Volume 5 Number 12 December 1978 Nucleic Acids Research

A protein linked to the 5′termini of both RNA components of the cowpea mosaic virus genome

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Received 3 November 1978

ABSTRACT

Evidence is presented for the presence of a protein covalently bound to the 5' termini of both M and B RNA components of CPMV. The protein is found to be linked in both cases to the 5' phosphate of the dinucleotide pUpAp, derived by ribonuclease digestion of the RNA. The intact protein is not required for infectivity or for in vitro translation of the RNA in cell-free extracts.

INTRODUCTION

The genome of CPMV comprises two separately encapsidated single-stranded RNAs (1,2) both of which are necessary for infection (3,4) inferring a distribution of genetic information between them. Both RNAs serve as messengers, giving products of high molecular weight when translated in vitro (5). The RNAs contain a 3' terminal poly(A) sequence (6) and in this respect resemble the animal picornavirus RNAs (7). Three members of the picornavirus group; poliovirus (8,9), encephalomyocarditis virus (10) and foot and mouth disease virus (11) have been shown to contain a protein associated with their RNA. The genome-associated protein of poliovirus has been demonstrated to be linked to the 5' terminus of the RNA, probably through a phosphodiester bond to a tyrosine residue (12).

Results have demonstrated that the 5' termini of CPMV RNAs do not contain a 'cap' structure as found on many eukaryotic and viral messengers and are not phosphorylated or polyphosphorylated (13). This raises the question as to the nature of the 5' termini. Are there proteins associated with the CPMV genome, if so, how are they linked to the RNA and can information be provided concerning the role of such structures during the processes associated with viral infection?

MATERIALS AND METHODS

In vitro iodination

CPMV was prepared as previously described (13) and the RNA isolated from purified virions by phenol extraction and ethanol precipitation (14). The RNA was further purified by dissolving the dried precipitate in detergent buffer (0.05 M Tris-HCl (pH 8.2), 0.1 M NaCl, 0.01 M EDTA containing 2% SDS, 2% sodium amino-salicylate, 1% sodium deoxycholate and 0.5% disodium triisopropylnaphthalene sulphonate), heating the solution to 60°C for 5 min and centrifuging the RNA after the addition of solid CsCl to a concentration of 1 g/ml (15). The pellet was dissolved in 0.1 M sodium borate (pH 8.5) and iodination performed using the Bolton and Hunter method (16). In a typical experiment, 350 µg RNA in 100 µl borate buffer was added to 140 µCi dried Bolton and Hunter reagent (Radiochemical Centre, Amersham) and the solution kept on ice for 30 min. 100 µl 0.2 M glycine in 0.1 M sodium borate (pH 8.5) was added and the mixture kept on ice for a further 20 min. Unincorporated radioactivity was removed by gel filtration on a Sephadex G25 column equilibrated with TNES buffer (0.01 M Tris-HCl (pH 7.5), 0.1 M NaCl, 0.001 M EDTA, 0.5% SDS).

Digestion of ^{125}I-labelled products

Proteinase K (Merck) digestion was carried out at an enzyme concentration of 750 µg/ml in TNES buffer, incubating for 2 h at 37°C. RNA was digested using a combination of pancreatic RNase (Worthington) and T_1 RNase (Calbiochem) at concentrations of 0.4 µg/µl and 0.2 unit/µl respectively in 0.01 M Tris-HCl (pH 7.4), 0.001 M EDTA. The mixture was incubated overnight at room temperature. Both digested and undigested RNA preparations were equilibrated with SPC buffer (0.15 M NaCl, 0.1 M Na_2HPO_4, 0.02 M sodium citrate (pH 7.1)) by gel filtration through an AcA 54 Ultrogel (LKB) column containing this buffer.

Isopycnic centrifugation of ^{125}I-labelled products

The RNA was analysed by Cs_2SO_4 density gradient centrifugation essentially as described (17). 1 ml samples in SPC buffer containing a maximum of 10 µg RNA were mixed with 1 ml 5% formaldehyde in water and left for 10 min at room temperature. With continuous stirring, 3.3 ml saturated Cs_2SO_4 solution in water was slowly added. Centrifugation

was carried out in an SW 50.1 rotor at 40,000 rpm for 67 h at 25°C. Fractions were collected from the bottom of the tube directly into scintillation vials and the ^{125}I radioactivity was estimated after the addition of 3 ml water and 20 ml Hydroluma (Lumac).

