Translation Products of Genome and Satellite RNAs of Tomato Black Ring Virus

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SUMMARY

In wheat germ extracts and reticulocyte lysates the genome RNA molecules of tomato black ring virus (TBRV), RNA-1 (mol. wt. 2.8 × 10^6) and RNA-2 (mol. wt. 1.6 × 10^6), were translated into products of maximum mol. wt. 2.2 × 10^5 and 1.6 × 10^5, respectively. These products represent about 80% and 100% of the coding capacity of the two RNA species. The 1.6 × 10^5 mol. wt. polypeptide reacted with antiserum to TBRV particles but the translation products of RNA-1 did not; this is additional evidence that RNA-2 contains the coat protein cistron.

Satellite RNA molecules (RNA-3, mol. wt. 4.8 × 10^5) associated with strain S of TBRV, like those associated with strain G, were translated in wheat germ extracts into a polypeptide of mol. wt. 4.8 × 10^4; this did not react with TBRV antiserum. Protease digestion released peptides from the translation product of strain S RNA-3 which were different from those released from the translation product of strain G RNA-3, suggesting that the two kinds of satellite RNA molecules differ in base sequence.

INTRODUCTION

The genome of tomato black ring virus (TBRV), like that of other nepoviruses, comprises two RNA molecules (Murant et al. 1973; Randles et al. 1977) of mol. wt. 2.8 × 10^6 (RNA-1; A. F. Murant, M. Taylor & G. H. Duncan, unpublished data) and 1.6 × 10^6 (RNA-2; Murant & Taylor, 1978). In addition, some isolates contain a satellite RNA (RNA-3) of mol. wt. 4.8 × 10^5 (Murant et al. 1973; Murant & Taylor, 1978).

We showed previously (Fritsch et al. 1978) that satellite RNA is translated in vitro and in vivo into a polypeptide of mol. wt. 4.8 × 10^4 and that unfractionated virus RNA directed the formation in vitro of numerous additional polypeptides among which the most prominent had a mol. wt. of 1.6 × 10^5 and the largest had a mol. wt. of 2.2 × 10^5. In this paper we show that the two large polypeptides are translated from RNA-2 and RNA-1 respectively, and that satellite RNA molecules from two strains of TBRV are chemically different.

METHODS

Virus isolates. Three isolates of TBRV contained satellite RNA: TBRV-G, an isolate of the German (potato bouquet) serotype of TBRV (Harrison, 1958); TBRV-E, an isolate obtained from Norfolk, England which was serologically close to TBRV-G; and TBRV-S, the stock culture from Arctium lappa (Harrison, 1958) of the Scottish (beet ringspot)
serotype of TBRV. An isolate lacking satellite RNA, TBRV-S12, was derived from TBRV-S using the procedure described by Murant et al. (1973). Tobacco rattle virus (TRV), PRN strain (Cadman & Harrison, 1959), was used in one experiment.

Purification. TBRV was extracted from systemically infected leaves of *Nicotiana clevelandii* by the method used previously (Fritsch et al. 1978). TRV was purified from systemically infected leaves of *N. clevelandii* by differential centrifugation of borax-clarified leaf extracts (Harrison & Woods, 1966).

RNA extraction. Virus suspensions (about 1 mg/ml) were shaken with phenol as described by Fritsch et al. (1978) and RNA was recovered from the aqueous phase by ethanol precipitation.

Separation of RNA species. Nucleoprotein components of TBRV-S12 were separated by centrifugation to equilibrium in caesium chloride followed by sedimentation in 10 to 40% sucrose gradients (Harrison & Barker, 1978). RNA-1 and RNA-2 were extracted from B and M nucleoprotein components respectively.

