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Antibodies Against the Genome-Linked Protein VPg of Cowpea Mosaic Virus Recognize a 60,000-Dalton Precursor Polypeptide

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We have prepared a rabbit antiserum specifically directed against the genome-linked protein (VPg) of cowpea mosaic virus by injecting an hydrolysate of purified virion RNA. Using this antiserum as a probe in combination with "Western" (protein) blots of subcellular fractions of cowpea mosaic virus-infected cowpea (*Vigna unguiculata*) cells, we have detected a bottom component RNA-encoded, 60,000-dalton polypeptide which is membrane bound and presumably represents the immediate precursor of VPg.

Since the discovery of a small protein (VPg) covalently linked to the 5′ end of the genome RNA of poliovirus (8, 17), several other animal viruses (5, 13, 14, 29) and plant RNA viruses (6, 7, 10, 18, 32, 33) with a genome-linked protein have been recognized. For picornaviruses, VPg has been proposed to act as a primer during initiation of viral RNA synthesis as soon as it is generated by proteolytic cleavage from a larger viral precursor protein (8, 15, 20–22, 24). However, a direct and obligatory role of VPg in viral replication remains to be proven. The genome of cowpea mosaic virus (CPMV) is composed of two single-stranded, plus-type RNA molecules with molecular masses of 2.4×10^6 daltons (2,400K) (B RNA) and 1,400K (M RNA), respectively, which are separately encapsidated (3, 19, 37) and which both possess a 4K to 5K protein (VPg) at the 5′ end (6, 7, 32, 33). Recent studies on the distribution of genetic functions between the two genome segments have shown that B RNA is capable of self-replication and codes for VPg and that M RNA requires expression of B RNA to be replicated and codes for the capsid proteins (11, 26, 28, 31). As antibodies which specifically recognize VPg sequences would greatly facilitate a functional analysis of VPg in viral replication, we have prepared a rabbit anti-VPg serum.

A New Zealand white rabbit was immunized by injecting (at an interval of 5 weeks) successively 15 and 45 mg of purified virion RNA which had been hydrolyzed in alkali to release VPg. Assuming an approximate molecular mass of 4K to 5K for VPg (7), this corresponds to the injection of approximately 30 and 90 μg of VPg, respectively.

Preliminary screening revealed that the antiserum thus obtained was reactive against VPg (see below), but in addition contained antibodies against both capsid proteins, VP37 and VP23. Apparently, spurious amounts of highly antigenic capsid proteins were tightly (noncovalently) associated to the RNA used in the immunization procedure, in spite of copious phenol-cresol extractions. However, antibodies against the capsid proteins were readily removed from the anti-VPg serum by adsorption to purified virion particles coupled to Affi-Gel 10. Anti-VPg serum thus purified was tested for immunoreactivity to VPg by incubation with ^{125}I-labeled VPg-pU released from virion RNA by complete nuclease P1 digestion. As shown in Fig. 1, anti-VPg serum was capable of specifically precipitating VPg, whereas normal rabbit serum did not precipitate any VPg. This demonstrates that the immunization with alkaline-treated RNA has been effective in raising antibodies which specifically react with the small, 4K to 5K, genome-linked protein of CPMV.

Having established the immunoreactivity of the anti-VPg serum to mature VPg, we next investigated whether this antiserum would recognize VPg sequences contained within larger, virus-encoded polypeptides. For this purpose we employed the so-called "Western" (protein) blotting technique (1, 2, 4, 25, 34–36). To test first whether this technique was also applicable to the analysis of CPMV-specific proteins in producing a faithful replica of the original gel pattern, [^{35}S]methionine-labeled proteins from CPMV-infected and mock-infected cowpea leaf cells were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose. By using the gel electrophoretic system of Sefton et al. (30) and