Growth and ^{32}P-labelling of CPMV

Vigna unguiculata (L) Walp variety 'Blackeye Early Ramshorn' plants were grown and inoculated with CPMV (yellow strain) as previously described (13). One day after inoculation, ten primary leaves were removed from the plants by cutting the petioles under water. With the leaves kept under water to prevent air entering, the ends of the cut petioles were inserted into water-filled micropipette tips (with the wider ends trimmed to a convenient size). Each leaf was transferred to a plastic microcentrifuge tube containing 1 mCi $^{32}PO_4$ (Radiochemical Centre, Amerham; supplied in 0.02 N HCl and subsequently neutralised with 0.1 volume 0.2 N KOH). When most of the liquid had been drawn into the micropipette tips, 100 µl phosphate-deficient Hoagland nutrient solution was introduced into each tube and allowed to be taken up by the leaves. When the level of air had almost reached the petiole, the micropipette tip was removed and the leaves rapidly transferred to a petri dish containing phosphate-deficient Hoagland nutrient solution. The leaves were kept at 30°C with 75% relative humidity and continuous light for 3 to 4 days (depending on the extent of leaf deterioration and virus multiplication).

Extraction and purification of CPMV ^{32}P-RNA

The virus was extracted from the leaves and purified as described (13). The virus pellet was dissolved in 0.05 M Tris-HCl (pH 8.0), layered over a 42% to 65% (w/v) CsCl gradient in the same buffer and centrifuged for 20 h at 4°C in an SW 27 rotor. Light-scattering bands containing M and B virus components visible in the gradient were removed, diluted with 3 volumes buffer and the virions recovered by centrifugation. The virions were disrupted with detergent and the RNA fractionated by sucrose gradient centrifugation (13). Peak fractions of M and B RNA were pooled, the RNA precipitated and washed twice with ethanol and dried under vacuum. The RNA was redissolved in water to a concentration of 2 pmole/µl and stored at -20°C. Yields of RNA (per 10 leaves) reached 450 µg (M RNA) and 650 µg (B RNA) with a specific

activity of 3 x 10^5 dpm/μg.

Digestion of ^{32}P-RNA and product fractionation

All reaction vessels and micropipette tips were treated with dimethyldichlorosilane solution and rinsed with water before use. Routinely, 10 pmole M or B RNA were digested for 2 h at 37°C in 50 μl TNES buffer in the presence (final concentration: 400 μg/ml) or absence of proteinase K. The digest was extracted with 2 volumes phenol/chloroform/isoamylalcohol (25:24:1), the phenol phase discarded and the RNA precipitated and washed with ethanol and dried. The RNA was digested at 37°C in 10 μl volumes for either 1 h using 2 μg pancreatic RNase in 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA or for 2 h using 2 units RNase U_2 (Sankyo) in 0.05 M sodium acetate, 0.002 M EDTA (pH 4.5). Products of digestion were fractionated by electrophoresis on Whatman 3MM paper at pH 3.5 (18) with the origin located across the middle of the paper.

Analysis of the ^{32}P-RNA moiety of digestion products

Appropriate bands were cut out and the RNA eluted from the paper with water. Carrier RNA (20 μg) was added before drying under vacuum. The nucleotide content of the products was examined by further digestion at 37°C in 10 μl volumes using 0.3 N NaOH for 16 h, 0.2 unit alkaline phosphatase (P L Biochemicals) in 0.01 M Tris-HCl (pH 9.0) for 0.5 h, 2 μg snake venom phosphodiesterase (Boehringer Mannheim) in 0.05 M Tris-HCl (pH 8.9), 0.01 M $MgCl_2$ for 2 h or 0.2 μg nuclease P1 (Boehringer Mannheim) in 0.05 M ammonium acetate (pH 5.3) for 4 h. Products were fractionated by electrophoresis on 3MM paper at pH 3.5.

Isolation of specific ^{32}P-labelled fragments by phenol extraction

M and B RNA were incubated in the presence or absence of proteinase K and subsequently digested with pancreatic RNase as described above. 100 μl TNES buffer was added and the digest extracted with phenol. The phenol layer was washed 3 times with buffer, 50 μg carrier RNA added and the RNA precipitated from the phenol with 2.5 volumes ethanol, washed and dried. The precipitate was redissolved in water, reincubated in the presence or absence of proteinase K and fractionated by electrophoresis on 3MM paper at pH 3.5.

In vivo ^{35}S-labelling

CPMV was labelled with ^{35}S-sulphate essentially as described for the ^{32}P-labelling procedure except that sulphate-deficient Hoagland nutrient solution was used. Labelling with ^{35}S-methionine in Vigna protoplasts was performed as previously described (19) using 8 x 10^6 protoplasts and 0.5 μCi ^{35}S-methionine in a 10 ml volume. Incubation was carried out for 65 h. Virus was isolated as described (13) and the RNA purified by phenol extraction and CsCl centrifugation (14,15).