Translation in wheat germ extracts. Commercial wheat germ (General Mills Inc., Vallejo, Calif.) was extracted as described by Marcu & Dudock (1974). RNA (5 to 10 μg) was added to 100 μl reaction mixture containing 30 μl wheat germ extract, 15 to 60 μCi ³⁵S-methionine (300 to 700 Ci/mmol, Radiochemical Centre, Amersham), 20 mM-HEPES (pH 7.8), 98 mM-potassium acetate, 2.75 mM-magnesium acetate, 0.65 mM-spermidine, 2.5 mM-ATP, 0.375 mM-GTP, 5 mM-phosphoenol pyruvate, 1.8 mM-dithiothreitol and each essential amino acid at 0.025 mM. The reaction mixtures were kept at 30 °C for 2 h.

Translation in reticulocyte lysates. Rabbit reticulocyte lysates were prepared as described by Mohier et al. (1975). RNA (5 to 10 μg) was added to 50 μl reaction mixture containing 20 μl lysate, 75 mM-KCl, 0.8 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, 15 μM-haemin, 15 mM-creatine phosphate, 2 units creatine phosphokinase, 0.1 mM-unlabelled amino acids and 0.5 μCi ³⁵S-methionine. Incubation was for 1 h at 27 °C. In some experiments lysates were treated with micrococcal nuclease (Pelham & Jackson, 1976) before adding radioactive amino acids and mRNA.

Analysis of polypeptides in polyacrylamide-SDS gels. Reaction mixtures were centrifuged at 105 000 g for 1 h and the supernatant fluids were treated with RNase (Mayo et al. 1976). Solutions were then made 2% SDS, 2% 2-mercaptoethanol and 10% glycerol, and heated in boiling water for 90 s. Samples were stored at −20 °C or analysed immediately on discontinuous slab gels of 7.5% acrylamide using buffers described by Laemmli (1970). After electrophoresis the gels were stained, dried and autoradiographed using Kodirex film (Kodak Ltd.). The proteins used to estimate mol. wt. were RNA polymerase from *Escherichia coli* (Boehringer; mol. wt. 165 000, 155 000, 95 000 and 39 500), bovine serum albumin (67 000), pyruvate kinase (57 000), ovalbumin (45 000) and carbonic anhydrase (29 000). Calibration lines of log (mol. wt.) against mobility were slightly curved; lines fitted by eye thus gave estimates with an accuracy of about ±5%.

Serological precipitation. After translation of RNA in wheat germ extracts the reaction mixtures were centrifuged at 105 000 g for 1 h. Trichloroacetic acid was added to the supernatant fluid to give a final concentration of 10% (w/v) and the mixture was kept at 4 °C for 1 h. The precipitate was washed with ethanol and dissolved in 0.1 to 0.2 ml 0.15 M-sodium chloride + 0.01 M-sodium phosphate, pH 7.0 (PBS) containing 0.2% SDS. Samples containing similar amounts of radioactivity were incubated at room temperature with different amounts of TBRV-S antisera (titre 1/2048 in double diffusion tests in agarose) for 2 h and normal rabbit serum was then added to bring each sample to the same serum concentration. Rabbit antibodies were then precipitated by adding an equal volume of goat anti-rabbit γ globulin serum (a gift from Dr M. van Regenmortel) and incubating the mixture overnight at 4 °C. The precipitate was sedimented, washed twice with 2% Triton
Translation of TBRV RNA

Fig. 1. Autoradiograms showing electrophoretic separations in 7.5% acrylamide gels of $^{35}$S-labelled polypeptides produced in wheat germ extracts; (a to c) are from the same gel and (d to f) are adjacent tracks. Reaction mixtures contained: (a) RNA from TBRV-S; (b) RNA from TBRV-E; (c) RNA from TBRV-G; (d) RNA from TBRV-S; (e) RNA-1 from purified B component of TBRV-S12; (f) RNA-2 from purified M component of TBRV-S12. Arrows 1, 2 and 3 indicate the largest products from RNA-1, RNA-2 and RNA-3, respectively. The bars indicate the tops of the gels and the position of TBRV-S coat protein (CP).

X-100 in PBS, dissolved in 50 µl 1 M NaOH and neutralized with HCl. Radioactivity was determined using Bray's scintillator.

