Gene Mapping and Expression of Tomato Bushy Stunt Virus

By R. J. HAYES,1 A. A. BRUNT2 AND K. W. BUCK1

1Department of Pure and Applied Biology, Imperial College of Science and Technology, London SW7 2BB and 2Institute of Horticultural Research, Littlehampton, West Sussex BN17 6LP, U.K.

(Accepted 15 August 1988)

SUMMARY

Cloned DNA species complementary to the RNA of tomato bushy stunt virus (TBSV) were produced and the location of each on the virus genome was determined. Polysomes were prepared from TBSV-infected plants and RNA, released from the polysomes by treatment with puromycin, was shown by Northern hybridization to contain TBSV genomic RNA (4.8 kb) and two subgenomic ssRNA species of 2.0 and 0.9 kb. Hybrid selection using clones corresponding to different parts of the virus genome showed that the two subgenomic RNA species are probably 3' coterminal with the genomic RNA. In vitro translation of hybrid-selected RNA showed that the genomic (4.8 kb), 2.0 and 0.9 kb RNA species encode proteins of M, 37000 (37K), 40K (the virus coat protein) and 22K respectively. Translation in the presence of calf liver tRNA produced, in addition to the other products, a protein of M, 90K which may be a readthrough product of the 37K protein. The results have enabled a model for the genome organization and expression of TBSV to be formulated.

INTRODUCTION

Tomato bushy stunt virus (TBSV) is the type member of the Tombusvirus group. It has a unipartite genome of ssRNA of about 4.8 kb and isometric particles about 30 nm in diameter with a capsid composed of one polypeptide species, M, 41000 (41K) (Matthews, 1982). Although the structure of TBSV particles has been determined at high resolution (Hopper et al., 1984), comparatively little is known about the virus gene products, their arrangement on the genome or their mode of synthesis. Several dsRNA species ranging in size from 4.8 to 0.44 kbp have been isolated from TBSV-infected plants (Henriques & Morris, 1979; Hayes et al., 1984; Hillman et al., 1985). However analysis of TBSV-specific ssRNA species in total RNA or polysomal RNA from infected plants has been hindered by electrophoretic artefacts attributed to interactions between fragments of viral RNA and plant RNA species (Hayes et al., 1984).

We now report the production and mapping of cloned DNA complementary to the TBSV genome and its use to isolate two subgenomic mRNA species by hybrid selection. The products of translation in vitro of the virus genomic and subgenomic RNA species have been identified and a model for the genome organization and expression of the virus is presented.

METHODS

Plant growth and virus isolation. TBSV (Type strain, from the culture collection of the Institute of Horticultural Research, Littlehampton, U.K.) (Smith, 1935) was propagated in Nicotiana clevelandii and N. benthamiana by inoculating the expanding upper leaves of plants grown to the six- to eight-leaf stage. Virus was isolated 10 days after inoculation by differential and sucrose density gradient centrifugation as described by Hayes et al. (1984), and further purified by centrifugation to equilibrium in caesium chloride (starting density 1.36 g/ml) in 50 mm-sodium phosphate buffer pH 7.8 at 126500 g for 18 h in a Beckman SW50.1 rotor at 4 °C.

RNA isolation and Northern blot analysis. RNA was isolated from purified virus particles as described by Hayes et al. (1984). The genomic RNA was further purified by centrifugation through a gradient of 2 to 10% (w/v) sucrose in formamide : 10 mm-Tris–HCl pH 7.5 (85 : 15 v/v) (Paucha et al., 1978), followed by electrophoresis through a 1%
low melting point agarose gel containing 5 mM-methylmercuric hydroxide and extraction as described by Miller et al. (1983).

Polyosomes were obtained from healthy or infected plants by the method of Hari (1980); mRNA was then released by treatment with a 10 mM-puromycin solution and purified as described by Palukaitis et al. (1983). Total RNA was isolated from infected plants by the method of Taylor & Powell (1982). After centrifugation through caesium chloride solution, ssRNA and dsRNA were separated using Whatman CF11 cellulose (Morris & Dodds, 1979).