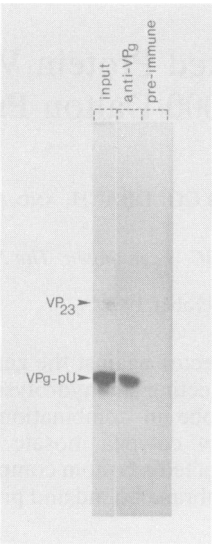

FIG. 1. Preparation of anti-VPg serum and immunoprecipitation of ^{125}I-labeled VPg-pU. CPMV was grown in cowpeas (*Vigna unguiculata*) and purified as previously described (16). A virus solution (4 to 5 mg/ml in water) was mixed with 2 volumes of 0.1 M glycine–NaOH (pH 9.0), 0.1 M NaCl, 0.01 M EDTA, 4% (wt/vol) Sarkosyl NL 97, 2% (wt/vol) sodium-triisopropylnaphthalene sulfonate, and 4% (wt/vol) *p*-aminosalicylate and heated at 60°C for 3 min to disrupt the virions, and the RNA was purified by four phenol-cresol extractions. For the first injection, 15 mg of purified RNA (corresponding to approximately 30 μg of VPg) was hydrolyzed for 60 min in 1 ml of 0.3 N KOH at 37°C and neutralized with HCl. After lyophilization, the residue was dissolved in 1 ml of water, emulsified with an equal volume of Freund complete adjuvant, and injected subcutaneously at several sites into the neck region of a male New Zealand white rabbit. Five weeks later, the rabbit was boosted with a similar antigen preparation containing 45 mg of hydrolyzed RNA emulsified with incomplete adjuvant. After 12 and 19 days, blood was collected from the ear vein and used for the isolation of the antibodies specific to VPg as described below. Subsequent injections with 35, 10, and 10 mg of hydrolyzed RNA were administered at intervals of 6 weeks. Immunoglobulin G was purified from the antiserum by DEAE-Affi-Gel Blue (Bio-Rad Laboratories) column chromatography using the experimental conditions as supplied by the manufacturer. To remove antibodies against the capsid proteins, the purified immunoglobulins were incubated in a rotary mixer for 16 h at 4°C with CPMV coupled to Affi-Gel 10 (*N*-hydroxysuccinimide derivative of Bio-Gel A-5m [Bio-Rad]; 1:1 [vol/vol] mixture of immunoglobulin solution and gel suspension) in PBSTDS (0.01 M sodium phosphate [pH 7.5], 0.1 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS). After incubation, Affi-Gel was removed by centrifugation, and the purified anti-VPg serum was stored in working samples at −80°C. Coupling of CPMV to Affi-Gel 10 was performed in 0.1 M $NaHCO_3$ (pH 8.0) by using 2.5 mg of CPMV per ml of gel. After coupling and subsequent incubation with 0.1 M ethanolamine-hydrochloride (pH 8.0) to block any remaining active esters, the gel was washed successively with 0.1 M $NaHCO_3$ (pH 8.0), water, phosphate-buffered saline, and PBSTDS. To prepare ^{125}I-labeled VPg-pU, purified virion RNA was subjected to the chloramine-T iodination reaction as described previously (7) and then digested with nuclease P1 at 37°C for 60 min with 1 μg of RNA in 25 μl of 0.05 M ammonium acetate (pH 5.3). Samples (2 μl) of the digest were incubated at 4°C for 18 h with either 5 μl of anti-VPg serum (lane anti-VPg) or 5 μl of preimmune serum (lane pre-immune) in 100 μl of PBSTDS. To adsorb immune complexes, 25 μl of a 10% (vol/vol) suspension of *S. aureus* cells (IgG Sorb; Enzyme Center Inc., Boston, Mass.) in PBSTDS containing 10 mg of bovine serum albumin per ml was added, and incubation was continued for 60 min at 4°C. Bacteria were then collected by centrifugation through a sucrose cushion (a 0.5-ml layer of 1 M sucrose and a 0.25-ml layer of 0.5 M sucrose, both in PBSTDS) and finally washed three times in PBSTDS. The precipitates were dissolved in sample buffer (1× SB: 0.01 M Tris-hydrochloride [pH 8.0], 1 mM EDTA, 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, and 0.001% [wt/vol] bromophenol blue) by heating for 5 min at 100°C, and the bacteria were removed by centrifugation. The solubilized immune precipitates were analyzed by electrophoresis on a polyacrylamide gel containing 20% acrylamide, 0.065% methylenebisacrylamide, 0.375 M Tris-hydrochloride (pH 8.8), and 0.1% SDS. The stacking gel contained 4% acrylamide. 0.1% methylenebisacrylamide, 0.125 M Tris-hydrochloride (pH 6.8), and 0.1% SDS. Electrophoresis was carried out in 0.05 M Tris, 0.384 M glycine, and 0.1% SDS. A third sample (2 μl) of the RNA digest was adjusted to 1× SB, heated for 5 min at 100°C, and applied directly to the gel (lane input). The gel was dried, and labeled polypeptides were visualized by autoradiography. In addition to VPg-pU, the position of the capsid protein VP23, a small amount of which was contaminating the RNA preparation, is indicated (see lane input).

