Proc. Natl. Acad. Sci. USA
Vol. 78, No. 7, pp. 4231–4235, July 1981
Biochemistry

Correlation between particle multiplicity and location on virion RNA of the assembly initiation site for viruses of the tobacco mosaic virus group

(cucumber green mottle mosaic virus/virus encapsidation)

MEGUMI FUKUDA*, TETSUO MESHI*, YOSHIMI OKADA*, YOSHIAKI OTSUKI†, AND ITARU TAKEBE‡

*Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo 113, Japan; †Institute for Plant Virus Research, Tsukuba Science City, Yatabe, Ibaraki 305, Japan; and ‡Biological Institute, Faculty of Science, Nagoya University, Nagoya 464, Japan

Communicated by Heinz Fraenkel-Conrat, April 6, 1981

ABSTRACT **The initiation site for reconstitution on genome RNA was determined by electron microscopic serology for a watermelon strain of cucumber green mottle mosaic virus (CGMMV-W), which is chemically and serologically related to tobacco mosaic virus (TMV). The initiation site was located at the same position as that of the cowpea strain, a virus that produces short rods of encapsidated subgenomic messenger RNA for the coat protein (a two-component TMV), being about 320 nucleotides away from the 3′ terminus, and hence within the coat protein cistron. Although CGMMV-W was until now believed to be a single-component TMV, the location of the initiation site indicated the presence of short rods containing coat protein messenger RNA in CGMMV-W-infected tissue, as in the case for the cowpea strain. We found such short rods in CGMMV-W-infected tissue. The results confirmed our previous hypothesis that the site of the initiation region for reconstitution determines the rod multiplicity of TMV. The finding of the second two-component TMV, CGMMV, indicates that the cowpea strain of TMV is not unique in being a two-component virus and that the location of the assembly initiation site on the genome RNA can be a criterion for grouping of viruses.**

The process of tobacco mosaic virus (TMV) reconstitution from its RNA and protein has been extensively studied since it was documented by Fraenkel-Conrat and Williams more than 25 years ago (1). Contrary to earlier belief (2–4), recent investigations established that reconstitution of virus rods of common TMV strains starts at an internal region of the viral RNA molecule, 800–1000 nucleotides away from its 3′ terminus, to proceed in two opposite directions (5–8). The rod elongation in the direction of 3′ to 5′ is rapid and reaches the 5′ terminus within 5–7 min, while the elongation in the other direction is much slower (9). We have shown that internal initiation followed by bidirectional elongation is a universal mechanism of particle assembly among various TMV strains, including a tomato strain (T) and a cowpea strain (Cc), but that different strains may have initiation sites at different locations on the RNA (10). The particle reconstitution started on T RNA at the same site as on RNA of common strains, whereas the initiation site on Cc RNA was located much closer to the 3′ terminus, being only about 320 nucleotides away from it, and hence within the coat protein cistron.

It is generally accepted that TMV coat protein is translated from a separate messenger RNA (11,12), which contains the 3′-distal 700 nucleotides of the genome RNA (8). Cc strain is characterized by the production of short rods containing the coat protein messenger RNA (13,14) in addition to the normal-sized particles. The location of the initiation site on Cc RNA explains why the Cc strain, but not the other strains, produces the short rods (10).

In the present study we determined the initiation site on the RNA of a watermelon strain of cucumber green mottle mosaic virus (CGMMV-W), which is chemically and serologically related to TMV (15). Although CGMMV-W has been believed to be a single-component-virus, the location of the initiation site for reconstitution indicated that CGMMV should have short particles, and these were actually found.

MATERIALS AND METHODS

Preparation of Viruses, Viral RNAs, and Viral Proteins. Three strains of TMV were used; a common strain (OM), a tomato strain (T) (10), and CGMMV-W. CGMMV-W was isolated from Japanese watermelon and is chemically and serologically related to TMV as well as to cucumber virus 4 (15).

