In vitro Encapsidation of Cucumber Mosaic Virus RNA Species

Akira KOBAYASHI* and Yoshio EHARA*

Abstract

RNAs and coat protein of cucumber mosaic virus (CMV) strain Y were dissociated with LiCl, and *in vitro* homologous reassembly was examined. The reassembly products obtained by various protein-RNA ratios were analyzed by analytical sedimentation and gel electrophoresis. When the reassembly was carried out with a deficit of protein against the RNA, both encapsidated and unencapsidated RNA were detected, and encapsidated RNAs consisted mainly RNAs 1, 2 and partly 3, but only little encapsidated RNA 4 and satellite RNA. With sufficient protein, not only RNAs 1-3 but also RNA 4 and satellite RNA were fully encapsidated. It seems that encapsidation efficiency is different for the various virus RNA species, the high molecular weight RNAs 1 and 2 being preferentially encapsidated. Besides, it was confirmed that each of CMV RNAs or the satellite RNA was able to be encapsidated independently and formed virions.

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INTRODUCTION

Cucumber mosaic virus (CMV), the type member of the cucumovirus group, is a spherical virus containing four single stranded RNA genomes and 180 identical protein subunits. The virus genomes designated RNA 1-4, in order of decreasing molecular weight. In addition to the genomic RNAs, some CMV strains contain satellite RNA(s). Similar to brome mosaic virus (BMV)⁹, it has been suggested that CMV RNAs 1 and 2 are encapsidated separately, whereas one molecule each of RNAs 3 and 4 are encapsidated in the same particle¹⁰. The encapsidation of satellite RNA is not clearly understood.

CMV is dissociated into its RNA and coat protein (CP) subunits by chloride salts and can then be reconstituted with the RNAs and CP *in vitro*⁶. In addition to homologous reassembly, heterologous reassembly is successful between the CP and RNAs of CMV and tomato aspermy virus⁴, between CMV CP and the RNA of turnip yellow mosaic virus⁸ and tobacco mosaic virus¹. Accordingly, it has been considered that CMV CP lacks specificity for RNA encapsidation. On the other hand, it has been reported that BMV CP displays *in vitro* a differential affinity for various BMV RNA species⁵. Thus, CMV CP may also have specificity of RNA encapsidation to some extent.

This report presents characteristics on the reassembly of CMV RNAs and the satellite RNA with the CP *in*

MATERIALS AND METHODS

Preparation of virus RNA and coat protein.

The yellow strain of CMV (CMV-Y) containing a satellite RNA was purified from infected tobacco (*Nicotiana tabacum* L. cv. Ky57) leaves by the method of Scott¹¹ and followed by sucrose density gradient ultracentrifugations. The virions were dissociated to their RNAs and CP by freezing and thawing in 3 M LiCl⁶, and the CP was prepared by a modification of the method by Chen and Francki¹¹; the mixture of dissociated CP and RNA in LiCl was centrifuged with 10,000×*g* and the supernatant CP fraction was dialyzed against 2 M LiCl in 0.5 M Tris-HCl pH 8.4 for 1 hr, 1 M LiCl in the same buffer for 1 hr, 0.5 M LiCl in 0.2 M Tris-HCl pH 8.4, containing 2.5 mM DTT for 1 hr, and finally 20 mM Tris-HCl pH 7.2, containing 80 mM KCl, 1 mM DTT and 10 mM EDTA for 30 min. The CP preparation was then clarified by centrifugation at 370,000×*g* for 25 min. The CP concentration in the preparation was determined using the Bradford assay (Biorad Laboratories). While precipitated RNAs obtained by centrifugation were further purified by phenol extraction, and were used as preparations which had all RNAs species. In addition, each RNA species was separated from low gelling/melting temperature agarose gel slices.

Virus assembly. Reassembly of CP and virus RNA was derived from the method of Chen and Fran-

* Laboratory of Plant Pathology, Faculty of Agriculture, Tohoku University, 1-1, Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981, Japan 東北大学農学部

cki¹¹). CP and virus RNA were mixed in various ratios (ranging from 2:1 to 8:1 by weight) and the mixtures were dialyzed overnight at 4°C against 20 mM Tris-HCl pH 7.2, containing 80 mM KCl, 1 mM DTT and 10 mM EDTA. The reassembly did not occur without the dialysis step. After dialysis, the CP-RNA mixture was ultracentrifuged on a 10-40% linear sucrose density gradient at 45,000 rpm for 1 hr in a Hitachi RPS 50-2 rotor. The gradient was monitored with an ISCO UA-5 absorbance monitor at 254 nm and fractionated into 12 fractions from top to bottom with an ISCO model 185 density gradient fractionator. RNAs in each fraction were analyzed by 1.5% TBE agarose gel electrophoresis.