Infectivity assays

RNA was treated with proteinase K as follows. The proteolytic enzyme at a concentration of 2.5 mg/ml was preincubated in 0.05 M HEPES-NaOH (pH 7.5) for 1 h at 37°C. 0.4 ml preincubated enzyme and 0.1 ml 0.15 M Tris-HCl (pH 8.8) were added to 0.5 ml RNA solution (1 mg/ml) containing 1% SDS. Proteolytic digestion was carried out for 18 h at 37°C. The infectivity of control and protease-treated RNA preparations was tested both at 5 μg/ml and 50 μg/ml by local lesion assay on primary leaves of Phaseolus vulgaris cultivar Pinto. Excised leaves were cut along the midrib into two halves. Each half was finely dusted with sterile carborundum and inoculated with two drops (approximately 0.1 ml) of RNA solution by wiping along the leaf with a small sterile plastic sponge. After 5 to 10 min, the leaves were rinsed with water and placed on moist filter paper in petri dishes. Local lesions were counted after 5 days incubation at 19°C under continuous light.

In vitro translation

Wheat germ from General Mills Inc. (Vallejo, Calif. USA) was used to prepare cell-free extracts with the appropriate modifications and incubation conditions for translation of CPMV RNAs (5). Treatment of RNA with proteinase K and pronase (protease V from Streptomyces griseus; Sigma) was performed under the conditions described for the infectivity assays.

RESULTS

In vitro iodination

In vitro iodination using the Bolton and Hunter reagent resulted in the introduction of ^{125}I into the RNA preparation to a specific activity of 6,070 cpm/μg. Treatment of the RNA with proteinase K prior

to labelling reduced the specific activity to 480 cpm/μg. Throughout the in vitro iodination experiments, the RNA invariably aggregated, precluding the use of techniques such as sucrose gradient centrifugation or polyacrylamide gel electrophoresis for the analysis of the iodinated products. The RNA preparations were therefore analysed by Cs_2SO_4 gradient centrifugation after equilibrating with SPC buffer by column chromatography (see Materials and Methods), in which they could be fixed with formaldehyde. The radioactivity remained associated with the untreated RNA upon isopycnic centrifugation, whereas little radioactivity banded in the position of RNA which had been treated with proteinase K (Fig. 1). RNA treated with ribonuclease resulted in a considerable proportion of the radioactivity eluting from the column in the void volume or immediately thereafter. This labelled material was no longer found at the buoyant density of RNA but appeared at the top

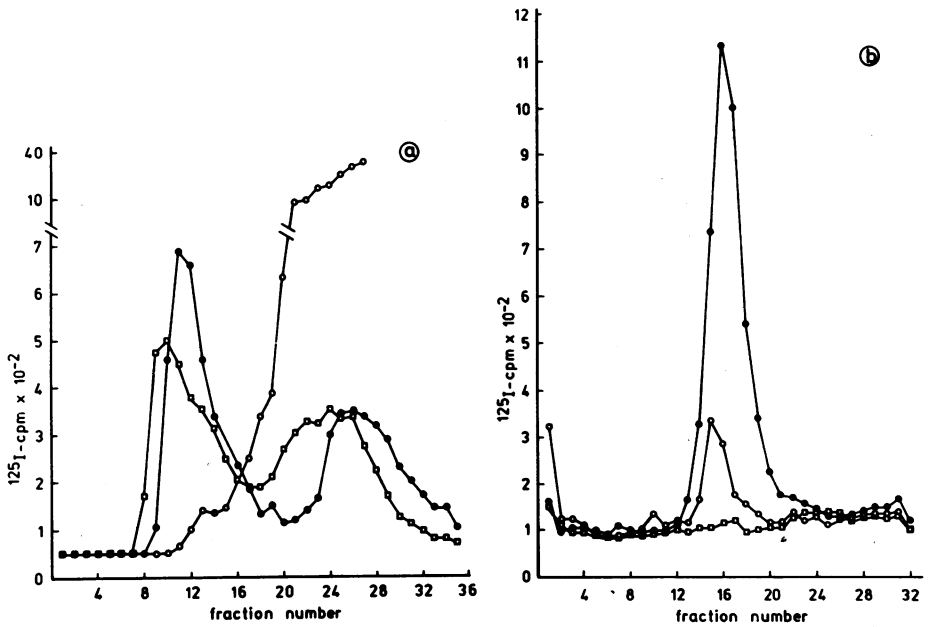

Fig. 1. a. Analysis of ^{125}I-labelled RNA preparations by gel filtration using Ultrogel type AcA 54 (fractionation range 6,000-70,000 daltons). 5 μl from each 6 drop fraction was counted in 6 ml Hydroluma.
b. Isopycnic centrifugation of the void volume collected from each sample after gel filtration as shown in (a). Fractions were collected from the bottom of the tube. ●—● untreated RNA; o—o RNA treated with proteinase K; □—□ RNA treated with a mixture of pancreatic and T_1 RNase.

of the gradient, mostly absorbed to the wall of the tube. This suggests that the observed aggregation phenomenon is probably due not to the RNA but to the protease-sensitive, iodinated moiety.

