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Cowpea Mosaic Virus RNA Replication in Crude Membrane Fractions from Infected Cowpea and Chenopodium amaranticolor

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SUMMARY

The replication of cowpea mosaic virus (CPMV) RNA was studied in crude membrane fractions prepared from leaves of CPMV-infected cowpea and Chenopodium amaranticolor. In vitro replicase assays showed that in the cowpea extract only the replicative intermediate (RI) and replicative form (RF) were synthesized. In the C. amaranticolor extract however, single-stranded progeny RNA was produced in addition to RI and RF. Production of the ssRNA in the C. amaranticolor extract was a result of the greater stability of the CPMV replication complex in this host. Comparison of the viral replicase activity and the amount of virus-encoded proteins in cowpea and C. amaranticolor crude membrane fractions indicated that only a small fraction of the non-structural proteins detected in cowpea is active in RNA replication. This suggests that viral replication proteins are used only once, perhaps because of a stringent coupling of polyprotein processing and replication.

INTRODUCTION

The comovirus cowpea mosaic virus (CPMV) resembles animal picornaviruses in having a plus-stranded RNA genome with a small protein (VPg) at the 5' end and a poly(A) tail at the 3' end, and which contains a single long open reading frame, coding for a polyprotein from which the functional polypeptides are derived by specific proteolytic cleavages. The structure and expression of the two genomic RNAs of CPMV, denoted M- and B-RNA, have been studied in detail (for recent reviews, see Goldbach & van Kammen, 1985; van Kammen et al., 1987) and are shown in Fig. 1.

Non-structural proteins of CPMV and poliovirus share significant amino acid sequence homology, display probably similar functions in RNA replication and are similarly arranged in the genome (Franssen et al., 1984; Argos et al., 1984; Goldbach, 1986, 1987). Therefore, it is tempting to assume that the mechanisms of replication of CPMV RNA and poliovirus RNA will be very similar.

The replication of CPMV RNA has been studied extensively with crude membrane fractions prepared from CPMV-infected cowpea plants (for reviews, see van Kammen & Eggen, 1986; Eggen & van Kammen, 1988). These studies showed the existence of two functionally distinct and physically separable RNA-dependent RNA polymerase (RdRp) activities. The first has been identified as a host-encoded RdRp, which can be readily released from the membranes by washing with a Mg2+-deficient buffer. Although the biological function of this host-encoded enzyme (M, 130K) still remains to be determined, it has been shown to transcribe plant RNAs, and in infected cowpea plants also viral RNAs, into short RNA molecules of negative polarity (Dorssers et al., 1982, 1983; Van der Meer et al., 1983, 1984). The second RdRp, representing only 5% of the total RdRp activity, is the virus-specific, tightly membrane-bound RNA.
replication complex. Protein analysis has demonstrated the presence in the complex of the B-RNA-encoded 110K polypeptide, constituting the viral replicase and two host proteins with M, values of 68K and 57K (Dorssers et al., 1984). Whether these host proteins are contaminants in the replication complex preparation or are functional subunits has not yet been investigated. In vitro the viral RNA replicase is capable of elongating only viral plus-sense RNA chains that have already been initiated in vivo. The completed chains are only detectable in double-stranded RNA (replicative form; RF). Moreover, the viral polymerase activity does not correlate with the amount of B-RNA-encoded 110K protein in CPMV-infected cowpea plants (Dorssers et al., 1984).

Since the viral polymerase isolated from cowpea plants has only low activity, almost overshadowed by the host RdRp, extracts from another systemic host of CPMV, Chenopodium amaranticolor, were prepared to examine whether in this host additional viral RNA species and lower amounts of host RdRp products are synthesized. In this paper we report that the viral RNA-synthesizing activity extracted from this plant can produce single-stranded progeny RNA. These ssRNA molecules were produced in addition to RF and the replicative intermediate (RI), which is in contrast to extracts from CPMV-infected cowpea leaves that synthesized only RF and RI. To understand this difference, the structure and stability of the replication complexes in cowpea and C. amaranticolor have been studied. The host-encoded RdRp activity was lower in the C. amaranticolor extract than in cowpea extracts. The viral RNA polymerase activity and the amount of viral-encoded proteins involved in viral RNA replication were compared in extracts of the two hosts. This analysis indicated that the bulk of viral replication proteins in cowpea are inactive in viral RNA replication.

