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Identification by immunoprecipitation of cauliflower mosaic virus *in vitro* major translation product with a specific serum against viroplasm protein

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A highly specific antiserum was prepared against purified cauliflower mosaic virus viroplasm-protein (VmP). A virus specific *in vitro* major translation product (TPmaj), encoded by the 19S poly(A)$^+$ RNA fraction from cauliflower mosaic virus infected turnip leaves, was recognized by this antiserum. The N-terminal sequence of TPmaj corresponds to the sequence following the first in-phase initiation codon in gene VI of the cauliflower mosaic virus genome. Both VmP and TPmaj have blocked termini and probably start from the same AUG codon.

Key words: cauliflower mosaic virus/viroplasm/gene/*in vitro*/translation/immunopreciptation

Introduction

Cauliflower mosaic virus (CaMV) contains double-stranded DNA as genetic material (for reviews, see Hull, 1979; Shepherd, 1979; Hohn *et al.*, 1981). As for all the caulimoviruses, CaMV particles in infected cells are embedded within the matrix of cytoplasmic inclusion bodies called viroplasms. The viroplasm matrix is proteinaceous (Martelli and Castellano, 1971; Shepherd *et al.*, 1980; Xiong *et al.*, 1982). Various authors have suggested that the major viroplasm protein, which will be referred to as VmP, is encoded by the viral genome (Al Ani *et al.*, 1980; Odell and Howell, 1980; Shockey *et al.*, 1980; Covey and Hull, 1981). We and other workers have detected a prominent *in vitro* translation product (TPmaj) synthesized under the direction of RNA from virus-infected but not from healthy turnip plants (publications as above). The apparent mol. wt. of this translation product has been estimated to lie between 61 K and 66 K by reference to mol. wt. standards during SDS-polyacrylamide gel electrophoresis (SDS-PAGE, this paper; Al Ani *et al.*, 1980; Odell and Howell, 1980; Shockey *et al.*, 1980; Covey and Hull, 1981). Hybrid-arrested translation experiments (Odell and Howell, 1980) have shown that TPmaj is encoded by the part of the viral genome encompassing open reading frame VI of the CaMV sequence (Franck *et al.*, 1980).

A non-spliced polyadenylated viral transcript, termed 19S RNA, which contains open reading frame VI and which can direct TPmaj synthesis *in vitro*, has been detected and mapped (Odell *et al.*, 1981; Covey and Hull, 1981; Guilley *et al.*, 1982). The close similarity in electrophoretic mobility of VmP and TPmaj suggests that the two polypeptides are related but up until now no conclusive evidence establishing this relationship has been published.

In this paper we describe: (i) techniques for the purification of VmP as well as preparation of an antiserum of very high specificity against this protein; and (ii) evidence that TPmaj is recognized by this antiserum.

Results

Purification and identification of VmP

The pattern of electrophoretic migration of semi-purified viroplasms is shown in Figure 1a. VmP is present with other plant and virus proteins. This protein, migrating with an apparent mol. wt. of 66 K, is absent from an extract of healthy plants treated in the same manner (see Xiong *et al.*, 1982). To purify this major protein, the corresponding band was sliced from the gel and eluted by electrophoresis (see Materials and methods) and its homogeneity was confirmed by another electrophoretic run. A single band was found as judged by SDS-PAGE (Figure 1b). We have purified ~120 μg of VmP in this fashion to prepare an antiserum.

Preparation and analysis of the specificity of the VmP antiserum

VmP purified as described above was used to immunize one rabbit (see Materials and methods). The level of VmP antibodies was measured by solid-phase radioimmunoassay

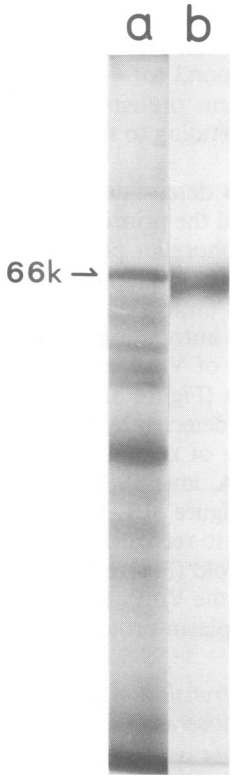

Fig. 1. Electrophoresis of semi-purified viroplasm and purified major viroplasm protein VmP in 10% SDS-PAGE. (**a**) 20 μl semi-purified suspension (1 ml/5 g leaves) were treated by boiling for 2 min in 2% SDS, 2% 2-mercaptoethanol before being loaded on the gel. (**b**) 5 μg purified VmP (see Materials and methods) were treated as for semi-purified viroplasm. The gel was stained with Coomassie blue.

