Translation of the Satellite RNA of Tomato Black Ring Virus

*in vitro* and in Tobacco Protoplasts

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**SUMMARY**

Translation of the satellite RNA (RNA-3) of tomato black ring virus (TBRV) in wheat germ extracts or reticulocyte lysates resulted in the synthesis of a polypeptide of mol. wt. about 48,000, both in the presence and in the absence of RNA-1 and RNA-2. The RNA-3 specific polypeptide of TBRV strain G was slightly larger than that of strain S. A polypeptide of the same electrophoretic mobility as the *in vitro* translation product of RNA-3 was found in extracts of protoplasts infected with an isolate of TBRV-S possessing RNA-3 but not in extracts of protoplasts infected with an isolate lacking RNA-3. The lack of phenotypic effect of RNA-3 raises the question of the function of this protein in the infected plant.

**INTRODUCTION**

In addition to the two functional RNA species (RNA-1 and RNA-2) characteristic of nepoviruses (Harrison & Murant, 1977), some naturally occurring isolates of tomato black ring virus (TBRV) possess a ‘satellite’ RNA (RNA-3) of mol. wt. about $5 \times 10^5$ (Murant *et al.* 1973; A. F. Murant & M. A. Mayo, unpublished data). A similar RNA was found in the distantly related virus, myrobalan latent ringspot (Delbos *et al.* 1976). The satellite RNA of TBRV was eliminated from a culture containing it and was found to have no effect on the types of symptom produced by the virus; however, when added to inocula containing RNA-1 and RNA-2 it decreased the number of local lesions induced in leaves of *Chenopodium amaranticolor*. TBRV RNA-3 multiplied in plants only in the presence of RNA-1 and RNA-2, and it became packaged in TBRV coat protein (Murant *et al.* 1973; A. F. Murant & M. A. Mayo, unpublished data).

TBRV RNA-3 is thus analogous to the satellite RNA species associated with tobacco ringspot and cucumber mosaic viruses (Schneider, 1969, 1971; Kaper *et al.* 1976), although much larger. Its size (coding potential of about 50,000 daltons of protein) suggests that it may function as a messenger RNA and this paper describes experiments to test this possibility, both *in vitro*, using wheat germ extracts and reticulocyte lysates, and *in vivo*, using tobacco protoplasts.

**METHODS**

*Virus isolates used:* TBRV-G, an isolate of the German (potato bouquet) serotype of TBRV (Harrison, 1958), containing RNA-1, RNA-2 and RNA-3; TBRV-G12, a single
lesion isolate of TBRV-G free of RNA-3 (Murant et al. 1973); TBRV-S, the stock culture from *Arctium lappa* (Harrison, 1958) of the Scottish (beet ringspot) serotype of TBRV containing three RNA species (RNA-1, RNA-2 and RNA-3); and TBRV-SI2, a single lesion isolate of TBRV-S that was freed of RNA-3 using the procedure described by Murant et al. (1973).

**Purification.** Systemically infected leaves of *Nicotiana clevelandii* were disrupted in 0.067 M-phosphate buffer, pH 7.0 (2 ml/g leaf), containing 0.1% thioglycollate and 0.01 M-EDTA using a mechanical blender. After filtering through muslin, the extract was clarified using 8.5% (v/v) n-butanol and the virus was concentrated and purified by precipitation with 10% polyethylene glycol + 1% NaCl followed by one or two cycles of differential centrifugation. Pellets of virus were resuspended in phosphate buffer.

**RNA extraction.** Virus (about 1 mg/ml) in phosphate buffer was shaken with an equal volume of phenol + m-cresol (9:1, v/v) containing 0.1% 8-hydroxyquinoline. After further extraction of the aqueous phase with the phenol mixture the RNA was precipitated from 70% ethanol. RNA was washed by dissolving it in 0.15 M-sodium acetate + 0.5% SDS (pH 6.0) and precipitating it with ethanol. RNA was stored as a precipitate in 70% ethanol, or dry in sealed ampoules, or as an aqueous solution at −20 °C.

**Separation of RNA species.** The three RNA species were separated by sedimentation in sucrose gradients or by electrophoresis in polyacrylamide gels. Gradients of 10 to 40% (w/v) sucrose in 0.15 M-tris-HCl, pH 7.5, were loaded with 0.5 ml buffer containing 0.25 mg RNA and centrifuged in a Beckman SW27.1 rotor for 15 h at 24000 rev/min at 12 °C. The RNA was recovered by precipitation from 70% ethanol.