the blotting procedure of Bowen et al. (2), small polypeptides as well as large polypeptides with molecular weights of up to 200,000 were readily transferred (Fig. 2A). Among the proteins transferred, all major CPMV-specific polypeptides were identified, including the B RNA-encoded 170K, 110K, 87K, and 32K polypeptides (11, 12, 26) as well as the M RNA-encoded capsid proteins VP37 and VP23 (9, 11, 28).

Nitrocellulose blots of unlabeled proteins from crude membrane fractions and soluble fractions of both CPMV-infected and mock-infected cowpea leaves were similarly prepared and incubated with either anti-VPg serum or preimmune serum. ^{125}I-labeled protein A from *Staphylococcus aureus* was then used to detect immune complexes. Anti-VPg serum appeared to react mainly with a single, 60K polypeptide (denoted as p60; Fig. 2B). In addition, a weak but significant signal was obtained with a 170K polypeptide (p170). Both immunoreactive polypeptides were specific for CPMV-infected cells and were

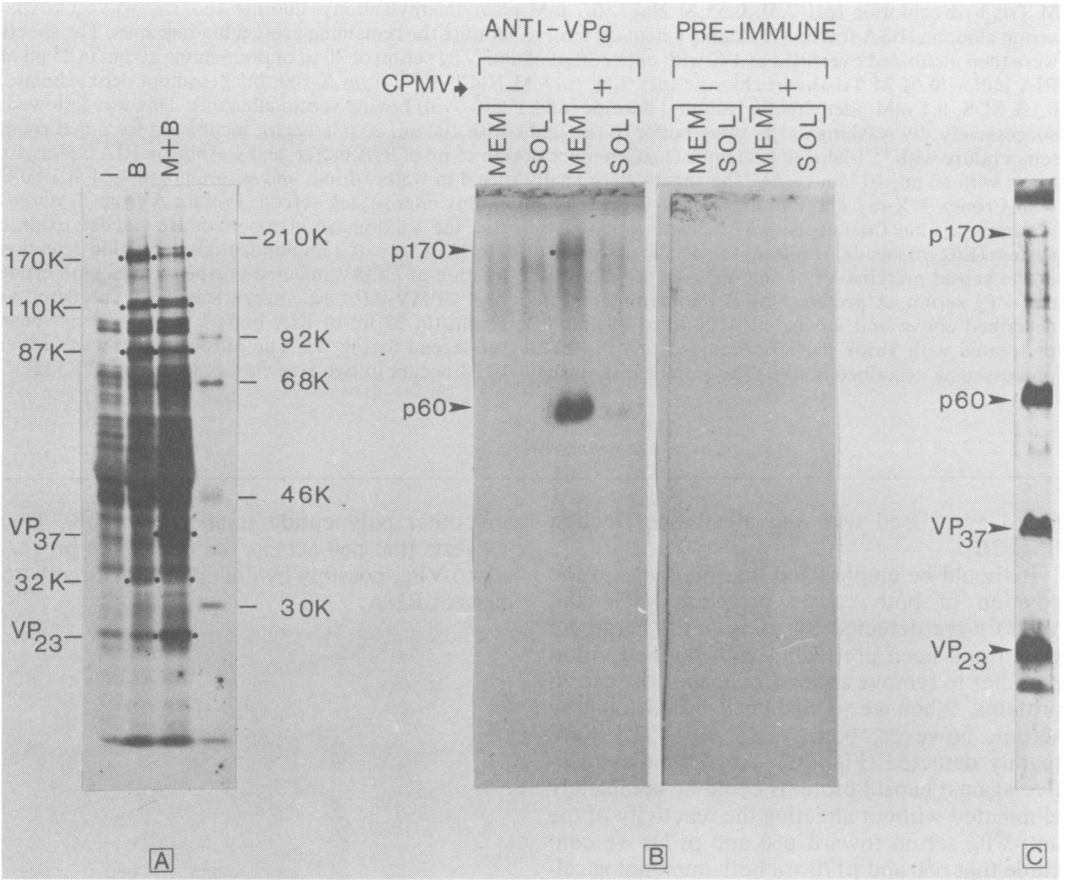

FIG. 2. (A) Autoradiograph of [^{35}S]methionine-labeled proteins of mock-inoculated and CPMV-inoculated cowpea mesophyll protoplasts transferred from a 12.5% SDS-polyacrylamide gel to a sheet of nitrocellulose. Cowpea mesophyll protoplasts were isolated and either mock inoculated (lane −) or inoculated with purified B components (lane B) or complete virus (lane M + B) as previously described (26, 27). For labeling of proteins, portions of protoplasts (2.5×10^6 in 5 ml of culture medium) (27) were incubated with 150 μCi of [^{35}S]methionine (Radiochemical Centre; 982 Ci/mmol) between 20 and 25 h after infection (26). Soluble protein fractions (31,000 × *g* supernatants) were prepared (26) and electrophoresed on a 12.5% SDS-polyacrylamide gel as previously described (26, 30). After electrophoresis, polypeptides were transferred to nitrocellulose (BA 85; Schleicher & Schuell) by the method of Bowen et al. (2). With this protein blotting procedure, two filter replicas of the original gel pattern were obtained. In brief, the gel was first immersed in urea buffer (0.01 M Tris-hydrochloride [pH 7.0], 0.05 NaCl, 