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In vitro processing of the RNA-2-encoded polyprotein of two nepoviruses: tomato black ring virus and grapevine chrome mosaic virus

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In vitro translation of RNA-2 of each of two closely related nepoviruses, tomato black ring virus (TBRV) and grapevine chrome mosaic virus (GCMV), in a rabbit reticulocyte lysate resulted in the synthesis of single polypeptides of 150K and 146K respectively. Processing of these polyproteins occurred after the addition of translation products of homologous RNA-1. The positions of the cleavage products within the polyproteins were determined. From the N to the C terminus, M_r values for the proteins were 50K, 46K and 59K for TBRV and 44K, 46K and 56K for GCMV. TBRV RNA-1 translation products also cleaved the polyproteins encoded by GCMV RNA-2 which suggests that the cleavage sites in the two polyproteins are similar.

Introduction

Nepoviruses have a bipartite genome consisting of two single-stranded mRNAs which possess a VPg at their 5′ end (Mayo *et al.*, 1982) and have polyadenylated 3′ ends (Mayo *et al.*, 1979). RNA-1 is able to replicate independently of RNA-2 in protoplasts and thus contains the information necessary for RNA replication (Robinson *et al.*, 1980); RNA-2 is needed for RNA encapsidation (Randles *et al.*, 1977) and for the spread of the virus in the plant (Meyer *et al.*, 1986). Tomato black ring virus (TBRV) and grapevine chrome mosaic virus (GCMV) are closely related nepoviruses. However, although pseudo-recombinants can be obtained between these two viruses (Doz *et al.*, 1980), they differ widely in their biological properties and thus constitute an interesting model for the study of the molecular basis of these properties.

The nucleotide sequences of the two RNAs of TBRV and GCMV have been established (Meyer *et al.*, 1986; Greif *et al.*, 1988; Le Gall *et al.*, 1989; Brault *et al.*, 1989). The RNA-1-encoded polyproteins, M_r 250000 (250K), of both viruses have 60% identical amino acid sequences and also resemble the 200K polyprotein encoded by the B-RNA of cowpea mosaic virus (Greif *et al.*, 1988). Alignment of the conserved domains suggested a tentative genetic map of the RNA-1 of TBRV and GCMV, including nucleotide binding, protease and polymerase domains (Greif *et al.*, 1988; Le Gall *et al.*, 1989). Recently we have shown that the 250K polyprotein of TBRV is autocatalytically cleaved when synthesized by *in vitro* translation (Demangeat *et al.*, 1990). The size of the proteins produced was in relatively good agreement with the genetic organization of RNA-1 suggested by the sequence data. In the same translation conditions, the TBRV RNA-2-encoded polyprotein remained stable. Earlier results based on *in vitro* translation of the RNAs of two nepoviruses, grapevine fan leaf virus (GFLV) (Morris-Krsinich *et al.*, 1983) and tobacco ringspot virus (TobRV) (Forster & Morris-Krsinich, 1985; Jobling & Wood, 1985) have shown that the RNA-2-encoded polyprotein is probably processed by a protease activity present in the *in vitro* translation products of RNA-1 but the cleavage products could not be located within the polyproteins.

In this paper we demonstrate that the polyproteins encoded by the RNA-2 of TBRV and GCMV are specifically cleaved to yield three mature products by an RNA-1-encoded proteolytic activity. A cleavage map for the RNA-2 polyprotein of these viruses is proposed.

Methods

Virus and RNA purification. The TBRV (L−) isolate from Lanarkshire (U.K.), free of satellite RNA (Greif *et al.*, 1990), was purified from infected *Nicotiana clevelandii* plants (Fritsch *et al.*, 1978). The two RNA species were extracted from purified virus particles that had been separated by CsCl equilibrium centrifugation (Demangeat *et al.*, 1990).

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The GCMV strain obtained from Dr G. P. Martelli (Bari, Italy), was propagated in *Chenopodium quinoa*. Virus and viral RNAs were purified as described by Doz *et al.* (1980).