Electrophoretic separations were made in composite gels of 2.2% acrylamide + 0.5% agarose in 0.09 M-tris + 0.0025 M-EDTA + 0.01 M-boric acid, pH 8.3. The gels were stained briefly along one side with toluidine blue; the partially stained bands were excised and the RNA eluted electrophoretically (L. Pinck, personal communication) and collected in Ultra Thimbles (Schleicher & Schuell, GmBH, W. Germany). The RNA was then treated with aqueous phenol and recovered by precipitation from 70% ethanol.

Judging from the absorbance profiles of polyacrylamide gels the isolates studied contained about 4 to 5 times as much RNA-2 as RNA-1; RNA-3 when present was about equal in amount to RNA-1.

**Translation in wheat germ extracts.** Commercial wheat germ (General Mills Inc., Vallejo, California) was extracted as described by Marcu & Dudoit (1974). RNA (5 to 10 μg) was added to 0.1 ml reaction mixture containing 0.03 ml wheat germ extract, 15 to 60 μCi 35S-methionine (sp.act. 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-pteridine, 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, 2 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, 15 mM-haemin, 15 mM-creatine phosphate, 40 units/ml creatine phosphokinase, 0.1 mM-unlabelled amino acids and 0.5 μCi 35S-methionine (as above). Incubation was for 1 h at 27 °C.

**Estimation of incorporation.** Samples (5 to 10 μl) were dried on disks of Whatman 3 MM paper. After washing once in boiling 5% trichloroacetic acid and several times in cold acid, then once each in ethanol and ethanol/ether (1:1, v/v), the radioactivity of dry disks was determined in 5 ml 0.5%, 2,5-diphenyloxazole in toluene using an Intertechnique SL30 counter. Counting efficiency was about 40% for 35S.
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Analysis of polypeptides in polyacrylamide-SDS gels. Reaction mixtures were centrifuged at 105000 g for 1 h and the supernatant fluids were treated with RNase as described by Mayo et al. (1976). Solutions were then made 2% SDS, 2% 2-mercaptoethanol and 10% glycerol, and heated in a boiling water bath for 90 s. Samples were stored at −20 °C or analysed directly on discontinuous slab gels of 7.5% or 12.5% acrylamide using buffers described by Laemmli (1970). Following electrophoresis, gels were stained with Coomassie blue, dried and placed in contact with Kodirex film (Kodak Ltd.). The proteins used to estimate mol. wt. were RNA polymerase from Escherichia coli (Boehringer; mol. wt. 165000, 155000, 95000 and 395000), bovine serum albumin (67000), pyruvate kinase (57000), ovalbumin (45000) and carbonic anhydrase (29000). Calibration lines of log (mol. wt.) against mobility were slightly curved; thus lines fitted by eye give estimates with an accuracy of no better than ± 5%.

Isolation and irradiation of tobacco protoplasts. Protoplasts were isolated by two-step enzyme digestion (Kubo et al. 1975b) from palisade cells of leaves of Nicotiana tabacum cv. Xanthi grown in a controlled environment cabinet (Kubo et al. 1975a). Protoplasts were irradiated in 0.7 M-mannitol at 10⁶ protoplasts/ml in approx. 2 mm deep layers under a Model 12 u.v. lamp (Hanovia Ltd., Slough, U.K.) with a dose of 420 μW/cm² estimated from potassium ferrioxalate actinometry (Hatchard & Parker, 1956). After irradiation protoplasts were washed with 0.7 M-mannitol and inoculated within 40 min.

Inoculation and culture of protoplasts. Protoplasts (0.5 × 10⁶ per ml) were inoculated for 10 min by the indirect method (Kubo et al. 1975b) using final concentrations of 1 μg/ml TBRV-S or TBRV-S12, 1 μg/ml poly-L-ornithine (type 1-C, mol. wt. about 120000; Sigma Biochemicals, London) and 0.025 M-potassium phosphate, pH 6. Following inoculation, protoplasts were washed and suspended in incubation medium (Kubo et al. 1975b) containing chloramphenicol at 100 μg/ml. Infection of non-irradiated protoplasts, assessed by counting the proportion of living protoplasts that stained with fluorescent antibody to TBRV-S after culture at 22 °C for 44 h, was between 80% and 90%. Irradiation decreased the amount of antigen in the protoplasts making accurate assessment of staining impracticable.

Samples of 9.6 μCi in 3 μl of ³⁵S-methionine (sp. act. 850 Ci/mmol, Radiochemical Centre, Amersham) were added 2.5 h after inoculation to 0.1 ml cultures in small conical tubes containing 2.5 × 10⁴ protoplasts. These were kept at an angle of about 45° to the light source at 22 °C, 3000 lux for 44 h.

