Replication of RNA-1 of Tomato Black Ring Virus Independently of RNA-2

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SUMMARY

In hybridization experiments, using complementary DNA (cDNA) copies of the two genome parts of tomato black ring virus (TBRV RNA-1 and RNA-2), no sequence homology between the two RNA species was detected.

When tobacco mesophyll protoplasts were inoculated with purified middle component particles, which contain only RNA-2, no replication of TBRV RNA could be detected. However, when they were inoculated with purified bottom component particles, which contain only RNA-1, extracts made 24 or 48 h later contained RNA that had the same mobility as RNA-1 in polyacrylamide–agarose gels, and that hybridized to cDNA copies of RNA-1. Thus RNA-1 can replicate in protoplasts that do not contain RNA-2. Moreover, this RNA-1 was capable, when mixed with nucleoprotein particles containing RNA-2, of inducing the formation of local lesions in Chenopodium amaranticolor leaves, and therefore was intact and attached to the genome-linked protein. The genome-linked protein of nepoviruses is probably virus-coded, and its production in protoplasts inoculated with bottom component particles therefore suggests that RNA-1 contains the gene for this protein.

INTRODUCTION

Particles of nepoviruses contain two major RNA species. The larger (RNA-1) has a mol. wt. of about $2.8 \times 10^6$, estimated by electrophoresis of denatured RNA in agarose gels, and the smaller (RNA-2) has a mol. wt. of $1.4 \times 10^6$ to $2.4 \times 10^6$, depending on the virus (Murant et al. 1979). In experiments with three members of this group, raspberry ringspot, tobacco ringspot and tomato black ring (TBRV) viruses, preparations of RNA-1 or nucleoprotein particles containing RNA-1, and preparations of RNA-2 or nucleoprotein particles containing RNA-2, induced very few lesions in test plants, whereas mixtures of preparations containing the two kinds of RNA induced many lesions (Harrison et al. 1972a; Randles et al. 1977). Moreover, further work, in which pseudo-recombinant isolates were produced by mixing RNA-1 and RNA-2 from different virus strains of either raspberry ringspot virus or TBRV, indicated that genetic determinants for various properties could be assigned to either RNA-1 or RNA-2 (Harrison et al. 1972b, 1974; Harrison & Murant, 1977). It was, therefore, concluded that the genome of these viruses is in two parts, both of which are needed for infection.

A few experiments have been done to test the possibility that either RNA-1 or RNA-2 of raspberry ringspot virus could replicate without inducing lesions in inoculated leaves, but the results did not support the idea that either RNA species can replicate independently of the other (Harrison et al. 1972a).
There are several general similarities in the genome structure and properties of nepoviruses and comoviruses (Bruening, 1977). For example, comoviruses, such as cowpea mosaic virus, resemble nepoviruses in producing two major RNA species, which are packaged in different nucleoprotein particles; both RNA species are needed to induce lesions in inoculated leaves (Wood & Bancroft, 1965; Bruening & Agrawal, 1967; van Kammen, 1968; Harrison et al. 1972a), both have polyadenylate at their 3' ends (El Manna & Bruening, 1973; Mayo et al. 1979a) and both are covalently linked to a small polypeptide (Daubert et al. 1978; Stanley et al. 1978; Mayo et al. 1979b).

Although none of this work provided direct evidence that nepovirus or comovirus RNA-1 or RNA-2 can replicate independently of each other, translation products of cowpea mosaic virus RNA-1 are synthesized in protoplasts inoculated with bottom component particles, which contain only RNA-1 (Rottier, 1980; Rottier et al. 1980). This suggests that the possibility should be examined further, and in this paper we describe tests of the ability of TBRV RNA-1 and RNA-2 to replicate in mesophyll protoplasts inoculated with bottom component particles alone or middle component particles alone. The results show unequivocally that RNA-1 can replicate independently, whereas RNA-2 is synthesized only when the cells also contain RNA-1.

METHODS

Purification of virus particles. TBRV (isolates A and GI2; Hanada & Harrison, 1977) was propagated in Nicotiana clevelandii. Preparations of virus particles were obtained using the method described by Mayo et al. (1979a). Middle (M) and bottom (B) component particles were obtained from preparations of virus particles as described by Harrison & Barker (1978). All experiments were done with TBRV-A unless otherwise stated.

