Analysis of the *in vitro* cleavage products of the tomato black ring virus RNA-1-encoded 250K polyprotein

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Tomato black ring virus RNA-1 was translated in a rabbit reticulocyte lysate. The primary translation product of M, 250K, which corresponds to its whole coding capacity, was synthesized within 45 min and, during further incubation in the translation medium, was proteolytically processed. Essentially, four cleavage products (P190, P120, P60 and P50) were detected and located within P250 by pulse–chase and immunoprecipitation experiments. P190 is an intermediate cleavage product which is further cleaved to form P60 and P120. P120, which contains the region that has been assigned to the virus protease and the virus polymerase, was not further cleaved *in vitro*.

**Introduction**

The genome of tomato black ring virus (TBRV), a member of the nepovirus group, consists of two separately encapsidated RNA molecules of 7359 nucleotides (nt) (RNA-1) and 4662 nt (RNA-2) respectively (Greif et al., 1988; Meyer et al., 1986). Both RNAs are polyadenylated at their 3' end (Mayo et al., 1979) and their 5' end is linked to a small protein (VPg) (Mayo et al., 1982; Koenig & Fritsch, 1982). Each RNA is translated *in vitro* to give one protein whose size corresponds to the whole coding capacity of the RNA (Fritsch et al., 1980). Analysis of the amino acid sequence of the 250K polyprotein encoded by TBRV RNA-1 revealed striking similarities with four regions of the non-structural 58K, VPg, 24K and 87K proteins of cowpea mosaic virus (CPMV) and the 2C, VPg, 3C and 3D proteins of poliovirus (Greif et al., 1988). These results suggested that similar functions are encoded in similar positions by TBRV RNA-1 and led us to propose a hypothetical gene organization for this RNA (Greif et al., 1988). We now report the results of *in vitro* translation experiments in rabbit reticulocyte lysate that were carried out in order to determine whether the predicted proteins are formed *in vitro* by processing of the 250K polyprotein encoded by RNA-1.

**Methods**

*Virus and RNA purification.* The TBRV-L isolate from Lanarkshire (Scotland) is of the Scottish serotype (Fritsch et al., 1984). TBRV-L (−) used in this work is free of satellite RNA and was a gift from M. A. Mayo (Scottish Crop Research Institute, Invergowrie, U.K.). TBRV was propagated in *Nicotiana clevelandii* and was purified as previously described (Fritsch et al., 1978). Particles containing either RNA-1 or RNA-2 were separated by equilibrium centrifugation of 2 mg samples of virus in aqueous CsCl (final density of 1.48 mg/ml) in a VTi-65 rotor (Beckman) for 16 h at 55000 r.p.m. at 20 °C. Fractions were pooled, recentrifuged in a 42.1 rotor (Beckman) for 16 h at 30000 r.p.m. and then resuspended in a 65 mM-phosphate buffer pH 6.8. RNA was extracted as previously described (Fritsch et al., 1978).

*In vitro translation.* TBRV RNA-1 was translated in a rabbit reticulocyte lysate as described by Hemmer et al. (1989). Generally, the reticulocyte lysate was used without nuclelease treatment but, in a few experiments, it was treated with 10 µg/ml micrococcal nuclease ( Worthington) by the method of Pelham & Jackson (1976). [35S]Methionine (1000 Ci/mmol; Amersham) and RNA (100 to 200 µg/ml) were added and incubated at 30 °C. Samples were mixed with 1 volume of 160 mM-Tris-HCl pH 6.8, 10% SDS, 25% 2-mercaptoethanol. After boiling for 90 s, samples of 8 µl were analysed by electrophoresis on SDS-polyacrylamide gels.

*Mapping of viral products.* The primary translation product was labelled in either its N- or C-terminal part by a method used by Goldbach & Rezelman (1983). For N-terminal labelling [35S]Methionine (15 mCi/ml) was present in the medium at time 0 and was chased after a short period of translation by addition of an excess (5 mM final) of unlabelled methionine. For pulse-labelling, translation was started in the absence of [35S]Methionine. [35S]Methionine was then added at the chosen time and chased 5 min later by the addition of non-labelled methionine (5 mM) and 10 µg/ml edeine. Preferential labelling of the C-terminal part of the polyprotein was achieved by the addition of edeine and [35S]Methionine, 10 min and 15 min after the start of translation respectively.