In vitro *translation*. Rabbit reticulocyte lysates purchased from Green Hectares were made mRNA-dependent by treatment with micrococcal nuclease (Worthington) as described by Pelham & Jackson (1976). *In vitro* protein synthesis was done as described previously (Hemmer *et al.*, 1989) in the presence of [^{35}S]methionine at 1000 Ci/mmol (Amersham), and of RNA at 100 μg/ml. Samples were incubated at 30 °C and analysed by PAGE (Fritsch *et al.*, 1980).

Mapping of the viral products. Preferential N- or C-terminal labelling of the *in vitro* synthesized polyproteins was obtained as described previously (Demangeat *et al.*, 1990). For N-terminal labelling, [^{35}S]-methionine was present in the medium at the beginning and was chased after 4 min by the addition of 5 mM unlabelled methionine. For C-terminal labelling, translation was started in the absence of methionine, edeine was added at 10 μg/ml after 8 min to prevent further initiation of translation and [^{35}S]methionine was added after 10 min.

Preparation of antisera. Antisera were raised against TBRV, GCMV and a synthetic peptide (T) in rabbits each injected four times at 2 week intervals. The antigen was emulsified with complete Freund's adjuvant for the first injection or with incomplete Freund's adjuvant for the following injections (Demangeat *et al.*, 1990). The 20 amino acid long synthetic peptide T corresponds to the amino acids 791 to 810 of the 150K TBRV RNA-2-encoded polyprotein (Meyer *et al.*, 1986). Each injection contained 400 μg of the peptide conjugated to ovalbumin.

Immunoprecipitation. Immunoprecipitation of dissociated translation products was done as described before (Demangeat *et al.*, 1990) except that, in competition experiments, 50 μg of the peptide or 100 μg of dissociated virus and 5 μl of their respective antiserum were incubated for 1 h at room temperature in 80 μl of immunoprecipitation buffer (10 mM-sodium phosphate pH 7·2, 150 mM-NaCl, 1% Triton X-100, 0·5% deoxycholate and 0·1% SDS) in a total volume of 100 μl, before addition of the translation products.

Densitometric tracings. Scanning of the autoradiograms was done at 600 nm with a Shimadzu CS900 dual wavelength flying spot scanner.

Results and Discussion

The experiments reported in this paper were undertaken to determine whether the proteolytic activities contained in the TBRV and GCMV 250K polyproteins are able to cleave their respective RNA-2-encoded polyprotein and whether these activities are virus-specific. For this purpose RNA-2 molecules of TBRV and GCMV were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine under the conditions described by Hemmer *et al.* (1989). After incubation for 45 min, the products were mixed with the unlabelled translation products (obtained after a 1 h incubation) of their respective RNA-1 in the ratio of 1:9 (v/v) and were further incubated for 2 h at 30 °C.

Fig. 1(*a*), lanes 1 and 2 show that the translation of TBRV RNA-2 resulted in a major protein of 150K and in a number of minor proteins corresponding to partial translation products. In the absence of RNA-1 translation products, no new proteins appeared even after an incubation period of 3 h (lane 2) whereas after addition of TBRV RNA-1 products and further incubation for 2 h, two new proteins of 59K and 46K appeared (lane 4). This effect is specific to the RNA-1 translation products since the pattern remained unmodified after addition of lysate incubated in the absence of exogenous RNA (lane 3). Comparison of the densitometric tracing of lane 3 (Fig. 1*b*, i) and lane 4 (Fig. 1*b*, ii) showed that in addition to peaks 13 and 15, corresponding to the 59K and 46K proteins, the size of peaks 6 and 14, which correspond to proteins of approximately 100K and 50K increased upon incubation. These results show that RNA-1 products contain a proteolytic activity able to cleave the RNA-2-encoded polyprotein.

To map the cleavage products inside the polyprotein, they were further characterized by immunoprecipitation. The 59K protein was immunoprecipitated by the serum directed against the virus (anti-CP serum) (Fig. 1*a*, lane 6), which reacts against the 57K protein obtained by dissociation of the virus (CP) (result not shown). The 46K protein was immunoprecipitated by the anti-T serum directed against the synthetic peptide T, located in front of the N terminus of the coat protein (Meyer *et al.*, 1986; Fig. 3) (Fig. 1*a*, lane 8). The proteins of 59K and 46K were not detected when non-processed RNA-2 translation products were immunoprecipitated by the anti-CP or anti-T sera (Fig. 1*a*, lanes 5 and 7). The protein of approximately 100K (corresponding to peak 6 on Fig. 1*b*), which increased after addition of the RNA-1 products was immunoprecipitable by the anti-T serum (Fig. 1*a*, lane 8) but not by the anti-CP serum (Fig. 1*a*, lane 6). No proteins were precipitated from processed RNA-2 products, such as shown in Fig. 1*a*, lane 4, by non-immune sera or by sera preincubated with their respective antigens (data not shown).

