JOURNAL OF VIROLOGY, May 1983, p. 614–619
0022-538X/83/050614-06$02.00/0

Vol. 46, No. 2

NOTES

Orientation of the Cleavage Map of the 200-Kilodalton Polypeptide Encoded by the Bottom-Component RNA of Cowpea Mosaic Virus

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Received 9 November 1982/Accepted 3 February 1983

The genomic organization of the bottom-component RNA of cowpea mosaic virus was studied. In vivo, this RNA encodes at least eight different polypeptides of 170, 110, 87, 84, 60, 58, 32, and 4 kilodaltons (K), the last polypeptide representing the genome-bound protein VPg. In rabbit reticulocyte lysates, bottom-component RNA is translated into a 200K polypeptide which is then processed to give the 32 and 170K polypeptides also found in vivo. By pulse-labeling the 200K primary translation product, we now show that the 32 and 170K polypeptides are derived from the NH_2-terminal and COOH-terminal parts of this polypeptide, respectively. Comparison of the proteolytic peptide patterns of 170K polypeptides synthesized in vitro and pulse-labeled at either the NH_2-terminal or the COOH-terminal end with the patterns of the 170 and 110K polypeptides found in vivo demonstrates that the order within the 200K primary translation product of cowpea mosaic virus bottom-component RNA is as follows: NH_2–32K polypeptide–58K polypeptide–VPg–24K polypeptide–87K polypeptide–COOH.

In the last few years, much information on the expression of cowpea mosaic virus (CPMV) has emerged from both in vivo and in vitro studies (8, 10–12, 18, 21–24, 32). Thus, it has been shown (10) that the bottom-component RNA (B-RNA) of CPMV is able to replicate independently of the middle-component RNA and encodes at least eight different polypeptides, among them the genome-bound protein VPg of approximately 4 kilodaltons (K) (27, 28). The relationships among the polypeptides have been elucidated by comparing their proteolytic peptide patterns (21) and by screening for the presence of VPg sequences with anti-VPg serum (11, 32). The information available to date fits a model in which B-RNA is translated into a 200K polyprotein, which is first cleaved into a 32 and a 170K polypeptide. Subsequently, the 170K polypeptide is further processed in one of two ways, to give either 60 and 110K polypeptides or 84 and 87K polypeptides (21). Finally, the 60K polypeptide undergoes proteolysis to give a 58K polypeptide and VPg (11). Although it has been shown that 84 and 60K polypeptides are derived from the same end of the 170K precursor polypeptide and that the 110 and 87K polypeptides are derived from the other end (21), so far the orientation (NH_2-terminally or COOH-terminally) of these polypeptides within the 170K polypeptides has not been determined. Moreover, although previous in vitro translation experiments (12, 18) suggest that the 32K polypeptide is derived from the NH_2-terminal part of the 200K polyprotein, this has not been firmly established.

We now describe experiments which allow all B-RNA-encoded polypeptides to be mapped within their common precursor. For this purpose, purified B-RNA was translated in a rabbit reticulocyte lysate, and the 200K primary translation product was pulse-labeled in either its NH_2-terminal or COOH-terminal end with [^{35}S]methionine used as the radioactive amino acid. Subsequently, the 170K polypeptides, derived from the pulse-labeled 200K polypeptides, were isolated from a polyacrylamide gel and subjected to limited proteolytic digestion by *Staphylococcus aureus* V8 protease. By comparing the radioactive peptide maps thus obtained with those of uniformly labeled 170 and 110K polypeptides isolated from bottom-component-infected cowpea protoplasts, the 110, 87, 84, and 60K polypeptides could all be mapped within the 170K polypeptide. B-RNA was isolated from purified bottom components and translated in rabbit reticulocyte lysates as described