RNA was electrophoresed through 1 to 1.8% agarose gels containing 5 mM- or 10 mM-methylmercuric hydroxide as a denaturant (Bailey & Davidson, 1976), transferred to GeneScreen, and hybridized according to the manufacturer's instructions (New England Nuclear) to labelled cDNA probes (Hayes et al., 1984).

Sizes of RNA species were determined from plots of the log of the number of bases versus electrophoretic mobility. The Bethesda Research Laboratories DNA kilobase ladder or RNA size markers (9.5 to 0.24 kb) were used for calibration. In agarose gels containing 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.

Plasmids and cloning. DNA manipulations were as described by Maniatis et al. (1982) unless otherwise stated. Plasmid DNA was purified from Escherichia coli by the alkaline lysis method (Birnboim & Dolby, 1979) and further purified by centrifugation to equilibrium in caesium chloride gradients containing ethidium bromide. E. coli JM101 cells (Messing, 1983) were used as recipients for transformation with recombinant plasmids.

A pUC/XhoI vector, designated pUC19X, which was capable of X-gal colour selection, was constructed by digesting 1 µg pUC19 (Messing, 1983) with PstI and SpH I, blunt-ending with 1 unit of mung bean nuclease (New England Biolabs) in 20 µl of 30 mM-sodium acetate pH 4.6, 50 mM-sodium chloride and 1 mM-zinc chloride for 10 min at 37 °C, which removed 10 bases in the polylinker region of the vector, and tailing with unphosphorylated XhoI linkers (10 mer; New England Biolabs) (Barnes, 1987). The product was used to transform competent E. coli JM101 cells. Nucleotide sequence analysis of the resultant recircularized plasmid, pUC19X, confirmed that 10 bases containing a XhoI site had been added (M. Siong, personal communication). Hence the reading frame of the β-galactosidase gene and the X-gal colour selection were unaltered.

First-strand cDNA synthesis was performed for 1 h at 42 °C in 100 µl of 50 mM-Tris—HCl pH 8.3, 8 mM-magnesium chloride, 100 mM-potassium chloride, 5 mM-dithiothreitol (DTT), 0.5-µg/ml salmon sperm DNA random primers (Maniatis et al., 1982), 1 mM-dATP, 1 mM-dGTP, 1 mM-dTTP (Pharmacia), 2 µCi [γ-32P]dCTP (410 Ci/mmol; Amersham), 100 µg/ml actinomycin D (Sigma), 250 units/ml reverse transcriptase (avian myeloblastosis virus; Life Sciences) and 100 µg/ml TBSV genomic RNA.

Second-strand synthesis and blunt ending of the double-stranded cDNA was performed as described by Gubler & Hoffman (1983). The double-stranded cDNA was then ligated to phosphorylated XhoI linkers (New England Biolabs) and ligated overnight to XhoI-digested pUC19X.

In a separate experiment, first-strand cDNA was synthesised after the RNA had been denatured with 10 mM-methylmercuric hydroxide (Krug & Berger, 1987). The double-stranded cDNA was then partially digested with Sau3A and ligated to BamHI-digested pUC19X.

The location of clones relative to the 5' and 3' ends of TBSV was achieved using RNase H (Ahlquist et al., 1984). DNA fragments from cDNA inserts were subcloned into M13mp8 (Messing, 1983). TBSV RNA (1 µg) was annealed to the single-stranded M13 clones (cDNA insert was in twofold molar excess) 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 the mixture, 0.5 unit of RNase H (Bethesda Research Laboratories) was added and the reaction was incubated for 15 min at 32 °C. Calf intestinal phosphatase (Boehringer) (1 unit) was then added and the incubation was continued for 5 min. RNA was recovered by phenol: chloroform extraction and ethanol precipitation. It was then dissolved in water, heated to 90 °C for 3 min and labelled with [γ-32P]ATP (3000 Ci/mmol; Amersham) using T4 polynucleotide kinase (Pharmacia) or with [5'-32P]pCp using RNA ligase (Cobianchi & Wilson, 1987).