Electron microscopy. The products of reassembly were examined in an electron microscope (JEOL 100B) after staining with 4% uranyl acetate.

RESULTS

Reassembly of CP and total virus RNAs

The RNA content of CMV virion used for the reassembly experiment was 18.5%, as shown by Kaper *et al.*⁷). Agarose gel electrophoresis profile of separated virus RNA is shown in Fig. 1; the ratios of RNA 1-4 and satellite RNA were 1:1:2:1:1. The total virus RNAs and CP were mixed for reassembly, and the mixture was density gradient centrifuged (sedimentation profiles were shown in Fig. 2), fractionated and analyzed with TBE-agarose gel electrophoresis as described in Materials and Methods. RNAs encapsidated were mainly detected in fractions 7 and 8 (Fig. 3a). Most of free RNAs were detected in fraction 1, and a little was in fractions 2 and 3 (Fig. 3b).

In the mixture of the CP/RNAs=2/1, with a deficit of CP, a small sedimentation peak showing the reassembled virions was detected, though the free RNA peak was predominant (Fig. 2). Agarose gel electrophoresis of RNAs separated from the reassembled virions showed that they were mainly RNAs 1 and 2, and partly 3. In this case, RNA 4 and satellite RNA were rarely encapsidated;

Fig. 1. 1.5% TBE-agarose gel electrophoresis pattern of the RNA species of CMV-Y.

sidated; free RNA fractions contained mostly RNAs 3, 4 and satellite RNA, and RNAs 1 and 2 were a little in the fraction (Fig. 3c). These results show that RNAs 1 and 2 were encapsidated predominantly, followed by RNA 3. Thus, although it has been suggested that RNAs 3 and 4 are encapsidated in the same particle¹⁰, it was shown that encapsidation efficiencies of RNAs 3 and 4 were different each other. As CP/RNAs ratios increased, with an excess of CP, the amount of reassembled virions increased and that of free RNAs decreased (Fig. 2), and RNAs 1-4 and satellite RNA were detected significantly in the reassembled virion fractions (Fig. 3d). On the other hand, free RNAs were mainly satellite RNA (Fig. 3d). In the mixture of the CP/RNAs=8/1, with a great excess of CP, on the contrary, reassembled virions decreased in comparison with that in the mixture of the CP/RNAs=6/1 (data not shown). In this case it seemed that CP was quantitatively in excess and aggregated without virion formation. These data show that encapsidation efficiencies of large CMV RNAs were higher and followed the order of the molecular weights.

Encapsidation of individual virus RNAs

Reassembly of each individual CMV RNA and the satellite RNA with CMV CP was carried out. Each of the RNAs was extracted from agarose gel after electrophoresis as described in Materials and Methods. RNAs 1 and 2 were difficult to extract separately due to their

Fig. 2. Sedimentation profiles of reassembled CMV virions as a function of the increasing CP/RNA ratio ranging from 2/1 to 6/1. Purified CMV virion, RNA and CP-RNA mixtures were layered over 10 to 40% sucrose density gradients and centrifuged at $300,000 \times g$ for 1 hr.

Fig. 3. Distribution of CMV RNA species in 10 to 40% sucrose density gradient. After density gradient centrifugation at $300,000 \times g$ for 1 hr, the gradients were fractionated into 12 fractions from top to bottom and the RNA species in each fraction were analyzed by 1.5% TBE-agarose gel electrophoresis. Lane numbers indicate the fraction numbers. Lane 1; top fraction, lane 12; bottom fraction. The positions of the RNAs were indicated in left. (a) purified CMV, (b) RNA. Reassembly was carried out with (c) CP/RNA=2/1 and (d) 5/1.

close position in the gel and thus the two RNAs were used together without separation. CP and RNA used in this reassembly experiment were in very small amounts, and it was expected that the amounts of reassembled products are also very small, and thus they had to be examined using an electron microscope. As a result, individual CMV RNA, each of RNAs 1+2, 3, 4, and the satellite RNA, was reassembled with CP and formed spherical particles. Thus it was confirmed that individual CMV RNA or the satellite RNA is encapsidated independently and able to constitute virions. The sizes (diameter) of reassembled virions ranged from 30 to 34.5

Table 1. The diameters of reassembled virions

Encapsidated RNA	Diameter (nm) ^{a)}
Purified native virion	29.7
RNAs 1+2+3+4+satellite RNA	33.2
1+2	34.5
3	33.1
4	31.6
Satellite RNA	30.2

a) The average diameter of one hundred virions.

nm (Table 1); diameters of those seemed to differ slightly corresponding to enclosed RNA molecules. While, number of each RNA molecules enclosed in a virion was no clear. Incidentally, diameter of purified native virion was 29.7 nm.