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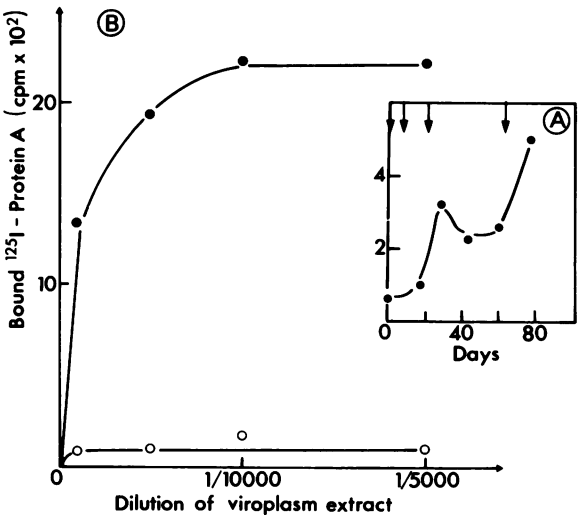

Fig. 2. Binding of VmP antiserum in RIA with semi-purified viroplasm extract. **A**: Production of VmP antibodies by immunization of a rabbit with purified VmP. Antibody levels were measured using semi-purified viroplasm diluted 1:20 000 as antigen and antiserum diluted 1:100. Arrows indicate the immunization schedule (30 μg VmP/injection). **B**: Binding of VmP antiserum (diluted 1:100) with increasing quantities of semi-purified viroplasm (●——●). Binding of normal rabbit serum was insignificant (○——○).

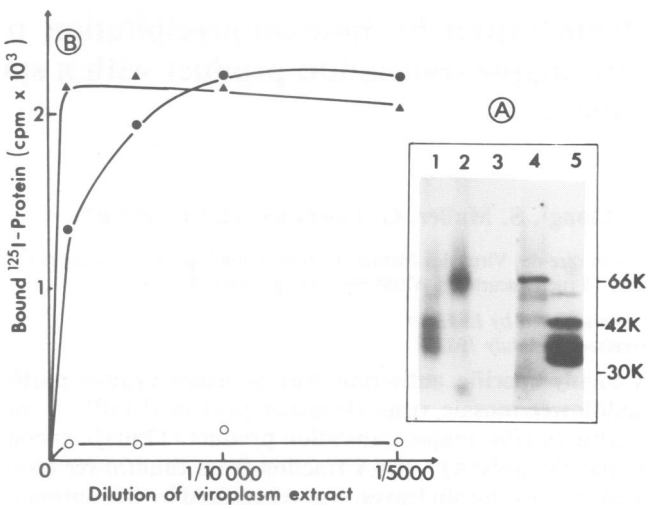

Fig. 3. Binding of VmP or CaMV antiserum with semi-purified viroplasm extract. **A**: Immunoautoradiographic detection of proteins contained in viroplasmic extracts after electrophoretic transfer from 10% polyacrylamide gel to nitrocellulose paper. After transfer according to the technique of Towbin *et al.* (1979), the nitrocellulose sheets were incubated with CaMV antiserum (**lane 1**), VmP antiserum (**lane 2**), or normal rabbit serum (**lane 3**) all diluted 1:100. Resulting antigen-antibody complexes were visualized by autoradiography after incubation with [^{125}I]protein A (5 x 10^5 c.p.m./ml) (exposure 36 h). Marker proteins migrated in the same run in starting 10% polyacrylamide gel, semi-purified viroplasm (**lane 4**) (see Figure 1) and viral-proteins (**lane 5**) (see Al Ani *et al.*, 1979). **B**: Binding in RIA of VmP antiserum (●——●), CaMV antiserum (▲——▲), or normal rabbit serum (○——○) all diluted 1:100 to increasing quantities of semi-purified viroplasm extract.