Southern blot analysis. DNA was electrophoresed in 1.2% agarose gels (Hayes et al., 1988), transferred to GeneScreen, prehybridized and hybridized to nick-translated plasmids (Rigby et al., 1977), or isolated labelled RNA fragments (Ogden & Adams, 1987), according to the manufacturer's instructions (New England Nuclear). DNA size markers (kilobase ladder) were obtained from Bethesda Research Laboratories.

Hybrid selection, translation and immunoprecipitation. Hybrid selections were performed using GeneScreen Plus (New England Nuclear) essentially as described by Jagus (1987) using high stringency conditions, 300 µg immobilized plasmid DNA/cm² and 10 mg/ml total RNA from infected or healthy plants.

RNA was translated in a rabbit reticulocyte lysate (Amersham; N. 90) according to the manufacturer's instructions. Usually 1 µl of RNA (1 µg) was added to 8 µl of lysate and 1 µl (10 µCi) [5, 3H]leucine (Amersham). In some instances 10 µg calf liver tRNA (Boehringer) was added. The mixture was incubated for 1 h at 30 °C, and then either analysed directly by electrophoresis in 12.5% or 15% polyacrylamide gels containing 0.1% SDS (Laemmli, 1970), or immunoprecipitated (Shelbourn et al., 1988) and then analysed by SDS—PAGE followed by fluorography (Chambertain, 1977). Protein M, markers were from Sigma.
Mapping and expression of TBSV

RESULTS

Preparation and analysis of TBSV cDNA clones

TBSV RNA was shown to lack a poly(A) sequence at its 3' end because it did not bind to either oligo(dT)-cellulose or poly(U)-Sepharose, and did not act as a template for cDNA synthesis using oligo(dT)_{10-12} as a primer for reverse transcriptase (R. J. Hayes, unpublished results). First-strand cDNA was therefore synthesized using random oligonucleotide primers prepared from salmon sperm DNA and double-stranded cDNA was cloned into pUC19X after addition of XhoI linkers. Twenty-four recombinant plasmids with inserts between 0-8 and 2-0 kbp were isolated. By restriction mapping and cross-hybridization of restriction fragments, overlapping clones were identified which accounted for more than 90% of the TBSV genome (Fig. 1).

To determine the location of two of the longest clones, pTBSV65 and pTBSV118 (Fig. 1), relative to the 5' and 3' ends of the genomic RNA, the recombinant plasmids were digested with HindIII which cuts once in the polylinker region of the vector moiety and once in each of the TBSV cDNA inserts. The orientations of the TBSV cDNA inserts in these two recombinant plasmids were such that the smaller of the two possible HindIII fragments was released in each case (Fig. 1). These two fragments were then subcloned into M13mp8 to give ssDNA recombinants designated mTBSV65HS(+) , mTBSV65HS(-), mTBSV118HS(+) and mTBSV118HS(-), where (+) and (−) indicate inserts in the same or opposite sense to the TBSV genomic RNA. Single-stranded DNA of the (−) sense clones was annealed to genomic RNA. After digestion with RNase H, the products were labelled at their 5' ends using T4 polynucleotide kinase and [γ-^32P]ATP, or at their 3' ends using RNA ligase and [5'-^32P]pCp, and then analysed by gel electrophoresis (Fig. 2).

RNase H digests the RNA portion of RNA/DNA hybrids, so that if the ssDNA clone is complementary to internal sequences in TBSV RNA, two RNA fragments will be generated, one corresponding to the 5' end and one to the 3' end of the genomic RNA. The 3' end of TBSV RNA was readily labelled (Fig. 2, lane 1). However, the 5' end of the RNA is blocked, preventing labelling with [γ-^32P]ATP (Fig. 2, lane 2). Therefore, although all RNA fragments produced by the RNase H digestion can be labelled at their 3' ends, only newly created 5' ends, derived from internal sequences, can be labelled.