METHODS

Plants and virus. The primary leaves of 8-day-old cowpea (Vigna unguiculata, 'California Blackeye') or all the leaves of 40-day-old C. amaranticolor plants were inoculated with sap from CPMV (Sb isolate)-infected cowpea leaves, extracted in 0.1 M-sodium phosphate pH 7.0. Healthy samples were taken from plants inoculated with buffer alone. Propagation of the virus was as described by Zabel et al. (1974).

Preparation of the crude membrane fractions. The method was modified from Zabel et al. (1974). All operations were performed at 4 °C. Portions of freshly harvested leaves (12 g), from which the biggest ribs had been removed, were rinsed with distilled water, blotted with paper and homogenized in 35 ml buffer (50 mM-Tris-acetate pH 7.4, 10 mM-potassium acetate, 1 mM-EDTA, 5 mM-dithiothreitol and 0.5 mM-PMSF).

The homogenate was filtered through two layers of "Miracloth" and subsequently centrifuged for 15 min at 1000 g. The supernatant fraction was adjusted to 20% (v/v) glycerol and was centrifuged for 30 min at 31000 g. The resulting pellet was resuspended thoroughly using a Dounce homogenizer in 0.5 ml buffer (50 mM-Tris-acetate pH 7.4, 10 mM-potassium acetate, 1 mM-EDTA, 5 mM-dithiothreitol and 0.5 mM-PMSF).
acetate pH 8.0, 25% glycerol, 50 mM-potassium acetate, 1 mM-EDTA, 5 mM-dithiothreitol and 0.5 mM-PMSF) per g of fresh leaf. Small samples were stored at −80 °C.

Micrococcal nuclease treatment. Calcium acetate was added to 30 μl crude membrane fractions of cowpea or C. amaranticolor to a final concentration of 1 mM. After the addition of 90 units micrococcal nuclease, the mixture was incubated for 30 min at 30 °C. The micrococcal nuclease was inactivated by the addition of EGTA (final concentration 5 mM).

RdRp assay and product purification. The standard assay (60 μl) contained the following: 30 μl of the crude membrane fraction, 50 mM-Tris-acetate pH 8.2, 8 mM-magnesium acetate, 1 mM-EDTA, 25 mM-ammonium sulphate, 1.25 μg actinomycin D, 2 μCi [α-32P]UTP (sp. act. 3000 Ci/mmol) and 1 mM each of ATP, CTP and GTP. The reaction mixtures were incubated at 30 °C for 60 min.

After incubation, the RNAs synthesized in vitro were extracted with an equal volume of a phenol/chloroform (1:1) mixture in the presence of 2 μg yeast RNA, 0.5% SDS and 10 mM-EDTA, and re-extracted without adding further SDS and EDTA. RNA was recovered by ammonium acetate/isopropanol precipitation (Maniatis et al., 1982) and washed twice with 70% ethanol.

RNA gel electrophoresis. RNA samples were analysed either native in a 1% agarose gel containing 40 mM-Tris-acetate, 20 mM-sodium acetate and 2 mM-EDTA, pH 7.4, or fully denatured, using glyoxal and DMSO, in a 1% agarose gel in 10 mM-sodium phosphate pH 7.0, according to McMaster & Carmichael (1977).

