Double-stranded, Replicative Form RNA Molecules of Cowpea Mosaic Virus Are Not Infectious

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SUMMARY

Virus-specific double-stranded replicative form (RF) RNA was isolated from cowpeas infected with cowpea mosaic virus. The RF was assayed for infectivity in a local lesion host and a systemic host. In neither host was undenatured RF infectious, although infectivity was restored upon denaturation.

Cowpea mosaic virus (CPMV) is a virus of legumes whose genome consists of two separately encapsidated, positive-sense RNA molecules, both of which are required for infection (van Kammen, 1967; de Jager, 1976). Both RNA molecules have recently been sequenced, the larger, B RNA having 5889 nucleotides, the smaller, M RNA having 3481 nucleotides (Lomonossoff & Shanks, 1983; van Wezenbeek et al., 1983). Each RNA has a small protein (VPg) covalently attached to its 5' terminus (Stanley et al., 1978; Daubert et al., 1978) and is polyadenylated at the 3' end (El Manna & Bruening, 1973). Translation in vitro and protoplast studies have shown that each RNA is initially translated into a large polypeptide which is subsequently cleaved by virus-specific proteases to functional virus proteins (Davies et al., 1977; Pelham, 1979; Rezelman et al., 1980; Rottier et al., 1980; Goldbach et al., 1981).

Replication of CPMV RNA in infected plants takes place in a membrane-associated replication complex containing a 110K polypeptide encoded by the B RNA and two host-specified polypeptides (Dorssers et al., 1984; Franssen et al., 1984). As intermediates in the replication process, partially double-stranded RNA molecules, termed replicative intermediates (RI), and completely double-stranded RNA molecules, termed replicative forms (RF), are produced (van Griensven & van Kammen, 1969; van Griensven et al., 1973). In this short communication we describe the isolation of RF molecules from CPMV-infected cowpeas and assays of their infectivity.

The method of purification of the two RF forms of CPMV, RF-M and RF-B, involving DNase I treatment and CF-11 and Sepharose column chromatography, is essentially a standard method for preparing double-stranded RNA and has been widely used in the preparation of plant virus RFs (Zelcer et al., 1981; Jackson et al., 1971) including those from CPMV (Dorssers et al., 1984). Primary leaves of Vigna unguiculata var. 'Blackeye Early Ramshorn' were harvested 5 or 6 days after inoculation with CPMV strain SB. The mid-ribs were excised and the leaf tissue was frozen in liquid nitrogen and the RNA extracted and treated with DNase I as described by Zelcer et al. (1981). After the elimination of the DNase I by phenol extraction, the RNA was precipitated with ethanol and redissolved in water. An equal volume of 4 M LiCl was added and the solution was kept at −20 °C overnight. After centrifugation, the supernatant containing double-stranded RNA and tRNA was precipitated with ethanol. Partially double-stranded RNA (RI) could be recovered from the LiCl-insoluble precipitate.

The LiCl-soluble RNA was dissolved in 100 mM-NaCl, 50 mM-Tris–HCl pH 7.4, 1 mM-EDTA (STE) and ethanol was added to a final concentration of 17.5% (v/v). The solution was loaded on to a CF-11 (Whatman) column (60 ml bed volume in a disposable syringe) and the column was washed with 10 column volumes of 17.5% ethanol–STE to elute single-stranded RNA. The double-stranded material containing RF-M and RF-B was then eluted with water.

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Agarose gel (1.2%) of CPMV dsRNA (lanes 1 and 2) or CPMV virion RNA (lanes 3 and 4). In each case 1 μg of RNA was loaded. The samples in lanes 2 and 4 were treated with 1 unit RNase T1 in 10 μl 50 mM-sodium phosphate pH 7-0 for 30 min at 37°C. The gel was stained with 2 mg/l ethidium bromide and photographed under u.v. light.

After concentration by lyophilization and ethanol precipitation, the RF sample was dissolved in 1 ml of a solution containing 10% (w/v) sucrose, 0.025% each of xylene cyanol FF and bromophenol blue and was then applied to a Sepharose CL-2B column which was run as described by Richards & Ehrenfeld (1980).

