terminal BMV sequences that were hybridized with single-stranded cDNA sequences (Ahlquist et al., 1984a). These experiments revealed that obstruction of the 3'-terminus by hybrid formation inhibited replication, presumably by preventing initiation. When cDNA sequences complementary to internal

regions of the 3'-end were hybridized, replication was generally unaffected. Therefore, it seemed likely that the replicase utilized the 3'-end for binding to the RNA template.

The three RNAs comprising the BMV genome share a conserved, highly structured 3'-terminal sequence approximately 200 bases long (Ahlquist et al., 1981). The last 134 bases in this region are base paired to form tRNA-like structure which can be esterified with tyrosine by aminoacyl tRNA synthetase (Hall et al., 1972) (Fig.1.2). Initiation of minus-strand synthesis occurs on the penultimate base of the plus-strand template (Miller et al., 1986), requiring that the terminal A residue of the mature BMV virion be added in a subsequent template independent reaction by ATP,CTP:tRNA nucleotidyl transferase (Deutscher, 1984). This is supported by the observation that double-stranded replicative form RNAs isolated from infected plants lack 3'-terminal A (Miller et al., 1986). A similar situation is found with QB (Weber & Weissman, 1970). The ds forms of both CARNA5 and RNA-3 of CMV contain an unpaired guanosine (G) at the 3' end of the minus-strand which is not found on the encapsidated plus-strand (Collmer & Kaper, 1985), a feature also present in the replicative forms of several animal alphaviruses (Wengler et al., 1979, 1982). Again synthesis of nascent plus-strands is initiated internally at the penultimate nucleotide. Although the function of such a post-transcriptionally added nucleotide is not understood, one possibility is that it serves as part of a recognition signal for the replicase that initiates nascent plus-strand synthesis at an internal site. Therefore, both the aminoacylation by aminoacyl tRNA synthetase and the adenylation with ATP,CTP:tRNA nucleotidyl transferase may be crucial in viral replication.

Isolation of cDNA clones to each of the BMV RNAs and the use of in vitro transcription systems, have allowed the synthesis of partial BMV RNA transcripts that are reactive in in vitro assays (Dreher et al., 1984) and complete BMV RNA transcripts that are infectious to barley plants (Ahlquist et al., 1984b) and protoplasts (French et al., 1986). Dreher et al. (1984) and Bujarski et al. (1985) introduced a series of mutations into the transcripts and measured their reactivity in vitro. Dreher et al. (1984) showed that when two bases in the anticodon in arm C of the tRNA like 3'-end were substituted with other different bases over 80% of the replicase template function was lost, while aminoacylation was affected by less than 10%. These results delineated arm C as being involved in replicase binding and strongly suggested that it had little function in aminoacylation. Subsequent experiments in which the small (4 bases - Dreher & Hall, 1987) or large (54 bases - Bujarski et al., 1985) deletions were made also resulted in little change in aminoacylation function, but effectively eliminated replicase template activity.

As would be expected, mutation of the 3'-terminal two nucleotides drastically reduced replicase template activity and eliminated the tyrosine acceptance function (Dreher et al., 1984). Other mutations in which various other parts of the tRNA-like structure were deleted (Bujarski et al., 1985) showed: (1) deletions in arm B led to a substantial inhibition of aminoacylation; (2) extensive deletions in arm D had little or no effect on aminoacylation and, in several cases, substantially increased the template activity of the transcribed RNA for replicase; and (3) the structural features recognised by nucleotidyl transferase overlap those recognized by the replicase, and differ significantly from those recognized by synthetase (Bujarski et al., 1986). The rough parallel

of replicase and adenylation activities of templates with mutations in the C and D arms may reflect interaction of nucleotidyl transferase with at least part of the anticodon arm, a region shown earlier (Dreher et al., 1984) to be important in replicase recognition, but not aminoacylation.

The BMV mutants were tested in vivo by their ability to act as a replicate in protoplasts (Bujarski et al., 1986). Only the transcript with a mutation in arm D proved to be infectious but was shown to replicate at lower levels than the wild type RNA. Other deletions proved to be non-infectious, including templates which showed near normal replication and adenylation but little or no aminoacylation in vitro. This lends support to the contention that aminoacylation is essential in vivo.

The aminoacylation reaction is specific for a given amino acid and, where the kinetics have been measured (Hall et al., 1972, Giege et al., 1978) they are similar to values obtained for aminoacylation of tRNAs. The role of aminoacylation in replication may revolve around the involvement of cell elongation factors in replication. This is suggested by analogy with the QB replicase which contains the bacterial host elongation host factors EF-Tu.Ts as part of the complex. Since, as in the case of tRNA, elongation factor binds only to aminoacylated viral RNAs (Bastin & Hall, 1976), aminoacylation of viral RNA could assist in binding of part of the replication complex. Recent experiments using EF-1a specific antibody have failed to show the presence of eukaryotic EF-1a in turnip yellow mosaic virus replicase (Joshi et al., 1986), but similar tests revealed its presence in BMV replicase (Hall et al., 1987)

Proteins Involved In Replication

Cornelissen & Bol (1984) and Haseloff et al. (1984) compared the amino acid sequences of the proteins encoded by RNA-1 and RNA-2 of AlMV and BMV (the only RNAs of these viruses required for replication in protoplasts) and the 126 kD and 183 kD proteins encoded by TMV. Three blocks of extensive homology of about 200 to 350 amino acids each were observed. Two of these blocks are located at the N and C terminus the AlMV and BMV RNA-1 encoded proteins and the TMV 126 kD protein. The third block is located in the AlMV and BMV RNA-2 encoded proteins (90 kD and 94 kD respectively) and in the C-terminal of the TMV 183 kD protein.

The most notable of the conserved amino acids in the third block of homology are a glycine and two aspartic acid residues flanked on either side by six hydrophobic residues, and glycine, threonine and asparagine residues situated about 24 to 32 amino acids on the N-terminal side of this region. These conserved amino acids are also found in the putative replicases of other plant viruses such as the 194 kD protein of tobacco rattle virus (TRV), the 94 kD protein of CMV, the 87 kD and/or 110 kD protein(s) of CPMV, as well as more divergent animal viruses such as the poliovirus, FMDV, EMCV, coxsackievirus, rhinovirus 2 and 14, black beetle, hepatitis A, semliki forest (SFV), sindbis (SIN) and middleberg viruses (Kaariainen et al., 1987 and references therein). The strong conservation between such proteins as the core polymerase 3D^{Pol} of poliovirus, the only protein required for in vitro RNA chain elongation on poliovirus minus-strand (Dasgupta et al., 1979; Flanagan & Baltimore, 1977), and certain nonstructural proteins in the plant viruses argues strongly that proteins such as the 183 kD of TMV and the 87 kD and/or 110 kD proteins of CPMV are involved in viral replication. Although the precise function of the

conserved domains is not known, it has been proposed that the gly-asp-asp motif may be a common nucleic acid recognition site and/or active processing region (Kamer & Argos, 1984).

Another poliovirus encoded protein thought to be involved in RNA replication is 2C, by virtue of the fact that mutations which map in this region lead to an altered phenotype of poliovirus RNA synthesis (Pincus & Wimmer, 1986; Pincus et al., 1986). When the sequence of this protein is compared to the AlMV 1a, BMV 1a and TMV 126 kD proteins, a region of homology is detected which maps in the 3'-terminus of RNA-1 of AlMV and BMV and the 3'-terminus of the TMV 126 kD gene and in the proteins from animal RNA viruses, including nsP2 from SFV and SIN and the 2C proteins from EMC and FMD (Kaariainen et al., 1987). This gly-x-x-x-x-gly-lys-ser-(thr) sequence is not found in VSV L-protein, but there is a closely related sequence GLYRVGRT in the N protein. In influenza A PB2 and PA proteins there are the related sequences GFLILGKE AND GFIKGRS respectively (Kaariainen et al., 1987).

Interestingly, computer searches of data banks has recently identified a number of other proteins which contained this conserved region (Hodgman, 1988). All these proteins are involved in nucleic acid replication and/or recombination. This group includes two E.coli ATP-dependent helicases, four essential human herpes virus proteins (probably also helicases), two exonuclease V subunits and the yeast PIF protein (involved in mitochondrial recombination). Regions of homology between these proteins and the RNA virus proteins such as AlMV 1a, BMV 1a, TMV 126 kD, SIN nsP2, SFV nsP2 and CMV 1a outside the gly-x-x-x-x-gly-lys-ser-(thr) sequence were also

found. These regions of homology probably represent common secondary structures that make up functional sites for pyrophosphate, magnesium or nucleic acid binding (Hodgman, 1988).

A clue to the function of these RNA virus proteins comes from studying two sets of SIN temperature sensitive (ts) mutants. One ts mutant, termed a group B mutant, stops minus-strand synthesis soon after shift to a non-permissive temperature (Sawicki et al., 1981a). However, under these conditions plus-strand synthesis is not effected, suggesting that the "group B protein" is the specific factor required for minus-strand initiation. If group A mutant infected cultures in which minus-strand synthesis has halted are shifted to the non-permissive temperature minus strand synthesis starts again (Sawicki et al., 1981b). This suggests that the "group A protein" is regulating the shut-off of minus-strand synthesis, perhaps by inhibiting the "group B protein". UV mapping of the ts mutants suggested that the group A mutations lay within the nsP2 gene (Fuller & Marcus, 1980).

1.8 FUNCTIONS OF VIRALLY ENCODED PROTEINS

The function suggested for TMV 126 kD and 183 kD proteins is that they are components of the virally encoded RNA-dependent RNA polymerase. Evidence for the involvement of the 183 kD protein in a polymerase comes from: (1) demonstration that the protein is present at high concentrations in partially purified RNA-dependent RNA polymerase (Palukaitis & Zaitlin, 1986); (2) the readthrough portion of the protein shows a high degree of homology with the core polymerase of poliovirus; and (3) that mutagenesis of the 183 kD ORF destroys infectivity (Ishikawa et al., 1986).

There is less evidence for the participation of the 126 kD protein in replication, namely: (1) the 126 kD protein has been found in high levels in partially purified RNA-dependent RNA polymerase (Young et al., 1987); and (2) the 126 kD protein shows a degree of homology with protein 1a from BMV and AlMV, which have both been shown to be required for replication in protoplasts (Haseloff et al., 1984; Cornelissen et al., 1984). However, direct evidence for its involvement in replication is lacking; indeed, mutation experiments in which the 126 kD ORF is destroyed (Ishikawa et al., 1986) suggest that it might be disposable for replication, but not for normal symptom development. Ishikawa et al. (1986) showed that when the amber termination codon at the end of the 126 kD ORF was changed to a tyrosine, thereby preventing the formation of the 126 kD protein but allowing the production of a normal 183 kD protein, the transcripts showed an altered behaviour. On the initially infected leaves the lesions were much smaller than with the wild type RNA and appeared later, suggesting that the mutant can replicate to some extent by itself but that symptom development was impaired. However, since the 183 kD protein has the whole sequence of the 126 kD protein in its molecule, the 183 kD protein might also perform the role of the 126 kD protein under such conditions, albeit at a low level.

The Role of the RNA-Dependent RNA Polymerase in Subgenomic RNA synthesis

The RNA-dependent RNA polymerase has been shown to be responsible for the synthesis of subgenomic RNAs in vitro. It was shown by Miller et al. (1985) that subgenomic BMV RNA-4 is synthesised using the BMV RNA-dependent RNA polymerase and the minus-strand of RNA-3 as a template. Watanabe & Okada (1986) analysed the in vitro replication products from an extract prepared from TMV-infected

protoplasts. As well as replicating the genomic RNA, the extract also synthesised the coat protein subgenomic RNA. The involvement of the TMV RNA polymerase in the production of the subgenomic RNAs of TMV has also been shown in experiments concerning attenuation of symptoms in some TMV strains.

When plants are inoculated with viable mild strains (protecting strains) of some viruses, the plants acquire resistance against closely related severe strains. An attenuated strain of TMV, L11A, has been used to protect tomatoes from the virulent strains such as L strain. If L11A is propagated singly in plants, the multiplication of the virus decreases drastically at later stages of infection to give a final yield of about 10% of that of the L strain. Symptoms with the L11A strain are very mild. However, in protoplasts the replication levels of the genomic RNA of both strains are very similar (Nishiguchi et al., 1978; Nishiguchi et al., 1980).

When the nucleotide sequences of the two strains were compared (Nishiguchi et al., 1985), three significant base differences were found which resulted in amino acid changes. All three of these base substitutions, which are responsible for attenuation, mapped in the 126 kD/183 kD ORF. When these strains were inoculated into protoplasts (Watanabe et al., 1987) a comparison showed a reduction in the level of both the I2-RNA and 30 kD protein synthesis in the attenuated strain. The amount of the coat protein subgenomic RNA and its product were the same for both strains. This result suggests that 126 kD and/or 183 kD proteins are also involved in the transcription of the I2-RNA. Although the genome RNA sequences neighbouring the transcription sites of the two subgenomic mRNAs show some resemblance, the 126 kD and/or 183 kD proteins differentially regulate the synthesis of the two subgenomic RNAs. The reduction of the 30 kD protein

probably prevents the L11A strain from moving in large quantities to other host cells, and thus infection is attenuated.

Evidence that it is 126 kD protein which effects subgenomic RNA synthesis comes from comparison of this protein with those from BMV and Sinbis virus (Kaariainen et al., 1987). There is a block of homology between the carboxy-termini of TMV 126 kD, BMV protein 1a and nsP2 of SIN, and between the amino termini of TMV p126, protein 1a of BMV and nsP1 of SIN. SIN nsP2 was shown to be involved in the regulation of minus-strand synthesis (Kaariainen et al., 1987). However, when a nsP2 ts mutant is shifted to the restrictive temperature, the synthesis of the 26S subgenomic RNA is rapidly shut off. Synthesis of minus-strands were unaffected. A similar situation is found with another ts mutant which maps in nsP1. Therefore, as these proteins regulate the subgenomic RNA synthesis it is likely that a similar function is performed by BMV 1a and the TMV 126 kD protein.

Proteases

The most distinctive aspect of the comovirus replication cycle is proteolytic processing. Proteolytic cleavage occurs at three different sites, namely at a glutamine-glycine, glutamine-methionine, and glutamine-serine amino acid pairs (Fig.1.1). The 32 kD protein, the first mature cleavage product from the 200 kD polyprotein, had been proposed by Franssen et al. (1984b) to represent the protease involved in the cleavage at the glutamine-methionine site in the polyproteins from the M-RNA. This proposal is based upon the action of antibodies raised to the 32 kD protein which are able to prevent cleavage at this site. If the 32 kD protein is responsible for cleavage at the glutamine-methionine pair, it is possible

that it is also responsible for the similar cleavage in the B polyprotein. However, Vos et al. (1988) created a set of cDNA deletion mutants which, when transcribed and translated in rabbit reticulocyte lysate, underwent specific proteolytic cleavages. The results indicated that the B encoded 24 kD polypeptide represents a protease responsible for all cleavages in the polyproteins produced by both CPMV M- and B-RNA. For efficient cleavage of the glutamine-methionine site the 32 kD polypeptide is essential, although the polypeptide itself was shown not to have a proteolytic activity.

The CPMV polyproteins contain several potential cleavage sites which are not cleaved. This indicates that apart from the dipeptide sequence the folding of the protein around the cleavage site is also important (Wellink et al., 1986). Furthermore, a weak consensus sequence for the amino acids surrounding the cleavage site has also demonstrated (Wellink et al., 1986). Similarly, in poliovirus only a few of the theoretically possible cleavage sites are recognised by the protease 3C^{Pro}. Arnold et al. (1987) have proposed that 3C^{Pro} requires structurally flexible contexts surrounding the active sites.

Possible Roles For VPg

The role of the VPg in plant viruses is ambiguous. However, in picornaviruses it has been shown that the viral polymerase is completely dependent on a primer, and evidence suggests that the VPg provides for the primer function needed, namely: (1) VPg is present at the 5'-ends of the minus-strands in RF molecules and also at the 5'-ends of very short nascent plus-stranded RI molecules (Nomoto et al., 1977a, 1977b; Flanagan et al., 1977; Pettersson et al., 1978); (2) VPg precursors are present

in extracts of poliovirus infected HeLa cells active in initiating viral RNA synthesis in vitro (Takegami et al., 1983a); (3) A uridylated form of VPg has been shown to be synthesized in both intact, infected HeLa cells and in extracts of such cells actively synthesizing viral RNA in vitro (Takegami et al., 1983b; Crawford & Baltimore, 1983). This uridylated form of the VPg may be part of the initiation complex in viral replication and may be regarded as an activated primer; (4) Anti-VPg antibodies have been shown to inhibit host factor stimulated *in vitro* synthesis by the polio replicase but not the oligo(U) primed replicase activity (Baron & Baltimore 1982; Morrow & Dasgupta 1983).

Although it would seem likely that the VPg is involved in poliovirus replication, the function is far from resolved. Young et al. (1985) reported that polio replicase (in the presence of a host factor) may synthesize in vitro dimer RNA products. The VPg found at the 5'-end of the minus-strand may be involved in the cleavage of the loop, and remains attached to the 5'-end of the product RNA after cleavage.

Lomonossoff et al. (1985) have shown that the majority of the minus-strands in the CPMV RF RNA have a VPg. As the VPg is encoded by the B-RNA and this RNA has been shown to replicate independently of M-RNA in protoplasts in the absence of the VPg, and a role for VPg in replication seems likely.

Other possible functions for the VPg include a role in the encapsidation and/or protection against nucleases. For picornaviruses it has been demonstrated that the viral RNAs in polysomes do not possess a VPg (Nomoto et al., 1977a, 1977b). The VPg may therefore discriminate between those molecules which should or should not be encapsidated. Mayo & Barker (1983) showed that the VPg of the nepovirus TBRV might be involved in encapsidation.

They showed that although a mixture of RNA-1 from tomato black ring virus (TBRV) strain A and RNA-2 from strain G does not infect leaves of Chenopodium, this mixture induces in protoplasts the synthesis of RNA-1 from strain A and top components built up from capsid protein specified by RNA-2 from strain G. Since the VPg is encoded by RNA-1, it was speculated that the produced RNA-1 from strain A could not assemble with capsids specified by strain G since it possessed a heterologous VPg.

Cell-to-Cell Transport

Evidence implicating the I2-RNA 30 kD protein in TMV cell-to-cell movement comes from studies with temperature sensitive (ts) mutants. At the restrictive temperature of 32°C TMV Ls1, a spontaneously occurring temperature sensitive mutant of the Japanese tomato strain L, can replicate and assemble normally in initially infected leaves but is incapable of spreading from cell-to-cell in the inoculated leaves (Nishiguchi et al., 1978, 1980). This defectiveness at high temperatures can be complemented not only by temperature insensitive strains but also by potato virus X (PVX), a virus belonging to another taxonomic group (Taliensky et al., 1982c). This indicates that a trans-acting factor is involved in virus cell-to-cell movement, and that this factor is rather general among plant viruses (Taliensky et al., 1982b; Atabekov & Dorokov, 1984).

A comparison of nucleotide sequences of the Ls1 and L strains revealed that the Ls1 virus had a single base change in the 30 kD protein ORF which resulted in a serine instead of a proline residue (Ohno et al., 1983). However, although this data strongly implicates the 30 kD protein in cell-to-cell movement, it was not conclusive as peptide mapping could not detect any mutations in the 126 kD and

183 kD protein ORFs. Therefore, neither the possibility that the other virally encoded proteins might play some role in transport, or that the 30 kD protein plays a role in replication could be ruled out. Similar observations have been reported for a nitrous acid-induced mutant, Ni2519, derived from the common strain A19 (Talian sky et al., 1982a; Zimmer n & Hunter, 1983)

These questions were recently addressed in two papers. Doem et al. (1987) introduced a cDNA clone of the 30 kD ORF into tobacco cells via the Ti plasmid mediated transformation system of Agrobacterium tumefaciens. Transgenic plants were found to complement the ts mutation at the restrictive temperature, allowing Ls1 to systemically spread at 32°C. Meshi et al. (1987) produced infectious TMV RNAs from cloned full-length cDNA copies of the L genome. Introduction of the Ls1 mutation into the cDNA resulted in the transcripts showing the ts phenotype. Therefore, the single base substitution in 30 kD ORF adequately explains the defectiveness in transport of the Ls1 strain.

The mechanism of viral transport remains unknown. The p30 of TMV shares homology with the 29 kD protein of TRV (Boc cara et al., 1986; Cornelissen et al., 1986) and the product of gene 1 of caulimoviruses (Hull et al., 1986). However, there is little homology between the 30 kD protein and the products of the tri-partite viruses; the proteins P3 of AlMV and 3A of BMV are also thought to be transport proteins (Ahlquist et al., 1985). Nevertheless, complementation in viral spread has been reported for a combination of TMV and BMV (Hamilton & Dodds, 1970; Talian sky et al., 1982b). The difference may be due to more than one mechanism by which viruses are transported. One hypothesis is that 30 kD protein modifies the plasmodesmata through which the viral genomes are thought to move (Talian sky et al., 1982c; Atabekov & Dorokov,

1984). Another mechanism proposes that 30 kD protein suppresses the host defence responses - virus infection often induces a host response, and certain factors may spread from infected to healthy cells to provide resistance. The 30 kD protein might interfere with such a mechanism, therefore making the surrounding tissue susceptible (Taliany et al., 1982c; Atabekov & Dorokov, 1984).

Evidence for the effect of 30 kD protein on plasmodesmata came from using antibodies raised against the protein (Tomenius et al., 1987). The 30 kD protein was found to be present in the plasmodesmata at least 16 hours post-inoculation, to increase to a maximum after 24 hours, and then to steadily decrease in accordance with the earlier finding that 30 kD protein is transiently expressed (Joshi et al., 1983; Watanabe et al., 1984). However, the amount of coat protein present at the plasmodesmata, as measured by the amount of labelling with the coat protein antibodies, was insignificant until 22 hours post-inoculation, and continued to increase after the amount of 30 kD protein had significantly decreased. Therefore the coat protein, or virus particles, probably pass through the plasmodesmata at a late stage in the cells infection. The 30 kD protein might be a protease having some effect on the structure of the plasmodesmata, or the viral RNA might be transported in conjunction with 30 kD protein as a ribonucleoprotein particle (Dorokov et al., 1983). Whereas the 30 kD accumulated in the plasmodesmata, there was no significant increase in the amount of labelling in the nuclei of infected tissue compared to healthy tissue, suggesting that earlier results in which the protein had been found in the nuclei of protoplasts were artifactual (Watanabe et al., 1986).

However, as the earliest time point studied in whole tissue infection was 16 hour post-inoculation, the possibility of an early transient reaction in the nuclei cannot be ruled out.

TABLE 1.1: SOME PROPERTIES OF TOMBUSVIRUSES
(adapted from Martelli et al. 1988)

Virus	S _{20w}	Bouyant density in CsCl (g/cm ³)	Size of Genomic RNA (kb)	Mr of coat protein (kd)	Estimated percent sequence homology with TBSV-type	Serological relationship with TBSV-type	Presence of multivesicular bodies in peroxisomes of infected cells
TBSV	130	1.35	4.7	42	100	++++	Yes
AMCV	132	1.35	4.7	43	7	+++	Yes
CIRV	135	1.36	4.7	37	11	+	No*
CyRSV	127	1.36	4.7	43	10	+/-	Yes
EMCV	132	1.34	4.7	41	7	++	Yes
GALV	128	1.34	4.7	37	15	-	Yes
MPV	140	1.36	4.7	43	10	++	Yes
PAMV	134	1.35	4.7	41	11	+++	Yes
PLCV	136	1.34	4.7	41	24	+++	Yes
TVN	133	1.36	4.7	45	15	+	Yes

CHAPTER 2
MATERIALS AND METHODS

2.1 ABBREVIATIONS

The following abbreviations have been used:

ATP	adenosine 5'-triphosphate
[γ - ³² P]ATP	adenosine 5'-[γ - ³² P] triphosphate
b	base(s)
bp	base pairs(s)
BSA	bovine serum albumin
cDNA	complementary DNA
cpm	counts per minute
d	daltons
dH ₂ O	distilled water
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
[α - ³² P]dCTP	deoxycytidine 5'-[α - ³² P] triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dNTPs	equimolar mixture of dATP, dCTP, dGTP & dTTP
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded
DEAE	diethylaminoethyl

DTT	dithiothreitol
EDTA	ethylene-diamine-tetraacetic acid
EGTA	ethylene-glycol-tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.
IPTG	isopropyl β -D-thiogalactopyranoside
K	1000
kb	1000 bases
kD	kilodalton
min	minute or minutes
Mr	relative molecular mass
β -NAD	nicotinamide-adenine dinucleotide
poly A	polyadenylic
poly(A) ⁺	possessing a polyadenylic tail
poly(A) ⁻	not possessing a polyadenylic tail
POPOP	1,4-bis(5-phenyl-2-oxazolyl)-benzene
PPO	2,5-diphenyloxazole
RF	replicative form
RNA	ribonucleic acid
mRNA	messenger RNA
RNase	ribonuclease
RI	replicative intermediate
rRNA	ribosomal RNA
rpm	revolution per minute
SDS	sodium dodecylsulphate
sgRNA	subgenomic RNA
ss	single stranded

TCA	trichloroacetic acid
TEMED	N,N'-tetramethylethylenediamine
Tris	trishydroxymethylaminoethane
U.V.	ultraviolet
X-Gal	5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

2.2 SAFETY CONSIDERATIONS

A protective laboratory coat was worn at all times. Gloves were worn when working with hazardous chemicals, radioisotopes, enzymes, nucleic acids and bacteria. U.V. filtering safety glasses were used when using the U.V. transilluminator. Radioactive work was carried out behind a perspex screen when practical, using monitors to check for spillages.

An Envair Class 2 Microbiological Safety Cabinet was used when working with recombinant bacteria. Media and contaminated waste were autoclaved prior to disposal. Spillages were cleaned using hypochlorite followed by methylated spirits. Registration with the Genetic Manipulation Advisory Group and a medical examination were completed for work with recombinant DNA.

All radioactive and toxic chemicals were disposed of in accordance with Imperial College Safety Committee regulations.

2.3 CHEMICAL COMPANIES

The following companies supplied chemicals or major items of equipment:

Aldrich Chemical Co. Ltd., New Road, Gillingham, Dorset. SP8 4JL.

Amersham International plc., P.O.Box 16, Amersham, Bucks. HP7 9LL.

Anglian Biotechnology Ltd., Hawkins Road, Colchester, Essex. CO2 8JX.

BDH Chemicals Ltd., Freshwater Road, Dagenham, Essex. RM8 1RZ.

Beckman-RIIC Ltd., Progress Road, High Wycombe, Bucks. HP12 4SL.

Bethesda Research Laboratories (BRL), Gibco Ltd., P.O.Box 35, Washington Road, Paisley. PA3 4EP.

Biorad Laboratories Ltd., Caxton Way, Watford, Herts. WD1 8RP.

Boehringer Corporation (London) Ltd. (BCL), Bell Lane, Lewes, E.Sussex. BN7 1LG.

Difco Ltd., P.O.Box 14B, Central Avenue, East Moseley, Surrey. KT8 OBE.

Eppendorf GmbH., Alpha Laboratories, Parham Drive, Eastleigh, Hants. SO5 4NU.

Gallenkamp Ltd., Belton Road West, Loughborough, Leicestershire. LE11 OTR.

Kodak Ltd., High Holborn Street, London WC1.

Lancaster Synthesis, Eastgate, Morecambe, Lancs. LA3 3DY.

MSE, Fisons Scientific Equipment, Fisons Ltd., Gatwick Road, Crawley, Sussex. RH10 2UL.

New England Biolabs, CP Laboratories Ltd., P.O.Box 22,
Bishops Stortford, Herts. CM23 3DH.

New England Nuclear, DuPont U.K. Ltd., NEN Research
Products, Wedgewood Way, Stevenage, Herts. SG1 4QN.

NBL Enzymes Ltd., South Nelson Industrial Estate,
Cramlington, Northumberland. NE23 9HL.

Pharmacia P-L Ltd., Midsummer Boulevard, Milton Keynes.
MK9 3HP.

Rose Chemicals Ltd., Darenth Road, London. N16 6EB.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset.
BH17 7NH.

Sterilin Ltd., Clockhouse Lane, Feltham, Middlesex. TW14
4QS.

Whatman LabSales Ltd., Coldred Road, Parkwood, Maidstone,
Kent. ME15 9XN.

2.4 GENERAL METHODS

Dialysis tubing was prepared by boiling in 10 mM EDTA pH 8.0 for 30 min followed by autoclaving in dH₂O for 15 min. The tubing was then stored in sterilized dH₂O at 4°C.

Plasticware, such as Eppendorf tubes, and chemicals were - where possible - sterilized and made DNase-free by autoclaving for 20 min at 121°C, 15 pounds per square inch.

Glassware was sterilized and rendered DNase/RNase-free by baking at 200°C overnight.

Sucrose and other chemicals sensitive to autoclaving were made RNase free by adding diethylpyrocarbonate to 0.1%, incubating for 6 hours at 37°C and then at 65°C overnight.

Phenol (BDH) was re-distilled at 180°C and stored in the dark at -20°C. Aliquots were thawed, equilibrated with 10 mM Tris.HCl pH 7.5, 0.1% hydroxyquinoline and stored in the dark at 4°C.

All chloroform used was a mixture of chloroform and isoamyl alcohol in a ratio of 24:1 v/v.

An MSE Hi-Spin 21 centrifuge was used for high speed centrifugation, a Beckman Microfuge B for the centrifugation of Eppendorf tubes, and a Beckman L8-55 for ultracentrifugation.

A Beckman Model 35 spectrophotometer was used to determine the concentration of DNA, RNA and viral preparations according to the relationship:

$$1 A_{260} = 50 \mu\text{g/ml dsRNA and dsDNA}$$

$$1 A_{260} = 40 \mu\text{g/ml ssRNA}$$

$$1 A_{260} = 0.22 \text{ mg/ml virus}$$

A relatively pure preparation of RNA and DNA was assumed to have $A^{260}/_{280}$ ratio > 1.9 (Maniatis et al., 1982), while the ratio for a relatively pure preparation of virus was assumed to be 1.66 (Ambrosino et al., 1967).

The refractive indices of samples were measured using an Abbe High Accuracy "60" refractometer (Bellingham and Stanley Ltd., London N5, England) provided with a sodium D1 line source (Gallenkamp). The refractometer was calibrated with a quartz block with a refractive index of 1.54422. The refractive index was converted to density using the expression:

$$p(25^\circ\text{C}) = a n_D 25^\circ\text{C} - b$$

$p(25^\circ\text{C})$ is the density of the solution at 25°C, $n_D 25^\circ\text{C}$ is the index of the the solution at 25°C at the wavelength of the sodium D1 line (589.6 nm), and $a = - 10.8601$ & $b = - 13.4974$ over the range 1.25 - 1.9 g/cm³.

2.5 COMMON BUFFERS

BE : 2.5 mM sodium borate, 25 mM boric acid pH 8.2, 0.5 mM EDTA made as a 10x solution.

TBE : 90 mM Tris, 90 mM boric acid pH 8.3, 2.5 mM EDTA made as a 10x stock solution.

TE : 10 mM Tris.HCl pH 8.0, 1 mM EDTA.

TAE : 40 mM Tris, 5 mM acetic acid pH 8.2, 1 mM EDTA made as a 25x stock solution.

SSC : 150 mM sodium chloride, 15 mM sodium citrate pH 7.0 made as a 20x stock solution.

2.6 PLANT PROPAGATION AND INOCULATIONS

Nicotiana clevelandii and Nicotiana benthamiana were used during this study. All plants were raised from seed obtained from Dr.A.A.Brunst or Dr.R.H.A.Coutts. Seeds were germinated on tap water saturated, acid-washed sand in a petri dish at room temperature. Germination usually required 7 days. Seedlings were then planted into John Innes Potting Compost No 2 in 4" square pots. These were kept covered with polythene for 1 week to prevent the seedlings from drying out. All plants were kept in a greenhouse maintained at 23-28°C with a relative humidity of at least 75%. Natural lighting was supplemented by mercury vapour and fluorescent strip lights, giving approximately 4000 lux for 16 out of every 24 hours. At 4-8 weeks post germination the plants were ready for inoculation; at this stage they were between 4-6" in height with 6-8 leaves.

Plants were inoculated using either ground up leaves of previously infected plants or freeze dried infected leaf material supplied by Dr.A.A.Brunst. The inoculum, in 10 mM

sodium phosphate buffer pH 7.0, was rubbed onto the upper surfaces of the plant leaves using carborundum as an abrasive. The plant leaves were then rinsed with tap water and placed under normal growth conditions.

2.7 VIRUS PURIFICATION

Method 1 (Martelli et al., 1971): 10 days after inoculation, leaves from N.clevelandii were homogenized using a Sorvall Omnimixer in 1.25 volumes of 50 mM sodium phosphate pH 7.8 containing 0.1% 2-mercaptoethanol. n-butanol was added drop-wise to 8.5%. The solution was then filtered through muslin and stirred for 24 hours at 4°C. After low speed centrifugation at 12000 rpm in a MSE HS21 8x50 rotor, the aqueous phase was centrifuged at 30000 rpm for 75 min in a Beckman Ti30 rotor at 4°C. The pellet was then resuspended in homogenization buffer.

Method 2: 10 days after inoculation, leaves from N.clevelandii or N.benthamiana were homogenized in 1.25 volumes of 50 mM sodium phosphate pH 7.8 containing 1 mM phenylmethanesulphonyl fluoride, 1 mM EDTA and 0.1% 2-mercaptoethanol. After filtration through muslin and clarification by low speed centrifugation the supernatant was stirred for 15 min with an equal volume of 1:1 v/v chloroform:n-butanol. After low speed centrifugation the aqueous phase was centrifuged at 30000 rpm for 75 min in a Beckman Ti45 rotor at 4°C and the sedimented virus resuspended in homogenization buffer.

After both of the above methods the virus was further purified by centrifugation through a linear gradient of 10 to 40% w/v sucrose in 50 mM sodium phosphate pH 7.8 (formed by overlaying equal volumes of 10, 17.5, 25, 32.5 and 40% w/v sucrose and allowing the sucrose to diffuse at room temperature for 3 hours or for 16 hours at 4°C) at 27000 rpm for 120 min at 4°C in a Beckman SW28 rotor. The

band of virus, usually half way down the gradient, was removed by side puncturing the centrifuge tube with a syringe. The virus solution was then diluted with an equal or greater volume of buffer, pelleted by centrifugation at 40000 rpm for 45 min in a Beckman Ti65 rotor at 4°C, and the virus resuspended in buffer. In some cases caesium chloride was then added to a density of 1.36 g/ml, and the solution centrifuged at 36000 rpm at 10°C in a SW50.1 rotor for 18 hours. Virus particles were collected by puncturing the tube, diluting the caesium chloride four fold and pelleting the virus particles by centrifugation for 45 min at 40000 rpm in a Beckman Ti65 rotor.

2.8 VIRAL RNA EXTRACTION

To isolate the virion nucleic acid, Tris.HCl pH 7.8, EDTA and SDS were added to 10 mM, 1 mM and 1% respectively and the solution extracted sequentially with an equal volume of phenol, 1:1 (v/v) phenol:chloroform and chloroform. The final aqueous phase was made 0.3 M in sodium acetate and the nucleic acid precipitated with 2½ volumes of ethanol overnight at -20°C.

The precipitated RNA was pelleted by centrifugation at 25000 rpm at 4°C in a Beckman SW28.1 rotor for 30 min. The supernatant was removed, and the pellet washed with 70% v/v ethanol before being dried briefly under vacuum, resuspended in sterile dH₂O and stored in aliquots at -70°C.

2.9 PREPARATION OF RNA FROM HEALTHY OR TBSV-INFECTED PLANTS

Method 1: Leaves from N.clevelandii or N.benthamiana plants, or plants infected 5-7 days earlier, were used. Five grams of leaf tissue were frozen in liquid nitrogen and, using a pestle and mortar, ground with 5 mls of 100 mM Tris.HCl pH 8.0, 80 mM 2-mercaptoethanol, 1% SDS and 10 mls of 1:1 (v/v) phenol:chloroform. The homogenate was then centrifuged at 8000 rpm for 5 min at 4°C in a MSE 8x50 ml rotor. The aqueous phase was re-extracted twice more with 1:1 (v/v) phenol:chloroform and then twice with chloroform. Nucleic acid was precipitated by adding sodium acetate to 0.3 M, 2½ volumes of ethanol and storing at -20°C overnight. The precipitated nucleic acid was then recovered by centrifugation at 25000 rpm in a Beckman SW28.1 rotor at 4°C for 30 min. The pellet was washed with 70% ethanol, dried under vacuum, resuspended in 50 mM Tris.HCl pH 7.5, 10 mM magnesium chloride and the nucleic acid incubated with 50 µg/ml of DNase 1 (BCL) for 40 min at room temperature. The solution was then extracted with an equal volume of 1:1 (v/v) phenol:chloroform, centrifuged and precipitated with ethanol in a dry ice/ethanol bath for 30 min. The RNA was pelleted, resuspended in dH₂O, made 2 M with lithium chloride and incubated at 4°C for 2 hours. After centrifugation the dsRNA and low molecular weight ssRNA was recovered from the lithium chloride soluble fraction by ethanol precipitation. The lithium chloride insoluble fraction, containing mostly mRNA, rRNA and viral RNA was redissolved in sterile dH₂O and stored in aliquots at -70°C.

Method 2 (Taylor & Powell, 1982): Leaves from N.clevelandii or N.benthamiana infected 5-7 days earlier were used. Five grams of leaf tissue were frozen in liquid nitrogen and ground to a fine powder. 2-mercaptoethanol was then added to 2% v/v together with an equal volume of

boiling extraction buffer containing 2% w/v cetyltrimethylammonium bromide (CTAB), 100 mM Tris.HCl pH 8.0, 20 mM EDTA, 1.4 M sodium chloride. The mixture was transferred to a water bath at 60°C and stirred until the temperature had reached 50°C. The homogenate was then shaken with an equal volume of chloroform and centrifuged at 13000 rpm in a MSE 8x50 rotor at room temperature. The aqueous phase was removed and a one-tenth volume of a solution containing 10% w/v CTAB, 0.7 M sodium chloride added. The chloroform extraction and centrifugation were then repeated. To the upper aqueous phase an equal volume of precipitation buffer was added containing 1% CTAB, 50 mM Tris.HCl pH 8.0, 10 mM EDTA and 1% 2-mercaptoethanol. The solution was mixed and allowed to stand for 30 min.

The precipitated nucleic acid was pelleted by centrifugation at 6000 rpm in a MSE 8x50 rotor for 5 min at room temperature. The pellet was resuspended in 3 mls of 1 M caesium chloride, 50 mM Tris.HCl pH 8.0, 5 mM EDTA, 50 mM sodium chloride and layered over 2 mls of a caesium cushion containing 5.7 M caesium chloride, 50 mM Tris.HCl pH 8.0, 5 mM EDTA, 50 mM sodium chloride. The caesium solution was then centrifuged at 30000 rpm for 17 hours in a Beckman SW50.1 rotor at 20°C. The pelleted RNA was resuspended in sterile dH₂O and reprecipitated with 2½ volumes of ethanol. After 1 hour at -70°C the RNA was pelleted, dried under vacuum, resuspended in dH₂O and stored in aliquots at -70°C.

When required single and double-stranded RNA were separated from each other using Whatman CF-11 cellulose (Morris & Dodds, 1979). After centrifugation through caesium chloride, washing with 70% v/v ethanol and reprecipitation as described above, the RNA was resuspended in approximately 5 mls of STE buffer (0.1 M sodium chlorided, 50 mM Tris.HCl pH 7.0, 1 mM EDTA) and made 15% v/v with ethanol. Cellulose powder was then added to a

concentration of 0.25 g/ml, and the mixture kept on ice for 10 min with occasional shaking. The cellulose was then pelleted by centrifugation for 10 min at 6000 rpm in a MSE HS21 rotor at 0°C. The supernatant was discarded and the pelleted cellulose powder resuspended in 5 mls of STE/15% v/v ethanol. The cellulose was then poured into a small column and washed free of ssRNA with 60 mls STE/15% v/v ethanol at room temperature. The dsRNA was eluted from the column with 5 mls STE buffer and precipitated by addition of 2½ volumes of ethanol.

2.5 POLYSOME ISOLATION

Polysomes were extracted essentially as described by Hari (1979). One gram of deribbed N.clevelandii or N.benthamiana leaves was ground with 6 ml of ice cold extraction buffer containing 200 mM Tris.HCl pH 9.0, 400 mM potassium chloride, 200 mM sucrose, 35 mM magnesium chloride, 25 mM EGTA, 25 mM sodium diethyldithiocarbamate, 0.5% Nonidet P40. The homogenate was filtered through muslin cloth and centrifuged at 15000 rpm in a MSE 8x50 rotor at 4°C. The supernatant was layered over a 2 ml cushion of 1.75 M sucrose, 40 mM Tris.HCl pH 9.0, 200 mM potassium chloride, 30 mM magnesium chloride, 5 mM EGTA and centrifuged at 49000 rpm for 2 hours at 4°C in a Beckman Ti65 rotor.

To analyze the intactness of the polysomes, the pellets were drained free of liquid and resuspended in 0.6 ml of 40 mM Tris.HCl pH 8.0, 200 mM potassium chloride, 30 mM magnesium chloride, 5 mM EGTA. The polysomes were then layered onto a 12.5-20% w/v sucrose gradient and centrifuged at 39000 rpm for 75 min at 4°C in a Beckman SW50.1 rotor. The gradients were fractionated using a LKB Uvicord II U.V. detection unit.

To release the mRNA from the polysomes (Blobel, 1971), the pellets were resuspended in dH_2O at a concentration of 200 A_{260} units/ml. 0.2 ml aliquots were then combined with 0.25 ml of 0.1 M Tris.HCl pH 7.5, 1 M potassium chloride, 10 mM magnesium chloride and 50 μl of 10 mM puromycin (Sigma), and incubated first at 0°C for 15 min and then at 37°C for 10 min. The reaction mixture was layered onto a 5-20% w/v sucrose gradient in 50 mM Tris.HCl pH 7.5, 0.5 M potassium chloride, 5 mM magnesium chloride and centrifuged at 4°C for 18 hours at 21000 rpm in a Beckman SW28.1 rotor at 4°C . The gradients were then fractionated using a LKB Uvicord U.V. detection unit.

2.11 SELECTION OF POLY(A)^+ RNA ON POLY(U) -SEPHAROSE

A 1 ml column of poly(U)-sepharose [Pharmacia P-L, binding capacity approximately 100 μg poly(A)⁺ mRNA] was poured into a Pasteur pipette, washed with 20 mls of elution buffer (50 mM HEPES pH 7.0, 10 mM EDTA, 90% v/v formamide) and then 20 mls of binding buffer (50 mM Tris.HCl pH 7.5, 0.7 M sodium chloride, 10 mM EDTA, 25% v/v formamide). The RNA, at a concentration of 1 mg/ml in sample buffer (10 mM Tris.HCl pH 7.5, 1 mM EDTA, 1% SDS) was heated for 5 min at 65°C , cooled on ice and added to 5 volumes of binding buffer. Approximately 100 μg of RNA was applied to the column, followed by 10 ml of binding buffer. The bound RNA was then eluted with 2 mls of elution buffer. At each stage the optical densities of the 0.5 ml fractions were measured at 260 nm, and those fractions required in each group were pooled, diluted 5 fold with 0.2 M sodium acetate and ethanol precipitated.

2.12 AGAROSE GEL ELECTROPHORESIS

Three types of agarose gels were used:

(1) TBE gels for DNA electrophoresis. These were agarose gels (0.8-2%) cast in various sizes and electrophoresed in 1xTBE buffer at between 10 and 70 volts. Agarose (Sigma, Type 1) was added to the buffer, the solution heated to 100°C, cooled to approximately 60°C and the gel poured and allowed to set for approximately one hour. Frequently ethidium bromide (10 mg/ml in dH₂O) was added at a final concentration of 0.5 µg/ml, thus enabling the migration of the DNA to be visualized directly during electrophoresis. A one-tenth volume of loading buffer (0.05% bromophenol blue, 50% glycerol) was added to the samples prior to loading them into slots in the gel. Gels were usually run submerged at 5-6 volts/cm in 1xTBE buffer. If ethidium bromide had not been added to the gel when it was cast, the gel was stained in the dye at a concentration of 0.5 µg/ml in dH₂O for 30 minutes at the end of electrophoresis. Size markers used were usually restriction fragments of lambda DNA (NBL) or Bethesda Research Laboratories (BRL) DNA kilobase ladder.

(2) Methylmercury gels for single-stranded RNA electrophoresis. Methylmercuric hydroxide (Lancaster Synthesis) is a powerful denaturant of dsRNA and dsDNA, allowing the molecular weight of single-stranded nucleic acids to be determined (Bailey & Davidson, 1976). It is also highly toxic and so great care must be taken at all stages when running this type of gel. All the following steps were therefore performed in a well ventilated fume cupboard, and all waste considered toxic and disposed of according to the regulations laid down by Imperial College Safety Committee.

Agarose was added (1-2%) to the 1xBE buffer and the solution heated to 100°C, cooled to 60°C and transferred to the fume cupboard where 1 M methylmercuric hydroxide was added to a final concentration of 10 mM. The gel was poured and allowed to set for approximately 30 min. Samples were added to 5x loading buffer (50 mM methylmercuric hydroxide, 5xBE buffer, 0.025% bromophenol blue and 25% v/v glycerol), loaded and electrophoresis carried out at 3 volts/cm until the bromophenol blue had migrated the desired distance. Gels were then stained in 100 mM ammonium acetate, 1 µg/ml ethidium bromide for one hour; as well as staining the nucleic acid this treatment drives off the methylmercury thus allowing the gel to be removed from the fume cupboard. Size markers were usually BRL DNA kilobase ladder or BRL RNA ladder (9.5 kb to 0.24 kb) In agarose gels containing 5 mM or 10 mM methylmercuric hydroxide DNA and RNA size markers fall on the same line (Miller et al., 1983), and this was verified by co-electrophoresis of both sets of markers.

(3) Alkaline gels. A 1-2% solution of agarose was prepared in dH₂O and cooled to just above gelling temperature before the addition of sodium hydroxide and EDTA to a final concentration of 30 mM and 10 mM respectively. The gel was then poured and allowed to set for 1 hour. Just prior to loading, samples were added to an equal volume of loading buffer (0.01% bromophenol blue, 10% glycerol) containing 60 mM sodium hydroxide and 20 mM EDTA.

The gel was run overnight at 5 volts/cm using wicks of Whatman 3MM paper and 30 mM sodium hydroxide, 10 mM EDTA running buffer. After electrophoresis the gel was neutralized and stained in TE buffer, 1 µg/ml ethidium bromide, or directly dried down on a Biorad Slab Gel Dryer. Size markers were usually restriction fragments of lambda DNA (NBL) or BRL DNA kilobase ladder.

In all cases the stained gel was then transferred to a U.V. transilluminator to allow the bands to be visualized at 300 nm. Gels were photographed using a Pentax K1000 camera fitted with one Y(K2) filter and one UV(0) filter (Hoya) and Ilford HP5 film.

2.13 FRACTIONATION OF RNA

RNA samples were fractionated by electrophoresis in 10 mM methylmercury gels or on 2-10% w/v sucrose/formamide gradients.

