Expression of High Molecular Weight Polypeptides by Carnation Mottle Virus RNA

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SUMMARY

In vitro translation of virion RNA from carnation mottle virus (CarMV) in a messenger RNA-dependent rabbit reticulocyte lysate (MDL) resulted in the synthesis of four virus-specific polypeptides of apparent M, 100000 (P100), 77000 (P77), 38000 (P38) and 30000 (P30). Partial peptide mapping experiments and in vitro translation in the presence of partially purified calf liver amber suppressor tRNA demonstrated that P30, P77 and P100 are a series of overlapping polypeptides generated by a double readthrough mechanism. In addition we report the infection of Chenopodium quinoa protoplasts with CarMV. The viral coat protein was detected in virus-infected protoplasts. Only one other infection-specific protein with an apparent M, of 100000 corresponded in size to any of the other in vitro translation products.

INTRODUCTION

The translation of carnation mottle virus (CarMV) virion RNA in a rabbit reticulocyte lysate results in the formation of three major virus-specific polypeptides of apparent M, 77000 (P77), 38000 (P38) and 30000 (P30) (Harbison et al., 1984) comparable to those reported previously (Salomon et al., 1978) in wheat germ extracts. The existence of a population of subgenomic RNAs isolated from CarMV particles and infected plant material has recently been demonstrated (Harbison et al., 1984; Carrington & Morris, 1984). At least one of these sub-genomic RNAs was found to encode the viral coat protein. At this time we also noted the presence of a minor virus-specific polypeptide of apparent M, 100000 (P100) during in vitro translation of unfractionated or genome-length CarMV RNA.

More recently, Carrington & Morris (1985) have found that the viral coat protein is encoded predominantly by an encapsidated subgenomic mRNA of 1-6 kb. A function for the larger subgenomic RNA of CarMV (Carrington & Morris, 1984) has not yet been found. No evidence was found for a virus-specific polypeptide of apparent M, 100000, although recent sequence data (H. Guilley, T. J. Morris & K. Richards, personal communication) predict the synthesis of such a product.

In this paper we present evidence for the synthesis of a product of M, 100000 (P100) in vitro which is structurally related to P77 and arises by a readthrough mechanism of translation.

In addition, we report the successful infection of Chenopodium quinoa Wild. protoplasts with CarMV. Among the proteins synthesized in infected protoplasts that were absent from healthy ones, we detected CarMV coat protein. A protein of apparent M, 100000 was also detected which may correspond to P100.

METHODS

In vitro translation and product analyses. CarMV was purified from infected C. quinoa leaves. Virion RNA was extracted and translated in a mRNA-dependent rabbit reticulocyte lysate (MDL) prepared according to Pelham & Jackson (1976) as described previously (Harbison et al., 1984). Partially purified, calf liver amber suppressor tRNA (kindly provided by R. Valle, Institut Jacques Monod, Paris, France) was added to some translation incubations to a final concentration of 2.5 µg/ml.
Peptide mapping by partial proteolysis. Peptide mapping was an adaptation of the method of Cleveland et al. (1977). Translation products were excised and the backing paper was removed. The dry gel pieces were placed separately in the wells of a second, 20% (w/v), polyacrylamide slab gel containing 0.1% (w/v) SDS. The pieces were allowed to swell in electrophoresis buffer, then covered with 10 μl of sample buffer (Laemmli, 1970) containing 5 μg α-chymotrypsin (Worthington) or 5 μg proteinase K (Worthington). The gel was electrophoresed at 50 V for 90 min during the stacking phase and 200 V for 3 h in the separating phase. The gel was then fluorographed (Bonner & Laskey, 1974).

Protoplasm isolation. Protoplasts were isolated from C. quinoa essentially as described by De Varennes et al. (1984).