When TBSV RNA was annealed to ssDNA of mTBSV65HS(−) followed by RNase H digestion and 3' end labelling, two major RNA species of 3-7 and 0-6 kb were produced, together with a fainter upper band which probably represents undigested genomic RNA (Fig. 2, lane 5). The only band detected after 5' end labelling was the 0-6 kb fragment (Fig. 2, lane 6). Therefore the 3-7 and 0-6 kb fragments correspond to the 5' and 3' ends of the genomic RNA respectively, locating the cloned sequence towards the 3' end of the genomic RNA. When the experiment was repeated using ssDNA of mTBSV118HS(−), RNA fragments of 3-5 kb and 1-0 kb were produced, but only the former could be labelled at its 5' end (Fig. 2, lanes 9 and 10), enabling the cloned sequence to be located towards the 5' end of the genomic RNA. As controls, TBSV RNA was annealed to ssDNA of mTBSV65HS(+) or mTBSV118HS(+). No new bands were
Fig. 2. Autoradiograph of RNA fragments generated by RNase H digestion of hybrids formed between TBSV genomic RNA and cloned cDNA and separated by electrophoresis in a 1% agarose gel containing 10 mM-methylmercuric hydroxide. TBSV genomic RNA was hybridized to ssDNA of (lanes 1 and 2) M13mp8, (lanes 3 and 4) mTBSV65HS(+), (lanes 5 and 6) mTBSV65HS(−), (lanes 7 and 8) mTBSV118HS(+) and (lanes 9 and 10) mTBSV118HS(−). RNA was labelled using [5'-32p]pCp (lanes 1, 3, 5, 7 and 9) or [γ-32p]ATP (lanes 2, 4, 6, 8 and 10). RNA sizes indicated on the side of the gel are in kb.

Fig. 3. 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 32P-labelled cDNA prepared using 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, TBSV genomic RNA (50 ng); lane 4, polysomal RNA from healthy plants (100 ng). RNA sizes on the side of the gel are in kb.
produced by RNase H digestion in either case (Fig. 2, lanes 3, 4, 7 and 8). The relative orientations and locations of the two clones with respect to the genomic RNA are shown in Fig. 1.

Clones corresponding to the 5' and 3' ends of TBSV RNA were produced by denaturing the RNA with methylmercuric hydroxide before first-strand synthesis and ligating Sau3A fragments of double-stranded cDNA into BamHI-digested pUC19X. Three recombinants designated pTBSVS1, pTBSVS2 and pTBSVS3, which had inserts of approximately 0-2, 0-4 and 0-6 kbp respectively, hybridized to TBSV RNA but not to pTBSV65 or pTBSV118. These plasmids were linearized with XhoI and electrophoresed in an agarose gel. Two blots were prepared. One was probed with the 3'-terminal 0-6 kb RNA fragment (isolated as shown in Fig. 2, lanes 5 and 6) and the other was probed with the 5'-terminal 1-0 kb RNA fragment (isolated as shown in Fig. 2, lane 9). The insert from pTBSVS2 hybridized only to the 3'-terminal probe, whereas the inserts from pTBSVS1 and pTBSVS3 hybridized only to the 5'-terminal probe (not shown).

Detection of TBSV subgenomic RNA in RNA isolated from polysomes

Polysomes, prepared from TBSV-infected plant tissue, were treated with puromycin and the released mRNA was purified by sedimentation in a sucrose density gradient. Three main peaks were observed in the u.v. profile. Gel electrophoretic analysis, together with tests for sensitivity to RNase A and S1 nuclease (Hamilton et al., 1982), showed that the middle peak contained ssRNA essentially free from ribosomal RNA. Electrophoresis of this RNA in a denaturing gel, followed by blotting and hybridization with a TBSV randomly primed cDNA probe, revealed TBSV genomic RNA and two subgenomic RNA species of 2-0 and 0-9 kb (Fig. 3, lane 2). No bands were detected in polysomal RNA from healthy plants (Fig. 3, lane 4), showing that the probe was free from cellular RNA.

It is noteworthy that the multiple artefactual electrophoretic bands previously reported in Northern blots of total RNA or polysomal RNA (Hayes et al., 1984) were not detected in mRNA released from polysomes by puromycin treatment. A similar observation was made by Palukaitis et al. (1983) in studies of subgenomic RNA species of tobacco mosaic virus.

