Expression of Middle-Component RNA of Cowpea Mosaic Virus: In Vitro Generation of a Precursor to Both Capsid Proteins by a Bottom-Component RNA-Encoded Protease from Infected Cells

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The expression of the middle-component (M) RNA of cowpea mosaic virus was studied by means of in vitro translation. In both the wheat germ extract and the rabbit reticulocyte lysate, M RNA was translated into two overlapping polypeptides of 95 and 105 kilodaltons. Incubation of these polypeptides with $30,000 \times g$ supernatant fractions from cowpea mesophyll protoplasts inoculated with complete virus or with separate bottom (B) components alone resulted in extensive processing, yielding polypeptides of 60, 58, 48, and 47 kilodaltons. Similar proteolytic activity was found associated with the in vitro translation products from the bottom-component RNA, demonstrating that the protease present in infected cells is encoded by B RNA. Using antisera raised against the separate capsid proteins VP23 and VP37, it was shown that the 60-kilodalton cleavage product is the precursor to both capsid proteins. Cleavage of nascent 95- and 105-kilodalton polypeptides by the in vivo protease demonstrated that this capsid protein precursor is located C terminally within both polypeptides and that the synthesis of these two overlapping polypeptides is the result of two initiation sites on middle-component RNA. In addition, a second virus-induced proteolytic activity, capable of releasing VP23 from the 95- and 105-kilodalton polypeptides, was detected in leaves of infected plants, but not in infected mesophyll protoplasts. A model for the expression of the middle-component RNA is presented.

In cowpea mesophyll protoplasts infected with cowpea mosaic virus (CPMV), at least nine virus-coded polypeptides have been detected, with sizes of 170, 110, 87, 84, 60, 37, 32, 23, and 4 kilodaltons (K) (9, 19). The 37K and 23K polypeptides represent the capsid proteins (denoted as VP37 and VP23) and the 4K polypeptide represents the genome-linked protein VPg, attached to the 5' ends of both bottom (B)- and middle (M)-component RNA (4, 27). Upon infection of protoplasts with purified B components, the seven noncapsid polypeptides are still synthesized, but VP37 and VP23 are lacking (9, 19, 26). On the other hand, inoculation with the M component does not result in detectable synthesis of any viral protein. These findings have been explained by the independent replication of B RNA (molecular weight, 2.02×10^6 [18]), a property not associated with M RNA (molecular weight, 1.37×10^6 [18]) (9). Therefore, direct in vivo studies on the expression of B RNA have been possible, and a model has been proposed in which this RNA is translated into a 200K polypeptide which is processed by three different

proteolytic cleavages to produce the 110K, 87K, 84K, 60K, and 32K polypeptides (19). Among these B RNA-encoded polypeptides the 60K polypeptide represents the direct precursor to VPg (P. Zabel, M. Moerman, F. van Straaten, R. Goldbach, and A. van Kammen, manuscript in preparation). As M RNA is not replicated in the absence of B RNA, the expression in vivo of this RNA cannot be studied directly. VP23 and VP37 are not formed in B-infected protoplasts and are therefore probably coded for by M RNA. Larger M RNA-coded (precursor-) polypeptides have not been detected in vivo (9, 19). Therefore, we have studied the expression of M RNA by in vitro translation, using two different cell-free systems, the wheat germ system and the rabbit reticulocyte lysate. In both systems M RNA is translated into two large polypeptides of approximately 95K and 105K with overlapping amino acid sequences (10, 16). Since B RNA is translated properly in these systems, resulting in a 200K polypeptide which is cleaved into 170K and 32K polypeptides also found in CPMV-infected cells (19), the M RNA-encoded 95K or

105K polypeptide, or both, may well represent proper primary translation products, which remain undetectable *in vivo*. Previously, it has been shown by Pelham (16) that either the 170K or the 32K polypeptide translated from B RNA in reticulocyte lysate possesses proteolytic activity which accomplishes cleavage of these two M RNA-encoded *in vitro* products. Here we show that this B RNA-encoded protease is actually produced in CPMV-infected cells and that it cleaves the M RNA-coded 105K and 95K primary translation products *in vitro*, generating a 60K precursor to both capsid proteins VP23 and VP37. Besides, a second virus-specific proteolytic activity, present in leaves of infected plants but not detectable in infected protoplasts, is able to cleave only VP23 from the M RNA-coded *in vitro* products. A model for the expression of M RNA is discussed.