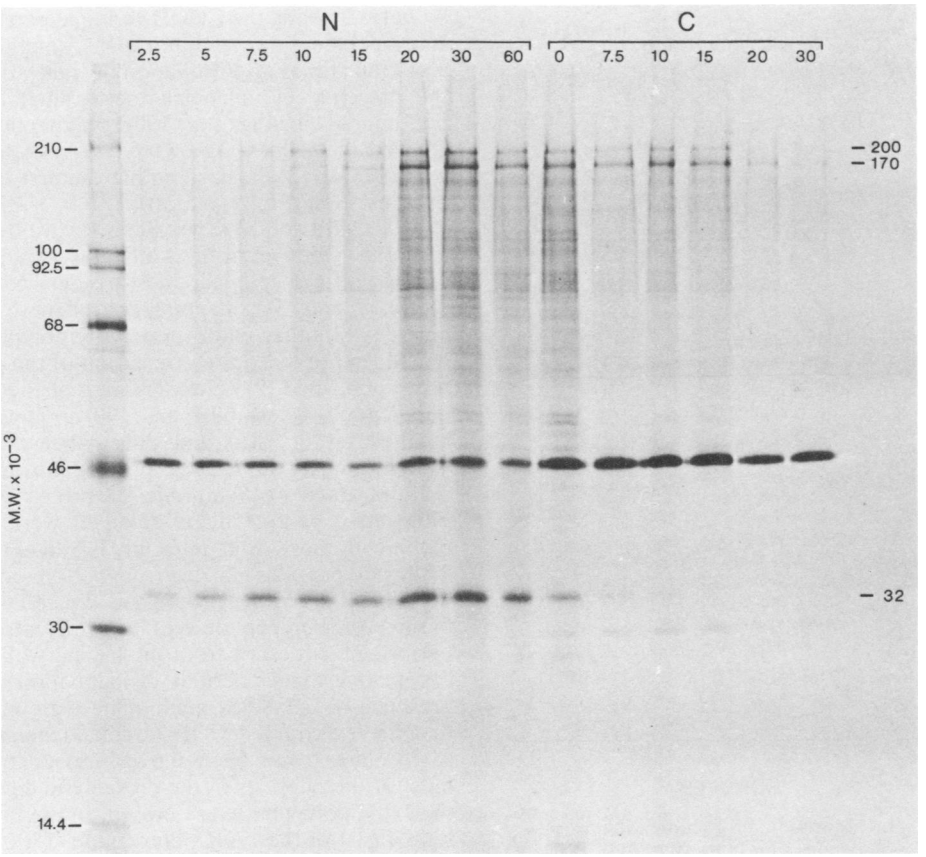

FIG. 1. In vitro translation of CPMV B-RNA. B-RNA was translated in rabbit reticulocyte lysate, and the 200K primary translation product was pulse-labeled with [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.; 1,267 Ci/mmol) in either its NH_2-terminal or COOH-terminal part. The labeled products were compared by electrophoresis in a 15% sodium dodecyl sulfate-polyacrylamide gel as described previously (16, 21). For NH_2-terminal labeling (lanes indicated with N), translation was started in the presence of [^{35}S]methionine, and after 6 min edeine (Calbiochem-Behring, La Jolla, Calif.; final concentration, 10 μg/ml) was added to prevent new initiations. At the times indicated in the figure (minutes), samples were removed, chased with excess unlabeled methionine (final concentration, 10 mM) and further incubated to complete a total translation period of 1 h. For COOH-terminal labeling (lanes indicated with C), translation was started with unlabeled methionine (final concentration, 3 μM), and after 6 min edeine (final concentration, 10 μg/ml) was added. At the times indicated in the figure (minutes), samples were removed, mixed with [^{35}S]methionine (final concentration, 9 μM), and further incubated to complete a total translation period of 1 h. The numbers at the left refer to the molecular weights ($\times 10^{-3}$) of the marker proteins used: myosin, 210,000; phosphorylase *b*, 100,000 and 92,500; bovine serum albumin, 68,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; and α-lactalbumin, 14,400. The polypeptide band of approximately 50K present in all lanes represents an endogenous product of the rabbit reticulocyte lysate and is the product of a ribosome-independent process (16).

previously (10, 15, 18, 19, 21, 29). For labeling the NH_2-terminal part of the 200K precursor polypeptide, [^{35}S]methionine was present from the start of translation. At various times, samples were removed from the translation mixture, chased with 10 mM unlabeled methionine to prevent further incorporation of labeled methionine, and further incubated to allow completion of the polypeptide synthesis.

