Expression of the Bottom-Component RNA of Cowpea Mosaic Virus: Evidence that the 60-Kilodalton VPg Precursor Is Cleaved into Single VPg and a 58-Kilodalton Polypeptide

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In cowpea protoplasts infected with cowpea mosaic virus, a bottom-component (B) RNA-encoded 60-kilodalton (60K) polypeptide is synthesized, which is membrane-bound and represents the direct precursor to the genome-bound protein VPg. The relationship between this VPg precursor and other B-RNA-encoded polypeptides was studied. Digestion of the B-RNA-encoded 170K and 84K polypeptides with *Staphylococcus aureus* protease V8 and subsequent analysis of the generated peptides with antiserum against VPg showed that a VPg sequence resides internally in these polypeptides. Furthermore, a new B-RNA-encoded polypeptide was detected, with a size of 58K, which differed from the 60K polypeptide only in the lack of VPg sequences. A model is presented in which the 60K VPg precursor is generated from the 200K primary translation product from B RNA and further processed to a 58K polypeptide and single VPg.

In cowpea mesophyll protoplasts, bottom-component (B) RNA of cowpea mosaic virus (CPMV) is able to replicate independently from middle-component (M) RNA, a property which has greatly facilitated direct studies on its expression in vivo (5). When protoplasts are inoculated with purified B components and incubated in the presence of [³⁵S]methionine, at least seven virus-induced polypeptides have been detected, with sizes of 170, 110, 87, 84, 60, 32, and 4 kilodaltons (13, 15). The 4-kilodalton (4K) polypeptide represents the genome-linked protein VPg, which is found attached to the 5' ends of both B and M RNA (2, 16). Comparison of the proteolytic peptide patterns of the proteins found in vivo with those of the polypeptides translated from B RNA in vitro has revealed that the 170K, 110K, 87K, 84K, 32K, and possibly 60K polypeptides are all coded for by B RNA and that they are probably derived from a 200K precursor polyprotein by three successive cleavages (13). The proteolytic peptide pattern of the 60K polypeptide, a polypeptide found predominantly in the membrane fraction of infected protoplasts, showed insufficient similarity with any of the other B-component-induced polypeptides to establish its genetic origin with certainty. Using Western blots of proteins from CPMV-infected cells, Zabel et al. (18) have recently detected a B-component-induced polypeptide of approximately 60 kilodaltons which reacts specifically with antiserum raised against VPg. Since VPg is coded for by B RNA (15), it has been proposed that the immunoreactive 60K

polypeptide is a B-RNA-encoded precursor to VPg. The only other polypeptide from CPMV-infected cells able to react with anti-VPg serum appeared to be the B-RNA-encoded 170K polypeptide, which showed only a very weak reaction, however (18). We have now further investigated the relationship between this 60K VPg precursor and the B RNA-encoded 170K, 110K, and 84K polypeptides. Furthermore, we have tested whether the previously described B-component-induced 60K polypeptide, detected upon labeling with [³⁵S]methionine (13), is identical to the equal-sized VPg precursor. The analyses are based on limited proteolytic digestion of the polypeptides (using *Staphylococcus aureus* V8 protease) and treatment of the generated peptides with anti-VPg serum to screen for the presence of VPg sequences. Furthermore, the experiments presented reveal the presence of a 58K polypeptide in B-component-inoculated protoplasts, which is closely related to the 60K VPg precursor but which does not react with anti-VPg serum. A refined model for the expression of B RNA is presented.

MATERIALS AND METHODS

Virus purification and separation of M and B components. CPMV (yellow strain) was grown in *Vigna unguiculata* L. (California Blackeye) and purified as previously described (8, 17). B and M components were separated by three cycles of centrifugation in a linear 15 to 30% (wt/vol) zonal sucrose gradient (13).