*Preparation of antisera.* Antisera were prepared against two synthetic peptides, C and P. The amino acid sequence of peptide C corresponds to amino acids 1421 to 1439 of the 250K polyprotein, the C-terminal region of the predicted protease, and that of peptide P corresponds to amino acids 2249 to 2265, the last 17 amino acids of 250K. Four-hundred µg of the peptide, conjugated to ovalbumin was emulsified either with complete Freund’s adjuvant and injected into rabbits subcutaneously (first injection) or with incomplete Freund’s adjuvant and injected intramuscularly. Rabbits were injected three times at 2
week intervals. Antisera were collected 2 weeks after the last injection and tested by ELISA, as described by Berna et al. (1984).

Immunoprecipitation. Ten μl of a dissociated translation medium was mixed with 5 μl preimmune serum or antiserum and adjusted to 1 x IB (10 mM-sodium phosphate pH 7.2, 150 mM-NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) in a final volume of 100 μl. In competition experiments, a mixture of 25 μg peptide and 5 μl antiserum was incubated for 1 h at room temperature before addition to the sample of translation medium. After incubation overnight at 4 °C, 20 μl of Pansorbin (Calbiochem) was added to the mixture which was further incubated at room temperature for 2 h. The samples were then centrifuged for 6 min at 6000 r.p.m, and the pellets resuspended in 200 μl of IB. After four washings, the pellets were resuspended in 80 mM-Tris-HCl pH 6.8, 5% SDS, 12.5% 2-mercaptoethanol and analysed on SDS-polyacrylamide gels. The gels, impregnated with Amplify (Amersham), were subjected to fluorography at −80 °C.

Results

Kinetics of synthesis of TBRV RNA-1 products

We have previously shown that TBRV RNA-1 is translated in wheatgerm extract, as it is in treated or untreated reticulocyte lysates, to give a protein of an estimated M, of 220K (Fritsch et al., 1980). The M, of this protein was later re-evaluated at 250K (P250) which is in better agreement with the M, deduced from the amino acid sequence data (Greif et al., 1988). Recently we showed that, during longer incubation periods, a non-specific proteolytic cleavage of viral polyproteins could occur in some micrococcal nuclease-treated lysates but that this effect could be avoided by using untreated lysates or lysates treated with the micrococcal nuclease supplied by Worthington (Hemmer et al., 1989). We therefore analysed the kinetics of the cleavage of P250 in both systems. In an untreated lysate (Fig. 1a, b), P250 was synthesized within 20 min and reached a maximum yield 45 to 60 min after the start of incubation. It then decreased in amount as proteins of M, 190K, 120K, 60K and 50K appeared in the medium, reaching a maximum yield after about 2 h. Then, the amount of P190 decreased too, but that of P120 and P60 remained constant. A number of less prominent proteins appeared during the first 30 min, and thereafter either decreased slightly or did not alter in amount. Most of these proteins are probably produced by premature termination or by internal initiations of translation. In treated reticulocyte lysates (Fig. 1c, d), the kinetics of formation of the different proteins were similar to those in untreated lysates, although the efficiency of translation was generally two- to fourfold higher than in untreated lysates. However, incomplete translation products were much more abundant than in untreated lysate and these obscured the accumulation of P60 and P50 at longer incubation periods. Proteins of lower M, (34K and 30K) increased in abundance in the medium after approximately 2 h, but they were insufficiently labelled to determine the kinetics of their synthesis.

Location of P190 and P120

To check that P190 and P120 result from cleavage of the fully synthesized P250 and not from delayed synthesis,
starting either from the same initiation site as P250 but terminated prematurely or from an internal initiation site, the following experiment was performed. TBRV RNA-1 was translated in the absence of labelled amino acid and, 10 min after the start of incubation, edeine was added to inhibit further initiation. [35S]Methionine was added 5 min after the addition of edeine, 15 min after the start of incubation, when we estimate that approximately half of the length of P250 has been synthesized. Fig. 2 (b) shows that, in these conditions, P190 and P120 were slightly less labelled than in standard conditions (Fig. 2(a), but became clearly predominant among the other proteins of similar Mr. Thus, it appears that P190 and P120 did not arise by delayed initiation or by premature termination (as the other proteins probably did), but arose by cleavage of P250 and originate from the C-terminal region of this protein.

