Arch Virol (1997) 142: 1051–1058

Archives of Virology

Evidence for in vitro and in vivo autocatalytic processing of the primary translation product of beet necrotic yellow vein virus RNA 1 by a papain-like proteinase

Brief Report

A. Hehn, C. Fritsch, K. E. Richards, H. Guilley, and **G. Jonard**

Institut de Biologie Moléculaire des Plantes, Strasbourg, France

Accepted October 30, 1996

Summary. Beet necrotic yellow vein virus RNA 1 contains a single long ORF corresponding to the theoretical translation product of 237 kDa which contains the information necessary for replication of the viral genome. This ORF contains a putative papain-like proteinase domain which has been localized, on the basis of sequence alignments, between the helicase and polymerase domains. Here we show that the RNA 1 primary translation product can be cleaved autocatalytically in vitro into two species of 150 kDa and 66 kDa, the latter of which probably contains the entire polymerase domain. A 66 kDa protein was detected immunologically in infected *C. quinoa* protoplasts using an antiserum specific for the C-terminal region of the RNA 1 primary translation product, confirming that processing also occurs in vivo.

*

Beet necrotic yellow vein virus (BNYVV), a tentative member of the furovirus group [14], has a genome composed of up to five single-stranded positive-sense RNAs which are 5′-capped and 3′-polyadenylated [19]. While RNAs 3, 4 and 5 intervene in the natural infection of sugar beet, RNAs 1 and 2 are necessary and sufficient for the development of infection when mechanically inoculated to leaves of *Chenopodium quinoa* [9, 18, 23]. Moreover, RNA 1 is able to replicate by itself when inoculated to *C. quinoa* protoplasts [1], demonstrating that this RNA contains all the information needed for replication. The long, unique open reading frame (ORF) present on RNA 1 extends from AUG (154–156) to UAA(6481–6483) and has the coding potential for a protein of 237 kDa [3]. A protein of this size, as well as species of ~220 000 apparent M_r, has been detected among the translation products of viral RNA 1 or a cDNA 1 transcript in wheat germ extract [7]. The 220 kDa species (P220) results from

translation initiation at AUG(496–498) rather than at AUG(154–156) and it, rather than the 237 kDa species, is the longest RNA 1 translation product synthesized in rabbit reticulocyte lysates [7]. Which initiation codon is used in vivo is unknown.

The RNA 1 ORF contains three distinct replication-associated domains: a methyl transferase domain, an NTP-binding/helicase domain and a polymerase domain [3, 10, 17]. Recently, Koonin and Dolja [10] have detected papain-like proteinase motifs between the NTP-binding/helicase and the polymerase domains, suggesting that the RNA 1 primary translation product could undergo post-translational cleavage. To test this possibility, we have searched for cleavage products of the RNA 1 putative polyprotein in rabbit reticulocyte lysate at different times.

RNA extracted from virions of the BNYVV isolate Stras 12, which contains only RNA 1 and 2 [18], was translated in vitro in rabbit reticulocyte lysates (Green Hectares, Madison, WI) which had been made mRNA-dependent by treatment with micrococcal nuclease (Worthington) [16]. Proteins were synthesized in 10 µl of lysate supplemented with 0.15 mM $MgCl_2$ and 0.125 mM KCl (final concentrations), 0.5 µCi/µl [^{35}S]-methionine, and 100 ng/µl Stras 12 RNA. Translations were incubated at 30°C and samples were removed at intervals after the start of incubation, diluted with an equal volume of dissociation buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 20% glycerol and 5% β-mercaptoethanol) and heated for 3 min at 90° before analysis by SDS-PAGE in an 8% gel and autoradiography. A band corresponding to the previously described 75 kDa readthrough protein (P75; 85 kDa apparent M_r) [2], the longest polypeptide encoded by RNA 2, appeared within 25 min (Fig. 1B, lane 1) and P220 appeared after 45 min (Fig. 1A, lane 1; Fig. 1B, lane 2). In addition to P75 and P220, a 'background' of minor translation products was also synthesized. These products may arise from translation of partially degraded viral RNA's and/or internal translation-initiation or premature translation-termination ('early quitters') on intact templates.