0.002 M EDTA, 4 M urea, and 0.1 mM dithiothreitol) for 3 h and then sandwiched between two sheets of nitrocellulose in transfer buffer (0.01 M Tris-hydrochloride [pH 7.0], 0.05 M NaCl, 0.002 M EDTA, and 0.1 mM dithiothreitol) to allow proteins to diffuse out of the gel onto the nitrocellulose. The sheet was then dried and exposed to Cronex 4 X-ray film. Molecular mass markers (Radiochemical Centre) included ^{14}C-methylated myosin (210K), phosphorylase b (92.5K), ovalbumin (46K), carbonic anhydrase (30K), and lysozyme (14.3K). The numbers at the left refer to the molecular masses (in K) of the B RNA-encoded polypeptides (26); VP37 and VP23 refer to the capsid proteins of 37K and 23K, respectively. (B) Immunoautoradiographic detection of VPg precursors in the membrane fraction of CPMV-infected cowpea leaves. Cowpea (*V. unguiculata*; L. Blackeye early Ramshorn) plants were grown and either mock inoculated (indicated with −) or inoculated with CPMV (indicated with +) as previously described (38, 39). The membrane fraction (31,000 × *g* pellet; lane MEM) and soluble fraction (31,000 × *g* supernatant; lane SOL) were prepared as described previously (38). Briefly, leaves were homogenized in 0.05 M Tris-acetate (pH 7.4), 0.01 M potassium acetate, 1 mM EDTA, 0.01 M dithioerythritol, and 0.5 mM phenylmethylsulfonyl fluoride, filtered through Miracloth, and centrifuged for 15 min at 1,000 × *g*. The supernatant was adjusted to 20% (vol/vol) glycerol and centrifuged at 31,000 × *g* for 30 min to give the 31,000 × *g* pellet and supernatant fraction, respectively. The pellet was suspended in 0.05 M Tris-acetate (pH 8.2), 25% (vol/vol) glycerol, 0.05 M potassium acetate, 1 mM EDTA, 0.01 M dithioerythritol, and 0.5 mM phenylmethylsulfonyl fluoride. Samples of the pellet and supernatant fraction containing 40 μg of protein were then adjusted to 1× SB (see legend to Fig. 1) and subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel (26, 30). Proteins were transferred from the gel to two sheets of nitrocellulose as described above. After transfer, the sheets were incubated at room temperature for 5 h with gentle rocking in 50 ml of 0.01