TMV-OM and TMV-T were purified from infected tobacco (*Nicotiana tabacum* Linnaeus cv. Xanthi) leaves as described (7). CGMMV-W was propagated in *Lagenaria siceraria* Stand var. *hispida* Hara. Systemically infected leaves harvested 3 weeks after inoculation were homogenized as described for Cc strain (10) except that borate/EDTA buffer was replaced by 0.1 M sodium phosphate/0.01 M EDTA (pH 7.2). Tissue debris and denatured materials were removed by centrifugation at 10,000 × *g* for 10 min, and virus was precipitated by centrifugation through 30% (wt/vol) sucrose in 0.1 M sodium phosphate buffer (pH 7.2) at 34,000 × *g* for 16 hr (13). Fractions enriched for short particles were obtained by differential resuspension of the precipitate (16) followed by differential centrifugation. Further purification of the short particles was achieved by centrifuging the fractions through 5–20% sucrose gradients in the phosphate buffer for 2 hr and 20 min at 63,000 × *g*.

RNA was isolated from the purified viruses by phenol/bentonite extraction (17). Coat protein was isolated by the acetic acid method (18).

Locating the Assembly Initiation Site on CGMMV-W RNA. Sequential reconstitution of virus particles from CGMMV-W RNA was started with OM protein and was completed with T protein as described for Cc RNA (10). The products were purified by centrifugation through 5–20% sucrose gradients and were examined by electron microscopic serology (7). About 7% of the CGMMV RNA was recovered in fully reconstituted particles.

Particle Length Measurement by Serologically Specific Electron Microscopy. Antisera to CGMMV-W were obtained from rabbits, and an immunoglobulin fraction was prepared by

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Abbreviations: TMV, tobacco mosaic virus; CGMMV, cucumber green mottle mosaic virus; SERF, specifically encapsidated RNA fragments.

the method described (19). Crude extracts of CGMMV-infected leaves were prepared as described above and were placed on electron microscope grids coated with antisera to CGMMV (20). The grids were washed with water, and the virus particles attached to them were negatively stained with 2% phosphotungstic acid (20) containing *ca.* 0.15% Driwell (Fuji Photofilm). The length of particle images was measured at a final magnification of × 200,000.

***In Vitro* Translation of CGMMV Short RNA.** Wheat germ S30 extract was prepared as described by Marcu and Dudock (21), except that potassium chloride was replaced by potassium acetate. Reaction mixtures contained, in a volume of 50 μl, 1 A_{260} unit of S30, 20 mM Hepes/KOH (pH 7.2), 2 mM dithiothreitol, 3 mM magnesium acetate, 100 mM potassium acetate, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, creatine kinase at 5 μg/ml, each of the 20 amino acids except leucine at 25 μM, 4 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]leucine (37.5 μM), and 2 μg of RNA of CGMMV-W short rod. After 60 min of incubation at 30°C, the translation products were precipitated with 10% trichloroacetic acid either directly or after treatment with antibodies. For immunoprecipitation, purified CGMMV-W protein was added to the reaction mixture to 0.03 mg/ml and was mixed with an excess amount of anti-CGMMV-W immunoglobulin or normal rabbit immunoglobulin in 10 mM sodium phosphate (pH 7.2)/0.85% NaCl/1% Triton X-100/0.4% sodium deoxycholate. The precipitates formed after incubation for 1 hr at 37°C and then standing at 4°C overnight were washed twice by centrifugation through a cushion of 0.5 M sucrose/10 mM sodium phosphate (pH 7.2)/0.85% NaCl/1% Triton X-100/0.4% sodium deoxycholate/1 mM leucine, once with 10% trichloroacetic acid, and then three times with acetone. They were resuspended in gel sample buffer and electrophoresed on 15% $NaDodSO_4$/polyacrylamide slab gels (22, 23). After fixing and staining with Coomassie brilliant blue, the gels were impregnated with sodium salicylate (24) and fluorograms were made on preexposed Kodak X-Omat R x-ray film at −70°C (25).

RESULTS

Location of Assembly Initiation Site on CGMMV-W RNA. Sequential reconstitution of virus rods with the proteins of different strains provided a means to locate the assembly initiation site on RNA of various TMV strains (7, 10). In our attempt to locate the assembly initiation site on CGMMV-W RNA by the same technique, we first started reconstitution with its own protein. Unfortunately, however, the reconstitution intermediates formed with CGMMV-W protein could not be elongated with protein of any other TMV strain tested. Therefore, we started reconstitution with protein of TMV-OM and completed it with protein of TMV-T. The same combination of proteins was successfully used to locate the assembly initiation site on TMV-Cc RNA (10).