Methylmercuric hydroxide gels were prepared and electrophoresed as described in Section 2.12 except that low melting temperature agarose (BRL) was used. After electrophoresis, tracks containing markers were stained in the usual way. The portions containing the fractionated RNAs were soaked in two changes of freshly prepared 100 mM DTT until the gel appeared totally clear (usually 30-60 min). The gel was then cut into 3 mm slices using a scalpel, and each slice placed into a 1.5 ml Eppendorf tube. To each tube 500 μ l of 0.5 M ammonium acetate, 2.5 mM EDTA was added, and the tubes heated to 65°C. After 3 min, when the agarose had melted, 500 μ l of phenol and 5 μ l of 10% w/v SDS were added and the tube vortexed and then centrifuged in a microfuge for 5 min. The aqueous phase was then extracted sequentially with 1:1 v/v phenol:chloroform and chloroform, and the RNA finally precipitated with the aid of 10 μ g of calf liver tRNA by adding 2½ volumes of ethanol and storing at -20°C overnight.

Separation of RNA by sucrose/formamide gradients was achieved using the method of Pauche et al. (1978). Up to 25 μ g of RNA was denatured by heating to 37°C for 15 min in 85% v/v formamide, 10 mM Tris.HCl pH 7.5, 1 mM EDTA. After cooling on ice the sample was layered onto a 2-10%

w/v sucrose gradient containing 85% v/v formamide, 10 mM Tris.HCl pH 7.5, 1 mM EDTA and centrifuged for 20 hours at 40000 rpm in a Beckman SW50.1 rotor at 20°C. Fractions of 200 µl were collected, diluted four fold with TE buffer, and then the RNA precipitated with 3 volumes of ethanol.

2.14 POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gels were used to separate DNA fragments of less than 500 bp and to analyze dsRNA. They were cast between two glass plates, 20 x 16.5 cm held apart by 1.5 mm spacers, at a variety of concentrations depending on the size of the nucleic acid of interest. A 13.2 x 2.4 cm slot was cut out of the top of one of the plates to allow contact between electrophoresis buffer and the gel. Slot widths were 0.85 cm allowing a sample volume of up to 100 µl.

The most frequently used gels were 4% polyacrylamide, made by adding to a Buchner flask 5 ml stock aqueous acrylamide (40% w/v acrylamide, 0.4% NN'-methylenebis-acrylamide), 2 ml 25xTBE, 0.5 ml 5% w/v TEMED and 42.5 ml dH₂O. After mixing, the solution was degassed for 30 min and then 2.5 ml of 1.5% ammonium persulphate added. The solution was poured between the glass plates and allowed to set for at least two hours. Gels were run vertically, with TBE buffer in both the upper and lower reservoirs, at 150 volts for 5 hours, or 50 volts overnight. Samples were loaded in 0.005% bromophenol blue, 5% v/v glycerol. After electrophoresis the gel was stained in ethidium bromide and photographed in the same manner as agarose gels.

To purify restriction fragments by polyacrylamide gel electrophoresis, the gel slice containing the fragment was placed inside dialysis tubing filled with TBE buffer which had been knotted at one end. The other end of the tubing was then knotted, and the bag placed in an electrophoresis

tank at 90° to the electrodes. The bag was just covered with TBE, and then electrophoresis at 7 volts/cm was carried out for 1 hour. The direction of the current was reversed for 30 seconds to detach any DNA which had become bound to the dialysis tubing. The buffer was then removed from the bag and filtered through glass wool in a pipette to remove contaminating polyacrylamide fragments. The DNA was then precipitated overnight at -20°C with the aid of 0.3 M sodium acetate and 2½ volumes of ethanol.

2.15 RESTRICTION ENZYME DIGESTS

Reactions were usually performed in 0.5 ml Eppendorf tubes. DNA was digested at 37°C at a concentration of 50-200 µg/ml in a reaction that contained 1x digestion buffer (see Table 2.1) and a 2-5 fold excess of enzyme. Double digestions were performed sequentially, starting with the enzyme requiring the lowest ionic strength buffer. After completion of the first reaction, the buffer was adjusted to the appropriate conditions for the second digest. Reactions were terminated by the addition of EDTA to a final concentration of 10 mM and, sometimes, extraction with 1:1 v/v phenol:chloroform.

TABLE 2.1 : RESTRICTION ENZYMES AND DIGESTION CONDITIONS

ENZYME	BUFFER
<u>Cla</u> 1	10 mM Tris.HCl pH 7.6, 10 mM magnesium chloride, 1 mM DTT.
<u>Sal</u> 1, <u>Xho</u> 1	10 mM Tris.HCl pH 7.6, 10 mM magnesium chloride, 1 mM DTT, 100 mM sodium chloride.
<u>Acc</u> 1, <u>Bam</u> H1, <u>Eco</u> R1, <u>Hind</u> 111, <u>Pst</u> 1, <u>Sau</u> 3A, <u>Xba</u> 1.	10 mM Tris.HCl pH 7.6, 10 mM magnesium chloride, 1 mM DTT, 50 mM sodium chloride.
<u>Sma</u> 1.	10 mM Tris.HCl pH 8.0, 7 mM magnesium chloride, 20 mM potassium chloride, 7 mM 2-mercaptoethanol.

All enzymes were purchased from Amersham Int. or Pharmacia P-L.

2.16 EXTRACTION OF DNA FROM AGAROSE

The most reliable method for extraction of DNA from agarose was found to be that of Dretzen et al. (1981). DEAE 81 discs (Whatman) were cut into slices slightly wider and deeper than the sample wells of a gel. They were then soaked in 2.5 M sodium chloride for 2 hours at 4°C and washed five times in dH₂O, twice in running buffer. The DNA samples were electrophoresed on a TBE gel, the gel stained in ethidium bromide and the bands visualized on the UV transilluminator. Using a scalpel blade cuts were made in the gel just below the band of interest and a piece of DEAE paper carefully inserted. If necessary this process was repeated above the band to prevent contamination from higher molecular weight DNA. Electrophoresis was then continued until the band had run onto the paper. The paper was then removed into a 0.5 ml Eppendorf tube containing a suitable amount - 100 µl for a 5 mm square piece of DEAE paper - of elution buffer (20 mM Tris.HCl pH 7.5, 1 mM EDTA and 1.5 M sodium chloride). The tube was vortexed in order to shred the paper and then incubated at 37°C for 1 hour.

A small hole was punched in the bottom of another 0.6 ml Eppendorf using a syringe needle and siliconized glass wool tightly packed into the bottom. This tube was then placed in a 1.5 ml Eppendorf, and the DEAE paper/buffer added to the upper tube. The tubes were then spun in a microfuge so that the buffer passed into the lower Eppendorf while the glass wool trapped the paper. Fresh buffer was then added to the paper and the tubes respun. The final buffer was then removed, butanol extracted and then 1:1 v/v phenol:chloroform extracted twice. The DNA was precipitated overnight in ethanol with the aid of 10 µg of tRNA.

2.17 DETERMINATION OF RADIOACTIVE INCORPORATION

In order to determine the percentage of incorporation in radioactive labelling reactions, aliquots (usually 1-2 μ l) were pipetted onto Whatman DEAE 81 discs (2.5 cm diameter) prior to enzyme addition, at time points during the course of the reaction and/or at its termination. The discs were then washed five times with 0.5 M disodium hydrogen orthophosphate, twice for 1 min with dH_2O and similarly in acetone. After drying the discs were added to 10 mls of scintillant (66% v/v toluene, 33% v/v ethanol, 0.4% 2,5-diphenyloxazole, 0.01% 1,4-bis[5-phenyl-2-oxazolyl]-benzene) and counted in a Packard Tricarb Scintillation Counter.

2.18 SPERMINE PRECIPITATION OF DNA

A restriction endonuclease or DNA ligase reaction was diluted to a 45 μ l volume with 0.1 M potassium chloride, 10 mM magnesium chloride. Five microlitres of 0.1 M spermine tetrahydrochloride were added, and then the mixture was incubated on ice for 15 min followed by centrifugation in a microfuge for 15 min. The supernatant was then removed and the pellet washed for one hour on ice with 75% v/v ethanol, 75 mM sodium acetate, 2.5 mM magnesium chloride. The pellet was then quickly washed with 70% v/v ethanol and dried.

2.19 FILLING RECESSED 3'-ENDS OF dsDNA WITH KLENOW FRAGMENT

Usually 1 μ g of dsDNA was end filled in a 20 μ l reaction which contained 50 mM Tris.HCl pH 7.2, 10 mM magnesium chloride, 0.1 mM DTT, 50 μ g/ml bovine serum albumin, 0.1 mM dNTPs and 1 unit of Klenow fragment (Amersham Int.). The reaction was incubated for 30 minutes at 37°C before being terminated by the addition of 1 μ l of 0.5 M EDTA pH 8.0.

2.20 RAPID END LABELLING

The recessed 3'-end of dsDNA can usually be labelled directly after restriction enzyme digest. The digested DNA, usually in a 20 μ l reaction volume was added to 10 μ Ci of dried down [α -³²P]dCTP (410 Ci/mmol) in an Eppendorf tube. One unit of Klenow fragment (1 μ l) was then added together with 1 μ l of a 1 mM solution of the other three unlabelled nucleotides. The reaction was incubated at room temperature for 30 min before being terminated by the addition of 1 μ l of 0.5 M EDTA pH 8.0.

2.21 LABELLING OF DNA RESTRICTION FRAGMENTS

Although the method of individual DNA fragment isolation described in Section 2.16 gives good yields of intact DNA, it is quite time consuming. In this project a large number of DNA fragments were to be labelled and therefore a more rapid method was sought. It was found that fragments separated by electrophoresis on low temperature melting agarose could be labelled in the presence of the agarose removing the necessity to extract the DNA from the gel.

TBE agarose gels were prepared and electrophoresis was carried out as described in Section 2.12, except low melting point agarose was used. After visualizing the DNA the band of interest was cut out with the minimum amount of agarose, placed in a 1.5 ml Eppendorf, melted in a boiling water bath for 5 min and then cooled to 37°C. To the melted agarose, usually a volume of about 40 μ l, 35 μ l of dH₂O and 35 μ l salmon sperm DNA primers (10 mg/ml) were added together with 15 μ l of 10x nick translation buffer (0.5 M Tris.HCl pH 7.2, 100 mM magnesium chloride, 10 mM DTT) and 15 μ l of 1 mM dNTPs-dCTP. Nuclease free bovine serum albumin (BRL) was added to a final concentration of 0.1%. The reaction mix was then transferred to a 0.6 ml

Eppendorf tube in which 20 μCi of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (410 Ci/mmol) had been dried down, and the reaction started by the addition of 2 units of Klenow fragment. This was incubated at room temperature for 2 hours and then 1:1 v/v phenol:chloroform extracted.

2.22 5'-LABELLING OF RNA WITH T4-POLYNUCLEOTIDE KINASE

Usually 10 μg of RNA in 10 μl of dH_2O was added to an equal volume of 100 mM Tris.HCl pH 9.4. The mixture was heated at 67°C for 15 min before the addition of 2 μl of 100 mM magnesium chloride and 1 μl of 100 mM DTT. This was then added to an Eppendorf tube containing the dried down label, usually 20 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol, Amersham Int.). One microlitre of T4-polynucleotide kinase (10 units, Amersham Int.) was added and the reaction incubated at 37°C for 60 minutes before being stopped with 2 μl of 0.5 M EDTA and passed down a column of Sephadex G-50 in TE buffer pH 8.0, 0.1% SDS.

2.23 3'-LABELLING OF RNA WITH RNA LIGASE

Bacteriophage T4 RNA ligase catalyzes the ligation of a 5'-phosphoryl donor to a 3'-hydroxyl acceptor. This allows the 3'-OH end of RNA to be labelled with mononucleoside 3',5'-biphosphates (pNps). One microgram of TBSV RNA (1 μl) was added to a 20 μl reaction containing 5 μM ATP, 50 mM HEPES pH 8.3, 10 mM magnesium chloride, 3 mM DTT, 3% v/v DMSO, 10 $\mu\text{g/ml}$ BSA and 20 μCi $[\text{5-}^{32}\text{P}]\text{pCp}$ (3000 Ci/mmol). Twelve units of T4 RNA ligase were then added and the reaction incubated overnight at 4°C.

2.24 NUCLEASE DIGESTIONS

The following enzymes were used:

RNase A (Sigma, type X1-A)

DNase 1 (BCL)

Nuclease S1 (BCL)

The RNase and DNase digestions were performed in 10 mM Tris.HCl pH 7.4, 10 mM magnesium chloride, while the S1 digestions were performed in 10 mM sodium acetate pH 5.0, 5 mM magnesium sulphate, 0.5 mM zinc chloride. One microgram of RNase and DNase and 15 units of S1 nuclease were used to digest 3 μ g of nucleic acid in 20 μ l at 37°C for 1 hour.

2.25 NORTHERN BLOTTING

The transfer of RNA from methylmercury gels to GeneScreen (New England Nuclear) was carried out according to the manufacturer's instructions. The efficiency of transfer of RNA from the gels is reduced by staining with ethidium bromide, and therefore to visualize the RNA it was necessary to run parallel tracks. After electrophoresis the GeneScreen was cut to the exact size of the gel to be blotted and soaked in 25 mM sodium phosphate pH 6.5 buffer for 15 min. The gel was placed on two sheets of Whatman 3MM paper saturated with 25 mM sodium phosphate buffer pH 6.5. The GeneScreen was laid over the gel, covered with two sheets of 3MM paper and a 5-7 cm thick layer of paper towels, a glass plate, and a weight. The whole apparatus was then covered with Sarenwrap to prevent evaporation, and the transfer allowed to proceed overnight.

After transfer the GeneScreen was baked for 2 hours at 80°C and then prehybridized in 50% v/v formamide (deionized by stirring with 5 mls per gram of BDH MB3 mixed bed ion exchange resin for 30 min), 0.2% polyvinylpyrrolidone (Sigma, Mr 40,000), 0.2% bovine serum albumin (Sigma), 0.2% ficoll (Sigma, Mr 400,000), 50 mM

Tris.HCl pH 7.5, 1 M sodium chloride, 0.1% sodium pyrophosphate, 1% SDS, 10% w/v dextran sulphate (Sigma, Mr 500,000) and 100 µg/ml denatured salmon sperm DNA (Sigma). Prehybridization was conducted in a sealed bag for 6 hours at 42°C with constant agitation.

Hybridization of the probe to the GeneScreen was performed in the same solution by adding the probe directly to the bag: the probe was denatured by heating to 100°C for 5 min and rapidly cooling on ice before addition. Due to the relatively small volume of the probe compared to the prehybridization solution, the changes in the concentration of the components already in the bag were insignificant. Hybridization was carried out at 42°C for 16 hours. The GeneScreen was then washed in two 100 ml washes of 2xSSC at room temperature for 5 min, each with constant agitation, and then two 100 ml washes of 2xSSC, 1% SDS at 65°C for 30 min each. Finally the GeneScreen was washed in two 100 ml washes of 0.1% SSC at room temperature for 30 min before drying and exposure to Kodak XRI X-ray film at -70°C.

2.26 SOUTHERN BLOTTING

The transfer of DNA from agarose gels to GeneScreen was performed according to manufacturer's recommendations (New England Nuclear). After staining the gel with ethidium bromide and visualization on the U.V. transilluminators, the gel was soaked in 0.2 M sodium hydroxide, 0.6 M sodium chloride at room temperature for 30 min. It was then neutralized by soaking in 25 mM sodium phosphate buffer pH 6.5 at room temperature for 1 hour, with three changes of buffer. The DNA was transferred to the GeneScreen, baked, prehybridized and hybridized as in the Northern blotting procedure. The blot was then washed with two changes of 0.3 M sodium chloride, 60 mM Tris.HCl pH 8.0, 2 mM EDTA at room temperature for 5 min with constant agitation, and then in a similar fashion at 60°C for 1 hour in 0.3 M

sodium chloride, 60 mM Tris.HCl pH 8.0, 2 mM EDTA. The GeneScreen was finally washed in 0.03 M sodium chloride, 60 mM Tris.HCl pH 8.0, 0.2 mM EDTA for 1 hour at room temperature before being dried and autoradiographed.

2.27 PREPARATION OF DENATURED DNA AND RANDOM PRIMERS

Salmon sperm DNA was dissolved at a concentration of 10 mg/ml in dH₂O and then sheared by passing it several times through a gauge 18 syringe needle. The DNA was then autoclaved for 15 min and stored at -20°C. Just prior to use it was denatured by boiling for 10 min in a water bath.

For random primers (Maniatis et al., 1982) approximately 1 gram of calf thymus DNA (Sigma) was dissolved in 30 ml of 20 mM Tris.HCl pH 7.4, 10 mM magnesium chloride, to which 2 mg of DNase 1 (BCL) was added. The mixture was incubated for 30 min at 37°C before the addition of 3 ml of 10% w/v SDS and 1.5 ml of 20 mg/ml pronase. Incubation was continued for another 45 min before extraction with 1:1 v/v phenol:chloroform. The aqueous phase was then autoclaved for 10 min. The DNA was cooled on ice and sodium chloride added to a final concentration of 1 M. The solution was then loaded onto a DEAE cellulose (Sigma) column equilibrated with 5 mM Tris.HCl pH 7.4, 1 mM EDTA, 0.1 M sodium chloride. The column was washed with 300 ml of the same buffer, and the primers eluted by 150 ml of 5 mM Tris.HCl pH 7.4, 1 mM EDTA, 0.3 M sodium chloride. The primers were then concentrated by ethanol precipitation overnight at -20°C, followed by centrifugation at 20000 rpm in a Beckman SW28.1 rotor for 1 hour. After washing with 70% v/v ethanol and re-centrifugation, the primers were dissolved at a concentration of 10 mg/ml in TE buffer pH 8.0 and stored at -20°C.

2.28 SYNTHESIS OF cDNA

Typically this was performed in 100 μ l of 50 mM Tris.HCl pH 8.3 (at 42°C), 8 mM magnesium chloride, 70 mM potassium chloride, 5 mM dithiothreitol (Pharmacia), 5 mg/ml random primers (0.5 mg/ml for long cDNA), 1 mM dATP, 1 mM dGTP, 1 mM dTTP (Pharmacia), 100 μ g/ml actinomycin D (Sigma) and 100 μ g/ml RNA. When preparing cDNA for probes [$-^{32}\text{P}$]dCTP (410 Ci/mmol, Amersham Int.) was included to give a specific activity of 10^7 - 10^8 cpm/ μ g cDNA, while for cloning experiments a concentration of 200 μ M of unlabelled dCTP (Pharmacia) was used together with enough labelled nucleotide to give a final cDNA specific activity of 10^5 - 10^6 cpm/ μ g cDNA. In both cases reverse transcriptase (Life Sciences Inc. Florida, supplied by NBL Enzymes) was added to 250 units/ml and the reaction incubated for 90 min at 37°C. In some cases human placental ribonuclease inhibitor (Amersham Int.) was included at a concentration of 500 units/ml.

Reactions were terminated by the addition of EDTA to a final concentration of 10 mM. The RNA template was then removed by the addition of sodium hydroxide to 300 mM and incubation at 65°C for 1 hour. The labelled DNA was then separated from the unincorporated dNTP's by chromatography through a column (2 ml bed volume) of Sephadex G-50 (Pharmacia) equilibrated in TE buffer pH 7.6. Fractions of 200 μ l were collected and the amount of radioactivity measured by Cerenkov counting. The desired fractions were then pooled, and the cDNA ethanol precipitated after the addition of 10 μ g/ml sheared denatured salmon sperm DNA.

The cDNA synthesized for cloning was converted into double-stranded cDNA (dscDNA) by two different methods. In the first method (Maniatis *et al.*, 1982) the alkali-treated cDNA (1 μ g) from the first-strand reaction was resuspended in 43 μ l dH₂O and added to an 50 μ l of 2x reaction buffer (0.2 M HEPES pH 6.9, 20 mM magnesium chloride, 5 mM DTT, 140 mM potassium chloride) and 2 μ l of

25 mM dNTPs. Twenty units (5 μ l) of Klenow fragment were then added and the reaction was incubated for 4 hours at 15°C. The low temperature stabilises the transient formation of the loop (Maniatis et al., 1982). The reaction was then stopped, extracted with phenol and chloroform and the cDNA was precipitated of ethanol in the presence of 2 M ammonium acetate to remove the nucleotides. The precipitated Klenow treated cDNA was then resuspended in 25 μ l dH₂O. An aliquot of 5 μ l was kept and the rest made, in a total volume of 50 μ l, 100 mM Tris.HCl pH 8.3 (at 42°C), 140 mM potassium chloride, 10 mM magnesium chloride, 10 mM DTT and 1 mM dNTPs. Forty units of reverse transcriptase were then added (2 μ l) and the reaction was allowed to continue for 1 hour at 42°C.

In the second method (Gubler & Hoffman, 1983), after the addition of EDTA to terminate the first-strand reaction, the solution was extracted with an equal volume of 1:1 v/v phenol:chloroform. Following centrifugation in a microfuge to separate the phases, the aqueous phase was removed and the organic phase re-extracted with an equal volume of TE buffer pH 8.0. The pooled aqueous phases were made 2 M with ammonium acetate and ethanol precipitated in a dry ice/ethanol bath for 15 min. After allowing the Eppendorf tube to warm to room temperature with gentle shaking, the RNA:cDNA hybrid was then pelleted by centrifugation for 15 min. The pellet was resuspended in TE buffer and the precipitation repeated. The hybrid was finally resuspended in 30 μ l TE buffer. A 2 μ l aliquot was electrophoresed on a 1.5% alkaline gel in order to estimate the average size of the first strand reaction products.

After calculating the yield from the first strand reaction, 1 μ g of the RNA:cDNA was converted into dscDNA in a 100 μ l reaction containing 100 mM HEPES pH 7.6, 4 mM magnesium chloride, 12 mM 2-mercaptoethanol, 100 mM potassium chloride, 250 μ M dNTPs, 150 μ M β -NAD (Pharmacia), and 10 μ Ci of [α -32P]dCTP (410 Ci/mmol).

Thirty units of DNA polymerase 1 (Amersham Int.), 2 units of RNase H (BRL) and 1 unit of E.coli DNA ligase (Pharmacia) were then added and the reaction incubated at 12°C for 1 hour, followed by 1 hour at 22°C. The reaction was terminated by the addition of EDTA to 10 mM, phenol extraction and precipitated as described above.

2.29 ADDITION OF LINKERS TO DOUBLE-STRANDED cDNA

Double-stranded cDNA was blunt-ended in a 30 µl reaction containing 33 mM Tris-acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.1 mg/ml BSA, 0.2 mM dNTPs and 0.5 mM DTT. Ten units of T4 DNA polymerase (Anglian) were added and the reaction incubated at 37°C for 30 min. The reaction was then terminated by the addition of EDTA to 10 mM, extraction with 1:1 v/v phenol:chloroform and ethanol precipitation.

Xho1 linkers were radiolabelled before being added to the dscDNA. A 50 µl reaction containing 5 µg of dephosphorylated Xho1 linkers (New England Biolabs) in 10 mM Tris.HCl pH 7.5, 10 mM magnesium chloride, 10 mM DTT and 10 µCi [γ -³²P]ATP (3000 Ci/mmol Amersham Int.) was incubated with 15 units of T4 polynucleotide kinase (Anglian) for 15 min at 37°C. Unlabelled ATP was then added to a final concentration of 1 mM and the reaction incubated for a further 30 min. Aliquots of 5 µl were then stored frozen at -20°C.

The pelleted dscDNA was resuspended in 5 µl of 10 mM Tris.HCl pH 7.5, 10 mM magnesium chloride, 10 mM DTT, 1 mM ATP, and then added to 5 µl of labelled linkers. Ten units of T4 DNA ligase (Anglian) were added and the reaction incubated overnight at 14°C. The reaction was terminated by extraction with 1:1 v/v phenol:chloroform and ethanol precipitation.

The ligase reaction was resuspended in 10 µl of dH₂O and heated to 65°C for 10 min and then added to an equal volume of 20 mM Tris.HCl pH 7.6, 100 mM sodium chloride,

20 mM magnesium chloride, 2 mM DTT. Twenty units of Xho1 were added and the reaction incubated for 1 hour at 37°C, followed by another 20 units of enzyme and further 1 hour incubation. The reaction was terminated by phenol:chloroform extraction and ethanol precipitation. Xho1 linkers were removed from the dscDNA by running the resuspended dscDNA down a 32 cm x 0.2 cm Bio-Gel A-50m column. The glass column was first siliconized (soaked in dichloromethylsilane for 2 min, washed 10 times with dH₂O and then baked at 180°C for 1 hour) and then packed with pre-swollen Bio-Gel A-50m (final bed volume of 1 ml) in the buffer of 10 mM Tris.HCl pH 7.5, 100 mM sodium chloride, 1 mM EDTA. After extensively washing the column, the precipitated dscDNA was resuspended in 20 µl of column buffer and then passed down the Bio-Gel A-50m. Three drop fractions (approximately 50 µl) were collected and Cerenkov counted. Aliquots of each fraction were then electrophoresed on a 1.4% agarose gel. The desired aliquots were precipitated with ethanol and ligated into Xho1 cleaved, alkaline phosphatased pUC19/Xho1.

2.30 CLONING OF DOUBLE-STRANDED cDNA USING RESTRICTION ENZYMES

To determine which restriction enzymes would cut the dscDNA, aliquots of dscDNA (approximately 10000 cpm) were digested in a volume of 10 µl with 5 units of enzyme. The digestions were then electrophoresed through a 1.2% agarose gel. After drying down the gel and autoradiography, it could be established which enzymes had cut the dscDNA.

One microgram of dscDNA was digested in a 50 µl volume with 10 units of Sau3A for 1 hour. After extraction with 1:1 v/v phenol:chloroform and precipitation, the digested dscDNA was resuspended in 20 µl of dH₂O. Similarly, 1 µg

of pUC/Xho1 was digested with BamH1, dephosphorylated with alkaline phosphatase (see Section 2.31), extracted with 1:1 v/v phenol:chloroform, precipitated with ethanol and redissolved in 20 μ l of dH₂O.

Vector and dscDNA fragments were then mixed at an approximate 1:3 ratio (vector DNA:dscDNA) in a buffer containing 50 mM Tris.HCl pH 7.6, 10 mM magnesium chloride, 20 mM DTT, 1 mM ATP, at a final DNA concentration of 10-50 μ g/ml. One unit of T4 DNA ligase (Anglian Biotech.) was added and the mixture incubated at 14°C overnight. The hybrid molecules were used the next day to transform competent E.coli JM101 cells.

2.31 ALKALINE PHOSPHATASE REACTION

Because of its ability to dephosphorylate the 5' end of dsDNA alkaline phosphatase treatment reduces the transformation background due to vector recircularization. One microgram of vector DNA, usually pUC/Xho1, was digested to completion with the required enzyme (checked by running 50 ng on a agarose gel). One unit of alkaline phosphatase (BCL) was then added and the reaction incubated at 37°C for a further 30 min. The DNA was then extracted three times with 1:1 v/v phenol:chloroform and ethanol precipitated.

2.32 ORIGIN AND STORAGE OF BACTERIA

E.coli DH-5 (F-, recA1, endA1, gyrA96, thi-1, hsdR17 r-m+, supE44, relA1, λ -) was obtained from Dr.D.Glover, Imperial College. E.coli JM101 (Δ lacproAB, thi, supE,[F', traD36, proAB lacI^QZ, Δ M15]) was obtained from Dr.W.D.O.Hamilton, Imperial College. Both strains were stored on minimal media plates at 4°C.

2.33 MEDIA FOR GROWTH AND STORAGE OF BACTERIA

Minimal agar consisted of:

- 500 ml 3% w/v Bacto Agar (Difco)
- 500 ml M9 salts (3 g potassium dihydrogen phosphate,
6 g disodium hydrogen phosphate,
0.5 g sodium chloride,
1 g ammonium chloride,
dH₂O to 1 litre)
- 1 ml 0.1 M calcium chloride
- 1 ml 1 M magnesium sulphate
- 0.5 ml 1% vitamin B (BDH)
- 1 ml 20% w/v glucose.

SOB broth contained:

- 2% w/v Bacto Tryptone (Difco)
- 0.5% Bacto Yeast Extract (Difco)
- 10 mM sodium chloride
- 2.5 mM potassium chloride
- 10 mM magnesium chloride
- 10 mM magnesium sulphate.

SOC broth: SOB broth plus 10 mM glucose

2xTY broth contained:

- 1.6% w/v Bacto Tryptone
- 1% Bacto Yeast Extract
- 0.5% sodium chloride

TY plates: TY containing 1.5% w/v Bacto Agar.

2xTY plates: 2xTY containing 1.5% w/v Bacto Agar.

Top Agar: TY containing 0.6% Bacto Agar.

TF buffer contained:

- 100 mM potassium chloride
- 45 mM manganese chloride
- 10 mM calcium chloride
- 3 mM hexamine cobalt (III) trichloride (Sigma)
- 10 mM 2(N-morpholino)etone sulphonic acid pH 6.3
(Sigma)

DnD solution contained:

- 1 M DTT (Sigma)
- 90% DMSO (BDH)
- 10 mM potassium acetate pH 7.5.

2.34 PREPARATION OF COMPETENT CELLS

Two strains of E.coli cells were used, JM101 (Messing, 1983) and DH-5 (Hanahan, 1985). They were made competent using the same method. A single colony was picked, dispersed in 10 ml of 2xTY broth and incubated overnight at 37°C with shaking. Usually 300 µl of this culture was then used to inoculate 30 ml of SOB media, which was then incubated at 37°C with shaking until the optical density at 550 nm had reached 0.45-0.55. The cells were cooled on ice for 10 min, harvested by centrifugation at 3000 rpm for 10 min at 4°C in a MSE HS21 rotor and gently resuspended in 10 ml of ice cold TF buffer. After 10 min they were re-centrifuged and resuspended in 2.4 mls of ice cold TF buffer. DnD solution was added to 3.5% and the cells incubated for a further 10 min before repeating this step. Usually 200 µl aliquots of cells were pipetted into chilled 1.5 ml Eppendorf tubes and used immediately for transformations.

In many cases when a lower average transformation frequency would suffice a simpler method for the preparation of cells was used. In this method DnD was not added, and instead of TF buffer, 50 mM calcium chloride was used.

2.35 TRANSFORMATION OF CELLS WITH PLASMID DNA

Up to 10 ng of DNA (either in TE or ligation buffer) at a concentration of $<1 \mu\text{g/ml}$ was added to 200 μl of competent JM101 cells. The mixture was left on ice for 40 min before being heat shocked at 42°C for 90 seconds. The cells were then added to 800 μl of SOC broth and incubated at 37°C with shaking for 30 min. Ten microlitres of 100 mM IPTG (Sigma) and 50 μl of X-Gal (Sigma, made up as a 2% solution in dimethylformamide) were then added, and the cells spread onto 2xTY plates containing ampicillin (Sigma, made up as 10 mg/ml in dH_2O) at a concentration of 50 $\mu\text{g/ml}$; these were allowed to dry before being incubated inverted overnight at 37°C .

When using DH-5 cells the same procedure was used except that neither IPTG or X-Gal were added.

2.36 ORDERED ARRAY OF COLONIES

Colonies were picked using sterile toothpicks and stabbed into a fresh 82 mm plate of 2xTY plus 50 $\mu\text{g/ml}$ ampicillin in an ordered array. The colonies were grown at 37°C until they were 1 mm in diameter. Sterile GeneScreen was then laid on the plate, removed and placed colony side upwards, for 5 min, upon Whatman 3MM paper saturated with 0.5 M sodium hydroxide. The filter was then transferred to another sheet soaked with sodium hydroxide for a further 5 min. This was followed by two neutralization steps, each of 5 min, first with 1 M Tris.HCl pH 8.0, and then with 1

M Tris.HCl pH 8.0, 1.5 M sodium chloride. The filter was then placed colony side up on dry Whatman 3MM paper and allowed to air dry for 1 hour. The filter was finally baked in an oven at 80°C for 1 hour before being prehybridised as described in Section 2.26.

2.37 DOT BLOTS

Two microlitres of plasmid DNA from a miniprep were spotted directly onto GeneScreen. The membrane was allowed to dry for 30 min, washed in 2xSSC for 10 min and treated with sodium hydroxide and Tris.HCl as described in Section 2.26. The GeneScreen was then baked, prehybridized, hybridized with labelled insert DNA, washed and then autoradiographed as described in Section 2.26.

2.38 PURIFICATION OF PLASMID DNA

Plasmid DNA was prepared either on a small scale - miniprep - in order to determine whether an E.coli colony contained recombinant DNA, or on a larger scale for use in restriction mapping, blotting and hybrid selection.

Minipreps: Colonies for plasmid extraction were picked into 2 ml 2xTY broth containing 50 µg/ml ampicillin in Sterilin tubes and grown overnight at 37°C with shaking. Next day 1 ml of each culture was transferred to a 1.5 ml Eppendorf tube and centrifuged in a microfuge for 1 min. The pellet was resuspended thoroughly in 100 µl of TE buffer and 200 µl of 0.2 M sodium hydroxide, 1% SDS was added. The sample was then mixed gently by inversion and held on ice for 5 min, after which 150 µl of 3 M potassium acetate was added and the mixture vortexed gently before being kept on ice for a further 5 min. The Eppendorf was then centrifuged for 5 min and the supernatant transferred to a fresh tube where it was extracted with an equal

volume of 1:1 v/v phenol:chloroform. After centrifugation two volumes of cold ethanol were added to the supernatant and the Eppendorf tube held at room temperature for 2 min before being centrifuged for 10 min. The nucleic acid pellet was washed twice with 70% v/v ethanol and then dried under vacuum before being finally resuspended in 50 μ l TE buffer pH 8.0, 20 μ g/ml RNase A (Sigma).

Large Scale Plasmid Preparation: A bacterial colony was added to 5 mls of 2xTY broth plus 50 μ g/ml ampicillin and grown at 37°C overnight with shaking. This starter culture was used to inoculate 500 mls of 2xTY plus ampicillin, which was then incubated with shaking at 37°C overnight. Next day the cells were harvested by centrifugation at 7000 rpm for 10 minutes in a MSE HS18 rotor, resuspended in 20 ml TE buffer pH 8.0, re-pelleted and then resuspended in 10 ml of TE buffer. Twenty millilitres of alkaline-SDS were then added, and the mixture placed on ice for 10 min. Fifteen millilitres of potassium acetate were added, followed by a further incubation on ice for 10 min and a 10 min centrifugation at 13000 rpm in a HS21 8x50 rotor. The supernatant was transferred to fresh tubes, and then 0.6 volumes of isopropanol were added. After standing at room temperature for 30 min the DNA was pelleted by centrifugation at 12000 rpm in a HS21 rotor. The pelleted DNA was then washed with 70% v/v ethanol and resuspended in 10 ml TE buffer pH 8.0. Ten grams of caesium chloride were added. The volume was then measured, and 0.8 mls of a 10 mg/ml solution of ethidium bromide was added for every 10 mls of caesium chloride solution. This gave a final density of 1.55 g/ml, which was checked using a Gallenkamp Sodium Lamp Refractometer. The solution was transferred into a Beckman Type-65 tube and centrifuged in a Beckman Type-65 rotor for 36 hours at 20°C. The lower band of covalently closed circular DNA was recovered by puncturing the side of the tube with a syringe. The

ethidium bromide was removed from the DNA solution by extraction with water-saturated butanol ten times and the caesium chloride removed by extensive dialysis against several changes of TE buffer pH 8.0.

2.39 SUBCLONING AND TRANSFORMATION WITH M13 RF DNA

DNA restriction fragments were ligated into the appropriately restricted M13mp18 (Biolabs) RF DNA as described in section 2.25.

Up to 10 ng (either in TE or ligation buffer) at a concentration of <1 µg/ml was added to 200 µl of competent JM101 cells. The mixture was left on ice for 40 min before being heat shocked at 42°C for 90 seconds. The 200 µl aliquots were added to 3 mls of top agar kept previously at 42°C, together with 10 µl of IPTG, 50 µl of X-Gal, and 200 µl of exponentially growing JM101 cells, and the mixture vortexed briefly before being spread onto 1xTY plates and incubated at 37°C overnight.

2.40 PREPARATION OF SINGLE-STRANDED M13 DNA

Colourless plaques from the transformation were picked individually into 2 ml of 2xTY medium in Sterilin tubes. The cultures were incubated with shaking for 8 hours and 1 ml samples transferred to 1.5 ml Eppendorf tubes. The cells were pelleted by centrifugation in a microfuge for 10 min and 0.8 ml of the supernatant was withdrawn into a fresh tube for M13 phage DNA purification as described below. The remaining supernatant was used to test for the presence and orientation of the inserts. Usually 20 µl of the supernatant was mixed with 1 µl of 2% w/v SDS and 3 µl loading buffer. Samples were then electrophoresed through a 0.7% agarose gel in TBE buffer at 7 volts/cm for 5 hours. The gel was then stained with ethidium bromide. As

with recombinant plasmids, the mobility of the recombinant phage was found to decrease proportionally to the length of the inserted DNA.

Because the plus-strand is always contained in the viral particle, two recombinant phage with a DNA fragment cloned in opposite orientations into the M13 RF will hybridise only via the insert region. To test whether two recombinant phage have DNA complementary to each other, 20 μ l of two supernatants were mixed with SDS and loading buffer as described above. After overlaying the mixture with paraffin oil, the sample was incubated for 1 hour at 65°C. The sample was then electrophoresed through an agarose gel as described above. If the two viral DNAs hybridise via their insert regions, they form a figure eight-like structure and migrate slower in the gel (Messing, 1985).

For phage DNA purification 200 μ l of a solution containing 2.5 M sodium chloride and 20% w/v polyethyleneglycol 6000 (BDH) was added to 800 μ l of supernatant and mixed by inversion several times. The mixture was allowed to stand for 30 min on ice before being centrifuged for 10 min. The supernatant was decanted and 50 μ l of TE buffer pH 8.0 added to the pellet. The pelleted M13 were resuspended and then extracted with 50 μ l of phenol; the mixture was vortexed and then allowed to stand for 30 min, followed by a 1 min centrifugation in a microfuge. The aqueous phase was then removed to a fresh tube and extracted three times with ether. Finally, 10 μ l of 1 M sodium acetate pH 5.5 and 140 μ l of ethanol were added and the mixture left at -20°C overnight. The DNA was collected by a 15 min centrifugation and then washed with 70% v/v ethanol, dried and resuspended in 50 μ l dH₂O.

2.41 RNase H DIGESTION OF TBSV RNA-cDNA HYBRIDS

TBSV RNA (1 µg) was annealed to a 2-fold molar excess of the desired sscDNA fragment in 20 µl of 40 mM Tris.HCl pH 7.9, 4 mM magnesium chloride, 1 mM DTT for 20 min at 60°C. After cooling, 0.5 units of RNase H (BRL) was added and the reaction incubated for 15 minutes at 32°C. Five minutes before the end of the reaction 1 unit of calf alkaline phosphatase (BCL) was added. RNA was recovered by 1:1 v/v phenol:chloroform extraction, ethanol precipitation and then labelled with [γ -³²P]ATP using T4 polynucleotide kinase or with [5'-³²P]pCp using RNA ligase.

2.42 TRANSFER OF DNA TO AMINOPHENYLTHIOETHER (APT) PAPER

Cellulose derivatized with o-aminophenylthioether groups bind DNA and RNA covalently. APT paper was prepared by a modification of the protocol described by B.Seed. Twenty grams of Whatman 50 filter paper were placed in a sealable bag which contained 70 ml 0.5 M sodium hydroxide and 30 ml of 1,4-butanediol diglycidyl ether (Aldrich) plus 2 mg/ml sodium borohydride. The bag was sealed and rotated end-over-end (axis of rotation in the plane of the bag) overnight at 40 rpm. The bag was then drained of the first solution and this replaced with 40 mls of acetone and 10 mls 2-aminothiophenol (Aldrich) The bag was resealed and rotated for a further 18 hours. The paper was then removed from the bag and washed thoroughly three times with acetone, twice with 0.1 M hydrochloric acid and finally with dH₂O. The paper was air dried and stored at -70°C. Just prior to use the paper was diazotized by soaking it for 30 min in a cold solution (4°C) of 1.2 M hydrochloric acid which contained 0.27 mg/ml sodium nitrite; the paper turned bright yellow within 5 min.

APT paper can bind 15-25 ug of ssDNA per cm² (Jagus, 1987). In order to bind DNA, the DNA had to be denatured prior to its application to the paper. DNA was linearised with Xho1, phenol extracted, precipitated with ethanol and resuspended. The DNA, in 10 µl of dH₂O, was then boiled for 1 min followed by snap freezing in a dry-ice/ethanol bath just prior to application to the filter.

The freshly diazotized DPT paper was cut into 5 mm squares and washed twice with ice cold dH₂O and then twice with 20 mM sodium acetate pH 4.0. After removing any excess liquid the paper was carefully placed horizontally in a vertically held 1.5 ml Eppendorf tube. The DNA was spotted directly onto the paper and allowed to dry in air for 2 hours. The top of the tube was then closed and left overnight at room temperature. Next day the filter was washed four times with dH₂O, four times with 0.4 M sodium hydroxide for 10 min at 37°C, four times with 2 M sodium acetate pH 5.5 and then four times with dH₂O. Finally the filter was washed four times in prehybridisation buffer (see Section 2.44 below).

2.43 TRANSFER OF DNA TO GENESCREEN-PLUS

GeneScreen-Plus (New England Nuclear) was cut into 5 mm squares, soaked in dH₂O for 5 min and then in 0.4 M sodium hydroxide for 30 min. DNA to be applied was linearised with Xho1, phenol extracted, precipitated with ethanol and resuspended in 0.4 M sodium hydroxide. The DNA was then applied to the filter by spotting 10 µl aliquots at a time, drying between applications. The filter was then washed twice in 1 M ammonium acetate pH 5.5 and twice in 2xSSC, followed by overnight drying at room temperature.

2.44 HYBRID SELECTION

GeneScreen-Plus or APT filters were soaked overnight at 37°C in prehybridisation buffer containing 50% v/v formamide, 0.75 M sodium chloride, 0.1 M PIPES-NaOH pH 6.4, 8 mM EDTA, 1% glycine (for APT filters), 0.5% non-fat dried milk (i.e. Marvel for GeneScreen-Plus), 100 µg/ml calf liver tRNA, 0.5% SDS. Afterwards the filters were washed 10 times at 60°C in 1xSSC, 2 mM EDTA, 0.5% SDS, and then briefly with one of the hybridisation buffers. RNA was hybridised, at a concentration of 10 mg/ml, to five filters in either 200 µl of low abundance RNA hybridisation buffer (50% v/v formamide, 0.4 M sodium chloride, 10 mM PIPES-NaOH pH 6.4, 8 mM EDTA, 0.5% SDS) or 200 µl of high abundance RNA hybridisation buffer (65% v/v formamide, 0.4 M sodium chloride, 10 mM PIPES-NaOH pH 6.4, 8 mM EDTA, 0.5% SDS) at 37°C or 48°C respectively.

After the optimum time the filters were washed ten times in 1xSSC, 0.5% SDS at 50°C (1.5 mls), five times with 1xSSC at 50°C, and then once in 5 mM Tris.HCl pH 7.5, 1 mM EDTA for 40 seconds at 50°C. The hybridised RNA was finally eluted from the filters by boiling in 0.5 ml of 5 mM potassium chloride, 2 mM EDTA, 10 µg/ml calf thymus tRNA for 1 min, followed by snap freezing in dry ice-ethanol. After removal of the eluate, the elution was repeated with another 0.5 ml of elution buffer. The eluates were then pooled and the RNA precipitated with ethanol and 0.2 M potassium acetate.

2.45 CELL-FREE TRANSLATION OF RNA

Rabbit reticulocyte lysate from Amersham (N90) was used according to the supplier's instructions. Typically, 2 µl of RNA solution (containing 1 µg of RNA) was added to 8 µl of the lysate in a 0.6 ml Eppendorf tube in which 10 µCi of L-[4,5-³H] leucine (Amersham; 128 Ci/mmol) had been

previously dried down. The mixture was incubated at 30°C for 75 min, after which an aliquot (2 μ l) was removed to determine TCA precipitable radioactivity. The remaining 8 μ l of the translation were then either immunoprecipitated or subject to SDS-polyacrylamide gel electrophoresis.

2.46 DETERMINATION OF AMINO ACID INCORPORATION

Typically a 2 μ l aliquot of the translation mixture was pipetted onto a Whatman No1 filter paper disc (2.5mm diameter). The disc were placed into a beaker of ice-cold 5% w/v TCA and washed with gentle swirling for 15 minutes. The discs were then transferred to a beaker of 5% TCA at 90°C and incubated to hydrolyze the charged tRNA. After 15 minutes the discs were added to fresh 5% w/v TCA at room temperature and washed for a further 15 minutes, and then washed successively with ethanol and acetone for 1 minute. The discs were then allowed to dry at room temperature before 40 μ l of 10% w/v hydrogen peroxide was pipetted onto the discs to bleach the sample, thus preventing colour quenching during scintillation counting. After 1 hour the discs were placed in 10mls of scintillant and counted as described in Section 2.17.

2.47 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins and in vitro translation products were analyzed on 12½ or 15% w/v polyacrylamide gels using the discontinuous buffer system of Laemmli (1970). The gel consists of two parts, a stacking gel and a resolving gel. The resolving gel was prepared using 12.5 ml or 15 ml of acrylamide-bisacrylamide (30:0.8), 3.75 ml of 3M Tris.HCl pH 8.8, 0.3 ml of 10% w/v SDS, 1.5 ml of 1.5% w/v ammonium persulphate and 11.95 or 9.45 ml of dH₂O. After degassing the solution for 20 min, 20 μ l of TEMED was added and the mixture poured between two glass plates leaving sufficient

space at the top for the stacking gel, usually 3 cm. The gel was then overlayed with 5 ml of 0.1% SDS and allowed to set for approximately 1 hour.

The stacking gel of 20 ml was prepared using 2.5 ml of the acrylamide, 5 ml of 0.125 M Tris.HCl pH 6.8, 0.2 ml of 10% w/v SDS, 1 ml of 1.5% w/v ammonium persulphate and 11.3 ml dH₂O. After degassing the solution for 30 min, 20 μ l of TEMED was added. The overlay on top of the resolving gel was removed, the stacking gel poured on top of the resolving gel and a gel comb with slot widths of 0.85 cm inserted.

After allowing the gel to set for at least an hour the comb was removed and the gel placed in a vertical electrophoresis tank with 0.025 M Tris, 0.192 M glycine pH 8.3 in both the upper and lower reservoirs. Samples were added to an equal volume of disruption buffer (0.125 M Tris.HCl pH 6.8, 2% w/v SDS, 5% v/v 2-mercaptoethanol, 20% v/v glycerol, 0.002% bromophenol blue) and heated to 100°C for 3 min. After cooling the samples were loaded onto the gel and electrophoresed at 10 volts/cm for 6 hours or 3 volts/cm overnight.

Coomassie blue R250 (0.1%) was dissolved in water:methanol:acetic acid (5:5:2 v/v/v) and filtered through Whatman No.1 filter paper. After electrophoresis the gel was stained in the coomassie for 2 hours, and then destained in 12.5% v/v isopropanol, 10% v/v acetic acid overnight.

2.48 FLUOROGRAPHY OF SDS-POLYACRYLAMIDE GELS

The method of Lasky and Mills (1975) was used. After electrophoresis the gel was placed in a polyethylene tray containing 10% w/v TCA, 10% v/v glacial acetic acid, 30% v/v methanol for 1 hour. The gel was then soaked twice in 100 ml DMSO for 30 min, followed by soaking for 3 hours in

100 ml of 22% w/v PPO in DMSO. Finally, the gel was washed four times in 250 mls dH₂O for a total of 2 hours before drying under vacuum on a BioRad Gel Drier. The dried gel was exposed to Kodak XR1 X-ray film.