Partial proteolysis of satellite RNA translation products. Regions of dried acrylamide gels corresponding to the 48000 mol. wt. band in autoradiograms were cut out and the protein was eluted with 0.1% SDS, 1 mM-EDTA, 0.125 M-tris-HCl, pH 4.8, for 24 h (Benicourt et al. 1978). Samples of 10 to 15 µl containing about equal amounts of radioactivity were digested for 30 min at 30 °C with 0.2 to 80 µg/ml protease V8 from Staphylococcus aureus (Miles) or 0.08 to 80 µg/ml subtilisin (Sigma; Cleveland et al. 1977). The reactions were stopped by boiling the samples and the polypeptide composition of each was analysed by electrophoresis in 16% acrylamide gels.
RESULTS

Translation in wheat germ extracts

TBRV RNA at about 100 μg/ml (the optimum concentration) stimulated incorporation of 35S-methionine by wheat germ extracts about 50- to 100-fold. Analysis by polyacrylamide gel electrophoresis of the translation products of RNA from TBRV-S, TBRV-E and TBRV-G is shown in Fig. 1(a to c). With all three strains one of the prominent bands (band 3, arrowed) is the translation product of satellite RNA described previously (Fritsch et al. 1978); the remainder result from translation of RNA-1 and RNA-2. The largest has a mol. wt. of about 2.2 × 10^6 (band 1), and a polypeptide of mol. wt. 1.6 × 10^6 (band 2) is prominent in the translation products of TBRV-S and TBRV-G and present in those of TBRV-E. Both band 1 and band 2 polypeptides of TBRV-S migrated slightly more slowly than those of TBRV-E and TBRV-G.

To determine which RNA species gave rise to the different polypeptides in Fig. 1(a), RNA was extracted from preparations of the B and M components of TBRV-S12 to give purified RNA-1 and RNA-2. Comparison of the translation products of mixed RNA from TBRV-S (Fig. 1d) with those of RNA-1 (Fig. 1e) and RNA-2 (Fig. 1f) shows that bands 1 and 2 are translated from RNA-1 and RNA-2 respectively. In addition the RNA-1 translation products contained a prominent 1.9 × 10^6 mol. wt. polypeptide.
Translation of TBRV RNA

Table 1. Precipitation of translation products by antiserum to tomato black ring virus (TBRV)

<table>
<thead>
<tr>
<th>Expt.</th>
<th>RNA translated*</th>
<th>µl TBRV antiserum added</th>
<th>% ct/min in pellet</th>
<th>Ct/min in supernatant fluid</th>
<th>% ct/min in pellet</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>TBRV-S RNA-1</td>
<td>10</td>
<td>24567</td>
<td>232</td>
<td>9:9</td>
</tr>
<tr>
<td></td>
<td>TBRV-S RNA-2</td>
<td>10</td>
<td>25586</td>
<td>1650</td>
<td>6:1</td>
</tr>
<tr>
<td></td>
<td>TBRV-S satellite</td>
<td>10</td>
<td>15260</td>
<td>170</td>
<td>1:1</td>
</tr>
<tr>
<td>2</td>
<td>TBRV-S RNA-2</td>
<td>0:1</td>
<td>43763</td>
<td>645</td>
<td>1:5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>41501</td>
<td>552</td>
<td>1:3</td>
</tr>
<tr>
<td></td>
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<td>10</td>
<td>39725</td>
<td>3162</td>
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<tr>
<td></td>
<td>TRV-PRN RNA-1</td>
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<tr>
<td></td>
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<td>1:6</td>
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<td></td>
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<td>1</td>
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<td>690</td>
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<td></td>
<td></td>
<td>10</td>
<td>48807</td>
<td>562</td>
<td>1:1</td>
</tr>
</tbody>
</table>

* RNA was translated in wheat germ extracts. Washed protein from translation mixtures was treated as described in Methods.

The patterns of bands produced by the translation of RNA from TBRV-S and TBRV-G (Fig. 1 a, c, respectively) showed many minor differences in the position of the bands, whereas those produced by the translation products of RNA from the serologically related strains TBRV-E and TBRV-G (Fig. 1 b, c, respectively) differed much less.