The distribution of OM and T proteins on the sequentially reconstituted CGMMV-W particles was studied by electron microscopic serology (7). OM-specific antibody molecules bound specifically to an internal region of the virus rod near its one end. In analogy with the previous findings with other TMV strains (5, 10), this end should correspond to the 3′ terminus of the RNA. In contrast, T-specific antibody molecules bound to the entire span of the virus rod, except the region where OM-specific antibody molecules bound (Fig. 1). This pattern of binding of strain-specific antibody molecules indicated that reconstitution of CGMMV-W also proceeds in two directions starting at an internal site on the RNA near its 3′ terminus, as was the case with other TMV strains (5, 6, 7, 10). Fig. 2 compiles the results of experiments in which various amounts of OM protein were used to start sequential reconstitution and the length of the portion of virus rods encapsidated with this protein was measured. The distance between the assembly initiation site and the 3′ terminus differed between the OM antibody labeling and T antibody labeling as shown in Fig. 2. The actual distance must be the mean values because antibody molecules at the borders of the binding region stick out into the adjacent nonbinding region as described previously (7, 10). The results show that reconstitution starts at a site on viral RNA about 5% of its full length, or 320 nucleotides, from the 3′ terminus. The location coincides with that of TMV-Cc previously studied (10) and hence is probably within the coat protein cistron.

Occurrence of Short Particles of CGMMV-W. Since its identification in 1971, CGMMV-W has been thought to have only 300-nm particles. However, the results described above raised the possibility that CGMMV-W may have short particles too, as does TMV-Cc, which has the same location of assembly initiation as CGMMV-W. All other TMV strains, which have the assembly initiation site outside the coat protein cistron, do not produce short particles.

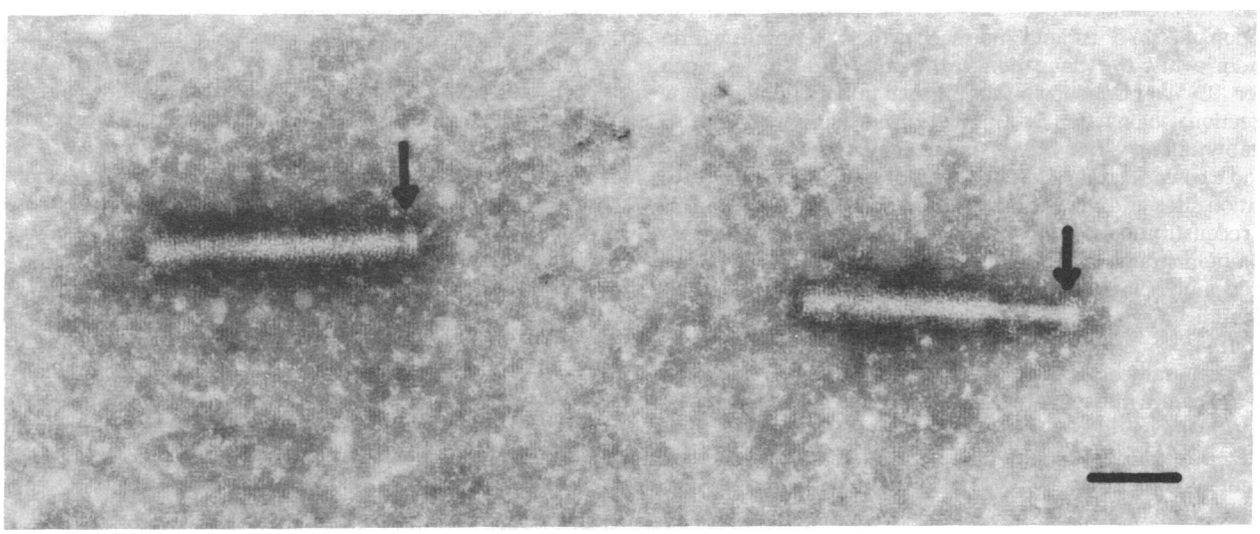

FIG. 1. Virus particle after sequential reconstitution from CGMMV-W RNA, first with OM protein and then with T protein in excess. Particles were treated with T-specific antibody on the electron microscopic grids and were negatively stained with phosphotungstic acid containing Driwell. Arrows indicate the initiation site for reconstitution. Bar represents 100 nm.