Agarose gel electrophoresis of the nucleic acid eluting in the void volume revealed two very sharp bands corresponding in size to those expected for RF-M and RF-B (Fig. 1, lane 1). The material in these bands was resistant to digestion with RNase T1 under conditions which degraded single-stranded virion RNA (Fig. 1), and was also resistant to digestion with DNase I (data not shown). When denatured by glyoxylation, the material shown in Fig. 1 migrated in electrophoresis as two bands corresponding in position to the two single-stranded viral RNAs; Northern blot analysis showed that the denatured RFs hybridized to strand-specific M13 clones of CPMV RNA of both orientations (data not shown). These lines of evidence demonstrate that the high molecular weight bands shown in Fig. 1 do indeed correspond to CPMV-specific double-stranded RNA molecules. The average yield of purified RFs was approximately 10 μg from 100 g of infected tissue.

The infectivity of the RF molecules was determined on detached half-leaves of *Phaseolus vulgaris* var. 'Pinto', a local lesion host, essentially as described by de Jager (1976). Primary leaves were detached from 6- to 8-day-old seedlings and divided into two halves. The four half-leaves from each plant were inoculated with 50 μl 10 mM-sodium phosphate pH 7-0 containing various amounts of either native RF, heat-denatured RF, virion RNA or heat-denatured virion RNA. Heat denaturation was achieved by boiling the samples for 2 min followed by quick cooling in ice. The RF of CPMV is not infectious; no lesions were observed after 6 days when up to 2 μg RF were inoculated on to a single half-leaf (Fig. 2). Denaturation of the RFs rendered them infectious. The mean number of lesions per half-leaf produced following inoculation with denatured RF was approximately one-third of that produced following inoculation with the
**Fig. 2.** Infectivity assay on *P. vulgaris* var. 'Pinto'. Opposite half-leaves were inoculated to compare virion RNA with boiled virion RNA and RF with boiled RF. Lesions were counted after 6 days and plotted against the amount of RNA inoculated per half-leaf. Each point is the mean of four half-leaves. ●, ssRNA; ○, ssRNA boiled; ▲, RF; △, RF boiled.

**Fig. 3.** Dot blots of homogenates from cowpea leaves inoculated with 1 μg of either CPMV virion RNA (ss), boiled virion RNA [ss(B)], CPMV RF (RF) or boiled CPMV RF [RF(B)]. The standards (M Stds or B Stds) were 5, 25, 100, and 250 ng of purified M or B components. Each dot represents the homogenate from one leaf. The M probe contained the sequence between nucleotides 190 and 695 of M RNA; the B probe contained bases 2141 to 3979 of B RNA.

The same concentration of boiled virion RNA (Fig. 2). Within the limits of the assay, this result is compatible with the expectation that the specific infectivity of denatured RF should be approximately half that of virion RNA. Jackson *et al.* (1971) obtained similar results when assaying the infectivity of the RF of TMV, denatured tobacco mosaic virus (TMV), RF being only 85% as infectious as half the concentration of heated virion RNA.
Since the RF was not infectious in the local lesion host *Phaseolus vulgaris* we decided to investigate its infectivity in a systemic host, the 'Blackeye Early Ramshorn' variety of cowpea. The primary leaves were detached from 1-week-old seedlings and inoculated with 1 μg of either RF or heat-denatured RF (four leaves each). As controls, the opposite leaves were inoculated with 1 μg of either virion RNA or heat-denatured virion RNA. The volume of each inoculum was 50 μl. The leaves were then placed on moist filter paper in Petri dishes and kept for 10 days at 30 °C under constant illumination.