SDS-polyacrylamide gels and immunoblot analysis. Protein samples were mixed with one-third volume of a fourfold concentrated sample buffer (4 × SB; 40 mM-Tris-HCl pH 8.0, 4 mM-EDTA, 40% v/v glycerol, 8% w/v SDS, 20% v/v 2-mercaptoethanol and 0.004% w/v bromophenol blue). After heating for 3 min at 100 °C, protein samples were separated by electrophoresis in a 12.5% SDS-polyacrylamide gel and blotted onto nitrocellulose. The resulting blot was subsequently incubated with antisera and 125I-labelled Protein A for the detection of immune complexes as described by Zabel et al. (1982). The antisera used were raised against purified viral protein (anti-VP23, Franssen et al., 1982), or synthetic peptides (anti-24K, Wellink et al., 1987; anti-VPg, M. Jaegle & M. Van den Broek, unpublished data).

RESULTS

Time course of development of enzyme activity in infected plants

To compare the viral replicase activities in C. amaranticolor and cowpea, crude membrane fractions containing viral replication complexes active in vitro were prepared from leaves at daily intervals after inoculation with CPMV. As with cowpeas, the replicase activity isolated from infected C. amaranticolor leaves could only be detected in the membrane fraction (Dorssers et al., 1983). The level of viral replicase activity in vitro in C. amaranticolor extracts was 25% or more of that in cowpea (data not shown). Differences in RNA polymerase activity and products made in the two hosts are shown in Fig. 2. In cowpea extracts there was a slower decrease of the viral replicase activity from days 2 to 6 after infection, and a stimulation of the host-encoded RdRp resulting in synthesis of products of low Mr. In C. amaranticolor extracts additional RNA species were synthesized; in addition to RI and RF, also produced in cowpea extracts (Dorssers et al., 1983), labelled RNAs comigrating with CPMV B- and M-RNA were detected, suggesting the in vitro production of ss progeny RNA.

Characterization of the ssRNA products synthesized in C. amaranticolor extracts

To identify the nature of the putative ssRNAs produced in vitro by the viral replication complex from C. amaranticolor leaves, harvested on day 3 after inoculation, the products comigrating with viral B- and M-RNA were isolated from an agarose gel (Fig. 2) by the freeze-squeeze method (Tautz & Renz, 1983) and subjected to electrophoresis under denaturing conditions. Under these conditions the putative ssRNA products comigrated with the corresponding ss viral RNAs (Fig. 3). This result indicates that they were genome-length and excludes the possibility that the ssRNA bands were formed as a result of aggregation of labelled, negative-sense RNA fragments (e.g. produced by the host-encoded RdRp) and unlabelled viral RNA, as seen by Dorssers et al. (1983) and Jaspars et al. (1985) with CPMV and alfalfa mosaic virus respectively.

Isolated labelled replication products hybridized with ss M13 DNA containing negative-sense sequences of CPMV M-RNA but not with DNA containing positive-sense sequences (data not shown). These experiments thus show that the label was incorporated into plus-stranded RNA.
Fig. 2. Autoradiogram showing the time course of development of RdRp activity in crude membrane fractions prepared from infected *C. amaranticolor* (a) and cowpea (b) leaves on successive days (indicated by lane number) after inoculation. Total RNA samples were analysed on a 1% agarose gel under non-denaturing conditions. RF<sub>B</sub> and RF<sub>M</sub> indicate the positions of the dsRNA; B-RNA and M-RNA indicate the ssB- and ssM-RNA respectively. L indicates the low M, RNA products produced by the host-encoded RdRp.

No labelled RI, RF or ssRNA could be detected when the *in vitro* RdRp assays were carried out in the presence of [³²P]UTP or [³²P]UTP plus ATP as the only ribonucleotides (data not shown). This result eliminated terminal addition of labelled UTP as a possible mechanism for the production of labelled ssRNA (Zabel et al., 1981), and indicates that the labelled ssRNAs arose by *in vitro* elongation and correct termination of shorter endogenous RNA chains already synthesized *in vivo*.