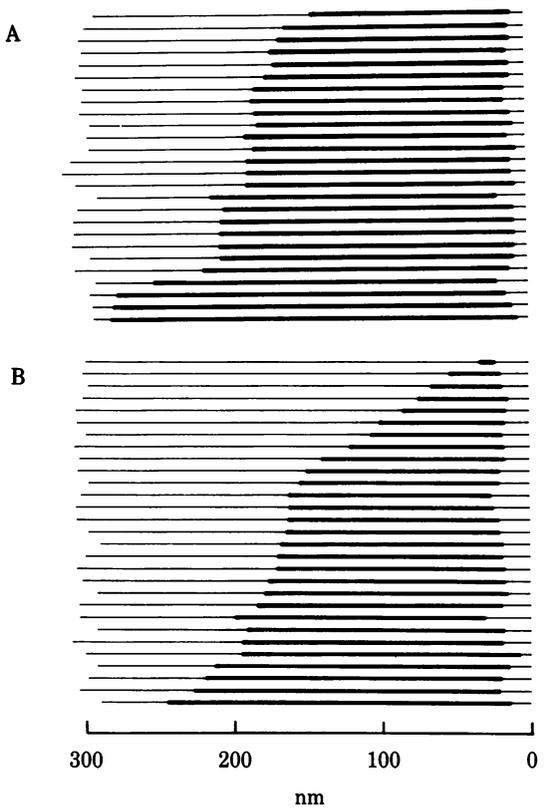

FIG. 2. Diagram of examples of TMV particles after sequential reconstitution from CGMMV-W RNA first with OM protein and then with T protein in excess as described for TMV-Cc RNA (10). Particles were treated with OM antibody (*A*) and with T antibody (*B*). The region encapsidated with OM protein is indicated by the thick lines, and it must be near the 3′ end, because the assembly initiation site should be in the 3′ half of the RNA as in the other strains (5, 10). Rods are arranged with their 5′ ends on the left side. Rods with the length of 300 ± 9 nm were assumed to be fully reconstituted and were selected on electron micrographs, and measurements were made of the length of T protein and OM protein regions.

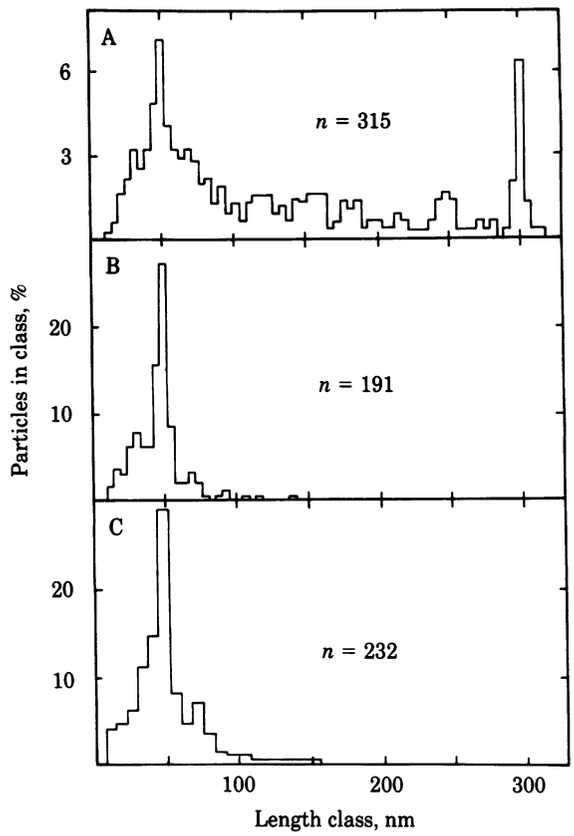

FIG. 3. Length distributions of CGMMV-W particles from the leaf extract (*A*), of the purified short rods of CGMMV-W (*B*), and of the reconstituted particles formed by the incubation of their RNA with CGMMV-W protein at the RNA-to-protein ratio of 1:30 in 0.1 M sodium phosphate buffer (pH 7.2) at 25°C overnight (*C*). Particles were loaded on electron microscopic grids pretreated with (*A*) or without (*B, C*) anti-CGMMV-W immunoglobulin and were negatively stained with phosphotungstic acid containing Driwell. Their length was measured at the nominal magnification of ×200,000 (*A, B*) or ×120,000 (*C*). *n* represents the number of particles measured.