(RIA) using semi-purified viroplasms as antigen. As shown in Figure 2A, VmP antibodies were detected after three injections. Figure 2B illustrates the great sensitivity of RIA for detection of the VmP: with a 100-fold dilution of serum (third bleeding) a binding of ~1350 c.p.m. of [^{125}I]protein A was still observed in the presence of 1/200 000 of semi-purified viroplasms (1 ml/5 g leaves). Such a dilution was estimated to correspond to ~2.5 ng VmP/ml if we assume that all the viroplasm protein was bound to the microtiter plate in RIA. The binding to normal rabbit serum was insignificant.

In an attempt to determine the specificity of VmP antiserum, we have used the principle of immunochemical detection after electrophoretic protein transfer described by Towbin *et al.* (1979) and adapted to viroplasms. The binding of CaMV or VmP antibodies was revealed by addition of [^{125}I]protein A and autoradiography. The results indicate a specific recognition of VmP contained in viroplasm extracts by anti-VmP serum (Figure 3A, lane 2). Characteristic viral protein bands were detected by CaMV-antiserum (Figure 3A, lane 1). No binding of normal rabbit immunoglobulins was observed (Figure 3A, lane 3). These data were confirmed by solid-phase RIA (Figure 3B). When purified VmP was used as antigen, little or no recognition occurred with CaMV antiserum diluted 100-fold (Figure 4), confirming, first, the high degree of purity of the VmP, and second, the negligible contamination by viroplasm-protein of virions used to prepare CaMV antiserum.

Recognition of a translation product from infected plant mRNA by VmP antiserum

In view of the great specificity of the antiserum for VmP it became feasible to use this antiserum to identify *in vitro* translation products. The 19S mRNA fraction was purified by sucrose gradient centrifugation of poly(A)$^+$ RNA from virus-infected plants and translated in the rabbit reticulocyte lysate (see Materials and methods). TPmaj with an apparent mol. wt. of 66 K was present along with the various translation products of RNA of plant origin (Figure 5A, lane b). No such protein was present among the translation products of the 19S RNA fraction of healthy plants (Figure 5A, lane c). These translation products were incubated with either VmP antiserum, CaMV antiserum, or normal rabbit serum and then with protein-A Sepharose. The immunoprecipitates were then analysed by SDS-PAGE. TPmaj is essentially the only protein that was precipitated from the translation products by VmP antiserum (Figure 5B, lane c). When rabbit CaMV or

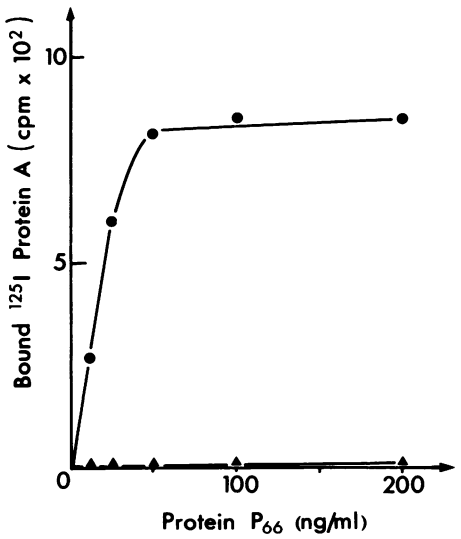

Fig. 4. Binding in RIA of VmP antiserum (●——●) or CaMV antiserum (▲——▲) with increasing quantities of purified VmP. Both sera were diluted 1:100.