**Analysis of RNA precursor-product relationships**

The elongation, termination and possible initiation of viral RNA synthesis in *C. amaranticolor* extracts (prepared 3 days after inoculation) were examined by investigating the kinetics of appearance of the different virus RNA species, including RI, RF and ssRNA. For this purpose RNA produced in crude membrane fractions after different times of incubation was analysed by agarose gel electrophoresis under native (Fig. 4a) and denaturing conditions (Fig. 4b). Analysis under native conditions showed that RI, forming a diffuse band migrating more slowly than RFs (Dorssers et al., 1983), had formed by 1 min of incubation and that the RI disappeared after prolonged incubation, apparently being chased into RF<sub>B</sub>, RF<sub>M</sub> and the ssB- and ssM-RNA (Fig. 4a). Analysis under denaturing conditions showed that during incubation the RNA products increased in size with time until full length genomic RNAs appeared (Fig. 4b, lane 5).

These results, together with those described in the previous paragraph, provide evidence that, following elongation, correct termination of viral RNA replication had taken place in membrane fractions from CPMV-infected *C. amaranticolor* leaves.

After an incubation of 30 min virtually all labelled RI was converted into ds- and ssRNA (Fig. 4a, lane 6), but no further increase of the amount of ssRNA was observed in the following 30 min (Fig. 4a, lane 7). This result indicates that if any *de novo* initiation of viral RNA replication *in vitro* had occurred, followed by elongation, it had been at a low undetectable frequency.

**Structure and stability of the replication complex**

For infected cowpea, it has been suggested that upon fractionation of the plant or during *in vitro* RNA synthesis, the nascent RNA chains in the RI molecule are released or degraded (Dorssers et al., 1983) resulting in most of the RNA synthesized in cowpea extracts being ds RF. To examine whether the CPMV replication complex in the membrane fraction of *C.*
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Fig. 3. Autoradiogram showing the characterization of ssRNA products synthesized in the *C. amaranticolor* extract, by electrophoresis in a 1% agarose gel under denaturing conditions. Lanes 1 and 2 contain isolated RNA products which comigrated with B- and M-RNA respectively (see Fig. 2). The positions of CPMV B- and M-RNA are indicated at the side.

Fig. 4. Autoradiogram showing the time course of RNA-dependent RNA synthesis by crude membrane fractions of infected *C. amaranticolor* leaves. Reaction mixtures were incubated for different times (lanes 1, 1 min; lanes 2, 2 min; lanes 3, 5 min; lanes 4, 10 min; lanes 5, 20 min; lanes 6, 30 min; lanes 7, 60 min). After 30 min of incubation, one mixture was adjusted to 1 mM-UTP and incubated for a further 30 min (lanes 8). After extraction of the RNA products, the RNA samples were subjected to electrophoresis in a 1% agarose gel under native (a) or denaturing (b) conditions. Labels are as in Fig. 2, RI_B and RI_M indicate the positions of the replicative intermediates.

*amaranticolor* is more resistant to degradation, the products synthesized in vitro in a mixture of both plant extracts were analysed (Fig. 5). The production of ssRNA in *C. amaranticolor* extract (lane 2) was not affected by the addition of cowpea extract to the assay mixture (lane 3). Thus the replication complex in *C. amaranticolor* is more stable and less susceptible to nuclease than that in cowpeas.

This interpretation was further supported by attempts to remove endogenous RNA in cowpea and *C. amaranticolor* replication complexes. Micrococcal nuclease treatment abolished the synthesis of any labelled RNA species in cowpea extracts, whereas in *C. amaranticolor*, although ssRNA synthesis was affected, some RF was produced (Fig. 6, lanes 1 to 6). Even after micrococcal nuclease treatment in the presence of the non-ionic detergents Triton X-100 or dodecyl β-D-maltoside than with Triton X-100, some viral RNA species were produced in the *C. amaranticolor* extract (lanes 8 and 10). Addition of the detergents alone reduced the RF and ssRNA production at the same rate (lanes 7 and 9).