The technique of serologically specific electron microscopy (20) was used to detect short particles of CGMMV-W. Extracts of leaves infected by this virus were placed on electron microscope grids that were precoated with anti-CGMMV-W antibody. Electron microscopic examination of the particles thus retained on the grids revealed the presence not only of the normal virus particles of 300-nm length but also of shorter rods with a modal length of 50 nm (Fig. 3*A*). The same technique applied to extracts of *Phaseolus vulgaris* infected by TMV-Cc showed the presence of short rods of 35 nm as reported previously (13, 16, 26), whereas no short rods were detected in extracts of tobacco leaves infected by TMV-OM or TMV-T. The results show that CGMMV-W in fact produces short rods *in vivo*. The length of CGMMV-W short rods is significantly larger than that of TMV-Cc, as is also confirmed by comparing the size of the RNA extracted from these rods (data not shown).

Short rods of CGMMV-W were purified from extracts of infected leaves (Fig. 3*B*), and RNA was isolated. When the RNA of short rods was reconstituted *in vitro* with CGMMV-W protein, 50-nm rods were formed (Fig. 3*C*). Protein of TMV-OM could substitute for CGMMV-W protein in reconstitution of the short rods (data not shown).

Translation of RNA from CGMMV-W Short Particles. In TMV-Cc, RNA of short rods directs the synthesis of coat protein in cell-free protein-synthesizing systems (13, 14). Experiments were therefore performed to see if this is the case in CGMMV-W also. When added to the wheat germ cell-free protein-synthesizing system, the RNA of CGMMV-W short particles induced the incorporation of [^{3}H]leucine into three species of polypeptides (Fig. 4, lanes A and B), of which a polypeptide with the highest radioactivity comigrated with CGMMV-W coat protein. When the products of *in vitro* translation were treated with antibody against CGMMV-W and the immunoprecipitate was analyzed by gel electrophoresis, only the polypeptide with the mobility of CGMMV-W coat protein was detected (Fig. 4, lane C). This product was not formed in the absence of RNA of short rods (Fig. 4, lane E) and was not precipitated with normal rabbit serum (Fig. 4, lane D). These results show that the RNA of the short particles of CGMMV-W contains the message for coat protein. The nature of the two other products of *in vitro* translation is not clear at present; they do not appear to contain coat protein sequences, because they are not precipitated by anti-CGMMV-W antibody (Fig. 4, lane C).

DISCUSSION

Previous studies from our group showed that internal initiation and two-way elongation are the general mechanism of particle assembly in TMV strains (7, 9, 10). The assembly initiation site of CGMMV-W, a member of the TMV group (15), was studied in the present work, and it was found that CGMMV-W is not an exception to this general rule. Furthermore, the present work demonstrated that this virus has the same location of the

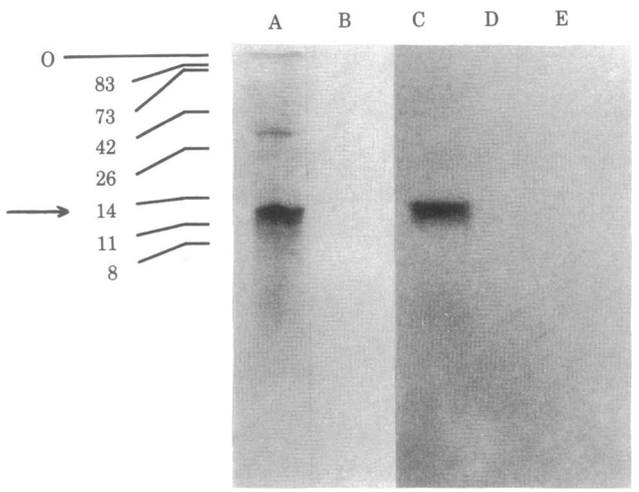

FIG. 4. Fluorograms of polypeptides formed by the incubation of short CGMMV RNA with wheat germ extracts. Lane *A*, products of translation of short CGMMV-RNA; lane B, wheat germ endogenous RNA products; lane C, products of translation of short CGMMV-W RNA precipitated with anti-CGMMV-W immunoglobulin; lane D, products of translation of short CGMMV-W RNA precipitated by immunoglobulin from a nonimmunized rabbit; lane E, endogenous products precipitated with anti-CGMMV-W immunoglobulin. The numbers refer to molecular weight $\times 10^{-3}$ of marker proteins. O and the arrow denote the origin and the position of the CGMMV-W coat protein, respectively.

assembly initiation site as TMV-Cc and produces short 50-nm particles in addition to the 300-nm particles typical of the TMV group.