Fig. 2 shows that the 146K GCMV RNA-2-encoded polyprotein was specifically cleaved, after addition of GCMV RNA-1 products into 90K, 56K, 46K and 44K proteins. This last protein, more visible on the densitometric tracing (Fig. 2*b*, ii, peak 15) migrates as a diffuse band and is usually merged with a partial translation product of similar mobility. The 56K cleavage product comigrates with the GCMV coat protein isolated from the virion (data not shown) and is immunoprecipitated by an anti-GCMV serum (Fig. 2*a*, lane 8). Preferential labelling of the N- or C-terminal parts of the 146K polyprotein (Fig. 2*a*) showed that neither the 46K nor the 56K proteins were labelled when [^{35}S]methionine was present only during the first 4 min of incubation whereas labelling of the 44K protein could be detected (Fig. 2*a*, lane 3). On the contrary, addition of [^{35}S]methionine 10 min after the start of incubation led to labelling of the 56K and 46K proteins (Fig. 2*a*, lane 5).

Analysis of the cleavage products of RNA-2-encoded polyproteins of both TBRV and GCMV processed by the

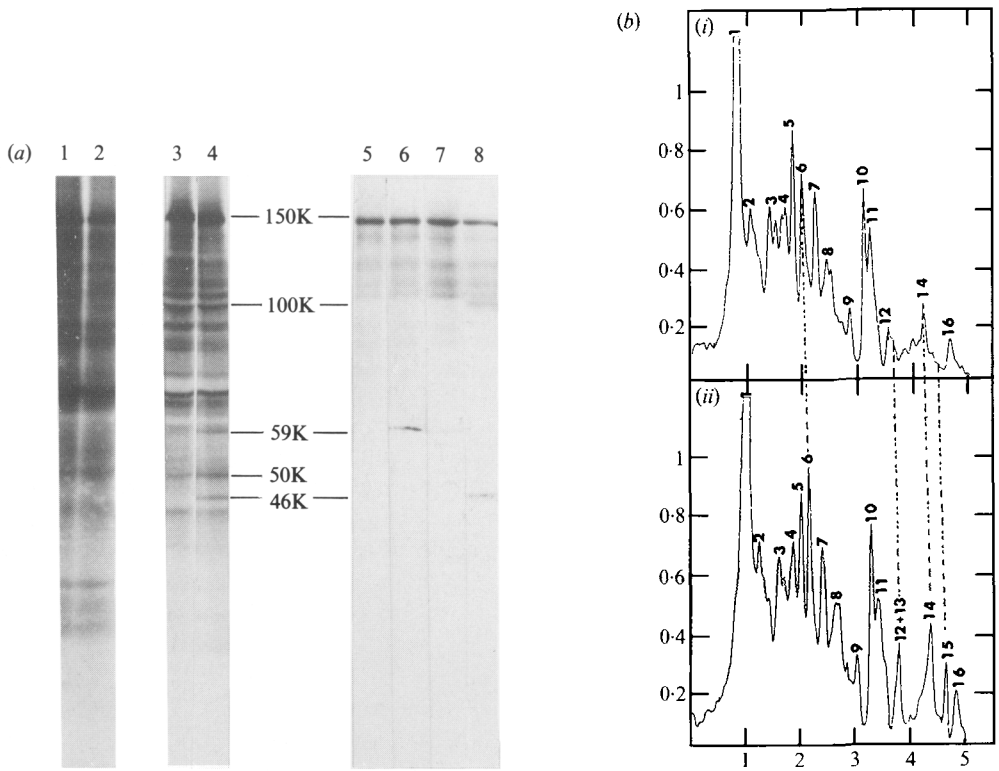

Fig. 1. *In vitro* processing of TBRV RNA-2 products. (*a*) Analysis by electrophoresis in an 8% polyacrylamide gel of the *in vitro* synthesized and processed TBRV RNA-2 polyprotein. [^{35}S]Methionine-labelled TBRV RNA-2 products synthesized in a rabbit reticulocyte lysate were analysed after incubation at 30 °C for 45 min (lane 1) and 3 h (lane 2). The translation medium containing products synthesized after 45 min was diluted 10-fold with translation medium containing 100 μM unlabelled methionine and incubated for 1 h without RNA (lane 3) or with RNA-1 (lane 4). The mixtures were further incubated for 2 h at 30 °C. Proteins immunoprecipitated by anti-CP serum (lanes 5 and 6) or anti-T serum (lanes 7 and 8) from samples from lane 3 (lanes 5 and 7) or from lane 4 (lanes 6 and 8) are also analysed. Autoradiography of the gel was performed overnight. (*b*) Densitometric tracing of lane 3 (i) and lane 4 (ii) from (*a*). Peaks 1, 6, 13, 14 and 15 correspond to proteins of 150K, 100K, 59K, 50K and 46K respectively.