M Tris-hydrochloride (pH 7.5), 0.35 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride and 3% (wt/vol) bovine serum albumin (BSA fraction V; Sigma Chemical Co.) to saturate the remaining protein binding sites. The sheets were then incubated overnight at 4°C with either 30 μl of anti-VPg serum or 30 μl of preimmune serum in 25 ml of RIA buffer (0.01 M Tris-hydrochloride [pH 7.5], 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, and 1% (wt/vol) bovine serum albumin). This was followed, successively, by washing in the same buffer (three times with 100 ml, 20 min each), incubation for 2 h at room temperature with ^{125}I-labeled protein A (1 μCi per gel slot) in 25 ml of RIA buffer, and washing in RIA buffer (six times with 50 ml, 10 min each). The sheets were finally rinsed in water, dried, and autoradiographed at −80°C with Cronex 4 X-ray film and a Du Pont Cronex Lighting-Plus intensifying screen. Protein A from *S. aureus* (Pharmacia Fine Chemicals) was labeled with ^{125}I by using the solid-phase lactoperoxidase-glucose oxidase system (Enzymobeads, Bio-Rad) to specific activities of 10 to 25 μCi/μg. (C) Immunoautoradiographic detection of the capsid proteins VP37 and VP23 in the membrane fraction of CPMV-infected cowpea leaves with crude anti-VPg serum. A protein blot of the membrane fraction of CPMV-infected cowpea leaves was prepared as described above and incubated with 30 μl of anti-VPg serum (in 25 ml of RIA buffer) which had not been pretreated with virion particles coupled to Affi-Gel 10 (see legend to Fig. 1). The nitrocellulose was further processed as described above. The small capsid protein VP23 occurs in two size classes (23K and 22K) (27).

found associated with the membrane fraction (Fig. 2B).

It should be emphasized that no bands at the position of both capsid proteins (VP37 and VP23) were detected by using anti-VPg serum which had been pretreated with purified virion particles to remove antibodies against the capsid proteins. When we applied unadsorbed anti-VPg serum, however, both VP37 and VP23 were readily detected (Fig. 2C). As antibodies reactive against capsid proteins could be completely eliminated without affecting the reactivity of the anti-VPg serum toward p60 and p170, we conclude that p60 and p170 are both immunologically unrelated to the capsid proteins, but related to the only noncapsid protein, VPg.

On the basis of previous evidence, indicating that B RNA encodes VPg (31), this would mean that p60 is encoded by B RNA. To test this hypothesis, protein blots were prepared from protoplasts which were inoculated with either purified B components or complete virus. In these experiments, p60 was dependent on the presence of only B components in the inoculum (Fig. 3). Surprisingly, p60 was more pronounced in B-infected than in B-plus M-infected protoplasts (Fig. 3). This apparent abundance of p60 in B-infected cells reflects the marked overproduction of B RNA-encoded polypeptides in cells only infected with B component (Fig. 2A; see also references 11 and 26). As M RNA encodes both capsid proteins (9, 11, 28), and p60 is found in protoplasts inoculated with only purified B components, these findings support the conclusion that the immunoreactive p60 represents a B RNA-encoded polypeptide containing VPg sequences. In an attempt to detect immunoreactive polypeptides smaller than p60, we also transferred proteins from a 20% polyacrylamide gel to nitrocellulose (data not shown). However, no evidence was obtained for either free VPg or any other polypeptide smaller than 60K. This suggests that p60 acts as the immediate precursor to VPg, possibly by donating VPg directly to nascent RNA.