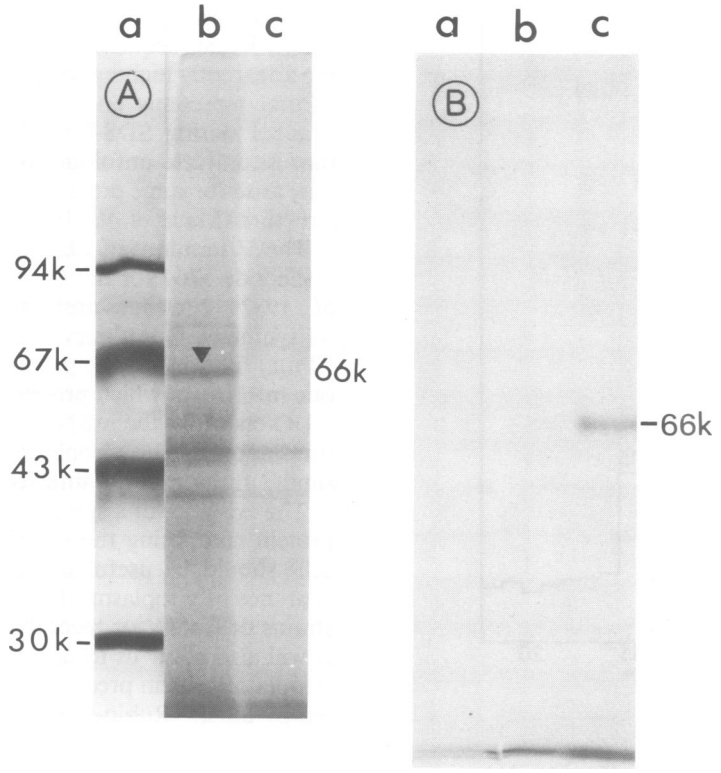

Fig. 5. A: *In vitro* translation products directed by the 19S mRNA fraction of a sucrose gradient from (**b**) infected plants, (**c**) uninfected plants. 2 μg mRNA was translated in a 10 μl rabbit reticulocyte lysate reactions mixture as described under Materials and methods. Translation products labelled with [^{35}S]methionine were separated on an 10% SDS-polyacrylamide gel and detected by autoradiography. (**a**): migration of mol. wt. standards: phosphorylase b (94 K); BSA (67 K); ovalbumin (43 K); carbonic anhydrase (30 K). **B**: Immunoprecipitation autoradiography of the *in vitro* translation products encoded by the 19S RNA sucrose gradient fraction against (**a**) normal serum, (**b**) CaMV antiserum, (**c**) VmP. Antiserum immunoprecipitates were co-precipitated with protein A-Sepharose and electrophoresed in reducing conditions in an 10% SDS-PAGE. Both stained standards and radioactive samples were run on the same gel.

normal antiserum were used, the 66-K band was absent (Figure 5B, lanes a and b). Thus we have confirmed that, among the 19S mRNA translation products, the only component which reacts with VmP antiserum is the TPmaj *in vitro* synthesized protein.

Analysis of the amino acid amino terminus of TPmaj and VmP

For amino acid amino terminus analysis, the translation mixtures were subjected to electrophoresis in a preparative gel. TPmaj was extracted from the gel slices, located using the autoradiogram as template, and recovered by acetone precipitation (see Materials and methods). The amino terminus of both TPmaj and VmP were analysed. TPmaj synthesized in reticulocyte lysate gave no significant peaks of radioactivity upon automated Edman degradation (data not shown) suggesting that TPmaj has a blocked amino terminus. The amino terminus of VmP likewise gave no N-terminal residue by the dansyl-Edman degradation procedure (Chang *et al.*, 1978; data not shown) suggesting that VmP also has a blocked amino terminus. There are only a few types of amino-terminal blocking group known, the most common being acetylation (Bloemendal, 1977). The acetyl group is derived from acetyl-CoA and is incorporated during the translation process. Acetylation can be prevented by metabolizing the available acetyl-CoA to citrate by addition of citrate synthetase and oxalacetate to the *in vitro* translation system (Palmiter, 1977). Figure 6A shows that, if the translation system was supplemented with an acetylation inhibitor, the protein can now be submitted successfully to automated Edman degradation with the first amino acid being Met derived from the initiator fMet: this suggests that the amino-terminal of TPmaj was blocked by acetylation. Also, [^{35}S]methionine is easily detected at residues 1, 9, 15 of the first 22 residues of TPmaj when *in vitro* translation was performed in the presence of citrate synthetase and oxalacetate. By comparison with the sequence of open reading frame VI (Figure 6B), it can be seen that this corresponds to expectation for the first 66 nucleotides following the first in-phase AUG of this open reading frame (Franck *et al.*, 1980). To verify this result, we used a computer program to compare the observed distribution of methionine residues with that expected on other portions of the CaMV sequence. This search revealed that there is no other continuous DNA sequence that could encode a polypeptide containing 22 amino acids in which the first, ninth and fifteenth residue is a methionine.