2.49 IMMUNOPRECIPITATION

Sixteen microlitres of TBSV-Type strain antiserum (a kind gift of Dr.A.A.Brunst, titre of 1/2048) was mixed with an in vitro translation sample (8 μ l), and the mixture was incubated overnight at 4°C. Thirty-two microlitres of formalin-fixed Staphylococcus A cells (Immuno-Precipitin, BRL) were pelleted in a microfuge for 2 min, washed twice in NP40 buffer (0.15 M sodium chloride, 0.5 mM EDTA, 0.05% Nonidet P40, 5mM Tris.HCl pH 7.9) containing 1% BSA, resuspended in 130 μ l of NP40 buffer plus 1% BSA and mixed thoroughly with the antiserum-sample mixture. After incubation at room temperature for 30 min and cooling on ice, the cells were spun down, washed twice in NP40 buffer plus 1% BSA, once with NP40 buffer and then resuspended in 16 μ l of denaturing buffer (2% w/v SDS, 5% v/v 2-mercaptoethanol in 6.25 mM Tris.HCl pH 6.8) and heated to 100°C for 3 min to release the bound antigen.

CHAPTER 3

RESULTS: DOUBLE-STRANDED AND SINGLE-STRANDED SUBGENOMIC RNAs FROM PLANT TISSUE INFECTED WITH TBSV

3.1 VIRAL PASSAGE AND SYMPTOMS

The original TBSV isolate, the type strain (Smith, 1935), was kindly supplied by Dr.A.A.Brunst of the Glasshouse Crops Research Institute, Littlehampton as freeze dried infected N.benthamiana leaf material. This material was ground in 10 mM phosphate buffer pH 7.0 in a mortar with a pestle using carborundum as an abrasive, and the supernatant inoculated mechanically onto the two largest lower leaves of four week old N.benthamiana or N.clevelandii plants. The symptoms produced in both plants were very similar. After two days local lesions began to appear on the inoculated leaves, and approximately five days later the first systemic symptoms could usually be seen as curling and chlorosis of the youngest leaves (Fig.3.1a). The chlorosis during the next two days developed into lesions on the young and lower, older leaves (Fig.3.1b), followed by withering of the stem and total plant collapse after two weeks (Fig.3.1c) in both N.clevelandii and N.benthamiana. No symptoms were seen on plants mock inoculated with 10 mM phosphate buffer pH 7.0.

Various parts of an infected N.benthamiana plant were ground in buffer and used to inoculate healthy N.benthamiana plants. All the newly infected plants developed the symptoms described above, irrespective of whether the inoculum originated from the systemically



FIG.3.1. TBSV induced symptoms.

(a) N.benthamiana plant 5 days post-inoculation showing systemic symptoms of curling and chlorosis on the youngest leaves.

(b) N.benthamiana plant 9 days post-inoculation showing lesions on the youngest leaves.

(c) N.benthamiana plant 14 days post-inoculation.



b



c

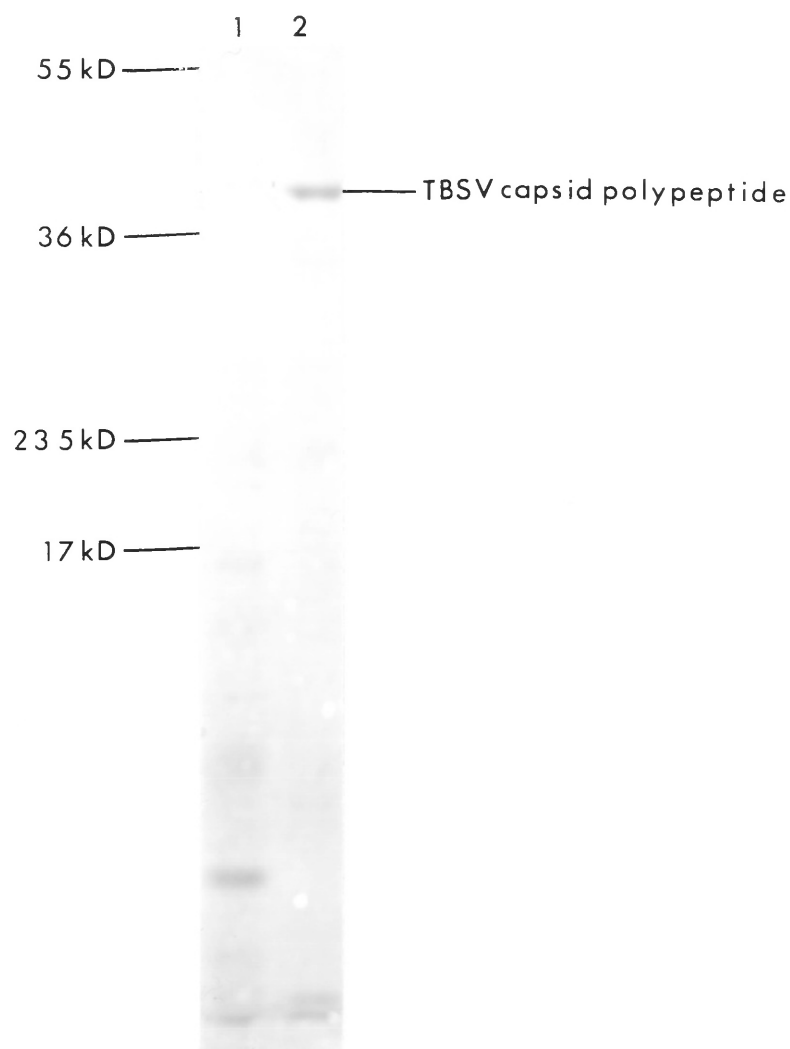


FIG.3.2. Capsid protein from TBSV particles (5 μ g) electrophoresed through a 12% SDS-polyacrylamide gel and stained with Coomassie Blue. Lane 1, TBSV purified by Method 1; lane 2, TBSV purified by Method 2. The positions of Mr markers, human gamma globulin (55 kD and 23.5 kD), lactate dehydrogenase (36 kD) and myoglobin (17 kD) are indicated on the left.

infected leaves, stems or roots, indicating that TBSV is present in all of these tissues. However, for routine inoculations and subsequent virus purification only the leaves of the infected plant were used.

3.2 VIRUS PREPARATION

Two methods for preparing virus were used (Section 2.7). Both methods yielded a clear virus pellet after the initial high speed centrifugation, and after resuspension and centrifugation through a 10-40% w/v sucrose gradient, a single well defined band approximately half way down the gradient was usually seen using overhead illumination. Aliquots of the band from both sucrose gradients were inoculated onto N.benthamiana plants. Typical symptoms were produced, confirming that the single band was indeed virus. Although virus yields of up to 250 mg virus per kilogram infected leaf material with Method 1 have been obtained (Dr.A.A.Brun - personal communication), the average yield with this method, calculated by measuring the absorbance of a sample at 260 nm, was 114 mg virus per kilogram of infected leaf. Method 2 typically yielded only 77 mg virus per kilogram of infected leaf.

Further purification of the virus by centrifugation to equilibrium in caesium chloride was then attempted. Virus from the sucrose gradient was pelleted after dilution with extraction buffer by high speed centrifugation. The virus was resuspended in caesium chloride at an initial density of 1.36 gm/ml and centrifuged for eighteen hours. Virus particles produced by Method 2 banded halfway down the gradient at a density of 1.36 g/ml (Martelli et al., 1971). However, repeated attempts to purify the virus obtained by Method 1 in caesium chloride gradients failed.

Sucrose gradient purified virus was resuspended in extraction buffer, denatured and electrophoresed through a

12% SDS-polyacrylamide gel (Laemmli, 1970; Fig.3.2). After staining with Coomassie Blue a single band with a Mr of approximately 40 kD (Butler, 1970) can be seen for virus prepared by Method 2 (lane 2). However, virus prepared by Method 1 (lane 1) gave only a faint band with Mr of approximately 40 kD, and numerous other bands of lower molecular weight. This result, together with the failure of attempts to purify virus from Method 1 on caesium chloride gradients, indicated that virus produced by Method 1 was substantially degraded. This is probably due to the high levels of proteases and oxidases present in plant material; Method 1 contains an overnight extraction step, while Method 2 is much shorter and includes in the extraction buffer phenylmethanesulphonyl fluoride, a irreversible inhibitor of serine proteases.

3.3 VIRAL RNA EXTRACTION

TBSV RNA was extracted from virus particles purified by both methods using an alkaline buffer, SDS as a detergent, EDTA as a metal chelator and phenol plus chloroform as organic denaturants. The extracted RNA was then electrophoresed through 1% agarose gels containing 5 mM methylmercuric hydroxide (Fig.3.3), a compound which denatures nucleic acids and protects RNA from the RNases naturally present in agarose (Bailey & Davidson, 1976).

As expected, RNA from virions prepared by Method 1 (Fig.3.3, lane 1) was substantially degraded, while RNA from virions prepared by Method 2 (lane 2) contained a dominant single intact RNA species. This was further proof that while Method 1 gave a higher virus yield, most of the virus particles were not intact. Therefore, purification Method 1 was abandoned in favour of Method 2.

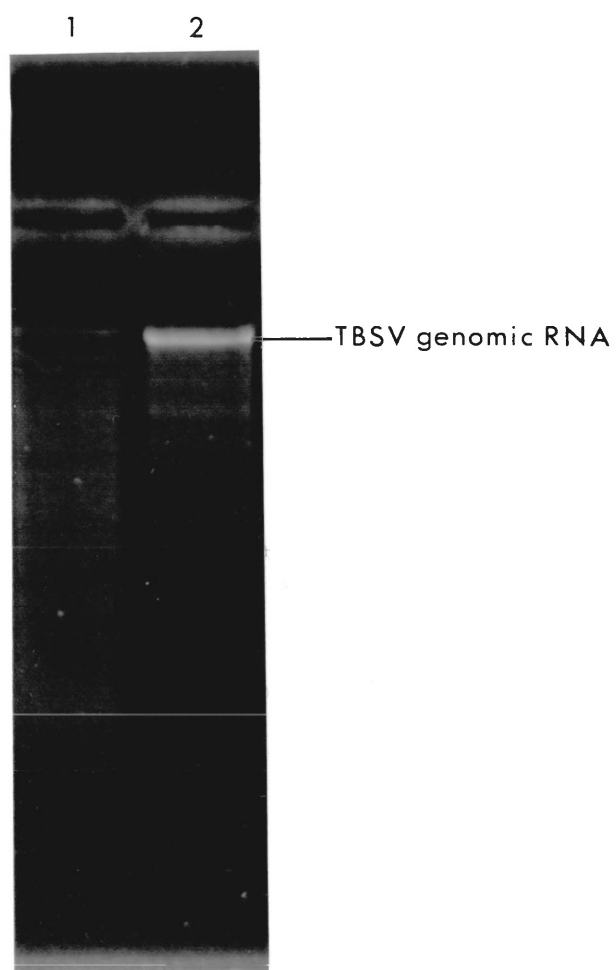


FIG.3.3. TBSV RNA (250 ng) prepared from virus purified by (lane 1) Method 1, and (lane 2) Method 2 after electrophoresis through a 1% agarose gel containing 5 mM methylmercuric hydroxide and staining with ethidium bromide.

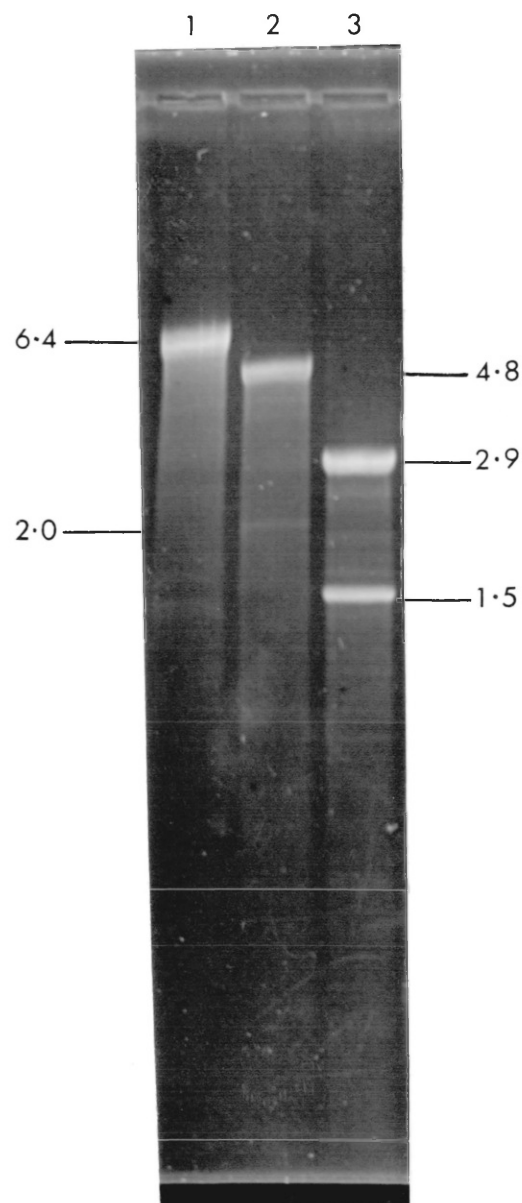


FIG.3.4a. Comparison of the mobility of TBSV RNA with ssRNA species of known molecular weight after electrophoresis through a 1% agarose gel containing 5 mM methylmercuric hydroxide and staining with ethidium bromide. Lane 1, tobacco mosaic virus RNA (250 ng); lane 2, tomato bushy stunt virus RNA (250 ng); lane 3, E.coli ribosomal RNA (500 ng). Lengths of RNA in kb.

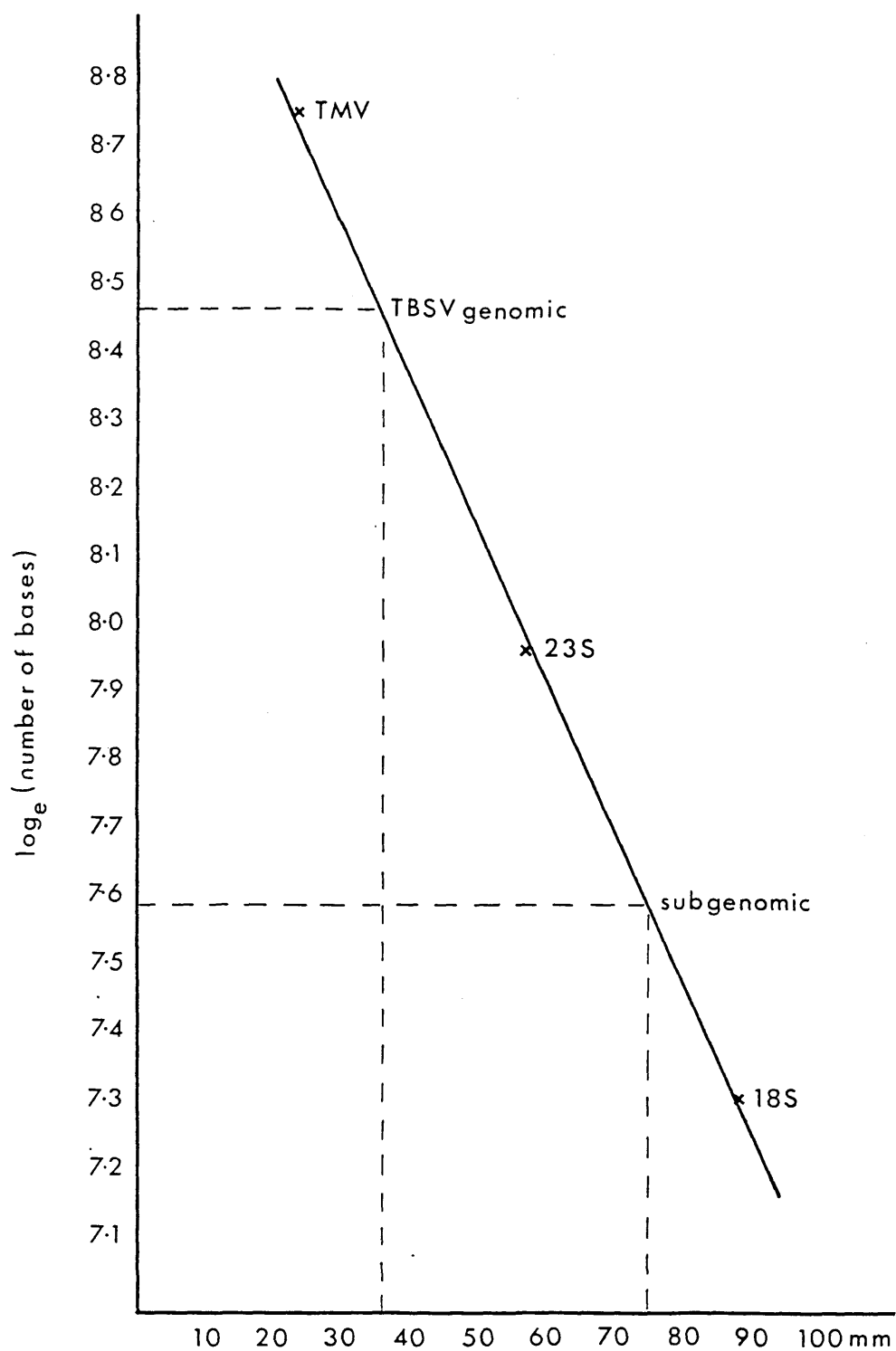


FIG.3.4b. Graph of $\log_e(\text{number of bases})$ vs. electrophoretic mobility from Fig.3.4a. TMV, *E.coli* 23S and 18 S RNAs are 6395, 2904 and 1542 nucleotides long respectively (Goelet *et al.*, 1982; Brosius *et al.*, 1978, 1980).

3.4 DETERMINATION OF GENOME SIZE

The electrophoretic mobility of nucleic acids in non-denaturing gels depends on both the molecular weight and the conformation. Methylmercuric hydroxide reacts with the purines and pyrimidines in a reversible manner, disrupting the secondary structure of the RNA, so that mobility depends solely on molecular weight (Bailey & Davidson, 1976).

To determine the size of the TBSV genome, the purified RNA was electrophoresed in a 1% agarose gel containing 5 mM methylmercuric hydroxide with ssRNA markers of known size (Fig. 3.4a). Graphs of $\log_e$ (number of bases) against mobility for five such gels allowed the mean length of TBSV to be calculated as 4800 bases (Fig.3.4b), which is similar to both the values determined for TBSV-Cherry and TBSV-BS3 strains in non-denaturing gel systems (Dorne & Pinck, 1971; Henriques & Morris, 1979), and to the genome sizes estimated for the other definitive tombusvirus group members (Gallitelli & Hull, 1985a).

3.5 ENCAPSIDATION OF LESS THAN GENOME SIZE RNA

In both Fig.3.3 and Fig.3.4a (lane 2), 250 ng of TBSV RNA had been electrophoresed through the agarose gel. When a greater amount (2 μ g) of TBSV RNA was loaded onto a 1.8% agarose gel containing 5 mM methylmercuric hydroxide, a number of other smaller RNA species could also be detected. Included among these smaller RNAs are two distinctive species approximately 2.0 kb (Fig.3.5, Fig.3.6 and also present in Fig.3.4a) and 0.9 kb (Fig.3.5 and Fig.3.6) in length. The 2.0 kb and other lower molecular weight RNA species were also detected in TBSV-Cherry strain, TBSV-Type strain and TBSV-BS3 strain virus

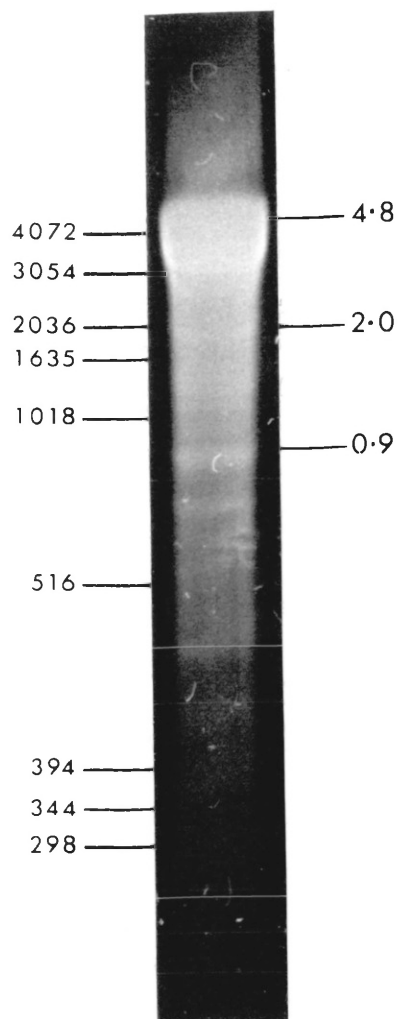


FIG.3.5. TBSV RNA (2 μ g) after electrophoresis through a 1.8% agarose gel containing 5 mM methylmercuric hydroxide and staining with ethidium bromide. The positions of BRL kb markers are shown on the left. Lengths of other RNAs are in kb.

particles prepared by Hillman et al. (1985) and Gallitelli & Hull (1985a). The 2.0 kb RNA may be the encapsidated ssRNA form of the 2.0 kbp dsRNA detected in infected tissue by Henriques and Morris (1979).

Although some of the smaller ssRNA bands could be genuine subgenomic RNAs and/or encapsidated host RNA, they might have arisen by degradation of the genomic RNA, or they may represent contamination of the virus preparation. Contaminating RNA would be expected to pellet when virus particles are centrifuged to equilibrium in caesium chloride. However, to ensure that RNA was not attached to the outside of the virions, the virus particles were treated with RNase A. Sucrose gradient purified TBSV virions in 50 mM sodium phosphate pH 7.8 were incubated for 30 minutes at 37°C with 1 µg/ml RNase A (Rochon & Siegal, 1984). Caesium chloride was then added to a density of 1.36 g/ml, and the virus particles were centrifuged to equilibrium. RNA extracted from the purified particles was then electrophoresed through a 1.8% agarose gel containing 5 mM methylmercuric hydroxide (Fig.3.6, lane 2). Comparison with RNA from non-treated virus particles (lane 1) shows that the RNase A treatment had little or no detectable effect on the pattern or concentration of the non-genomic RNA species. As a control, genomic RNA was treated with and without RNase A under the same conditions as the virus particles. The treated RNA, electrophoresed through a denaturing gel immediately after the RNase treatment, was found to be completely degraded (data not shown). Therefore, the non-genomic RNAs are encapsidated RNA species and not external contaminants.

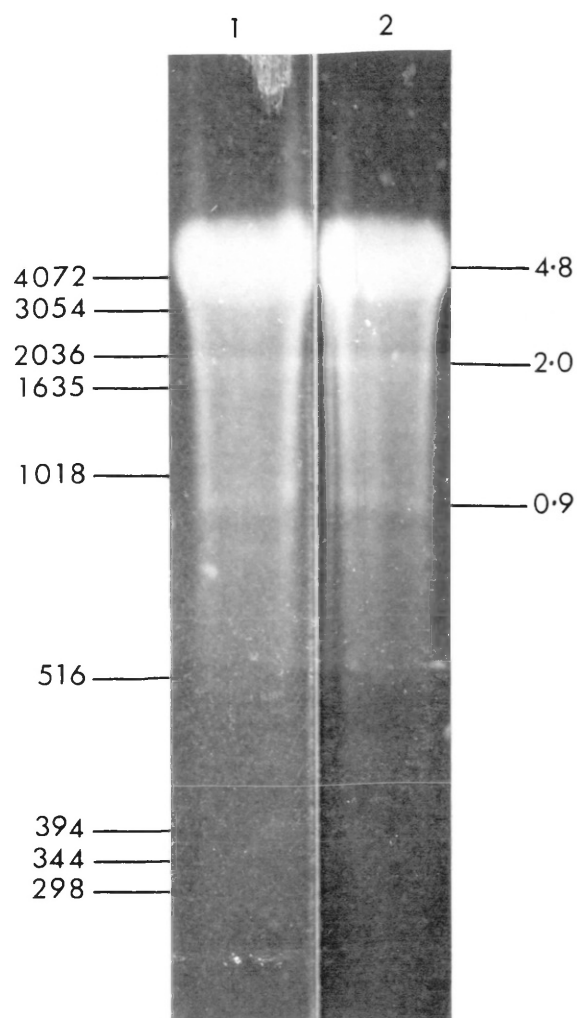


FIG.3.6. TBSV RNA prepared from (lane 1) virus particles purified by caesium chloride and (lane 2) RNase treated particles purified by caesium chloride, after electrophoresis through a 1.8% agarose gel containing 5 mM methylmercuric hydroxide and staining with ethidium bromide. The positions of BRL kb markers are shown on the left. Lengths of other RNAs are in kb.

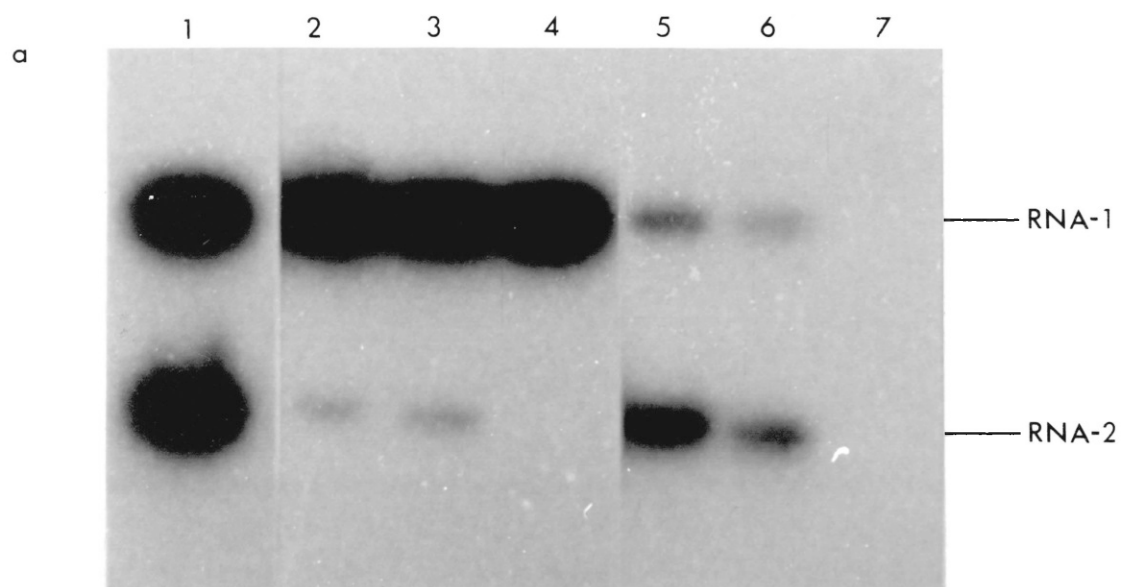
3.6 PURIFICATION OF TBSV GENOMIC RNA

Initially it was thought that purification by two cycles of sucrose/formamide gradient centrifugation (Paucha et al., 1978; Osman et al., 1986) would be sufficient to purify the genomic 4.8 kb band. However, parallel experiments with red clover necrotic mosaic virus (RCNMV) showed this not to be the case. RCNMV is a bipartite virus with RNA segments of approximately 4.5 kb and 1.5 kb (Osman et al., 1986; Fig.3.7a, lane 1). Component-specific clones (a kind gift of Dr.A.C. Marriot) were used to detect the degree of contamination of RNA-1 with RNA-2 (a kind gift of Mr.T.A.M. Osman) after various methods of purification. After a single round of sucrose/formamide purification, significant amounts of RNA-2 contaminated RNA-1, although the yield was nearly 60% (determined by dot blotting a sample of the RNA, probing with ³²P-labelled nick-translated component specific clones, and then scintillation counting) of the amount of RNA loaded onto the gradient (lanes 2 and 5). Applying the RNA-1 fractions to a second sucrose/formamide gradient still did not free RNA-1 completely from contamination, and the yield of RNA decreased to 10% of the original RNA (lanes 3 and 6). Therefore, the RNA-1 fraction from the first sucrose/formamide gradient was electrophoresed through a 1% low melting temperature agarose gel containing 10 mM methylmercuric hydroxide. The band of RNA-1 was then eluted from the gel. A total yield of 30% was routinely obtained, and RNA-1 was free from RNA-2 as judged using Southern blot hybridisation analysis (lanes 4 and 7).

The procedure was then repeated using TBSV encapsidated RNA (Fig.3.7b). Comparison of the encapsidated RNA (lane 1) with RNA after purification (lane 2) shows that most of the less than genome sized RNA has been removed. Genomic RNA prepared by this method was regarded as purified genomic RNA.

FIG.3.7 (a). Northern blot analysis of RNA purified by various methods. The two RCNMV species (lane 1) were separated by: lanes 2 & 5, a single cycle of centrifugation through a sucrose/formamide gradient; lanes 3 & 6, two cycles of centrifugation through sucrose/formamide gradients; lanes 4 & 7, a single cycle of centrifugation through a sucrose/formamide gradient, followed by purification by electrophoresis through a 1% low melting agarose gel containing 10 mM methylmercuric hydroxide. Lane 1 was hybridised to ^{32}P -labelled RCNMV cDNA, lanes 2, 3 & 4 were hybridised to a ^{32}P -labelled nick translated clone containing a insert specific to RNA-1 (pBM3; Osman & Buck, 1987), and lanes 5, 6 & 7 were hybridised to a ^{32}P -labelled nick translated clone containing a insert specific to RNA-2 (pGC9; Osman & Buck, 1987).

(b). Northern blot analysis of purified TBSV genomic RNA. Lane 2, RNA was prepared from TBSV virions purified by sedimentation in caesium chloride gradients. Lane 1, the TBSV genomic RNA was further purified by a single cycle of centrifugation through a sucrose/formamide gradient, followed by purification by electrophoresis through a 1% low melting agarose gel containing 10 mM methylmercuric hydroxide



3.7 PURIFICATION OF SINGLE-STRANDED RNA FROM HEALTHY OR TBSV-INFECTED PLANTS

Although many subgenomic RNAs generated during infection are encapsidated into virions, other subgenomic RNAs can only be detected by Northern blot analysis of RNA extracted from infected tissues. Plant tissues contain a large number of RNA species which are often at a higher concentration than the viral RNAs, and therefore direct visualisation of the smaller subgenomic RNAs on gels is not usually possible.

Two methods were used to prepare RNA from healthy or infected plants (Section 2.9). Method 1 is relatively simple and quick while Method 2, although being far more time consuming, yields more nucleic acid per gram of tissue - 10 g of N.clevelandii leaf tissue typically yielded 0.25 mg of single-stranded RNA with Method 1 and 3 mg with Method 2. Single-stranded RNA prepared by both methods subjected to electrophoresis on denaturing 1.2% agarose gels was found to be intact as judged by the ribosomal bands (Fig.3.8). RNase and DNase digestion showed that all the bands were single-stranded RNA (data not shown). Due to the superior yield, Method 2 was routinely used to purify RNA from plant material.

Fig.3.9, lane 1 contains RNA from TBSV infected plants prepared by Method 2. Comparison with RNA from healthy plants (lane 2) clearly demonstrates the presence of the 4.8 kb genomic RNA (lane 3). Any lower molecular weight specific for TBSV infection, such as coat protein subgenomic RNA, probably cannot be detected due to low abundance and/or co-migration with the host ribosomal RNAs. This contrasts with the TMV coat protein subgenomic RNA which is clearly visible among ssRNA from infected plants (Hunter et al., 1976).

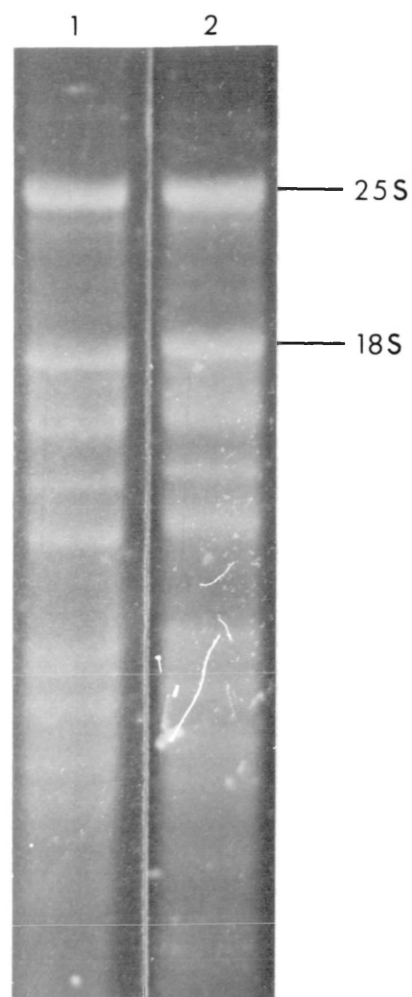


FIG.3.8. Total ssRNA from a healthy N.clelandii plant prepared by (lane 1) Method 1 and (lane 2) Method 2 after electrophoresis through a 1.2% agarose gel containing 5 mM methylmercuric hydroxide and staining by ethidium bromide. The RNA species below the 18S rRNA are chloroplast and mitochondrial rRNAs.

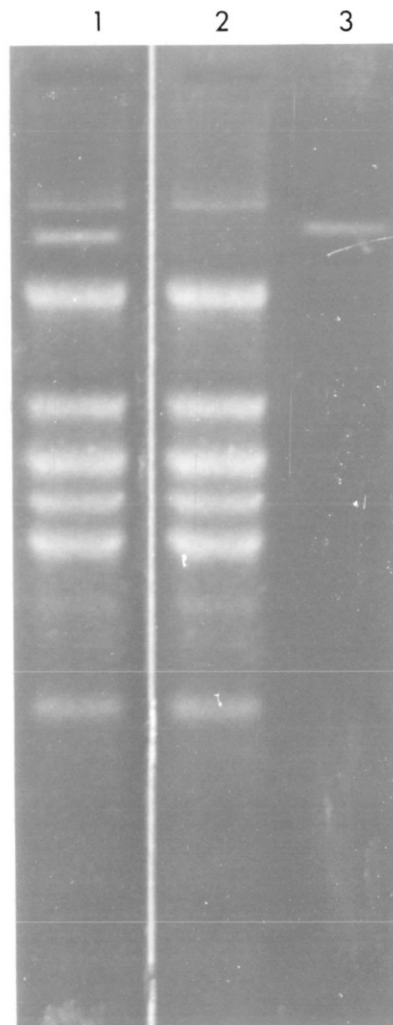


FIG.3.9. Electrophoresis of total ssRNA prepared from (lane 1) TBSV infected N.clevelandii plant, (lane 2) healthy N.clevelandii plant and (lane 3) caesium chloride purified TBSV particles through a 1.2% agarose gel containing 5 mM methylmercuric hydroxide, followed by staining with ethidium bromide.

3.8 PURIFICATION OF mRNA FROM POLYSOMES

The question of which subgenomic RNAs are really mRNAs and not breakdown products is best answered by analysing which RNAs are found on polyribosomes, on the presumption that they are mRNAs. The polyribosome fraction should be enriched in subgenomic RNAs compared to the total RNA present in the cells.

Polysomes were extracted from infected and healthy fully expanded N.clevelandii leaves (Hari, 1980) and sedimented in a 12.5-20% w/v sucrose gradient. Polysomes containing up to 10 ribosomes were isolated from healthy plants (Fig.3.10). Because sedimenting virions obscure the UV profile, it was not possible to make a similar qualification of polysomes from virus infected tissue. Therefore, the fractions to be retained from these gradients were determined by studying the UV profile of parallel sucrose gradients of healthy material.

The method for preparing polysomes (Hari, 1980) contrasts with earlier methods because both fully expanded and younger leaves from infected plants can be used as a source (Jackson & Larkins, 1976). Older leaves contain high levels of RNase which are inhibited by the high salt concentrations, metal ion chelators, inhibitors and the alkaline pH used in the extraction buffer. The larger peaks towards the top of the gradient (Fig.3.10) represent the ribosomal subunits and monomers, while the strongly absorbing material at the top of the gradient is probably mostly single-stranded RNA and tRNA.

The mRNA was then released from the polysomes (Blobel, 1971; Palukaitis et al., 1983). Polysomes from healthy N.clevelandii (fractions represented by the bar in Fig.3.10) and from TBSV-infected N.clevelandii (fractions from a 12.5-20% w/v sucrose gradient run in parallel to the sucrose gradient shown in Fig.3.10 - see above) were

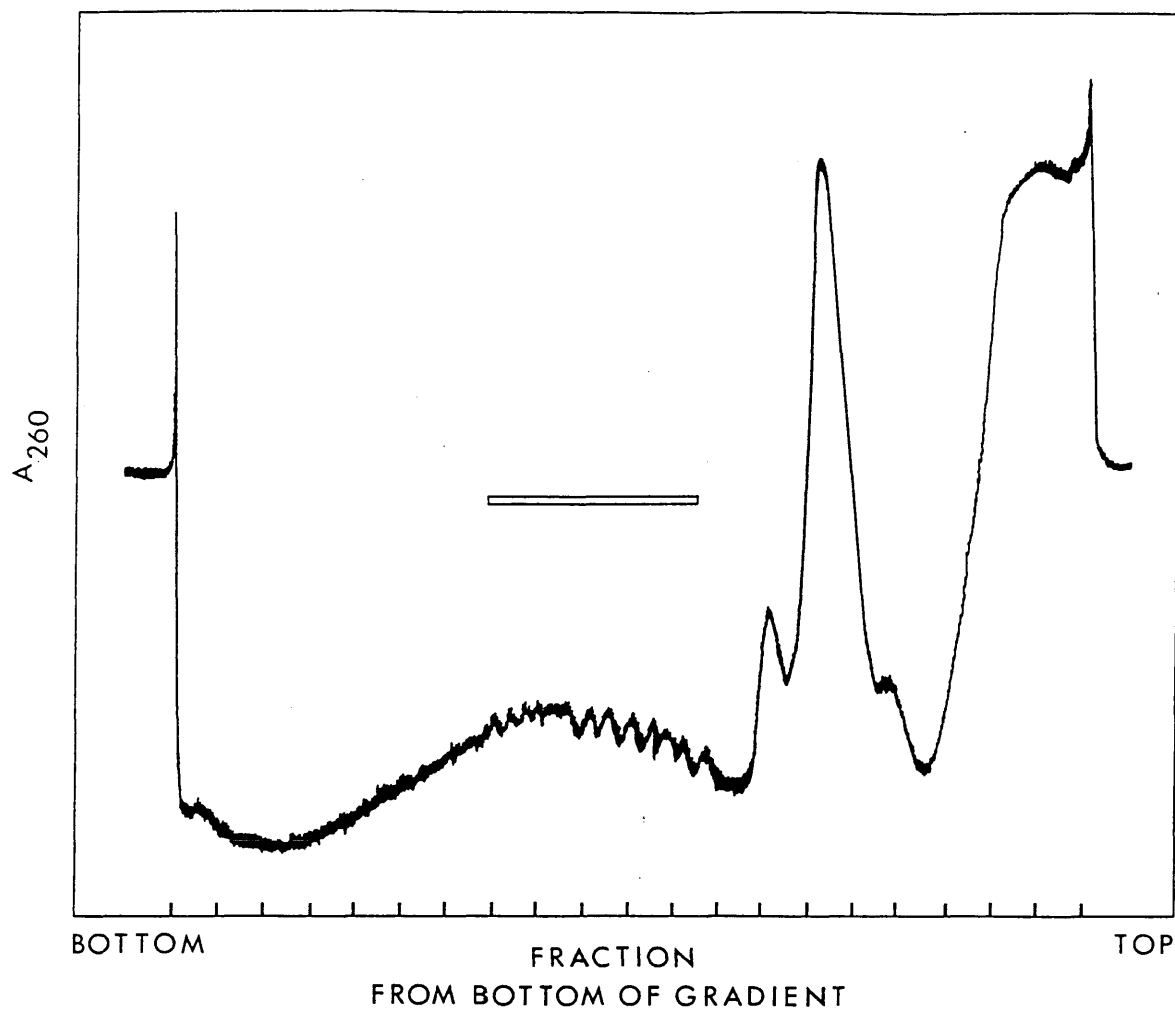


FIG.3.10. UV profile of polysomes from healthy N.clevelandii after sedimentation in 12.5 - 20% w/v sucrose gradient. The fractions pooled for later treatment with puromycin (Fig.3.11) are indicated by a bar.

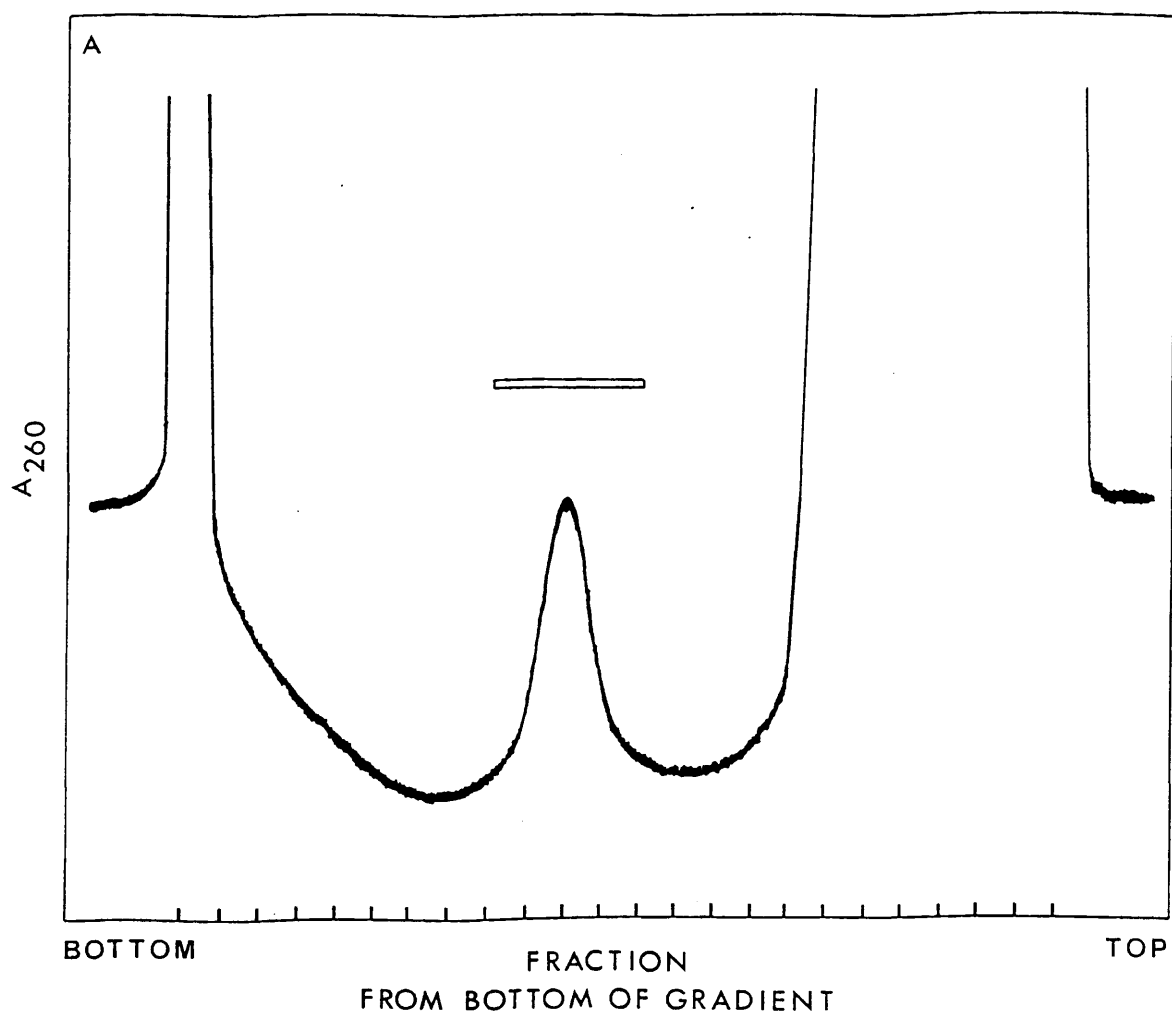
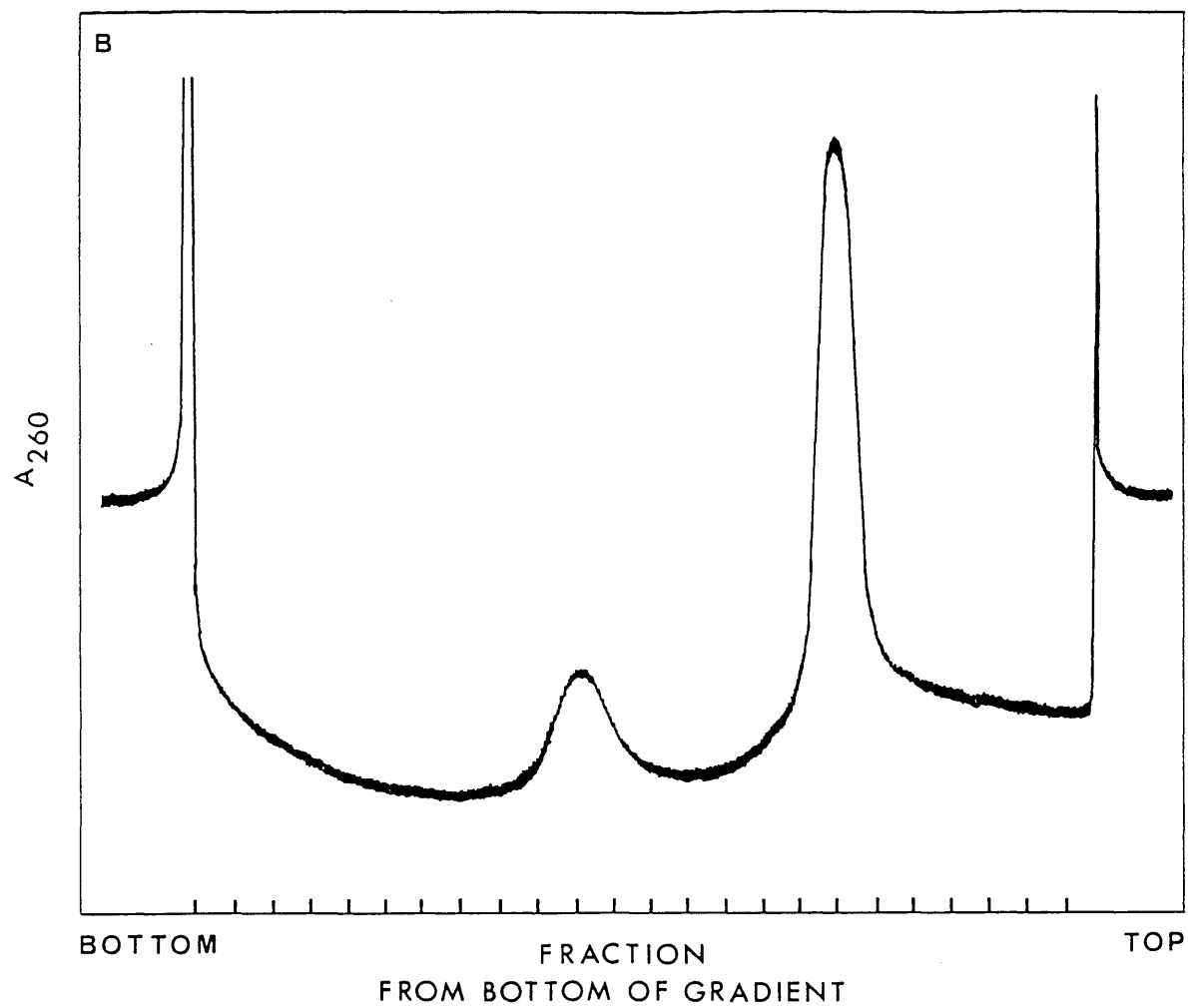


FIG.3.11. (a) UV profile of puromycin-treated polysomes and (b) non-puromycin treated polysomes after centrifugation through a 5 - 20% w/v sucrose gradients. The mRNA pooled for Fig.3.12, lane 1 is indicated by a bar.



treated with puromycin and then centrifuged through a 5-20% w/v sucrose gradient (Fig.3.11a). Selected polysomes from TBSV-infected N.clevelandii which had not been treated with puromycin were also centrifuged through a 5-20% w/v sucrose gradient (Fig.3.11b). The UV absorbing material at the top of the gradients shown in Fig.3.11 consists of puromycin and tRNA (Blobel, 1971). Comparison of the Fig.3.11a with Fig.3.11b shows that puromycin treatment results in a much increased middle peak (represented by the bar in Fig.3.11a). This represents both the mRNA from the polysomes and some degraded RNA. Under these centrifugation conditions the virus particles are pelleted.