MATERIALS AND METHODS

Virus and RNA. CPMV was grown in cowpea plants (*Vigna unguiculata* L., "California Blackeye"), and B and M components were purified and separated as previously described (9, 12). CPMV RNAs were extracted from separated components as described by Davies et al. (6). Quality and purity of B and M RNA preparations were tested by electrophoresis in 1% agarose gels (8).

Translation in wheat germ extracts. Wheat germ (General Mills Inc., Vallejo, Calif.) was extracted as described by Davies et al. (5). RNA (0.5 μ g) was added to a 15- μ l reaction mixture containing 7.5 μ l of wheat germ extract, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH, pH 7.5, 2.9 mM magnesium acetate, 90 mM potassium acetate, 0.4 mM spermidine-hydrochloride, 2.5 mM ATP, 0.375 mM GTP, 10 mM creatine phosphate, 10 μ g of creatine kinase per ml, 2 mM dithiothreitol, 5 μ g of human placental RNase inhibitor (2, 21) per ml, 25 μ mol of each amino acid (except methionine), and 2 to 5 μ Ci of [35 S]methionine (1,000 to 1,100 Ci/mmol, Radiochemical Centre, Amersham, England). Incubation was for 1 h at 30°C.

Translation in reticulocyte lysates. Translation in an mRNA-dependent rabbit reticulocyte lysate (a generous gift of H. R. B. Pelham and R. J. Jackson, Department of Biochemistry, University of Cambridge, Cambridge, England) was performed as previously described (10, 14, 16). [35 S]methionine (2 to 5 μ Ci per 15- μ l reaction mixture) was used as radioactive amino acid, and incubation was for 1 h at 30°C.

Preparation of subcellular fractions from uninfected and CPMV-infected cells. Extracts were prepared from both uninfected and CPMV-infected cowpea leaves and cowpea mesophyll protoplasts. Extracts from leaves were prepared by the method of Zabel et al. (30, 31) for the preparation of solubilized viral replicase. Portions of 10 to 12 g of freshly harvested primary leaves of 13-day-old cowpea plants (uninfected or infected on day 9) were homogenized at 0°C in 35 ml of homogenization buffer (HB buffer), containing Tris-acetate (pH 7.4), 10 mM potassium acetate, 1 mM

EDTA, 10 mM dithioerythritol, and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was filtered and centrifuged at $1,000 \times g$ for 15 min at 4°C. The supernatant was adjusted to 20% (vol/vol) glycerol and centrifuged at $30,000 \times g$ for 30 min at 4°C. The $30,000 \times g$ pellet was extracted by resuspension in TGKEDP buffer, containing 50 mM Tris-acetate (pH 8.2), 25% (vol/vol) glycerol, 50 mM potassium acetate, 1 mM EDTA, 10 mM dithioerythritol and 0.5 M phenylmethylsulfonyl fluoride (1 ml for each gram of leaf tissue used), and was centrifuged at $30,000 \times g$ for 60 min at 4°C. The resulting $30,000 \times g$ pellet extract was used for the *in vitro* processing experiments and has been stored at -80°C for at least several months without any loss of proteolytic activity. Extracts from protoplasts were prepared as follows. Mesophyll protoplasts were prepared and infected with either complete virus (B + M) or purified B component as previously described (19, 20). Forty hours after infection, portions of 2.5×10^6 protoplasts were collected by centrifugation (2 min, $600 \times g$) and disrupted by homogenization for 5 min in a small Thomas tissue homogenizer with 0.5 ml of HB buffer containing 10% sucrose. Intact cells were removed by centrifugation for 15 min at $1,000 \times g$ and 4°C. The homogenate was then centrifuged for 30 min at $30,000 \times g$ and 4°C to give the $30,000$ supernatant, to be used in the *in vitro* processing experiments. Such preparations have been stored at -80°C for at least several months without any loss of proteolytic activity.