Discussion

We have shown conclusively that CaMV open reading frame VI carries the information for VmP. A number of workers have measured the apparent mol. wt. of VmP by polyacrylamide gel electrophoresis. The reported mol. wts. were 66 K (this paper, Odell and Howell, 1980), 62 K (Al Ani *et al.*, 1980; Covey and Hull, 1981) and 61 K (Shockey *et al.*, 1980). The variation in these mol. wt. estimates may in part reflect the choice of size markers used during the electrophoretic migration (typically bovine serum albumin (BSA) with a mol. wt. taken to be between 66 000 and 68 000 daltons). The purification procedure used to prepare the

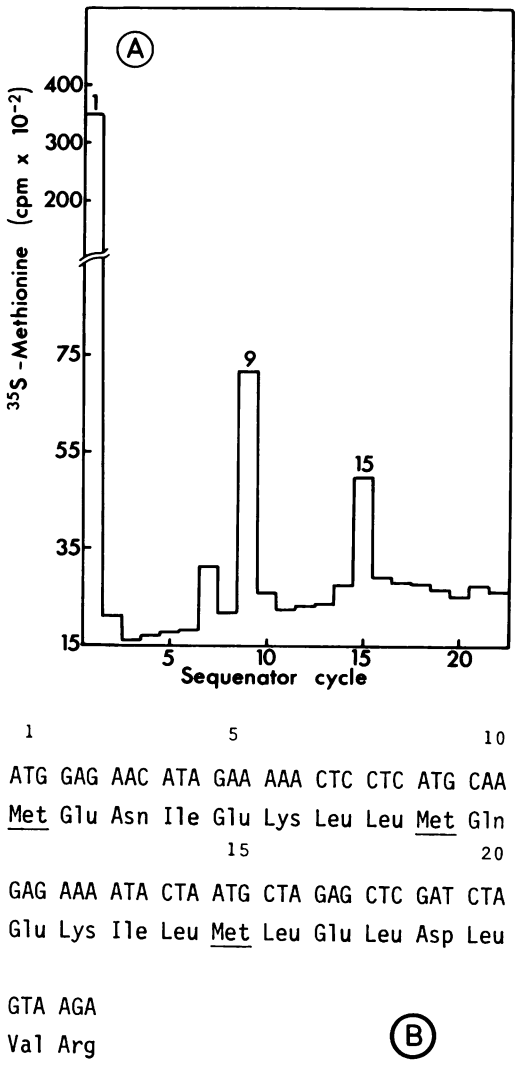

Fig. 6. A: Edman degradation of TPmaj synthesized in a reticulocyte lysate in the presence of citrate synthetase and oxalacetate. The input was 610 000 c.p.m. of TPmaj. **B**: amino terminus of amino acid sequence coded by open reading frame VI of CaMV-DNA sequence.

viroplasms may also be important. Thus Shepherd *et al.* (1980) have used a rather different method of purifying the viroplasm structural protein and report a mol. wt. of 55 K for the major species from four different CaMV isolates. In any event, it is noteworthy that, in all but the last case, the estimated mol. wt. for VmP is superior to the theoretical coding capacity of open reading frame VI, which corresponds to 58 K rather than 61 K reported by Franck *et al.* (1980) if one takes into account the exact amino acid composition of the protein rather than using an average value for the residue mol. wt. as in Franck *et al.* (1980).

There are several lines of evidence proving that the 19S RNA is not spliced (Odell *et al.*, 1981; Covey and Hull, 1981; Guilley *et al.*, 1982). Thus it is reasonable to calculate the amino acid composition of the open reading frame VI protein from the nucleotide sequence.