Translation in reticulocyte lysates

In untreated lysates there was considerable endogenous protein synthesis (Fig. 2 a); nevertheless a polypeptide of mol. wt. 1.6 × 10^5 was detected in lysates containing purified RNA-2 from TBRV-S12 (Fig. 2 c), although no new products were detected in lysates containing RNA-1 (Fig. 2 b). However, when the lysates were pre-treated with nuclease (Pelham & Jackson, 1976) endogenous protein synthesis was abolished and autoradiographs of controls without added RNA showed no bands. In the treated lysates RNA-2, as before, was translated into a polypeptide of mol. wt. 1.6 × 10^5 (Fig. 2 e) and RNA-1 was translated into many polypeptides (Fig. 2 d), of which the largest had a mol. wt. of 2.2 × 10^5; there was also a prominent product of mol. wt. 1.9 × 10^5 (Fig. 2 d). These three polypeptides co-migrated with similar products produced in wheat germ extracts containing TBRV-S RNA (Fig. 2 f). No attempt was made to suppress the formation of lower mol. wt. bands by adding extra tRNA (Pelham & Jackson, 1976).

Detection of coat protein sequences in the translation product of RNA-2

Experiments with pseudo-recombinant isolates (Randles et al. 1977) indicated that TBRV RNA-2 contains the cistron for the virus coat protein. However, although RNA-2 translation products made in nuclease-treated reticulocyte lysates included a minor polypeptide that migrated to the position of TBRV coat protein (Fig. 2 e), no similar product was produced when RNA-2 was added either to untreated lysates (Fig. 2 c) or to wheat germ extracts (Fig. 1 f). We conclude therefore that there was no significant translation into coat protein molecules.

In experiments to detect coat protein sequences the products of translation of different RNA species in wheat germ extracts were recovered by acid precipitation, washed and incubated with rabbit antiserum to TBRV-S. Addition to the mixtures of goat antiserum to rabbit γ-globulin precipitated up to about 7% of the radioactivity in TBRV RNA-2.
Fig. 3. Autoradiograms showing electrophoretic separations in 16% polyacrylamide gels of polypeptides released by partial proteolysis from: (a, c, e, g) 48,000 mol. wt. polypeptide translation product of satellite RNA from TBRV-S; (b, d, f, h) 48,000 mol. wt. polypeptide translation product of satellite RNA from TBRV-G. (a, b) Undigested polypeptide; (c, d) polypeptide incubated with 80 μg/ml Staphylococcus aureus V8 protease; (e, f) polypeptide incubated with 0.08 μg/ml subtilisin; (g, h) polypeptide incubated with 0.4 μg/ml subtilisin. Incubation was for 30 min at 30 °C. Arrows indicate the position of undigested 48,000 mol. wt. polypeptide, arrow heads indicate the position of the main products of enzyme digestion.

Comparison of translation products of satellite RNA species from different TBRV strains

We showed previously (Fritsch et al. 1978) that the 48,000 mol. wt. polypeptides translated from satellite RNA molecules associated with TBRV-G and TBRV-S migrated at slightly different rates in 7.5% acrylamide gels. Further evidence for differences between these polypeptides came from experiments in which the 48,000 mol. wt. bands were cut from acrylamide gels of RNA-3 translation products, treated with proteases and the resulting peptides compared by electrophoresis in 16% acrylamide gels (Fig. 3). The number and position of the peptides produced from either translation product by V8 protease (Fig. 3c, d) or subtilisin (Fig. 3e to h) were different. Increasing the duration of protease treatment or the concentration of enzyme used did not affect this result; we conclude that the satellite RNA species from the two TBRV strains produce different translation products, and therefore probably have different base sequences.
DISCUSSION

Translation of TBRV RNA-1 in vitro resulted in the synthesis of many polypeptides although fewer were produced in reticulocyte lysates than in wheat germ extracts. Two high mol. wt. polypeptides were produced by both systems, the larger being of mol. wt. $2.2 \times 10^5$ which represents about 80% of the coding capacity of the RNA molecule. The other large polypeptide produced in both translation systems was of mol. wt. $1.9 \times 10^5$ and must therefore contain much of the sequence of the $2.2 \times 10^5$ mol. wt. polypeptide. It may be produced by some specific breakdown of the larger polypeptide or by partial translation.