Processing of *in vitro* translation products by subcellular fractions from CPMV-infected cells. One volume of *in vitro* translation products from CPMV M RNA (as obtained after 1 h of translation at 30°C in either wheat germ extract or reticulocyte lysate) was mixed with an equal volume of extract from either CPMV-infected or uninfected cells and incubated for 1 h at 30°C. As a control, a 1:1 (vol/vol) mixture of M RNA *in vitro* products and TGKEDP buffer (see above) was incubated under the same conditions. Processing was followed by electrophoresis of samples in polyacrylamide gels.

SDS-polyacrylamide slab gel electrophoresis. Samples of radiolabeled proteins were mixed with one-third volume of a fourfold concentrated sample buffer (4 $\times$ SB: 40 mM Tris-hydrochloride (pH 8.0), 4 mM EDTA, 40% [vol/vol] glycerol, 8% [wt/vol] sodium dodecyl sulfate [SDS], 20% [vol/vol] β -mercaptoethanol, and 0.004% [wt/vol] bromophenol blue). After heating for 3 min at 100°C, samples were analyzed in polyacrylamide gels containing 12.5% acrylamide (with 0.09% bisacrylamide) as previously described (13), using spacers of 4% acrylamide with 0.10% bisacrylamide. Gels were dried and autoradiographed with Kodak Royal X-Omat film.

Antisera. Antisera against the electrophoretically separated capsid proteins VP23 and VP37 were prepared by directly immunizing rabbits with polyacrylamide containing denatured antigen, in principle as described by Tjian et al. (29) and Schiff and Grandgenett (22) but with some modifications. CPMV particles (1.5 mg) were diluted in 1 $\times$ SB (see above), heated for 3 min at 100°C, and electrophoresed in a 15% polyacrylamide gel (containing 0.10% bisacrylamide). After electrophoresis, capsid proteins were visualized by staining with Coomassie brilliant blue. The separate

VP37 and VP23 bands were excised from the gel and washed successively with 25% isopropanol and 10% methanol. Washed gel slices were then chopped up, lyophilized, and ground in a mortar. The resulting powder was mixed with approximately 1 to 2 ml of 10 mM sodium phosphate (pH 7.2)–0.9% NaCl and emulsified with an equal volume of Freund complete adjuvant (final volume, 2 to 4 ml). The emulsion was injected subcutaneously in the neck region of New Zealand White rabbits; injection was repeated twice, at 4-week intervals and using Freund incomplete adjuvant. Specificity of the anti-VP23 and anti-VP37 sera was tested as described later in the text.

Immunoprecipitation. For immunoprecipitation 5 to 10 μ l of protein sample was adjusted to PBSTDs (10 mM sodium phosphate, pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) in a total volume of 100 μ l and was incubated for 16 h at 4°C in the presence of 5 μ l of preimmune serum, anti-VP23 serum, or anti-VP37 serum. Finally, 25 μ l of a 10% (wt/vol) suspension of *Staphylococcus aureus* cells (IgG-sorb from the Enzyme Center Inc., Boston, Mass.) in PBSTDs (containing 10 mg of bovine serum albumin per ml) was added, and incubation at 4°C was continued for 1 h. Samples were then centrifuged through a sucrose cushion, consisting of a 0.5-ml layer

