Accumulation of different types of raspberry ringspot nepovirus particle in infected *Nicotiana* protoplasts

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*Accumulation of different types of raspberry ringspot nepovirus particle in infected* *Nicotiana* *protoplasts*

Raspberry ringspot virus (RRV) is characteristic of nepoviruses in that three differently sedimenting types of particle are synthesized in infected cells (Murant *et al.*, 1972). The particle types are top (T), middle (M) and bottom (B), named according to their relative positions during sedimentation through sucrose gradients. They contain no RNA (T), about 25% (w/w) RNA [one molecule of RNA 2 (4 kb); M] or about 45% RNA [one molecule of RNA-1 (8 kb) or two molecules of RNA-2; B] (Murant *et al.*, 1972). The proportions of T, M and B particles in purified preparations differ among different strains of RRV and can be characteristic for one strain in one host but can differ in different hosts (Barker, 1980). Also it is known for RRV (Barker, 1980) and for tobacco ringspot nepovirus (Schneider & Diener, 1966) that the relative proportions of T, M and B in infected leaf tissue can differ at different times after inoculation, although this was not so for another isolate of tobacco ringspot virus in a different host (Rezaian & Francki, 1973). Coat protein of RRV is encoded by RNA-2 (Harrison *et al.*, 1972) but *in vitro* translation of RRV RNA yielded only large polypeptides (Jones *et al.*, 1985). By analogy with other nepoviruses (Mayo, 1987) coat protein probably arises by proteolytic processing of a large polyprotein precursor.

RRV multiplies in inoculated tobacco protoplasts (Kubo *et al.*, 1975) and Barker & Harrison (1977) showed that infective virus accumulated in protoplasts at 20 °C to reach a maximum level after about 75 h culture. However the accumulation of the different particle types was not measured. Most RNA-2 molecules are encapsidated in pairs in B particles (Mayo *et al.*, 1973) and M component may therefore be formed when there is an excess of coat protein over RNA molecules. Moreover it is known that T particles can arise from B particles as a result of physical effects such as freezing (Quacquarelli *et al.*, 1976). In order better to understand the relationships between the different particle types we have measured the amounts of each particle type in *Nicotiana* protoplasts at intervals after inoculation.

RRV-S (Harrison, 1958) was propagated in *Nicotiana clevelandii* and purified as described by Mayo *et al*. (1982). Protoplasts were isolated either from *N. tabacum* cv. Xanthi as described by Kubo *et al*. (1975), or from *N. clevelandii* essentially as described by Barker & Harrison (1982) except that 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout and the enzyme solution was 0-6 M-mannitol was used throughout. At the start of the work three isolates were prepared from single local lesions induced by an inoculum of the stock culture. Protoplasts were inoculated with phosphate-buffered mixtures of poly-L-ornithine and purified virus particles, cultured at 20 °C in continuous light and stained with fluorescein isothiocyanate (FITC)-labelled antisera to RRV as described by Barker & Harrison (1977). Viability was measured by the ability of protoplast samples to take up and hydrolyse fluorescein diacetate (Widholm, 1972). At intervals, after inoculation, samples of protoplasts were collected by centrifugation and either kept at −20 °C before lysis or lysed immediately in 0-05% Tween-20 in 0-8% NaCl, 0-02% KCl, 1-44% Na₂-
HPO$_4$.2H$_2$O, 0.02% KH$_2$PO$_4$ (PBS/Tween). Samples were then layered over sucrose gradients and centrifuged. Fractions of the gradients were analysed by ELISA as described by Mayo & Barker (1983). The particle type in the fractions was determined by parallel sedimentation of purified RRV particles and spectrophotometric analysis of the resulting fractions. In control experiments it was found that the proportions of T, M and B particles were not affected by freezing protoplast pellets or storing them for up to 15 days at $-20 \, ^\circ\mathrm{C}$. However, storage of protoplast extracts in PBS/Tween at 4 $^\circ\mathrm{C}$ or $-20 \, ^\circ\mathrm{C}$ resulted in a decrease in the abundance of B particles and a corresponding increase in the abundance of particles that co-sedimented with T particles (data not shown). In subsequent experiments, tests were therefore made on fresh extracts of protoplasts or protoplast pellets kept frozen for less than 15 days.

ELISA was by the indirect method (Barbara & Clark, 1982). Samples were diluted to make the background reaction of samples of healthy material insignificant and readings were converted to an antigen concentration using the ELISA reactions of dilutions of a preparation of unfractionated purified RRV particles, with a concentration determined by spectrophotometry assuming $E_{1\,\text{cm},\,260} = 7$ (Murant et al., 1972). When assessing antigen content, estimates were multiplied by 0.75 to correct for the average RNA content of purified virus.

Mean amounts of virus particles per protoplast were estimated by correcting the yield of virus per protoplast for the proportion of protoplasts stained with FITC-conjugated RRV antibodies in samples taken 2 days after inoculation. The amount of each particle type was calculated by plotting the ELISA values for each gradient fraction and determining the area under the peak corresponding to each particle type.