Inoculation of protoplasts with poly-L-ornithine. CarMV (10 μg/ml) and poly-L-ornithine (PLO; 1 μg/ml) were incubated in 0.6 M-mannitol, 10 mM-potassium citrate, pH 5.2, at room temperature for 10 min. Freshly sedimented protoplasts were resuspended in this mixture to a concentration of 3 × 10⁵ protoplasts per ml. After 15 min, inoculated protoplasts were washed three times in 0.6 M-mannitol containing 10 mM-CaCl₂. Protoplasts were cultured (3 × 10⁵ per ml) in 0.6 M-mannitol, 0.2 mM-KH₂PO₄, 1 mM-KNO₃, 1 mM-MgSO₄, 10 mM-CaCl₂, 1 μM-KI, 0.01 μM-CuSO₄ and 25 μg/ml gentamicin (Rottier et al., 1979), at 25°C with 2.5 klx continuous lighting.

Healthy protoplasts were either uninoculated or mock-inoculated with CarMV pretreated with RNase A. Protoplast viability was assessed using phenosaframine (Widholm, 1972). Percentage infection was determined by the indirect fluorescent antibody staining technique (Maule et al., 1980).

Nucleic acid synthesis in protoplasts. Protoplasts were disrupted in TNE buffer (10 mM-Tris–HCl pH 8.0, 1 mM-EDTA, 0.1 M-NaCl) containing 2% (w/v) SDS. Nucleic acid was extracted with buffer-saturated phenol/chloroform (1:1), precipitated with ethanol and stored in water at −20°C. Samples were spotted onto nitrocellulose filters presoaked in 20 × SSC (3 M-NaCl, 0.3 M-sodium citrate), the filters were air-dried, baked in vacuo at 80°C, prehybridized and then hybridized with [35S]labelled cDNA (Thomas, 1980). Complementary DNA was prepared essentially as described by Kummert & Kettmann (1978). The standard reverse transcription reaction contained 3 μg CarMV RNA, 50 μg calf thymus DNA primer (Taylor et al., 1976), 2.5 mM-DTT, 25 mM-KCl, 10 mM-MgCl₂, 25 mM-Tris–HCl pH 8.3, 1 mM-dATP, 1 mM-dGTP, 1 mM-dTTP, 20 μCi [α-32P]dCTP (New England Nuclear; 3200 Ci/mmol) and 18 units reverse transcriptase (P&S Biochemicals, Liverpool, U.K.) in a final volume of 25 μl. After incubation at 42°C for 1 h, RNA templates were hydrolysed by incubation at 65°C in 50 mM-NaOH for 30 min. The cDNA preparation was separated from unincorporated dNTPs and hydrolysed template molecules by spin-column chromatography on Sephadex G-50 in TNE (Maniatis et al., 1982) and stored at −20°C.

Protein synthesis in infected protoplasts. Protein synthesis was assessed by incubating protoplasts in 2 μCi/ml [35S]methionine (New England Nuclear; 1100 Ci/mmol). Synthesized proteins were analysed by lysing protoplast samples in buffer (Laemmli, 1970) then fractionating them in SDS-polyacrylamide gels (Harbison et al., 1984). Protein bands were detected by fluorography (Bonner & Laskey, 1974).

Immunoprecipitation of CarMV coat protein from protoplasts. Twenty-four h after inoculation, 50 μl samples of [35S]methionine-labelled healthy or infected protoplasts were mixed with 150 μl 1 mM-NaCl in 1% Triton X-100, 8 μl rabbit polyvalent serum and 60 μl of a freshly prepared suspension of Protein A–Sepharose (62.5 μg/μl of 25 mM-Tris–HCl pH 7.5. The mixture was shaken at 4°C for 2 h, then centrifuged (13000 g, 4 min, 4°C). The supernatant fluid and three washes of the pellet with 60 μl 0.75 M-NaCl in 1% Triton X-100 were pooled and incubated at 4°C overnight with 20 μg CarMV-specific IgG and 60 μl Protein A–Sepharose suspension. The Protein A–Sepharose-containing pellets were collected by centrifugation as described above and washed five times with 60 μl portions of 0.75 M-NaCl in 1% Triton X-100. Suspensions of each pellet in 20 μl sample buffer were boiled for 5 min before loading onto a 15% (w/v) polyacrylamide slab gel for electrophoresis (Harbison et al., 1984).