No symptoms were observed on leaves inoculated with RF; however, those leaves inoculated with denatured RF or virion RNA displayed similar symptoms to those obtained following inoculation with virus, namely diffuse yellow-green chlorotic patches. To confirm that no virus was produced in 'Blackeye Early Ramshorn' leaves inoculated with RF, segments of tissue were removed with a No. 10 cork borer and homogenized in 0.5 ml 10 mM-sodium phosphate pH 7. Five μl of this homogenate was then applied to nitrocellulose filters and probed with nick-translated RF DNA from M13 clones containing sequences from the M or B RNA of CPMV (Kafatos *et al.*, 1979; Lomonossoff & Shanks, 1983). No CPMV sequences were detected in 'Blackeye Early Ramshorn' leaves inoculated with RF (Fig. 3). CPMV-specific sequences were present in leaves inoculated with virion RNA as well as with heat-denatured RF. Since dot blotting of crude homogenates from infected leaves only detects encapsidated RNA (Evans, 1985), we repeated the above experiment with the exception that RNA was phenol-extracted from leaves 10 days post-inoculation. This allows unencapsidated RNA to be detected. The results were identical to those shown in Fig. 3, no CPMV-specific RNA being detected in leaves inoculated with RF. The sensitivity of the blot assays was such that a specific infectivity of non-denatured RF of less than 1% of that of single-stranded RNA would easily have been detected.

The evidence presented in this communication indicates that full-length, double-stranded, RF RNA molecules isolated from cowpeas infected with CPMV are not infectious unless first denatured. RFs have been isolated following infection of plants with a wide range of viruses including TMV (Ralph *et al.*, 1965; Jackson *et al.*, 1971), alfalfa mosaic virus (Pinck *et al.*, 1968), barley stripe mosaic virus (Pring, 1972), brome mosaic virus (Phillips *et al.*, 1974), pea enation mosaic virus (German & de Zoeten, 1975), cucumber mosaic virus (Kaper & Diaz-Ruiz, 1977) and tomato bushy stunt virus (Hayes *et al.*, 1984). However, only Jackson *et al.* (1971) investigated the infectivity of the RFs. They found that, as is the case with CPMV, the RF of TMV was not infectious unless first denatured. Amongst the animal viruses the RFs of Semliki Forest virus were shown not to be infectious (Friedman, 1968) whereas the RFs of all picornaviruses tested are infectious (Montagnier & Sanders, 1963; Brown & Cartwright, 1964; Pons, 1964; Perez-Bercoff *et al.*, 1974). Indeed, picornavirus RF has a 30-fold greater specific infectivity than the virion RNA (Bishop & Koch, 1967). However, the mechanism whereby double-stranded RNA can initiate infection is obscure. Unlike infection with virion RNA, infection with picornavirus RF is sensitive to actinomycin D and is greatly reduced in enucleated cells (Bishop & Koch, 1967; Detjen *et al.*, 1978). This implies a role for the nucleus during infection with RF which is not required for normal infection.

That the RF of picornavirus is infectious whereas that of CPMV is not is of interest since the two viruses have much in common. The virion RNA of both viruses is infectious, possesses a VPg at the 5' terminus and is polyadenylated. The translation strategy of both viruses is that of the initial synthesis of a precursor polypeptide which is cleaved by virus-specified proteases into the viral proteins. Recently, it has been shown, by computer-assisted comparison of the RNA sequences, that there is a degree of homology between the non-structural proteins of CPMV and poliovirus (Franssen *et al.*, 1984).

There are three possible explanations for this difference. Firstly, the structures of CPMV and picornavirus RFs could be radically different. However, detailed structural studies on CPMV RFs (G. P. Lomonossoff, unpublished data) show them to be remarkably similar to their picornavirus counterparts, making this explanation unlikely. Secondly, plant cells and/or their nuclei lack the functions necessary to melt dsRNA, in contrast to animal cells. Thirdly, the RFs fail to reach sites in cells such as the nuclei which would render them infectious. It might be possible to discriminate between the last two possibilities by assaying the ability of RFs to
replicate in protoplasts and/or isolated nuclei. Whatever the explanation of the lack of infectivity of dsRNA from CPMV it may well have a bearing on the ability of ds cDNA clones of CPMV to infect plants.

REFERENCES


Short communication


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