homologous RNA-1 translation products suggests in each case the presence of three genes on RNA-2 localized as shown on Fig. 3. On the basis of sequence data, the N terminus of the CP of TBRV was first predicted to be at position 832 by Meyer *et al.* (1986). It has now been determined precisely at position 838 by automated Edman degradation using the technique described by Serghini *et al.* (1990). This result clearly positions the 59K protein at the C terminus of the polyprotein. The fact that the 100K cleavage product reacts with the anti-T serum but not with the anti-CP serum indicates that it is located at the N terminus of the polyprotein and that it is likely to be produced from it by removal of the 59K protein. Since it does not react with any of the antisera used, the 50K protein observed after addition of the RNA-1 product probably corresponds to the N-terminal part of the 150K (and 100K) protein. In the model presented in Fig. 3, the 100K protein corresponds to the sum of 50K and 46K proteins but from our results we cannot conclude whether there is a precursor–product relationship between these proteins or if they are produced directly by cleavage of the 150K polyprotein.

For GCMV the known location of the 56K coat protein at the C terminus of the RNA-2-encoded polyprotein (Brault *et al.*, 1989) and the lack of labelling of the 46K protein when [^{35}S]methionine was present only during the first minutes of incubation suggest a map of the 44K, 46K and 56K proteins within the 146K polyprotein as shown in Fig. 3. The 90K protein not immunoprecipitated by anti-CP serum could, by analogy with TBRV, correspond to the precursor of the 44K and 46K proteins.

To determine whether the processing of RNA-2 products is a specific event, the cleavage of the products of GCMV RNA-2 and of those of GFLV RNA-2 by TBRV RNA-1 translation products were studied. GCMV RNA-2 146K polyprotein was cleaved to give the same proteins as those obtained after maturation by