RNA extraction from preparations of virus particles. Preparations of the two RNA species (RNA-1 and RNA-2) were obtained by extracting RNA from separated B and M component particles respectively, using the phenol/SDS method described by Harrison & Barker (1978). Following precipitation with ethanol at −18°C, RNA was collected by sedimentation at 3000 g for 5 min. The RNA was resuspended in 0.15 M-sodium acetate plus 0.5% SDS, pH 6 (acetate-SDS), to which was added an equal volume of 4 M-LiCl. After 18 h at 4°C, the precipitated RNA was collected by sedimentation at 10000 g for 10 min, resuspended in acetate-SDS and precipitated at −18°C by adding 3 vol. ethanol.

Complementary DNA-RNA hybridization. 3H-cDNA to each TBRV RNA species was prepared essentially by the method of Taylor et al. (1976). Reaction mixtures of 50 µl contained 50 mM-tris-HCl, pH 8.3, 8 mM-dithiothreitol, 3 mM-MgCl₂, 0.67 µM each of dATP, dCTP and dGTP, 50 µCi Me-³H-TTP (sp. act. 60 Ci/mmol; ICN Pharmaceuticals, Irvine, Calif., U.S.A.), 33 µg/ml oligomycin (Calbiochem, San Diego, Calif., U.S.A.), 20 µg/ml TBRV RNA-1 or RNA-2, 500 µg/ml DNA primer mixture and 16 units of avian myeloblastosis virus reverse transcriptase (kindly donated by Dr G. E. Houts, Bethesda, Md., U.S.A.). Incubation was for 2 h at 37°C. ³H-cDNA was purified from the reaction mixtures as described by Gould & Symons (1977), and dissolved in hybridization buffer (0.18 M-NaCl, 0.01 M-tris-HCl, pH 7, 1 mM-EDTA, 0.05% SDS; Gonda & Symons, 1978) to give about 1000 cpm/min/µl.

For hybrid formation, RNA samples were serially diluted in hybridization buffer, and duplicate 10 µl samples of each dilution were mixed with 2 µl ³H-cDNA. The mixtures were sealed into 25 µl capillary tubes, immersed in boiling water for 5 min, incubated at 60°C for 2.5 h and chilled on ice.

Hybrid formation was assayed using S1 nuclease, purified from Taka-diastase powder (Uniscience, Cambridge, U.K.) by the method of Vogt (1973), up to and including the
Independent replication of TBRV RNA-1

DEAE-cellulose column step. The capillaries were opened and washed out with 200 μl 50 mM-sodium acetate, pH 4.5, plus 100 mM-NaCl plus 2 mM-ZnSO₄ plus 10 μg/ml degraded DNA (Sigma, Type IV). Samples (100 μl) were transferred on to DE-81 filter discs (Maxwell et al. 1978) before and after digestion with S₁ nuclease at 45 °C for 30 min. As Gonda & Symons (1978) have shown that both the extent of resistance to digestion of hybrids and estimates of sequence homology decrease with increasing S₁ nuclease concentration, the amount of S₁ nuclease used was the minimum required to digest at least 95% of the cDNA in control experiments without RNA. The DE-81 discs were washed ten times in 0.4 M-Na₂HPO₄, once in water, twice in ethanol and once in diethyl ether. After drying, remaining radioactivity was counted in 3 ml toluene containing 0.5% diphenyloxazole, using an Intertechnique SL30 liquid scintillation counter.

Preparation, inoculation and culture of protoplasts. Mesophyll protoplasts were prepared from tobacco (Nicotiana tabacum cv. Xanthi) plants grown in controlled conditions (Kubo et al. 1975b). Protoplasts were prepared and inoculated by the indirect method as described by Kubo et al. (1975a); inocula contained M component (final concentration 0.1 μg/ml), B component (0.01 μg/ml), or both. After inoculation, protoplasts were incubated for periods up to 48 h at 22 °C in continuous light (Kubo et al. 1975a). Protoplast samples were stained with fluorescein-conjugated antibody to TBRV particles as described by Kubo et al. (1975a).