In vivo ^{35}S-labelling

After ^{35}S-labelling of CPMV-infected cowpea leaves or protoplasts, a small amount of ^{35}S radioactivity was detectable in the purified CPMV RNA fraction. The low specific activity (30 cpm/μg) was insufficient to allow further detailed analysis of the labelled moiety. Sufficient radioactivity was obtained, however, to enable its detection at the position of the RNA after Cs_2SO_4 isopycnic centrifugation.

Isolation of ^{32}P-labelled proteinase K-sensitive products

The products obtained after digestion with pancreatic RNase and RNase U_2, with and without pretreatment with proteinase K, were fractionated as shown in Fig. 2. For both M and B RNA, treatment with proteinase K followed by pancreatic RNase digestion yields two products with a net positive charge at pH 3.5, denoted P_a and P_b, that are absent without prior treatment with proteinase K. Similarly, two products are observed for both M and B RNA when digested with RNase U_2 following proteinase K treatment, denoted U_a and U_b, again absent when proteinase K treatment is omitted. In any one experiment in which the proteinase K digestions were carried out simultaneously in preparation for subsequent pancreatic RNase and RNase U_2 digestion, the ratios of P_a to P_b and U_a to U_b were similar. These ratios, however, were observed to vary considerably between separate experiments, often the amounts of P_a and U_a exceeding those of P_b and U_b. Three additional products with a net positive charge at pH 3.5 are present in the pancreatic RNase digests (indicated by arrows in Fig. 2), irrespective of proteinase K treatment. To test if these products were specific to CPMV RNA, *E. coli* rRNA was digested with pancreatic RNase under the same conditions (Fig. 2, track 1). Three products showing anomalous behaviour, migrating to positions similar to those within the CPMV digests, were present. These products are presumably derived from a close association of fragments of RNA with pancreatic RNase or a contaminant within the enzyme preparation. Alkaline hydrolysis indicates that the RNA moieties are pyrimidine-rich but are insensitive to further digestion using snake venom phosphodiesterease or alkaline phosphatase (data not given). As will be

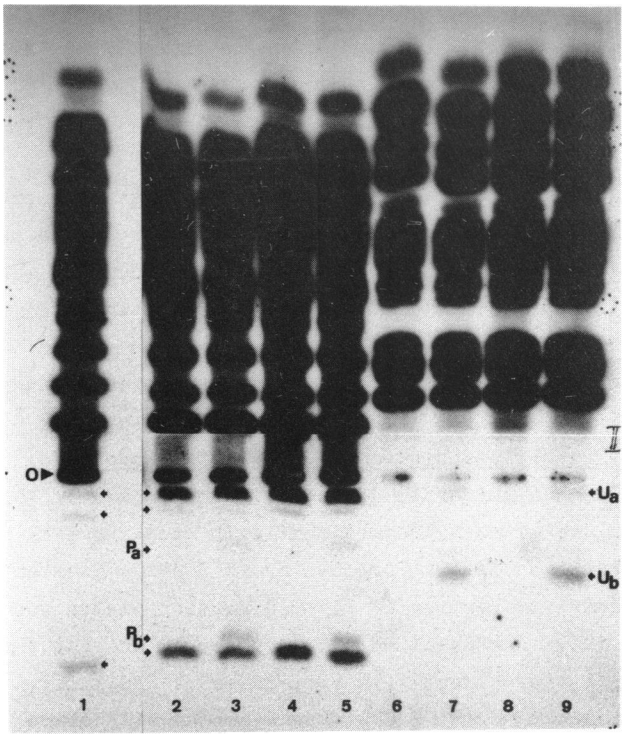

Fig. 2. Fractionation of pancreatic RNase and RNase U_2 digestion products on 3MM paper at pH 3.5. 1, pancreatic RNase digest of E. coli rRNA; 2, pancreatic RNase digest of M RNA; 3, pancreatic RNase digest of M RNA after proteinase K pretreatment; 4 and 5, digestion of B RNA as for 2 and 3 respectively; 6, RNase U_2 digest of M RNA; 7, RNase U_2 digest of M RNA after proteinase K pretreatment; 8 and 9, digestion of B RNA as for 6 and 7 respectively. O indicates the origin.

shown, treatment of the pancreatic RNase digest with phenol is sufficient to disrupt these (presumed) protein-RNA complexes thus preventing the associated radioactivity from entering the phenol phase.