Double-stranded RNA in plants infected by TBSV genomic RNA

Analysis of dsRNA, prepared from plants infected by purified TBSV genomic RNA, by electrophoresis in a denaturing agarose gel, blotting and probing with TBSV cDNA, revealed three RNA species which comigrated with the 4-8, 2-0 and 0-9 kb TBSV-specific polysomal RNA species (Fig. 3, lanes 1 and 2). When plants were infected by virus particles, three smaller dsRNA species (0-44, 0-50 and 0-58 kbp) were detected in addition to the 4-8, 2-0 and 0-9 kbp species. These smaller species, which were also found in previous studies (Hayes et al., 1984), are probably double-stranded forms of defective interfering RNA of the type described by Hillman et al. (1985, 1987). No dsRNA species of less than 0-9 kbp could be detected, either by Northern hybridization (Fig. 3) or by non-denaturing PAGE with ethidium bromide staining (not shown), in dsRNA isolated from plants infected by the genomic RNA, even after 10 successive passages of the progeny virus in N. benthamiana. Subsequent analyses of TBSV subgenomic RNA were carried out using RNA isolated from plants infected by inoculation with the purified genomic RNA.

Hybrid selection of TBSV subgenomic RNA from total plant RNA

TBSV-specific RNAs were selected from total RNA of infected plants by hybridization to immobilized recombinant plasmids and subsequent elution. Gel electrophoresis, followed by blotting and hybridization with a TBSV cDNA probe (Fig. 4) showed that pTBSVS1, pTBSVS3 and pTBSV118 hybrid-selected only TBSV genomic RNA (lanes 3 to 5), whereas pTBSV65 and pTBSVS2 hybrid-selected genomic RNA and subgenomic RNA species of 2-0 and 0-9 kbp (lanes 7 and 8). The specificity of the hybrid selection was confirmed by appropriate controls (lanes 1, 9 and 10).

Hybrid selection of the 2-0 kb subgenomic RNA by pTBSV65 but not by pTBSV118 locates it within the 3'-terminal 2-2 kb of the TBSV genomic RNA. The 0-9 kb RNA must lie in the same
TBSV virions were purified by sucrose density gradient centrifugation and incubated with ribonuclease A (1 μg/ml, 37 °C, 30 min) to remove any RNA bound to their surface. Under these conditions, a control sample of TBSV genomic RNA was completely degraded. The virions were then further purified by isopycnic cesium chloride density gradient centrifugation. When RNA prepared from such virions was electrophoresed in an agarose gel, blotted and probed with cDNA prepared to purified TBSV genomic RNA, bands of 4.8, 2.0 and 0.9 kb were detected (Fig. 5, lane 1). When similar blots were probed with nick-translated TBSV-specific recombinant plasmids, pTBSV118 detected only the genomic RNA, pTBSV65HL detected the genomic and 2.0 kb RNA and pTBSVS2 detected all three RNA species (not shown). It is clear, therefore, that the 2.0 and 0.9 kb subgenomic RNA species are all 3' coterminus.

Detection of subgenomic RNA in TBSV virion RNA

When blots were probed with cDNA prepared using total virion RNA, several minor RNA species were observed in addition to the 4.8, 2.0 and 0.9 kb species (Fig. 5, lane 3). These minor species were not detected when the probe was prehybridized with an excess of RNA from healthy plants (Fig. 5, lane 2) and therefore probably represent encapsidated host RNA.

In vitro translation of TBSV genomic and subgenomic RNA species

RNA prepared from virions, purified by a cesium chloride density gradient centrifugation, or isolated by hybrid selection to pTBSV65, was translated in a rabbit reticulocyte system and the products were analysed by SDS-PAGE (Fig. 6). The hybrid-selected translation products consisted of four polypeptides with Mr 40K, 37K, 22K and 20K (lane 2), whereas the virion RNA gave additionally a 28K polypeptide (lane 3). There was no indication of any polypeptides
Mapping and expression of TBSV

Fig. 5. Northern blot analysis of RNA encapsidated in TBSV virions. RNA (50 ng) was electrophoresed through a 1-2% agarose gel containing 5 mM-methylmercuric hydroxide, transferred to GeneScreen membrane and hybridized to (lane 1) 32P-labelled cDNA prepared using purified genomic RNA, (lane 2) 32P-labelled cDNA prepared using total encapsidated RNA and prehybridized to an excess of total RNA from healthy N. clevelandii and (lane 3) 32P-labelled cDNA prepared using total encapsidated RNA. RNA sizes on the side of the gel are in kb.