For incubation times ranging from 45 min to 4 hr, the amounts of P220 and 'background' translation products remained approximately constant whereas a protein of apparent M_r 150 000 (mobility identical to that of the P150 primary translation product of tomato black ring nepovirus RNA 2 [4] (data not shown)) appeared at 90 min and remained abundant at latter times (Fig. 1A, lanes 2 and 3; Fig. 1B, lane 3). At the same time as the 150 kDa species appeared, a new band of about 66 kDa could also be detected (Fig. 1A, lanes 2 and 3; Fig. 1B, lane 3). The appearance of the 150 kDa and 66 kDa species was almost completely inhibited if 2 mM $ZnCl_2$, an inhibitor of thiol proteinases [13], was included in the translation mix (Fig. 1A, lanes 4–6).

To determine the relationship between the 150 kDa and 66 kDa products and P220, samples were pulse-labelled to preferentially label either the N-terminal or C-terminal portion of P220. For N-terminal labelling, Stras 12 RNA was translated in the presence of 1 µCi/µl ^{35}S-methionine for 15 min, and then the mix was supplemented with a 10 000-fold excess of unlabelled methionine

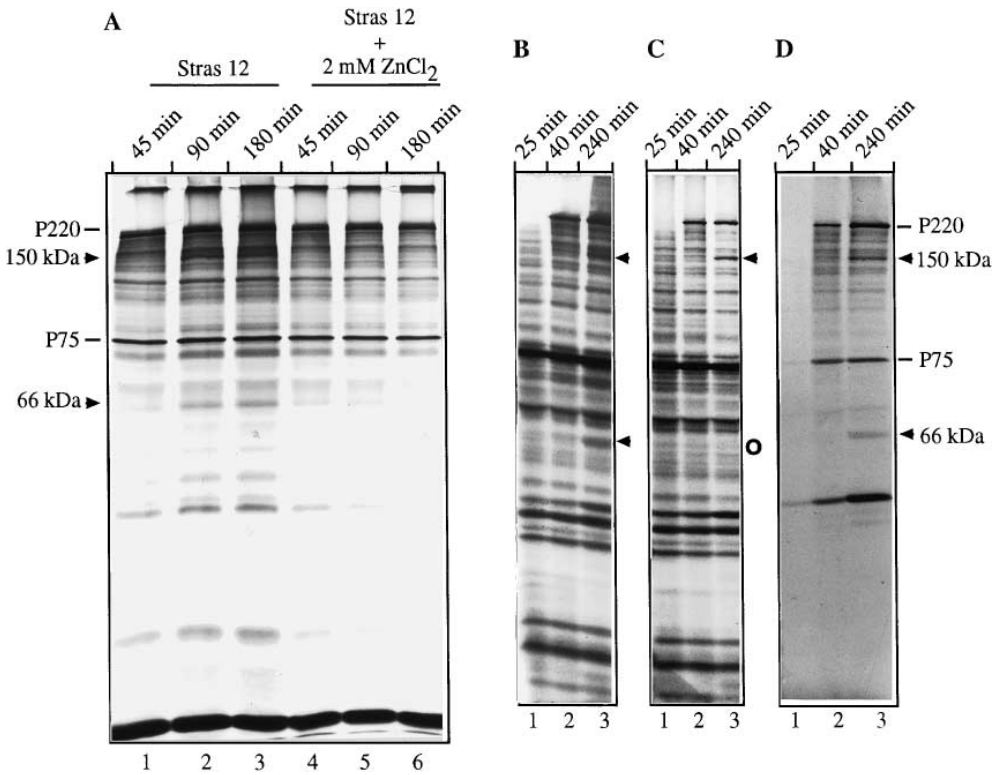


Fig. 1. Proteolytic processing of the ^{35}S-methionine-labelled primary translation product (P220) of BNYVV RNA 1 following in vitro translation in rabbit reticulocyte lysate. **A** Time-course of appearance of BNYVV RNAs 1 and 2 translation products. The RNA template was RNA extracted from virions of BNYVV isolate Stras 12 and translated in the absence (*1–3*) or presence (*4–6*) of 2 mM $ZnCl_2$, an inhibitor of thiol-proteinases. Samples were removed from the translation mix 45 min (*1*, *4*), 90 min (*2*, *5*) and 180 min (*3*, *6*) after the start of incubation and analyzed by SDS-PAGE in an 8% polyacryamide gel followed by autoradiography. The apparent M_r of the translation products were estimated by comparison to nonlabeled marker proteins (Promega) loaded in an adjacent lane (not shown). The positions of the major RNA 1 and 2 translations products P220 and P75 and the putative 150 kDa and 66 kDa cleavage products of P220 are indicated to the left. **B** Translation of Stras 12 RNA as in **A**, *1–3*. Samples were removed 25 min (*1*), 40 min (*2*) and 240 min (*3*) after the start of incubation and analyzed by SDS-PAGE as in **A**. **C** N-terminal pulse labeling of the RNA 1 and 2 translation products. After 15 min translation in the presence of ^{35}S-methionine plus the other 19 amino acids, nonradioactive methionine and edeine were added and incubation was continued. Samples were removed at the indicated times and analyzed as in **B**. The position where the 66 kDa maturation product would