RESULTS

Time course of CarMV RNA translation

Translation of unfractionated virion RNA from CarMV yielded four major products (Fig. 1) with apparent Mr 100 000 (P100), 77 000 (P77), 38 000 (P38) and 30 000 (P30). Since CarMV RNA is too small to contain independent genes for P100 and P77, their coding sequences must overlap in some way. A time course of polypeptide synthesis (Fig. 1) showed that the two high molecular weight products were first detected together after 15 min (visible on original fluorograph) and more strongly after 30 min incubation. This suggested that P77 did not arise as a result of proteolytic cleavage of P100.

Peptide mapping of translation products

Comparative peptide mapping with α-chymotrypsin (Cleveland et al., 1977) revealed that P77 and P100 contained similar-sized peptides (Fig. 2a) indicating that they are encoded in part by
Fig. 1. Time course of CarMV RNA-directed protein synthesis in vitro. Samples were taken at the times (min) indicated above each lane. Numbers on the left indicate the apparent $M_r$ values of the major translation products.

Fig. 2. (a) Peptide mapping of CarMV RNA translation products of apparent $M_r$, 100000 (lane 1) and 77000 (lane 2). Each product was digested with 5 µg α-chymotrypsin in 10 µl sample buffer during electrophoresis and analysed in a 20% (w/v) polyacrylamide slab gel. (b) Peptide mapping of CarMV RNA translation products of apparent $M_r$, 38000 (lanes 1 and 4), 30000 (lanes 2 and 5) and 77000 (lanes 3 and 6) with 5 µg α-chymotrypsin per sample (lanes 1 to 3) or 5 µg proteinase K per sample (lanes 4 to 6). Digestion and peptide separation were as in (a).
Fig. 3. (a) Fluorogram showing the effect of Mg\(^{2+}\) concentration on the translation of CarMV RNA for 60 min in a MDL that contained (lane 1) 1·0 mM excess EDTA, (lane 2) no MgCl\(_2\), (lane 3) 0·5 mM-MgCl\(_2\), (lane 4) 1·0 mM-MgCl\(_2\), (lane 5) 1·5 mM-MgCl\(_2\), (lane 6) 2·0 mM-MgCl\(_2\), (lane 7) 2·5 mM-MgCl\(_2\), (lane 8) 3·0 mM-MgCl\(_2\). (b) Suppression of the P30 and P77 termination codons. CarMV RNA was translated in the presence (lane 1) or absence (lane 2) of calf liver amber suppressor tRNA (2·5 μg/ml). Numbers indicate apparent \(M_r\) values of the major translation products.

the same region of RNA in the same reading frame. The same technique using α-chymotrypsin or proteinase K revealed that P30 and P77 also contained similar-sized peptides (Fig. 2b) which were distinct from those of the viral coat protein (P38).

**Effect of Mg\(^{2+}\) on the translation of CarMV RNA**

The results obtained were suggestive of a readthrough mechanism of translation, similar to that reported for a number of viruses including tobacco mosaic (Pelham, 1978), tobacco rattle (Pelham, 1979) and lucerne transient streak (Morris-Krsinich & Forster, 1983). It has been shown that readthrough of leaky termination codons can be increased by supraoptimal Mg\(^{2+}\) concentrations (Pelham, 1978). Fig. 3(a) shows the effect of Mg\(^{2+}\) concentration on the termination of CarMV RNA-directed protein synthesis. Translation was initiated in the absence of added MgCl\(_2\) by the addition of CarMV RNA. After 10 min incubation, edeine was added to a final concentration of 7 μg/ml to inhibit further initiation. Five min later, portions of the translation mix were adjusted to various concentrations of Mg\(^{2+}\). The MDL itself contributed 1 mM-Mg\(^{2+}\), final concentration (Pelham, 1979; confirmed by flame atomic absorption spectroscopy). To obtain final concentrations below 1 mM-Mg\(^{2+}\), appropriate
amounts of EDTA were added. The ratio of P100 to P77 was found to be altered by the Mg²⁺ concentration. The level of synthesis of P100 relative to P77 increased with increasing Mg²⁺ concentration between 0 and 2.0 mm-final Mg²⁺ concentration. The level of synthesis of P77 relative to P30 also increased with increasing Mg²⁺ concentrations.