Fig. 6. SDS-PAGE in a 12.5% gel of the products of in vitro translation in rabbit reticulocyte lysate programmed with (lane 1) no added RNA, (lane 2) RNA from an infected plant, hybrid-selected by pTBSV65 and (lane 3) TBSV virion RNA. Mr values are shown on the side of the gel.

produced by the endogenous activity of the lysate (lane 1). A time course study of the translation of virion RNA showed that the amounts of each of the polypeptides increased concomitantly from 5 min up to 120 min after the start of translation (not shown).

When the products produced after translation of virion RNA for 1 h (Fig. 7, lane 1) were mixed with an antiserum to TBSV, only the 40K polypeptide was immuno-precipitated (lane 2), thereby identifying it as the capsid polypeptide. No radioactive polypeptides were precipitated by the preimmune serum (lane 3).

When TBSV genomic RNA, hybrid-selected by pTBSV118, was translated, only the 37K polypeptide was produced (Fig. 8, lane 1). Translation of the mixture of the genomic RNA and 2-0 kb RNA, hybrid-selected by pTBSV65HL, produced both the 37K polypeptide and the 40K capsid polypeptide (lane 2). Hence the 2-0 kb RNA encodes the capsid polypeptide. Translation of the mixture of the genomic, 2-0 and 0-9 kb species, hybrid-selected by pTBSV65, produced additionally the 22K and 20K polypeptides (lane 3). These two polypeptides are therefore encoded by the 0-9 kb RNA. As this RNA is not large enough to encode two non-overlapping polypeptides of this size, it is possible that the 20K polypeptide is a late initiation or premature
Fig. 7. SDS–PAGE in a 12.5% gel of the products of in vitro translation programmed with TBSV virion RNA and analysed directly (lane 1) or after immunoprecipitation with an antiserum to TBSV particles (lane 2) or preimmune serum (lane 3). Mr values are shown on the side of the gel.

Fig. 8. SDS–PAGE in a 12.5% gel of the products of in vitro translation programmed with mRNA from infected plants hybrid selected by: lane 1, pTBSV118; lane 2, pTBSV65HL; lane 3, pTBSV65. Mr values are shown on the side of the gel.

termination product of the 22K polypeptide, or that the 20K polypeptide arose by degradation of the 22K polypeptide.

RNA, hybrid-selected by pTBSV65, was translated in the presence of calf liver tRNA, and samples were analysed after different intervals (Fig. 9). The 40K, 37K, 22K and 20K polypeptides were detected after 5 min (lane 2). However, after 15 min an additional polypeptide of Mr 90K was detected (lane 3) and the amounts of all five polypeptides increased during the following 75 min (lanes 4 and 5).

DISCUSSION

Translation of RNA species, hybrid-selected by TBSV-specific cloned DNA, has shown that the genomic, 2-0 and 0.9 kb RNA species encode polypeptides of Mr 37K, 40K (the capsid polypeptide) and 22K respectively. The 2-0 and 0.9 kb subgenomic RNA species were found both in RNA from infected plants and in virions. Their detection in mRNA released from polysomes by puromycin (Fig. 3) suggests that they are genuine mRNA. Subgenomic RNA species of 2.2 and 1.0 kb have been detected in ssRNA from plants infected by the cherry strain of petunia asteroid mosaic virus (also referred to as the cherry strain of TBSV) (Hillman et al., 1987).

The translation data, together with mapping of the subgenomic RNA species on the genomic RNA, have enabled us to propose a model for the genome organization and expression of TBSV (Fig. 10). In this model the 90K polypeptide is shown as a readthrough product of the 37K polypeptide cistron. This seems likely because the 90K polypeptide was produced only in the
Mapping and expression of TBSV

Fig. 9. Time course of in vitro translation in the presence of calf liver tRNA of mRNA from infected plants hybrid-selected by pTBSV65. The products were analysed by SDS-PAGE (10% gel). Lane 1, 0 min; lane 2, 5 min; lane 3, 15 min; lane 4, 60 min; lane 5, 90 min. Mr values are shown on the side of the gel.