Analysis of the ^{32}P-RNA moiety of the proteinase K-sensitive products derived from pancreatic RNase digestion

P_a and P_b were digested with alkali and alkaline phosphatase and the products fractionated as shown in Fig. 3. Alkaline hydrolysis in both cases (tracks 1 and 2) gave a single product migrating slightly slower than free phosphate to a position corresponding to that of

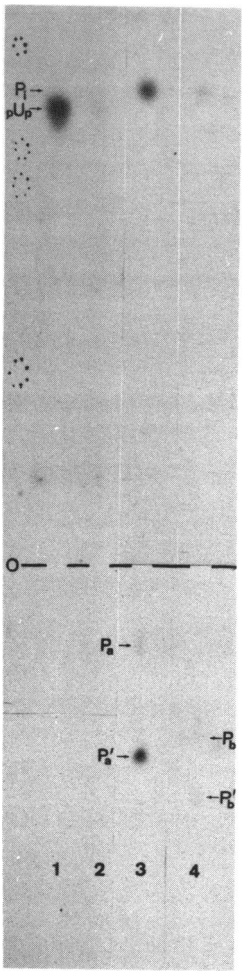

Fig. 3. Fractionation of digestion products of P_a and P_b from M RNA on 3MM paper at pH 3.5. 1, alkaline hydrolysis of P_a; 2, alkaline hydrolysis of P_b; 3, alkaline phosphatase digestion of P_a; 4, alkaline phosphatase digestion of P_b. The positions of undigested P_a and P_b are given. O indicates the origin.

uridine 3'5' diphosphate. Both P_a and P_b, on treatment with alkaline phosphatase (tracks 3 and 4), gave equimolar amounts of free phosphate and a product with a greater net positive charge than the undigested material (denoted P_a' and P_b'). Although only data for M RNA are given in Fig. 3, the digestion products of P_a and P_b from B RNA were identical in yield and mobility.

Analysis of the ^{32}P-RNA moiety of the proteinase K-sensitive products derived from RNase U_2 digestion

Figure 4 shows the products derived from U_a and U_b from both M and B RNA after digestion using nuclease P1, alkali and snake venom phos-

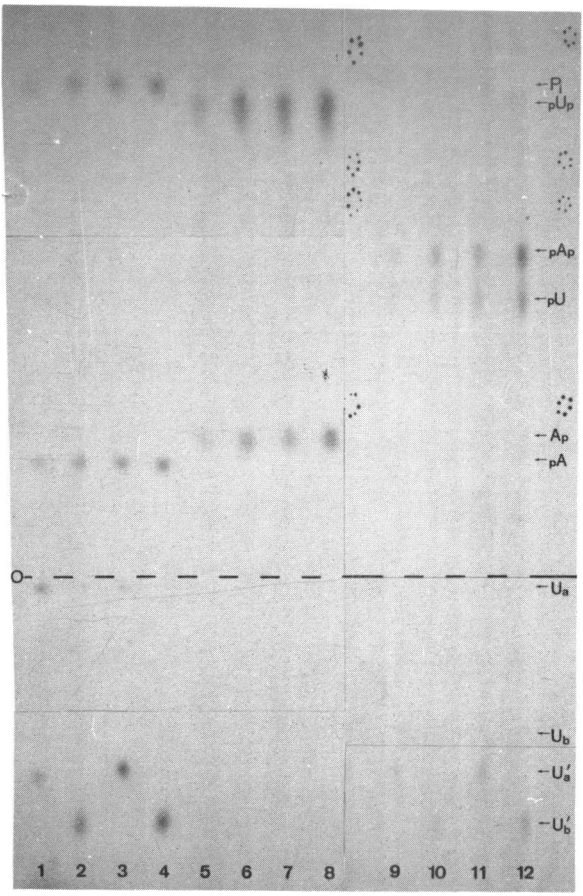

Fig. 4. Fractionation of digestion products of U_a and U_b from M and B RNA on 3MM paper at pH 3.5. 1 to 4, nuclease P1 digests of U_a (M RNA), U_b (M RNA), U_a (B RNA) and U_b (B RNA) respectively; 5 to 8, alkaline hydrolyses of U_a (M RNA), U_b (M RNA), U_a (B RNA) and U_b (B RNA) respectively; 9 to 12, snake venom phosphodiesterase digestion of U_a (M RNA), U_b (M RNA), U_a (B RNA) and U_b (B RNA) respectively. O indicates the origin.