We have shown that synthesis of the TPmaj polypeptide in the rabbit reticulocyte system begins with the first in-phase AUG codon in the open reading frame VI. If, as seems likely to be the case, translation proceeds from this point to the first in-phase termination codon at nucleotide 7334 (Franck *et al.*, 1980) the mol. wt. of the polypeptide chain predicted from the nucleotide sequence is 58 K. It thus appears probable that the apparently higher mol. wt. measured by us and others for TPmaj represents anomalous behaviour of the translation product during SDS-PAGE. Indeed, others have reported that incomplete unfolding of the polypeptide chain by SDS may arise for some proteins due to their charge and secondary structure (Mayo *et al.*, 1974).

The 5′ terminus of 19S viral transcript has been mapped at nucleotide 5764 for the Cabb-S isolate of CaMV (Guilley *et al.*, 1982) 12 residues upstream from the beginning of protein VI synthesis. Our observations thus constitute proof that the TPmaj follows the rule proposed by Kozak (1981) for eucaryotic mRNAs in which protein synthesis is initiated at the first AUG codon in the mRNA sequence. We think it likely that translation of the viroplasm structural protein occurs in a similar fashion in the infected cell.

The existence of a specific serum directed against the major protein composing the viroplasms found in CaMV-infected cells should be useful in following the time course of appearance of viroplasms during the infection cycle of different strains of CaMV or recombinants between different strains. It will also allow us to determine whether the appearance of viroplasm protein precedes that of virions as has been suggested by others (Rubio-Huertos *et al.*, 1968; Martelli and Castellano, 1971).

Materials and methods

Virus isolates and plants

The CaMV isolate used in this study was cabbage-Strasbourg (Cabb-S) (Lebeurier *et al.*, 1978). The isolate was propagated in turnip plants (*Brassica rapa* L., c.v. Just Right) grown under green-house conditions, as described by Xiong *et al.* (1982). Infected leaves were taken 14 days after inoculation for RNA extracts and 21 days for viroplasm extracts.

Purification of virus

Virus was prepared by the method of Hull *et al.* (1976).

Extraction of viroplasms

Semi-purified viroplasms were extracted by the method described by Al Ani *et al.* (1980).

SDS-PAGE

The semi-purified viroplasms were subjected to electrophoresis as described previously by Xiong *et al.* (1982). For recovery of VmP, we used a 10% SDS-polyacrylamide preparative slab gel with 124 mM boric acid and 91 mM Tris as a buffer system. In order to locate the protein band to be extracted, the gels were chilled to 4°C and put in 0.5 M KCl. After 10 min, the appropriate opaque bands were cut out of the gel and rinsed three times with 0.5 M NaCl at room temperature and stored at −20°C.

Electrophoretic apparatus and the protein recovery procedure

A hollow plastic tube with a tapered end was used for recovery of the protein (Figure 7). The tube was prepared as follows: the tapered end was covered with a nylon screen and then fitted with a closed dialysis tube (UH 100/25 purchased by Schleicher and Schüll) held in place with a rubber ring. The gel slices were put into the tubes and electrophoresed at 100 V overnight at 4°C with 0.047 M Tris-HCl buffer pH 8.8. After the run, the buffer containing the eluted polypeptide in the dialysis tube was transfererd to another dialysis tube and dialyzed against several changes of deionized water. The solution of the polypeptide was concentrated by lyophilization and stored at −20°C.

Antisera

Anti-CaMV sera was raised in two rabbits by i.m. injection of 1 mg purified CaMV emulsified in Freund's incomplete adjuvant. Further booster doses were given in the same conditions every 2 weeks.

Anti VmP serum was produced in one rabbit by four i.m. injections of 30 μg VmP purified as described above and emulsified in Freund's incomplete adjuvant.

Solid-phase RIA

The antigen was prepared in the following manner: the semi-purified viroplasm was treated with 2% SDS and 2% 2-mercaptoethanol at 100°C 2 min.