Sampling and analysis of polypeptides in protoplasts. Protoplasts were sedimented at about 150 g for 1 min, washed with 1 ml of 1 M-methionine in 0.7 M-mannitol and dissolved in 0.1 ml hot 2% SDS, 2% 2-mercaptoethanol, 15% glycerol in 0.05 M-tris-HCl, pH 6.8. Samples were heated in boiling water for 90 s and subjected to electrophoresis in 8% polyacrylamide gels as described above. In these gels RNA polymerase and pyruvate kinase were replaced as markers by α-phosphorylase (mol. wt. 94000) and β-galactosidase (mol. wt. 135000; Sigma).

RESULTS

When TBRV RNA was added to wheat germ extracts, the incorporation of methionine was stimulated about 50-fold (Table 1, Expt. 1). RNA from strain G or strain S was as effective as that from tobacco mosaic virus, showing that TBRV RNA is relatively efficient as a messenger RNA. When a range of concentrations of RNA from strains S and S12 were tested, maximum incorporation was stimulated by either kind of RNA preparation at 80 μg/ml and Table 1, Expt. 2 shows that RNA-3 did not affect the efficiency of incorporation.
Table 1. Translation of TBRV RNA species in wheat germ extracts*

<table>
<thead>
<tr>
<th>Expt</th>
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<th>Concentration (µg/ml)</th>
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<tr>
<td>1</td>
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<td>---</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>TBRV-G</td>
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</tr>
<tr>
<td></td>
<td>TBRV-S</td>
<td>100</td>
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</tr>
<tr>
<td></td>
<td>TMV</td>
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<td>2.0</td>
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<tr>
<td>2</td>
<td>TBRV-S</td>
<td>80</td>
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</tr>
<tr>
<td></td>
<td>TBRV-S12</td>
<td>80</td>
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</table>

* Reaction mixtures (0.1 ml) contained 35S-methionine (met) at 21.6 Ci/mmol (Expt. 1) or 7 Ci/mmol (Expt. 2) and were kept at 30 °C for 2 h.
† RNA-3 was extracted from bands in acrylamide/agarose gels.

Fig. 1. Autoradiograms of in vitro translation products after electrophoresis in acrylamide gels. (a-e) Comparison in 7.5 % gels of wheat germ translation products of TBRV RNA lacking or containing RNA-3. (a) RNA from TBRV-S12 (no RNA-3); (b) RNA from TBRV-S (+ RNA-3); (c) RNA from TBRV-G (+ RNA-3); (d) RNA from TBRV-S (+ RNA-3); (e) RNA-3 from TBRV-S purified by electrophoresis in polyacrylamide/agarose composite gels. Arrows indicate the 200000 mol.wt., 160000 mol.wt, and 48000 mol.wt, bands. (f-i) Comparison in 7.5 % gels of reticulocyte lysate translation products. (f) Control, incubation without added RNA; (g) RNA from TBRV-G (+ RNA-3); (h) RNA from TBRV-S (+ RNA-3); (i) RNA-3 from TBRV-S as in (e) above. Arrows indicate the 160000 mol.wt. and 48000 mol.wt, bands.

Purified RNA-3 also stimulated incorporation although residual acrylamide caused some inhibition, particularly at RNA concentrations greater than 40 µg/ml.

Analysis in polyacrylamide gels of the 35S-labelled products of translation of TBRV RNA revealed many bands of protein with a maximum mol. wt. of about 200000 and a prominent band of mol.wt. 160000 (Fig. 1a to d, arrowed). Preliminary experiments suggest that the
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Fig. 2. Comparison in 8% acrylamide gels of 35S-methionine labelled polypeptides synthesized in tobacco protoplasts with translation products synthesized in vitro. (a-c) Polypeptides extracted from protoplasts inoculated with (a) a virus-free inoculum, (b) TBRV-S12 (no RNA-3), or (c) TBRV-S (+RNA-3). (d) Translation products from TBRV-S RNA (+RNA-3) as in Fig. 1(d).

CP indicates the position of TBRV coat protein and S indicates the 48,000 mol.wt. band. (a'—d') A photographic enlargement of the part of the autoradiogram containing the coat protein and the 48,000 mol.wt. band (indicated by dashes).

160,000 mol.wt. protein is the translation product of RNA-2; if so, this protein would represent a complete translation of RNA-2 (mol.wt. 1.63 x 10^6, Murant & Taylor, 1978). As with other nepoviruses (Harrison & Murant, 1977), RNA-2 is thought to code for coat protein (Randles et al. 1977) and if the pattern of translation in vitro is reproduced in vivo the 160,000 mol.wt. protein must be processed by proteolytic cleavage to produce coat protein.