phodiesterase. In addition to a small amount of undigested material, nuclease P1 digestion (tracks 1 to 4) gave equimolar amounts of free phosphate, adenosine 3' phosphate and a product with a mobility similar to either P_a' or P_b' (Fig. 3) denoted U_a' or U_b'. Alkaline hydrolysis in each case (tracks 5 to 8) gave equimolar amounts of uridine 3'5' diphosphate and adenosine 3' phosphate. Digestion using snake venom phosphodiesterase (tracks 9 to 12) gave two major products migrating

to the positions of adenosine 3'5' diphosphate and uridine 5' phosphate and also a small amount of either U_a' or U_b'. These data are consistent with the sequence XpUpAp for U_a and U_b from both M and B RNA, where X represents as yet uncharacterised proteolytic digestion products of differing composition in U_a and U_b. The bond between X and pUpAp is resistant to nuclease P1 digestion and partially resistant to digestion with snake venom phosphodiesterase under the conditions described.

Isolation of specific fragments by phenol extraction

The ^{32}P-labelled products precipitated from phenol following pancreatic RNase digestion both with and without prior treatment with proteinase K were fractionated as shown in Fig. 5. In the case of B RNA, insignificant quantities of material were precipitated from the phenol layer when the RNA was pretreated with proteinase K (tracks 6

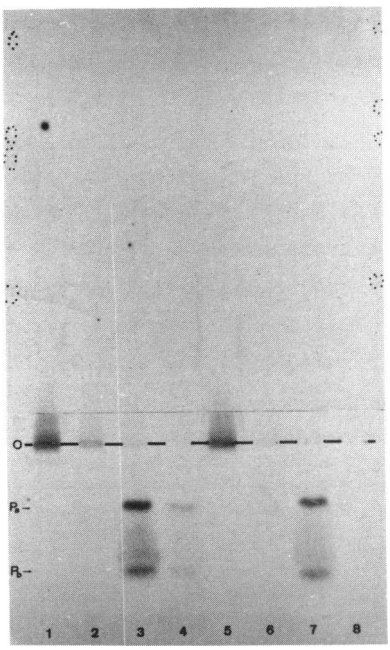

Fig. 5. Pancreatic RNase digestion products isolated from phenol by ethanol precipitation, fractionated on 3MM paper at pH 3.5. 1, pancreatic RNase digest of M RNA; 2, pancreatic RNase digest of M RNA with proteinase K pretreatment; 3 and 4, as for 1 and 2 respectively with additional proteinase K treatment after isolation from phenol; 5 to 8, treatment of B RNA as for 1 to 4 respectively. O indicates the origin.

and 8) when compared with the untreated RNA (tracks 5 and 7). For M RNA, although some label has been derived from the proteinase K-treated RNA (tracks 2 and 4) presumably due to incomplete digestion, the yield is greatly diminished when compared with the untreated RNA (tracks 1 and 3). When applied directly to 3MM paper, most of the material from both M and B RNA remains at the origin with slight smearing towards the anode (tracks 1 and 5). When digested with proteinase K before application to the paper, two bands appear (tracks 3 and 7) with mobilities similar to those of products P_a and P_b (Fig. 2).

What is the relationship between products P_a and P_b?

Products P_a and P_b were reincubated in the presence or absence of proteinase K. The samples were reapplied to 3MM paper and the products fractionated by high voltage electrophoresis at pH 3.5. No change of mobility was observed for P_a but P_b, on reincubation with proteinase K gave, in addition to undigested P_b, a second product with a mobility similar to that of P_a (greater than 50% conversion).

Is the genome-linked protein required for infection?

Proteinase K-treated CPMV RNA was tested for infectivity on Pinto bean half-leaves. At an RNA concentration of 5 μg/ml, 65 local lesions/6 half-leaves developed compared with 68 appearing after inoculation with untreated RNA. At an RNA concentration of 50 μg/ml, 290 and 307 local lesions developed after inoculation with treated and untreated RNA respectively. These results indicate that an intact protein moiety is not required for infection.

Is the genome-linked protein required for translation?

A comparison was made of the *in vitro* translation capabilities of CPMV M and B RNA, with and without pronase or proteinase K pretreatment. No loss of amino acid incorporation activity in wheat germ extracts was observed as a result of digestion of the protein. Indeed, in several experiments a two to four-fold increase in activity was observed subsequent to proteolytic treatment. This effect was found to be dependent on the RNA concentration. At low RNA concentrations, untreated and treated RNA were translated with equal efficiency. As the RNA concentration was increased, a difference in efficiency became evident as shown in Fig. 6. RNA with the genome-linked protein exhi-