Incubation of products, made by translation for 1 h and 5 h, with antisera directed against synthetic peptides P and C resulted in immunoprecipitation of P250, P190 and P120 (Fig. 3). No products were precipitated when non-immune serum or competing peptide were added to the reaction, which shows that the precipitations were specific. As peptide P corresponds to the C terminus of P250, we conclude that both P190 and P120 contain the C-terminal part of P250 and that P190, which must overlap P120, is an intermediate precursor. There was no evidence of immunoprecipitation of a protein with an Mr of about 90K, the size predicted for the C-terminal protein, the putative polymerase. Peptide C is located in the region assigned to the protease (Greif et al., 1988). The immunoprecipitation, by the serum directed against peptide C, of P120 shows that it is a precursor containing both the polymerase and the protease regions. This protein was stable in the translation medium; no further cleavage occurred even after incubation for 10 h.

Location of P60 and P50

In order to locate the other cleavage products of P250, the protein was labelled for periods of 5, 10 or 15 min,
Fig. 4. Mapping of TBRV RNA-I products by pulse-chase labelling. Incubation was in standard conditions (a). \[^{35}S\]Methionine was added either at time 0 and chased at 5 min (b), at 10 min (c) or at 15 min (d), by the addition of 5 mM unlabelled methionine and 10 μg/ml edeine. \[^{35}S\]Methionine was added at 5 min and chased at 10 min (e) or added at 10 min and chased at 15 min (f). The bottom of the gel was exposed for twice as long as the top. Incubation times: lanes 1, 30 min; lanes 2, 45 min; lanes 3, 60 min; lanes 4, 120 min; lanes 5, 240 min.

after which time an excess of unlabelled methionine was added and processing was allowed to proceed for a chase period (in this way processing of the proteins labelled during the pulse period could be followed).

Fig. 4(b) shows that a pulse from 0 to 5 min was sufficient to label P250 but neither P190, P120 nor P60 were labelled. P60, like P190, began to be labelled when the pulse was between 5 and 10 min (Fig. 4e) after the start of translation and incorporated more label with pulses from 10 to 15 min (Fig. 4f), from 0 to 10 min (Fig. 4c) and from 0 to 15 min (Fig. 4d). Addition of \[^{35}S\]methionine after 15 min resulted in little or no labelling of P60 (Fig. 2b). P120 was labelled only by a later pulse, from 10 to 15 min (Fig. 4f), or when \[^{35}S\]methionine was added to the medium 15 min after the start of incubation (Fig. 2b). These results show that P60, which could not be labelled within the first minutes of incubation, is not the N-terminal cleavage product of P250. Similarly, P190 began to be labelled by a pulse from 5 to 10 min (Fig. 4e), but could be labelled by later pulses (Fig. 4f, Fig. 2b) and would therefore appear to be the precursor of P60 and P120.

Proteins of \(M_r\) lower than 60K were all labelled when \[^{35}S\]methionine was added to the medium after the first 10 min. These proteins (P30, P34 and the most prominent, P50) (Fig. 1 and 4) appear to correspond to the N-terminal region of P250, but it was not possible to decide whether they arose by premature termination of translation or by cleavage of P250.