FIG. 1. Detection of a B RNA-encoded protease activity in extract of CPMV-infected cowpea mesophyll protoplasts. Panel A: M RNA was translated in rabbit reticulocyte lysate, and portions (4 μ l) of the products obtained were added to an equal volume of HB buffer (lane 1) or 30,000 $\times$ g supernatant fraction from protoplasts which were inoculated with either complete virus (lane 2) or B components alone (lane 3) or were uninfected (lane 4). Incubation was continued for 1 h at 30°C, and samples were analyzed in a 12.5% polyacrylamide gel. Panel B: B RNA was translated for 1 h in reticulocyte lysate either in the presence of [35 S]methionine or unlabeled methionine. The products formed were added to [35 S]methionine-labeled in vitro translation products from M RNA, and incubation was continued for 1 h at 30°C. Lane 1, labeled in vitro translation products from B RNA; lane 3, labeled in vitro translation products from M RNA; lane 2, same as lane 3 but mixed with an equal volume of labeled in vitro translation products from B RNA; lane 4, same as lane 3 but mixed with an equal volume of 30,000 $\times$ g supernatant from B-inoculated protoplasts; lanes 5 and 6, same as lane 3 but mixed with 1 (lane 6) or 10 (lane 5) volumes of unlabeled in vitro translation products from B RNA. Lane 7 contains [35 S]methionine-labeled polypeptides from CPMV-infected protoplasts. Samples were analyzed on a 12.5% polyacrylamide gel. Numbers indicated at the left side of the gel refer to the molecular weights ($\times 10^{-3}$) of the in vitro polypeptides; numbers indicated at the right side of the gel refer to the molecular weights ($\times 10^{-3}$) of the viral polypeptides visible in lane 7. The endogenous activity (no RNA added) of the reticulocyte lysate used was undetectable (data not shown).

FIG. 2. Characterization of the antisera against the separate capsid proteins VP23 and VP37. Unlabeled proteins from CPMV-infected protoplasts were separated in a 12.5% SDS-polyacrylamide gel and blotted onto nitrocellulose filters (0.45- μ m pore size) by the method of Bowen et al. (3). The nitrocellulose filters were incubated with preimmune serum (lane 1), anti-VP23 serum (lane 3), or anti-VP37 serum (lane 4), and immunocomplexes were visualized by using 125 I-labeled protein A from *S. aureus* as described in detail elsewhere (P. Zabel, M. Moerman, F. van Straaten, R. Goldbach, and A. van Kammen, manuscript in preparation). To test the fidelity of the protein-blotting technique 35 S-labeled proteins from CPMV-infected protoplasts were included in the original gel (lane 2). The small capsid protein VP23 occurs in two size classes (23K and 22K) (20).

of 1 M sucrose and a 0.25-ml layer of 0.5 M sucrose, both in PBSTDs, at $17,000 \times g$ for 30 min. The precipitate was washed three times in PBSTDs, dissolved in $1 \times$ SB, heated for 3 min at 100°C , and analyzed in polyacrylamide gels.

RESULTS

B RNA-coded protease active in CPMV-infected cells. Pelham (16) has shown that one of the in vitro translation products from B RNA (i.e., either the 170K or the 32K polypeptide) is able to cleave the M RNA-encoded 95K and 105K polypeptides. To verify whether this B RNA-encoded protease occurs in vivo, we prepared $30,000 \times g$ supernatant fractions from both infected and uninfected protoplasts. M RNA in vitro products (as obtained after 1 h of translation) were mixed with these protoplast extracts and incubated at 30°C for another hour. The results shown in Fig. 1A indicate that indeed a proteolytic activity was present in cells infected with complete virus (lane 2) or with B component alone (lane 4), but not in uninfected protoplasts (lane 3). This B component-induced activity was capable of cleaving both 95K and 105K polypeptides into polypeptides of 60K, 58K, and 48K. To test whether this activity was identical to the proteolytic activity of B RNA in vitro products previously described (16), the 95K and 105K polypeptides were mixed with B RNA in vitro products. As in vitro translation of isolated B RNA yielded, along with the 200K, 170K, and 32K polypeptides, a large number of minor products (Fig. 1B, lanes 1 and 2) (16, 17) which obscured the emergence of new polypeptide bands in acrylamide gels, B RNA was translated in an unlabeled reaction. The unlabeled products were mixed with labeled M RNA products, and the mixture was incubated for 1 h at 30°C . Significant processing occurred only upon addition of 10 volumes of unlabeled B RNA products to 1 volume of labeled M RNA products (cf. lanes 5 and 6 in Fig. 1B), resulting in the same cleavage products (60K, 58K, 48K, and sometimes 47K) as obtained with extract of B-infected protoplasts (cf. lanes 4 and 5 in Fig. 1B). This experiment proves that the protease present in infected cells is indeed coded for by B RNA. Additional support for this conclusion comes from the fact that the in vivo and in vitro protease activities were both sensitive to the same inhibitors: ZnCl_2 , *N*-ethylmaleimide, and chymostatin (results not shown).