The fractions represented by the bar in Fig.3.11a were electrophoresed through a 10 mM methylmercuric hydroxide agarose gel (Fig.3.12, lane 1). Comparison with non-puromycin treated polysomal RNA (lane 2) from TBSV-infected N.clevelandii (from the fractions selected after centrifugation through a 12.5-20% w/v sucrose gradient) shows that, while both lanes contain the distinctive 4.8 kb band, the RNA released from puromycin-treated ribosomes contains little, if any, ribosomal RNA, and can be considered as mostly mRNA.

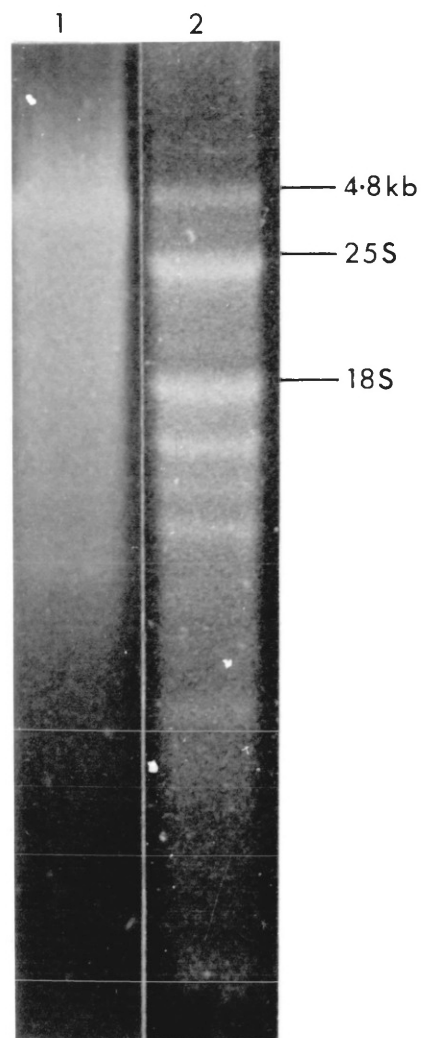


FIG.3.12. Purification of mRNA from polysomes. RNA was electrophoresed through a 1% agarose gel containing 5 mM methylmercuric hydroxide, followed by staining with ethidium bromide. Lane 1, mRNA (2 μ g) from puromycin-treated polysomes from TBSV-infected N.clevelandii plant (Fig.3.11); lane 2, total polysomal RNA (5 μ g) from TBSV-infected N.clevelandii plant.

3.9 ISOLATION AND CHARACTERISATION OF DOUBLE-STRANDED RNA FROM INFECTED PLANTS

As the amount of dsRNA present in infected tissue is small compared to the amount of ssRNA (Morris & Dodds, 1979), the second method of RNA extraction from plants was used to extract the nucleic acid from healthy or infected tissue (Murray & Thompson, 1980; Taylor & Powell, 1982). Initially, the dsRNA was separated from the ssRNA by 2 M lithium chloride (Baltimore, 1966). However, when the dsRNA prepared by this method was analysed by electrophoresis in 4% polyacrylamide gel (Fig.3.13; Buck & Ratti, 1977), the lithium chloride prepared dsRNA appeared to be contaminated with low molecular weight nucleic acid (Fig.3.13, lane 1). Repeated treatment with lithium chloride was required to remove most of this contaminating nucleic acid. Therefore, in later experiments the separation of dsRNA from ssRNA using 2 M lithium chloride was abandoned in favour of Whatman CF11 columns (Fig.3.13, lane 2; Morris & Dodds, 1979). Apart from a slow migrating band of dsDNA, all the bands in Fig.3.13, lane 2 were shown to be dsRNA by S1, RNase and DNase digestions (Fig.3.14). The typical yield of 10 µg dsRNA per gram of infected tissue was routinely achieved, which is approximately a ten-fold improvement in the yield obtained by Morris & Dodds (1979). The improvement in yield, using a combination of the method of RNA extraction developed by Taylor & Powell (1982) and Whatman CF11 columns, is probably due to concentrating and purifying the RNA before the application to the Whatman CF11 column.

Analysis of the dsRNA from infected plants by polyacrylamide gel electrophoresis revealed seven components which ranged in size from 4.8 kbp to 0.44 kbp (Fig.3.15). Unlike Phaseolus vulgaris (Wakerchuk & Hamilton, 1985), no dsRNA forms were found in uninfected tissue or mock infected N.benthamiana plants grown under

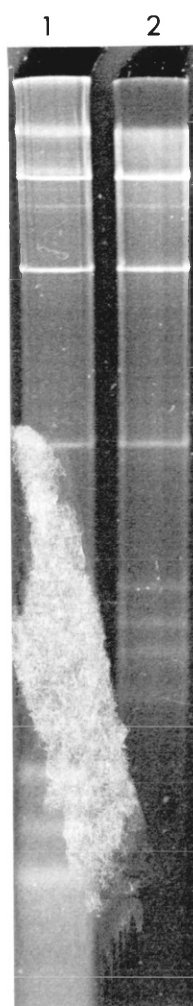


FIG.3.13. Double-stranded RNA from TBSV infected N.clevelandii plants purified using (lane 1) 2 M lithium chloride and (lane 2) Whatman CF11 after electrophoresis through a 4% polyacrylamide gel and staining with ethidium bromide.

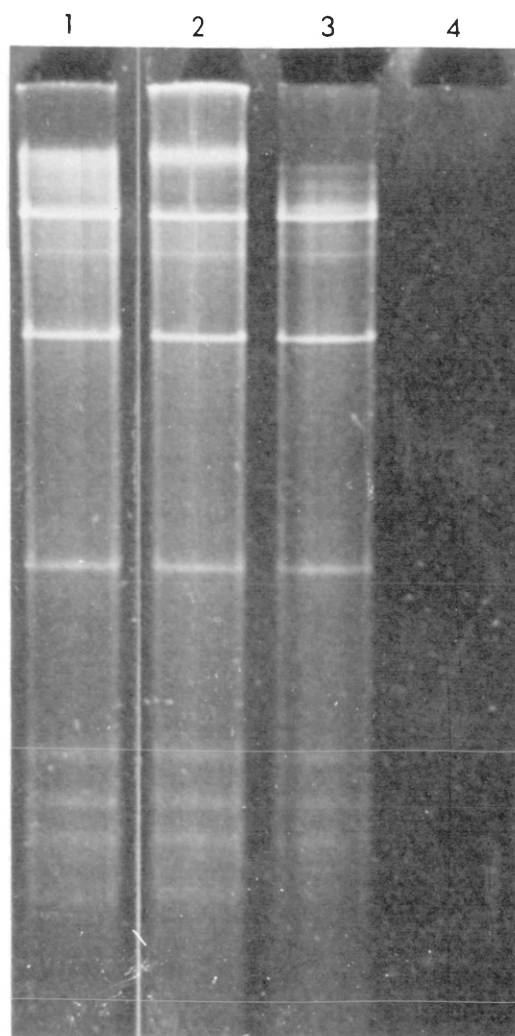


FIG.3.14. Nuclease treatment of dsRNA from TBSV-infected N.clevelandii. Double-stranded RNA (lane 1) was treated with (lane 2) S1 nuclease, (lane 3) DNase 1 or (lane 4) RNase A plus DNase 1, and then electrophoresed through a 4% polyacrylamide gel and stained with ethidium bromide.

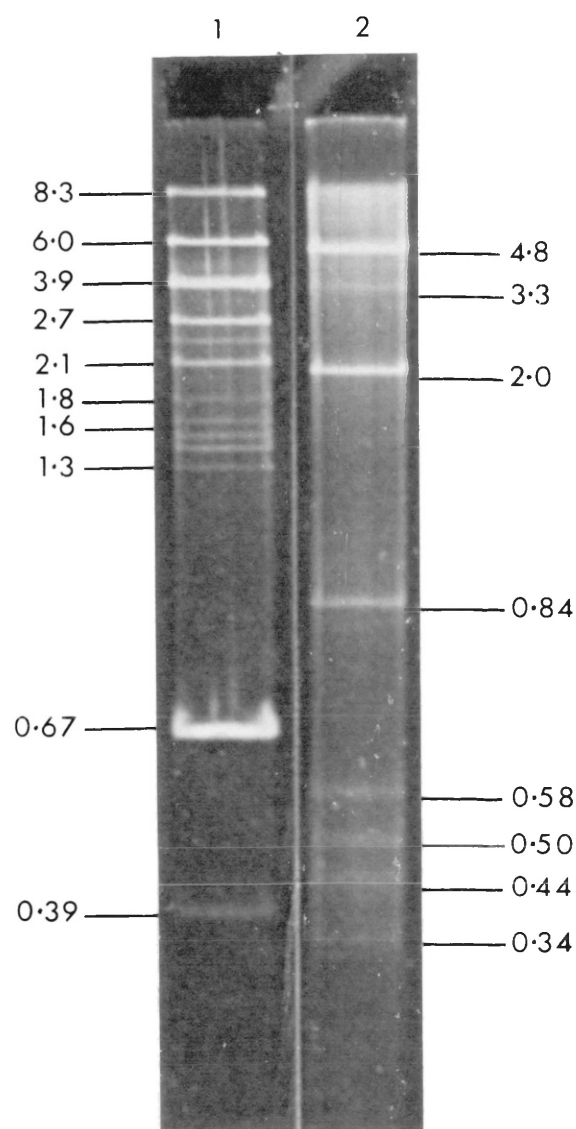


FIG.3.15. Double-stranded RNA from (lane 1) viruses of Helminthosporium maydis, Aspergillus foetidus and Penicillium stoloniferum as standards (Bozarth & Harley, 1976; Ratti & Buck, 1972, 1975; Buck & Ratti, 1977) and (lane 2) TBSV infected N.clevelandii. Lengths of dsRNAs are in kbp.

the same conditions, and the same size dsRNA species were found in both TBSV-infected N.benthamiana and N.clevelandii plants (data not shown). Therefore, all the dsRNA forms in Fig.3.15 are probably virus specific. The 4.8 kbp and the 2.0 kbp components correspond to the dsRNA species described by Henriques & Morris (1979), but the remaining five dsRNA species of 3.3 kbp, 0.84 kbp, 0.58 kbp, 0.50 kbp and 0.44 kbp have not been described before. Some, but not all, preparations also contained two slower migrating components (present in Fig.3.14, lane 3) which, because of their sensitivity to S1 nuclease, probably correspond to the replicative intermediates described by Henriques & Morris (1979).

Hillman et al. (1985) studied dsRNA present in tissue infected with TBSV-Cherry strain. Their results also show the presence of the three higher molecular weight dsRNAs (with estimated molecular weights 4.8 kbp, 2.0 kbp and 1.0 kbp instead of the 4.8 kbp, 2.0 kbp and 0.84 kbp), together with a number of other lower molecular weight dsRNA species. Different isolates were shown to have differing numbers of lower molecular weight dsRNAs; some of the isolates had three 0.3 kbp - 0.6 kbp bands similar in size to those shown in Fig.3.15, while other isolates only had a 0.4 kbp band

Hillman et al. (1985) noticed that the presence of the 0.4 kb ssRNA species in the TBSV-Cherry strain virus particles reduced the severity of symptoms, allowing the plants to survive for at least 4 weeks instead of a maximum of 2 weeks. However, plants which contained more than one dsRNA band survived for at least two months longer. Although a 0.4 kb ssRNA was shown to be encapsidated, no comparison of the encapsidated RNAs was made between isolates of the virus showing heterogeneity in the 0.3 kbp - 0.6 kbp dsRNA forms and those which had the single 0.4 kbp dsRNA band. Therefore it is not known

if all the lower molecular weight ssRNA forms are encapsidated (Hillman et al., 1985), or indeed even if the encapsidated 0.4 kb RNA is the single-stranded form of the 0.4 kbp dsRNA. The presence of the the 0.44 - 0.58 kbp dsRNA forms in Fig.3.15 suggest that ssRNA forms should be present in infected tissue and that at least one of the single-stranded forms should be encapsidated in the virus particles. However, they cannot be seen among the virion ssRNA in Fig.3.5 or detected on Northern blots of encapsidated RNA (Fig.3.7b), even when large amounts are loaded onto the gels, suggesting that they may be encapsidated inefficiently. The severity of the viral infection, with plants surviving for no more than 2 weeks, suggests that these lower molecular weight forms afforded little, if any, protection to the plant. The situation may be analogous to virus satellites (Murant & Mayo, 1982; Francki, 1985), different strains of which can affect the severity of the symptoms in different ways. For example, some strains of the CMV satellite RNAs such as the S-CARNA5 reduce the severity of the viral infection, while other strains such as the D-CARNA5 elicit lethal necrosis in tomato upon co-infection with the helper virus (Kaper et al., 1988).

Interestingly, Hillman et al. (1985) noticed that raising the temperature at which the plants grew from normal greenhouse levels (approximately 16°C) to 27°C resulted in a change in the dsRNA profile, and enabled the plants to survive for much longer periods. The 0.4 kbp dsRNA band were no longer detected, the level of the 1.0 kbp band (probably equivalent to the 0.87 kbp dsRNA band in Fig.3.15) band was reduced and a new band at 3.3 kbp appeared. In the dsRNA lane shown in Fig.3.15 the 3.3 kbp band is present, together with the 0.87 kbp band. This may be due to the conditions under which the plants in this study were grown. The temperature in the greenhouse was

not regulated and so, during the summer months, the temperature often varied between 20°C and 30°C.

The effect of temperature on viral symptoms occurred irrespective of whether the lower molecular weight dsRNAs were present or not (Hillman et al., 1985). Symptom modulation might be caused by temperature affecting the transcription of a subgenomic RNA. The replicase of BMV has been shown to transcribe the subgenomic RNA-4 from the negative strand of RNA-3 (Miller et al., 1985) and in a similar way the TBSV replicase could transcribe TBSV subgenomic RNA(s). Elevated temperatures might affect the ability of the replicase to bind to the correct site on the minus-strand of the genomic RNA. If the polymerase no longer bound to the 1.0 kb subgenomic RNA promotor but bound to a site closer to the 3'-end of the template, a longer subgenomic RNA would be produced. This in turn would result in a longer dsRNA.

3.10 NORTHERN BLOT ANALYSIS OF ENCAPSIDATED RNA

To detect TBSV-specific subgenomic RNA in virus particles, RNA from RNase A treated, caesium chloride purified virus particles was subjected to Northern blot analysis (Fig.3.16) with three different cDNA probes (for preparation of cDNA probes, see Section 4.1):

Lane 1. Genomic probe. A cDNA was prepared from purified genomic RNA and used without further treatment.

Lane 2. Prehybridised probe. A cDNA probe prepared from RNA encapsidated in RNase A treated, caesium chloride purified virus particles was first prehybridised at 42°C with a 1000-fold excess of RNA isolated from healthy plants (Odel & Hari, 1983) in prehybridisation buffer. After 18 hours the prehybridised cDNA was added to the prehybridised Northern blot.

Lane 3. Non-prehybridised probe. A cDNA probe was prepared from RNase A treated, caesium chloride purified virus particles. The probe was then used without further treatment.

Only the genomic RNA species could be identified in the initial exposures of Fig.3.16, but longer exposures allowed the detection of a number of faster migrating bands (Fig.3.16, lanes 1, 2 and 3), including two bands corresponding to RNAs of 2.0 kb and 0.9 kb..

RNA species detected by probing a Northern blot with cDNA prepared from total encapsidated virion RNA (Fig.3.16, lane 3) may or may not be related to the TBSV genomic RNA; as they are present when the cDNA is prepared, their sequences are represented in the probe. In lanes 1 and 2 the number of bands present in the blots have been reduced leaving only the genomic, 2.0 kb and 0.9 kb bands. Any bands that are present in lane 3 but not in lane 2 represent encapsidated host RNAs; any cDNA prepared to these sequences would have hybridised to the healthy plant RNA during the prehybridisation step. In lane 1, any bands present must be directly related to the genomic RNA, either as subgenomic RNA molecules, or as encapsidated degraded genomic RNA. By comparing lanes 1 and 2 with lane 3, it can be concluded that the 2.0 kb and 0.9 kb bands are directly related to the TBSV genomic RNA, and that the other RNA species present in the virions are encapsidated host RNA.

In vitro assembly of tobacco mosaic virus nucleoprotein rods is initiated by reaction between a capsid protein oligomer and a specific internal region on viral RNA called the encapsidation initiation site (ei) (Butler & Klug, 1971; Correia et al., 1985). In the case of the cowpea and cucumber strains both the I2-RNA and the coat protein subgenomic RNA (Okada et al., 1980; Higgins et al., 1976) are also encapsidated because they contain the

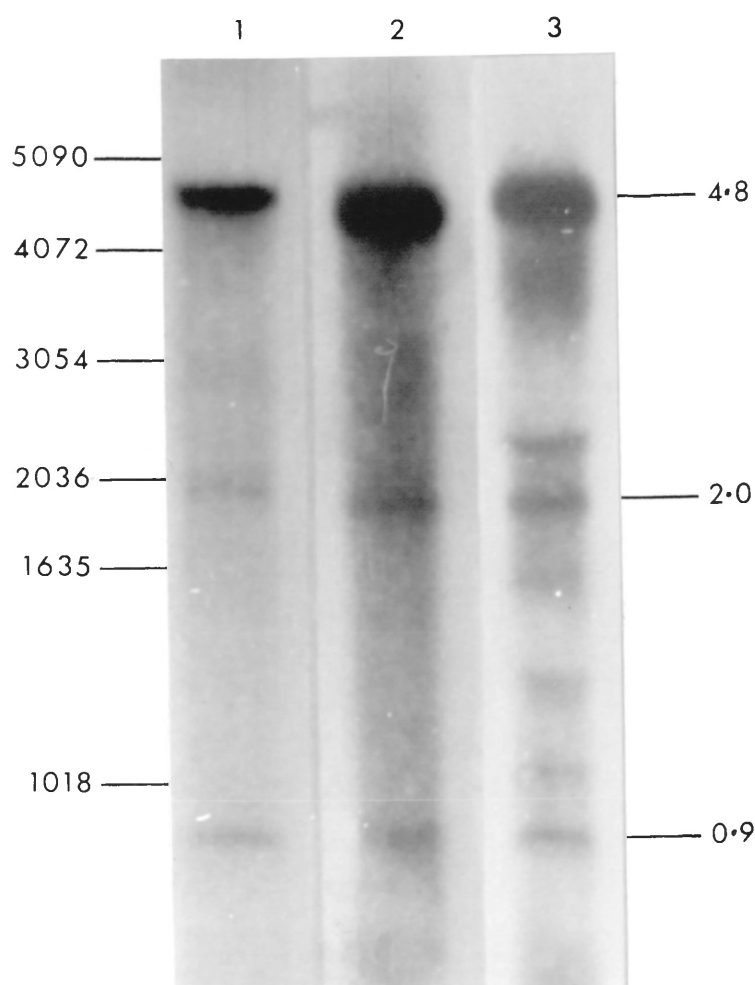


FIG.3.16. Northern blot analysis of TBSV encapsidated RNA (50 ng) electrophoresed through a 1.2% agarose gel containing 5 mM methylmercuric hydroxide. RNA was transferred to GeneScreen and hybridised to (lane 1) ^{32}P -labelled cDNA prepared to purified genomic RNA, (lane 2) ^{32}P -labelled cDNA prepared to total encapsidated RNA and prehybridised to an excess of total RNA from healthy *N.clevelandii*, and (lane 3) ^{32}P -labelled cDNA prepared to total encapsidated RNA. The positions of BRL kb markers are shown on the left. Lengths of other RNAs are in kb.

ei site. Similarly, both the TBSV 2.0 kb and 0.9 kb RNAs are encapsidated, probably because, together with the genomic RNA, they also contain some sequence and secondary structure that interacts with the coat protein and determines encapsidation. During caesium chloride purification TBSV-Type strain virus particles form a single band, suggesting that subgenomic RNA is encapsidated in multiple copies.

Some TMV preparations also contain a small proportion of host RNA packaged by TMV protein termed pseudovirions (Siegal, 1971; Rochon & Siegal, 1984). The host RNA has been shown to consist primarily of many discrete sized mRNA transcripts derived from different chloroplast DNA regions with little or no chloroplast or cytoplasmic ribosomal RNA being present (Rochon & Siegal, 1984). It has been proposed that the encapsidated host RNA contains a sequence similar to the ei site, and that it is encapsidation in a manner similar to the virus RNA (Rochon & Siegal, 1984). The identity of the TBSV encapsidated host RNA has not been determined, but the virus need not be assembled at intracellular location of the host RNA for the host RNA to become encapsidated; the capsid protein may be transported across intracellular membranes in a fashion similar to the TMV capsid protein (Rochon & Siegal, 1984).

3.11 NORTHERN BLOT ANALYSIS OF RNA FROM INFECTED PLANTS

Fig.3.17 shows a Northern blot of total, polysomal (non-puromycin treated) and healthy ssRNA probed with prehybridised cDNA used in Fig.3.16, lane 2. Both total ssRNA (lane 4) and polysomal RNA (lane 5) contain RNA with the mobility of TBSV genomic RNA (lane 1) and several smaller species which are not detected in RNA from healthy plants (lane 3). To establish whether these bands were due

to specific cleavage of the TBSV genomic RNA during the extraction procedure, purified genomic TBSV RNA was added to a homogenate of healthy N.clevelandii leaves prior to ssRNA extraction. The absence of detectable amounts of the of lower molecular weight RNA species (lane 2) indicates that degradation of TBSV genomic RNA into specific fragments during extraction is unlikely to account for the relatively large amounts of lower molecular weight species detected in lanes 4 and 5, but does not exclude degradation in vivo.

To determine whether some of the ssRNAs detected on the Northern blot in Fig.3.17 (lanes 4 & 5) correspond in size to TBSV dsRNAs (Fig.3.15), total polysomal RNA and dsRNA were compared by electrophoresis in adjacent tracks of an agarose gel containing 10 mM methylmercuric hydroxide, a concentration shown to be sufficient to denature dsRNA (Jordan & Dodds, 1983). RNA was then transferred to GeneScreen and hybridized to TBSV cDNA prehybridised to healthy plant RNA. Three clear dsRNA bands of 4.8, 2.0, and 0.8 kb, and two fainter bands of 1.0 and 0.4 kb were detected (Fig.3.18, lanes 5 & 9). The two largest bands (4.8 & 2.0 kb) correspond in size to the 4.8 and 2.0 kbp dsRNA species detected by ethidium bromide staining in non-denaturing polyacrylamide gels (Fig.3.15). The 0.4 kb band may represent one or more of the lower molecular dsRNA bands, or may represent a dsRNA band too faint to be detected by ethidium bromide staining of polyacrylamide gels; because the resolving power in this size range is better in polyacrylamide gels than in agarose gels, it is possible that the smallest dsRNA bands are all present in this band. The 0.8 kb band probably represents the 0.84 kbp dsRNA band. Because the dsRNA in Fig.3.18 was prepared using 2 M lithium chloride, the fainter 1.0 kb band could be an artifact caused by some ssRNA remaining in the preparation.

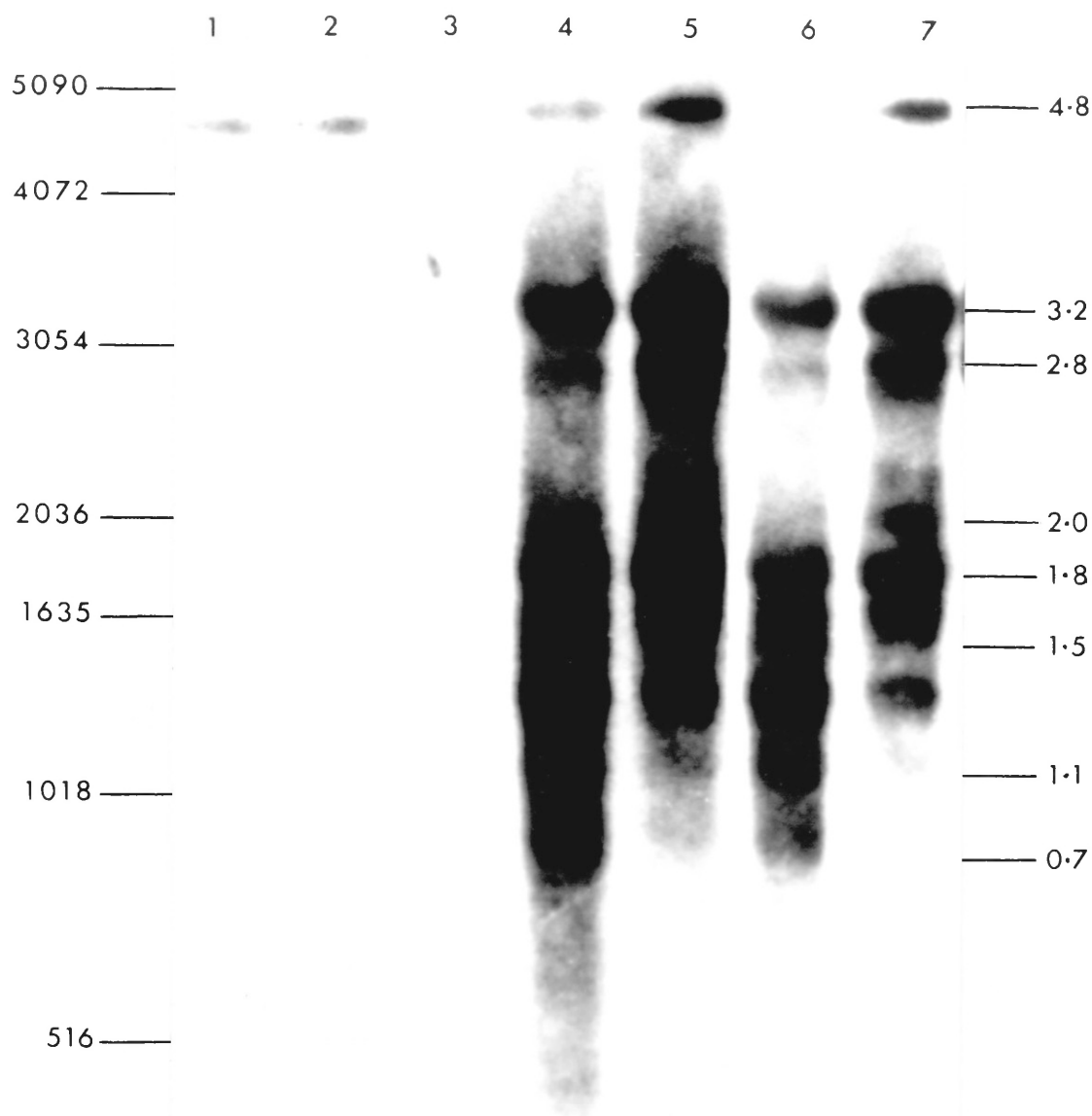


FIG.3.17. Northern blot analysis of ssRNA from TBSV infected plants. RNA was electrophoresed through a 1% agarose gel containing 5 mM methylmercuric hydroxide, transferred to GeneScreen membrane, hybridised to ^{32}P -labelled TBSV cDNA and autoradiographed for 24 hours (lanes 1 to 5) or 12 hours (lanes 6 & 7). Lane 1, TBSV genomic RNA (50 ng); lane 2 RNA extracted from a homogenate of healthy plant leaves to which TBSV genomic RNA had been added prior to extraction (equivalent to 2.5 ug healthy plant RNA plus 30 ng TBSV RNA); lane 3, healthy plant RNA (1 μg); lanes 4 & 6, virus infected plant RNA (1 μg); lanes 5 & 7, polysomal RNA (1 μg) from virus infected plants. The positions of BRL kb markers are shown on the left. Lengths of other RNAs are in kb.

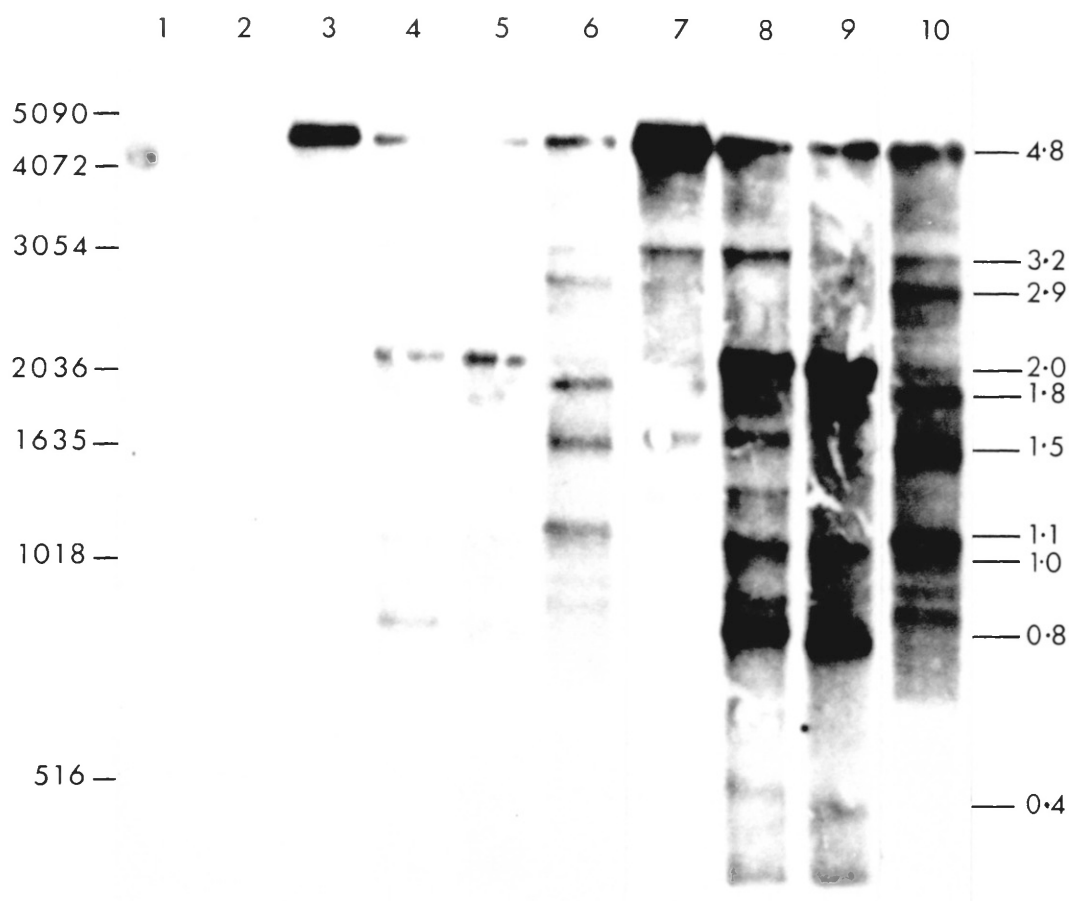


FIG.3.18. Northern blot analysis of ssRNAs and dsRNAs from TBSV infected plants. Procedure as in Fig.3.17 except that the agarose gel contained 10 mM methylmercuric hydroxide. Lane 1, TBSV genomic RNA (50 ng); lane 2, healthy plant RNA (5 μ g); lanes 3 & 7, healthy plant RNA (5 μ g) plus TBSV genomic RNA (50 ng); lanes 4 & 8, healthy plant RNA (5 μ g) plus dsRNA (50 ng) from virus infected plants; lanes 5 & 9, dsRNA (50 ng) from virus infected plants; lanes 6 & 10, polysomal RNA (1 μ g) from virus infected plants. Autoradiography was for 8 hours (lanes 1 to 6), 24 hours (lanes 7 to 9) or 14 hours (lane 10). TBSV genomic and subgenomic ssRNA are indicated by single headed arrows and subgenomic (denatured) dsRNA by double headed arrows. The positions of BRL kb markers are shown on the left. Lengths of other RNAs are in kb.

The 3.3 kbp dsRNA, detected as a minor component on non-denaturing polyacrylamide gel, could not be detected on the denaturing agarose gel, possibly because this molecule has one or more single-strand breaks in its plus-strand which are hidden in the native dsRNA. Whether or not the minus-strands of this or any of the dsRNAs were intact could not be established from this experiment because the cDNA probe detected only plus-strands of denatured dsRNA. However, the resistance of all the native dsRNAs to S1 nuclease implies that they are fully base paired structures without single-stranded tails. Another explanation is that the 3.3 kbp dsRNA is not related in sequence to the genomic RNA. It could be a the dsRNA form of a host RNA replicated by the viral polymerase in a manner similar to satellite RNAs, the dsRNA of a large satellite RNA or perhaps some artefact created from the host nucleic acid during RNA extraction.

It is noteworthy that, of the polysomal RNAs (lanes 6 & 10), only the genomic TBSV genomic RNA clearly correspond in size to a band of denatured dsRNA; the only bands which correspond directly to the denatured subgenomic dsRNAs are rather faint and obscured by other strongly hybridising RNA species.

The Northern blot in Fig.3.18 shows that several bands smaller than TBSV genomic RNA were formed when TBSV genomic RNA was added to healthy plant RNA prior to electrophoresis (lanes 3 & 7); none of these was seen in healthy plant RNA alone (lane 2) or TBSV RNA alone (lane 1). Bands of this mobility detected in polysomal RNA (lanes 6 & 10) are therefore probably artefacts. The artefactual bands are probably caused by interaction of fragments of TBSV RNA with plant RNA species. Isolated TBSV genomic RNA would be expected to contain only a very small proportion of fragmented RNA, which would account for the low intensities of the artefactual bands relative

to the genomic band in the reconstitution experiment (Fig.3.18, lane 3) and the failure to detect them in a similar experiment when the intensity of the TBSV genomic RNA band was much lower (Fig.3.17, lane 2). In contrast, total ssRNA and polysomal RNA from virus infected plants gave rise to artefactual bands of relative high intensity compared to the genomic RNA (Fig.3.17, lanes 4 & 5; Fig.3.18, lanes 6 & 10) implying that a greater degree of RNA fragmentation. Since it has been shown that significant fragmentation of TBSV genomic RNA does not occur during extraction, it is likely that this fragmentation had occurred in vivo.

Evidence for this hypothesis was shown by creating artefactual bands in vitro (Fig.3.19). All three lanes contain 50 ng TBSV RNA. Lanes 2 and 3 also contain 5 µg of healthy plant RNA. However, the TBSV RNA in lane 3 was extracted from virions that had been prepared by virus purification Method 1, and was therefore partially degraded. As can be seen, fragmentation results in the formation of several artefactual bands. Interestingly, these artefactual bands had the same sizes as the strongest hybridising bands in Fig.3.18, lane 10. No distinct bands were seen in Northern blots of the degraded RNA.

Palukaitis et al. (1983) and Dougherty (1983) have also described the generation of artefactual bands following electrophoresis of mixtures of plant RNA with RNA from tobacco mosaic or tobacco etch viruses respectively, and in both papers similar explanations for the phenomena were advanced. Formamide/formaldehyde gels were used by both investigators, ruling out the possibility that these artefacts are a result of using methylmercury as a denaturant.

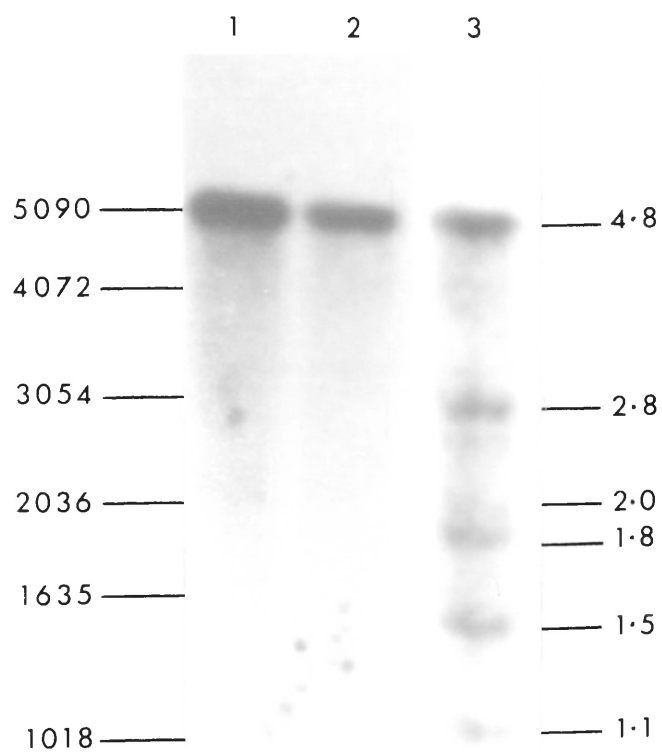


FIG.3.19. Interaction of TBSV RNA with host RNA. Procedure as in Fig.3.18. Lane 1, TBSV RNA (50 ng); lane 2, TBSV RNA (50 ng) plus healthy plant RNA (5 μ g); lane 3, partially degraded TBSV RNA (50 ng) plus healthy plant RNA (50 ng). Autoradiography was for 12 hours (lane 3) or 24 hours (lanes 1 & 2). The positions of BRL kb markers are shown on the left. Lengths of other RNAs are in kb.

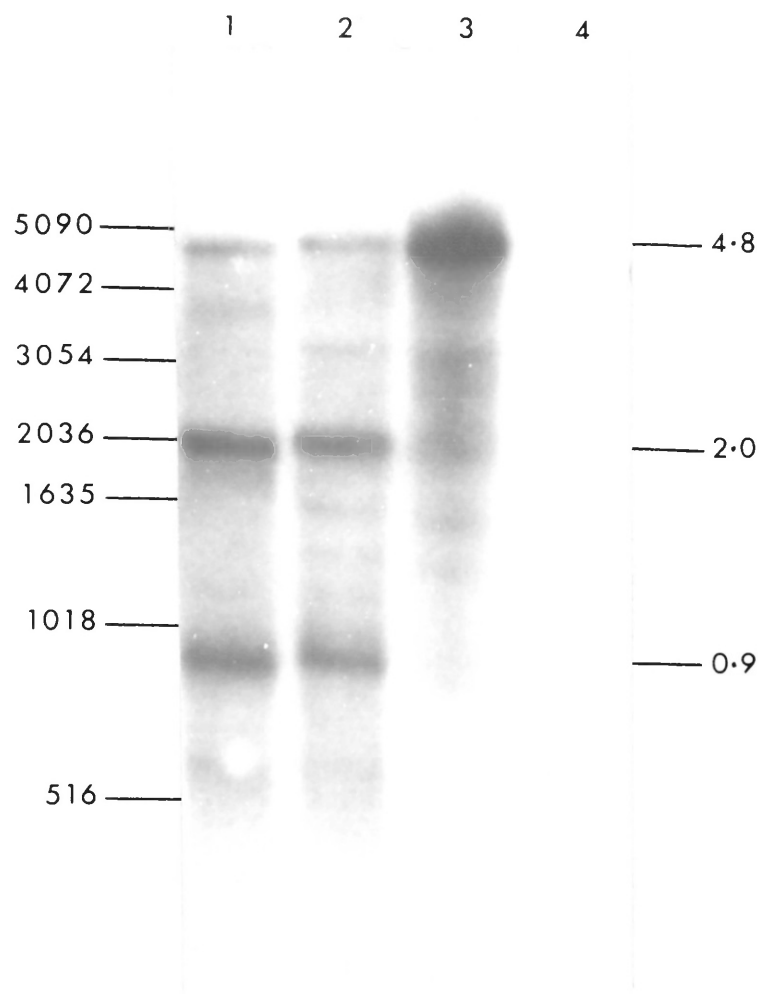


FIG.3.20. Northern blot analysis of RNA from TBSV-infected plants. RNA was electrophoresed through a 1.2% agarose gel containing 10 mM methylmercuric hydroxide, transferred to GeneScreen membrane, probed with ^{32}P -cDNA prepared to TBSV genomic RNA and autoradiographed. Lane 1, dsRNA from infected plants (50 ng); lane 2, puromycin-released mRNA from polysomes isolated from infected plants (100 ng); lane 3, genomic TBSV RNA (50 ng); lane 4, polysomal RNA from healthy plants (100 ng). The positions of BRL kb markers are shown on the left. Lengths of other RNAs are in kb.

When Palukaitis et al. (1983) treated polysomes from TMV-infected tissue with puromycin and compared the released TMV ssRNAs with the dsRNA forms on a denaturing gel, they found that the four polysome-associated ssRNAs co-migrated with the four dsRNAs. This procedure was repeated with polysomes from TBSV-infected material. Lane 1 of Fig.3.20 contains dsRNA and lane 2 contains puromycin treated polysomal. Although not all the artefactual bands have been removed, lane 2 contains far less bands than non-puromycin treated polysomal RNA. The dsRNA forms now co-migrate with strongly hybridising ssRNA forms.

Initially it was thought that the only possible subgenomic RNAs in Fig.3.16 were the 1.8 kb, 1.5 kb and 1.1 kb forms (lane 10), because the RNA species which did co-migrate with the denatured dsRNA forms were very faint, and in some cases (such as the 2.0 kb RNA) were also found in the healthy RNA plus genomic RNA lane (lane 7). However, the presence of an artefact with this size does not preclude the existence of a genuine subgenomic RNA with the same size. The demonstration that artefacts could also be created with sizes similar to the presumed subgenomic RNAs (Fig.3.19), and that puromycin treatment not only removes one of the presumed subgenomics, but also results in co-migration of ds and ssRNA forms (Fig.3.20), casts doubt on the identity of the 1.8 kb, 1.5 kb, and 1.1 kb RNAs as genuine subgenomic RNAs. From these set of results it can be concluded that the 2.0 kb and 0.9 kb ssRNAs are possibly genuine subgenomics, and that the genuine subgenomics in the total (non-puromycin treated) polysomal ssRNA are partially obscured by artefactual bands.

3.12 SATELLITE AND DEFECTIVE INTERFERING RNAs

During the course of this work there were two reports of satellite RNAs associated with TBSV-Cherry strain (Hillman et al., 1985), TBSV-Type strain and TBSV-BS3 strain (Gallitelli & Hull, 1985b). Both reports showed that the RNAs attenuated the symptoms of TBSV, and that they were dependent upon TBSV for their replication, both characteristics of satellite RNAs (Murant & Mayo, 1982; Franki, 1985). However, while the 0.7 kb RNA detected by Gallitelli & Hull (1985b) did not show any homology to the genomic RNA, the 0.4 kb species described by Hillman et al. (1985) did. This was confirmed later (Hillman et al., 1987) when it was shown that the 0.4 kb species was a defective interfering RNA (DI RNA).

One of the features of the DI RNA described by Hillman et al. (1985) was heterogeneity within the population, resulting in a number of different dsRNA forms in infected tissue. These dsRNA forms are approximately the same size as those shown in Fig.3.15, and one or more of the lower molecular weight dsRNAs might also hybridise to the genomic RNA (Fig.3.17 & 3.18), suggesting that at least one may be a DI RNA. However, when encapsidated RNA was analysed on denaturing gels, Hillman et al. (1985) showed that the ssRNA form of the DI RNA was clearly visible. No RNA of this size can be seen in Fig.3.5 and Fig.3.6. This prevented purification of the DI RNA in order to assay for ability of DI RNA to attenuate TBSV symptoms (Hillman et al., 1987).

The satellite reported by Gallitelli & Hull (1985b) showed no homology to the genomic RNA. The satellite RNA was present in low amounts in virions purified from N.clevelandii. However, when the virus was passaged through through N.benthamiana large amounts of the satellite were detected. Examination of Fig.3.5 and Fig.3.6 also shows a very faint band of 0.7 kb. However,

this band appeared to vary in intensity in different virus preparations, and in some preparations could not be detected at all, suggesting that it may simply represent degraded genomic RNA. Attempts to repeat the experiments of Gallitelli and Hull (1985b) by passaging TBSV through N.benthamiana ten times, failed. No increase in the amount of the 0.7 kb RNA species was detected. As the smaller dsRNAs (putative DI RNAs) might interfere with the acquisition and/or replication of satellite RNA, the experiment was repeated using purified genomic RNA as an original inoculum. No satellite RNAs or smaller dsRNAs were detected after 10 passages of the resultant virus.

When the total ssRNA from plants free from smaller dsRNAs was used to inoculate 10 N.benthamiana and 10 N.clevelandii plants, the plants all died within 14 days. No satellite RNA or smaller dsRNAs were detected in these plants. When plants were inoculated with an equal amount of total ssRNA from plants containing the smaller dsRNAs, the plants were also killed within 14 days, suggesting that the smaller dsRNAs do not attenuate TBSV symptoms. The smaller dsRNAs were present in these plants.

CHAPTER 4

RESULTS: COMPLEMENTARY DNA CLONING OF TBSV RNA

4.1 PREPARATION OF cDNA

Subgenomic RNA and putative host RNA species have been detected encapsidated in TBSV and in total RNA from infected plants by first fractionating the plant nucleic acid by gel electrophoresis, transferring the RNA from the gel to a binding medium, and then incubating with radiolabelled probes prepared from the genomic RNA (see Chapter 3). These DNA probes, complementary (cDNA) to the subgenomic RNA and the genomic RNA, were prepared using reverse transcriptase.

Like all DNA polymerases, reverse transcriptase requires a primer (Verma, 1981). In most cases, such as with mRNA, the primer used is oligo(dT) which binds to the 3' poly(A) tail of RNA (Green & Gerard, 1974). In ideal conditions the enzyme can then proceed from the 3'-end to the 5'-end of the RNA synthesising full-length cDNA copies. However, in cases where the RNA species lack a poly(A) tail, other methods have to be used:

- 1) Random primers can be prepared from salmon sperm or calf thymus DNA by the action of DNase and boiling. Because of the complexity of these oligonucleotides, they have a number of sequences which are complementary to sequences within the virus genome. These single stranded primers, usually 8 to 16 bases, bind to the virus RNA, anywhere between the 5' and 3'-end depending on their sequence, and prime the action of reverse

transcriptase (Taylor et al., 1976). The cDNA will be composed of a population of mostly less than full-length molecules because most of the primers will bind internally.

2) A poly(A) tail can be added in vitro to the 3'-end of the RNA by poly(A) polymerase (Sippel, 1973), or an oligo(C) can be ligated onto the 3'-end of the RNA using RNA ligase (Imai et al., 1983). Either oligo(dT) or oligo(dG) is then used to prime the action of reverse transcriptase. A large proportion of full-length cDNA should then be obtainable.

3) If the sequence of the 3'-end of the RNA is known, a specific primer can be synthesised corresponding to the known sequence. Full-length cDNA can then be obtained (Ahlquist et al., 1981, Goelet & Karn, 1982).

TBSV was tested for the presence of a poly(A) tail by attempting a reverse transcriptase reaction using oligo(dT) as a primer, and by studying the behaviour of TBSV RNA on poly(U)-sepharose columns (Palatnik et al., 1979):

(1) Oligo(dT) primed cDNA reactions.

A number of cDNA reactions with various primers were performed. In half the cDNA reactions the TBSV RNA was denatured using methylmercuric hydroxide. After one hour the level of incorporation in each reaction was calculated by spotting duplicate aliquots of the reactions onto DE81 discs. The results, shown in Table 4.1, show that TBSV cDNA synthesis is not primed to any significant level by oligo(dT), suggesting that TBSV lacks an poly(A) tail. The methylmercuric hydroxide did not stimulate incorporation by any significant amount, showing that the lack of cDNA synthesis is probably not due to any secondary structure around the RNA 3'-end. The levels of incorporation obtained in the oligo(dT) priming reactions and in

Table 4.1. cDNA Synthesis using TBSV Virion RNA*

No pretreatment. Randomly primed	37100cpm
Methylmercury treated. Random primed	38280cpm
No pretreatment. Oligo(dT) primed	1880cpm
Methylmercury treated. Oligo(dT) primed	1910cpm
No pretreatment. No primers	1760cpm
Methylmercury treated. No primers	2060cpm

* See Materials and Methods for experimental details.