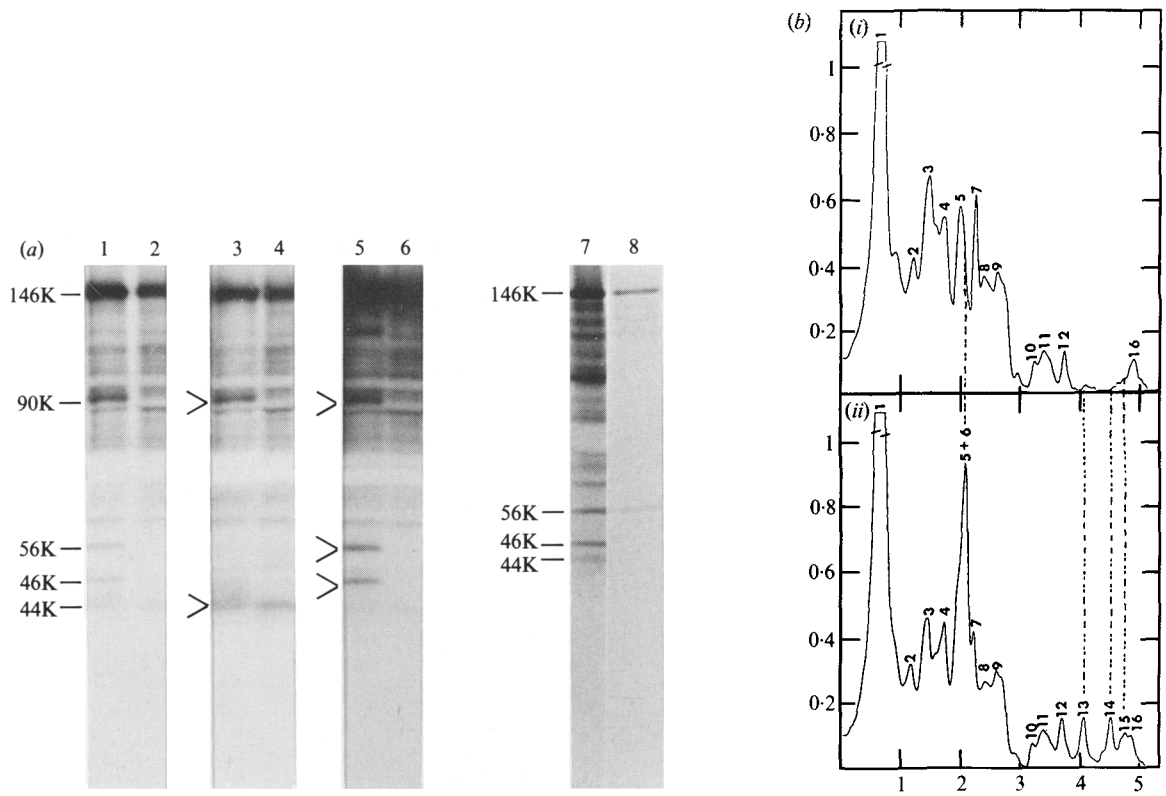

Fig. 2. Translation and processing of GCMV RNA-2-encoded polyprotein. (*a*) Autoradiography of an 8% polyacrylamide gel after electrophoresis of GCMV RNA-2-encoded proteins synthesized after 45 min incubation at 30 °C in a reticulocyte lysate. After this time the medium was diluted 10-fold with translation medium which had been incubated either with GCMV RNA-1 for 1 h (lanes 1, 3, 5 and 7) or without RNA (lanes 2, 4 and 6) and then further incubated for 2 h at 30 °C. [^{35}S]Methionine was present during the whole incubation period (lanes 1, 2 and 7) or chased after 4 min of incubation by the addition of 5 mM unlabelled methionine (lanes 3 and 4), or added after only 10 min of incubation (lanes 5 and 6). In this last case, edeine at 10 μg/ml had been added 8 min after the start of translation. The GCMV RNA-2 translation products processed by GCMV RNA-1 translation products (lane 7) (under the same conditions as described in Fig. 1) were immunoprecipitated by the antiserum raised against GCMV coat protein (lane 8). (*b*) Densitometric tracing of lane 1 (i) and lane 2 (ii) was obtained as described in Fig. 1. Peaks 1, 6, 13, 14 and 15 correspond to proteins of 146K, 90K, 56K, 46K and 44K respectively.

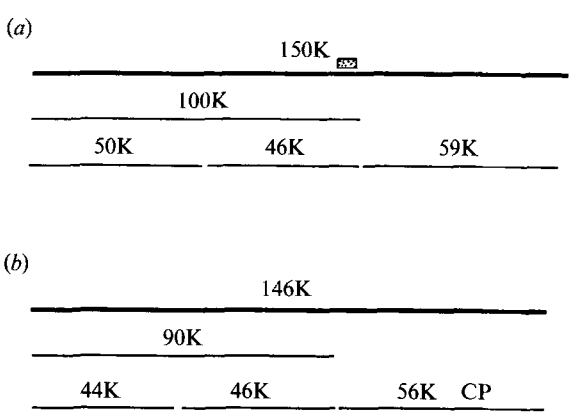

Fig. 3. Hypothetical genetic maps of TBRV (*a*) and GCMV (*b*) RNA-2. Position of peptide T is shown by (▒).

GCMV RNA-1 products (Fig. 4, lanes 1 and 2), whereas the 130K GFLV RNA-2-encoded protein was not cleaved (Fig. 4, lanes 4 and 5).