The cowpea strain has been thought to be a unique virus of the TMV group because of its production of short rods. However, in the present work we found another short-rods-forming virus. Our comparative studies of TMV strains and related viruses indicate that they can be divided into two groups according to the location of the assembly initiation site. The common (OM) and the tomato (T) strains belong to group 1, in which the assembly initiation site is 800 nucleotides away from the 3′ terminus of RNA and hence outside the coat protein cistron, which corresponds to the 3′-distal 700 nucleotides of the viral RNA (8). The coat protein messenger RNA, which makes up the 3′-distal 700 nucleotides, does not contain the assembly initiation site and is not separately assembled *in vivo* with coat protein. Viruses of group 1 are therefore single-component viruses. In contrast, group 2 viruses include TMV-Cc and CGMMV-W, and the assembly initiation site of this group is located much closer to the 3′ terminus of RNA, being only 320 nucleotides away from it. The site falls within the coat protein cistron, and is present also in the coat protein messenger RNA. The group 2 viruses are two-component viruses, because the assembly of coat protein messenger RNA results in the production of short

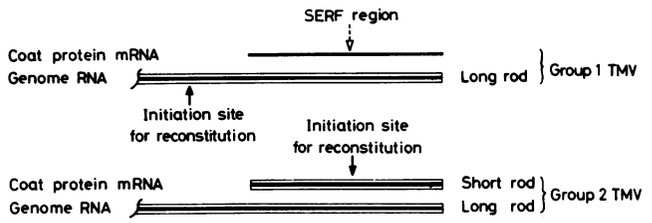

FIG. 5. Schematic representation of correlation between particle multiplicity and the site of assembly initiation. Common strains, tomato strains, and maybe U2 and HR strains belong to group 1. TMV-Cc, CGMMV-W, and CGMMV-C belong to group 2. The drawing is not to scale. SERF, specifically encapsidated RNA fragments.