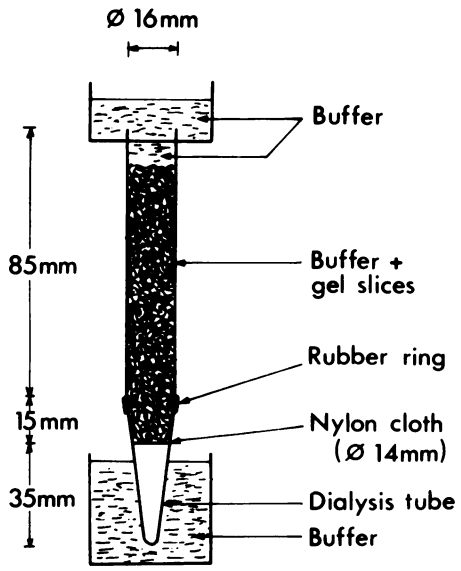

Fig. 7. Diagram of the apparatus used to recover the protein from preparative polyacrylamide gel system.

After centrifugation in an Eppendorf tube, the precipitate was discarded and the supernatant was used as antigen for measuring the level of antibodies by solid-phase RIA.

The assay was performed as described by Muller *et al.* (1982). Briefly, flexible microtitre plates were coated overnight at 37°C with 250 μl of antigen diluted in 0.05 M sodium carbonate buffer, pH 9.6. Washed coated wells were then incubated for 3 h at 37°C with 250 μl antiserum diluted in phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 and 1 mM EDTA (PBS-T-E). After further washing, 250 μl (4 x 10^4 c.p.m./ml) of ^{125}I-labelled protein A (purchased from Amersham International, Amersham, UK) were added. After 1 h incubation at 37°C and extensive rinsing, the individual wells were cut out and the bound radioactivity directly counted in a gamma counter.

Protein blotting procedure

Protein blotting was performed as described by Towbin *et al.* (1979). Briefly, the semi-purified viroplasms were subjected to electrophoresis in a 10% SDS-polyacrylamide slab gel as described above and then electrophoretically transferred to nitrocellulose sheets (0.45 μm pore size: Schleicher and Schüll) wetted with 25 mM Tris-192 mM glycine. Additional protein binding sites were saturated with 3% BSA for 2.5 h at 37°C. After three washings in PBS-T-E, the nitrocellulose sheets were independently incubated with various specific antisera diluted 100 times in PBS-T-E containing polyvinylpyrolidone (PVP) (0.02%), Ficoll (0.02%) and BSA (0.02%). The reaction mixtures were gently shaken on a plane shaker during 9 h at room temperature. Following extensive rinsing with BBS-T (borate buffered saline pH 8.5 containing 0.1% Tween 20) and PBS-T-E, ^{125}I-labelled protein A (60 – 100 mCi/mg) diluted in PBS-T-E-PVP-Ficoll-BSA to a final concentration of 5 x 10^5 c.p.m./ml was added. After 45 min incubation at 37°C, the sheets were washed until unbound [^{125}I]protein A was removed; the blots were air dried and exposed for autoradiography at −70°C.

Preparation of 19S mRNA fraction by sucrose gradient centrifugation

19S mRNA was extracted by a modification of the method of Howell and Hull (1978) as described by Guilley *et al.* (1982). Briefly, 50 g leaf tissue was quick frozen in liquid nitrogen and then immediately ground at high speed in a Waring Blendor with 75 ml TNS buffer plus 1% diethylpyrocarbonate and 50 ml phenol per g tissue (TNS buffer: 1% sodium triisopropylnaphthalene sulfonate, 6% *p*-amino-salicylic acid, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5). After low-speed centrifugation the aqueous phase was reextracted with phenol and then three times with ether. Nucleic acid was ethanol precipitated, collected by centrifugation, and the pellet was resuspended in 25 ml of ice-cold 3 M sodium acetate, pH 6. The high mol. wt. RNA, which does not dissolve, was collected by centrifugation and washed twice more with 3 M sodium acetate. Finally, the residual RNA was dissolved in water and ethanol precipitated. RNA was prepared from healthy turnip plants in a similar manner to serve as a control.

Poly(A)$^+$ RNA was separated from nonpolyadenylated molecules by two cycles of chromatography on oligo dT cellulose (P.L. Biochemicals Milwaukee,WI) and then concentrated by ethanol precipitation. Sucrose gradient centrifugation was for 16 h at 30 000 r.p.m. in a Beckman SW 41 rotor. The gradient was 5 – 20% sucrose made up in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% SDS. The poly(A)$^+$RNA sample, no more than 200 μg, was heated to 50°C for 2 min in the gradient buffer and then quick-chilled before being layered on the gradient. 19S gradient fractions of 0.5 ml were ethanol precipitated with 20 μg carrier tRNA and the precipitate, after washing with 70% ethanol, was taken up in 30 μl sterile water.