DISCUSSION

Virus assembly is a process involving the direct interaction between the RNAs and the viral CP. Heterologous CP-RNA reassembly between CMV and other viruses has been demonstrated^{1,8)} and it has been suggested that CMV CP lacks of specificity for RNA encapsidation. However, with turnip crinkle virus (TCV), which is a spherical virus having a single-component genome, ribonucleoprotein complex represented the initiation structure required for the assembly process, and binding sites of CP on viral RNA were identified¹²⁾. In addition, it has been suggested that TCV CP contains both specific and non-specific RNA binding activities located at physically distinct sites¹³⁾. With BMV, some specificity of recognition and encapsidation of BMV RNA by BMV CP and differential affinity for various BMV RNA species has been demonstrated^{2,5)}. Furthermore, domains of BMV RNA 1 responsible for specific binding to CP have been identified³⁾. From these results, it was considered that CMV RNA may have some specific binding site to the CP and it is necessary to reexamine whether CMV CP has specificity for its homologous RNAs. Thus we investigated the encapsidation of CMV RNA species and satellite RNA.

In native CMV particles, the ratio of CP to RNA is a constant (4 : 1). Theoretically, when the ratio of the CP to RNA in the reassembly mixture is smaller than a value 4 : 1 (CP/RNA < 4/1), parts of RNAs might remain as free RNA, without encapsidation. In this case, if encapsidation efficiency is different in individual RNA species, competition on binding with CP might occur among them.

At CP and total virus RNA ratio 1 : 2, RNAs 1 and 2 were encapsidated predominantly, followed by RNA 3. Thus RNA 4 and especially satellite RNA were encapsidated very little. Increasing CP content, encapsidation of RNA 4 and satellite RNA advanced and as a whole the amount of free RNAs decreased. Accordingly, the encapsidation affinity of each CMV RNA species followed the order RNAs 1 and/or 2, RNA 3, RNA 4 and

satellite RNA. Thus it seemed that there is specific binding between the CP and the RNAs.

The fact that CMV RNAs 1 and 2 has a preferential affinity for CP was contrast to BMV RNAs 1 and 2. The reason of this difference between these two viruses is unknown.

In the present experiments, it was confirmed that CMV RNA individual or the satellite RNA alone is able to encapsidate and form spherical virions of approximately 30 nm in diameter. In this case, numbers of each RNA species enclosed in a virion are unknown, though it might be plural molecules. Besides, the CP may play a role for the spherical virion construction of a regular size.

RNA distribution in CMV virions extracted from infected cells has been presumed that RNAs 1 and 2 were each encapsidated in separate virions, whereas RNA 3 is coencapsidated together with RNA 4 in a virion, though encapsidation of satellite RNA was unclear¹⁰. Further study is needed to confirm whether virions enclosed only each of RNAs 3, 4 or satellite RNA are produced actually in the infected cells.

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和 文 摘 要

小林 晃・江原淑夫：キュウリモザイクウイルスRNAの試験管内におけるビリオン形成

キュウリモザイクウイルス黄斑系 (CMV-Y) の RNA と外被タンパク質の混合比率を変えて再構成を行い、ショ糖密度勾配遠心分離ならびにアガロースゲル電気泳動により再構成産物の解析を行った。外被タンパク質量が少ない条件下では再構成産物中よりウイルス粒子を形成した RNA と遊離の RNA の両者が検出され、形成された粒子中から主として RNA1, 2 さらに RNA3 が認められ、RNA4, サテライト RNA の量は少なかった。十分量の外被タンパク質により再構成を行うと、形成されたウイルス粒子中からは RNA1 から 3 に加え、RNA4, およびサテライト RNA も検出されるようになった。この結果より、RNA1, 2 が優先的に粒子化されることが示唆された。次いで RNA1+2, 3, 4 およびサテライト RNA の各単独での再構成を試みた。その結果、電子顕微鏡観察より、いずれにおいても粒子の形成が認められた。以上の結果より、CMV RNA の各セグメントおよびサテライト RNA と外被タンパク質との親和性はそれぞれ異なるが各 RNA は単独でも粒子を形成しうることが示唆された。