The 32K polypeptide was labeled within 2.5 min, whereas the 170K polypeptide was labeled only after 10 min (Fig. 1, lanes N). Variation of the sample volumes used did not make the kinetics of the formation of both the 32 and 170K polypeptides immediately clear (Fig. 1). Therefore, the incorporated radioactivity in each of the 200, 170, and 32K polypeptides was estimated throughout the time course and normalized for each sample (Fig. 2A). Incorporation of [^{35}S]methionine into the 32K polypeptide

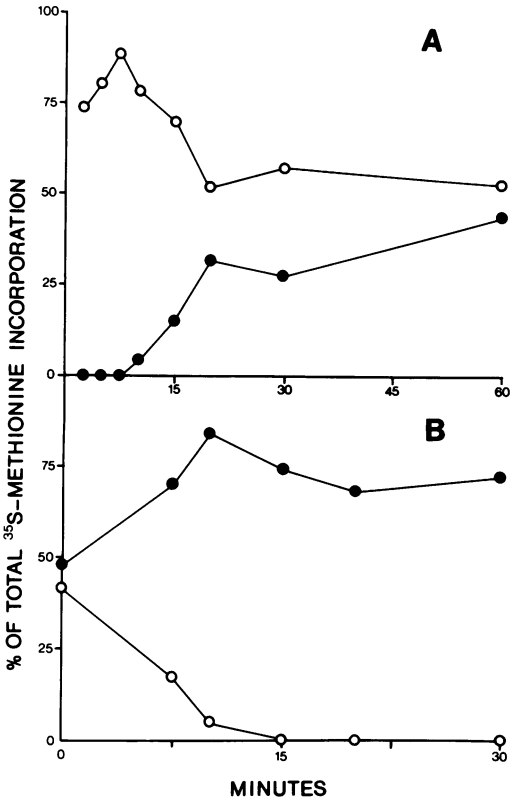

FIG. 2. Incorporation of [^{35}S]methionine into 32 and 170K polypeptides upon translation of B-RNA in vitro. Bands containing 32, 170, or 200K polypeptides were excised from the gel shown in Fig. 1 and treated with 0.5 ml of 90% (vol/vol) Soluene (Packard Instrument Co., Inc., Rockville, Md.) in water for 2 h at 50°C. After the addition of 7 ml of Instafluor (Packard), the amount of radioactivity was estimated by scintillation counting. For each time point, incorporation of [^{35}S]methionine into the 32 or 170K polypeptides is expressed as a percentage of total incorporation into the 200, 170, and 32K polypeptides. (A) Incorporation of radioactivity upon NH_2-terminal labeling for the times (minutes) indicated. (B) Incorporation of radioactivity upon COOH-terminal labeling. [^{35}S]methionine was added to unlabeled translation mixture at the times (minutes) indicated. The total incorporation into the 200, 170, and 32K polypeptides ranged between 150 and 2,930 cpm (net) in (A) and between 90 and 820 cpm (net) in (B). Symbols: ○, 32K polypeptide; ●, 170K polypeptide.

reached a maximum value within 10 min, whereas incorporation of this label into the 170K polypeptide did not reach a plateau within 60 min (Fig. 1 and 2A). These results indicate that the 32K polypeptide is derived from the NH_2-terminal part and the 170K polypeptide is derived from the COOH-terminal part of the 200K precursor. This orientation was fully confirmed by pulse-labeling the COOH-terminal part of the 200K polypeptide. Addition of [^{35}S]methionine at various times after initiation of translation (in the presence of unlabeled methionine) led to preferential labeling of the 170K polypeptide and showed that almost all of the 32K polypeptide sequence was sythesized within the first 10 min of translation (Fig. 1 and 2B).