Radioactive labelling experiments. Protoplasts (1 × 10⁶ to 2 × 10⁶) in 1 ml incubation medium were mixed with 5 to 10 μl ³H-uridine (28 Ci/mmol, 1 mCi/ml; ICN Pharmaceuticals) either 2 h or 19 h after inoculation. RNA was extracted as described below, usually 19 h or 44 h after inoculation. After precipitation from ethanol plus acetate–SDS (5:2, v/v), RNA was dissolved in 25 μl 2% SDS plus 0.036 M-tris plus 0.03 M-NaH₂PO₄ plus 0.01 M-EDTA, pH 7.8 (TPE), containing 5% sucrose (RNase-free grade; Sigma). Samples of 10 μl were analysed in 1 mm-thick slab gels of 2.4% polyacrylamide plus 0.5% agarose by electrophoresis at about 7 V/cm in TPE buffer. Gels were then fixed overnight in methanol plus water plus acetic acid (5:5:1, by vol.) at 4 °C, infiltrated for 60 to 90 min with 4 to 5 vol. of scintillator solution (En³Hance; New England Nuclear, Boston, Mass., U.S.A.) and for 60 min with water and then dried. Fluorographs were made by exposing pre-flashed (Laskey & Mills, 1975) Kodak XRP5 film at −70 °C for 1 to 5 days.

Extraction of RNA from protoplast samples. Protoplast samples were resuspended in 0.01 M-tris-HCl plus 1% SDS, pH 7.5, and emulsified with an equal volume of water-saturated phenol plus m-cresol (9:1, v/v) containing 0.1% 8-hydroxyquinoline. The aqueous phase was re-extracted with phenol mixture and the RNA was precipitated twice from 70% ethanol at −18 °C, once from 2 M-LiCl at 4 °C and once again from 70% ethanol, as described above.

Infectivity assay. The relative infectivity of RNA-containing extracts or TBRV particles was estimated from the number of lesions produced in inoculated leaves of Chenopodium amaranticolor. The samples were diluted in 0.01 M-tris-HCl, 0.015 M-NaCl, pH 7.6, containing bentonite (20 μg/ml); leaves were dusted ‘Corundum’ (UK Optical Co., London, U.K.) before inoculation; plastic gloves were worn during inoculation; the fore-finger was rinsed and dried after inoculating each leaf; and a Latin square design was used to allocate treatments to different leaves on a group of plants. After inoculation, plants were kept for 3 days in a glasshouse at 15 to 20 °C and were then transferred to a cabinet in which they were illuminated (5000 lux) for 16 h at 24 °C with alternating periods of 8 h in darkness at 20 °C. These conditions favour the production of readily countable lesions (R. L. S. Forster, personal communication).

To assay samples that contained only one species of TBRV genome RNA, the infectivity of each sample was tested with and without added M component or B component particles.
RESULTS

Hybridization of cDNA to purified TBRV RNA species

Fig. 1 shows the kinetics of hybridization of each of the cDNA preparations to the two TBRV genome RNA species in excess. At the highest concentrations of RNA, about 60% of the homologous cDNA became resistant to S1 nuclease, and this value is taken to represent 100% hybridization. Conversely, in the absence of RNA, less than 5% of the cDNA was resistant, and this is taken to represent 0% hybridization.

For the hybridization of each cDNA to its homologous RNA species, a single sharp transition was observed. Values of Rot (initial RNA concentration × time, for 50% hybridization) were estimated from experiments similar to those shown in Fig. 1 but including more determinations near the midpoint of the curve, and were 1.42 × 10^{-2} mol. s/l for cDNA-I versus RNA-I and 1.29 × 10^{-2} mol. s/l for cDNA-2 versus RNA-2.

Reaction of cDNA-I with RNA-2 and of cDNA-2 with RNA-1 resulted in very little hybridization, and this occurred only at high values of Rot. The curves are consistent with mutual contamination of each RNA preparation with the other RNA species to the extent of between 0.1 and 0.5%. No evidence for sequences common to the two RNA species was obtained, and it is estimated that such sequences, if present at all, account for less than 5% of either species.

Hybridization of cDNA to RNA extracted from protoplasts

Hybridization of cDNA to RNA preparations extracted from protoplasts followed kinetics similar to those shown in Fig. 1. Concentrations of TBRV genome RNA species in the extracts were estimated by multiplying the concentration of purified RNA (R0) required to hybridize a chosen percentage of the cDNA by the dilution of the protoplast extract required to reach the same percentage hybridization. Results of three typical experiments are given in Table 1. As far as possible, calculations were based on 50% hybridization of the cDNA, but with extracts that contained only small amounts of the TBRV RNA, this extent of hybridization was not reached, and estimates were based on the lower part of the curves. Such estimates are less reliable and are indicated by parentheses in Table 1.

