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Evidence for the existence of a coat protein messenger RNA associated with the top component of each of three tymoviruses

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ABSTRACT

On centrifugation in a CsCl density gradient the three tymoviruses, eggplant mosaic, wild cucumber mosaic, and okra mosaic, separate into a major bottom component and several less dense minor components. The RNA of the top component is composed of about 90 % tRNA and 10 % of an approximately 250 000 dalton messenger RNA. The latter induced the synthesis of coat protein when translated in wheat-germ and rabbit-reticulocyte cell-free systems.

INTRODUCTION

Work in this laboratory [1] and in others [2, 3] has shown that turnip yellow mosaic virus (TYMV), the type species of the tymoviruses, has, in addition to a heavy infectious RNA, a light RNA of 220 000 dalton molecular weight (Briand, personal communication). Although both of these RNAs contain the nucleotide sequence corresponding to the coat-protein cistron [4], only the light RNA can induce the synthesis of coat protein in cell-free extracts of wheat-germ. Pleij *et al.* [3] found this light RNA both in particles sedimenting to equilibrium near the bottom of a CsCl density gradient and in minor components near the top of the gradient but never in the top (which is completely free of any RNA).

In order to find out if the presence of a light RNA is a common feature among the tymoviruses, we investigated the messenger functions of three other members of the group : eggplant mosaic virus (EMV), wild cucumber mosaic virus (WCMV), and okra mosaic virus (OMV).

MATERIALS AND METHODS

Virus purification

Viruses were grown in *Nicotiana glutinosa* (EMV), *Cucurbita pepo* (WCMV), and *Cucumis sativus* or *Hibiscus esculentus* (OMV). They were isolated as described by Bouley *et al.* (EMV) [5], Yamazaki and Kaesberg (WCMV) [6], and Givord and Hirth (OMV) [7].

CsCl density-gradient fractionation

Viruses were centrifuged in a solution of CsCl in distilled water (initial density = 1.39 g/ml) for 45 h at 35 000 rpm in a R50 Ti Beckman rotor. The different nucleoproteins were observed by their light scattering and collected by careful aspiration with Pasteur pipettes. An equal volume of water was added to each sample, which was then centrifuged for 4 h at 250 000 g. The nucleoprotein pellet was resuspended in 20 mM phosphate buffer, pH 7.0, and sometimes was centrifuged again through a CsCl density gradient as above.

RNA extraction and analysis

RNA was extracted from virus preparations or fractionated viral components with water-saturated phenol and analyzed by polyacrylamide-gel electrophoresis on slab or cylindrical gels [8].

The slab gels consisted of two layers of polyacrylamide, made from 10 % and 3 % acrylamide respectively (acrylamide:bisacrylamide ratio 20:1) containing 8 M urea. For electrophoresis in slab gels, RNA was dissolved in 90 mM Tris, 90 mM boric acid, 2.5 mM Na_2EDTA, 8 M urea, 50 % sucrose buffer, whose pH is about 8.3, and heated for 10 min at 60°C. Electrophoresis lasted for 5 h at 80 V (20 mA). Gels were stained with 0.05 % o-toluidine blue and destained in 10 mM sodium acetate, 1 mM magnesium acetate buffer, adjusted to pH 5.5 with acetic acid.

For electrophoresis in cylindrical tubes, the RNA was resuspended in 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, 50 mM 2-mercaptoethanol, 1 M urea, 10 % glycerol buffer and then incubated for 10 min at 60°C. Samples were layered on gels 8 cm long and 1 cm in diameter, of 2.4 % polyacrylamide and 0.5 % agarose [8] ; After electrophoresis for 2 h at 70 V (5 mA/gel), gels were stained and destained as above.

Cell-free systems

Commercial wheat germ (General Mills Inc., Vallejo, California) was purified by flotation and extracted according to the method of Marcu and Dudock [9]. Incubation mixtures were prepared according to Mayo *et al.* [10] with some modifications. A 50 µl sample contained 15 µl of S_{23} wheat-germ extract, 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate), 98 mM potassium acetate, 2.75 mM magnesium acetate, 0.65 mM spermidine, pH 7.6 2.5 mM ATP, 0.375 mM GTP, 5 mM Na_3 phosphoenolpyruvate, 1.8 mM dithiothreitol, 19 unlabelled amino acids (0.025 mM each), and 10 µCi of ^{35}S-methionine (SJ 204 Amersham). The optimal concentration of RNA ranged from 50-100 µg/ml. RNA was incubated in this extract for 2 h at 30°C. Amino acid incorporation was determined by measuring the radioactivity retained on Whatmann 3 MM paper

discs after they had been washed with hot and cold trichloroacetic acid, ethanol, and ether. Scintillation counting was in 5 ml of 0.5 % 2.5-diphenyl-oxazole in toluene.