The major product of translation of TBRV RNA-2 (mol. wt. $1.6 \times 10^5$; Murant & Taylor, 1978) in both in vitro systems was a polypeptide of mol. wt. $1.6 \times 10^5$. The RNA molecule is therefore translated into a single polypeptide. RNA-2 determines the serological specificity of TBRV coat protein (Randles et al. 1977) and our tests showed that some of the $1.6 \times 10^5$ mol. wt. polypeptide reacted with antiserum prepared against virus particles. In contrast the translation products of RNA-1 and satellite RNA failed to react with TBRV antiserum. The serological reaction was inefficient, possibly because the coat protein sequence was in a relatively denatured state within the larger polypeptide; however, the results provide direct evidence that RNA-2 is a plus strand and that the mRNA used in vitro could be that used in vivo.

As was shown previously (Fritsch et al. 1978) the band 2 polypeptide translated from TBRV-S RNA migrated in gels slightly more slowly than that from TBRV-G RNA (Fig. 1a, c) and the general patterns of polypeptide bands produced in wheat germ extracts by RNA from TBRV-G and TBRV-S were similar. However in the present work the correspondence of the bands of RNA translation products was closer between TBRV-G and TBRV-E than between TBRV-G and TBRV-S. Presumably most of these polypeptides arise from premature termination, translation of degraded RNA or proteolysis of larger translation products, and their sizes depend on the primary or secondary structure of the RNA molecules. It is interesting that the degree of similarity between these translation artifacts should reflect the serological relatedness of the viruses.

We showed previously that TBRV satellite RNA (RNA-3, mol. wt. $4.8 \times 10^5$; Murant & Taylor, 1978) is translated in both in vitro systems into a polypeptide of mol. wt. $4.8 \times 10^4$. The results of partial proteolysis of the translation products provide the first chemical evidence that satellite RNA molecules associated with different strains of TBRV are themselves different. It is not possible to measure the extent of the difference using this technique but few if any peptides produced by the proteases were common to the two polypeptides suggesting that they had significantly different amino acid sequences. The 48000 mol. wt. polypeptide translated from the satellite RNA associated with TBRV-S was detected in extracts of infected protoplasts (Fritsch et al. 1978) suggesting that relatively large quantities were synthesized. Moreover, all or nearly all of the coding potential of the satellite RNA is used during translation. Taken together, these results suggest that the protein may have a specific role, perhaps facilitating the replication of the satellite RNA in some way.

In most plant viruses so far studied, coat protein is produced in vivo by translation of a sub-genomic monocistronic mRNA (e.g. Lane, 1974; Hunter et al. 1976; Siegel et al. 1976). With TBRV, if it can be assumed that translation behaviour in vitro is followed in vivo, it seems that a different strategy is adopted: the genome RNA is translated in its entirety into a large polypeptide from which the coat protein is presumably cleaved by protease action. In this respect TBRV appears to resemble cowpea mosaic virus (CPMV). CPMV M-RNA (equivalent to RNA-2) codes for the smaller coat protein polypeptides of mol. wt. about 25000 (Gopo & Frist, 1977), whereas both in reticulocyte lysates (Pelham...
& Stuik, 1977) and in wheat germ extracts (Davies et al. 1977) it is translated into two large polypeptides of mol. wt. about 110000 but not into coat protein. Recent work has shown other similarities between RNA molecules of TBRV and CPMV. RNA molecules from both viruses contain polyadenylate (El Manna & Bruening, 1973; Mayo et al. 1979a) and also have small proteins covalently attached (Daubert et al. 1978; Stanley et al. 1978; Harrison & Barker, 1978; Mayo et al. 1979b). Perhaps the similarities between nepoviruses and comoviruses in their translation strategies are related to the structural features common to the two groups of viruses.

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