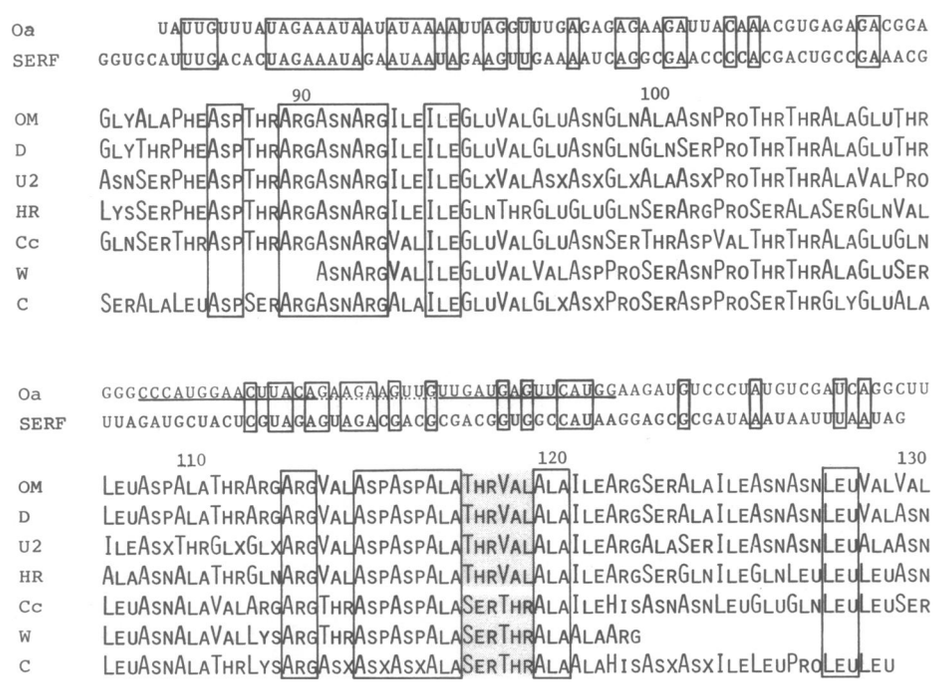

FIG. 6. Comparison of the RNA sequence at the initiation site for assembly with a SERF portion of the coat protein gene of the common strain, and the coat protein sequences of some strains of TMV between amino acids 85 and 130. Oa is the RNA sequence of the initiation site for assembly of a common TMV strain, vulgare (31). The region indicated by solid underline is the hairpin stem and the region indicated by the dotted underline is the loop of the hairpin that is supposed to be necessary for assembly initiation (31). SERF is a portion of the coat protein gene of vulgare (29, 30). Boxes around the RNA sequences show homologous nucleotides in the alignment proposed by Zimmern (31). OM is a common strain used in our study and is very closely related to vulgare (32). D is a tomato strain, dahlemense, closely related to T. W and C represent CGMMV-W and CGMMV-C, respectively. Boxes around the protein sequences show amino acids common to all strains described here. Putative key amino acids for determining the particle multiplicity, 118 and 119, are shadowed. For the amino acid sequences refer to refs. 15 and 32–37.

particles. These relationships are schematically illustrated in Fig. 5. Thus we have added a criterion for grouping of the TMV group viruses: the site of assembly initiation on its RNA, which may lead to production of short rods.

The first step of assembly of TMV particles consists in the interaction of the assembly initiation site on the viral RNA with the 20S aggregates of coat protein (2, 27, 28). Although the initiation site of TMV common strains is outside the coat protein coding region of viral RNA, the same region is known to have some affinity to the 20S aggregate. T1 RNase digestion of viral RNA thus led to the isolation of "specifically encapsidated RNA fragments" (SERF) derived from the coat protein cistron (29, 30). Zimmern (31) pointed out that the portion of the coat protein cistron represented by these fragments has extensive sequence homology with the authentic assembly initiation site (Fig. 6). Based on this observation, he put forward a hypothesis that the authentic assembly origin of the common strain is a duplicate of an ancestral assembly initiation site that is located within the coat protein cistron and that is no longer used by some strains. In support of this hypothesis we showed that two TMV strains (OM and T) of group 1 do not use this ancestral site (7, 10). Moreover, our previous work (10) and the present work established that the group 2 viruses still use the initiation site within the coat protein cistron; the location of the functional assembly initiation site in these viruses (320 nucleotides from the 3′ terminus) closely agrees with that of the cryptic initiation site of the common strain [300 nucleotides from the 3′ terminus (8)].

While the nucleotide sequence of the RNA of TMV common strain is known in some detail (8), little information is available about the primary structure of the RNA of other strains. Consequently, we do not know what kind of base exchanges are responsible for determining whether the ancestral initiation site is functional or not. However, such exchanges must be reflected in the primary structure of the coat protein, because this site is within the coat protein coding region. Fig. 6 compares seven strains of TMV with respect to the amino acid sequence of a segment of the coat protein polypeptide that corresponds to part of the SERF region of the RNA. According to Zimmern (31) the stable hairpin structure of RNA required for the interaction with the 20S protein aggregate corresponds to the portion of the SERF region coding for amino acids 109–124 of the coat protein. Analyses of TMV particles by using x-ray and other physicochemical techniques indicate that amino acids 113–122, as well as amino acids 87–94, represent the regions of coat protein polypeptide responsible for the binding to viral RNA (38, 39). As is shown in Fig. 6, the sequences between amino acids 87 and 94 and between 113 and 122 are highly conserved among TMV strains. However, among them, some amino acids are characteristic for the subgroups mentioned above. For example, 118 and 119 are threonine and valine, respectively, in four strains including two from group 1 (OM and D), whereas they are serine and threonine, respectively, in three other strains of which two are from group 2 (TMV-Cc and CGMMV-W). Certain combinations of base sequences specifying the amino acids characteristic for group 2 viruses, such as amino acids 118 and 119, may well affect the secondary structure of the RNA and thus the function of the ancestral initiation site.

Assuming that the above hypothesis is correct, some prediction can be made of particle multiplicity of the TMV group. Because CGMMV-C has the same amino acids at 118 and 119 as the group 2 viruses, the initiation site should be in the coat protein cistron and it should have short particles. Extracts of cucumber leaves infected with CGMMV-C were examined by serologically specific electron microscopy for the presence of short particles, and they were in fact present (unpublished results). On the other hand, in U2 and HR, assembly probably should start at the same site as in TMV OM and T and no short particles should be produced.

We thank Dr. J. Hashimoto of Institute for Plant Virus Research for his suggestions about the *in vitro* protein synthesizing system. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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