Rabbit-reticulocyte lysates were prepared and assays were performed according to Mohier *et al.* [11].

Preparation of viral protein

EMV protein was prepared as described by Briand *et al.* [12]. Before fingerprint analysis, the protein was oxidized with performic acid [13].

Analysis of synthesized products

After incubation in the wheat-germ or rabbit-reticulocyte system, samples were analyzed by polyacrylamide-gel electrophoresis, in the presence of sodium dodecylsulfate. Samples were layered on 12.5 % gels prepared as described by Laemmli [14], or on gels containing a linear concentration gradient of acrylamide (8 %-15 %) and of urea (0-4 M). Electrophoresis and subsequent autoradiography were done as described by Paterson *et al.* [15].

Tryptic peptide maps

^{14}C-labelled polypeptides synthesized *in vitro* were analyzed as described by Klein *et al.* [1]. A mixture of protein synthesized *in vitro* and authentic coat protein added as a carrier was treated overnight at 37°C with 2 % trypsin-TPCK (Merck). The resulting digestion products were separated by electrophoresis and subsequent chromatography on thin layers of cellulose (Macherey-Nagel Polygram Cell 400). The radioactive peptides revealed by autoradiography on Kodirex (Kodak) film were compared with the peptides of the carrier coat protein stained with fluorescamine.

RESULTS

Fractionation of viral components

On centrifugation in a CsCl density gradient, the particles of EMV banded in three main fractions, described in detail by Bouley *et al.* [5] (Fig. 1). Similar fractions, in the same relative proportions, were also found for WCMV and OMV. All three viruses had a predominant bottom fraction containing infectious, high-molecular-weight RNA.

The middle component consisted of several fairly well defined types of nucleoprotein particles. The mean molecular weight of their RNA was between 1.3 and 1.5 x 10^6 daltons. The top component occurred in varying amounts depending on the temperature at which the virus had been multiplied. In an earlier study Bouley [1] found that low molecular weight RNA species were also present

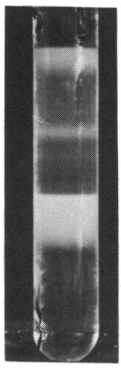

Figure 1. Photograph of a centrifuge tube in which EMV has been centrifuged to equilibrium in a CsCl gradient.

in the three components of EMV. Some of this RNA was identified as tRNA, but the recent results obtained for TYMV [2, 3] made it seem worth examining the possibility that a coat protein messenger might also be present.

Translation of EMV RNAs

Wheat-germ system. When RNA from unfractionated EMV or from bottom component was incubated in the wheat-germ system, there was a 20- to 30-fold increase in the amount of ^{35}S-methionine incorporated. Analysis by electrophoresis in polyacrylamide gels of the synthesized products showed polypeptides ranging in molecular weight from 10 000 to 180 000 daltons. However, the three main bands corresponded to proteins of 100 000, 35 000, and 22 000 daltons (Figs. 2a, 2b). Translation of the RNA from the middle component resulted in a pattern with main products of the same molecular weight (Fig. 2c), like that from the heavy RNA, although much smaller amounts of high-molecular-weight proteins were obtained.

Translation of the heavy RNA never yielded a polypeptide comigrating with the coat protein : heating the RNA in the presence of 1 mM EDTA [1] several times failed to change this result. By changing the concentrations of potassium and magnesium in the incubation medium, we determined the optimal conditions (see Materials and Methods) for translation of the heaviest products but did not achieve coat-protein synthesis.

On the other hand, RNA from the top component, when translated, usually gave rise to only one product, a 20 000-dalton polypeptide which comigrated in the gel with control coat protein (Fig. 2d) ; for some rare preparations, however, a 22 000-dalton polypeptide was the main translation product, and only a small amount of material comigrated with the coat protein (Fig. 2e).