In half the reactions the RNA was made 10 mM with methylmercuric hydroxide and kept at room temperature for 10 minutes. The methylmercury was then inactivated by adding 2-mercaptoethanol to 35 mM final concentration, and then the other reaction components were added 5 minutes later. Two control reactions were also performed - the first contained random primers in order to test the activity of the enzyme, and the second reaction did not contain any primers at all. After incubation for 1 hour the level of incorporation was calculated by spotting equal amounts of each reaction onto separate DE81 discs, washing the discs in 0.5 M disodium hydrogen orthophosphate and measuring cpm in a scintillation counter.

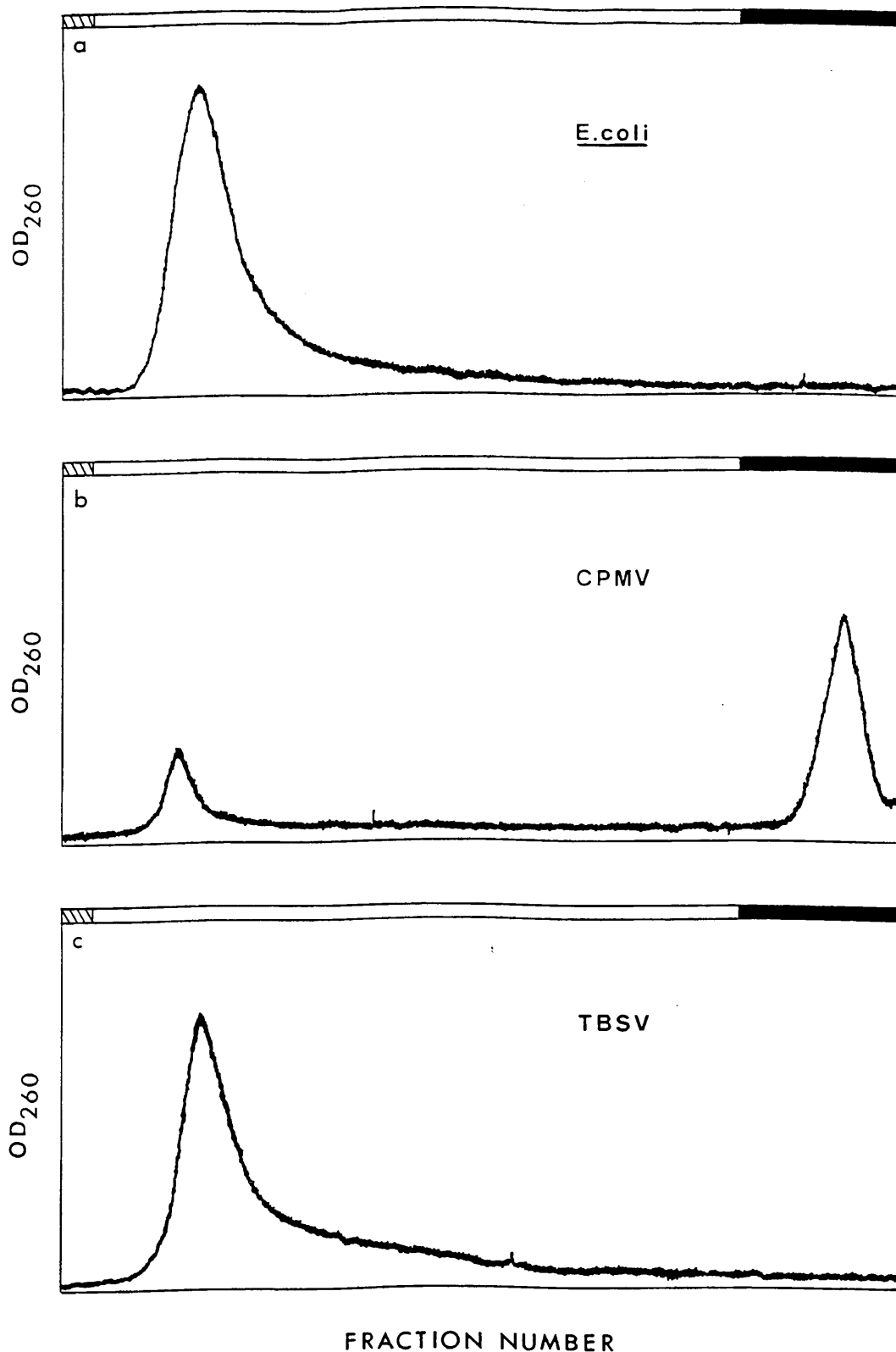


FIG. 4.1. UV profiles of (b) CPMV RNA, (a) *E.coli* rRNA and (c) TBSV RNA applied to a poly(U)-sepharose columns. RNA was applied to the column in high salt/low formamide buffer (hatched box). The column was then washed with binding buffer (open box), and then any remaining RNA was eluted with elution buffer (closed box).

reactions without primers are probably due to priming by small pieces of RNA. Slight degradation of the RNA is common in reverse transcriptase reactions, usually due to RNase contamination of one or more of the reaction constituents.

(2) Poly(U)-Sephadex.

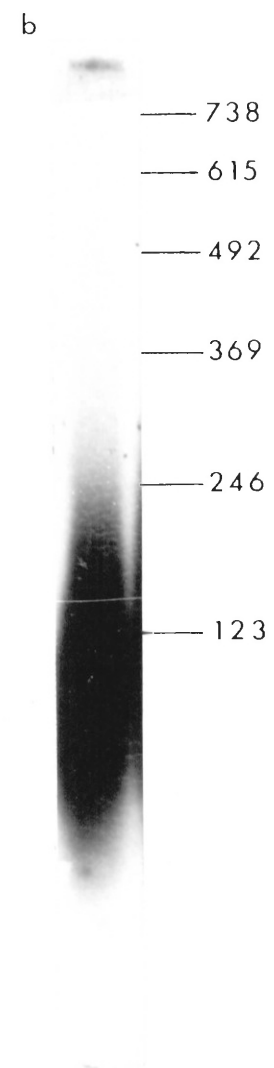
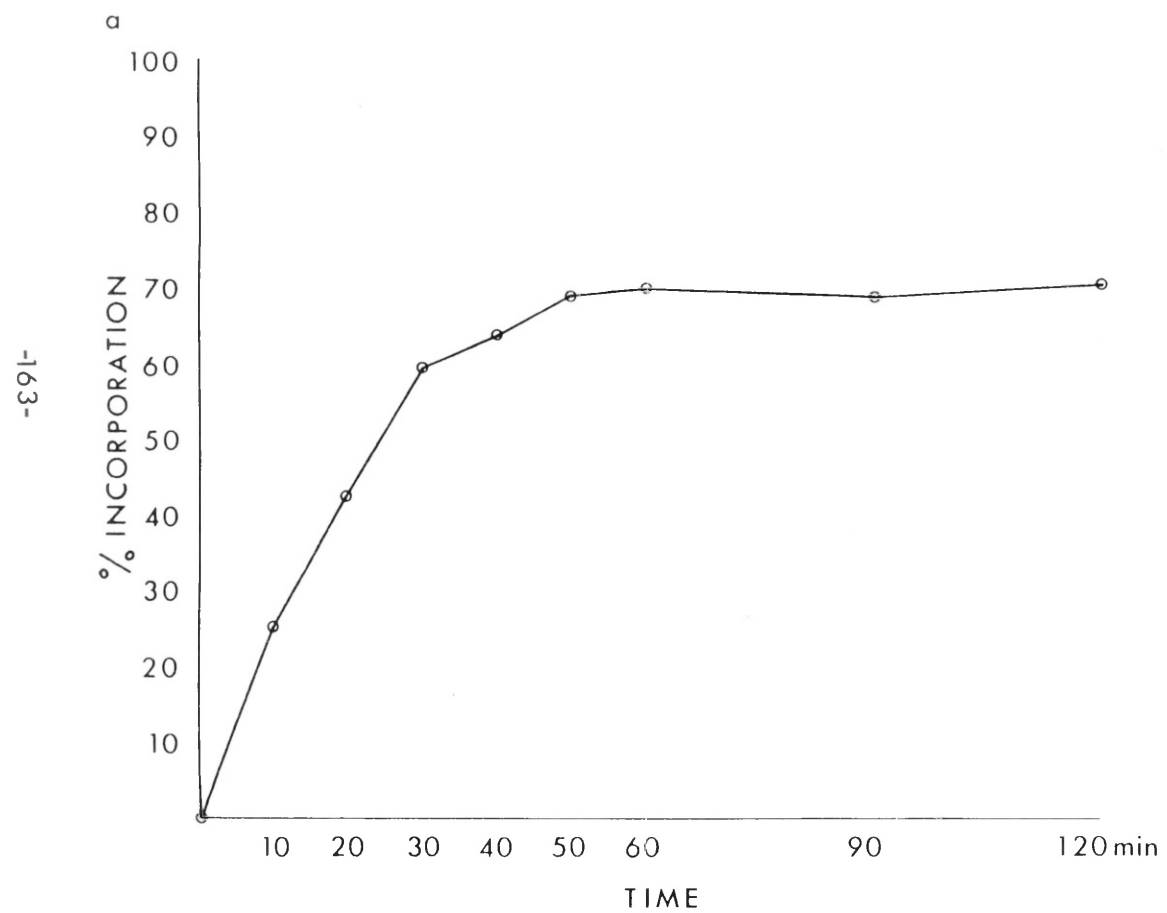
Polyadenylated RNA can be purified from total RNA by oligo(dT) cellulose or poly(U)-sephadex columns; approximately 90% of RNA, mostly in the form of ribosomal RNA and histone mRNA, is non-adenylated (Jacobson, 1987). The major disadvantage of oligo(dT) is the short length of oligomers generally used to prepare the resin (usually a maximum of 18-30 dT residues). This leads to inefficient binding of RNA molecules with relatively short poly(A) tails (less than 20 residues). Poly(U)-sephadex has molecules of poly(U) which are approximately 100 nucleotides long, providing more efficient binding of polyadenylated RNA (Jacobson, 1987). Total TBSV virion RNA was dissolved in dH₂O, heated to 65°C for 3 minutes in sample buffer to denature any secondary structure, and applied to a poly(U)-sephadex column. The column was then washed with binding buffer, and the optical density of fractions of the flow-through were measured. Any remaining RNA was then eluted from the column with low salt/high formamide elution buffer. As a control the experiment was repeated with CPMV RNA which is polyadenylated (Lomonossoff & Shanks, 1983; Van Wezenbeek *et al.*, 1983) and with non-adenylated E.coli ribosomal RNA. The results shown in Fig. 4.1 confirm the conclusions drawn from the cDNA experiments above, ie TBSV RNA is not polyadenylated to any detectable degree. As expected, unlike E.coli ribosomal RNA, polyadenylated CPMV bound to the column

under high salt/low formamide conditions. Electrophoresis of the eluted RNA through a 1.2% agarose gel containing 5 mM methylmercuric hydroxide confirmed that the TBSV RNA had not been degraded (data not shown).

From the above results it is clear that the direct preparation of cDNA probes using oligo(dT) primers was not possible. However, cDNA probes labelled to high specific activity could be prepared using labelled dCTP, random primers and a standard set of conditions (Maniatis et al., 1982). A time course of incorporation showed that the routine maximal incorporation of 70% occurred after approximately 60 minutes (Fig.4.2a), and that the probe length ranged from a few nucleotides to approximately 250 nucleotides (Fig.4.2b). Both unfractionated virion RNA or genomic RNA, purified as described in Section 3.6, could be used as a template with equal efficiency, showing that any agarose contaminants from the genomic RNA purification method were present in too low a level to effect the reverse transcriptase reaction (Ogden & Adams, 1987).

FIG.4.2. Preparation of random primed cDNA using TBSV RNA. Panel (a) shows the time course for a typical reaction determined by spotting and scintillation counting aliquots of the reaction on DE81 discs.

(b) autoradiograph of the cDNA (60 minutes reaction time) after electrophoresis through a 2% agarose-alkaline gel. The positions of the relevant markers (BRL 123 bp ladder) were determined by staining the gel with ethidium bromide prior to autoradiography.



4.2 END-LABELLING OF GENOMIC RNA

RNA was labelled at the 3'-end with RNA ligase, an enzyme which catalyzes the addition of [5'-³²P]pCp to the 3'-OH group. After the reaction the labelled RNA was electrophoresed through a 1.4% agarose gel containing 10 mM methylmercuric hydroxide. A single band was detected which corresponded to an RNA species of 4.8 kb, showing that the genomic RNA was not degraded during the reaction (see Fig.4.29, lane 1) and that the 3'-end of TBSV is a hydroxyl group.

T4 polynucleotide kinase should label the 5'-end of RNA using [γ -³²P]ATP as a substrate. When this reaction was performed TBSV RNA failed to become labelled; after electrophoresis the intact RNA could be seen on a ethidium bromide stained denaturing gel, but prolonged autoradiography failed to detect any bands (see Fig.4.29, lane 2). However, when the RNA was cleaved to expose internal phosphate groups, label became incorporated in the T4 polynucleotide kinase reaction (Fig.4.29, lanes 4, 6, 8 and 10).

These results show that TBSV does not possess an accessible 5'-phosphate group. As TBSV RNA is infectious after treatment with proteinase K (Mayo et al., 1982), and cannot be labelled at the 5'-end by ¹²⁵I (Dr.A.C.Marriot, personal communication), it would seem likely that the 5'-end of TBSV is capped.

4.3 ATTEMPTED POLYADENYLATION OF THE TBSV RNA 3'-END

The advantages of using cloned probes rather than non-cloned cDNA probes are that: (1) by virtue of their method of preparation, cloned probes should not be contaminated with other viral or host plant RNA sequences; (2) once cloned the viral RNA sequence can be amplified to high levels in bacteria, removing the need for lengthy virus

preparations; (3) by subcloning fragments it is possible to prepare probes to specific regions of the viral genome; and (4) single-stranded bacteriophage M13 clones can be prepared, specific not only to defined regions of the genome, but also to either the plus-strand viral RNA molecule or the minus-strand viral RNA.

One of the other major uses of dscDNA clones is their ability to hybrid-select specific RNAs from a population of RNAs. This would allow the subgenomic RNAs to be purified from infected plants and then translated in vitro. By comparing the translation products with the in vivo proteins, the functions of each of the subgenomics can be identified.

As discussed in Section 4.1, the production of cDNA is usually primed by binding oligo(dT) to the poly(A) tail of the mRNA. However, as shown in Section 4.1, TBSV lacks a poly(A) tail, and therefore the addition of an oligonucleotide directly to the 3' end of the RNA was attempted

Poly(A) polymerase was purchased from BRL and used according to the manufacturer's instructions. These conditions were essentially the same as described by Devos et al. (1976) and Gould et al. (1978), ie. the reaction mixture (50 μ l) contained 50 mM Tris-HCl pH 7.9, 250 mM sodium chloride, 10 mM magnesium chloride, 2.5 mM manganese chloride, 50 μ g/ml BSA, 1 mM EDTA, 1 mM DTT, 0.1 mM ATP, 10 μ g of TBSV RNA and 3 units of poly(A) polymerase. Usually 10 μ Ci of [32 P]ATP was added in order to determine whether enzyme was active.

Initially the enzyme failed to incorporate any ATP. Poly(A) polymerase requires a 3'-OH on the template RNA. As TBSV had been shown in Section 4.2 to have this 3'-terminal group, attention was paid to the reaction conditions. It was found that the level of sodium chloride



FIG.4.3. Electrophoresis of TBSV RNA, after a polyadenylation reaction, through a 1% agarose gel containing 5 mM methylmercuric hydroxide and staining with ethidium bromide. TBSV RNA (1 μ g, lane 1) was incubated in a polyadenylation reaction mixture in the presence (lane 4) or absence (lane 2) of RNasin. As a control, an equal amount was incubated in the reaction buffer alone (lane 3).

in the reaction had to be adjusted; because the poly(A) polymerase activity has a very narrow salt optimum (Sippel, 1973), and the enzyme itself was supplied at a dilute concentration in 0.7 M sodium chloride, it was necessary to adjust the added sodium chloride to compensate for the amount present with the enzyme. After altering the salt concentration the enzyme did incorporate ATP. Initially it was thought that 50 A residues had been added per TBSV RNA 3'-end in 30 minutes (determined by spotting an aliquot onto DE81 disc, washing the disc and scintillation counting). However, when the poly(A) polymerase treated RNA was electrophoresed through a denaturing gel, the vast majority of the RNA appeared degraded (Fig.4.3 lane 2). Incubating the RNA in the reaction buffer without the enzyme did not result in degradation (lane 3), and therefore the enzyme preparation must have contained significant levels of RNase. Attempts to inhibit the RNase by adding RNasin up to a level of 1 unit/ul were unsuccessful (lane 4). As commercial preparations of poly(A) polymerase from Pharmacia P-L were also tried without success, attempts to polyadenylate TBSV RNA were abandoned.

4.4 LIGATION OF A OLIGONUCLEOTIDE TO THE TBSV RNA 3'-END

Imai et al. (1983) added an oligo(C)₁₀₋₁₅ tail to the 3'-OH ends of dsRNA by using RNA ligase. The first-strand cDNA was then prepared using oligo(dG) as a primer for reverse transcriptase. The reaction requires that the donor and acceptor molecules be at high concentration, and ideally both molecules should be present in approximately equal molar amounts. The high Mr of acceptor molecule (TBSV RNA) compared to the donor molecule rendered this impractical, due to the very large amount of TBSV RNA that would be required in the reaction. Therefore, to prevent all the oligo(C) molecules from ligating to each other,

the oligo(C) 3'-end must be altered from a hydroxyl group to a phosphate group. The oligo(C) should then only be able to ligate to the 3'-OH end of the TBSV RNA.

Oligo(C)₁₅ was a kind gift of Dr.S.Delany (Dept. of Biochemistry, Imperial College). Poly(C) (Pharmacia P-L, 100mg) was dissolved in 10 mls TE buffer and partially digested with RNase A (10µg/ml, Sigma). The oligo(C) chains of various lengths were then separated by HPLC chromatography. As RNase A leaves 5'-OH and 3'-phosphate groups, 25 nmoles of oligo(C)₁₅ were phosphorylated for 45 minutes at 37°C in a 50 µl reaction mixture containing 50 mM Tris.HCl pH 7.5, 10 mM magnesium chloride, 10 mM DTT, 1 mM ATP, 2 mg/ml BSA, 10 µCi [γ -³²P]ATP (3000 Ci/mmol, Amersham Int.) and 10 units of T4 polynucleotide kinase from T4 am N81 pse T1 phage infected E.coli (BCL). The phosphorylated oligo(C)₁₅ was separated from the unincorporated label on a Sephadex G-10 column and then lyophilized overnight.

Oligo(C)₁₅ (300 pmoles) was ligated to TBSV RNA (60 pmoles) overnight at 4°C in a 75 µl reaction mixture containing 50 mM HEPES pH 7.5, 20 mM magnesium chloride. 3 mM DTT, 10 µg/ml BSA, 10% v/v DMSO, 60 µM ATP and 30 units T4 RNA ligase. Aliquots of each reaction were then electrophoresed on a 1.2% agarose gel containing 5 mM methymercuric hydroxide. When the gel was stained with ethidium bromide, the genomic RNA was found to be intact. However, when the gel was dried down and autoradiographed, no labelled RNA was detected, even after prolonged exposure (data not shown).

The reactions were repeated after denaturing the TBSV RNA at 90°C, with different preparations of oligo(C)₁₅ and with lower concentrations of either the donor or acceptor molecule. In each case the TBSV RNA was found to be intact, but oligo(C)₁₅ failed to ligate to the TBSV RNA at

any detectable level. However, the enzyme was shown to ligate [5'-³²P]pCp to the 3'-end of TBSV RNAs (Fig.4.29, lane 1), showing that the the enzyme was active. Therefore the method was abandoned.

4.5 PRIMING OF cDNA WITH RANDOM PRIMERS

In agreement with Taylor et al. (1976), the random primers from salmon sperm DNA prepared by DNase 1 digestion were found to be 10-20 nucleotides long (Dr.S.M.Dodd, personal communication). The cDNA prepared using such DNA fragments has been shown to be representative of the whole RNA template from which it is transcribed and is not grossly enriched for one or more regions (Gould & Symons, 1977). If such cDNA could be cloned then it is possible that the clones could be used either directly for hybrid selection, as a source of sequence specific primers for primer extension, or as templates for labelled probes.

The yield of long cDNA often depends upon the quality of the reverse transcriptase, the length of the RNA to be copied, the degree to which the RNA secondary structure interferes with extension, and the reaction conditions (Maniatis et al., 1982). The length of cDNA produced for probes was, at its maximum length, only approximately 250 bases long (Fig.4.2b). However, the reaction conditions such as the concentrations of salts, primers and nucleotides used to make short, highly labelled probes are considerably different to those used for the production of full-length cDNA; a number of papers describing optimization of conditions for producing full-length cDNA transcripts have been published (Efstratiadis et al., 1976; Buell et al., 1978; Retzel et al., 1980). Different mRNAs are copied into cDNA with different efficiencies, and therefore conditions that are optimal for copying one

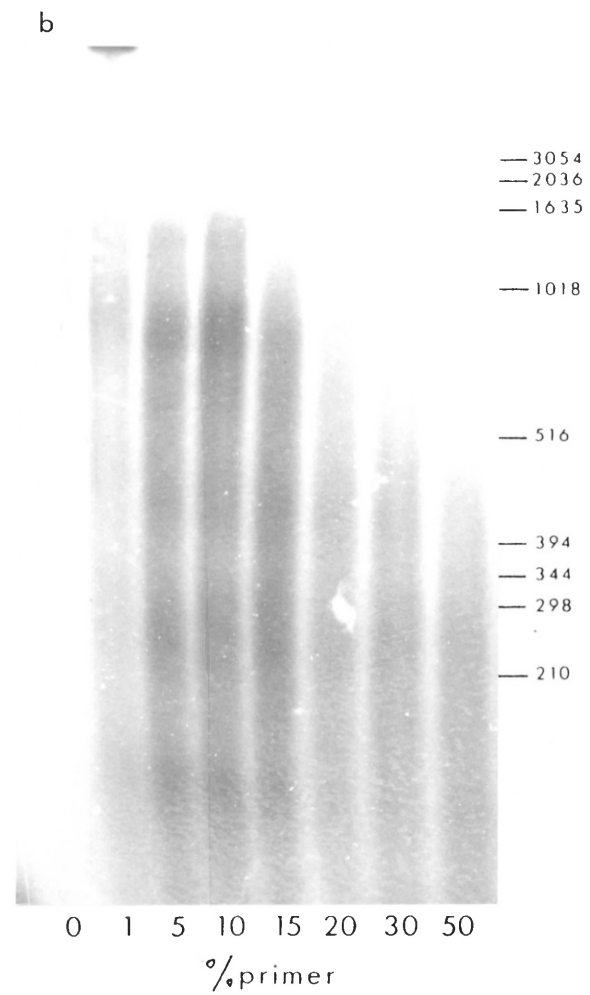
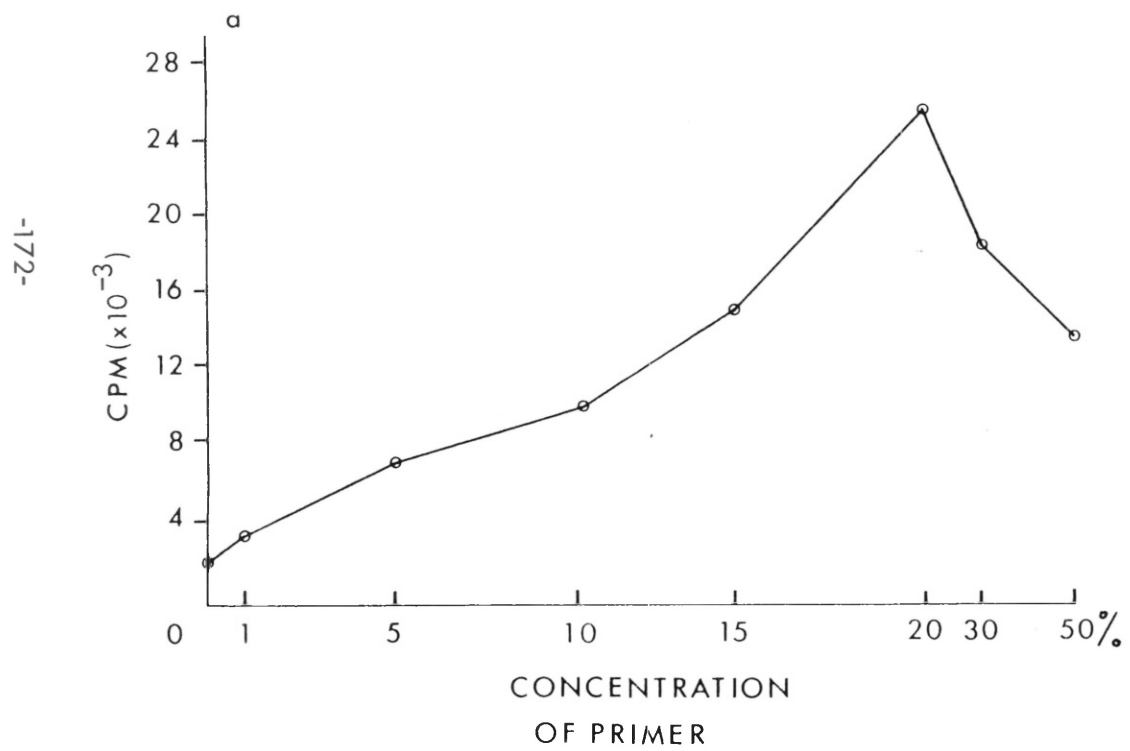
species of mRNA may not work well for another. A systematic evaluation of the reaction conditions was undertaken in an attempt to increase the length of randomly primed cDNA.

Primer Concentration.

As the concentration of random primers used in the reaction would be expected to have a significant effect on the length of the cDNA, the standard cDNA reaction was repeated using different dilutions of a standard primer solution (10 mg/ml), ranging from 1% v/v of the reaction volume (ie. in the standard reaction volume of 100 μ l only 1 μ l of random primers was added) up to 50% v/v. As shown in Fig.4.4 the concentration had a significant effect. The longest cDNA, up to 2500 nucleotides, was obtained when the concentration was 1-10% v/v, with decreasing cDNA length at higher concentrations. The cDNA produced when no DNA fragments had been added (0%) may be due to small oligoribonucleotides acting as primers. The incorporation reached a peak before greatest concentrations of primers had been reached, suggesting saturation of the RNA molecule (Fig.4.4b). Although As the highest levels of incorporations were reached near 20% v/v primers, a final concentration of 10% v/v was used which allowed optimum cDNA length with a reasonable incorporation.

FIG.4.4 (b). Effect of primer concentration on cDNA length. TBSV cDNA was prepared using the standard reaction conditions (Section 2.28) with varying primer concentrations. A concentration of 50% v/v primer is equal to the level normally used to prepare cDNA for probes (Fig. 4.2). After 1 hour the reactions were electrophoresed through a 2% agarose-alkaline gel and autoradiographed. The positions of the relevant markers (BRL kb ladder) were determined by staining the gel with ethidium bromide prior to autoradiography.

(a). Incorporation of label into cDNA with different concentrations of primers. Reaction conditions as in Fig.4.4a. After the reaction, duplicate aliquots were spotted onto DE81 discs. These were then washed and scintillation counted.



Salt concentration.

The salt concentration in the reaction can have a significant effect on cDNA length, and has been identified as one of the main factors influencing the synthesis of full-length cDNA (Retzel *et al.*, 1980). The cDNA reaction was repeated with 10% v/v primers and varying salt concentrations. As shown in Fig.4.5a, until an inhibitory concentration was reached, varying the salt concentration had little effect on cDNA length when using random primers. However, the yield of cDNA was significantly altered as shown in Fig.4.5b, dropping drastically above 100 mM potassium chloride. Therefore, a concentration of 70 mM potassium chloride was maintained.

Reaction Temperature and Time.

Reverse transcriptase reactions are usually performed for 1 hour at 42°C, but longer times, up to 3 hours, have been used, together with both lower and higher temperatures (Maniatis *et al.*, 1982). A time course of the standard reaction was performed using 10% v/v primers, 70 mM potassium chloride and ensuring that the reaction had been preincubated at the required temperature for 5 minutes prior to adding the enzyme. The highest yield was obtained at 30 minutes at 42°C, and at 45 minutes at 37°C (Fig.4.6b). There was no significant difference in length (Fig.4.6a) or yield of the cDNA between the two temperatures. As shorter primers might not anneal to the RNA at 42°C, a separate reaction was performed in which the reaction was kept at room temperature for 5 minutes after addition of enzyme, at 37°C for 5 minutes and then finally at 42°C for 30 minutes. Again there was no significant increase or decrease in length or yield of cDNA (Fig.4.6a, lane X).

FIG.4.5 (b). Effect of potassium chloride concentration on cDNA length. TBSV cDNA was prepared using the standard reaction conditions (Section 2.28), 10% v/v primers and varying concentrations of potassium chloride. A concentration of 70 mM is equal to the level normally used to prepare cDNA for probes. After 1 hour the reactions were electrophoresed through a 2% agarose-alkaline gel and autoradiographed. The positions of the relevant markers (BRL kb ladder) were determined by staining the gel with ethidium bromide prior to autoradiography.

(a). Incorporation of label into cDNA with different concentrations of potassium chloride. Reaction conditions as in Fig.4.5a. After the reaction duplicate aliquots were spotted onto DE81 discs. These were then washed and scintillation counted.

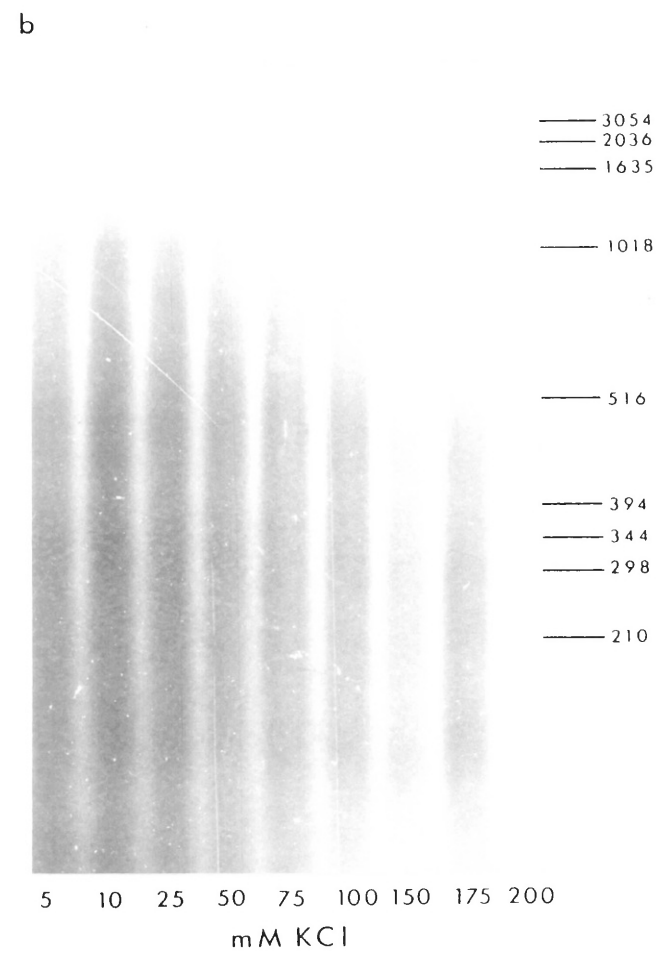
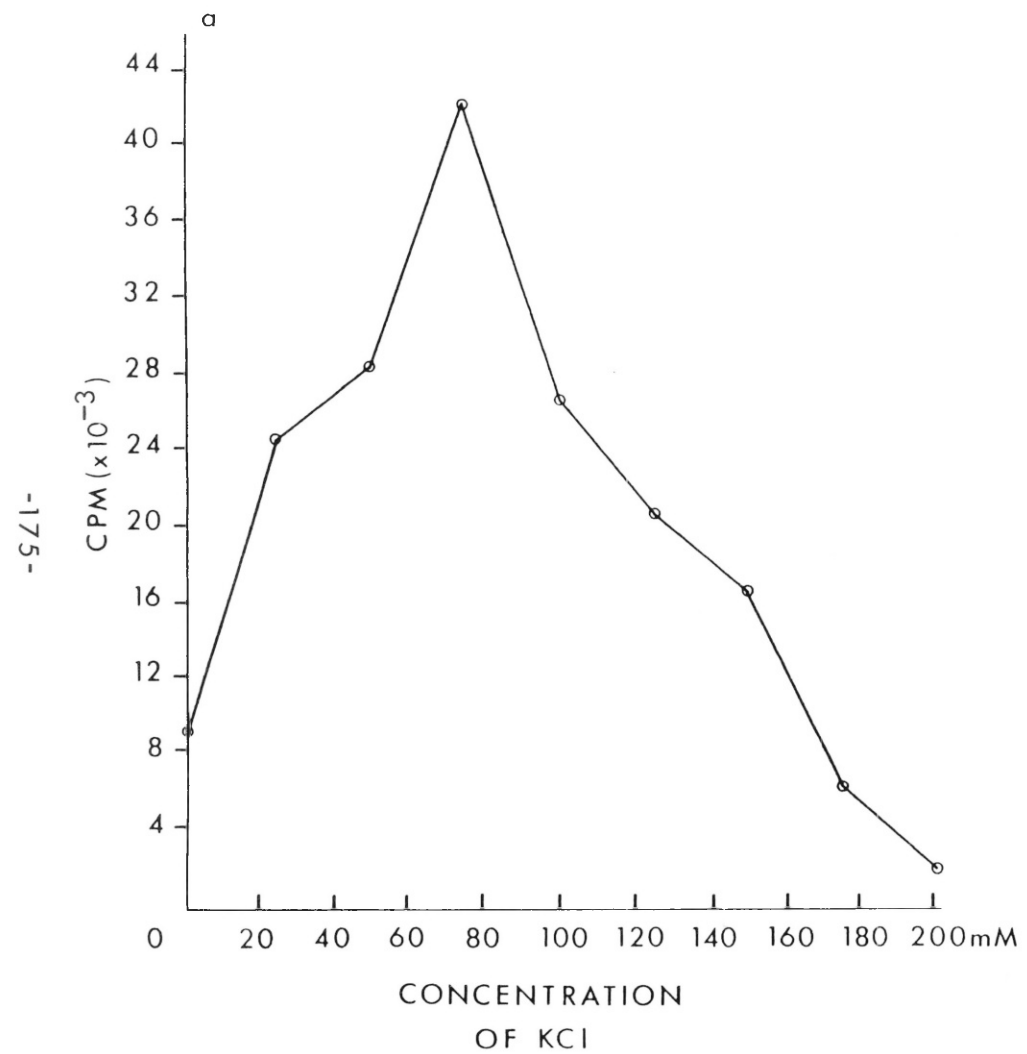
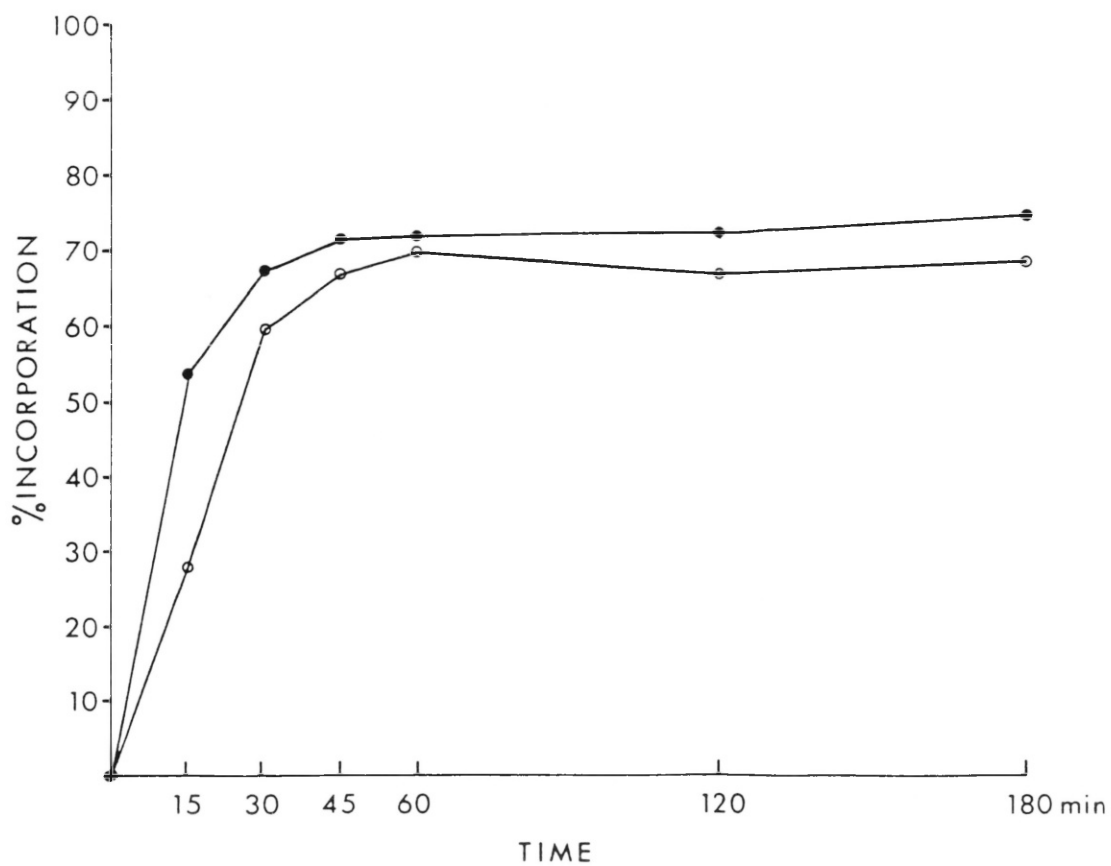
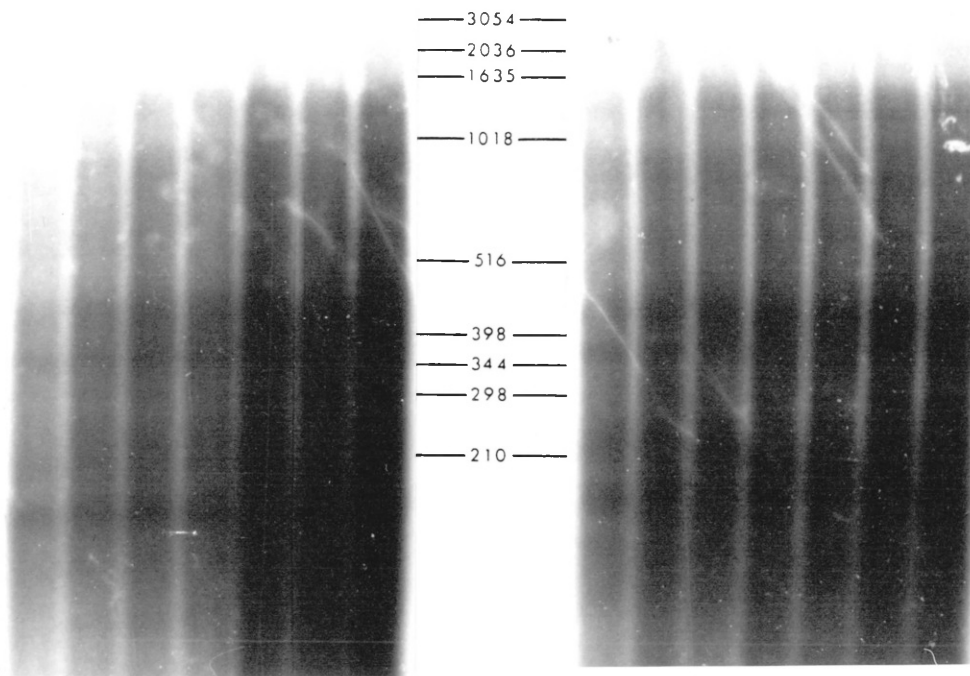


FIG.4.6 (a). Time course for cDNA production at 37°C and 42°C. TBSV cDNA was prepared using the standard reaction conditions (section 2.28), 10% v/v primers and 70 mM potassium chloride. After various times the reactions were stopped with 10 mM EDTA. All the samples were then electrophoresed through a 2% agarose-alkaline gel and autoradiographed. The positions of the relevant markers (BRL kb ladder) were determined by staining the gel with ethidium bromide prior to autoradiography.

(b). Time course for incorporation of label into cDNA at 37°C (◦) and 42°C (•). Reaction conditions as in Fig.4.6a. After the reaction duplicate aliquots were spotted onto DE81 discs. These were then washed and scintillation counted.

37°C
15 30 45 60 120 180 x

42°C
15 30 45 60 120 180 x



Reverse Transcriptase.

Like many polymerases, reverse transcriptase catalyzes more than one reaction. The enzyme embodies both a polymerase and ribonuclease H (RNase H) activity in vitro (Verma, 1981). During the polymerase reaction the RNase H, an exonuclease that degrades RNA in DNA-RNA hybrids, is fully active. The products of the exonucleolytic cleavage reaction are oligomers 2-30 nucleotides in length which can act inefficiently as primers. When these oligoribonucleotides are reverse transcribed the resultant DNA oligomers are effective primers (Krug & Berger, 1987). From the above results this increase in primer concentration would be expected to have an adverse effect on the length of the cDNA.

As yet there are no methods available to selectively inhibit the RNase H activity. RNasin has no effect on the RNase H activity when high concentrations of deoxyribonucleotides are included in the reaction, but does have an effect on any contaminating RNase present (Krug & Berger, 1987). High levels of deoxyribonucleotides do not inhibit RNase H, but do enhance the polymerase by supplying limiting substrates. Therefore, the concentrations of all the nucleotides were maintained at the high levels of 1 mM dATP, dGTP and dTTP and 0.2 mM dCTP. Different preparations of reverse transcriptase may differ in the ratio of the two activities. Such dissimilarities may be a consequence of partial protease degradation of the subunits or holoenzyme, dissociation of subunits, or the presence of unidentified components in the enzyme solution (Krug & Berger, 1987).

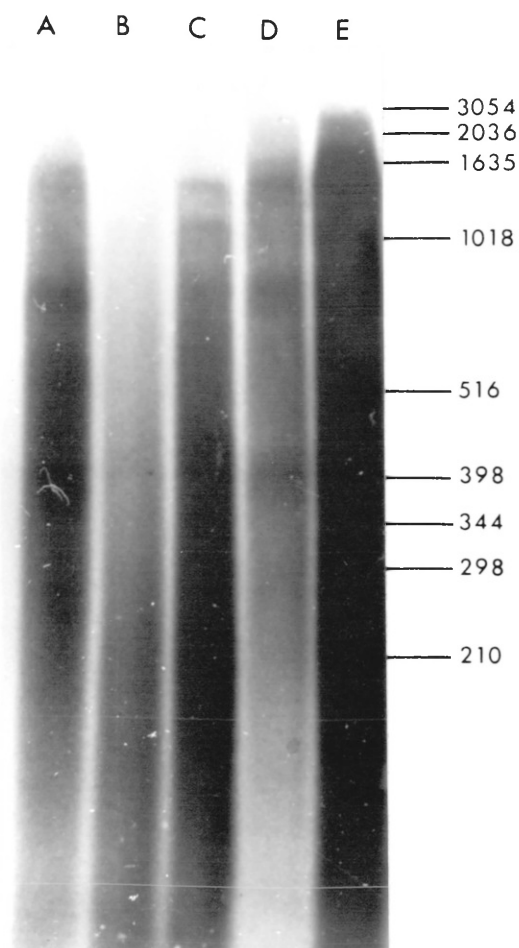


FIG.4.7. Effect of different preparations of reverse transcriptase on cDNA length. TBSV cDNA was prepared using the standard reaction conditions (Section 2.28), 10% primers and 70mM potassium chloride and five different preparations of reverse transcriptase for 1 hour at 42°C. The reactions were then electrophoresed through a 2% agarose-alkaline gel and autoradiographed. Preparation E (from NBL) was used in Figs.4.4 to 4.6. The positions of the relevant markers (BRL kb ladder) were determined by staining the gel with ethidium bromide prior to autoradiography.

The cDNA reaction was performed using reverse transcriptase from a number of sources. Fig.4.7a shows that there was a significant difference in the length of the cDNA depending on the commercial preparation used. The yield of cDNA also varied between the enzymes (Fig.4.7b), with the enzymes which produced the longer cDNA also tending to give better yields. There are a number of possible reasons for this, including stability of the enzyme in the reaction conditions, processivity of the enzymes and the relative activity of the RNase H.

RNasin and Methylmercury

In an attempt to increase the cDNA length further, two other alterations to the reaction conditions were tried. The first was the addition of RNasin (to a final concentration of 1 unit/ μ l) to inhibit any RNase present in the reaction buffer (Retzel et al., 1980) This was found to have no effect on the length or the cDNA yield (Fig.4.8).

The second alteration involved the use of methylmercuric hydroxide as a denaturant. When the template RNA has extensive secondary structure, the length of full-length cDNA is often increased by destroying the base pairing (Maniatis et al., 1982; Krug & Berger, 1987). Methylmercuric hydroxide was added to a final concentration of 10 mM (10 μ g RNA in 10 μ l dH₂O). After 10 minutes 2-mercaptoethanol was added to a final concentration of 35 mM to neutralise the methylmercuric hydroxide, followed 5 minutes later by the rest of the reaction components. Denaturing the TBSV RNA was found to have no significant effect on cDNA length or yield. Even boiling the RNA in methylmercuric hydroxide for 3 minutes failed to effect the cDNA length (Fig.4.9).

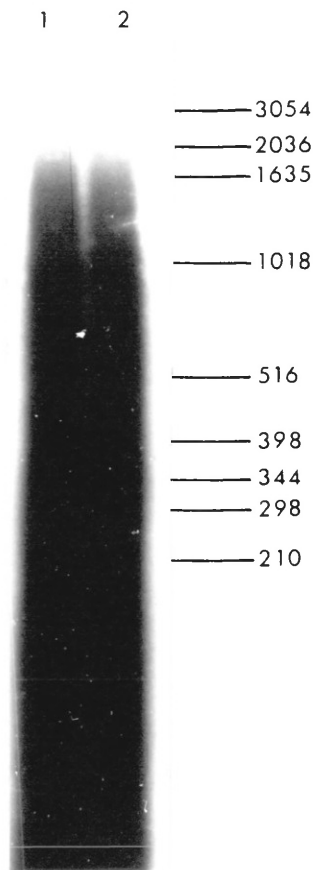


FIG.4.8. Effect of RNasin on cDNA length. TBSV cDNA was prepared as in Fig.4.7 with (lane 1) or without (lane 2) 1 unit/ul RNasin. The reactions were then electrophoresed through a 2% agarose-alkaline gel and autoradiographed. The positions of the relevant markers (BRL kb ladder) were determined by staining the gel with ethidium bromide prior to autoradiography.