Incubation of protoplasts and labeling of proteins. Cowpea mesophyll protoplasts were prepared, inocu-

lated with purified B components, and incubated in culture medium, as previously described in detail (6, 13). For labeling of proteins, portions of 150 μCi of [^{35}S]methionine (New England Nuclear Corp.; 1,100 Ci/mmol) were added to protoplast suspensions (5 ml, 5×10^5 cells/ml) 18 and 26 h after infection. Forty-four hours after infection, protoplasts were collected by centrifugation (2 min, $600 \times g$) and directly fractionated.

Subcellular fractionation of protoplasts. Protoplast samples (2.5×10^6 cells) were disrupted by homogenization for 1 min at 0°C in a small Thomas tissue homogenizer with 0.5 ml of homogenization buffer containing 50 mM Tris-acetate (pH 7.4), 10 mM potassium acetate, 1 mM EDTA, 10 mM dithioerythritol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% (wt/vol) sucrose. Cell debris was removed by low-speed centrifugation (30 s, $500 \times g$, 4°C). The homogenate was centrifuged for 30 min at $30,000 \times g$ and 4°C to give the $30,000 \times g$ supernatant fraction. The $30,000 \times g$ pellet was frozen and, after thawing, extracted twice with 0.1 ml of homogenization buffer containing 0.2% digitonin (13, 14). The suspension was centrifuged for 30 min at $30,000 \times g$ and 4°C, and the resulting supernatant, containing solubilized membrane-bound proteins, is referred to as the $30,000 \times g$ pellet extract.

Immunoprecipitations. Samples of $30,000 \times g$ pellet extracts from [^{35}S]methionine-labeled protoplasts were incubated with anti-VPg serum (18) as previously described in detail (4). In brief, 30 μl of protein sample was adjusted to 300 μl of PBSTDS (10 mM sodium phosphate, pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]), and incubated for 16 h at 4°C in the presence of 10 μl of either preimmune serum or anti-VPg serum. Immunoprecipitates were collected by addition of a suspension of Sepharose-bound protein A from *S. aureus* (Pharmacia) and centrifugation at $17,000 \times g$ for 3 min. Immunoprecipitates were washed three times in PBSTDS and suspended in 120 mM Tris-hydrochloride (pH 6.8), 1 mM EDTA, and 0.5% SDS (for limited proteolytic digestion) or in sample buffer (for analysis in a polyacrylamide gel).

SDS-polyacrylamide slab gel electrophoresis. Labeled protein fractions were mixed with one-third volume of a fourfold-concentrated solution of sample buffer (13), heated for 3 min at 100°C, and analyzed in 12.5 or 20% polyacrylamide gels (containing 0.09 or 0.07% bisacrylamide, respectively) as previously described (9), using spacers of 4% acrylamide with 0.10% bisacrylamide.

Isolation of polypeptides from polyacrylamide gels. Polypeptides, localized by autoradiography, were eluted from dried, unstained gels as described before (13).

Peptide mapping. Polypeptides, diluted in 120 mM Tris-hydrochloride (pH 6.8), 1 mM EDTA, and 0.5% SDS, were digested with *S. aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) at 30°C for 30 min with various enzyme concentrations as indicated in the figure legends. Peptide patterns were compared in 20% polyacrylamide gels containig 0.07% bisacrylamide.

RESULTS

The previously detected B-component-induced 60K polypeptide represents the VPg precursor. When B-component-inoculated protoplasts are