It is clear that in protoplasts inoculated with both M and B components, substantial replication of both RNA species takes place. For RNA-I, at least, more synthesis occurs in
Table 1. *Virus RNA content, estimated from hybridization kinetics, of extracts from protoplasts inoculated with M and B particles, separately or together*

<table>
<thead>
<tr>
<th>Protoplasts inoculated with*</th>
<th>Time extracted (h after inoculation)</th>
<th>No. of viable protoplasts (× 10⁴)</th>
<th>Wt. (ng) of</th>
<th>Copies (× 10⁻⁴) per infected protoplast† of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RNA-1</td>
<td>RNA-2</td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M + B</td>
<td>24</td>
<td>1.4</td>
<td>325</td>
<td>346</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>1.3</td>
<td>353</td>
<td>12 (12)†</td>
</tr>
<tr>
<td>M</td>
<td>24</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M + B</td>
<td>24</td>
<td>0.8</td>
<td>134</td>
<td>–</td>
</tr>
<tr>
<td>M + B</td>
<td>48</td>
<td>0.7</td>
<td>455</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>0.8</td>
<td>160</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>48</td>
<td>0.6</td>
<td>93</td>
<td>–</td>
</tr>
<tr>
<td>Expt. 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>1.5</td>
<td>1.5</td>
<td>–</td>
<td>61</td>
</tr>
<tr>
<td>M</td>
<td>24</td>
<td>0.8</td>
<td>–</td>
<td>(17)</td>
</tr>
</tbody>
</table>

* Inocula contained M component at 0.1 µg/ml and/or B component at 0.01 µg/ml.
† The proportion of viable protoplasts infected in each experiment was assumed to be equal to the proportion that were stained with fluorescein-conjugated antibody to TBRV 48 h after inoculation with both components. This proportion was 84% (expt. 1), 60% (expt. 2) and 59% (expt. 3). Less than 1% of protoplasts inoculated with either component separately could be stained.
‡ Numbers in parentheses were calculated from less than 50% hybridization of cDNA.

the second than in the first day after inoculation, with a fourfold increase between 24 and 48 h in the amount present. In protoplasts inoculated with B component only, and cultured for 24 h, RNA-1 was found in amounts comparable to or slightly greater than those in protoplasts inoculated with both components. However, in these protoplasts, the amount of RNA-1 decreased slightly between 24 and 48 h; the small amount of RNA-2 found can be attributed to the few doubly infected protoplasts, detected by fluorescent antibody staining, that resulted from the remaining slight contamination of the inoculum with M component.

Measurable amounts of RNA-2 were found in protoplasts 24 h after inoculation with purified M component, but these amounts were less than those in extracts made 1.5 h after inoculation. Very little RNA-2 was found in such protoplasts 48 h after inoculation (results not given). These measurements indicate a relatively slow degradation of RNA derived from the inoculum, and provide no evidence for replication of RNA-2 in protoplasts inoculated with purified M component.

Incorporation of ³H-uridine into virus RNA

RNA was extracted from protoplasts after incubation in a medium containing ³H-uridine, and analysed by electrophoresis in acrylamide–agarose gels. When ³H-uridine was added 19 h after inoculation and RNA was extracted 44 h after inoculation, three major and three minor radioactive bands were detected in gels of RNA from mock-inoculated protoplasts (Fig. 2h). Only in RNA from protoplasts inoculated with both M and B components (Fig. 2g) were bands of virus-specific radioactive RNA detected consistently. These corresponded in mobility to RNA-1 and RNA-2 (both arrowed) extracted from purified virus. When ³H-uridine was added to cultures 2 h after inoculation and RNA was extracted 19 h after inoculation a faint RNA-1 band was produced by extracts from protoplasts inoculated with both M and B components (Fig. 2c) and also by extracts from protoplasts inoculated with B component only (Fig. 2b). RNA-2 was found only in extracts from protoplasts inoculated with both M and B components but was partly obscured by a
Fig. 2. Fluorogram of polyacrylamide-agarose gel electrophoresis of RNA extracted from $^3$H-uridine-labelled protoplasts. Protoplasts were inoculated with (a, e) M component; (b, f) B component; (c, g) M and B components; (d, h) buffer only. For samples (a) to (d), $^3$H-uridine was added 2 h, and RNA extracted 19 h after inoculation. For samples (e) to (h), $^3$H-uridine was added 19 h, and RNA extracted 44 h after inoculation. Arrows mark the positions of TBRV RNA-1 and RNA-2.

band of protoplast RNA. No virus-specific bands were produced by extracts from protoplasts inoculated with M component only (Fig. 2a, e). In some experiments a band of RNA-1 was produced by extracts of protoplasts inoculated with B component when $^3$H-uridine was supplied between 19 h and 44 h after inoculation, but this band was always much less radioactive than the RNA-1 band produced by extracts from protoplasts inoculated with both M and B components.