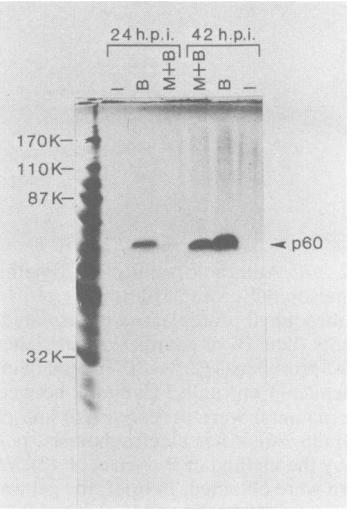

FIG. 3. Immunoautoradiographic detection of a 60K VPg precursor in cowpea mesophyll protoplasts inoculated with purified B components. Cowpea mesophyll protoplasts were either mock inoculated (lane −), inoculated with purified B components (lane B) or inoculated with complete virus (lane B + M) as described previously (26, 27) and incubated for 24 h (indicated as 24 h.p.i.) and 42 h (42 h.p.i.), respectively. Portions of protoplasts (2.5×10^5 to 3.0×10^5) were frozen, thawed, adjusted to 1× SB (see legend to Fig. 1), and heated for 5 min at 100°C. Extracts were clarified by centrifugation (5 min, 10,000 × *g*) and resolved by electrophoresis on a 12.5% SDS-polyacrylamide gel (26, 30). Protein blots were then prepared and incubated with anti-VPg serum as described in the legend to Fig. 2. Molecular mass markers (in K) included ^{35}S-labeled polypeptides from the 31,000 × *g* supernatant fraction of B-inoculated protoplasts (26) as indicated in the left lane.

When we fit our data on the immunoreactivity of p60 and p170 into the proposed translation map of B RNA (26), we arrive at the model depicted in Fig. 4. B RNA is translated into a 200K polypeptide which is cleaved into 32K and 170K polypeptides, the latter of which is further processed to generate a 110K polypeptide and the VPg precursor (60K). Alternatively, the 60K polypeptide can be generated from the 170K polypeptide via an 84K polypeptide. In view of the marked difference in immunoreactivity between p170 and p60, we anticipate that VPg sequences reside internally on the 170K polypeptide and terminally on the 60K polypeptide. Accordingly, VPg sequences would be internally located within the 84K polypeptide, which is not as abundant as other B RNA-encoded polypeptides (11, 26), however, and may therefore have been undetected.

For picornaviruses, RNA sequencing (15) and tryptic mapping (21) have recently revealed the presence of VPg sequences within the replicase precursor polypeptide. If the current hypothesis (8, 15, 20–22, 24) on the function of VPg is valid, then our results imply that the 60K polypeptide should play a crucial role in viral RNA replication as VPg donor and possibly replicase precursor. In this context, it is worth mentioning that among all B RNA-encoded polypeptides, only the 60K VPg precursor exclusively occurs in the membrane fraction, which has previously been shown to contain the viral replicase activity (39).

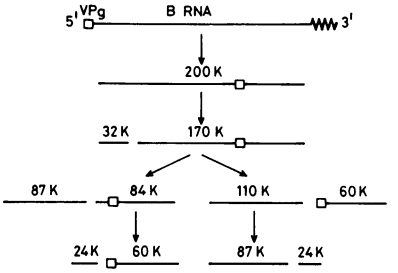

FIG. 4. Proposed position of VPg within the polypeptides processed from the B RNA-encoded 200K primary translation product. B RNA has previously been shown (12, 23, 26) to encode a 200K polypeptide which is processed by proteolytic cleavage into a 32K and a 170K polypeptide. The latter is then further cleaved to generate either a 110K plus a 60K polypeptide or, in an alternative route, an 84K plus an 87K polypeptide (26). It is now proposed that the immunoreactive p60 and p170 both contain VPg sequences and are similar to the 60K and 170K polypeptides previously detected in B-inoculated protoplasts (12, 26). To account for the marked differences in immunoreactivity between 60K and 170K VPg sequences are supposed to reside terminally on the 60K polypeptide and internally on both the 84K and 170K polypeptides. VPg has been indicated with a box and the poly(A) tail at the 3' end with a zigzag line. It is not known whether translation of CPMV RNA is preceded by removal of VPg.

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