**Addition of suppressor tRNAs**

Both increases obtained above are consistent with a readthrough mechanism of translation. To examine this further, partially purified calf liver amber (UAG) suppressor tRNA (R. Valle et al., unpublished) was added to MDL to a final concentration of 2.5 μg/ml. Fig. 3(b) shows that the suppressor tRNA preparation increased the yield of P100 relative to P77 and P30, demonstrating that both P30 and P77 are terminated by leaky UAG termination codons.

**Infection of protoplasts**

*C. quinoa* protoplasts were efficiently infected with CarMV. In some preparations up to 80% of protoplasts were infected. Inoculation in the absence of PLO resulted in only about 50% infection, or less. About 60% inoculated and mock-inoculated protoplasts survived for 48 h after inoculation; about 75% of uninoculated protoplasts survived for 48 h. Inoculation using higher concentrations of PLO or virus did not result in greater percentage infection and was often detrimental to the system.
Detection and time course of virus-specific RNAs in protoplasts

Nucleic acid samples from healthy and CarMV-infected protoplasts at various times after inoculation were spotted onto nitrocellulose filters (Thomas, 1980) and probed with 32P-labelled cDNA prepared to viral RNA by random priming (Taylor et al., 1976). The accumulation of viral RNA (Fig. 4a) paralleled the production of virus-specific antigen (data not shown). Viral RNA and virus antigen could be detected as early as 7 h post-inoculation. For quantification of viral RNA, the radioactive areas were cut from the nitrocellulose filters, assayed by scintillation counting and compared with blots of known quantities of purified viral RNA. Forty-eight h after inoculation, CarMV-infected protoplasts contained approximately $3 \times 10^7$ genome equivalents per viable protoplast or 6.4 pg of progeny RNA per viable protoplast (Fig. 4b). These values are higher than those obtained with cowpea mosaic virus or cymbidium ringspot virus in C. quinoa protoplasts (De Varennes et al., 1984).
CarMV-specific protein synthesis

Fig. 6. Gene organization and potential open reading frames derived from the RNA sequence of CarMV (H. Guilley, T. J. Morris & K. Richards, personal communication) aligned with our in vitro translation products (open boxes). The positions of the AUG and termination codons are marked.

Synthesis of virus-specific proteins in protoplasts

The incubation of healthy or infected protoplasts in the presence of [35S]methionine permitted detection of two proteins in infected protoplasts which were absent from healthy ones (Fig. 5a). The protein of apparent Mr 38000 was specifically precipitated by antiserum to CarMV (Fig. 5b). Another protein of apparent Mr 100000 was detected at low levels in infected protoplasts 37 h after inoculation. This protein corresponds in molecular size to the P100 product from in vitro translations of genome-length CarMV RNA.

DISCUSSION

We have identified, by in vitro translation, two high molecular weight polypeptides encoded by CarMV RNA. The larger product (P100) was shown to be derived by readthrough of the leaky amber termination codon of P77. This product was in turn derived by readthrough of the P30 stop codon. These results confirm the prediction from the sequence data (Fig. 6) that three such related products are made.

The MDL system used in our laboratory contains exogenous calf liver tRNA (50 µg/ml). Some commercial preparations of MDL, including that used by Carrington & Morris (1985), do not contain any added tRNA. The absence of this component may explain why no P100 was detected in the latter system. The addition of total calf liver tRNA (Boehringer) to a final concentration of 50 µg/ml has an effect on the readthrough of the tobacco mosaic virus RNA in vitro translation product of Mr 126000 similar to that of addition of 2.5 µg/ml partially purified suppressor tRNA (A.-L. Haenni, personal communication).

The detection of a protein of apparent Mr 100000 in CarMV-infected protoplasts suggests that the double-readthrough mechanism of translation for CarMV RNA may be operative in vivo.

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