Reticulocyte lysate system. Preliminary results with EMV RNAs translated in the rabbit-reticulocyte system showed that in addition to endogenous transla-

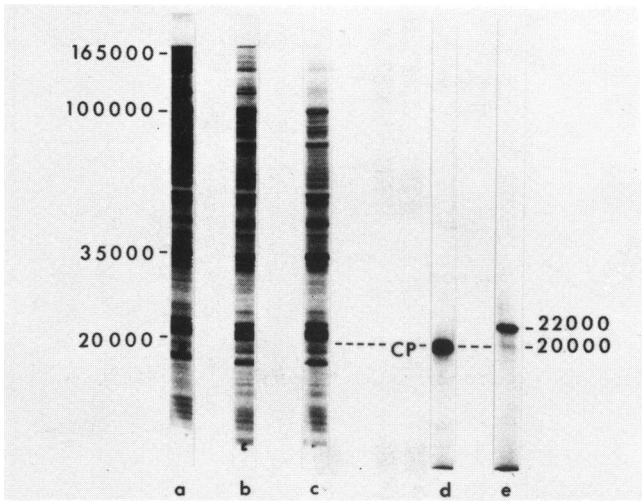

Figure 2. Polyacrylamide-slab-gel electrophoresis of the translation products of EMV RNA in wheat-germ extracts.
50-µl reaction mixtures containing 10 µCi of ^{35}S-methionine were incubated for 2 h at 30°C in the presence of 5 µg of (a) total RNA, and RNA from the (b) bottom, (c) middle, and (d, e) top components of the virus. A control gel loaded with a sample incubated in the same conditions, without messenger RNA, gave no detectable darkening of the film upon autoradiography for a similar period of time. After incubation, samples were treated with pancreatic RNase ; then 2 % sodium dodecylsulfate, 2 % 2-mercaptoethanol, and 15 % glycerol, were added ; then they were heated to 100°C, 10-µl samples were layered on slab gels containing a linear 8-15 % polyacrylamide gradient and electrophoresed for 4-5 h at 120 V. Kodirex films (Kodak) were exposed to the dried gels for 2 days.
Protein markers run on the same gel are : β subunit of E. coli RNA polymerase 165 000 daltons, AMV (alfalfa mosaic virus) RNA-2 and 3 *in vitro* translation products (100 000 and 35 000 daltons) EMV coat protein 20 000 daltons.

tion products, the bottom-component RNA induced the synthesis of polypeptides of 180 000, 150 000, and 70 000 daltons (Fig. 3b). The largest product resembles that protein in wheat-germ extracts, but the two other products were different and specific to this system.

As in the wheat germ system top component RNA induced the synthesis of the 20 000-dalton product comigrating with coat protein (Fig. 3c) or occasionally the 22 000-dalton product (not shown).

Translation of WCMV and OMV RNAs

The RNAs from the bottom components of OMV and WCMV, as in the case of EMV, induced the synthesis of polypeptides with molecular weight up to approximately 180 000 daltons. A product comigrating with coat protein was never observed when RNA from the bottom or middle components of these two viruses was translated. But WCMV and OMV top-component RNA, like that of EMV, induced

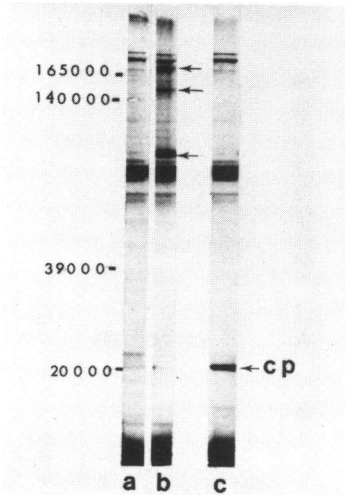

Figure 3. Polyacrylamide-slab-gel electrophoresis of the translation products of EMV RNA in a rabbit-reticulocyte-lysate system.

Cell-free synthesis was performed as described in Materials and Methods. 20-µl reaction samples were incubated : (a) without RNA, and then in the presence of 3 µg of RNA from (b) the bottom component, and (c) the top component of the virus. After incubation and further treatment at 37°C for 3 min in the presence of 50 mM EDTA and 1 µg of pancreatic RNase, each sample was mixed with 12 µl of a solution containing 10 % sodium dodecylsulfate, 10 % 2-mercaptoethanol, and 30 % glycerol, and was then heated to 100°C for 3 min. 6-µl samples were layered on slab gels containing a linear 8-15 % polyacrylamide gradient.
Protein markers run on the same gel are : TMV RNA translation products 165 000 and 140 000 daltons, α subunit of E. coli polymerase 39 000 daltons, EMV coat protein 20 000 daltons.

a single polypeptide of approximately 20 000 daltons that comigrated with their respective coat proteins (Figs. 4a, b).

Analysis of the 20 000- and 22 000-dalton products by tryptic fingerprints

Fig. 5a is a schematic drawing of a tryptic fingerprint of the 20 000-dalton protein synthesized under the direction of EMV top-component RNA in the presence of seven ^{14}C-labelled amino acids. All radioactive spots, except two which were only slightly labelled, correspond to fluorescamine-stained spots from the added EMV coat-protein carrier.