When RNA-3 was present, an additional prominent band of mol.wt. 48,000 was found (Fig. 1b to d, arrowed) which corresponded with the sole translation product from purified RNA-3 (Fig. 1e). A similar mol.wt. estimate was obtained using 12.5% acrylamide gels, suggesting that 48,000 is close to the true value for this protein. The other bands produced by wheat germ extracts were presumably either the result of premature termination, or translation of RNA fragments produced by nuclease action (Hunter et al. 1977). The patterns of these bands produced by TBRV-S RNA (Fig. 1b) and TBRV-G RNA (Fig. 1c) are very similar, suggesting that there may be similarities in secondary structure of the RNA of the two strains.

Translation in reticulocyte lysates gave very similar results. Although there was considerable endogenous synthesis of protein (Fig. 1f), TBRV-RNA containing RNA-3 stimulated the synthesis of a 160,000 mol.wt. band and a 48,000 mol.wt. band (Fig. 1g, h, arrowed) and purified RNA-3 from TBRV-S stimulated synthesis of the 48,000 mol.wt. band (Fig. 1i). Both in wheat germ extracts and reticulocyte lysates the apparent mol.wt. of the RNA-3 specific band of TBRV-G was slightly greater (Fig. 1c, g) than that of TBRV-S (Fig. 1b, h).
Extracts of $^{35}$S-methionine-labelled tobacco protoplasts were also examined for the presence of an RNA-3 specific protein. Protoplasts were u.v.-irradiated for 6 to 10 min (0.15 to 0.25 J/cm²) to diminish the synthesis of tobacco proteins (Sakai et al. 1977) and inoculated with TBRV-S or TBRV-S12. Comparison of extracts of protoplasts inoculated with a virus-free inoculum and those inoculated with TBRV-S12 showed a single virus-induced band of the same mobility as TBRV-S coat protein (estimated mol. wt. about 52,000 in these gels; CP in Fig. 2). Extracts of protoplasts inoculated with TBRV-S contained very similar amounts of $^{35}$S-labelled coat protein and in addition a band of mol. wt. 48,000 (S in Fig. 2) which corresponded in position to the RNA-3 specific band synthesized in wheat germ extracts (Fig. 2d).

**DISCUSSION**

The results show that RNA-3 of TBRV is translated both in vitro and in vivo into a polypeptide of mol. wt. about 48,000. Recent estimates of the mol. wt. of RNA-3 made in acrylamide gels containing 8 M-urea at 60 °C were $4.8 \pm 0.1 \times 10^5$ (Murant & Taylor, 1978). Thus the translation product represents virtually 100% of the coding potential of the RNA. There is little (< 25%) homology between the base sequences in TBRV-G RNA-3 and those in RNA-1 + RNA-2 of TBRV-G (A. C. Minson et al. unpublished data). Therefore RNA-3 is not analogous to the genome fragments that act as efficient coat protein messenger RNA of tobacco mosaic virus (Hunter et al. 1976) or turnip yellow mosaic virus (Klein et al. 1976; Pleij et al. 1976). TBRV RNA-3 is therefore, as suggested by Murant et al. (1973), a true satellite RNA. Although producing a sizeable protein in vivo TBRV satellite is coated by TBRV protein and not by its translation product (Murant et al. 1973). In contrast, RNA of tobacco necrosis satellite virus is translated in vitro and in vivo into a satellite specific protein (Salvato & Fraenkel-Conrat, 1977) and is coated in this protein and not that of tobacco necrosis virus (Kassanis, 1966).

TBRV RNA-3 resembles more closely the satellite RNA molecules associated with tobacco ringspot and cucumber mosaic viruses. However, the satellite RNA of tobacco ringspot virus (mol. wt. about 10⁶; Schneider, 1971) is not translated in vitro (Owens & Schneider, 1977) and satellite RNA-5 of cucumber mosaic virus (mol. wt. about 10⁶; Kaper et al. 1976) is translated into proteins of < 5000 mol. wt. (Owens & Kaper, 1977). TBRV satellite RNA is therefore distinct in being of much larger mol. wt. and in being almost completely translated into a large protein; however, although it is synthesized in about the same amount as TBRV coat protein, there is no evidence as to its biological function. By definition, TBRV satellite RNA depends for its multiplication on the functioning of TBRV RNA-1 and RNA-2, presumably on the synthesis of a replicase; possibly the 48,000 mol. wt. protein is required to modify the TBRV replicase so that it will replicate the satellite RNA. If so, it is possible to speculate that the modified replicase molecules might have a decreased affinity for TBRV RNA-1 and RNA-2; this could explain why RNA-3 inhibits local lesion formation when added to inocula containing RNA-1 + RNA-2 (Murant et al. 1973).

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REFERENCES


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