Fig. 10. Model for the genome organization and expression of TBSV. The thin lines represent the 4·8 kb genomic RNA and 2·0 and 0·9 kb subgenomic mRNAs. The thick lines represent the polypeptides with Mr 90K (p90), 40K (p40, the capsid polypeptide), 37K (p37) and 22K (p22).

presence of added calf liver tRNA and only the genomic RNA is large enough to encode a polypeptide of this size. Furthermore such a readthrough product accounts for the approx. 1·8 kb of sequence between the end of the 37K polypeptide and the start of the 40K capsid polypeptide. However the possibility that the 90K and 37K polypeptides are encoded by overlapping reading frames and that the calf liver tRNA causes a translational frameshift cannot be eliminated. Confirmation of the model will require sequence analysis of TBSV RNA, peptide mapping and sequence analysis, and evidence that the 90K, 37K and 22K polypeptides are synthesized in vitro.

A similar model has recently been proposed for another tombusvirus, cymbidium ringspot virus (Russo et al., 1988), except that the 90K polypeptide has not been described previously. For
both viruses the coat protein gene is located internally in the RNA and not close to the 3' end, in contrast to several other plant viruses whose coat protein is translated from a subgenomic RNA (Dougherty & Hiebert, 1985). This arrangement of genes may be characteristic of members of the *Tombusvirus* group.

No definitive evidence was obtained concerning the origin of the 28K polypeptide. Translation of cymbidium ringspot virus RNA produced a 34K polypeptide which was shown to be related to the 40K polypeptide (equivalent to the 37K polypeptide of TBSV) (Burgyan et al., 1986). The small amount of the 28K polypeptide made precluded its peptide mapping, but its production by translation of TBSV virion RNA, but not hybrid-selected RNA, suggests that it is more likely to arise from an encapsidated host mRNA or a satellite RNA, than from a genome-related RNA.

Gallitelli & Hull (1985) detected a satellite RNA in isolates of TBSV and other tombusviruses. When plants were inoculated with either our isolate of TBSV or its genomic RNA and the progeny virus was passed 10 times in *N. benthamiana*, a host reported to favour replication of tombusvirus satellite RNAs (Gallitelli & Hull, 1985), no satellite RNA could be detected in agarose gels stained with ethidium bromide (R. J. Hayes, unpublished results). The isolate of the type strain used here therefore differs from that used by Gallitelli & Hull (1985) in which a satellite RNA was clearly demonstrated by a similar method. However the possibility that our isolate contained a weakly replicating satellite cannot be eliminated.

Minor RNA species were detected in virions, in addition to the genomic RNA and 2-0 and 0-9 kb subgenomic RNA species, when Northern blots were probed with cDNA to total virion RNA (Fig. 5). The failure to detect these minor RNA species when the probe was prehybridized with RNA from healthy plants suggests that they are more likely to be host RNA than satellite RNA. Encapsulation of host mRNA has been described for tobacco mosaic virus (Rochon & Siegel, 1984). Further work, including hybridization of blots with labelled host DNA and preparation of cDNA clones to the minor TBSV RNA species, will be required to prove unequivocally whether these minor species are host or satellite RNA and whether one of them encodes the 28K polypeptide.

The three RNA species released from polysomes, or hybrid-selected from total RNA, i.e. genomic RNA and 2-0 and 0-9 kb subgenomic RNA, correspond in size to the three major dsRNA species isolated from TBSV-infected plants (Hayes et al., 1984; Hillman et al., 1985). Smaller dsRNAs found in plants infected by the virus in both the present and previous studies (Hayes et al., 1984) were not detected in plants infected by purified genomic RNA.

We thank the Science and Engineering Research Council for the award of a CASE Studentship (to R.J.H.).

REFERENCES


Mapping and expression of TBSV


(Received 13 June 1988)