Detection of virus-encoded proteins in crude membrane fractions

In an attempt to understand the difference between the RNA-synthesizing activities in replication complexes isolated from cowpea and *C. amaranticolor*, the viral protein constituents of the replication-active membrane fractions of the respective hosts were analysed by immunoblotting (Fig. 7). Incubation with anti-VP23 serum revealed that in cowpea extracts two to three times more structural proteins could be detected than in *C. amaranticolor* extracts (Fig.
Fig. 5. Autoradiogram showing the analysis of RNA species produced in vitro using 15 μl crude membrane fractions prepared from CPMV-infected leaves of cowpea (lane 1), C. amaranticolor (lane 2), and a mixture of 15 μl as in lane 1 and 15 μl as in lane 2 (lane 3). Labels are as in Fig. 2.

Fig. 6. Autoradiogram showing the effect of micrococcal nuclease and/or detergent on the RNA synthesis in cowpea (lanes 1 to 3) or C. amaranticolor (lanes 4 to 10) extracts in vitro. Crude membrane fractions were incubated with (lanes 3, 6, 8 and 10) or without (lanes 1, 2, 4, 5, 7 and 9) micrococcal nuclease. To some of these incubations with C. amaranticolor extract, 0.1% (w/v) dodecyl β-D-maltoside (lanes 7 and 8) or 0.1% (v/v) Triton X-100 (lanes 9 and 10) was added. The RNA products were analysed on a 1% agarose gel under non-denaturing conditions. Labels are as in Fig. 2.

7a, lanes 2 and 4). The 23K and 22K bands represent VP23 and its shortened product VP22 respectively (Franssen et al., 1982). We do not know the identity of the more slowly migrating bands.

The quantities of the non-structural viral proteins, however, were strikingly different; that in C. amaranticolor was at most 5% of that found in cowpea. Anti-VPg serum reacted weakly with
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170K and 84K proteins and very strongly with the 60K VPg precursor proteins. These proteins were only detected in the crude membrane fractions prepared from inoculated cowpea leaves (Fig. 7b). Also, the non-structural polypeptides visualized with the anti-24K serum (e.g. 170K, 110K and 84K) were detectable only in extracts from inoculated cowpea leaves (Fig. 7c). The nature of the immunoreactive proteins also present in crude membrane fractions prepared from both healthy and inoculated C. amaranticolor leaves is not known (Fig. 7c, lanes 1 and 2).

The polypeptide with an $M_r$ of about 100K that reacted with both the anti-VPg and anti-24K sera might represent a B-RNA-encoded protein arising by an alternative cleavage of the 170K polypeptide (Fig. 7b and c).

Radiolabelled virus-encoded non-structural proteins produced in cowpea protoplasts were degraded more rapidly when added to extracts of C. amaranticolor leaves than when added to extracts of cowpea leaves (data not shown). This indicated that the different amounts of non-structural proteins in the two hosts could be explained by rapid proteolytic breakdown occurring in C. amaranticolor. When added to extracts of both plants, labelled viral structural proteins were unaffected by the higher proteolytic activity in C. amaranticolor, which is not surprising because such stability would reflect their genome-protecting function.

**DISCUSSION**

Viruses with ss plus-sense RNA genomes that infect eukaryotic cells replicate via the synthesis of a minus-sense strand, which in turn is the template for the production of progeny virus RNA strands. Replication intermediates include RI and RF molecules. In extracts of CPMV-infected cowpea leaves RI and RF have been detected in *in vitro* replicase assays but viral ssRNA has not (Dorssers *et al.*, 1983). In this paper we show that crude membrane fractions prepared from CPMV-infected C. amaranticolor leaves contained CPMV replication complexes able to produce full length progeny ssRNA as well as the RI and RF synthesized in cowpea extracts.