Characterization of the cleavage products. As the capsid proteins VP23 and VP37 are absent in protoplasts inoculated with only B components, suggesting that they are coded for by M RNA (9), the in vitro cleavage products were analyzed by using antisera raised against the separate capsid proteins. To test the specificity of the antisera used in these experiments, the anti-VP23 and anti-VP37 sera were incubated with proteins from virus-infected cells which were separated in a 12.5% SDS-polyacrylamide gel and subsequently blotted onto a nitrocellulose

FIG. 3. In vitro processing and immunoprecipitation of translational products from CPMV M RNA. M RNA was translated for 1 h in either reticulocyte lysate (lanes 3–7) or wheat germ extract (lanes 8–12). Portions (2 μ l) of the translation products were mixed with an equal volume of either HB buffer (lanes 7 and 8), 30,000 $\times$ g supernatant fraction from B-inoculated protoplasts (lanes 5, 6, 9, and 10), or unfractionated homogenate of M + B-inoculated protoplasts (lanes 3, 4, 11, and 12). Incubations 4 and 11 included unfractionated CPMV RNA (250 μ g/ml). All mixtures were incubated for 1 h at 30°C, and samples 5 and 10 were immunoprecipitated with anti-VP23 serum. Molecular weight markers included 35 S-labeled polypeptides from CPMV-infected protoplasts (lane 1) and (in lane 2) 14 C-methylated myosin (210,000), phosphorylase *b* (100,000 and 92,500), bovine serum albumin (68,000), ovalbumin (46,000), and carbonic anhydrase (30,000).

filter (as described by Bowen et al. [3]). Of these polypeptides only VP23 reacted with anti-VP23 serum and only VP37 reacted with anti-VP37 serum, without any cross-reaction (Fig. 2). Of the processed M RNA in vitro products the only polypeptide precipitating with anti-VP23 serum was the 60K polypeptide, except for some minor polypeptides, which were also present in untreated M RNA product (Fig. 3, lanes 5 and 10).

The 58K and 48K cleavage products did not react with anti-VP23 serum. The 60K polypeptide was also the only cleavage product precipitated by anti-VP37 serum (Fig. 4, lane 1). The results shown in Fig. 3 and 4 demonstrate conclusively that M RNA encodes both capsid proteins. In view of its size, it is attractive to propose the 60K polypeptide as the common precursor to VP23 and VP37. Since the 95K and

FIG. 4. Immunoprecipitation of CPMV M RNA translational products. M RNA was translated in reticulocyte lysate, and the products obtained were incubated for 1 h at 30°C with either HB buffer (lanes 4–6) or 30,000 $\times$ g supernatant fraction from B-inoculated protoplasts (lanes 1–3). Samples 1 and 6 were immunoprecipitated with anti-VP37 serum; samples 2 and 5, with anti-VP23 serum. Analysis was on a 12.5% polyacrylamide gel.

105K polypeptides were both efficiently precipitated by at least the anti-VP23 serum (Fig. 3, lane 5, and Fig. 4, lanes 2 and 5), the 60K polypeptide must originate from both these primary products. The size difference between the 95K and 105K polypeptides should therefore correlate with the difference in size between the cleavage products of 58K and 48K. A reason that anti-VP37 serum reacted only weakly with 95K and 105K may be the considerably lower titer of this antiserum compared with that of the

anti-VP23 serum used, and possibly the internal location of the VP37-sequence within both 95K and 105K (see below).

Mapping of the cleavage products. To map the cleavage products more precisely within 95K and 105K, M RNA was translated in a wheat germ extract, and samples were taken at intervals of 10 min and processed by addition of 30,000 $\times$ g supernatant of B-inoculated protoplasts. Full-length (95K and 105K) products were detectable only after 30 min (Fig. 5). Processing of the 20-min sample, exclusively containing incomplete primary products, revealed that the 48K and 58K sequences were synthesized first (and simultaneously) and are therefore located N terminally within both the 95K and 105K polypeptides, respectively (Fig. 5). The 60K capsid protein precursor clearly appeared last, when full-size 95K and 105K polypeptides were produced, indicating that its sequence is located C terminally within both 105K and 95K polypeptides. This finding implies that the full-size 95K and 105K polypeptides differ in their N termini and confirms that there are two initiation sites on M RNA *in vitro*. Whether two different initiation sites on M RNA are actually used *in vivo* or are just the result of artificial events in the cell-free systems remains to be answered, however.