Fig. 1 and 2 show the distribution of RRV antigen in centrifuged extracts of protoplasts of $N. \, \text{tabacum}$ or $N. \, \text{clevelandii}$ at two times in the multiplication cycle. The only antigen detected corresponded in position to T, M and B particles of purified RRV centrifuged in a parallel gradient. There was no evidence for non-sedimenting antigen, such as individual subunits or small aggregates of protein. The amount of RRV detected in centrifuged extracts was similar to the amount assessed by ELISA of a non-centrifuged sample. Somewhat different results were obtained with the different species of Nicotiana (Fig. 1, 2; Table 1). Whereas the ratios of T/B in extracts of $N. \, \text{tabacum}$ protoplasts changed little during virus multiplication, in $N. \, \text{clevelandii}$ protoplasts T particles became relatively more abundant as infection progressed. The yields of each particle type, derived from plots similar to those shown in Fig. 1 and 2 for several times after inoculation of protoplasts from either plant species, are also given in Table 1 (isolate 1) and Fig. 3.
Table 1. Yields and ratios between amounts of T, M and B particles of raspberry ringspot virus in protoplasts from *N. tabacum* and *N. clevelandii*

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>N. tabacum protoplasts</th>
<th>N. clevelandii protoplasts</th>
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<tbody>
<tr>
<td></td>
<td>Amount of antigen* in particle type (isolate 1)</td>
<td>Ratio</td>
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<tr>
<td></td>
<td>T</td>
<td>M</td>
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<td>170</td>
</tr>
<tr>
<td>73</td>
<td>1280</td>
<td>160</td>
</tr>
</tbody>
</table>

* Nanograms per 10^6 protoplasts estimated from areas under peaks in plots such as those shown in Fig. 1.
More virus was synthesized in each *N. clevelandii* protoplast than in each *N. tabacum* protoplast, although this may perhaps only reflect the approximately 50% greater diameter of *N. clevelandii* protoplasts. However, many more T particles were made in *N. clevelandii* protoplasts and this is reflected in all the ratios in which T appears. The extra synthesis of T particles is evident at later times after inoculation; thus the T/B ratio changes little in *N. tabacum* protoplasts but increases sharply between 52 h post-infection (p.i.) and 65 h p.i. in *N. clevelandii* protoplasts. The ratio of B/M also changes differently with time after inoculation of protoplasts of the two plant species. Whereas B/M increased with time after inoculation of *N. tabacum* protoplasts, it decreased for *N. clevelandii* protoplasts. However the kinetics of accumulation of B particles in the two species are similar (Fig. 3). With all three isolates tested it was possible to detect some virus particles bound to inoculated *N. clevelandii* protoplasts sampled immediately after inoculation, but not to inoculated *N. tabacum* protoplasts. Possibly this difference was caused by different surface change properties of protoplasts of the two species causing different amounts of inoculum to bind.

There is no evidence from these results of T particles arising from other particle types. T particles first appear at the same time after inoculation as B particles and then accumulate either in proportion with B particles (*N. tabacum* protoplasts) or at a greater rate than B particles (*N. clevelandii* protoplasts) (Fig. 3). However, as found by Quacquarelli et al. (1976), nucleoprotein particles of RRV can be made to yield T particles by certain conditions of storage.

The unexpected result was that although M and B particle synthesis in *N. clevelandii* protoplasts had largely, or in some experiments completely, ceased by about 72 h p.i., T particles continued to accumulate. Thus either coat protein was being made from an antigenically non-reactive precursor or the coat protein-containing polyprotein precursor was being synthesized when no more genome RNA was being encapsidated, because either the genome RNA or some factor needed for its encapsidation was not being synthesized. This suggests that RNA-2 used as mRNA may differ from RNA-2 destined for encapsidation. A relatively stable mRNA population was one explanation given by Rottier et al. (1979) to explain synthesis of cowpea mosaic virus (isolate 2). Essentially similar results were obtained for isolate 3.

Fig. 3. Plots of the accumulation of RRV particles (antigen) against time after inoculation of (a) *N. tabacum* protoplasts or (b) *N. clevelandii* protoplasts. Values are derived from ELISA data corrected for the RNA content of reference virus preparations. △, T component, ○, M component, □, B component.
proteins in protoplasts inhibited by actinomycin D from synthesizing virus RNA. Similarly, thiouracil treatment inhibited synthesis of infective turnip yellow mosaic virus particles, but not of RNA-free T particles (Francki & Matthews, 1962; Ralph et al., 1965). A further possibility is that functional mRNA differs from genome RNA. For example, although protease treatment greatly decreases the infectivity of RNA of some nepoviruses (Mayo et al., 1982) similar treatment has no effect on its translation in vitro (Chu et al., 1981; Koenig & Fritsch, 1982). Another type of difference is exemplified by the mRNA for tobacco rattle virus coat protein which is identical to genome RNA-2 except that the 5' leader sequence is much shorter (Cornelissen et al., 1986).

Much more detailed analysis is needed to distinguish between these, and indeed other more elaborate explanations. However the results emphasize that there are subtle mechanisms that remain to be discovered controlling RRV multiplication in protoplasts.

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References


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