labeled with [^{35}S]methionine, a 60K polypeptide can be detected which is apparently tightly bound to membranes as it is mainly found in extracts of the $30,000 \times g$ pellet fraction (13, 14). To investigate whether this ^{35}S-labeled polypeptide is identical to the equal-sized VPg precursor, which has been recently detected by Western blotting using anti-VPg serum (18), the $30,000 \times g$ pellet extract was treated with anti-VPg serum, and the immunoprecipitate was analyzed in a polyacrylamide gel (Fig. 1). Of all ^{35}S-labeled polypeptides present in this fraction, including the B RNA-induced 170K, 110K, 87K,
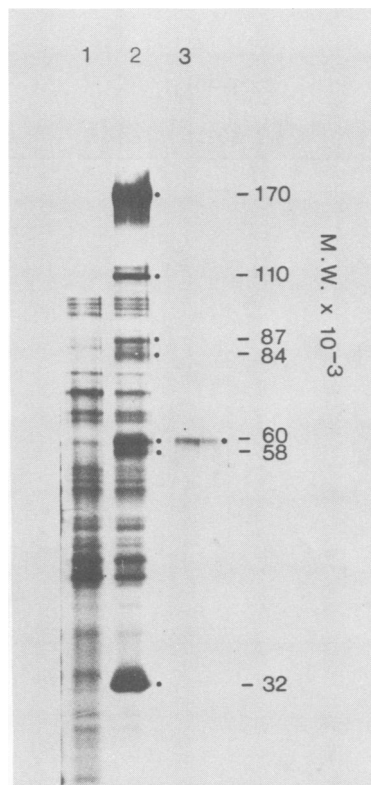

FIG. 1. Immunoprecipitation with anti-VPg serum of [^{35}S]methionine-labeled proteins from B-component-inoculated cowpea protoplasts. Protoplasts were either inoculated with B components or mock-inoculated and incubated for 18 to 44 h postinfection in the presence of [^{35}S]methionine (for details, see Materials and Methods). Protein fractions were analyzed in a 12.5% polyacrylamide gel. Lane 1, Solubilized membrane proteins ($30,000 \times g$ pellet extract) from mock-inoculated protoplasts; lane 2, solubilized membrane proteins from B-component-inoculated protoplasts; lane 3, immunoprecipitate with anti-VPg serum of solubilized membrane proteins from B-component-inoculated protoplasts. The numbers indicated at the right side of the gel refer to the molecular weights ($\times 10^{-3}$) of the B-component-induced polypeptides.

84K, 60K, and 32K polypeptides, only a 60K polypeptide appeared to precipitate with anti-VPg serum (Fig. 1, lanes 2 and 3). To test whether this polypeptide was similar to the 60K polypeptide detected in the membrane fraction (lane 2), both 60K polypeptide species were subjected to partial proteolysis, using *S. aureus* V8 protease. The resulting digestion patterns were identical (Fig. 2), indicating that the ^{35}S-labeled 60K polypeptide detectable in B-component-inoculated cells indeed represented the VPg precursor described elsewhere (18). Since VPg is coded for by B RNA (15), this 60K polypeptide must therefore also be encoded by this RNA.
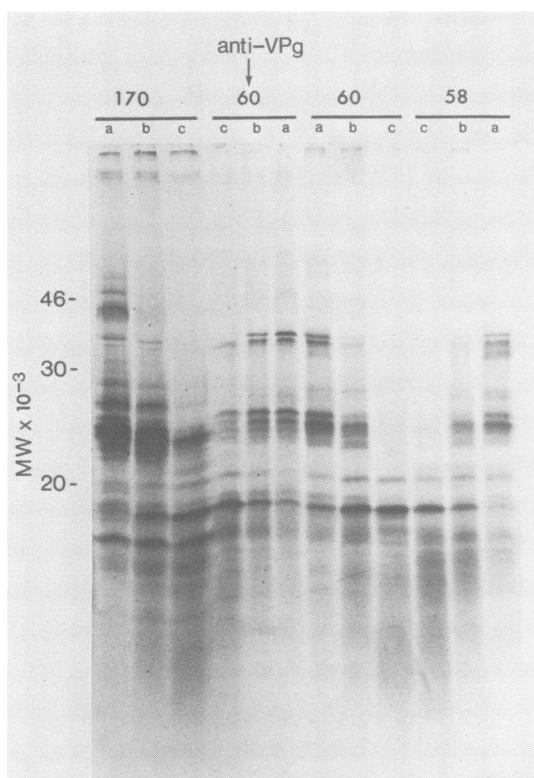