The yield of cleavage products obtained was however relatively low in spite of attempts to increase the efficiency of the processing by varying the incubation conditions (i.e. length of incubation, relative amounts of RNA-1 to RNA-2 products). It is likely that the poor efficiency of processing of the 250K polyprotein and the instability of the *in vitro* cleavage products observed by Greif (1989) and Demangeat *et al.* (1990) are responsible for the low proteolytic activity.

Among the three proteins produced *in vitro*, only the CP which is C-terminally located has been detected *in vivo*. Surprisingly, for TBRV, the C-terminal 59K CP obtained *in vitro* does not comigrate with the 57K protein

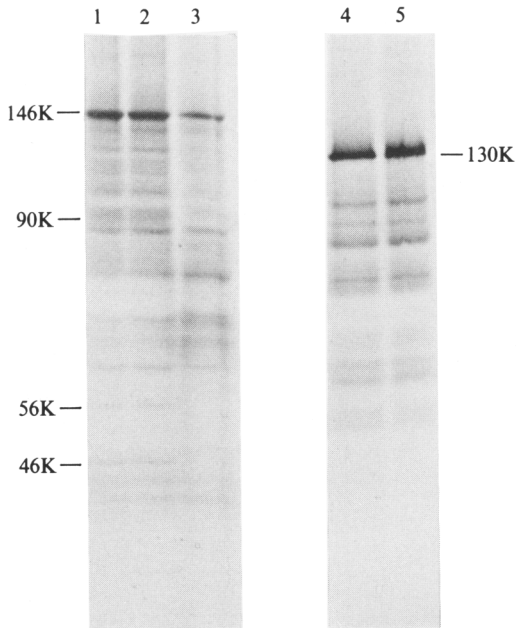

Fig. 4. Processing of GCMV RNA-2 (lanes 1, 2 and 3) and GFLV RNA-2 (lanes 4 and 5) polyproteins by TBRV RNA-1 (lanes 1 and 4) or GCMV RNA-1 (lane 2) translation products. Conditions were as in Fig. 1 and 2. Lanes 3 and 5 are control samples which correspond to incubation in the presence of lysate without any RNA-1 products.

obtained from dissociated virus. Results of preliminary experiments show that the 59K protein is also present in TBRV-infected *Chenopodium quinoa* leaves and therefore is not an artefact occurring *in vitro*. Further experiments will be needed to determine whether the 59K protein is a precursor of the 57K CP.

A 46K protein was recently detected in protoplasts infected with TBRV (G. Demangeat, unpublished results), which suggests that the 46K protein observed *in vitro* also corresponds to a mature protein.

The presence of three genes in RNA-2 of two nepoviruses is in good agreement with the earlier results obtained by Forster & Morris-Krsinich (1985) who showed that processing of the 116K TobRV RNA-2-encoded polyprotein yielded three proteins. They differ from those reported by Morris-Krsinich *et al.* (1983) for GFLV where the 125K RNA-2-encoded polyprotein is cleaved into only two proteins of 68K and 58K. This of course raises the question of the function of the N-terminal protein encoded by RNA-2 of the nepoviruses.

The cleavage maps of TBRV and GCMV RNA-2 polyproteins which have been deduced from the experiments described in this paper are very similar, which extends at the molecular level the relationships known to exist between these two viruses. The ability of the TBRV-encoded protease to process the GCMV RNA-2 polyprotein into products similar to those produced by the homologous protease, suggests that similar recognition and/or cleavage sites are present at similar positions in both polyproteins. The proteolytic activities detected in polyproteins of nepoviruses (Morris-Krsinich *et al.*, 1983), comoviruses (Goldbach & Krijt, 1982) and potyviruses (Garcia *et al.*, 1989) were always found to be highly specific and unable to recognize translation products of RNA of different viruses belonging to the same virus group. The ability of the TBRV-encoded protease to process correctly the TBRV as well as the GCMV polyproteins, but not that of a more distantly related nepovirus, GFLV, is probably related to the viability of TBRV–GCMV pseudo-recombinants. Indeed, correct processing of the viral polyproteins is probably a prerequisite for the expression of the viral functions harboured by these proteins, such as cell-to-cell movement or RNA encapsidation.

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