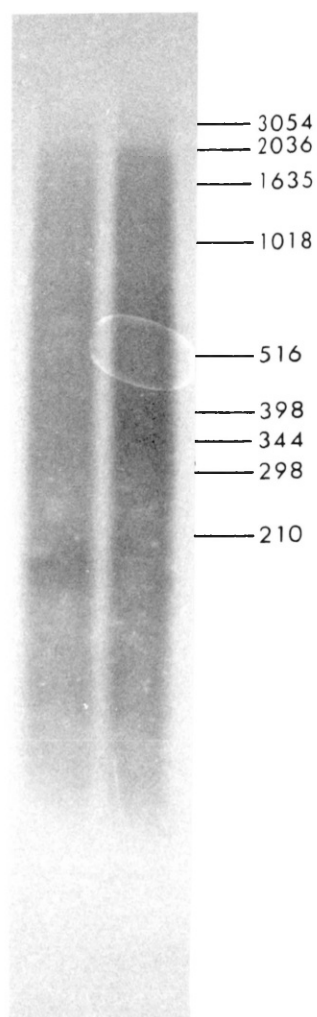


FIG.4.9. Effect of methylmercuric hydroxide on cDNA length. TBSV cDNA was prepared as in Fig.4.7 with or without prior denaturation using 10 mM methylmercuric hydroxide. The reactions were then electrophoresed through a 2% agarose-alkaline gel and autoradiographed. The positions of the relevant markers (BRL kb ladder) were determined by staining the gel with ethidium bromide prior to autoradiography.

The above results show that primer concentration has the greatest effect on cDNA length. Therefore, for cloning the primer concentration was reduced. Retzel et al. (1980) found that in order to synthesize long cDNA a high concentration of KCl must be used. However, the above results show that when using random primers the salt concentration has a greater effect on cDNA yield than length. The amount of label used when testing the second strand conditions was approximately 50 μ Ci-100 μ Ci [α -³²P]dCTP (410 Ci/mmol) which gave up to 10⁶ cpm/ μ g. However, when generating long cDNA for cloning the amount of label was kept down to 2 μ Ci per reaction because highly labelled molecules are unstable. A first-strand cDNA reaction using the optimised conditions and the best commercially available reverse transcriptase is shown in Fig.4.10. Routine incorporation, determined by spotting duplicate aliquots of the reaction onto DE81 discs, was determined to be 35-40% ie. approximately 4 μ g of cDNA was generated for every 10 μ g TBSV RNA added to the reaction. Actinomycin D was added to the reaction, thereby reducing incorporation by preventing formation of hairpin loops which allow the enzyme to use newly synthesized cDNA as a template, and preventing the primers themselves being used as a template (Maniatis et al., 1982). The level of incorporation showed the reaction to be very efficient, approaching the levels obtained when making cDNA probes (40-45%) in the presence of actinomycin D.



FIG.4.10. Length of cDNA using optimum conditions. TBSV cDNA was prepared using the standard conditions (Section 2.28), 10% v/v primers, 70 mM potassium chloride and the best commercially available reverse transcriptase (Life Sciences, supplied by NBL) for 1 hour at 42°C. The reactions were then electrophoresed through a 1.5% agarose-alkaline gel and autoradiographed (lane 1). The lengths of HindIII digested lambda markers (lane 2) are shown on the right.

4.6 SECOND-STRAND cDNA

Two methods are usually used for second-strand synthesis. The first method, often termed the "classical" method utilizes a hairpin loop that occurs as a transient structure at the 3'-end of the newly synthesised first strand (Efstratiadis et al., 1976; Rougeon & Mach, 1976). This is used as a primer by reverse transcriptase or Klenow fragment to ultimately generate a double-stranded cDNA molecule that is covalently closed at the end corresponding to the 5'-end of the original RNA molecule. The short single-stranded cDNA within the hairpin loop can then be cleaved with either S1 nuclease or mung bean nuclease. Commonly both Klenow fragment and reverse transcriptase are used in the second-strand reaction in a stepwise manner; it has been found that the two enzymes stop at different secondary structures in single-stranded cDNA (Maniatis et al., 1982).

In the second method, often termed the RNase H method (Gubler & Hoffman, 1983), after completion of the first-strand reaction the RNA-cDNA hybrid is left intact. E.coli RNase H is used to nick the RNA in the hybrids, leaving only small RNA primers with free 3'-OH groups attached to the cDNA. These 3'-OH groups can subsequently be used by DNA polymerase 1 to efficiently synthesize a second-strand all along the length of the cDNA first-strand. E.coli DNA ligase then seals any nicks in the second-strand. In general there is no need for hairpin loop cleavage.

The efficiency of second-strand synthesis using both methods was analysed using S1 nuclease. Fractions of aliquots taken after first-strand cDNA synthesis, after both the Klenow fragment and the reverse transcriptase stages in the classical method, and after the RNase H method were digested in 100 µl of S1 buffer at 37°C for 60 minutes with 10 units of S1. Carrier DNA (25 µg of calf thymus DNA) was added to the reaction to buffer the excess

S1 nuclease added. The control reaction used was an aliquot of nick translated pUC9 plasmid, and each reaction was performed twice and the average result calculated. Half the reactions were boiled for 5 minutes and then cooled rapidly on ice prior to the addition of the S1 nuclease.

Comparison of the effect on pUC9 before and after boiling (Table 4.2, samples 2 and 1) shows that the majority of double-stranded DNA is resistant to S1 nuclease under these conditions. S1 nuclease tends to be an aggressive enzyme, and will degrade fully base paired DNA when the enzyme is present in excess. The 5% of pUC9 not degraded after boiling can be taken as the background, or amount of DNA that is not digested by the enzyme. The high level of counts in the reactions where S1 nuclease has not been added show that boiling results in very little degradation (sample 3), and that the S1 nuclease buffer contains little, if any, contaminating nucleases (sample 4).

As expected the first-strand cDNA is resistant to S1 nuclease when in the form of the RNA-cDNA hybrid (sample 6). Denaturation of the hybrid results (sample 5) in degradation by S1 nuclease and only slightly more than background cpm.

The Klenow reaction of the "classical" second-strand reaction has converted approximately 40-45% of the sscDNA template into dscDNA (compare samples 1 & 10). The second stage, in which reverse transcriptase is added, only converts another 5% of the sscDNA into dscDNA (compare samples 10 & 14). This could be because the reverse transcriptase reaction was inefficient, and/or because most of the sscDNA that was available as a template had already been converted into dscDNA.

Table 4.2. S1 nuclease analysis of cDNA reaction products
(5000cpm)

Sample	S1	Boil for 5 mins	cpm (average)	% resistant to S1
1) pUC9	+	+	240	5%
2) pUC9	+	-	4620	92%
3) pUC9	-	+	4840	97%
4) pUC9	-	-	4870	97%
5) 1st strand cDNA	+	+	400	8%
6) 1st strand cDNA	+	-	4380	88%
7) 1st strand cDNA	-	+	4840	97%
8) 1st strand cDNA	-	-	4930	99%
9) 2nd strand-Klenow	+	+	790	16%
10) 2nd strand-Klenow	+	-	2430	49%
11) 2nd strand-Klenow	-	+	4780	96%
12) 2nd strand-Klenow	-	-	4850	97%
13) 2nd strand-R.T*.	+	+	920	18%
14) 2nd strand-R.T.	+	-	2680	54%
15) 2nd strand-R.T.	-	+	4760	95%
16) 2nd strand-R.T	-	-	4890	98%
17) 2nd strand-RNase H	+	+	480	10%
18) 2nd strand-RNase H	+	-	4450	90%
19) 2nd strand-RNase H	-	+	4860	97%
20) 2nd strand-RNase H	-	-	4820	96%

* R.T = reverse transcriptase

Samples 14 and 18 allow the comparison between the efficiency of the two second-strand methods using these reaction conditions. The maximum conversion of sscDNA to dscDNA with the classical method is approximately 50-55%, while up to 85-90% of the RNA-cDNA hybrid has been converted to dscDNA with the RNase H method. This is probably due to the relative efficiency of the primer used - the hairpin loops are formed rather transiently in the classical method, and therefore the chance of the Klenow enzyme having a suitable primer at the end of the cDNA when it is present is low. On the other hand, with the RNase H method the primer (RNA fragment) is bound to the cDNA relatively strongly and probably for a much longer period (Krug & Berger, 1987).

Interestingly, after boiling samples of either sscDNA and dscDNA are still partially resistant to S1 after boiling. If the strands were not covalently attached by hairpin loops, they would not be expected to re-anneal during the quick cooling on ice. Therefore, they would stay susceptible to S1 nuclease. However, hairpin looped molecules would quickly re-anneal (at least partially), and therefore the dscDNA would remain partially resistant to S1. The higher levels of S1 resistance with the classical method shows that loopback is occurring, leading to the production of dscDNA. These hairpin loops are then usually removed by S1 digestion in the next step of cloning. Although the level of loopback with the RNase H method is relatively small when compared to the classical method, the method does not utilise a single-stranded cutting enzyme after dscDNA formation. Therefore, these molecules cannot be cloned. A S1 or mung bean nuclease step could be introduced at the end of the protocol to circumvent this problem. However, these enzymes would also degrade some of the dscDNA, and therefore it was decided to ignore these covalently-closed dscDNA molecules.

Although S1 nuclease digestion allows the determination of the proportion of ssDNA that has been converted to dsDNA, it does not show the length of the second-strand cDNA. If the first-strand cDNA is unlabelled, adding label to the second-strand reaction will allow the length of the second-strand cDNA to be estimated. Fig.4.11, lane 1 and lane 3 contain labelled second-strand cDNA prepared by the classical and the RNase H methods respectively. Note that the longest dsDNA molecules in lanes 1 and 3 are longer than any cDNA in the first-strand reaction (lane 2), due to the covalent attachment of both strands by the hairpin loop.

The range of lengths of the second-strand cDNA prepared by the RNase H method (Fig 4.11 lane 3) are very similar to the first-strand cDNA (lane 2). This shows the reaction to have been efficient at making dsDNA mostly without hairpin loops. In lane 1, the majority of the second-strand should be twice the length of the first-strand due to the hairpin loops present. However, this is not the case, with most of the second-strand being only a few hundred bases long. Together with the degree of S1 resistance (Table 3.1), lane 1 shows that the processivity of the Klenow fragment and reverse transcriptase used has been low, perhaps due to secondary structure and/or the reaction conditions. However, when the reaction was repeated using fresh buffers and different aliquots of enzymes from other commercial sources, the results were no better. Because of the yield of S1 resistant DNA, the length of the second strand cDNA, and the fact that a S1 cleavage step was not required, the RNase H method was used to produce dsDNA for cloning.

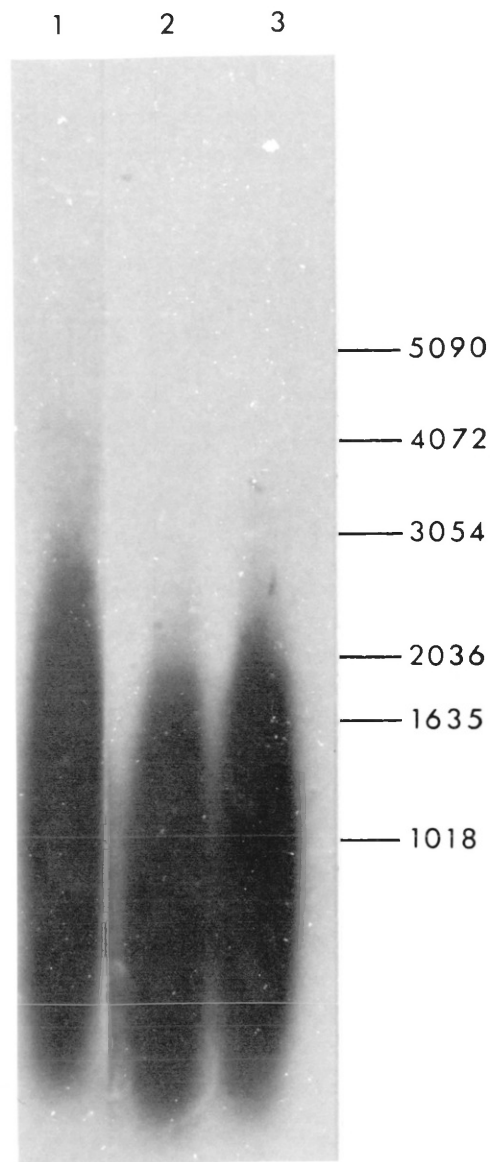


FIG.4.11. Length of second-strand cDNA. Second-strand cDNA was prepared by the classical (lane 1) or RNase H (lane 3) method as described in Section 2.28. The reactions were then electrophoresed through a 1.5% agarose-alkaline gel and autoradiographed. Comparison with the length of the first-strand cDNA reaction (lane 2) allows the length of the second strand to be estimated. The positions of the relevant markers (BRL kb ladder) were determined by staining the gel with ethidium bromide prior to autoradiography.

4.7 INTRODUCTION OF DOUBLE-STRANDED cDNA INTO THE VECTOR

After second-strand synthesis, a method had to be selected to introduce the dscDNA into the vector. Three approaches were available; (1) tailing the dscDNA with terminal transferase and annealing to a cleaved plasmid possessing complementary homopolymeric tails; (2) ligating synthetic symmetrical double-stranded oligomers (linkers) containing a recognition site for a restriction endonuclease onto the cDNA; and (3) digesting the dscDNA with restriction endonucleases and ligating the fragments directly into the plasmid. All three have their advantages and disadvantages.

Terminal Transferase

The tailing reaction is catalyzed by terminal transferase in which nucleoside monophosphate residues donated by dNTPs are polymerised onto the 3'-OH termini of either single-stranded or double-stranded cDNA. A gapped plasmid capable of transforming bacteria can be constructed by annealing cDNA molecules with vector molecules bearing complementary tails. The main advantage of tailing is that blunt-ending of the dscDNA and ligation steps are not required. However, the length of the homopolymeric tails on both the vector and the cDNA must be rigorously controlled in order to clone efficiently. The size and distribution of homopolmeric tails are a function of the concentration of the enzyme, the concentration of the dNTP and the concentration of the termini to be tailed. As the enzyme acts progressively, a high concentration of the enzyme is required to prevent synthesising long tails on a few termini, and no tails on others. With a excess of enzyme the factors governing tail length are temperature, the chosen dNTP and its concentration. Usually, with dGTP as the substrate the

reaction is self-limiting because the secondary structure of polymers of dGTP inhibits polymerisation. By limiting the concentration of dGTP, the rate of elongation can be reduced further, favouring a fairly narrow size distribution. Therefore, cDNA is best tailed with dGTP, while the more abundant vector is best tailed with dCTP.

The method often used for determining tail length depends on performing the tailing reaction in the presence of labelled dNTP. Incorporation is then determined, and by knowing the concentration of both the termini and the unlabelled dNTP, the tail length can be calculated. In theory, with cDNA this method works reasonably well because, as mentioned above, the reaction with dGTP is self-limiting. For efficient tailing the dscDNA must first be purified from the small single and double-stranded DNA fragments which are invariably produced during dscDNA synthesis. However, with dCTP tailing the vector determination of tail length is considerably more difficult. The results only give the average tail length, not the size range, and therefore homopolymer tails need to be sized (by tailing the plasmid, cutting with a restriction enzyme and electrophoresis on a sequencing gel).

The second major disadvantage of the terminal transferase method is that gaps or nicks in the cDNA may be tailed, with resultant loss in cloning efficiency. The E.coli DNA ligase used in the RNase H method seals these nicks, but of course not the gaps.

Addition of Linkers

Adding linkers to the end of blunt-ended dscDNA introduces restriction sites at their ends which can be ligated to compatible termini in the vector. The first stage is to blunt-end the cDNA using T4 DNA polymerase, an

enzyme that fills in receding 5'-ends by a 5' to 3' polymerase activity, and blunt ends protruding 3'-ends by a 3' to 5' exonuclease activity. This step has been shown not to be necessary as the 3' to 5' exonuclease activity of DNA polymerase 1 in the second-strand synthesis results in the blunt ends of a number of molecules. However, because the 3' to 5' exonuclease activity of T4 DNA polymerase is far greater than that of DNA polymerase 1 (Maniatis et al., 1982), the efficiency of cloning is greatly reduced compared to when a T4 DNA polymerase blunt ending step is included (D'Alessio & Gerard, 1988).

The use of linkers involves two ligation reactions. The first is the ligation of the linker to the blunt-ended DNA, and the second is the ligation of the tailed DNA into the vector. Linkers also ligate to one another to form blunt-ended concatamers consisting of linkers alone or linkers attached to cDNA. To generate cohesive ends flanking each DNA fragment, the linkers must be digested with the corresponding restriction enzyme, and the excess linkers no longer attached to the cDNA must be removed to prevent them interfering with the ligation of the tailored cDNA into the vector. If the cDNA contains the same restriction site as the linker it will also be cleaved. This can be prevented in some cases by methylating the cDNA prior to addition of linkers, thereby rendering the cDNA resistant to subsequent restriction endonuclease digestion. If practical, the obvious other way around the problem is to choose linkers which contain a restriction site not present in the dsDNA.

As full-length cDNA copies of TBSV were not being cloned, the theoretical problems favoured the use of linkers or restriction digestion of the dscDNA. Indeed, during a number of attempts when Pst1 cleaved pUC9 supposedly tailed with 20 C residues (as described by Land, 1984) was digested with Sau3A and run on a sequencing gel, the tail length was found to vary from 0-85 residues.

In order to test which restriction endonuclease sites were present in the dscDNA, the products of the T4 DNA polymerase reaction were resuspended in dH₂O. Aliquots were digested with restriction enzymes compatible with the cloning vector pUC19 (Vieira & Messing, 1982) and then electrophoresed through a 1.4% agarose gel (Fig.4.12). As expected, a number of enzymes which have few restriction sites in lambda DNA, such as Xba1 (Sanger *et al.*, 1982), failed to cleave the dscDNA; in the Xba1 lane the dscDNA is no shorter than the dscDNA in the control lane. Other enzymes, such as EcoR1 and HindIII, clearly cut the DNA.

Unfortunately, with the possible exception of BamH1, no common enzyme failed to cut the dscDNA. Although Xba1 does have a site in pUC19, it is dam methylated in E.coli JM101 (Marinus & Morris, 1973; Geier & Modrich, 1979). Sph1 and Sac1 are rather expensive for routine use, and high concentrations of Sal1 and Sma1 are required for efficient digestion. Therefore, it was decided to modify the vector and ligate on linkers which did not have a compatible site in the native pUC19 DNA (see section 4.6).

As the dscDNA was resistant to Xho1 (Fig.4.12), linkers containing this restriction site were ligated onto the ends of the blunt-ended dscDNA. The ligation reaction was subjected to prolonged digestion with Xho1, and the excess linkers and fragments were then removed by passing the

1 2 3 4 5 6 7 8 9 10 11 12 13



FIG.4.12. Digestion of dscDNA with restriction enzymes. TBSV dscDNA prepared by the RNase H method were digested with restriction enzymes, electrophoresed through a 1.4% agarose gel and autoradiographed. Samples in lane 1 and 13 were undigested dscDNA; lane 2, dscDNA digested with Sau3A; lane 3, dscDNA digested with Acc1; lane 4, dscDNA digested with BamH1; lane 5, dscDNA digested with EcoR1; lane 6, dscDNA digested with Hinc11; lane 7, dscDNA digested with Hind111; lane 8, dscDNA digested with Kpn1; lane 9, dscDNA digested with Pst1; lane 10, dscDNA digested with Sal1; lane 11, dscDNA digested with Xba1; lane 12, dscDNA digested with Xho1.

1 2 3 —————→ 22

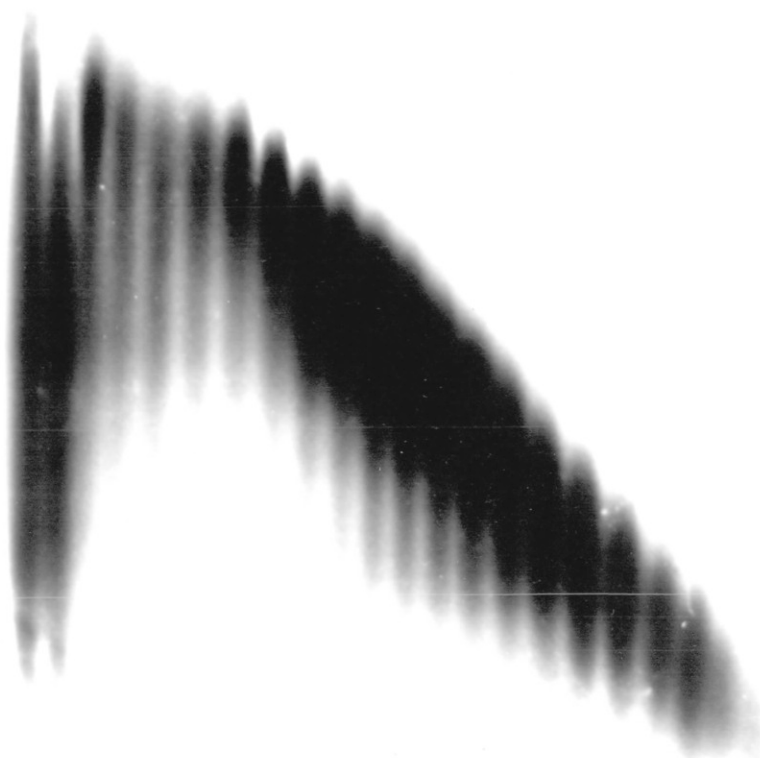


FIG.4.13. Separation of dscDNA from linkers. TBSV dscDNA was blunt-ended using T4 DNA polymerase, ligated to phosphorylated Xho1 linkers, digested with Xho1 and then separated from excess linkers on a Bio-Gel A-50m column. Fractions of the dscDNA were then electrophoresed through a 1.4% agarose gel and autoradiographed. Lane 1 shows the dscDNA prior to blunt-ending, lane 2 shows the dscDNA after blunt-ending, and lanes 3 to 22 are fractions from the Bio-Gel column.

digest down a Bio-Gel A-50m column and collecting fractions. As shown in Fig.4.13, this has the added advantage of crudely fractionating the dscDNA by size. Linkers are often removed by a quicker spermine precipitation, but with such a method all the DNA over 100 nucleotides is recovered.

4.8 MODIFICATION OF pUC19

The two most commonly used cloning vectors are probably pBR322 (Bolivar et al., 1977) and the pUC series of vectors (Vieira & Messing, 1982). The pUC plasmids have largely supplanted pBR322 and related vectors because they have a multiple cloning site (polylinker), containing unique sites for a number of restriction enzymes, contained within part of the lac operon allowing the identification of plasmids containing inserts. The assay for inserts in the polylinker is based on the in situ colour reaction of Xgal. The compound itself does not induce the E.coli lac operon, but is cleaved by β -galactosidase to produce deep blue dibromdichloroindigo. IPTG is added as an inducer of the operon and is not cleaved by the active β -galactosidase. Therefore, under these conditions a defective β -galactosidase is constitutively synthesised under the control of the F' episome, which is complemented by the β -galactosidase fragment produced by the plasmid. This results in blue colonies. The polylinker is a oligonucleotide inserted within the plasmid β -galactosidase gene. Because it is not too long and does not disturb the open reading frame, its presence does not interfere with the plasmid β -galactosidase production. However, when long DNA is

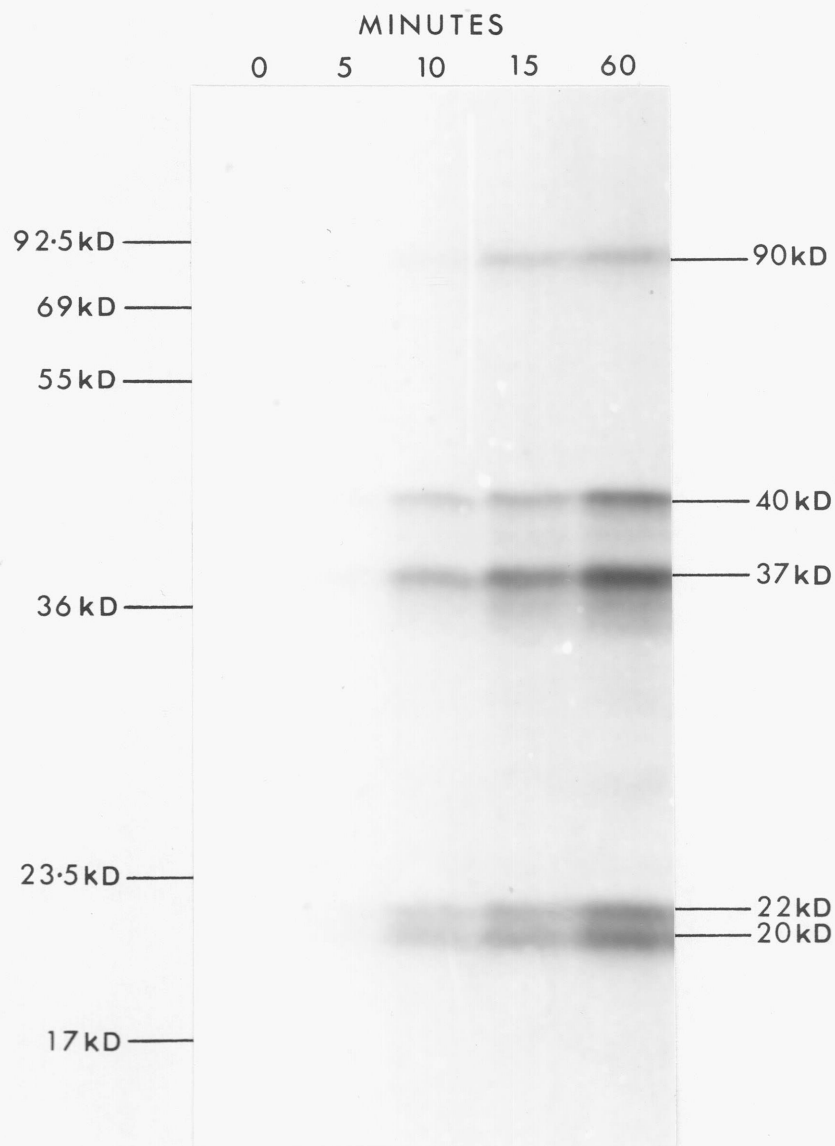


FIG.5.9. Time course of in vitro translation in the presence of 1 mg/ml calf liver tRNA of mRNA from infected plants hybrid-selected by pTBSV65. The positions of Mr markers, phosphorylase-b (92.5 kD), bovine serum albumin (69 kD), human gamma globulin (55 kD and 23.5 kD), lactate dehydrogenase (36 kD) and myoglobin (17 kD) are indicated on the left, and the estimated Mr of the translation products are shown on the right.

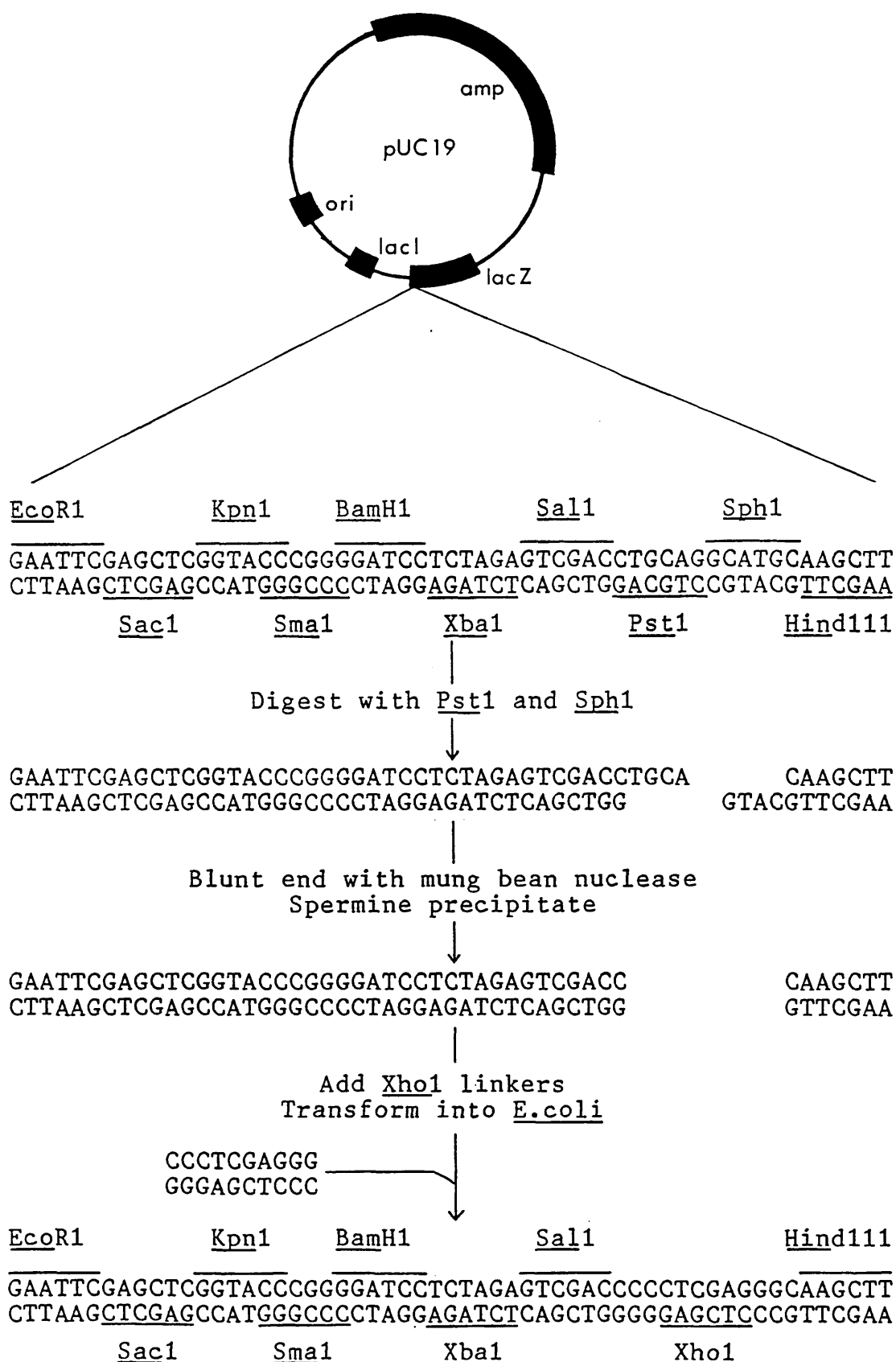


FIG.4.14. Construction of pUC/Xho1 from pUC19.

of tomato golden mosaic virus DNA (Hayes et al., 1988). Plasmid from one of the colonies, designated pUC/Xho1, was then prepared on a large scale and purified by sedimentation in caesium chloride gradients.

The Xho1 tailed dscDNA was ligated into Xho1 digested, alkaline phosphatase treated pUC/Xho1 at a molar ratio of approximately 3:1. The overnight ligation reaction was then diluted 5-fold and transformed into competent E.coli JM101 cells. The transformation efficiencies per microgram of DNA shown in Table 4.3 were obtained. Approximately 95% of the colonies obtained from ligating the dscDNA into the vector were white, a figure which was compatible with the efficiency of the phosphatase reaction. A total of 144 white colonies and one blue colony were picked, and their plasmids isolated by the miniprep method. Two microlitres of the plasmids were then spotted directly onto GeneScreen and hybridised to cDNA prepared to the purified TBSV genome or to nick-translated pUC/Xho1. As shown in Fig.4.15, all the plasmids hybridised to the vector, and the vast majority hybridised to TBSV RNA. The blue colony control failed to hybridise to TBSV RNA, showing that the positive plasmids contained TBSV dscDNA. The 48 most strongly hybridising plasmids were then digested with Xho1 and electrophoresed through 1.2% agarose gels (Fig.4.16a). Southern blotting confirmed the identity of the inserts (Fig.4.16b).

Table 4.3 Transformation frequencies of ligations

	Transformants/ug DNA
Supercoiled pUC9-----	3 x 10 ⁸
Supercoiled pUC/ <u>Xho</u> 1-----	1 x 10 ⁸
Linearised pUC/ <u>Xho</u> 1-----	2 x 10 ²
Linearised and religated pUC/ <u>Xho</u> 1-----	9 x 10 ⁶
Linearised, phophatased and religated pUC/ <u>Xho</u> 1-----	3 x 10 ⁴
Linearised, phosphatased ligated pUC/ <u>Xho</u> 1 dscDNA-----	6 x 10 ⁶

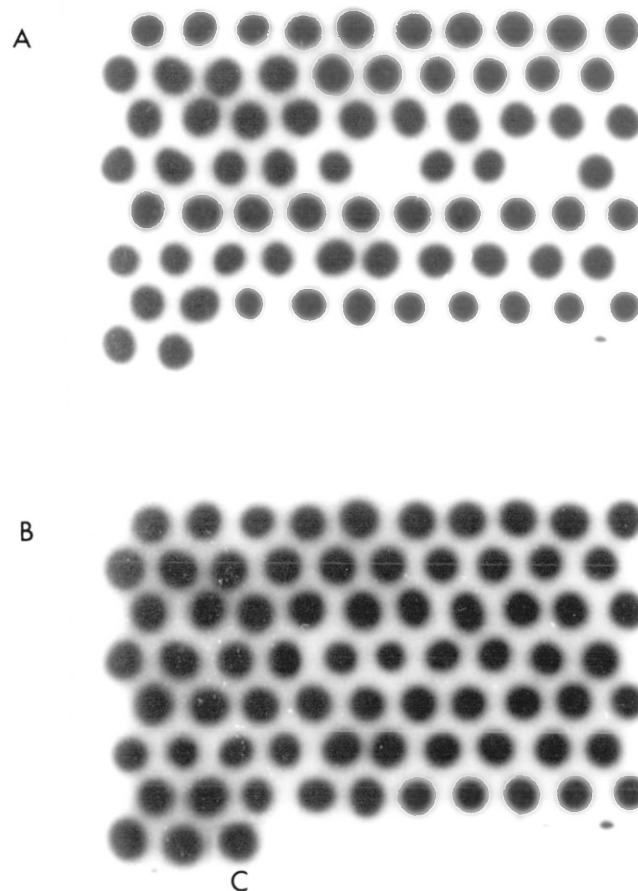


FIG.4.15. Dot blots of putative TBSV clones. Xho1 tailed dscDNA was ligated into pUC/Xho1 and transformed into competent E.coli JM101 cells. Plasmids from white colonies were dotted onto GeneScreen and hybridised to (a) cDNA prepared from genomic RNA or to (b) nick-translated labelled pUC/Xho1. The dot labelled C in (b) represents pUC/Xho1 DNA isolated from a blue colony.

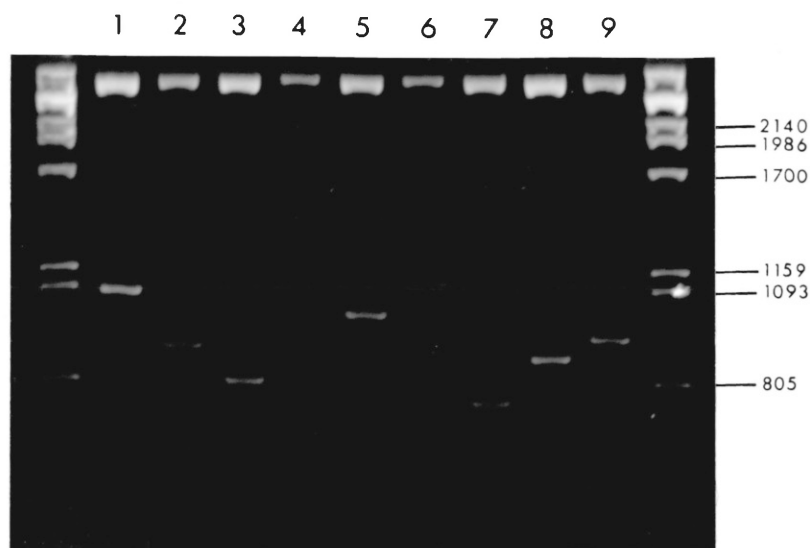


FIG.4.16a. An example of Xho1 digested recombinant plasmids. Plasmids which hybridised to cDNA in Fig.4.15 were digested with Xho1, and then electrophoresed through 1.2% agarose gels and stained with ethidium bromide.

(b) The plasmids in Fig.4.16a were Southern blotted onto GeneScreen and hybridised to cDNA prepared from TBSV genomic RNA.

4.9 RESTRICTION MAPPING OF cDNA CLONES

The 21 longest clones, all of 700 bp or longer (Table 4.4), were digested with Xho1, electrophoresed on 1.2% gels (Fig.4.17a) and Southern blotted. A number of clones were not digested to completion, and therefore these lanes contain a small amount of the open-circle and linear form of the plasmid. One of the longest clones, pTBSV118, was labelled by nick translation and used as a probe (Fig.4.17b). As well as hybridising to the vector DNA, pTBSV118 hybridised to a few inserts from other clones. The Southern blotting was then repeated using pTBSV41. This clone hybridised to most of the inserts (Fig.4.17c), including a number which did not hybridise to pTBSV118. The procedure was again repeated with pTBSV65, one of the clones which hybridised to the insert of pTBSV41 but not to the insert of pTBSV118 (Fig.4.17d).

All the 23 clones hybridised to at least one of the three probes (Table 4.4). The lack of cross hybridisation between pTBSV118 and pTBSV65 show their inserts to be derived from different sequences of the TBSV RNA template. Like many of the other clones, both pTBSV65 and pTBSV118 overlap with pTBSV41, showing that this clone contains sequences between pTBSV118 and pTBSV65. The sequences of any insert which hybridised to pTBSV41 must be therefore be contained within either pTBSV118 or pTBSV65, as these latter two clones were much longer than the others. Only four clones - pTBSV10, pTBSV66, pTBSV99 and pTBSV104 - hybridised to either pTBSV65 or pTBSB118 but not to pTBSV41. Therefore, their inserts might also contain a portion of the TBSV sequence not found within the inserts of either pTBSV118 or pTBSV65.

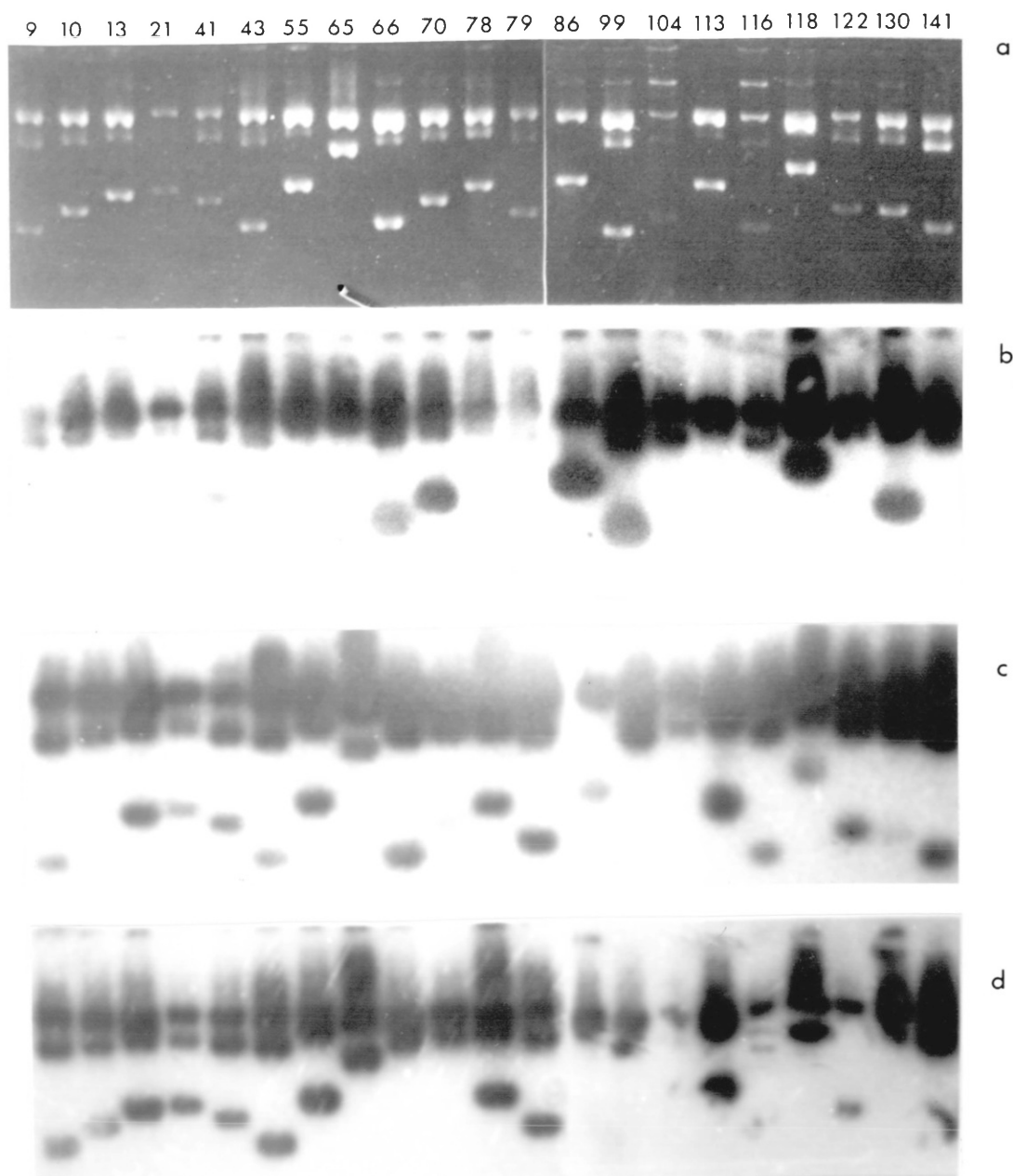


FIG.4.17. Cross-hybridisation of TBSV clones. The 21 longest clones were digested with Xho1, electrophoresed through a 1.2% gel (a) and then Southern blotted onto GeneScreen. The plasmids were then hybridised to (b) ^{32}P -labelled nick-translated pTBSV118, (c) ^{32}P -labelled nick-translated pTBSV41 or (d) ^{32}P -labelled nick-translated pTBSV65.

Table 4.4 Cross-Hybridisations of TBSV Clones

Clone	Length (bp)	Hybridises to		
		118	41	65

9	750	-	+	+
10	880	-	-	+
13	1030	-	+	+
21	1060	-	+	+
41	940	+	+	+
43	700	-	+	+
55	1060	-	+	+
65	1710	-	+	+
66	770	+	-	-
70	950	+	+	-
78	1110	-	+	+
79	850	-	+	+
86	1210	+	+	-
99	720	+	-	-
104	820	-	-	+
113	1110	-	+	+
116	800	-	+	+
118	1390	+	+	-
122	870	-	+	+
130	840	+	+	-
141	780	+	+	+

In order to more precisely estimate the degree of overlapping of the clones, pTBSV41, pTBSV65 and pTBSV118 were restriction endonuclease mapped. All the single enzyme digests were performed on Xho1 digested clones (Figs.4.18, 4.19 and 4.20). Single enzymes allowed the restriction site(s) relative to the ends of the insert to be estimated, and allowed the orientation of the insert relative to the vector polylinker to be established, while digestion with pairs of enzymes allowed the position of the sites relative to each other to be calculated. The orientation of pTBSV41, pTBSV65 and pTBSV118 relative to each other was established by digesting each clone with a couple of restriction enzymes (Fig. 4.21a), Southern blotting, and then probing with each clone in turn (Figs.4.21b, c & d).

Sizes of restriction fragments produced in single and double enzyme digestions of the three clones are given in Table 4.5. Whilst locating most of the restriction sites was quite straight forward, three sites required some extra mapping:

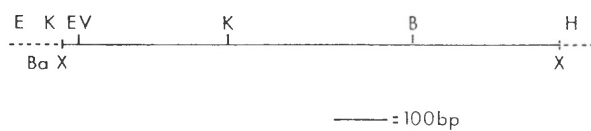
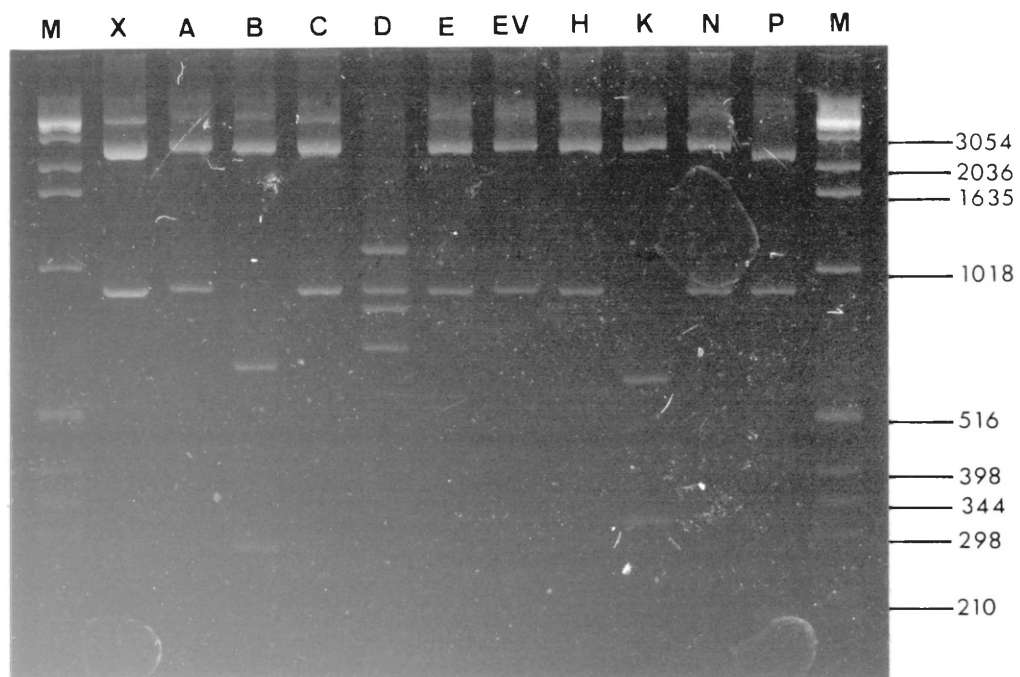
(1) Initially the Kpn1 digestion of pTBSV65 proved to be rather puzzling. Only one fragment of approximately 780 bp seemed to be produced when the insert was digested with Kpn1, and digestion of the uncut clone with Kpn1 also produced a fragment of the same size (Fig.4.19). This suggested that there was a single Kpn1 site in the centre of the insert. In the case of the Xho1 digested plasmid, the Kpn1 site in the centre of the insert would result in two insert restriction fragments of approximately the same size upon further digestion with Kpn1. Because of the presence of the Kpn1 site in the polylinker, digestion of the clone with only Kpn1 results in a single insert fragment. However, the existence of a Kpn1 site in pTBSV41 suggested that there may be a second site near the end of the pTBSV65 insert. Therefore, pTBSV65 was digested with

Table 4.5. Restriction Endonuclease Fragment Sizes of TBSV
Clones

Clone	Restriction Enzyme	Fragment Sizes (base pairs)			
pTBSV41	<u>Bgl</u> 111	660	+	280	
	<u>Kpn</u> 1	610	+	320	
pTBSV65	<u>Bgl</u> 111	1220	+	500	
	<u>Eco</u> RV	1110	+	330	+ 260
	<u>Hind</u> 111	1290	+	430	
	<u>Hpa</u> 1	1150	+	400	+ 180
	<u>Kpn</u> 1	790	+	790	+ 150
	<u>Nco</u> 1	1240	+	470	
pTBSV118	<u>Eco</u> R1	880	+	500	
	<u>Eco</u> RV	1180	+	220	
	<u>Hind</u> 111	1200	+	190	

None of the clones contained inserts that were digested with Acc1, BamH1, Cla1, Dra1, Pvu1, Sac1, Sal1, Sca1, Sma1, Xba1 or Xho1.

FIG.4.18. Restriction mapping of pTBSV41 using single restriction enzyme digestions of Xho1-digested pTBSV41. The lower diagram shows the positions of the restriction sites. The solid line represents the dscDNA insert, and the broken line (not to scale) represents the pUC/Xho1 polylinker. A, Acc1; Ba, BamH1; B, Bgl11; C, Cla1; D, Dra1; E, EcoR1; EV, EcoRV; H, Hind111; K, Kpn1; N, Nco1; P, Pst1; X, Xho1; M, BRL kb ladder.