FIG. 2. Proteolytic peptide patterns of virus-specific polypeptides in a 20% polyacrylamide gel. The 170K, 60K, and 58K polypeptides were eluted from an unstained 12.5% polyacrylamide gel containing [35S]methionine-labeled proteins from the 30,000 × g pellet extract of B-component-inoculated protoplasts. The immunoreactive 60K polypeptide was obtained from the same extract by precipitation with anti-VPg serum (lanes indicated anti-VPg). The isolated polypeptides were digested with 30 (a), 150 (b), or 750 (c) μg of V8 protease from *S. aureus* per ml. The numbers indicated at the left side of the gel refer to the molecular weights (×10⁻³) of the marker proteins used (ovalbumin, 46,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000).

Detection of a B-RNA-encoded 58K polypeptide. Closer analysis of the polypeptide patterns in Fig. 1 revealed the existence of a B-component-induced polypeptide of approximately 58K, which is absent in noninfected protoplasts (cf. lanes 1 and 2 in Fig. 1) and has not been detected before. This polypeptide appears to be mainly present in the membrane fraction (30,000 × g pellet extract) and is not precipitable with anti-VPg serum. To elucidate a possible relationship between the 60K and 58K polypeptides, the 58K polypeptide, eluted from an unstained gel, was treated with *S. aureus* V8 protease, and the electrophoretic digestion pattern was compared with that of the 60K polypeptide (Fig. 2). The almost complete similarity in digestion pattern (Fig. 2) indicated that the 58K and 60K polypeptides had fully overlapping amino acid sequences. The small difference in size (~2 kilodaltons) and the lack of VPg (molecular weight, 4,000 [2, 18]) sequences in the 58K polypeptide (Fig. 1) indicated that the VPg sequence was terminally located within the 60K polypeptide.