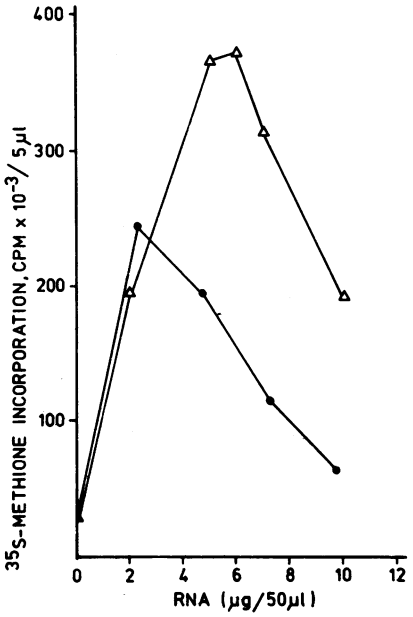

Fig. 6. Translation of pronase-treated CPMV RNAs. A mixture of CPMV M and B RNA was digested with pronase as described in Materials and Methods. The treated and control RNAs were translated in wheat germ cell-free extracts, using various concentrations of RNA. Trichloroacetic acid insoluble radioactivity was determined (5). • control (untreated); Δ pronase treated.

bited maximum translation at 2 µg RNA/50 µl reaction, above which concentration protein synthesis declined. Proteinase K-treated RNA however could be added to a concentration of 6 µg/50 µl before a similar decline in protein synthesis was observed.

The products of translation of CPMV RNAs, both untreated or treated with pronase or proteinase K, were analysed at several RNA concentrations. Electrophoresis was performed on two types of gel system, at different gel concentrations. Under the conditions used, no differences in products could be found owing to proteolytic digestion of the genome-linked protein. An example is given in Fig. 7. The large polypeptides of 185,000 daltons (coded by B RNA), and 130,000 and 118,000 daltons (coded by M RNA) were detected in all cases. It is not yet known however, if post-translation processing of these (presumed) large precursor proteins is different owing to the absence of the intact genome-

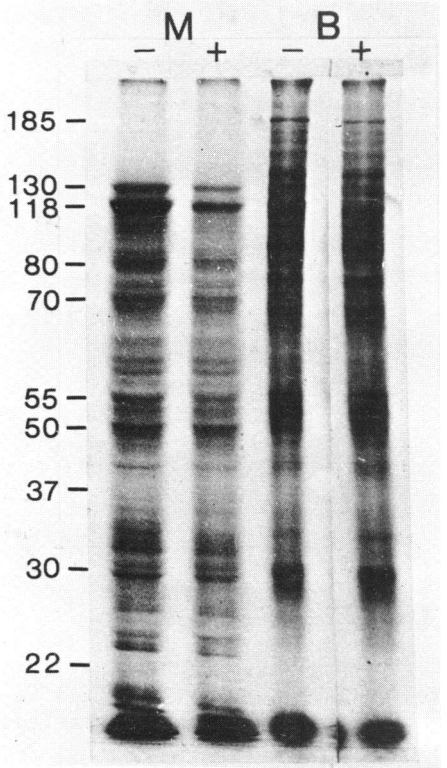

Fig. 7. In vitro translation of CPMV RNAs (6 μg/50 μl reaction mixture) in wheat germ cell-free extracts, with and without proteolytic digestion of the genome-linked protein. Analysis was on a 10% polyacrylamide gel using Tris-buffer at pH 8.8 and a stacking gel at pH 6.8, both containing 1% SDS. M, M RNA, B, B RNA; -, without intact genome-linked protein (proteinase K-treated); +, with intact genome-linked protein. The numbers on the left-hand side indicate the approximate molecular weight (x 10^{-3}) of the polypeptides. 37,000 and 22,000 are the expected positions of the coat proteins.

linked protein, but it is unlikely. No differences were detected in any of the smaller polypeptides, some of which may be post-translational cleavage products.

DISCUSSION

The results presented in this paper are consistent with the existence of proteins covalently linked to the 5' termini of CPMV RNAs. In vivo labelling of infected cowpea plants or cowpea protoplasts using

$^{35}SO_4$ or ^{35}S-methionine respectively leads to the recovery of ^{35}S radioactivity in the purified RNA fraction as shown by Cs_2SO_4 density gradient centrifugation. It has further been demonstrated that label may be introduced into the RNA fraction *in vitro* using the Bolton and Hunter reagent, a reagent specific for the iodination of proteins (16). The possibility of contamination of the RNA preparations with exogeneous proteins was reduced as far as possible by extensive purification of the RNA including pelleting through CsCl. Furthermore, treatment of the RNA with proteinase K prior to *in vitro* iodination led to a reduction of the amount of label recovered with the RNA by over 90%. Additional evidence for the presence of a protein associated with the RNA was obtained by isolation of proteinase K-sensitive products derived by ribonuclease digestion of uniformly ^{32}P-labelled RNA. The fact that the products P_a and P_b (Fig. 5) can be selectively isolated by ethanol precipitation of material entering the phenol phase during extraction of a pancreatic RNase digest is consistent with the above data and parallels the behaviour of the 5' terminal ribonucleoprotein fragment from poliovirus (12). That such protease-sensitive structures linked to a mononucleotide can be isolated from phenol under conditions that would normally disrupt non-covalently associated material suggests the covalent nature of the bond. In addition, equilibrium centrifugation of both the *in vivo* ^{35}S-labelled and *in vitro* iodinated RNA preparations in Cs_2SO_4 density gradients results in the radioactivity banding with the RNA. Under these conditions, non-covalently bound protein would be expected to dissociate from the RNA.