Analysis of the stability of the replication complex, using micrococcal nuclease and non-ionic...
detergents, showed that the structure and membranous environment may be responsible for the differences detected. Besides the greater stability of the replication complex in *C. amaranticolor* extract, the lower level of host-encoded RdRp activity is an improvement in comparison with the cowpea extract.

Examination of the precursor–product relationships of the different virus RNA species produced in *C. amaranticolor* extracts suggests that RI is the functional intermediate in viral RNA replication *in vivo*, as indicated by its immediate appearance and efficient chase into RF and ssRNA *in vitro*.

After prolonged incubation *in vitro*, RF and ssRNA were produced in equal amounts. From this one can speculate that at least two (as labelled RF and ssRNA are produced), but probably less than five polymerase molecules will be present per RI structure. This number has been estimated assuming that the viral polymerases work processively and taking into account the possible lower intensity of ssRNA bands due to degradation. This estimate is in agreement with the analysed RI structures of poliovirus, for which four to eight polymerase molecules per RI have been proposed (Richards *et al.*, 1984).

RF labelled *in vitro* is detectable only after increasing incubation times, which suggests that CPMV RF is probably a 'dead-end' molecule. This conclusion is supported by the observation that CPMV RF cannot infect cowpea plants unless it is first denatured (Shanks *et al.*, 1985). Earlier suggestions for functions of RF are that it represents a functional replication intermediate (Koch & Koch, 1985), an isolation artefact (Hall *et al.*, 1982; Richards *et al.*, 1984) or a 'dead-end' molecule arising either in non-optimally replicating *in vitro* systems (Chu & Westaway, 1987; Hall *et al.*, 1982; Jaspars *et al.*, 1985; Kuhn & Wimmer, 1987; Morrow *et al.*, 1985; Mouches *et al.*, 1974; Watanabe & Okada, 1986; Young & Zaitlin, 1986) or accumulating *in vivo* at the end of the infection cycle (Koch & Koch, 1985).

*Chenopodium amaranticolor* extracts did not support initiation of RNA synthesis, because no new RI molecules were produced during the replication assay. The initiation of viral RNA replication of VPg-containing viruses is poorly understood. Even in the intensively studied poliovirus RNA replication system this aspect is still a matter of debate (for a recent review, see Kuhn & Wimmer, 1987).

Although there was a striking difference between the amount of non-structural proteins present, the viral replicase activities in both plant extracts were of the same order of magnitude. This supports the proposal presented previously (Dorssers *et al.*, 1984) that only a small proportion of the non-structural proteins detected in cowpea plants are active. The bulk of such molecules found in infected cells is either irrelevant or, as we think, had been functional earlier during the infection and accumulated as inactive molecules in the cytoplasm (Wellink *et al.*, 1988). This idea has led to a replication model in which viral replication proteins are used only once by a stringent coupling of polyprotein processing and replication (van Kammen & Eggen, 1986; Eggen & van Kammen, 1988). The hypothesis of viral proteins with limited activity is supported by the lack of success in our laboratory of attempts to prepare a template-dependent *in vitro* RNA replicating system, for which re-usable polymerase molecules would be necessary (R. Eggen, unpublished results).

Cowpeas of cv. Arlington do not support detectable production of CPMV (Eastwell *et al.*, 1983). For this lack of CPMV multiplication three possibilities have been suggested: inhibition of the CPMV-encoded protease, inhibition of the CPMV RNA translation, or a general proteolytic degradation of CPMV proteins (Ponz *et al.*, 1987). The last possibility seems very unlikely in view of our results on CPMV replication in two different hosts. Although in *C. amaranticolor* the virus-encoded non-structural proteins could hardly be detected, as a result of proteolytic degradation, the amount of virus-encoded structural proteins accumulating in *C. amaranticolor* and also the level of replicase activity *in vitro* were of the same order of magnitude as those in cowpea plants. This suggests that high turnover levels do not necessarily result in lower virus yields.

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REFERENCES


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