Relationship between the 170K, 60K, and 58K polypeptides. Since VPg is encoded by B RNA (15) and a VPg sequence is present within the 60K polypeptide, both 60K and 58K polypeptides must be encoded by B RNA. According to our translation model for B RNA (13), the 170K polypeptide is cleaved to give the 60K and 110K polypeptides, or, alternatively, it is cleaved to give 84K and 87K polypeptides. If this model holds, then the 170K and 84K polypeptides should contain the VPg sequence, but the 110K and 87K polypeptides should not. Intact 170K and 84K polypeptides did not significantly precipitate with anti-VPg serum (Fig. 1). This may have been due to an internal position of the VPg sequence within these polypeptides. To screen for the presence or absence of VPg sequences, [35S] methionine-labeled 170K, 110K, 84K, 60K, and 58K polypeptides, eluted from unstained gels, were therefore first partially digested with *S. aureus* V8 protease before treatment with anti-VPg serum. Indeed, the digests of the 170K and the 60K polypeptides both contained peptides which specifically precipitated with anti-VPg serum (Fig. 3A). On the other hand, as expected, the digests of the 110K and the 58K polypeptides (Fig. 3A), as well as the digest of the 87K polypeptide (data not shown), failed to react with anti-VPg serum. This experiment demonstrates that the 60K polypeptide indeed must be processed from the B RNA-encoded 170K polypeptide, a conclusion which could not be drawn with certainty from our previous peptide mapping experiments (13). Since the 84K polypeptide also contained VPg sequences and even shared its immunoreactive proteolytic peptides with the 170K polypeptide (Fig. 3B), the
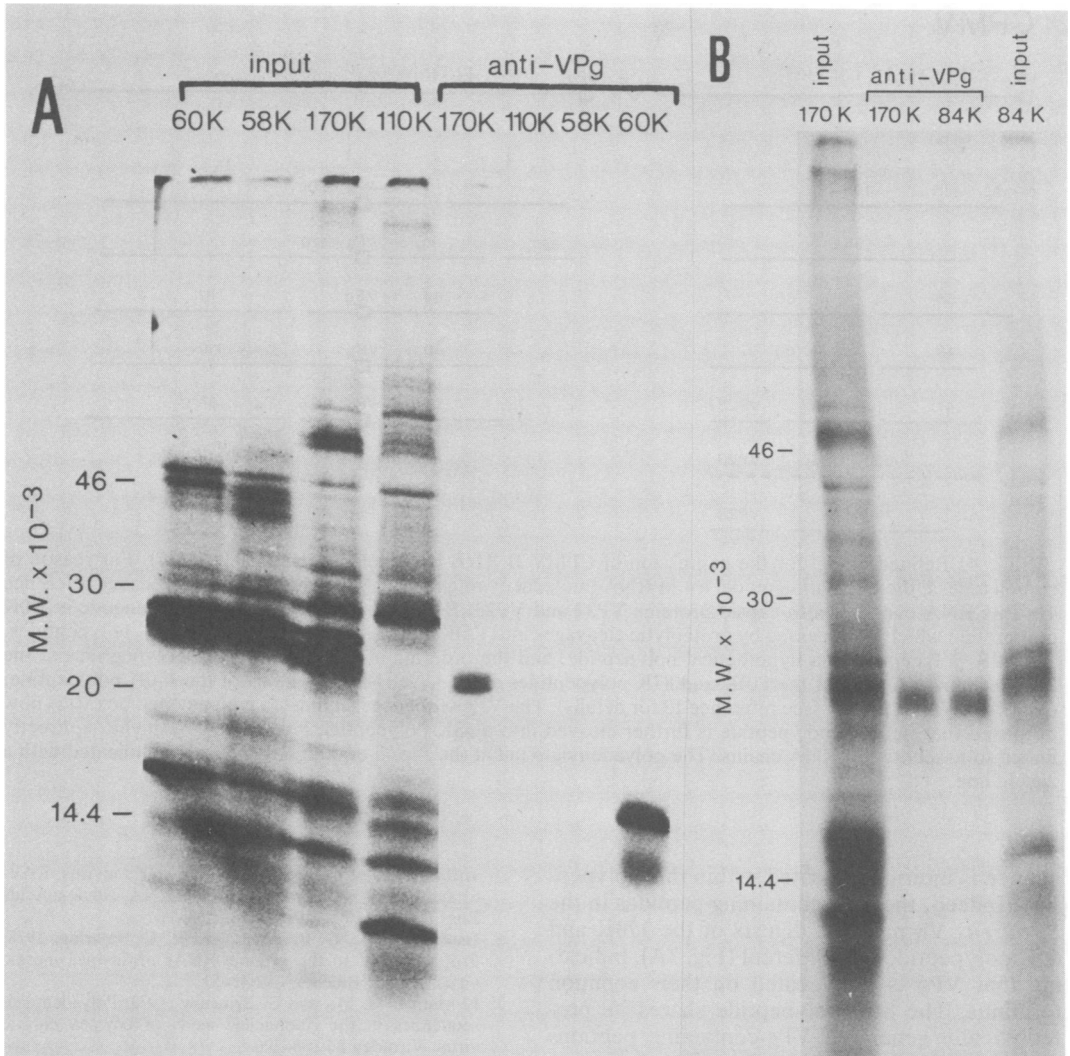
FIG. 3. Detection of VPg sequences in virus-specific polypeptides. [^{35}S]methionine-labeled polypeptides were digested with 150 μg of *S. aureus* V8 protease per ml, and the generated peptides either were directly electrophoresed in a 20% polyacrylamide gel ("input") or were first immunoprecipitated with anti-VPg serum ("anti-VPg"). (A) Peptide patterns of the 170K, 110K, 60K, and 58K polypeptides; (B) peptide patterns of the 170K and 84K polypeptides. The numbers indicated at the left side of both gels refer to the molecular weights (×10^{-3}) of the marker proteins used.

previously supposed translation model for B RNA (13) has now been fully confirmed.