Attempts to cleave the 60K polypeptide into mature capsid proteins. The finding that a B RNA-coded protease present *in vivo* is capable of specifically releasing a 60K capsid protein precursor from M RNA *in vitro* products strongly suggests that the *in vitro* studies reflect the situation within the infected cell. The question arises then of why the 60K precursor was not further processed into the mature capsid proteins. A possibility might be that this final cleavage is generated by a second protease which is membrane bound and therefore absent from the 30,000 $\times$ g supernatant fraction of CPMV-infected protoplasts. Unfractionated homogenate of protoplasts infected with complete virus (M + B) displayed only the same proteolytic activity as was found in the 30,000 $\times$ g supernatant fraction, however (Fig. 3, lanes 3 and 12). A second possibility is that the 60K polypeptides have to be assembled into a procapsid structure and that the presence of unpackaged viral RNA is necessary for maturation of this procapsid. Addition of excess viral RNA to a mixture of M RNA *in vitro* product and unfractionated homogenate of CPMV-infected protoplasts did not show any effect (Fig. 3, lanes 4 and 11). On the other hand, when M RNA products were incubated with a membrane protein fraction (the so-called 30,000 $\times$ g pellet extract; for details, see Materials and Methods) prepared from intact leaves of infected plants, 93K, 79K, and 23K

FIG. 5. Time course of appearance of in vitro translation products. CPMV M RNA was translated in wheat germ extract. Samples were removed from the translation mixture at the times indicated and were either treated with an equal volume of $30,000 \times g$ supernatant fraction from B-inoculated protoplasts (+) or left untreated (-); they were analyzed on a 12.5% polyacrylamide gel.

polypeptides were generated, in addition to the 60K, 58K, and 48K products (Fig. 6, lanes 4 and 5). Extracts from uninfected leaves did not contain this proteolytic activity (Fig. 6, lane 2). The 23K cleavage product comigrated with the small capsid protein VP23 (cf. lanes 3–5 in Fig. 6) and precipitated with anti-VP23 serum (result not shown). Since VP37 was not produced by this leaf extract, the 95K and 105K polypeptides were apparently cleaved into a polypeptide of 79K and VP23 and into a polypeptide of 93K and VP23, respectively, by a proteolytic activity recognizing the cleavage site between the VP23 and VP37 sequences. This activity seemed to be independent from the activity present in infected protoplasts, as incubation of the 95K and 105K polypeptides in a 1:1 (vol/vol) mixture of leaf and protoplast extracts did not result in further cleavage of the 60K polypeptide (data not shown). The release of VP23 and not of VP37 from 95K and 105K polypeptides enables us to map both capsid proteins within the 60K precursor, as summarized in the cleavage model of Fig. 7.

DISCUSSION

We have previously found (9, 19) that in cowpea protoplasts B RNA is replicated inde-

pendently, i.e., in the absence of M RNA, allowing in vivo studies on the expression of this RNA. On the other hand, M RNA is not replicated independently from B RNA. Therefore, a direct study of the expression of M RNA in vivo has been impossible. The results presented in this paper show that the translation strategy and coding function of M RNA can be studied by supplying extracts of CPMV-infected plant cells to the M RNA-encoded 95K and 105K polypeptides synthesized in cell-free systems. It has been demonstrated now that a B RNA-coded protease present in both infected leaves and infected mesophyll protoplasts is capable of cleaving the M RNA in vitro translation products, generating a 60K precursor to both capsid proteins VP23 and VP37. Complementation studies using particles from different CPMV isolates have suggested that at least one of the capsid proteins is coded for by M RNA (11, 28). We have now definitely established that M RNA encodes both VP23 and VP37. The cleavage map shown in Fig. 7 summarizes the results described in this paper. The model proposes that M RNA is translated in vitro into two overlapping polypeptides of 95K and 105K which are cleaved into pieces of 60K, 58K, 48K, and 47K by a protease translated from B RNA in vivo. Of