As the 110 and 87K polypeptides are derived from the same end of the 170K polypeptide and the 84 and 60K polypeptides are derived from the other end (21), mapping any of these polypeptides in either end of the 170K polypeptide would lead to a definite orientation of the previously published (21) cleavage map of the 170K polypeptide. To establish this, 170K polypeptide synthesized in vitro and pulse-labeled either NH_2-terminally or COOH-terminally was partially digested by *S. aureus* V8 protease. The radioactive peptide maps obtained were compared with those from uniformly labeled 110 and 170K polypeptides isolated from bottom-component-infected cowpea protoplasts. Pulse-labeling of the 170K polypeptide synthesized in vitro was performed as described in the legend to Fig. 1, except that longer periods of incubation in the presence of unlabeled methionine were chosen to achieve extreme COOH-terminal labeling (up to 50 min). It has been shown previously that most of the peptides in the proteolytic digest of the 110K polypeptide are present in the proteolytic digest of the 170K polypeptide (11, 21). In Fig. 3, which shows the proteolytic digests of both polypeptides (lanes 170K and 110K), these common peptides are indicated with asterisks, whereas peptides present only in the 170K polypeptide digest and specific for the sequence of the 60K polypeptide are indicated with arrows. Analysis of the proteolytic digests of 170K polypeptides pulse-labeled in their NH_2 termini indicated that the sequence of the 60K polypeptide is located NH_2-terminally (e.g., in Fig. 3, lane N-10). On the other hand, digests of 170K polypeptides pulse-labeled in their COOH termini (e.g., Fig. 3, lane C-50) consisted completely of peptides specific for the 110K polypeptide sequence. Thus, the results of both NH_2-terminal and COOH-terminal pulse-labeling experiments are consistent and indicate that the sequence of the 60K polypeptide is located NH_2-terminally and the sequence of the 110K polypeptide is located COOH-terminally within the 170K polypeptides. Since the relationship of the 110 and 60K polypeptides to all other known B-RNA-encoded polypeptides has been fully elucidated (11, 21) the present mapping studies allow the orientation of the complete B-RNA expression map (Fig. 4). According to this map, the 32K polypeptide is derived from the NH_2-terminal part of the 200K polyprotein. The sequences of