Attempts to inhibit proteolytic cleavage of P250

In order to test whether one of the N-terminal proteins was a cleavage product of P250, we analysed the effect of Zn\(^{2+}\) on the proteolytic processing of P250 (Fig. 5). This ion was shown to be able to inhibit the activity of viral proteases of encephalomyocarditis virus (Jackson, 1986) and CPMV (Franssen \textit{et al.}, 1984). Addition of Zn\(^{2+}\) within the first 20 min of incubation resulted in inhibition of the translation of P250 (not shown) and addition of Zn\(^{2+}\) after the synthesis of P250 had been completed (30 min) inhibited the cleavage of this protein. In fact P190, P120 and P60 did not appear in the medium and P50, which was present in a low amount at 30 min, did not increase in amount after 30 min. This result suggests that the higher amount of P50 in the control samples analysed after 30 min of incubation may come from the cleavage of P250. It is also possible, however, that the effect of Zn\(^{2+}\) on the amount of P50 corresponds to an inhibition of the translation of this protein taking
Inhibition of the proteolytic cleavage of the 250K protein. Proteins synthesized by TBRV RNA-1 in standard conditions (a) or in a medium supplemented with 2 mM-ZnCl₂ 30 min after the start of the translation (b). Exposure of the bottom of the gel was for twice as long as that of the top. Incubation times: lane 1, 15 min; lanes 2, 30 min; lanes 3, 60 min; lanes 4, 120 min; lanes 5, 240 min.

Discussion

The results reported here show that the 250K polyprotein encoded by TBRV RNA-1 has, when synthesized in a reticulocyte lysate, a proteolytic activity which causes the formation of smaller proteins. The proteins, of Mr 250K, 190K, 120K, 60K and 50K, have been mapped within the 250K protein (Fig. 6b) and, although there are some disparities, the results are in relatively good agreement with the genetic organization model of RNA-1 proposed by Greif et al. (1988) (Fig. 6a) and based on the comparison of the amino acid sequence of P250 with other viral proteins.

P50, which becomes labelled very soon after translation starts, could be either a premature termination product or an N-terminal cleavage product formed before the synthesis of P250 has been completed. The results shown in Fig. 5(b) suggest that P50 may be partially produced by cleavage but did not provide unequivocal evidence and this part of the cleavage scheme therefore remains speculative (Fig. 6). Moreover, by amino acid sequence comparison of the TBRV and CPMV polyproteins, it appears that the N-terminal region of the TBRV polyprotein may correspond to a protein of approximately 60K or to two proteins of approximately 30K (Greif et al., 1988; Le Gall et al., 1989). In vitro there is no evidence for the release of two proteins, although the small decrease of P50 in the medium with incubation periods of longer than 2 h may reflect a faint secondary cleavage.

Results of pulse-chase experiments (Fig. 4) showed that P60 is located in front of P120. Attempts to measure the molar proportion of P60 to P120 by densitometry of autoradiograms, taking into account the known methionine content, gave values of 1:2 rather than 1:1. However, prolonged incubation for 10 h showed that P120 degraded less than all the other products and any adjustment for this differential stability would probably yield a ratio closer to the predicted value.

Greif et al. (1988) have shown that P250 contains a consensus sequence characteristic of the cysteine proteases of CPMV and picornaviruses. This region is also contained in P190 and P120. Nevertheless P120 is not further cleaved, so the putative Q-S cleavage site between the protease and the polymerase domains is not recognized. This site was chosen by alignment with the Q-G observed in the CPMV 200K protein and the Q-G observed in the 246K protein of poliovirus (Greif et al., 1988). This result differs from those obtained in vitro by Franssen et al. (1984) for CPMV and by Carrington & Dougherty (1987) for tobacco etch virus, where the processing of the polyproteins leads to complete release of the protease. The efficiencies of cleavage of TBRV P250 at the different sites differ and it is possible that the cleavage between protease and polymerase is particularly inefficient. It seems more likely, however, that the protease of TBRV, which like that of grapevine chrome mosaic virus (GCMV) (Le Gall et al., 1989) and grapevine fanleaf virus (GFLV) (C. Ritzenthaler, personal communication) contains a leucine in place of a
histidine in its putative active site, does not preferentially cleave Q/E-G/S as the processing site (Bazan & Fletterick, 1989). Determinations of the N termini of three nepovirus coat proteins suggest that they arise by cleavage of the dipeptides R-A (GCMV; Brault et al., 1989), R-G (GFLV; Serghini et al., 1989) and K-A (TBRV; G. Demangeat, unpublished results). P250 may be cleaved at similar sites. Determination of the N-terminal amino acids of the cleavage products will be needed to determine why correct sites are cut.

References


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