The fingerprint of the 22 000-dalton protein (Fig. 5b) shows no peptides in common with real coat protein, suggesting that the two cannot be related.

Analysis of the top-component RNA

Since the top-component RNA of all three viruses induced the synthesis of a protein comigrating with authentic coat protein, we re-examined this

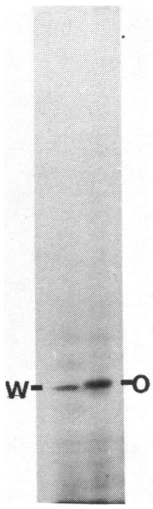

Figure 4. Translation products of WCMV (a), and OMV (b) top-component RNAs in a wheat-germ system.
Samples were prepared as described in Materials and Methods (see also legends to Fig. 2) and analysed in 12.5 % polyacrylamide gels.
Markers : O = OMV coat protein
W = WCMV " "

RNA, which was earlier described for EMV as being only tRNA [5].

Using the two-layer polyacrylamide gels we found that EMV and WCMV top component RNA (Fig. 6a and b) consisted of a major band comigrating with tRNA (Fig. 6c) in the lower polyacrylamide layer and another RNA called light RNA (l) which stayed in the upper layer of the gel. We assume that these light RNAs (representing about 10 % of the top RNA) are responsible for the synthesis of the 20 000-dalton polypeptide (shown in Fig. 2d (EMV) and 4a (WCMV)). The rare EMV top RNA component that induced the 22 000-dalton component as well as the coat protein contains in addition to this light RNA several bands of heavier RNA (Fig. 6d).

The molecular weights of RNA as well as the heavier component were more precisely determined by electrophoresis in cylindrical tubes. Fig. 7 shows that top RNA of EMV (a) and WCMV (c) comigrated with AMV-RNA-4 and so must have molecular weights of about 250 000 daltons. Occasional preparation of EMV top RNA which could also induce the 22 000-dalton protein always contained a major band comigrating with AMV-RNA-3 (about 650 000 daltons).

The nature of this RNA and the reason for its presence in top component is unclear but it seems possible that it represents the very upper fraction of the middle component which could easily contaminate top component.
It seems very likely that this RNA is responsible for the synthesis of the 22 000 d polypeptide. In this case the relative large amount of this RNA present in some preparations would explain the predominance of 22 000-dalton protein over coat protein when the RNA is translated (Fig. 2e).

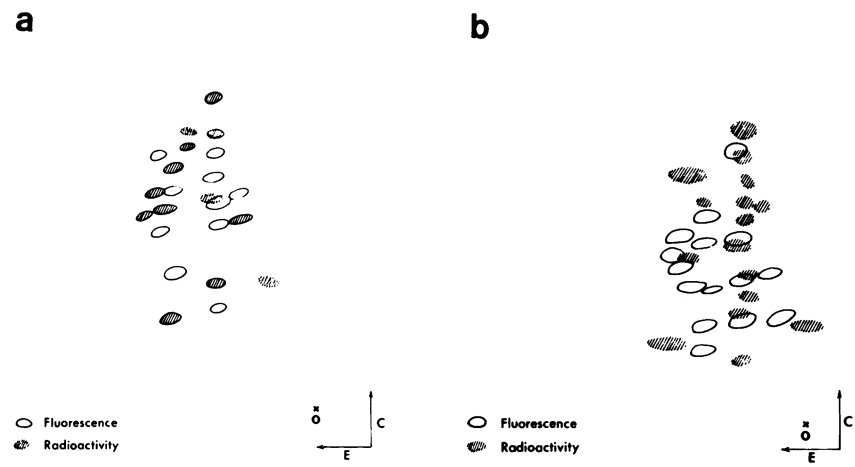

Figure 5. Fingerprints of the translation products of EMV top-component RNA in a wheat-germ system.
(a) 20 000-dalton product, (b) 22 000-dalton product.
Composite drawing of fluorescamine spots corresponding to EMV coat protein tryptic peptides (open ovals) and radioactive spots (cross-hatched ovals) corresponding to the *in vitro* synthesized products. Incubation mixtures contained ^{14}C arginine, ^{14}C leucine, ^{14}C phenylalanine, ^{14}C proline, ^{14}C serine, ^{14}C threonine, and ^{14}C lysine [1].