be expected to migrate is indicated by an empty circle to the right. **D** C-terminal pulse-labeling of the RNA 1 and 2 translation products. After 10 min translation in the presence of 1 μM methionine and 1 mM of each of the other amino acids, edeine and, five minutes later, ^{35}S-methionine, were added to the reaction mix. Samples were removed at the indicated times and analyzed by SDS-PAGE as in **B**

and 10 ng/μl edeine (Calbiochem), to inhibit further initiation. In this case, both P220 and the 150 kDa polypeptide were detected after 4 h incubation (Fig. 1C, lane 3), demonstrating that synthesis of both proteins was initiated early and that the 150 kDa product did not arise from delayed initiation at an internal site. The failure to label the 66 kDa product in this experiment also suggests that, if the 150 kDa and 66 kDa proteins arose from P220 by post-translational cleavage, the smaller species represents the C-terminal portion.

Preferential C-terminal was achieved by initiating translation in the presence of unlabelled methionine (1 μM), with addition of edeine after 10 min and ^{35}S-methionine (1000 Ci/mol; 1 μm) five minutes later. In this case, the 150 kDa protein appeared four hours after translation-initiation (Fig. 1D, lane 3) but in reduced amounts relative to that of P220 (compare the ratio of the two products in Fig. 1D, lane 3, to the ratio in Fig. 1C, lane 3). The 66 kDa species, on the other hand, was efficiently labelled (Fig. 1D, lane 3). Taken together, the above results suggests that P220 undergoes post-translational proteolytic cleavage to produce an N-terminal 150 kDa protein and a C-terminal 66 kDa protein. The fact that not all the P220 is cleaved a later times in these experiments may indicate that conditions in the in vitro translation system are subobtimal for proper folding of the protein or for the proteinase activity.

To determine if P220 is also processed in vivo, 2×10^5 *C. quinoa* protoplasts were inoculated with 1 μg of Stras 12 RNA as described [25] aliquots corresponding to 40 000 protoplasts were harvested by centrifugation 2, 3, 8, 10 and 24 h post-inoculation (pi). The protoplast pellets were resuspended in 40 μl dossociation buffer and heated for 3 min at 90°C. After a short centrifugation at 17000× **g**, 10 μl of the proteins in the supernatant were fractionated by SDS-PAGE in an 8% gel and electro-transferred to an Immobilon-P membrane (millipore). RNA 1-specific proteins were detected by western blotting using an antiserum directed against a fusion protein containing the C-terminal 578 amono acid residues of P220 [15] and staining by enhanced chemiluminescence (ECL) as described by the supplier (Amersham).