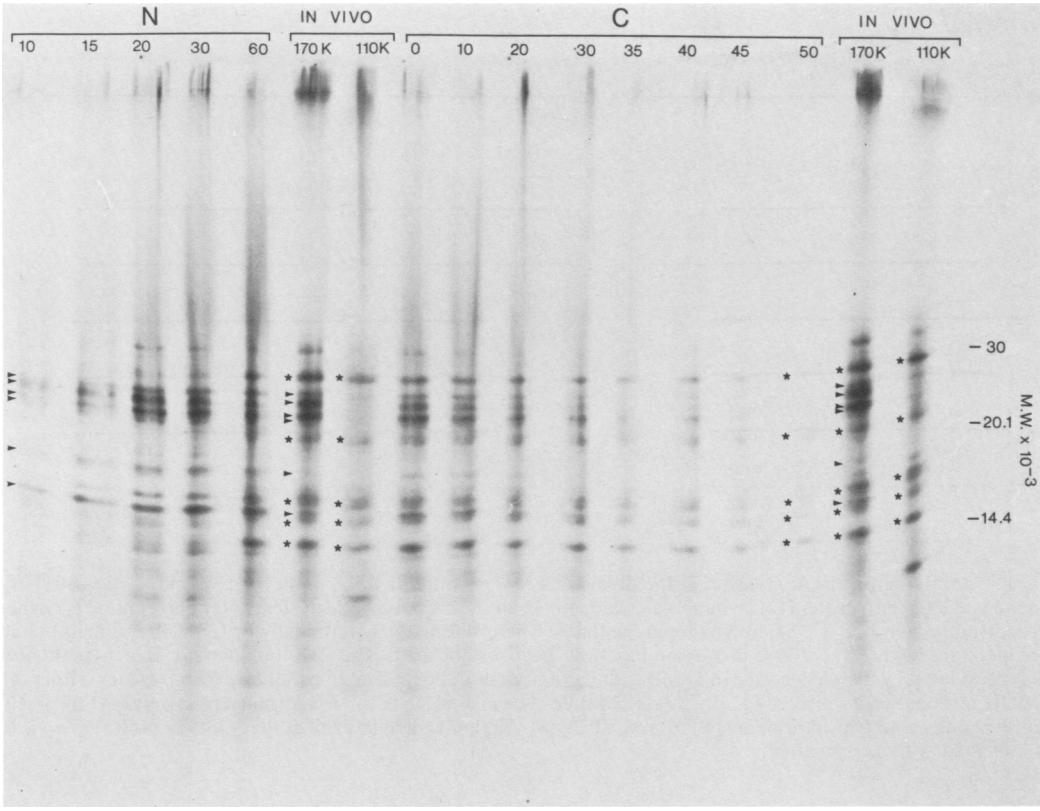

FIG. 3. Proteolytic peptide patterns of pulse-labeled 170K polypeptides. B-RNA was translated in vitro, and the 170K polypeptide was pulse-labeled with [^{35}S]methionine in either its NH_2-terminal or COOH-terminal part. After electrophoresis in a 15% polyacrylamide gel, bands containing the 170K polypeptides were excised from the dried, unstained gel and were swollen in buffer (120 mM Tris-hydrochloride [pH 6.8], 1 mM EDTA, 0.4% sodium dodecyl sulfate) containing 150 μg of *S. aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) per ml. After 1 h of incubation at 30°C, the gel pieces were transferred into slots of a 20% sodium dodecyl sulfate-polyacrylamide gel and were covered with a layer of sample buffer (20 mM Tris-hydrochloride [pH 8.0], 2 mM EDTA, 20% [vol/vol] glycerol, 4% [wt/vol] sodium dodecyl sulfate, 10% [vol/vol] β-mercaptoethanol, 0.004% [wt/vol] bromophenol blue). Electrophoresis was performed at 150 V; radioactively labeled peptides were visualized by autoradiography. Lanes N: Digests of 170K polypeptides obtained by NH_2-terminal labeling of B-RNA-encoded in vitro product for the times (minutes) indicated. Lanes C: Digests of 170K polypeptides obtained by COOH-terminal labeling of B-RNA-encoded in vitro product; radioactivity was added at the times (minutes) indicated. Lanes labeled 170K and 110K contain proteolytic digests of [^{35}S]methionine-labeled 170 and 110K polypeptides, respectively, isolated from bottom-component-inoculated cowpea protoplasts. The numbers at the right refer to the molecular weights ($\times 10^{-3}$) of the marker proteins used (see Fig. 1). Symbols: *, peptides specific for the sequence of the 110K polypeptide; ▶, peptides specific for the sequence of the 60K polypeptide.

the VPg-bearing 84 and 60K polypeptides are contained within the NH_2-terminal part of the 170K polypeptide, whereas the sequences of the 110 and 87K polypeptides reside within the COOH-terminal part of this polypeptide. Using anti-VPg serum, we recently determined (11) that the sequence of VPg resides terminally within the 60K polypeptide and internally within the 84 and 170K polypeptides. Since the 60K polypeptide has now been shown to be derived from the NH_2-terminal part of the 170K polypeptide, VPg must be located in the COOH-terminal end of its 60K precursor, as indicated in Fig. 4.

From previous experiments it has been deduced that CPMV, although having a divided genome, shares some remarkable features with the animal picornaviruses: (i) the capsids of both CPMV and picornaviruses have icosahedral symmetry and contain 60 copies of each of the capsid proteins (9, 26, 30); (ii) their RNAs have a protein (VPg) covalently linked to the 5′ end (4, 7, 17, 28) and a polyadenylate tail at the 3′ end (1, 2, 6, 31); and (iii) translation occurs via