Similar results were obtained when $^3$H-labelled RNA from protoplasts inoculated with B and M nucleoprotein components of TBRV-G12 was analysed.

When protoplasts were transferred to non-radioactive medium after $^3$H-uridine had been supplied between 2 h and 19 h after inoculation, and cultured for a further 24 h before RNA was extracted, the fate of $^3$H-labelled RNA-1 differed between protoplasts inoculated with both M and B components and those inoculated with B component only. Whereas RNA-1 and RNA-2 remained labelled in doubly inoculated protoplasts, no labelled virus-specific RNA was detected in extracts from protoplasts inoculated with B component.

**Local lesion formation by TBRV RNA from inoculated protoplasts**

RNA extracted from protoplasts inoculated with either M component or B component, or a mixture of the two, was tested for its ability to induce local lesion formation. Table 2 gives the results of assays on extracts of protoplasts sampled 24 h after inoculation. In this experiment fewer than 0.1% of the protoplasts inoculated with either M or B component were stained by treatment with fluorescent antibody to TBRV particles, whereas 60% of those inoculated with the mixture of M and B components were stained in parallel tests.
### Table 2. Local lesion induction by RNA extracted from protoplasts inoculated with M and B components, separately or together

<table>
<thead>
<tr>
<th>Component added to inoculum*</th>
<th>Buffer control</th>
<th>M component</th>
<th>B component</th>
<th>M + B components</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>1/8†</td>
<td>1/40</td>
<td>1/8</td>
</tr>
<tr>
<td>B</td>
<td>1/8†</td>
<td>19</td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td>M</td>
<td>1/8†</td>
<td>8</td>
<td>0</td>
<td>535</td>
</tr>
<tr>
<td>B + M</td>
<td>1/8†</td>
<td>1050</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* M and B component particles were used at final concentrations of 0.5 and 0.25 μg/ml, respectively.
† Dilution of protoplast extract. Undiluted extract contained RNA from about 8 x 10⁵ protoplasts/ml, harvested 24 h after inoculation.
‡ Number of lesions in three Chenopodium amaranticolor leaves.

Similarly, RNA extracted from protoplasts inoculated with either M or B component produced few lesions, whereas that from protoplasts inoculated with both kinds of particle produced many. However, when M particles were added to RNA from protoplasts inoculated with B component, the number of lesions increased more than 50-fold. No such increase occurred when B particles were added to this RNA, or when either kind of particle was added to RNA from protoplasts inoculated with M component. The lesion-forming ability of RNA from protoplasts inoculated with both M and B components was also increased by the addition of M particles, but only about threefold or less, and the number of lesions produced by this mixture was only slightly greater than that produced by the mixture containing M particles together with RNA from protoplasts inoculated with B component. Indeed the lesion-forming ability of RNA from protoplasts inoculated with M and B components was increased as much by the addition of B particles as by that of M particles.

These results indicate that the RNA-1 synthesized in protoplasts inoculated with B component is biologically active. They cannot be explained by the survival of B particles or RNA-1 from the inoculum, because RNA extracted from protoplasts immediately after inoculation with B component induced few or no local lesions either on its own or after adding M particles. Moreover, because the ability of RNA-1 of TBRV to take part in local lesion formation depends on the presence of a virus-specific genome-linked protein (Harrison & Barker, 1978; Mayo & Barker, 1980), our results indicate that the gene for this protein is in RNA-1.

In similar tests on RNA extracted from protoplasts 2 days after inoculation, the results followed the same general trends as those in Table 2 but the enhancement of lesion number produced by adding M particles to RNA from protoplasts inoculated with B component was somewhat smaller. Between 1 and 2 days after inoculation the amount of active RNA-1 in these protoplasts decreased by about half, whereas the amount of active RNA extracted from protoplasts inoculated with both M and B components increased about fourfold.

### DISCUSSION

In kinetic hybridization experiments between cDNA-1 and RNA-2 and between cDNA-2 and RNA-1 (Fig. 1), no reaction was observed in the range of Rₐ values where hybridization due to sequences common to the two RNA species would be expected to occur. We estimate that hybridization involving no more than 5% of the cDNA sequences would be reliably observable under our conditions, and therefore conclude that common sequences, if they exist at all, comprise about 250 nucleotide residues or less of RNA. Both species of RNA contain polyadenylate tracts at their 3'-ends (Mayo et al. 1979a) but this 'common
sequence' is probably too small to be detectable. Rezaian & Francki (1974), using RNA-
RNA hybridization, similarly concluded that there were few sequences common to RNA-1
and RNA-2 of another nepovirus, tobacco ringspot virus, although their limit for the maxi-
num amount of homology was about 900 nucleotide residues.