Analysis of products P_a and P_b derived by pancreatic RNase digestion shows the protein to be linked to pUp, conferring resistance to alkaline phosphatase on one phosphate group. Products U_a and U_b, produced by RNase U_2 digestion, contain the sequence pUpAp showing the phosphate, linked to the 3' of the uridine residue, to be involved in a normal phosphodiester bond between adjacent nucleotides. These results, together with a knowledge of the enzymic digestion products of U_a and U_b, all of which can be accounted for in the sequence pUpAp, infer that the protein is bound to the dinucleotide through the 5' terminal phosphate. If the protein was linked to the RNA at a point other than this phosphate or at additional points within the nucleotide chain, further products of analysis would be expected due to the specificity of the ribonucleases used to digest the RNA initially. These

data therefore imply that proteins are associated with the 5' termini of both components of the genome.

The nature of the residual product remaining bound to the RNA after proteinase K digestion is difficult to rationalise. The conversion of the pancreatic RNase-derived product P_b into P_a has been observed, inferring that P_b is a result of partial protease digestion or P_a a result of overdigestion. The latter possibility appears unlikely when the net charge of the product is considered. The anomolous mobilities of P_a and P_b under the conditions of fractionation used cannot readily be interpreted in terms of a single amino acid covalently bound to pUp, compensating for the charge of -3 assuming the linkage occurs through the 5' phosphate. Incomplete protease digestion of the protein will explain the high net positive charge of the products, but infers that the residual structures adjacent to the RNA are inaccessible or insensitive to proteolytic digestion. Incomplete pronase digestion of the protein associated with the poliovirus genome has been reported (20). Based on the observation that the mobilities of the mono- and dinucleotides bound to the residual products of protease digestion are constant, irrespective of whether they are derived from M or B RNA, it would appear likely that these residual products are identical for both RNAs. Furthermore, as the products exhibit similar sensitivities towards digestion using various nucleases, the bond between the protein and RNA is probably identical for M and B RNA. The characteristics of the bond between the RNA and protein in CPMV in many respects resemble those for poliovirus. Both are labile under alkaline conditions and when treated with snake venom phosphodiesterase but are resistant to mild digestion with nuclease P1 (9,20). Although the region of the protein moiety adjacent to the point of attachment is partially insensitive to digestion with proteolytic enzymes for both CPMV and poliovirus, the protease digestion products, containing an identical nucleotide component, differ considerably as judged by their electrophoretic mobilities. In both cases, the link is through the 5' terminal phosphate of a uridine residue and for poliovirus has been identified as a phosphodiester bond to tyrosine (12).

Infectivity studies on CPMV RNA demonstrate that the efficiency of infection is not impaired by treatment of the RNA with proteinase K. This result is similar to that for poliovirus RNA (21) but contrasts with the finding for nepovirus RNA in which a protease-sensitive struc-

ture associated with the RNA is a prerequisite for infection (22). As discussed above however, CPMV RNA may be associated with more than one amino acid residue after protease digestion and consequently their possible contribution to the infectivity requirements must not be overlooked.

In vitro translation of CPMV RNA in wheat germ cell-free extracts gives identical products, as judged by gel electrophoresis profiles (Fig. 7), both with and without protease pretreatment. Again, the effect of the residual product remaining attached to the RNA after protease digestion on the translational capabilities of the RNA is unknown. However, it can be concluded that an intact protein attached to the genome is not required for efficient translation in vitro. That the efficiency of translation is not impaired is shown in Fig. 6. The results indicate that above certain RNA concentrations (see Results section) the protease-treated RNA serves as a more efficient messenger for protein synthesis than the untreated RNA. Poliovirus mRNA lacks a 5' end protein (21). We do not yet know if the CPMV RNA found in polysomes from infected cells also lacks the protein moiety.

The possible absence of a translation function suggests a replication or virus assembly function, or both.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. C.P. de Jager for his helpful advice during the infectivity studies. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for the Advancement of Pure Research (ZWO).

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