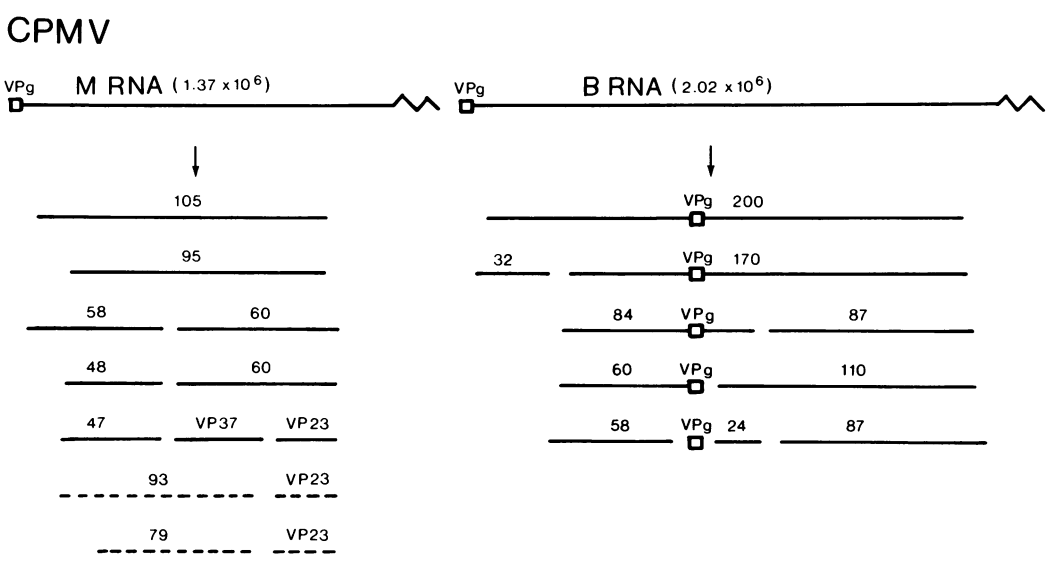

FIG. 4. Model for the expression of the divided CPMV genome. B-RNA (molecular weight, 2.02×10^6 [20]) encodes a 200K polyprotein (21) which is cleaved into 32 and 170K polypeptides. The latter polypeptide is further processed to give 110, 87, 84, and 60K polypeptides. The ordering (NH_2-terminally or COOH-terminally) of all polypeptides within the 200K precursor has now been established. The 24K polypeptide is a hypothetical polypeptide (21) which has not been found with certainty yet. VPg (indicated by an open box) resides within the COOH terminal of its direct 60K precursor. Middle-component RNA (M RNA; molecular weight, 1.37×10^6 [20]) encodes both capsid proteins (VP37 and VP23) (8). The polyadenylate tail at the 3' end of both RNAs (1, 6) is indicated with a zigzag line.

polyproteins which are post-translationally cleaved to generate the functional proteins. Furthermore, the CPMV genome (i.e., B-RNA), like the picornaviral genome, encodes at least one protease involved in the post-translational cleavages (8). However, with regard to translation strategy there are clear differences between CPMV and the picornaviruses. First, it has been shown for picornaviruses that the capsid proteins are derived from the extreme (e.g., poliovirus [5, 14]) or almost extreme (e.g., foot-and-mouth disease virus, encephalomyocarditis virus [3, 13, 25]) NH_2-terminal part of the primary translation product. The capsid proteins of CPMV (VP23 and VP37; see Fig. 4), however, are not derived from the NH_2-terminal part of their precursor but are preceded by large (58 and 48K) polypeptides. Second, picornaviral VPg is contained within the NH_2-terminal end of its precursor (e.g., poliovirus, polypeptide P3-1b [14]), which, in turn, is derived from the COOH-terminal part of the primary translation product (NCVPOO). In contrast, VPg of CPMV is located at the COOH-terminal end of its 60K precursor, as inferred from previous experiments (11, 32) and the mapping studies presented above. Therefore, the genomes of CPMV and picornaviruses appear to differ at least partly in gene order, whereas their primary translation products are processed via different proteolytic pathways.

We thank Jan Verver for the gift of B-RNA, Robert Pels Rijcken for help in initial experiments, Hugh Pelham and Richard Jackson for the gift of rabbit reticulocyte lysate, Pim Zabel for critical reading of the text, Piet Madern for printing the photographs, and Marie-José van Neerven for typing the manuscript.

This work was supported by the Netherlands Foundation for Chemical Research, with financial aid from the Netherlands Organization for the Advancement of Pure Research.

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