The conclusion that there is little or no sequence common to RNA-1 and RNA-2 of
TBRV depends on the assumption that the cDNA copies represent the entire RNA sequence
in each instance. Although this has not been demonstrated for these cDNA preparations,
cDNA copies of other virus RNA molecules prepared under essentially similar conditions
have been shown to be fully representative (Gould & Symons, 1977).

Experiments described in this paper show that, in protoplasts inoculated with purified B
component, RNA is synthesized that migrates in polyacrylamide-agarose gels at the
mobility expected for TBRV RNA-1, forms hybrids with cDNA copies of TBRV RNA-1,
and when mixed with M particles induces local lesions in C. amaranticolor. We conclude,
therefore, that RNA-1 is able to replicate in protoplasts that do not contain RNA-2. This
implies that RNA-1 contains all the information required for its own replication. Although
nothing is known about the enzymes involved in TBRV RNA replication, any virus-coded
replicase or replicase subunit must be coded by RNA-1. Alternatively, if TBRV RNA is
replicated by a host-coded enzyme, such activity must be induced by RNA-1. Alternatively, if TBRV RNA is
replicated by a host-coded enzyme, such activity must be induced by RNA-1 but not by
RNA-2.

It has been shown previously (Harrison & Barker, 1978) that the genome-linked protein
must be attached to each TBRV RNA species for them to be able to induce formation of
local lesions. Because the RNA-1 synthesized in protoplasts inoculated with purified B
component is active in this respect, it too must have this protein attached to it. The genome-
linked proteins of different nepoviruses can be distinguished by their mobilities in poly-
acrylamide gels and by the patterns of peptides they produce after treatment with proteases.
In contrast, no differences were found between the genome-linked proteins in preparations
of the same virus grown in different host plants or between the proteins attached to RNA-1
and to RNA-2 of TBRV (Mayo & Barker, 1980). These results suggest that the genome-
linked protein is virus-coded and, taken together with the results reported in this paper, that
a nucleotide sequence in RNA-1 codes for it. It is apparently a small protein, probably of
less than 50 amino acid residues (Mayo et al. 1979 b), so that we cannot rule out the possibility
that the coding sequence also occurs in RNA-2; such a small common sequence might be
below the limit of detection by the present methods.

The amounts of RNA-1 in protoplasts 24 h after inoculation either with B component
only or with M and B components are similar. However, the further four- or fivefold increase
in RNA-1 during the next 24 h in protoplasts containing both RNA species is in strong
contrast to the lack of any net synthesis of RNA-1 during the second day in protoplasts
inoculated with B component only. Although the labelling experiments with 3H-uridine
show that both synthesis and degradation of RNA-1 are occurring during the second day of
infection with RNA-1 only, such experiments cannot be interpreted quantitatively, and it is
not possible to tell whether the true rate of synthesis of RNA-1 is affected by the presence
of RNA-2. One obvious effect of the absence of RNA-2, which codes for TBRV coat protein
(Randles et al. 1977), is that none of the RNA-1 can be incorporated into nucleoprotein
particles and thus be protected from degradation.

It now seems likely that the ability of RNA-1 to replicate on its own is yet another
similarity between nepoviruses and comoviruses, since Goldbach et al. (1980) have recently
obtained results showing that RNA-1 (B RNA) of cowpea mosaic virus can replicate in
protoplasts independently of RNA-2 (M RNA). In this respect, both groups of viruses
behave like tobacco rattle virus (TRV), the RNA-1 of which has long been known to be
capable of replication in cells not containing RNA-2 (Harrison & Nixon, 1959). However,
RNA-1 of TRV, unlike that of TBRV, can induce local lesions and systemic symptoms in appropriate host plants, and is readily detected by infectivity tests on phenol extracts of infected plants (Singer & Brandenburg, 1961). These differences could be explained if, in infections with RNA-1 only, the infective RNA of TRV can move from cell to cell whereas that of TBRV cannot, or if the infective RNA of TRV is more protected from degradation by cellular enzymes than is that of TBRV. In this connection it is worth noting that the infective RNA of TRV is associated with rapidly sedimenting structures in tissue extracts and is much more stable in these extracts than is free RNA (Cadman, 1962). The outcome of inoculation of purified TBRV B component to whole plants will require further study.

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