In vitro translation of 19S mRNA fraction from sucrose gradients

The 19S mRNA fraction from sucrose gradients was translated in rabbit reticulocyte lysates. The translation kit was purchased from New England Nuclear. We adapted the conditions described by the manufacturer with a modification of the concentration of ^{35}S-labelled methionine. A reaction mixture of 10 μl contained 0.5 μl ^{35}S-labelled methionine, 2.2 μl cocktail, 0.8 μl 1 M potassium acetate, 0.13 μl 50 mM magnesium acetate, 0.37 μl water, 4 μl lysate, and 2 μl mRNA.

Immunoprecipitation

The translation mixture was centrifuged at 40 000 *g* for 60 min and the supernatant was removed to a Eppendorf tube. In each 35 μl supernatant, either 10 μl of non-diluted VmP antiserum, 10 μl of CaMV with antiserum, or 10 μl normal rabbit serum was added. The mixture was diluted to 80 μl in 2.3-fold concentrated PBS. After incubation at room temperature for 1 h, 15 μl 30 mg/ml protein A-Sepharose cl-4B (Sigma) containing 1% Triton X-100 1 mg/ml BSA and PBS were added and incubated at room temperature for 3 h. The precipitate was collected by centrifugation and washed with PBS containing 1% Triton and 0.1% 2-mercaptoethanol till no radioactivity could be detected in the supernatant (C. Fritsch, personal communication). The precipitate was removed to a new Eppendorf tube and dissociated in 2% SDS, 2% 2-mercaptoethanol at 100°C for 2 min and electrophoresed in a SDS-PAGE as described previously. Gels were stained with Coomassie brilliant blue, de-stained and dried as described previously (Xiong *et al.*, 1982). Dried gels were exposed to Kodak NS-2T film at −70°C.

Prevention of amino-terminal acetylation during in vitro translation

We adapted the method described by Palmiter (1977). A reaction mixture of 100 μl contained 5 μl ^{35}S-labelled methionine (8 – 10 mCi/ml), 22 μl cocktail, 8 μl 1 M potassium acetate, 1.3 μl 50 mM magnesium acetate, 40 μl lysate, 1 mM oxalacetate, 30 units/ml citrate synthetase, and 19 μg mRNA. The other conditions were the same as in the supplier's instructions.

Analysis of products of cell-free synthesis

Analysis of newly synthesized protein was performed in SDS-acrylamide gels prepared as described by Laemmli (1970) and modified by Burgess and Jendrisak (1975). Aliquots of the translation mixture were dissociated in 2% SDS, 2% 2-mercaptoethanol at 100°C for 2 min and electrophoresed in an SDS-polyacrylamide gel (5% stacking gel; 10% resolving gel) using Tris/glycine buffer, pH 8.3, containing 0.1% SDS. Gels were stained, de-stained, dried, and exposed as described above. For extraction of P66, gels with one well were used. After electrophoresis, the gels were washed with destaining solution overnight and dried. [^{35}S]Methionine labelled P66 was sliced from the gels after location of the band upon the autoradiogram. The gel slices were eluted by a modification of the method of Hager and Burgess (1980) (L. Pinck, personal communication). Briefly, the gel slices were dipped into sterilized water for separating the gels from the paper and then crushed by forcing them to pass through two nylon screens within a syringe treated with a buffer containing 1 mg/ml BSA and 0.15 N NaCl. The gel slurry was incubated in the elution buffer at 25°C overnight. The slurry was then centrifuged at 1200 *g* for 20 min through a glass wool cushion to eliminate the gel particles. P66 protein was recovered from the eluate by acetone precipitation and stored at −20°C. For amino-terminus analysis, P66 was dissolved in 50% formic acid and added to the undercut-cup of a Beckman sequentor, model 890 C. The radioactive products of each degradation cycle were dried under nitrogen, dissolved in methanol and counted in a scintillation spectrometer.

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