DISCUSSION

We have previously shown that in CPMV-infected cells, a VPg precursor of 60 kilodaltons is synthesized (18). The experiments described here unequivocally demonstrate that this precursor is coded for by B RNA and that it is derived from the 170K polypeptide. Moreover, a 58K polypeptide has been described which probably only differs from this 60K polypeptide by the lack of VPg sequences. Since the molecular weight of VPg is approximately 4,000 (2, 18), it is tempting to assume that the VPg sequence resides terminally within the 60K polypeptide and that this sequence is cleaved off to give the 58K polypeptide and VPg, as shown in the extended translation model for B RNA in Fig. 4. As no free VPg has been detected in CPMV-infected cells (18), this polypeptide is probably directly linked to nascent RNA chains. The weak reaction of the 170K polypeptide with the anti-VPg serum (Fig. 1; [18]) suggests that the VPg se-
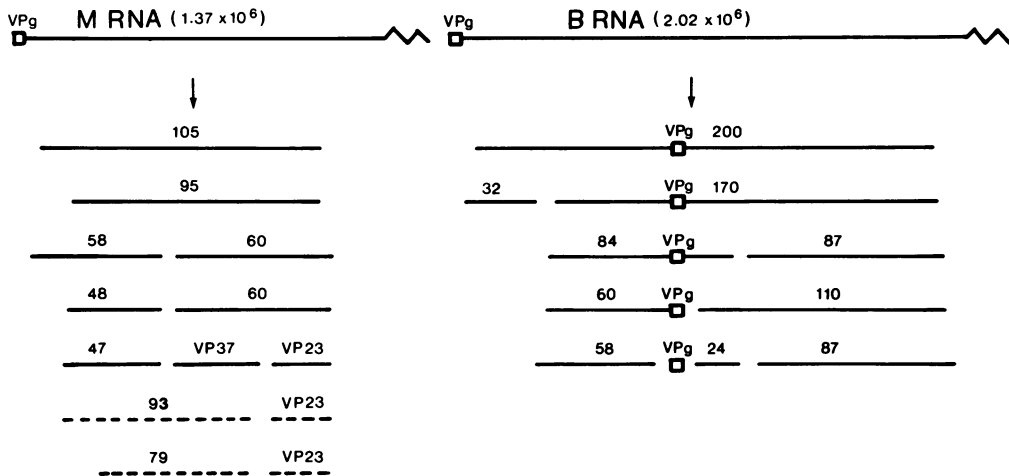
CPMV

FIG. 4. Refined model for the expression of CPMV B RNA (molecular weight, 2.02×10^6). For reason of completeness, the translation model for M RNA (molecular weight, 1.37×10^6) has been included (see reference 4). This RNA encodes both capsid proteins VP37 and VP23. B RNA has been shown (13) to encode a 200K polyprotein which is processed by proteolytic cleavages into 170K, 110K, 87K, 84K, 60K, and 32K polypeptides. The 24K polypeptide is a hypothetical polypeptide, and the ordering of the 84K and 60K polypeptides at the NH_2-terminal part and of the 110K and 87K polypeptides at the COOH-terminal part of the 170K polypeptide, respectively, is arbitrary (see reference 13 for details). The VPg sequence is indicated with an open box. It is now proposed that the 60K polypeptide is further cleaved into a 58K polypeptide and VPg. VPg might be directly linked to nascent viral RNA chains. The polyadenylate tail at the 3' end of both RNAs (1, 3) is indicated with a zigzag line.

quence is internally located within this polypeptide. Indeed, the VPg-containing peptides in the *S. aureus* V8 protease digests of the 170K and 60K polypeptides are different (Fig. 3A), indicating that VPg is not located on their common terminus. The 84K polypeptide shares its proteolytically generated VPg-containing peptides with the 170K polypeptide (Fig. 3B), confirming its previously assigned (13) position within the processing scheme. For picornaviruses, VPg has been demonstrated to be contained in the direct replicase precursor, suggesting that it plays an essential role in viral RNA replication (7, 10–12). If the same holds for CPMV, then the 58K polypeptide or a further cleavage product should possess RNA polymerase activity. This hypothesis is currently under investigation.

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