The western blot (Fig. 2A) reveals, in addition to strong but nonspecific 'background' bands which are also found in the mock-inoculated control (Fig. 2A, lane 1), an infection-specific protein of apparent M_r 66 000 which appeared at 8h pi and increased until 24 h pi (Fig. 2A, lanes 4–6). We assume that this species is identical to the 66 kDa polypeptide detected in vitro since both proteins have the same mobility and are derived from the C-terminal portion of P220. Note that the predicted 150 kDa N-terminal cleavage product seen in in vitro translation experiments cannot be detected with the C-terminal-specific antiserum. The 66 kDa species also appeared when BNYVV cDNA 1 transcript was inoculated to protoplasts (Fig. 2B, lanes 3–4), demonstrating that RNA 2 is not essential for its production. It is not known if the fall-off of the 66kDA protein at 32–48 h in Fig. 2B is due to turn-over of the protein or protoplast mortality. The cleavage event which generates the 66 kDa protein

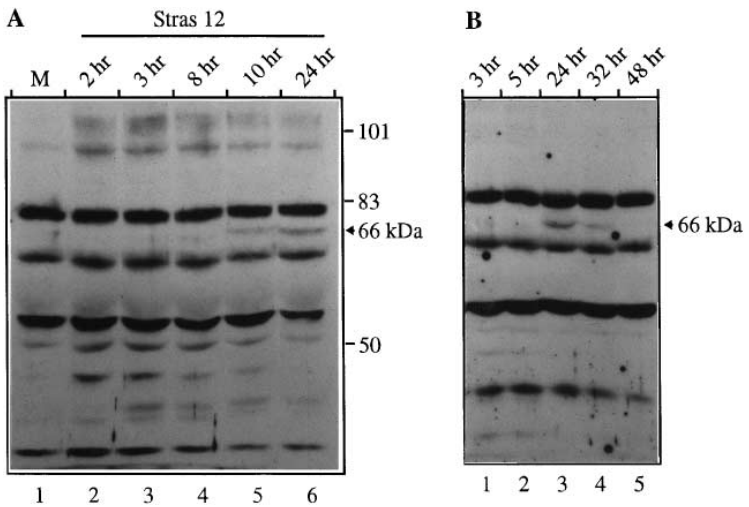
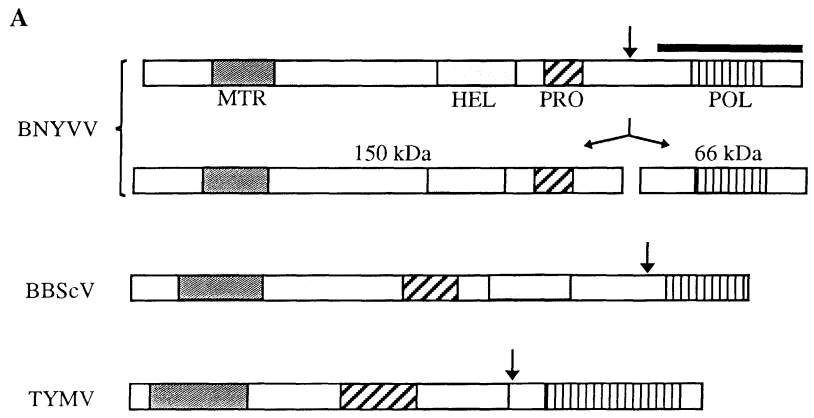


Fig. 2. Immunodetection of a protein encoded by BNYVV RNA 1 in infected *C. quinoa* protoplasts. **A** Protoplasts inoculated with Stras 12 RNA were harvested 2, 3 8, 10 and 24 h pi (*2–6*) and total proteins were separated by PAGE in an 8% SDS-polyacrylamide gel, followed by electrotransfer to Immobilon-P. Immunodetection was with a serum directed against a fusion protein containing the last 578 amino acids of the primary translation product of RNA 1 [15]. M Sample from mock-inoculated protoplasts (*1*). The positions of unlabelled markers are indicated in kDa to the right. The arrowhead indicates the position of the 66 kDa product discussed in the text. **B** The protoplasts were infected with cDNA 1 transcript and samples were prepared and analyzed as in **A**

from P220 must occur rapidly in vivo as no P220 could be immuno-detected by western blot, even at early times pi (data not shown).

Taken together, the foregoing results argue strongly that a papain-like proteinase cleaves the BNYVV RNA 1 primary translation product to produce an N-terminal 150 kDa protein and a C-terminal 66 kDa protein. Site-directed mutagenesis experiments on the putative proteinase domain in P220 will be necessary to confirm definitively that this domain is responsible for the cleavage we observe. If, as we consider likely, however, the aforesaid proteinase domain is responsible for the cleavage, this implies that the expression strategy of BNYVV RNA 1 resembles that of tymoviruses [13, 20] and carlaviruses [5, 11, 12, 24], except that the proteinase is located between the helicase and polymerase domains (Fig. 3A) as in picorna-like viruses instead of being located on the N-proximal side of the helicase domain.