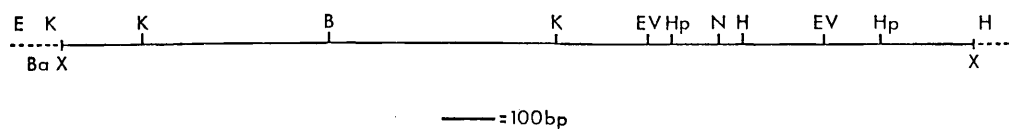


FIG.4.19. Restriction mapping of pTBSV65 using (a) single restriction enzyme digestions of Xho1-digested pTBSV65, and (b) single and double restriction enzyme digestions of undigested pTBSV65. The diagram shows the positions of the restriction sites. The solid line represents the dscDNA insert, and the broken line (not to scale) represents the pUC/Xho1 polylinker. Ba, BamH1; B, Bgl111; C, Cla1; E, EcoR1; EV, EcoRV; H, Hind111; Hp, Hpa1; K, Kpn1; N, Nco1; P, Pst1; X, Xho1; M, BRL kb ladder.

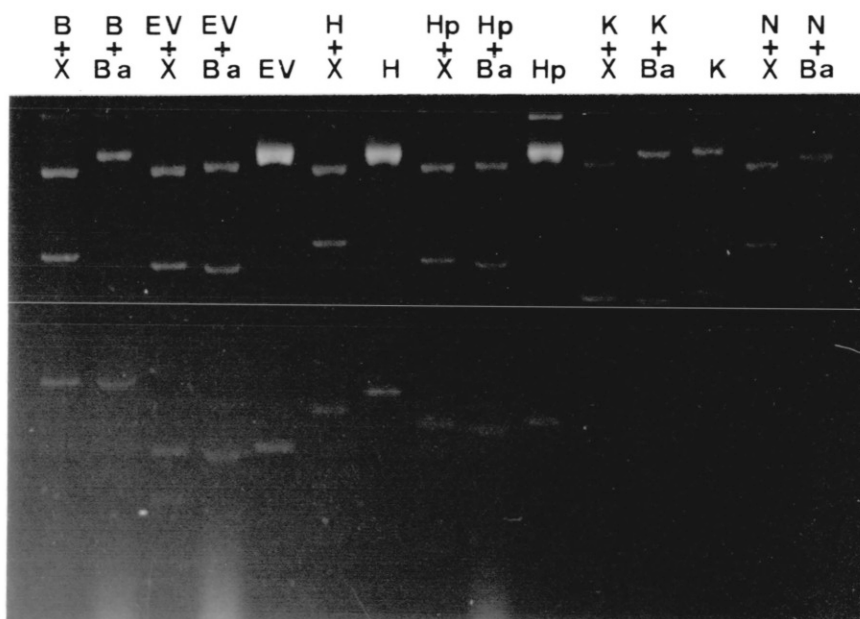
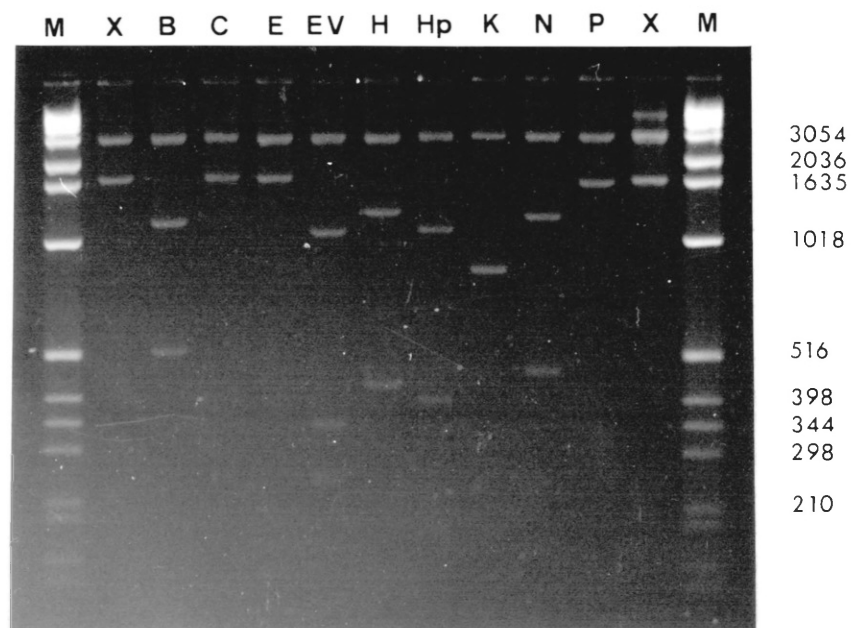
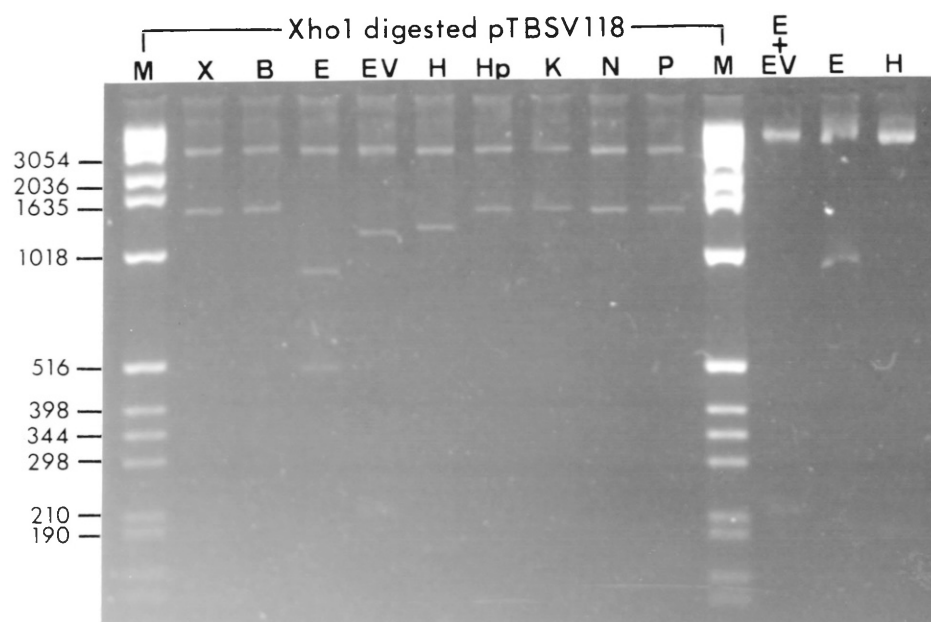


FIG.4.20. Restriction mapping of pTBSV118 using single enzyme restriction digestions of Xho1 digested pTBSV118 and enzyme restriction digestions of undigested pTBSV118. The lower diagram shows the positions of the restriction sites. The solid line represents the dscDNA insert, and the broken line (not to scale) represents the pUC/Xho1 polylinker. Ba, BamH1; B, Bgl111; E, EcoR1; EV, EcoRV; H, Hind111; Hp, Hpa1; K, Kpn1; N, Nco1; P, Pst1; X, Xho1; M, BRL kb ladder.



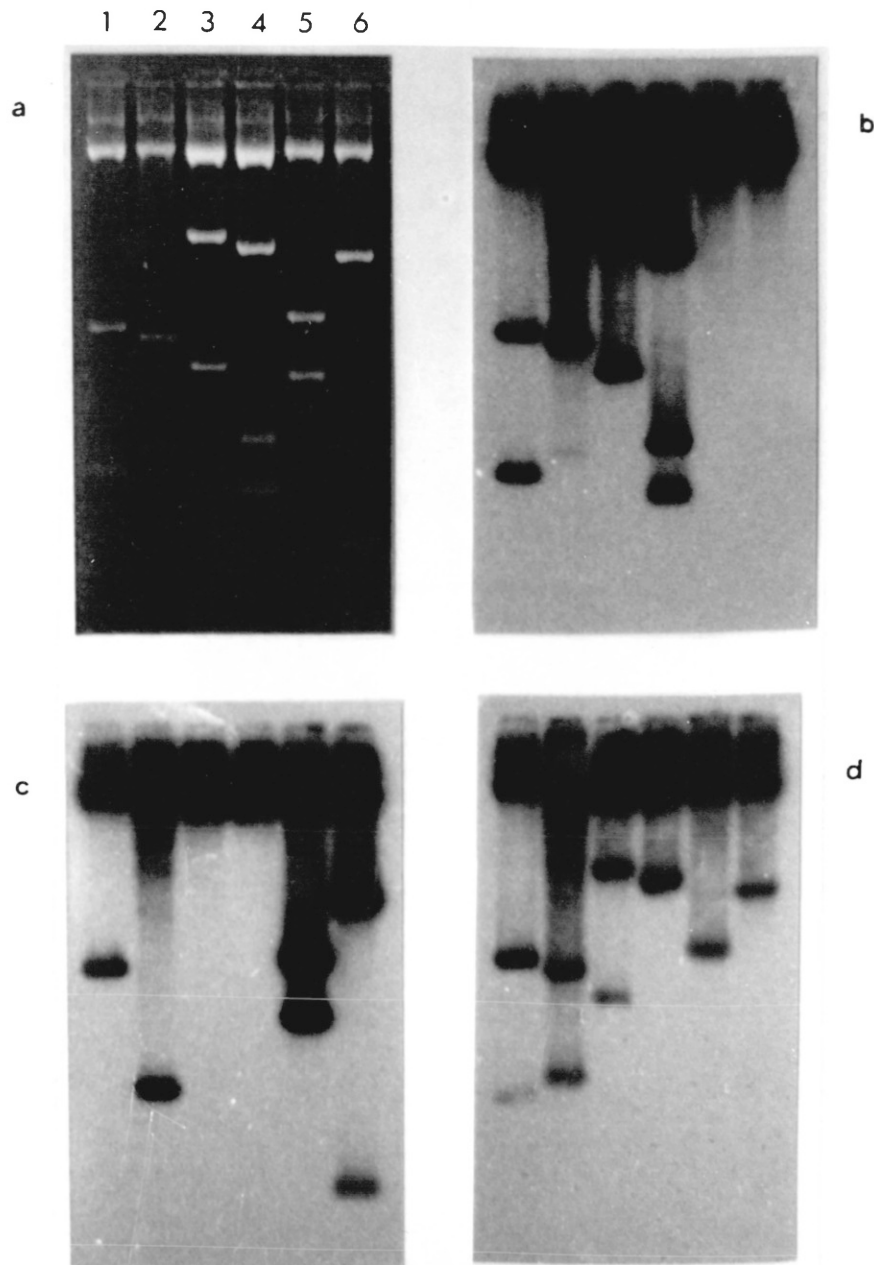


FIG.4.21. Cross-hybridisation of pTBSV41, pTBSV65 and pTBSV118. pTBSV41 was digested with Bgl11 (lane 1) or Kpn1 (lane 2); pTBSV65 was digested with Bgl11 (lane 3) or EcoRV (lane 4); pTBSV118 was digested with EcoR1 (lane 5) or Hind111 (lane 6). Each digested clone was then electrophoresed through a 1.4% agarose gel and stained with ethidium bromide (a). The gel was then Southern blotted and hybridised in turn to ^{32}P -labelled nick-translated (b) pTBSV65, (c) pTBSV118, or (d) pTBSV41.

Asp718 (because Kpn1 creates a 3' protruding end, this restriction sites cannot easily be end-labelled using Klenow fragmnet, and therefore the Asp718 isoschizomer was used), end-labelled using Klenow fragment and electrophoresed through a 8% polyacrylamide gel (Fig 4.22, lane 1). The upper band represents end-labelled vector, the middle band represents the larger 780 bp Kpn1 (Asp718) fragment and the lower band represents the 140 bp Kpn1 (Asp718) fragment. Presumably this band was too small to be detected by ethidium bromide staining in Fig.4.19. As expected, digestion of pTBSV65 with Asp718 and Xho1 resulted in the same size fragments (lane 2). However, the 780 bp band is approximately twice as intense in lane 2., confiming that two insert fragments of approximately the same size are produced after digestion of pTBSV65 with Kpn1 and Xho1.

(2) Initially it was thought that there was no Pst1 site in the insert of pTBSV65, as digestion of the Xho1 cut clone with Pst1 did not change the mobility of the insert significantly (Fig.4.19). However, when the uncut pTBSV65 was digested with Pst1 (Fig.4.23, lane 1), the clone was linearised. As the Pst1 site in the vector had been destroyed (Fig.4.14), there must be a Pst1 site within the insert. Digestion of pTBSV65 with EcoR1 and Pst1 gave a fragment approximately the same size as the insert (Fig.4.23), and therefore the Pst1 site must be close to the end of the insert. This was confirmed by digesting pTBSV65 with Pst1 and Hind111, end-labelling and electrophoresis through a 20% polyacrylamide gel. A labelled fragment of approximately 40bp was observed (Fig.4.24, lane 1). No such fragment was produced by Hind111 digestion.

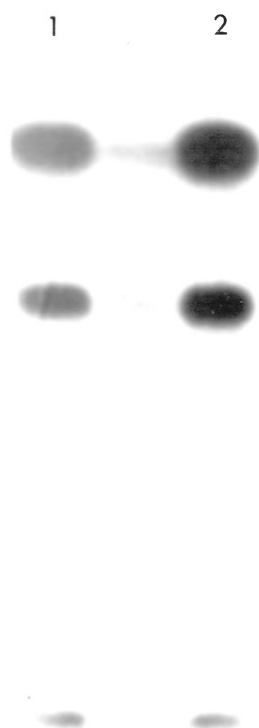


FIG.4.22. pTBSV65 was digested with (lane 1) Asp718 or (lane 2) Asp718 and Xho1 and ^{32}P -labelled using Klenow fragment. The DNA was then electrophoresed through a 8% polyacrylamide gel and autoradiographed.

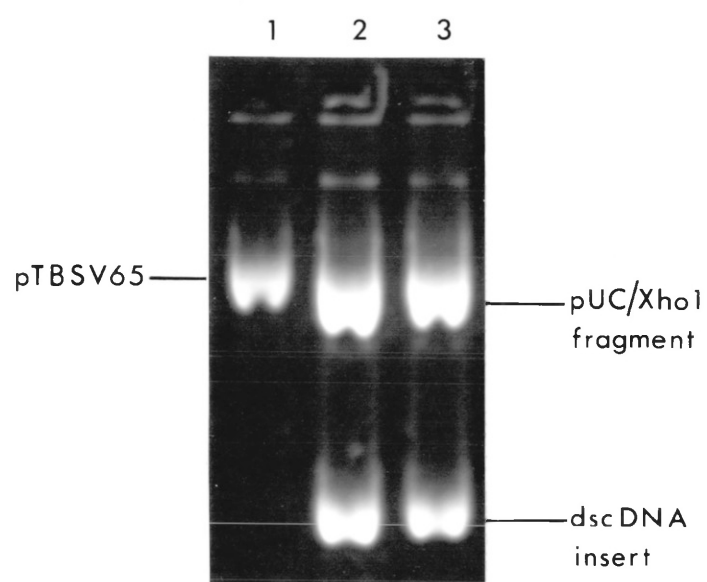


FIG.4.23. pTBSV65 was digested with (lane 1) PstI, (lane 2) XhoI, or with (lane 3) PstI and EcoRI, electrophoresed through a 1% agarose gel and stained with ethidium bromide.

(3) After a EcoRV site had been found in pTBSV118, an equivalent site was found in pTBSV41 (Fig.4.18). Using end-labelling and electrophoresis through a 20% polyacrylamide gel, the EcoR1 and EcoRV digested pTBSV41 fragment was shown to be approximately 30bp (Fig.4.24, lane 3). pTBSV118 was digested with Xho1 and EcoRV, electrophoresed through a 1.4% agarose gel (Fig.4.25, lane 1) and Southern blotted. pTBSV41 was digested with EcoRV and Hind111 and electrophoresed through a 1% agarose gel. The band corresponding to the vector plus the 30 bp insert fragment was then purified, end-labelled and used to probe the Southern blot of pTBSV118. As expected the 30 bp insert of pTBSV41 only hybridised to the larger insert fragment of pTBSV118 (Fig.4.25, lane 2).

As mentioned above, the cross-hybridisation results indicated that only four clones could contain TBSV RNA sequences not contained within pTBSV41, pTBSV65 or pTBSV118. However, when they were restriction mapped it was found that their inserts only contained sequences found within pTBSV65 or pTBSV118. The relative positions of the clones are shown in Fig.4.26.

Although approximately 3100 bp of the TBSV genome are represented in the three clones pTBSV118, pTBSV41 and pTBSV65, it is interesting that the 21 clones should all be grouped around one region of the genome. Contrary to the results of Gould and Symons (1977), this would suggest that most of the cDNA produced during first-strand synthesis was not representative of the whole RNA template from which it is transcribed. This may be a function of the primers, or more likely, there may be extensive secondary structure within the RNA template preventing transcription of certain regions.

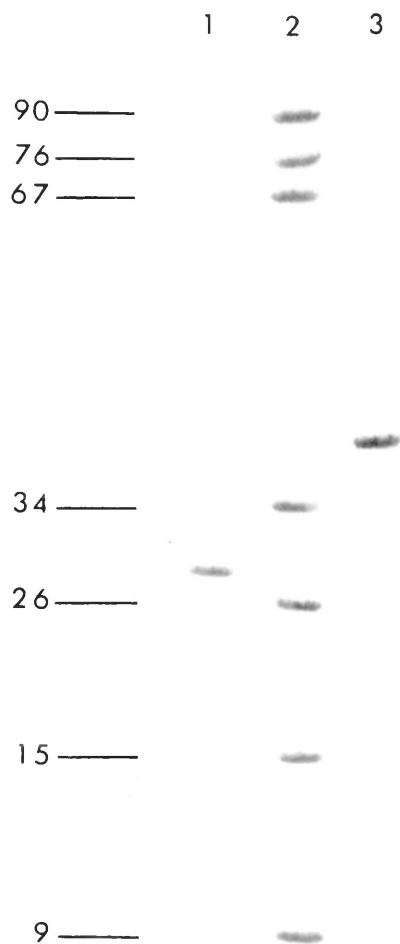


FIG.4.24. Autoradiograph of end-labelled DNA fragments after electrophoresis through a 20% polyacrylamide gel. Lane 1, pTBSV65 digested with Hind111 and Pst1; lane 2, pBR322 digested with Msp1; lane 3, pTBSV41 digested with EcoR1 and EcoRV. The lengths of the pBR322 fragments are shown in bp.

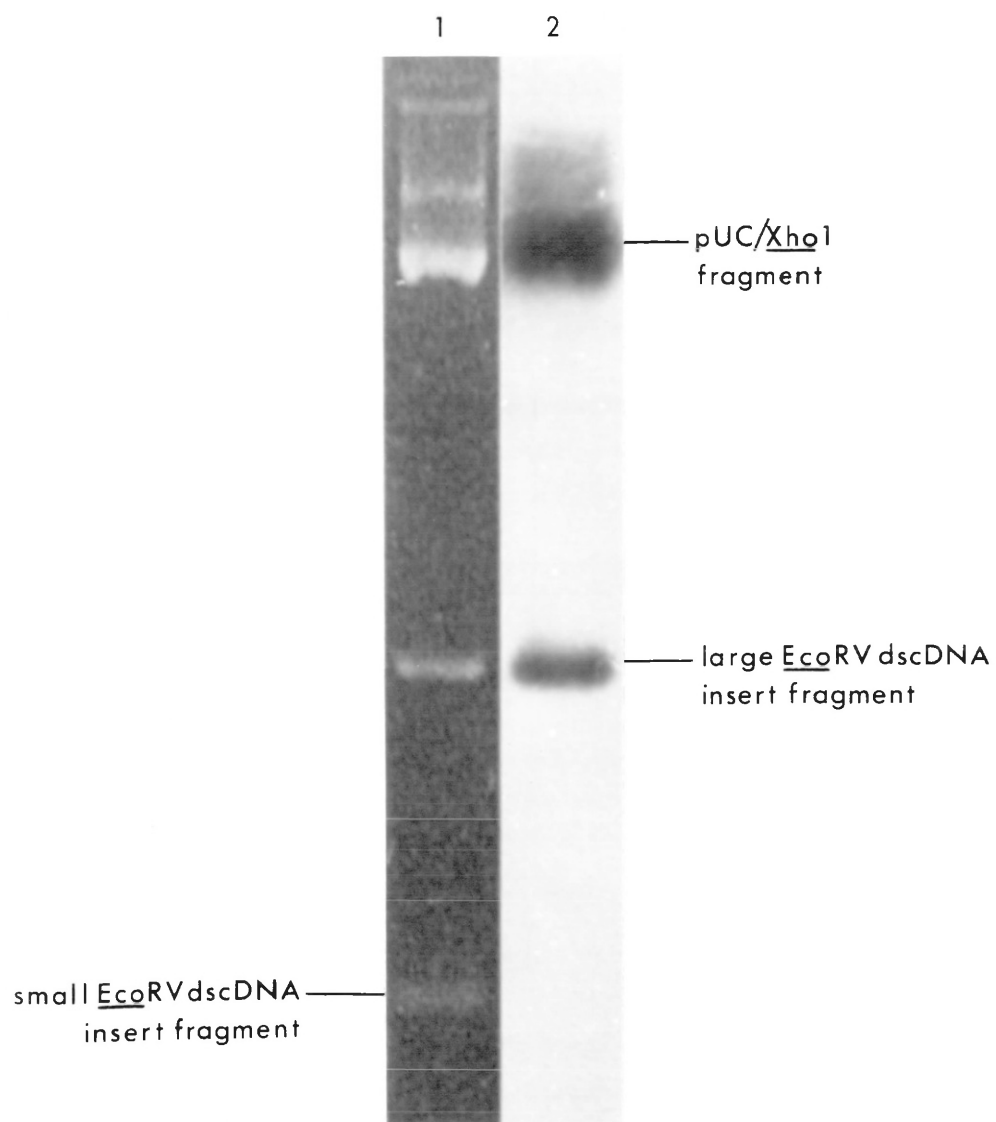


FIG.4.25. XhoI digested pTBSV118 was further digested with EcoRV and electrophoresed through a 1.4% agarose gel and stained with ethidium bromide (lane 1). The gel was then Southern blotted and hybridised to the ^{32}P end-labelled 30bp EcoRV-EcoRV pTBSV41 fragment (Fig.4.24, lane 3).

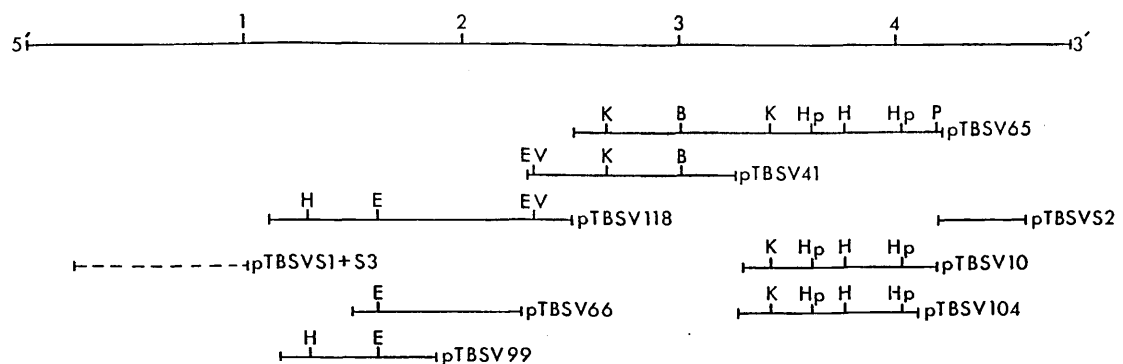


FIG.4.26. Map of selected clones of TBSV cDNA and their relation to the virus genome (upper line), calibrated in kb. The relative positions of pTBSVS1 and pTBSVS3 within the 5'-terminal 1 kb of the genomic RNA were not determined and these clones are indicated by a broken line. Nomenclature for restriction endonucleases as in Figs.4.18, 4.19 and 4.20.

4.10 DETERMINATION OF THE LOCATION OF CLONE INSERTS

To determine the orientations of the clone inserts relative to the 5'- and 3'-ends of the genomic RNA, E.coli RNase H was used. This enzyme is an exonuclease which specifically degrades the RNA portion of a RNA-DNA hybrid (Maniatis et al., 1982). By hybridising the clones to the RNA, adding RNase H and then analysing the resultant RNA on a denaturing gel it was hoped the clone closest to the 3'-end of the RNA could be identified. A similar technique was used to obtain the minimal length of the 3'-end of BMV which would react with BMV RNA-dependent RNA polymerase (Ahlquist et al. 1984a). RNA-DNA hybrids are often used for S1 mapping of transcripts and primer extension (Calzone et al., 1987). Ideally the DNA should be in single-stranded form; techniques exist for using the double-stranded form, with selective annealing temperatures which promote the formation of the RNA-DNA hybrid after thermal denaturation at the expense of the double-stranded DNA form (Calzone et al., 1987). However, single-stranded DNA overcomes the problem of determining the optimum annealing temperature.

The smaller HindIII fragments of pTBSV118 and pTBSV65 were subcloned into the HindIII site of M13mp8 RF (Messing, 1983). After transformation, white plaques were picked and the single-stranded DNA forms isolated. Only the plus-strand of the M13 RF form is packaged. As the insert could ligate into the M13 vector in either orientation, the isolated phage ssDNA can contain either strand of the insert. When M13 clones containing the opposite strands of the same insert are annealed, only the inserts hybridise. As hybridisation lowers the mobility of the annealed M13 clones, clones with opposite inserts can be detected by electrophoresis through agarose gels. Therefore, one of the M13 clones was hybridised to the ssDNA from a number of other clones. Fig.4.27 shows one

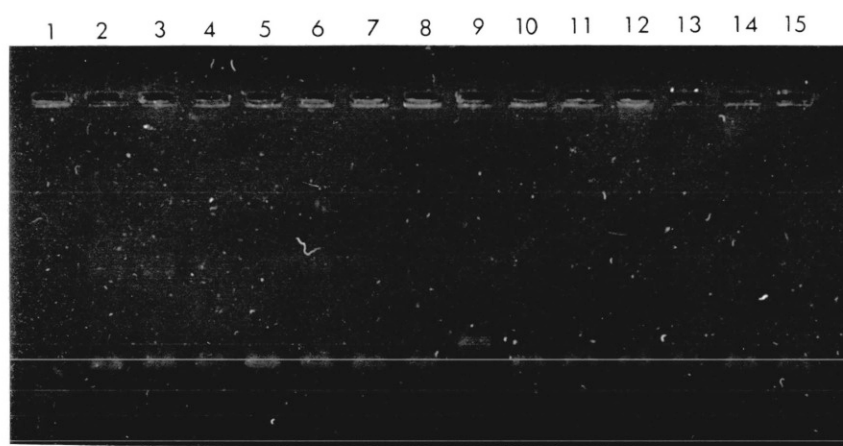


FIG.4.27. Single-stranded recombinant M13 DNA from different white plaques (lanes 2 to 15) was hybridised to the ssDNA in lane 2, electrophoresed through a 0.8% agarose gel and stained with ethidium bromide. Lane 1 contains M13mp8 DNA isolated from a blue plaque.

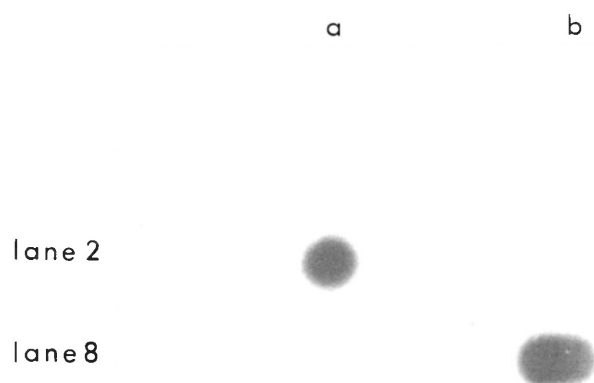


FIG.4.28. Recombinant M13 DNA (Fig.4.27, lanes 2 and 8) were dotted onto GeneScreen, hybridised to [5-³²P]pCp labelled genomic RNA (lane a) or cDNA prepared from genomic RNA (lane b) and autoradiographed.

set of clones (M13mp8 containing the smaller Hind111 fragment of pTBSV65 - designated mTBSV65HS) after hybridisation to the M13 clone in lane 2 and electrophoresis through a 0.8% agarose gel. Note the lower mobility in lane 8, showing that the M13 clone in lane 2 has hybridised to the M13 clone in lane 8. These two M13 clones, with inserts in the opposite orientations, were then dotted onto Genescreen and hybridised to [5'-³²P]pCp labelled genomic RNA or cDNA prepared from genomic RNA (Fig.4.28). Those that hybridised to the labelled RNA were designated mTBSV65HS(-) and mTBSV118HS(-), and those which hybridised to the cDNA (ie. had inserts in the same sense as the genomic RNA) were designated mTBSV65HS(+) and mTBSV118HS(+).

Each of the M13 clones was hybridised to the genomic RNA and treated with RNase H and alkaline phosphatase. The reaction was then divided into two aliquots. After phenol extraction, precipitation, resuspension in dH₂O and heating to 90°C for 3 minutes, the RNA in the first aliquot was labelled with [γ -³²P]ATP using T4 polynucleotide kinase, and the RNA in the second aliquot was labelled with [5'-³²P]pCp using RNA ligase. The reaction products were then electrophoresed in a 1.4% agarose gel containing 10 mM methylmercuric hydroxide (Fig.4.29). Lane 1 shows TBSV genomic RNA labelled at the 3'-end, again showing that the RNA has a 3'-OH group. However, the RNA fails to be labelled with [γ -³²P]ATP (lane 2) due to the blocked 5'-end. The same results are seen with mTBSV65HS(+) and mTBSV118HS(+) (lanes 3 & 4 and lanes 7 & 8), showing that these clones do not hybridise to the RNA. However, mTBSV65HS(-) and mTBSV118HS(-) do hybridise to the RNA, resulting in cleavage by the RNase H. Lanes 5 & 9 contain three bands. The upper band co-migrates with the labelled genomic band, indicating that this band represents undigested RNA. The two lower bands

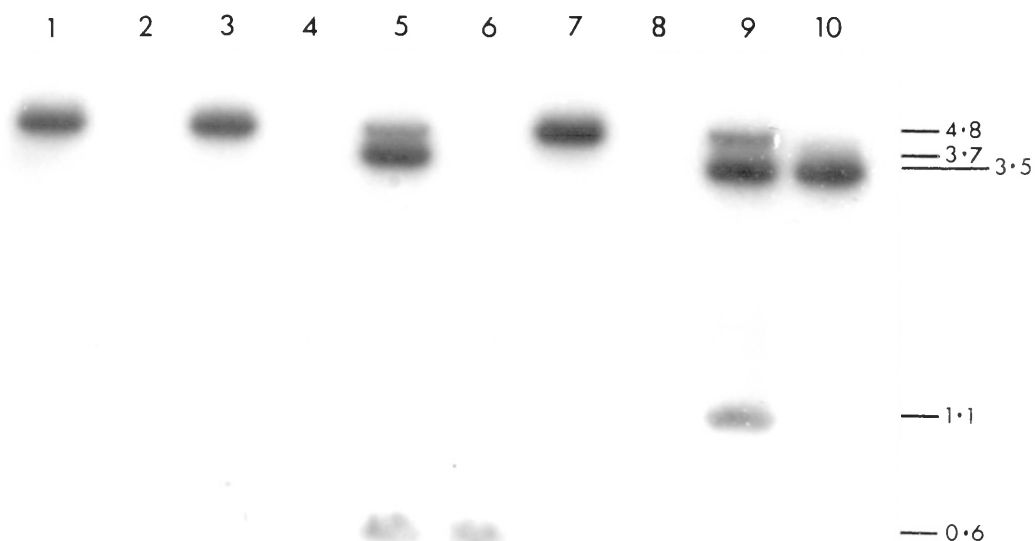


FIG.4.29. Autoradiograph of RNA fragments generated by RNase H digestion of hybrids formed between genomic RNA and cloned cDNA and separated by electrophoresis in a 1% agarose gel containing 10 mM methylmercuric hydroxide. TBSV genomic RNA was hybridised to ssDNA of: Lanes 1 and 2, M13mp8; lanes 3 and 4, mTBSV65HS(+); lanes 5 and 6, mTBSV65HS(-); lanes 7 and 8, mTBSV118HS(+); lanes 9 and 10, mTBSV118HS(-). RNA was labelled using $[5-^{32}\text{P}]\text{pCp}$ (lanes 1, 3, 5, 7 and 9) or $[\gamma-^{32}\text{P}]\text{ATP}$ (lanes 2, 4, 6, 8 and 10). RNA sizes on the side of the gel are in kb.

represent smaller 3'-end labelled RNA; one of these will be the original genomic 3'-end, and the other will be the new 3'-end created by the action of RNase H on the RNA-DNA hybrid. Only one band is present in lanes 6 & 10. Since the original 5'-end is blocked, this represents a newly created 5'-end.

As well as determining which clone is closest to the 5'-end and 3'-end of the RNA, comparing lanes 5 & 6 and lanes 9 & 10 allows the distance of the clones from both ends of TBSV to be estimated. This is shown diagrammatically in Fig.4.26.

4.11 CLONING OF THE 5' AND 3'-ENDS OF TBSV RNA

Although the majority of the TBSV sequence was contained within two clones, approximately 1.1 kb at the 5'-end and 0.6 kb at the 3'-end remained to be cloned. When methylmercuric hydroxide was used to denature the RNA prior to the first-strand reaction, there was shown to be no increase in the length of the cDNA (Fig.4.9). However, denaturation might allow the reverse transcriptase to transcribed a wider range of template sequences. Therefore, the first-strand cDNA reaction was repeated after denaturation. The dscDNA was shown earlier to be susceptible to digestion with restriction endonucleases. The newly synthesised dscDNA was digested with Sau3A, ligated into BamH1-digested, phosphatase treated pUC/Xho1 and transformed into E.coli JM101 cells. Resultant white colonies (96) were picked, stabbed into a fresh 2xTY plate, transferred to Genescreen and lysed with sodium hydroxide. The colonies were then probed with the labelled inserts of pTBSV65 and pTBSV118, or with random primed cDNA prepared from denatured RNA. A number of clones which hybridised to the TBSV RNA but not to pTBSV65 or pTBSV118 were identified (Fig.4.30). Twelve of these were

linearised with Xho1 and electrophoresed through a 1% agarose gel (Fig.4.31a). The clones had one of three different sized TBSV dscDNA (Fig.4.31b) inserts of approximately 0.2 kb (lanes 2, 4, 7, 9, 10 and 13), 0.4 kb (lanes 3, 5, 8 and 11) or 0.6 kb (lanes 6 and 12). The clone in lanes 2, 3 and 6 were termed pTBSVS1, pTBSVS2 and pTBSVS3 respectively.

pTBSVS1 was digested with Sau3A, end-labelled and electrophoresed through a 5% polyacrylamide gel. The dscDNA insert was located by autoradiography and isolated from the gel. Using the pUC/Xho1 fragments as size markers, the size of the insert was calculated to be approximately 220 bp. This insert was then used in Southern blot analysis of the other Sau3A clones. The Xho1 linearised clones were electrophoresed through an agarose gel (identical to Fig.4.31a), transferred to GeneScreen and hybridised to the pTBSVS1 insert (Fig.4.31b). Only the clones with inserts of approximately 200 bp hybridised to the pTBSVS1 insert, indicating that the clones of this size contained the same Sau3A dscDNA fragment.

Fig.4.31a was then Southern blotted and probed with the isolated 3'-end 0.6 kb RNA fragment shown in Fig.4.29, lanes 5 and 6. Only the 0.4 kb insert of pTBSVS2 and the clones in lanes 5, 8 and 11 hybridised to the 3'-terminal RNA fragment (Fig.4.31d). This indicated that the 0.4 kb inserts of these four clones were the same Sau3A fragments, and derive from the 3'-end of the RNA.

Additionally, the same clones hybridised to the small 40 bp Pst1-Hind111 insert fragment of pTBSV65 isolated from the polyacrylamide gel shown in Fig.4.24 (Fig.4.31e), but not to the larger Pst1-EcoR1 insert fragment (Fig.4.31f), showing that pTBSVS1 overlaps with pTBSV65, but by no more than 40 bp.

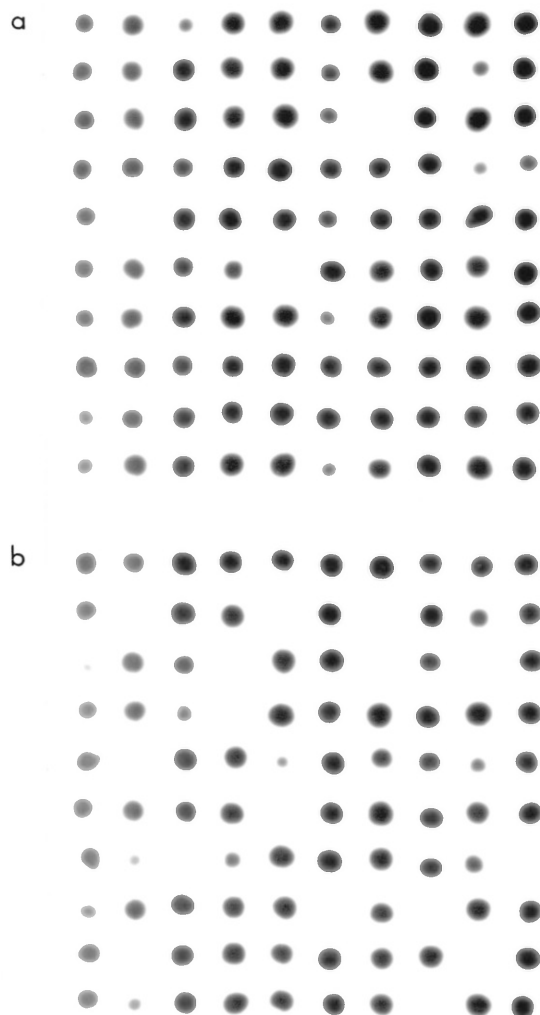


FIG.4.30. Colony blots of recombinant clones. White colonies were stabbed onto fresh 2xTY agar plate, lifted onto GeneScreen, lysed and hybridised to (a) cDNA prepared from genomic RNA, or (b) ³²P-labelled nick-translated dscDNA inserts from pTBSV65 and pTBSV118.

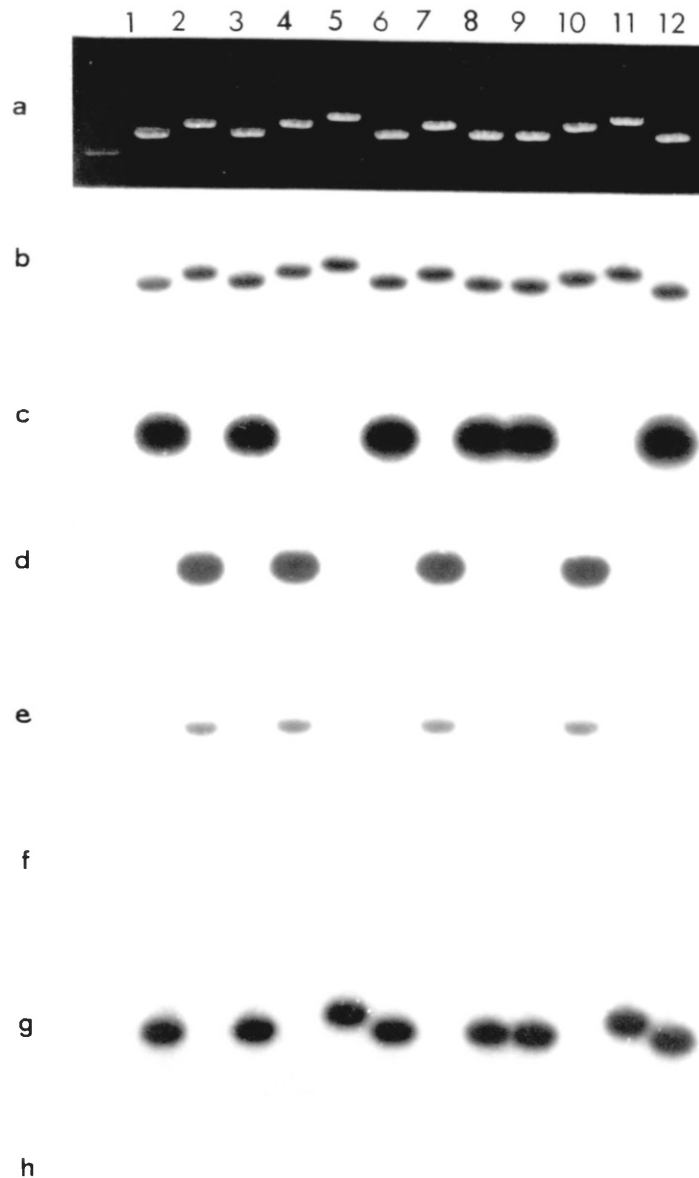


FIG.4.31. Southern blot analysis of Sau3A clones. Twelve Sau3A clones were linearized with XhoI, electrophoresed through a 1% agarose gel and stained with ethidium bromide (a). The gel was then Southern blotted and hybridised to: (b) cDNA prepared from genomic RNA; (c) pTBSVS1 Sau3A dscDNA insert; (d) 3'-terminal 0.6 kb RNA fragment (Fig.4.29, lanes 5 and 6); (e) 40 bp PstI-HindIII fragment of pTBSV65; (f) large PstI-EcoRI fragment of pTBSV65; (g) 5'-terminal 1.0 kb RNA fragment (Fig.4.29, lanes 9 and 10); (h) the dscDNA insert of pTBSV118.

The 5'-end 1.1 kb RNA fragment in Fig.4.29, lane 9 was then used to probe Fig.4.31a. Both pTBSVS1 and pTBSVS2 hybridised, showing that these clones were definitely derived from the 5'-end of TBSV RNA (Fig.4.31g). Confirmation that these clones did not overlap pTBSV118 was obtained by using the nick-translated insert as a probe (Fig.4.31h).

In all the hybridisations shown in Fig.4.31, all the clones with inserts of the same size hybridise to the same probe. This indicates that the clones of the same size contained the same Sau3A inserts. Although the genome had not be cloned in its entirety, a number of non-overlapping clones with total insert size of approximately 4300 bp, or 90% of the genome, had been obtained. The sizes of the Sau3A inserts of pTBSVS2 and pTBSVS3 are not very accurate because they were determined by calculating the difference between the clone size and vector size, Also, the distances of the Sau3A fragments of pTBSVS1 and pTBSVS3 from the 5'-ends of the genomic RNA were not determined. Therefore, only approximate locations and sizes are given in Fig.4.26.

CHAPTER 5

RESULTS: HYBRID SELECTION AND TRANSLATION OF TBSV RNA

5.1 NORTHERN BLOT ANALYSIS OF TBSV RNA USING TBSV CLONES

The TBSV dsDNA clones were used to probe Northern blots of ssRNA from puromycin-treated polysomes and the encapsidated RNA from RNase A treated caesium chloride purified virus particles (Fig.5.1). Clones pTBSVS1, pTBSVS3 and pTBSV118 hybridised only to the genomic RNA in both the virion RNA and RNA from puromycin-treated polysomes. However, as well as hybridising to the genomic RNA, pTBSV65 and pTBSVS2 also hybridised to the TBSV 2.0 kb and 0.9 kb subgenomic RNAs. Therefore, it would seem likely that the 4.8 kb genomic, 2.0 kb and 0.9 kb RNA species are 3'co-terminal, similar to the genomic and subgenomic RNA species of many other monocomponent viruses such as tobacco mosaic (Beachy et al., 1976; Pelham, 1978), turnip crinkle (Dougherty & Kaesberg, 1981; Altenbach & Howell, 1982), turnip rosette (Morris-Krsinich & Hull, 1981), turnip yellow mosaic (Mellema et al., 1979), southern bean mosaic (Salerno-Rife et al., 1980) and carnation mottle (Carrington & Morris, 1985).

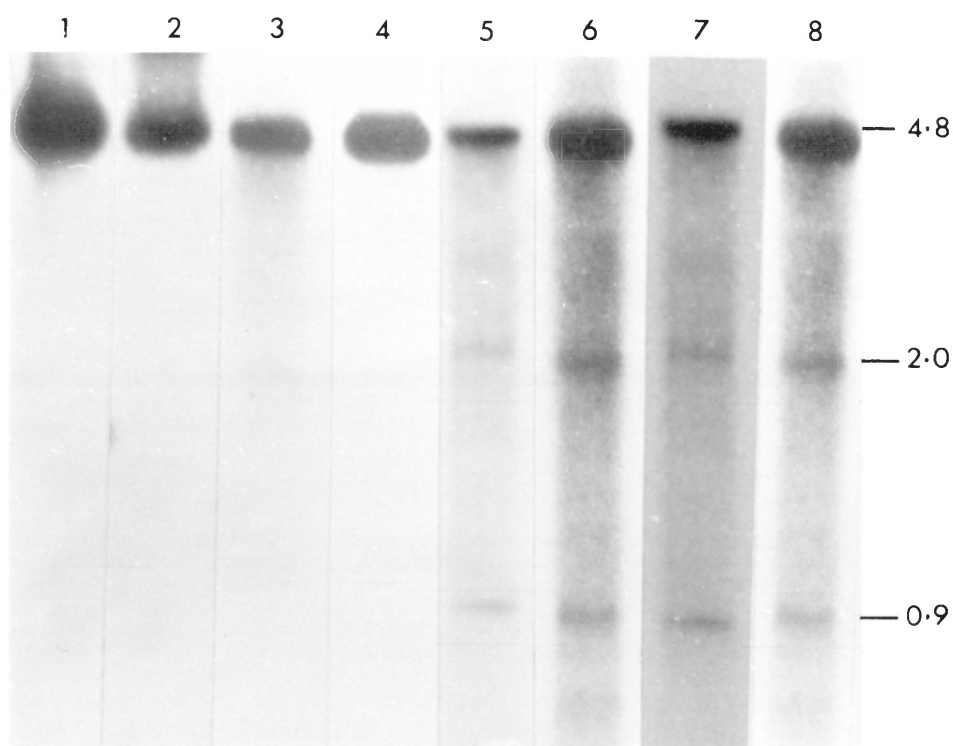


FIG.5.1. Northern blot analysis of TBSV encapsidated RNA and RNA from puromycin-treated polysomes from infected N.benthamiana. Encapsidated RNA (lanes 1, 3, 5 and 7) and polysomal RNA (lanes 2, 4, 6 and 8) was electrophoresed through a 1.4% agarose gel containing 10 mM methylmercuric hydroxide and then Northern blotted onto GeneScreen. Lanes 1 and 2 were hybridised to ^{32}P -labelled, nick-translated pTBSVS1 and pTBSVS3; lanes 3 and 4 were hybridised to ^{32}P -labelled, nick-translated pTBSV118; lanes 5 and 6 were hybridised to ^{32}P -labelled, nick-translated pTBSV65; lanes 7 and 8 were hybridised to ^{32}P -labelled, nick-translated pTBSVS2. RNA sizes on the side of the gel are in kb.

It is well documented that RNA viruses produce proteins required for their encapsidation, replication and transport (Hull & Maule, 1985). To begin to study the proteins produced by a virus, it is often necessary to translate the genomic RNA and any subgenomics, and then to compare the in vitro products to those proteins produced by the virus in vivo.

Comparison of the total RNA from a TBSV-infected plant with RNA from a healthy plant allows the genomic RNA to be easily detected, but the subgenomic RNAs are hidden by the host RNA (Fig.3.9). Therefore, in order to translate these RNAs, they were purified using hybrid-selection. The TBSV cDNA clones were bound to an inert support and hybridised to RNA under conditions of DNA excess. The RNA which hybridised specifically to the immobilised DNA was then recovered and translated in rabbit reticulocyte lysate.