FIG. 6. In vitro processing of CPMV M RNA translational products using extracts from CPMV-infected cowpea leaves. M RNA was translated in wheat germ extract, and portions (4 μ l) of the products obtained were mixed with an equal volume of either TGKEDP buffer (sample 1) or 30,000 $\times$ g pellet extract (for details, see Materials and Methods) from CPMV-infected (samples 4 and 5) or uninfected (sample 2) cowpea leaves. They were incubated for 1 h at 30°C. Samples 4 and 5 were treated with different leaf extracts. Products were analyzed in a 12.5% polyacrylamide gel. Lane 3 contains [35 S]methionine-labeled polypeptides from CPMV-infected protoplasts. The numbers indicated at the right side of the gel refer to the molecular weights ($\times 10^{-3}$) of the (processed) in vitro products.

these cleavage products the 60K polypeptide represents the direct precursor to VP23 and VP37, whereas the function of the other products is as yet unknown. Although a second virus-specific protease activity has been detected in extracts from infected leaves, capable of

generating cleavage between the VP23 and VP37 sequence within 95K and 105K, the conditions necessary for final processing of the 60K precursor are not yet understood and are the subject of further investigation. Neither mixing of leaf and protoplast extract nor addition of excess viral RNA resulted in cleavage of the 60K polypeptide. A possibility is that the 60K polypeptides should be arranged in a specific procapsid structure to allow final cleavage and that this assembly does not occur in vitro. A similar phenomenon has been described for the assembly and maturation of picornaviruses (for review, see 23). After the viral RNA has been packaged, the final maturation step in poliovirus (and encephalomyocarditis [EMC] virus) particles is cleavage of VP0 (EMC virus: ϵ) into the mature capsid proteins VP2 and VP4 (EMC virus: $\epsilon \rightarrow \beta + \delta$).

CPMV

FIG. 7. Model for the expression of CPMV M RNA. In cell-free protein-synthesizing systems M RNA is translated into two overlapping polypeptides of 95 and 105K, which differ in their N termini as a result of two active initiation sites on M RNA in vitro. Both polypeptides are cleaved by a B RNA-encoded protease present in infected cells, resulting in polypeptides of 60, 58, 48, and 47K. The 60K polypeptide represents a precursor to both capsid proteins VP23 and VP37. As this precursor is generated by a viral protease from infected cells, it is proposed that the model presented reflects the translation strategy of M RNA in vivo. The dotted lines represent the products generated by a second virus-induced protease detectable in infected leaves but not in infected protoplasts. The genome-linked protein VPg at the 5'-end of both CPMV RNAs (4, 27) has been indicated with a box, and the polyadenylate tail at the 3' end (1, 7) has been indicated with a zigzag line. It is not known whether translation of CPMV RNA is preceded by removal of VPg.

In reticulocyte lysates polioviral (and EMC viral) RNA is translated into a large polypeptide which is then processed extensively (15, 24, 25). One of the products is VPO (EMC virus: ϵ), which is not processed further, however, indicating that final assembly of virions also has been impeded.

The experiments presented in this paper confirm the observation of Pelham (16) that in vitro M RNA contains two active initiation sites. The overlapping 95K and 105K polypeptides are synthesized in two different cell-free systems (Fig. 3) (10), but it is not known whether they are both produced in vivo. The production of polypeptides with completely overlapping amino acid sequences does not appear useful and might be the result of the artificial conditions in cell-free extracts. In view of the faithful translation of B RNA in both in vitro translation systems (10, 19) and the generation of a specific 60K capsid protein precursor upon cleavage by an in vivo B RNA-coded protease, at least one of the M RNA in vitro products probably represents a correct primary translation product even if it has not been detected in vivo. The use of in vitro protein-synthesizing systems, in combination with well-defined subcellular fractions from infected cells, may provide the means for studying the remaining coding functions of M RNA and the mechanism of CPMV assembly.

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