The size of the 66 kDa C-terminal product indicates a cleavage site approximately 600 amino acids from the C-terminus of P220, i.e., between the helicase and polymerase domains (Fig. 3A). Analysis of the cleavage sites of six alphavirus papain-like proteinases [22] revealed that gly is invariably present at position P2 (two residues upstream of the cleavage site), Ala is usually at position P3 (three residues upstream of the cleavage site), and an amino acid with a short side chain often immediately precedes the cleavage site (position P1). The sequence in P220 which best conforms to this consensus is



B

```
BBScV                ..1465-GFQKNLLGADEGKREC..
BNYVV ..1537-NMAGGKTDA...KEFDLDNGADEYQCAY-1615.
TYMV                 ..1251-TRGPKLNGATPSASPT..
```

Fig. 3. A Schematic representation of the BNYVV RNA 1 polyprotein and the replicase polyproteins P206 [8] of turnip yellow mosaic tymovirus (TYMV) and P223 [11] of blueberry scorch carlavirus (BBScV). Corresponding domains of the different replicases are symbolized by identical shading. *MTR* Methyltransferase domain; *HEL* NTP-binding/helicase domain: *PRO* papain-like proteinase domain; *POL* polymerase domain. The approximate location of the predicted cleavage site for each polyprotein is indicated by an arrow and the resulting cleavage products of the BNYVV RNA 1 polyprotein are shown below. The thick line indicates the portion of the BNYVV RNA 1 primary translation product used to raise the C-terminus-specific antiserum. **B** Amino acid sequences flanking two possible cleavage sites of the BNYVV 237 kDa polyprotein and comparison with sequences flanking the cleavage site of TYMV P206 and that proposed for BBScV P223

the G/K dipeptide at positions 1541–1542, where the sequence at positions P3, P2 and P1 is AGG (Fig. 3B). Cleavage at this site would release a C-terminal protein with a molecular mass of 66 kDa. Another possible cleavage site is the A/D dipeptide at positions 1608–1609, where the flanking sequences resemble the cleavage sites determined for turnip yellow mosaic virus (TYMV) [8] and proposed for blueberry scorch virus (BBScV) [11] (Fig. 3B). In this case, the C-terminal cleavage product would have a molecular mass of 52 kDa.

Our evidence indicates that a BNYYV proteinase cleaves the RNA 1 polyprotein to generate discrete polypeptides carrying functionally distinct domains presumably active in viral replication. This expression strategy differs significantly from that of the accepted furoviruses soil-borne wheat mosaic virus (SBWMV) and peanut clump furovirus (PCV), both of which employ translational readthrough to synthesize two separate replicative proteins [6, 12]. This distinction, along with previously noted differences between BNYVV and other furoviruses [6, 19], confirms that the furovirus group is not a uniform taxon.