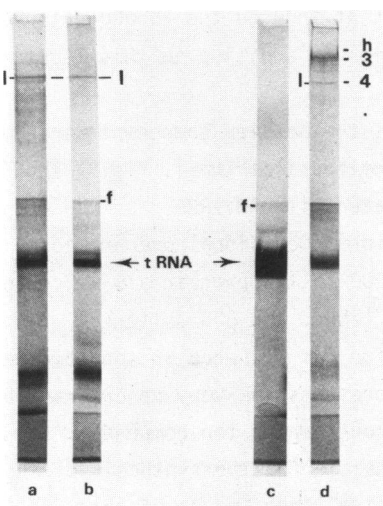

Figure 6. Analysis of EMV and WCMV top-component RNAs in a two-step polyacrylamide gel.
(For conditions, see Materials and Methods).

(a) EMV top-component RNA,
(b) WCMV top-component RNA,
(c) tRNA marker,
(d) the EMV top-component RNA that induces synthesis of the 22 000-dalton polypeptide.

l = light RNA
f = front between the 2 layers
h = heavy EMV RNA
3 - 4 = AMV RNA 3 and 4.

Scanning of the stained gel allowed evaluation of the relative proportions of tRNA and light RNA as a) tRNA 63 % ; lRNA 7 % - b) tRNA 86 % ; lRNA 13 % - d) tRNA 60 % : lRNA 7 % ; heavier RNA 20 %.

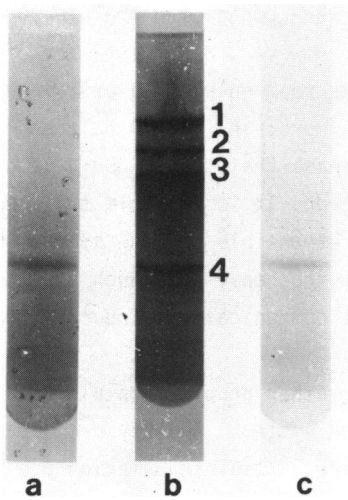

Figure 7. Analysis of light RNA on polyacrylamide-agarose (2.4 %/0.5 %) gels.
(a) Light RNA from EMV.
(b) Marker-AMV RNAs :
1) 1.2-1.04 x 10^6 daltons
2) 1.0-0.73 x 10^6 daltons
3) 0.7-0.62 x 10^6 daltons
4) 0.28-0.25 x 10^6 daltons [16, 17]
(c) Light RNA from WCMV.
In the conditions used, EMV and WCMV top-component tRNA ran off the gel after electrophoresis.

DISCUSSION

Our results show that like the type virus TYMV, our preparations of EMV, OMV and WCMV contained, besides the heavy genomic RNA able to induce virus infection, a light RNA which directed the coat protein synthesis *in vitro*.

It appears that for all tymoviruses studied until now this RNA is encapsidated in a nucleoprotein particle. Such a situation is different from the TMV system where the coat protein mRNA is present only in infected tobacco plants [18, 19], but resembles the case of the cowpea strain of TMV where such an RNA is also present in a special class of short particles [20, 21, 22].

Taken as a whole our observations suggest that the coat protein messengers of tymovirus are confined exclusively to top component where they are either encapsidated with one or more molecules of tRNA or encapsidated by themselves. TYMV seems to be an exception to this rule as its top component contains no RNA at all ; instead the small RNA is present in bottom fractions. It is not clear in the later case if one molecule of the light RNA is associated with one molecule of heavy RNA or if several (6 to 7) molecules of light RNA are in the same capsid.

Do tymoviruses contain other cistrons than those for the coat protein and the 180 000-dalton protein we detect in both protein synthesizing systems ? The figure of 2.3 x 10^6 daltons for the molecular weight of EMV-heavy RNA determined by Bouley *et al.* [5] suggests that tymoviruses might have a genome like tobacco mosaic virus whose RNA probably encodes three independent proteins, one of 165 000 daltons, one of 35 000 daltons, and the coat protein [23]. For instance, the 22 000-dalton protein we occasional-

ly find when protein synthesis is primed by an RNA containing the 650 000 dalton species could represent a third cistron. However, a new estimate of EMV RNA (T. Hall, personal communication) allows room only for a protein of molecular weight of 180 000 daltons and the coat protein (20 000 daltons) with no space for an additional cistron. This means that the 22 000-dalton product cannot correspond to an additional cistron. It is possible that the unusual RNA of 650 000 daltons which could be responsible for the synthesis of the polypeptide could be a 5'OH end piece of the heavy RNA. Such an RNA was found in the case of TYMV (Briand, personal communication) inducing the synthesis of a 35 000-dalton product.

We do not yet know the significance of the other products obtained with heavy RNA in wheat-germ and in reticulocyte systems. As they are different in the two systems we assume that they are partial translation products. But we cannot exclude the possibility that one or the other corresponds to a viral functional protein which could be made *in vivo* either by cleavage of the 180 000-dalton product or by reading from an internal initiation site.

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