5.2 IMMOBILISATION OF DNA

Traditionally, hybrid selection has been performed with DNA immobilised on nitrocellulose filters or activated cellulose such as APT (O-aminophenylthioether) paper. Nitrocellulose has a higher DNA binding capacity than APT paper and the application of the DNA is simpler (Jagus, 1987). However, unlike APT paper, nitrocellulose is rather fragile, and the immobilised DNA is not covalently bound, resulting in the gradual washing off of the DNA. The latter characteristic prevents the filters from being used more than a few times. Therefore, initial attempts at hybrid selection were made using APT paper.

Plasmids (10 µg) to be immobilised onto APT filters (5mm square) were digested with Xho1, precipitated and then resuspended in 20 µl of 25 mM potassium phosphate pH 6.2. After adding four volumes of DMSO the DNA was denatured by heating to 80°C for 10 minutes, and then applied to a

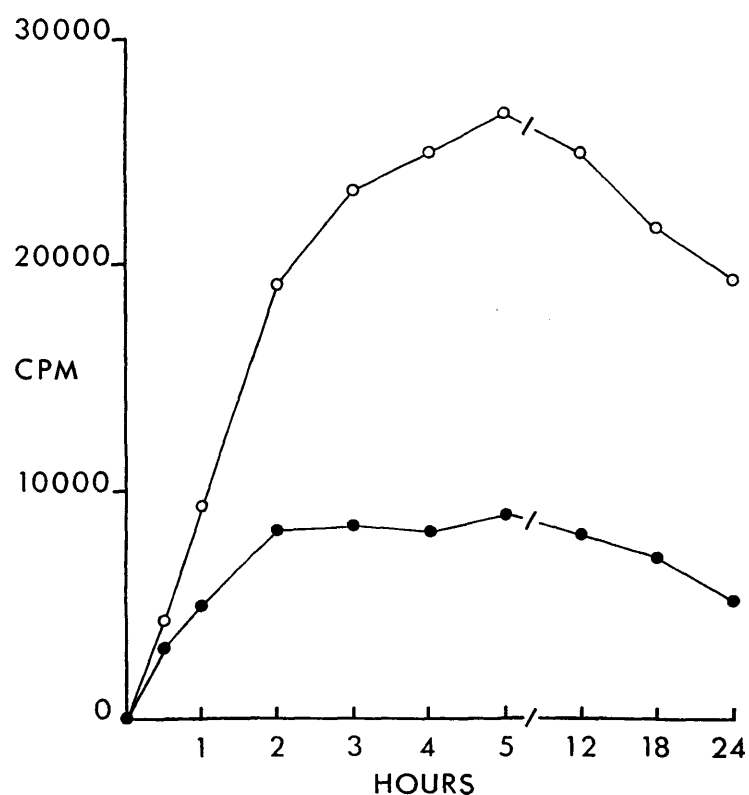
activated APT filter. After allowing the filter to dry overnight, the filter was washed to remove loosely bound DNA. Five filters were then hybridised to total plant RNA (10 mg/ml) in a volume of 200 μ l. Afterwards, the filters were washed to remove non-hybridised RNA, and then washed to elute the hybridised RNA.

When using total RNA instead of mRNA in hybrid selection, a far higher concentration of RNA is required due to the high proportion of ribosomal RNA. The non-ribosomal RNA from puromycin-treated polysomes could be used which would be enriched for TBSV RNAs. However, the quality of such RNA seems to be relatively low, with a small but significant level of degradation (Fig.3.20). Two sets of hybridisation conditions were used, low stringency conditions for rare RNA species and high abundance conditions which are more stringent, using higher levels of formamide and temperature. Less RNA is selected, but the eluted RNA should be purer than the RNA obtained using the low abundance conditions.

The factors effecting the success of hybrid selection are the hybridisation conditions, the length of hybridisation, the amount of DNA immobilised onto the filter and the concentration of input RNA. A time course was performed with total RNA from infected plants to determine the optimum time of hybridisation using both sets of conditions; excess time often results in increased RNA degradation without any substantial increase in final RNA yield. After the two sets of hybridisations had been performed for various times, aliquots of the hybridised RNA eluted from the filters were spotted onto GeneScreen. The dot blots were then probed with cDNA to TBSV genomic RNA or nick translated total DNA from a healthy plant, and then cut into portions and counted in a scintillation counter. The results, shown in Fig.5.2, show that after 5 hours with the low abundance conditions the degree of

hybridisation reaches a near plateau, while with the high abundance conditions the plateau is reached after only 2 hours. While the amount of RNA hybridised with the high abundance conditions is far less than that obtained with the low abundance conditions, the proportion of contamination with host RNA - judged by using the plant DNA probe - is 5 fold less when compared to the low abundance conditions (Fig.5.2b).

As the degree of contamination was very important, the high abundance conditions were selected in further experiments. However, it soon became apparent that the lower amount of selected RNA was to become a limiting factor. To probe the hybridised RNA in a single Northern blotting experiment, all the selected RNA had to be used. One of the reasons for this low yield, apart from the stringent hybridising conditions, was the amount of DNA immobilised to the filter. The amount of DNA that could be bound was determined by adding a aliquot of nick translated plasmid to the bulk of the DNA to be bound. After binding, the filters were washed and scintillation counted. The APT paper was found to only bind about 20 μg DNA/cm². Fortunately, while these experiments were being conducted a method for using GeneScreen-Plus (NEN) in hybrid-selection became available (Jagus, 1987). GeneScreen-Plus has a much higher binding capacity for DNA, and is far less fragile than nitrocellulose. It was found that this membrane would bind approximately 300 μg DNA/cm², resulting in a significant increase in the amount of final RNA obtained (at least 15 fold).



	Low Abundance (5 hours)	High Abundance (2 hours)
Probed with pTBSV65	32100 cpm	8120 cpm
Probed with total DNA	6890 cpm	310 cpm

FIG.5.2. Hybridisation of RNA to immobilised DNA. A time course (a) for the hybridisation of RNA under low abundance (○) or high abundance (●) conditions was established by probing dot blots of eluted RNA with ^{32}P labelled, nick-translated pTBSV65. The dot blots were then probed (b) with ^{32}P labelled, nick-translated genomic DNA from healthy *N.benthamiana* to establish the degree of contamination with host RNA.

5.3 HYBRID-SELECTION OF RNA

Fig. 5.3 shows Northern blot analysis of hybrid-selected RNA from infected and healthy plants. In order not to hybrid-select the lower molecular weight RNAs described in Chapter 3, thereby preventing any possible confusion about the origin of translation products, the RNA from infected plants was prepared from those plants which had been inoculated with the genomic RNA only. The genomic RNA and two lower molecular weight species of 2.0 kb and 0.9 kb hybridised to the immobilised pTBSV65 and pTBSVS2 (lanes 7 and 8) clones. However, as in Fig.5.1, only the genomic RNA species hybridised to the pTBSVS1, pTBSVS3 and pTBSV118 (lanes 3, 4 and 5). No RNA species hybridised to pUC/Xho1 (lane 9), and when pTBSV65 was used to hybrid-select RNA from healthy plants, no RNA species were detected by Northern blotting (lane 10), showing that all the RNA species selected are related to the TBSV genomic RNA. Northern blot analysis using a cDNA probe prepared from total RNA of a healthy plant showed that the eluted RNA which hybridised to pTBSV65 was essentially free from any detectable host RNA species (lane 1).

If the locations of the clones shown in Fig.4.28 are correct, it would be expected that the 1.3 kb HindIII-EcoRI insert fragment of pTBSV65 (Fig.4.19) should hybrid-select only the genomic and 2.0 kb RNA species. This was indeed found to be the case after the fragment was subcloned into pUC9 (Messing, 1983) to create pTBSV65HL (Fig.5.3, lane 6).



FIG.5.3. Northern blot analysis of hybrid-selected RNA. RNA was electrophoresed through a 1.2% agarose gel containing 10 mM methylmercuric hydroxide and Northern blotted onto GeneScreen. Lanes 1 and 10, RNA hybrid selected from healthy plants by pTBSV65; lane 2, TBSV genomic RNA; lanes 3 to 9, RNA hybrid selected from infected plants by pTBSVS1 (lane 3), pTBSVS3 (lane 4), pTBSV118 (lane 5), pTBSV65HL (lane 6), pTBSV65 (lane 7), pTBSVS2 (lane 8), pUC/Xho1 (lane 9). Lane 1 was hybridised to ^{32}P -cDNA prepared using healthy plant RNA as a template, and lanes 2 to 10 were hybridised to ^{32}P -labelled cDNA prepared using purified genomic RNA as a template. RNA sizes on the side of the gel are in kb.

5.4 TRANSLATION OF VIRION RNA

In rabbit reticulocyte lysates total TBSV virion RNA stimulated incorporation of [³H]leucine up to 10 times the amount in the control containing no added RNA. Analysis of the translation products by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of five major proteins with an estimated Mr of 40 kD, 37 kD, 28 kD, 22 kD and 20 kD, termed p40, p37, p28, p22 and p20 respectively (Fig.5.4 lane 3). No bands were present due to the endogenous activity of the lysate (Fig.5.4 lane 1). In addition to the major protein species there were a number of minor products which varied in intensity between different translations.

The proteins produced by in vitro translation of TBSV total virion RNA are very similar to those produced by CyRSV (CyRSV produces proteins with a Mr of 43 kD, 40 kD, 34 kD and 22 kD which here will be termed p43, p40, p34 and p22 respectively - Burgyan et al., 1986). The main difference between the two sets of data lies in the 22 kD region. Burgyan et al. (1986) note only one protein in this region, but in Fig.5.4 there are clearly two bands of equal intensity with molecular weights of 22 kD and 20 kD. This might be due to a genuine difference between the translation strategies of the two viruses, but more likely it is a result of either; (1) the interpretation of the data, which can be confused by the presence a number of minor bands, whose intensities vary and sometimes approach those obtained by the major products; (2), the reaction conditions of the in vitro translations leading to premature termination of p22 synthesis to give p20; (3) slight RNA degradation to expose an internal initiation codon; (4) electrophoresis conditions of the SDS-PAGE gel; or (5) that leucine was used in these experiments to label the encoded polypeptides, an amino acid which usually occurs far more frequently than methionine in proteins. If

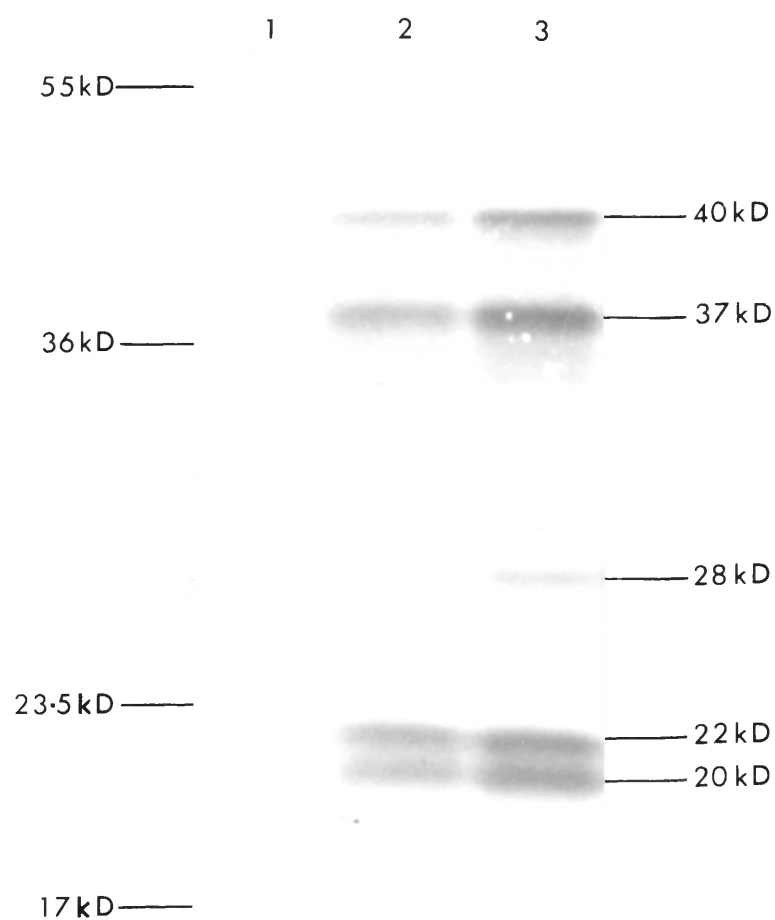


FIG.5.4. Products of in vitro translation in rabbit reticulocyte lysate programmed with: lane 1, no added RNA; lane 2, RNA from infected plant hybrid selected by pTBSV65; lane 3, TBSV virion RNA. The positions of Mr markers, human gamma globulin (55 kD and 23.5 kD), lactate dehydrogenase (36 kD) and myoglobin (17 kD) are indicated on the left, and the estimated Mr of the translation products are shown on the right.

the results of Burgyan et al. (1986) are studied carefully (see Fig.3a, lane 1 and Fig.5a in CyRSV paper) two bands are visible in this region. Unlike with TBSV, one of the bands is much more prominent than the other. Therefore, there may be two CyRSV proteins in this region of the gel. The number and prominence of the so-called minor bands in Fig.5.4 is significantly less than those obtained with CyRSV (Burgyan et al., 1986). This may be due to the intactness of the RNA and/or the quality of the rabbit reticulocyte lysate.

It should be noted that translation in vitro may not mirror the actual situation in vivo. It is wise to use both a wheat germ cell free system and rabbit reticulocyte lysate to translate RNA in vitro, and then to compare the products with those produced in vivo. While the rabbit reticulocyte lysates have the advantages of high activity and a capability to synthesise full-length, high molecular weight polypeptides, the wheat germ cell free system will translate a wider variety of mRNAs. When Burgyan et al. (1986) infected protoplasts with virus particles three proteins were observed with molecular weights of 45 kD, 43 kD and 22 kD. The p45 was shown to be an artifact, and p43 was shown to be the coat protein by immunoprecipitation. It is presumed that both the in vivo proteins p43 and p22 were the same as the in vitro translation products of the same molecular weight, but interestingly neither the p40 or p34 in vitro translation products were observed in infected protoplasts. It may be that these proteins are found in the protoplasts, but at a very low level, and that the concentrations of the proteins relative to each other differ between in vivo and in vitro experiments. On the other hand, CyRSV p40 and p34 (and presumably the TBSV p37 and p28 translation products) may not be normally found in vivo, and may be artifacts created by a lysate system from a very specialised cell type. In order to test for the

translational faithfulness of the particular batch of lysate used, TMV RNA was translated (Dr.S.Shelbourn & Dr.A.C.Marriot, personal communication). Both the 126 kD and 183 kD proteins were produced as predicted from the sequence (Goelet et al., 1982).

5.5 TIME COURSE OF TRANSLATION OF TOTAL VIRION RNA

When the products of the in vitro translations of CyRSV were subjected to peptide mapping, p34 was found to have some peptides in common with the p40, indicating that the p34 is partially contained within the p40 and is produced by translation of part of the same coding region (Burgyan et al., 1986). Unfortunately, time did not permit this experiment, but considering the similarity between the two viruses, it is likely that the same result would be obtained upon peptide mapping p37 and p28. The CyRSV p40 protein may result from readthrough of the p34 stop codon in a fashion analogous to the TMV 183 kD and 126 kD proteins (Pelham, 1978). Another possibility is that the p34 results from the proteolytic cleavage of p40. If this were the case then incubation of the translation reaction over a period of time should result in the decrease of p40 with a concomitant increase in the level of p34. This was not observed (Burgyan et al., 1986). Fig.5.5 shows that the amounts of p37 and p28 increase with time - there is no increase in the p28 protein at the expense of the p37. Unlike the situation with CyRSV, all the TBSV proteins appear at approximately the same time. Burgyan et al. (1986) found with CyRSV that the first protein to appear during a time course was p22.

In contrast to total virion RNA, translation of pTBSV65 hybrid selected RNA failed to produce p28 (Fig.5.4). It is tempting to speculate that p28 is produced from an encapsidated RNA (Chapter 3). However, the peptide mapping

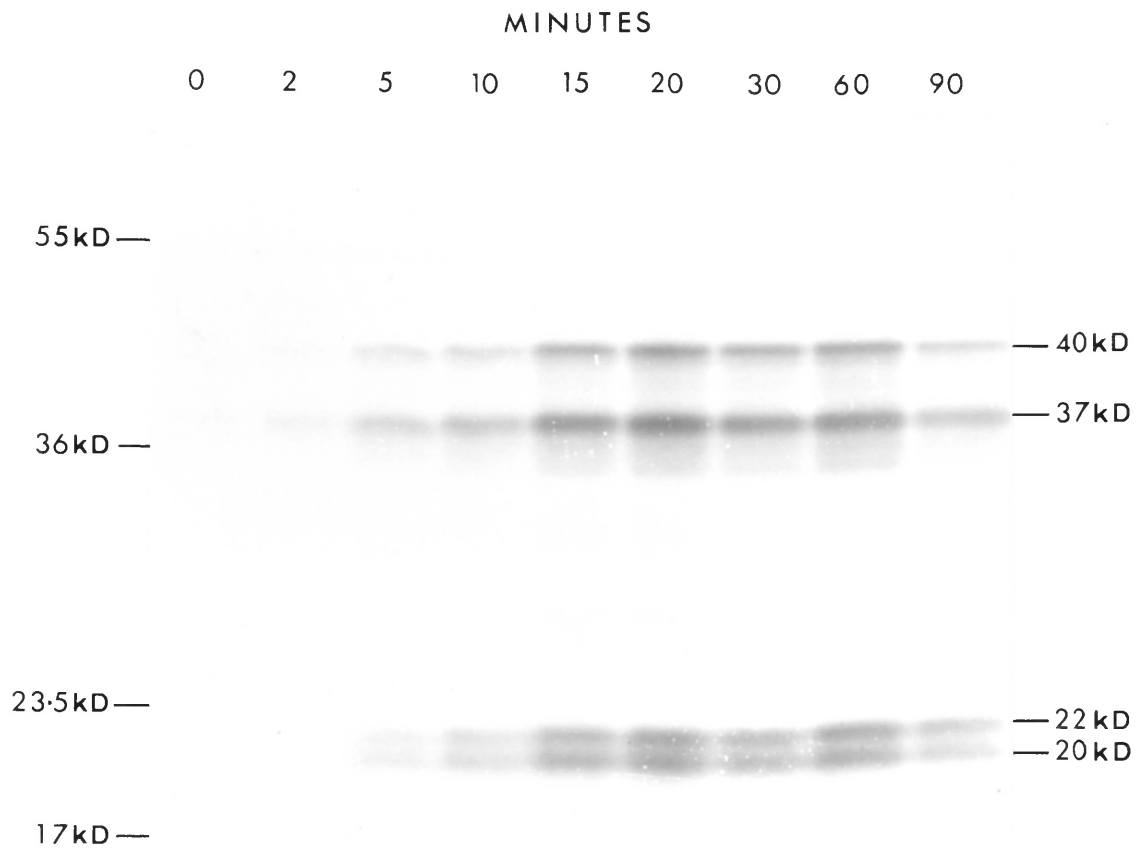


FIG.5.5. Time course of in vitro translation in rabbit reticulocyte lysate programmed with TBSV virion RNA. The positions of Mr markers, human gamma globulin (55 kD and 23.5 kD), lactate dehydrogenase (36 kD) and myoglobin (17 kD) are indicated on the left, and the estimated Mr of the translation products are shown on the right.

of CyRSV argues against such an explanation. The virus may contain a factor which acts to stimulate the production of p28, and purification during hybrid selection results in the loss of such a factor. Another possibility is that the protein is an artifact created by either premature termination or degraded RNA; there may be a second initiation codon downstream of the p37 initiation codon which is used if the 5'-end of the RNA is degraded. It has been noticed with CyRSV (Russo *et al.*, 1988) that the amount of p34 varies from one experiment to another. A similar situation is seen in Fig.5.5 where, although the translation has been programmed with total virion RNA, very little p28 is produced.

5.6 IMMUNOPRECIPITATION

When the products from a 1 hour translation of total TBSV virion RNA (Fig.5.6, lane 1) were incubated with the antiserum, only the 40 kD product was immunoprecipitated (lane 2). This protein also co-migrated with the capsid protein of disrupted caesium chloride purified virions, thereby confirming the 40 kD translation product to be the capsid protein. No proteins were immunoprecipitated when the pre-immune serum was used (lane 3).

5.7 TRANSLATION OF-HYBRID SELECTED RNA

When RNA hybrid selected by pTBSV118 was translated and the products separated by SDS-PAGE (Fig.5.7, lane 1) only p37 was detected. Translation of pTBSV65HL produced both p37 and the coat protein (lane 2). Comparison with the pTBSV65 hybrid-selected RNA products (lane 3) with the RNA species hybrid-selected by these clones (Fig.5.3) shows that the 2.0 kb RNA encodes the coat protein and the 0.9 kb RNA encodes p22 and p20.

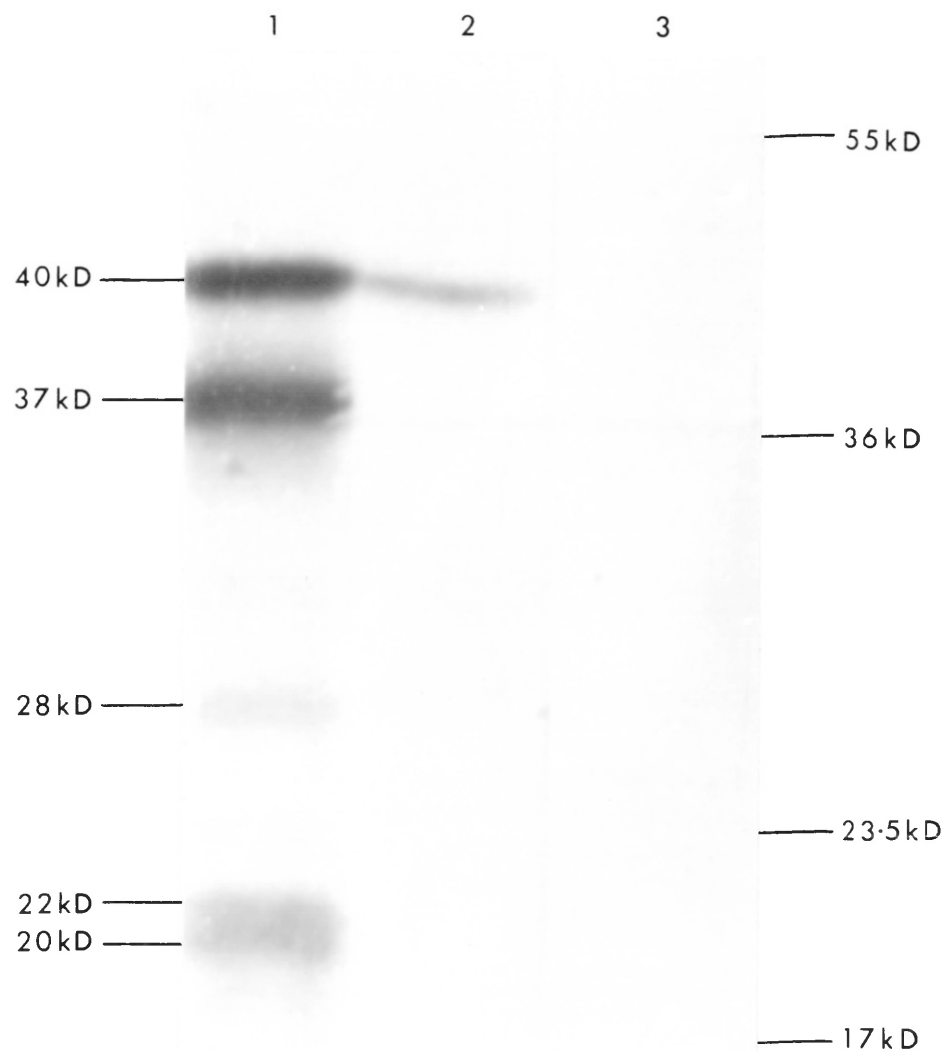


FIG.5.6. Products of in vitro translation programmed with TBSV virion RNA and analysed directly (lane 1) or after immunoprecipitation with an antiserum to TBSV virus particles (lane 2) or preimmune-serum (lane 3). The positions of Mr markers, human gamma globulin (55 kD and 23.5 kD), lactate dehydrogenase (36 kD) and myoglobin (17 kD) are indicated on the right, and the estimated Mr of the translation products are shown on the left.

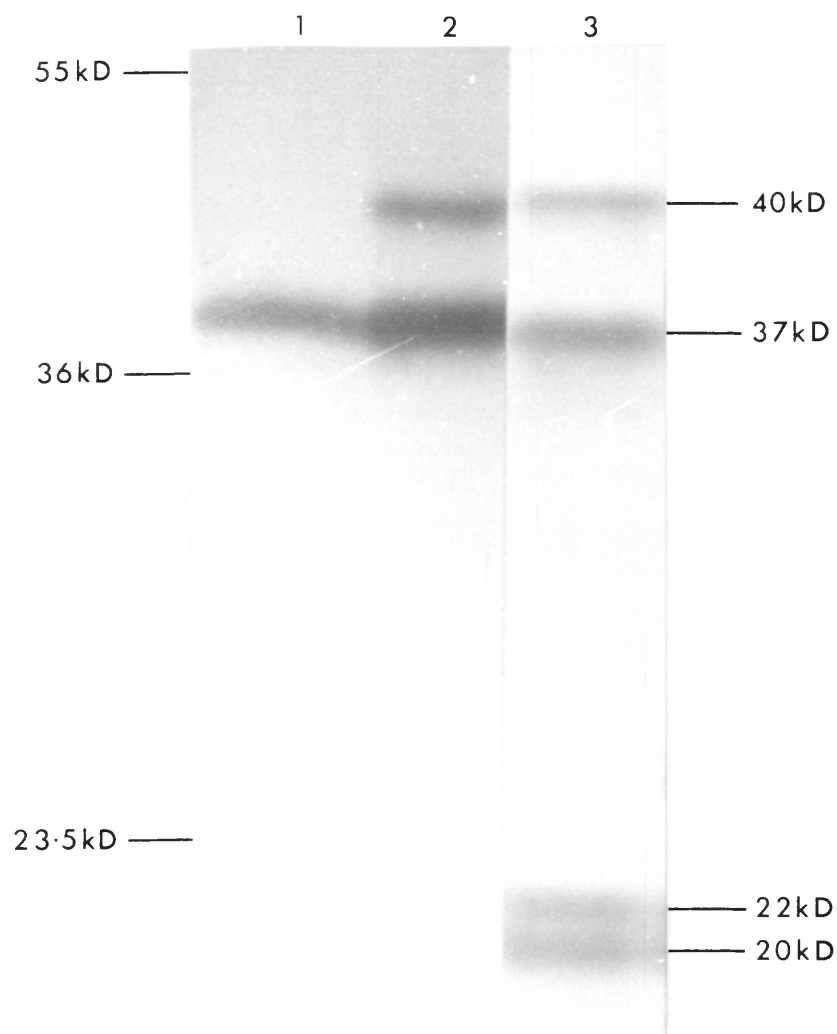


FIG.5.7. Products of in vitro translation programmed with mRNA hybrid selected by: lane 1, pTBSV118; lane 2, pTBSV65HL; lane 3, pTBSV65. The positions of Mr markers, human gamma globulin (55 kD and 23.5 kD) and lactate dehydrogenase (36 kD) are indicated on the left, and the estimated Mr of the translation products are shown on the right.

5.8 PRODUCTION OF HIGHER Mr POLYPEPTIDES

When the TBSV RNA hybrid selected by pTBSV65 was translated in the presence or absence of 1 mg/ml calf liver tRNA, the usual four polypeptides were produced. However, in the presence of the tRNA a higher Mr protein of approximately 90 kD was also produced in small amounts (Fig.5.8). Aliquots of the translation reaction containing tRNA were taken after 5, 10, 15 and 60 minutes and analysed by SDS-PAGE (Fig.5.9). The p40, p37, p22 and p20 polypeptides were produced after 5 minutes. However, by 10 minutes a small amount of the 90 kD polypeptide had been produced.

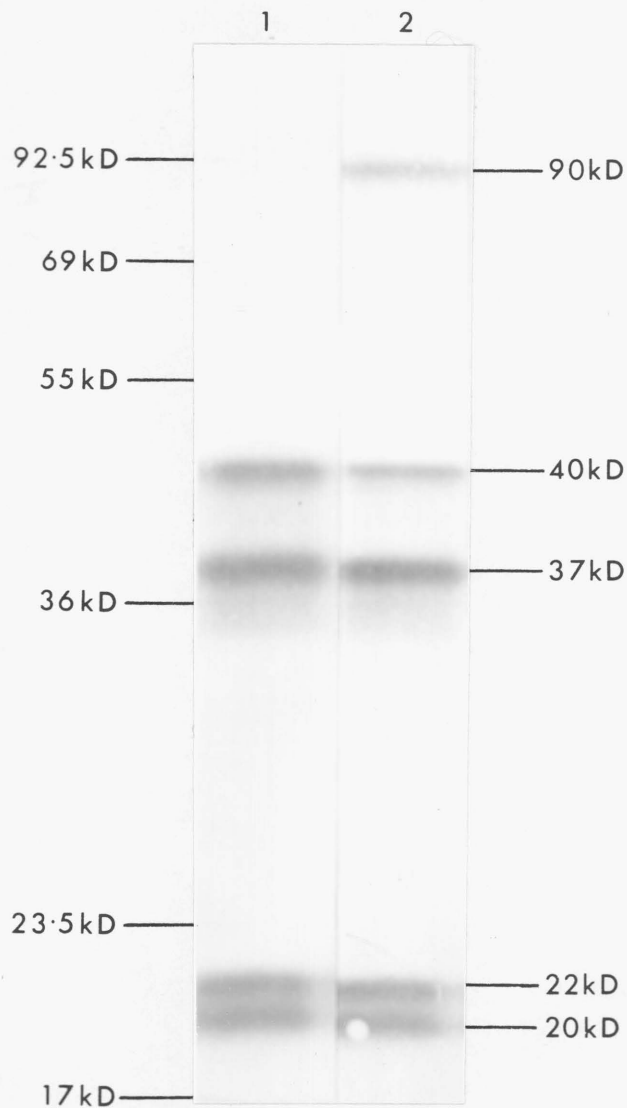


FIG.5.8. Products of in vitro translation programmed with mRNA hybrid-selected by pTBSV65 in the absence (lane 1) or presence (lane 2) of 1 mg/ml tRNA. The positions of Mr markers, phosphorylase-b (92.5 kD), bovine serum albumin (69 kD), human gamma globulin (55 kD and 23.5 kD), lactate dehydrogenase (36 kD) and myoglobin (17 kD) are indicated on the left, and the estimated Mr of the translation products are shown on the right.

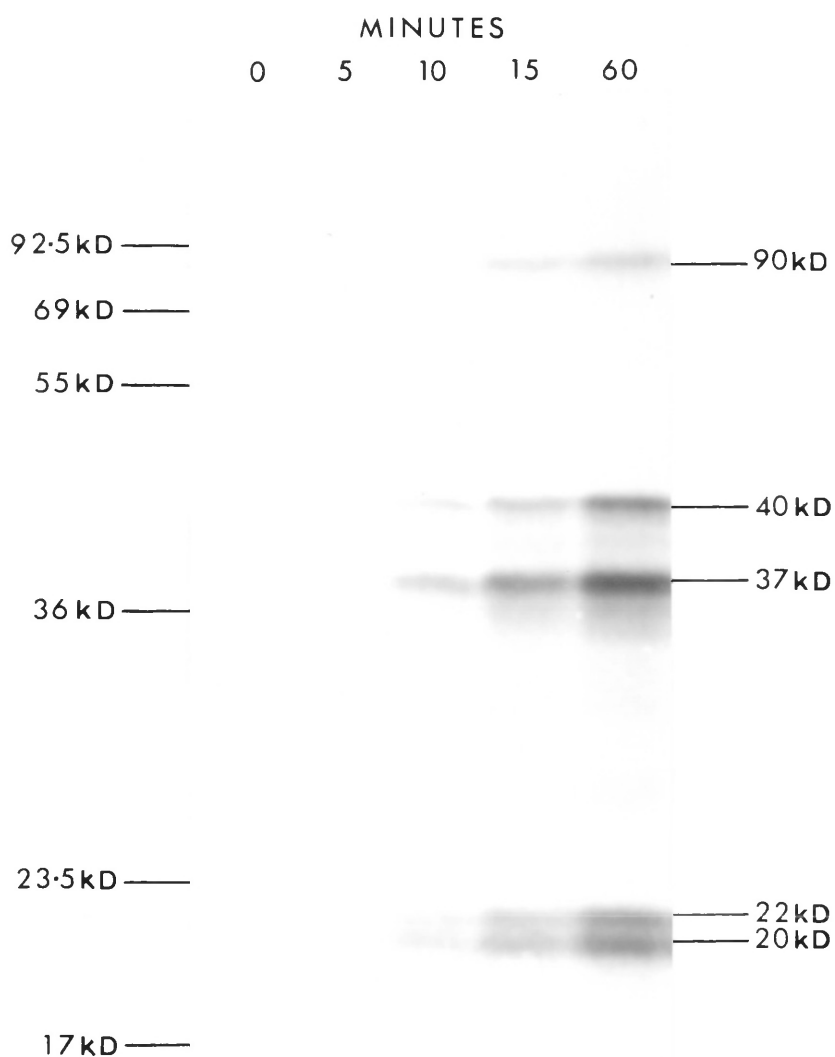


FIG.5.9. Time course of in vitro translation in the presence of 1 mg/ml calf liver tRNA of mRNA from infected plants hybrid-selected by pTBSV65. The positions of Mr markers, phosphorylase-b (92.5 kD), bovine serum albumin (69 kD), human gamma globulin (55 kD and 23.5 kD), lactate dehydrogenase (36 kD) and myoglobin (17 kD) are indicated on the left, and the estimated Mr of the translation products are shown on the right.

CHAPTER 6

DISCUSSION

When the contents of TBSV particles were analysed, a number of different ssRNA species were found in addition to the 4.8 kb genomic RNA. These can be divided into two groups; (1) those which hybridised to cDNA from healthy plants but not to cDNA prepared to the TBSV genomic RNA; and (2) the 2.0 kb and 0.9 kb species which only hybridised to the genomic RNA. It was initially thought that the first group of RNAs were contaminants of the virus preparation. However, these RNAs were still present after the virus particles had been treated with RNase A and further purified by sedimentation in caesium chloride. Therefore it must be concluded that they are encapsidated. Whether these host-related RNAs are normal host RNAs freely available within the cell that became accidentally encapsidated, or whether they have evolved from the host RNAs and are being carried by the virus cannot be determined. In either case they are likely to contain a sequence which allows them to be encapsidated, similar to the genomic RNAs. It is thought that satellite RNAs have evolved from host RNAs, and it is known that they require the virus proteins to replicate (Murant & Mayo, 1982; Francki, 1985). These RNAs may be very similar, even though they are not present at the levels usually seen with satellite RNAs. Another possibility is that they represent part of a host response to infection; the encapsidation of these RNAs might result in less efficient packaging of the viral genome and the subgenomic RNAs.

Encapsidation of such RNAs may also have an effect on the host; sequestration of host RNA may be harmful to the plant, perhaps giving rise to some of the symptoms seen during infection (Rochon & Seigal, 1984).

Satellite RNAs are a fairly common phenomena in RNA plant virus infections. Far less common are defective interfering RNAs. During the course of this work both types of RNA have been reported in the RNA from various tombusviruses. Although the aim of this project was to characterize TBSV subgenomic RNAs, the interesting nature of both satellite and defective interfering RNAs encouraged additional experiments. The only indication of an encapsidated satellite RNA in this work was a faint band of approximately 0.7 kb in some virus preparations, e.g. Fig.3.5. Gallitelli and Hull (1985b) proposed that the failure of other workers in the field to notice satellite RNAs was simply due to an unfortunate choice of N.clevelandii as the host plant. They claimed that these RNAs could be detected in much higher concentrations after a single passage of the virus through N.benthamiana. Unfortunately, when this was attempted, up to ten times, large quantities of the satellite did not appear in virus preparations. Also, contrary to Gallitelli and Hull's work (Gallitelli & Hull, 1985b), the satellite could not be detected by comparing total RNA from healthy and infected plants, suggesting that the lack of viral satellite in the virus particles was not a result of inefficient encapsidation. The isolate of the type-strain used here therefore differs from that of Gallitelli & Hull (1985b).

The origin of satellite RNA is of considerable interest. Baulcombe et al. (1986) showed that virus particles can encapsidate RNAs translated from the host plant. It may be that the satellite RNA in N.benthamiana derives from the host DNA, and becomes replicated and encapsidated in the presence of the genomic RNA. This RNA may be present in

different levels and/or have a slightly different sequence between the two sets of plants, which would account for the replication of the satellite in N.benthamiana but not in N.clevelandii. However, no explanation was given by Gallitelli & Hull (1985b) for the failure of cDNA prepared from the satellite RNA to hybridise to healthy plant RNA (Gallitelli & Hull, 1985b, Fig.5). Inoculation of the N.benthamiana stock used by Gallitelli and Hull (1985b) with the virus used in these experiments might help to resolve these differences in results.

Hillman et al. (1985, 1987) showed the presence of a 0.4 kb mosaic of TBSV-Cherry strain in infected tissue. The DI RNA was initially noticed encapsidated in high concentrations in purified virions, and in a double-stranded form in infected tissue (Hillman et al., 1985). Northern blot analysis later showed the presence of the DI RNA in ssRNA isolated from plants, and that the RNA has an attenuating effect on TBSV infections (Hillman et al., 1987). A large degree of heterogeneity in the DI RNA population was shown, with some containing repeats of the genomic sequence, and others containing deletions not found in other DI RNA molecules.

When the dsRNAs from TBSV-infected tissue were analysed in this study, a number of lower molecular weight RNAs with different molecular weights were observed. From Northern blot analysis of the dsRNA and ssRNA from infected plants, it would seem that at least one of these lower molecular weight RNAs may show some degree of homology to the genomic RNA. However, unlike those DI RNAs reported by Hillman et al. (1985), ssRNA forms of the dsRNAs could not be detected encapsidated in virus preparations, and they were not shown to have an attenuating influence on symptom development in infected plants. Therefore, the putative DI RNA(s) observed here with the type-strain are different to those reported by

Hillman et al. (1987) with TBSV-Cherry strain. However, as with the results of Gallitelli and Hull (1985b), the host plants may have some effect on the replication and expression of these RNAs. The lack of detectable DI RNA in TBSV-Type strain virus particles probably reflects inefficient encapsidation rather than inefficient replication; the levels of DI RNA dsRNA forms in these studies do not seem significantly less than those reported by Hillman et al. (1985).

Apart from the genomic RNA, two other TBSV specific RNAs of 2.0 kb and 0.9 kb are encapsidated. There is abundant evidence that these RNAs are genuine subgenomic RNAs and not degradation products of the genomic RNA:

- (1) They are the only small TBSV related RNA species found encapsidated.
- (2) They are the only small TBSV related RNA species found in total ssRNA and polysomal ssRNA.
- (3) dsRNA forms of both RNAs are found in infected plants. It would seem unlikely that ssRNA and dsRNA degradation products would be the same length.
- (4) The RNAs only hybridise to certain clones, and therefore to be degradation product they would have to be derived from the same portion of the genome each time
- (4) The small RNAs translate to produce proteins. One of the products is known to exist in vivo, ie. the capsid protein.

The double-stranded form of the 2.0 kb subgenomic RNA has been previously reported (Henriques & Morris, 1979). During the course of this work Hillman et al. (1985) and Gallitelli & Hull (1985) also showed that an RNA species of approximately 2.1 kb is also encapsidated in TBSV-Cherry strain (Hillman et al., 1985, 1987), TBSV-BS3 strain and the TBSV-Type strain (Gallitelli & Hull, 1985).

In addition, Hillman et al. (1987) showed that the TBSV-Cherry strain also encapsidates a 1.0 kb RNA species which, together with the 2.1 kb RNA, hybridised to cDNA prepared from the genomic RNA.

Evidence has been presented that another tombusvirus, CyRSV also utilises subgenomic RNA during genome expression. When the genomic RNA of the definitive tombusvirus CyRSV is translated only a 40 kD protein is produced (Burgyan et al., 1986). However, translation of encapsidated lower molecular weight RNA resulted in the production of the capsid polypeptide, suggesting that the capsid protein is produced from a subgenomic RNA or from fragmented genomic RNA. When total and encapsidated CyRSV RNA were subjected to Northern blotting and probing with CyRSV cDNA (Russo et al., 1988), two distinct RNA species of 2.1 kb and 1.0 kb were detected. In common with the subgenomic RNAs of monopartite virus so far identified, e.g. tobacco mosaic (Beachy et al., 1976; Pelham, 1978; Goelet et al., 1982), turnip crinkle (Carrington et al., 1987), turnip rosette (Morris-Krsinich & Hull, 1981), turnip yellow mosaic (Mellema et al., 1979), southern bean mosaic (Salerno-Rife et al., 1980), carnation mottle (Carrington & Morris, 1985) and barley yellow dwarf (Miller et al., 1988), the 2.0 kb and 0.9 kb subgenomic RNA species described here and the CyRSV subgenomics described by Russo et al. (1988) are 3' co-terminal with their genomic RNA.

Translation of the purified genomic RNA resulted in the production of p37. However, translation of total encapsidated RNA in the presence of tRNA also produced a 90 kD protein. It would seem likely that p90 is a read-through product of p37, because p90 was only produced in the presence of added calf liver tRNA and only the genomic RNA is large enough to encode a polypeptide of this size. This may be the first evidence for read-through in the

tombusviruses. As mentioned above, the only polypeptide translated from the purified CyRSV genomic RNA had a Mr of 40 kD, with no indication of any higher molecular weight readthrough proteins (Burgyan et al., 1986). However, the positive control used in these experiments was carnation mottle virus (CarMV). The production of the CarMV 77 kD read-through product (Carrington & Morris, 1985) and none higher than 40 kD with CyRSV under the same conditions was taken to indicate that readthrough of the CyRSV 40 kD gene does not occur (Burgyan et al., 1986). However, Harbison et al. (1985) showed that by using calf UAG suppressor tRNA or calf liver tRNA in the in vitro translation of CarMV, a read-through 100 kD polypeptide can be produced. Therefore, translation of CyRSV genomic RNA in the presence of calf liver tRNA might also result in a read-through product.

While the 90 kD polypeptide may be a termination codon read-through product, further work on the origin of this polypeptide is required. The very high levels of tRNA required before the polypeptide was seen - approximately 20 fold more than required for carnation mottle virus (Harbison et al., 1985) - may be due to the low amounts of suppressor tRNA in the batches of calf liver tRNA used. However, high levels of tRNA may effect fidelity of translation by the ribosomes, perhaps causing a translational frameshift or suppression of termination at a genuine stop codon. As the hybrid-selected RNA is essentially free from contamination, the polypeptide is unlikely to be host encoded.

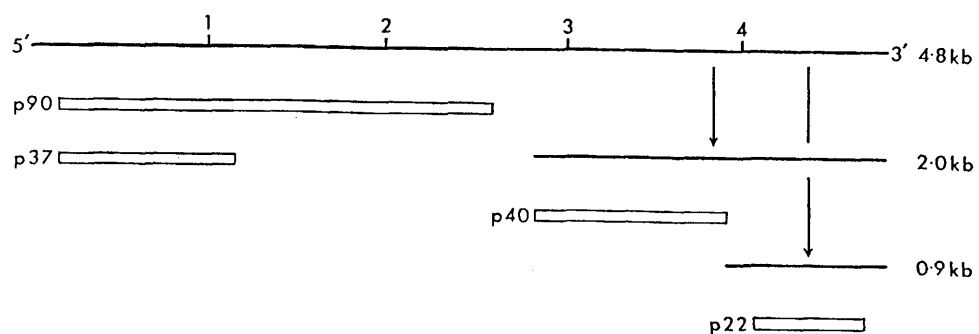


FIG.6.1. Model for the genome organisation and expression of TBSV. The thin lines represent the 4.8 kb genomic RNA and 2.0 kb and 0.9 kb subgenomic mRNA. The thick lines represent the polypeptides with Mr 90 kD (p90), 40 kD (p40, the capsid polypeptide), 37 kD (p37) and 22 kD (p22).

There is no evidence so far for proteolytic processing in the tombusviruses. Time courses for the production of the polypeptides in this study and with CyRSV (Burgyan et al., 1986) failed to show any decrease in the amounts of the larger polypeptides in favour of smaller polypeptides; all the peptides were produced at the same time. As the putative read-through product p90 was the last polypeptide to be produced in a time course, it is also unlikely to be a precursor of lower Mr proteins.

Translation of the 2.0 kb and 0.9 kb subgenomic RNAs revealed that the coat protein is encoded by the larger of the two molecules. This shows that the coat protein gene is situated internally and not at the 5' or 3'-end as with most other plant RNA viruses. The location of the coat protein near the middle of the genome has recently been reported for barley yellow dwarf virus (BYDV) (Fig.1.1: Miller et al., 1988), suggesting that the two viruses share a common origin. As the BYDV internal 60 kD ORF is thought to be expressed by translational frameshift at the end of the 39 kD ORF, there is a possibility that the TBSV 90 kD polypeptide may be a frameshift polyprotein.

The translation data, together with mapping of the subgenomic RNA species on the genomic RNA, enables a model for the genome organisation and expression of TBSV to be proposed (Fig.6.1). A similar model has recently been proposed for CyRSV (Russo et al., 1988). This arrangement of genes may be characteristic of members of the tombusvirus group. Apart from the 40 kD coat protein, the functions of the encoded proteins are unknown, but possible functions can be suggested.

- (1) As the polypeptides encoded by the 5'-end of the genomic RNA of monopartite RNA viruses such as TMV are likely to be involved in a RNA-dependent RNA polymerase, both the 37 kD polypeptide and the 90 kD polypeptide may

be involved in genomic RNA replication and subgenomic RNA synthesis.

(2) Raising the temperature at which the plants are grown during infection with TBSV results in attenuated symptoms and a decrease in the level of the 1.0 kbp (0.87 kbp) dsRNA. Hillman et al. (1985) speculated that the ssRNA form of this dsRNA may encode a product essential for symptom development, such as the cell-to-cell transport protein. It is therefore possible that p22 and/or p20 are involved in virus movement.

No definitive evidence was obtained concerning the origin of p28. Translation of CyRSV RNA produced p34 which was shown to be related to p40 (equivalent to p37 of TBSV) (Burgyan et al., 1986). The small amount of p28 precluded its peptide mapping, but its production by translation of TBSV virion RNA, but not hybrid-selected RNA, suggests that it might arise from an encapsidated host mRNA or a weakly replicating satellite RNA, rather than from a genomic-related RNA.

Although this work has shed some light on the expression strategy of TBSV, much work remains to be done. Direct sequencing of the 5' and 3'-ends would enable the construction of full-length clones. These could then be sequenced allowing the locations of the genes and subgenomic promoters on the genome to be accurately determined. Together with peptide mapping and peptide sequencing, this would establish the nature of p90. Determination of the sequence would also allow comparison with the known sequences of other viruses, and this, together with infection of whole plants and protoplasts with transcripts of full-length clones and transcripts containing mutations, might allow the functions of various polypeptides to be studied in more detail. The sequence would also allow cloned copies of TBSV genes to be

inserted into the plant genome behind constitutive promoters. The orientation of the TBSV genes behind such promoters would allow the production of both sense and antisense RNA. Translation of sense RNA encoding TMV coat protein has reduced the susceptibility of transgenic plants to TMV infection (Abel et al., 1986). It is envisaged that antisense RNA against certain TBSV genes might prevent the expression of these genes, thereby affording some protection against infection by the virus.

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