References

1. Bouzoubaa S, Scheidecker D (1990) Infection of protoplasts with beet necrotic yellow vein virus RNA. In: Koemig R(ed) Proceedings of the first symposium of the international working group on plant viruses with fungal vectors. Verlag Eugen Ulmer, Stuttgart, pp 16–20
2. Bouzoubaa S, Ziegler V, Beck D, Guilley H, Richards K & Jonard G (1986) Nucleotide sequence of beet necrotic yellow vein virus RNA-2. J Gen Virol 67: 1689–1700
3. Bouzoubaa S, Quillet L, Guilley H, Jonard G, Richards K (1987) Nucleotide sequence of beet necrotic yellow vein virus RNA-1. J Gen Virol 68: 615–626
4. Demangeat G, Hemmer O, Fritsch C, Le Gall O, Candresse T (1991) In vitro processing of the RNA 2 encoded polyprotein of two nepoviruses: tomato black ring virus and grapevine chrome mosaic virus. J Gen Virol 72: 247–252
5. Foster GD, Mills PR (1992) Translation of potato virus S RNA in vitro: evidence of protein processing. Virus Genes 6: 47–52
6. Herzog E, Guilley H, Manohar SK, Dollet M, Richards K, Fritsch C, Jonard G (1994) Complete nucleotide sequence of peanut clump virus RNA 1 and relationships with other fungus-transmitted rod-shaped viruses. J Gen Virol 75: 3 147–3 155
7. Jupin I, Quillet L, Ziegler-Graff V, Guilley H, Richards K, Jonard G (1988) In vitro translation of natural and synthetic beet necrotic yellow vein virus RNA 1. J Gen Virol 69: 2 359–2 367
8. Kadare G, Rozanov M, Haenni AL (1995) Expression of turnip yellow mosaic virus polymerase in *Escherichia coli* and determination of the cleavage site within the 206 kDa protein. J Gen Virol 76: 2 853–2 857
9. Koening R, Burgermeister W, Weich H, Sebald W, Kothe C (1986) Uniform RNA patterns of beet necrotic yellow vein virus in sugar beet roots, but not in leaves from several plant species. J Gen Virol 67: 2 043–2 046
10. Koonin EV, Dolja VV (1993) Evolution and taxonomy of positive strand RNA viruses: implication of comparative analysis of amino acid sequences. Crit Rev Biochem Mol Biol 28–5: 375–430
11. Lawrence DM, Razanov MN, Hillman BI (1995) Autocatalytic processing of the 223 kDa protein of blueberry scorch carlavirus by a papain-like proteinase. Virology 207: 127–135
12. Meehan BM, Mills PR (1991) Cell-free translation of carnation latent virus RNA and analysis of virus specific dsRNA. Virus Genes 5: 175–181
13. Morch MD, Drugeon G, Szafranski P, Haenni AL (1989) Proteolytic origin of the 150-kilodalton protein encoded by turnip yellow mosaic virus genomic RNA. J Virol 63: 5 153–5 158
14. Murphy FA, Fauquet C, Bishop DH, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD (1995) Virus Taxonomy. Classification and Nomenclature of Viruses. Sixth Report of the International Committee on Taxonomy of Viruses. Springer, Wien New York (Arch Virol [Suppl] 10)
15. Niesbach-Klösgen U, Guilley H, Jonard G, Richards K (1990) Immunodetection in vivo of beet necrotic yellow virus encoded proteins. Virology 178: 52–61
16. Pelham HRB, Jackson RJ (1976) An efficient mRNA-dependent translation system from reticulocyte lysate. Eur J Biochem 67: 247–256
17. Poch O, Sauvaget I, Delarue M, Tordo N (1989) Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J 8: 3 867–3 874
18. Quillet L, Guilley H, Jonard G, Richards K (1989) In vitro synthesis of biologically active beet necrotic yellow vein virus RNA. Virology 172: 293–301

19. Richards K, Tamada T (1992) Mapping functions on the multipartite genome of beet necrotic yellow vein virus. Annu Rev Phytopathol 30: 291–313
20. Rozanov MN, Drugeon G, Haenni AL (1995) Papain-like proteinase of turnip yellow mosaic virus: a prototype of a new viral proteinase group. Arch Virol 140: 273–288
21. Shirako Y, Wilson TMA (1993) Complete nucleotide sequence and organization of the bipartite RNA genome of soil borne wheat mosaic virus. Virology 195: 16–32
22. Strauss JH, Strauss EG (1990) Alphaviruses proteinases. Semin Virol 1: 347–356
23. Tamada T, Abe H (1989) Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus Polymyxa betae. J Gen Virol 70: 3 391–3 398
24. Tavantzis SM (1991) Coat protein and protease activity as in vitro translation products of potato carlavirus M RNA. Arch Virol 120: 241–252
25. Veidt I, Bouzoubaa S, Leiser RM, Ziegler-Graff V, Guilley H, Richards K, Jonard G (1992) Synthesis of full-length transcripts of beet western yellows virus RNA: messenger properties and biological activity in protoplasts. Virology 186: 192–200

Authors' address: Dr. A. Hehn, IBMP, 12 rue Général Zimmer, F-67084 Strasbourg Cedex, France.

Received September 10, 1996