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Functions of the Two Particles of Tobacco Rattle Virus

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Functions of long and short particles of five different tobacco rattle virus (TRV) systems were studied by complementation experiments with the corresponding long and short species of ribonucleic acid (RNA). The progeny of long RNA species alone was proteinless or "free" infectious long RNA, whereas short RNA species alone did not replicate by themselves but appeared to be dependent on long RNA for replication. When both types of RNA derived from the same isolate were inoculated together, particulate virus with long and short particles was produced in more than 50% of the resulting primary infections. These virus systems obtained by homologous complementation resembled the parent isolates in all their characteristics. In addition, heterologous complementation tests were performed with long and short RNA, each derived from another isolate. Heterologous interaction could be observed in only 2 out of 20 possible combinations. As a result, two "mixed" TRV systems with respect to their particle length distributions were obtained, since their long and short particles resembled the ones from the other isolate. The symptoms produced by these mixed viruses were determined by the corresponding long RNA and appeared not to be influenced by the heterologous short one. However, the protein coat of both particles of the "mixed" viruses was specified by the corresponding noninfectious short RNA. Therefore, TRV is a system of at least two functionally defective and mutually complementing components which appear to be specialized in early and late functions.

Tobacco rattle virus (TRV) is a tubular plant virus consisting of at least two elements: infectious "long" particles and noninfectious "short" particles (8, 18). Thus, it may be considered a multicomponent virus system (20). Recent work has demonstrated that these stable particles of the "complete" TRV system (C-TRV) can only be synthetized through the synergistic interaction of their functionally defective "long" and "short" ribonucleic acid (RNA) (5, 13, 15, 21). The infectious long RNA appears to be defective in coat protein production (13) and its progeny is proteinless or "free" infectious RNA (22). This "naked" progeny virus will be called incomplete or "defective" TRV (D-TRV). Noninfectious short RNA, on the other hand, appears to be defective in RNA replication and is apparently dependent on long RNA for its own replication. Furthermore, it was shown that the short particles or their short RNA must be in some way involved in the production of the viral protein coat for both particles of C-TRV (5, 13, 15).

The interaction in the C-TRV system has been called complementation, because its two species of RNA are functionally defective in different cistrons, each of which is required for a complete cycle of viral replication (20, 21). Complementation tests have successfully been employed to study the functional interaction of bacterial viruses (10). This report describes a series of complementation experiments undertaken to determine the functions of the components of the TRV system. It presents evidence that coat protein specification is a function of noninfectious short RNA of TRV.

MATERIALS AND METHODS

Viruses. The properties and the origin of the five different C-TRV isolates used in this study are summarized in Table 1. Pea early browning virus (PEBV; 3) can be regarded as a strain of TRV because it exhibits all the essential properties of the TRV system. Its restricted host range and its distant serological relationship (1, 2, 3, 16) do not appear to justify its classification as a separate virus.

Propagation and isolation of viruses. The C-TRV strains were propagated in *Nicotiana tabacum* L. var. *Xanthi-nc*, and for pea early browning virus *N. clevelandii* Gray was used as host plant. The leaves

were harvested 6 to 8 days after inoculation. Further steps of purification were essentially as previously described (21), but in place of the borax solution, 10 ml of a 1.0 M sodium citrate solution was added per 100 ml of sap. Furthermore, 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 8.0) containing 0.05 M ethylenediaminetetraacetic acid (EDTA) and 0.01 mercaptoethanol (TEM buffer) was used to suspend the concentrated and purified viruses throughout all experiments.

Purification of short particles. The population of short particles to be used in complementation tests was freed from long particles by four serial centrifugations in 10 to 40% sucrose gradients, and isolated from each gradient with the aid of a tube slicer, as described previously (21). The isolated virus was dialyzed against TEM buffer.

Preparation of RNA. Short RNA was extracted from solutions of highly purified short virus particles with water-saturated phenol and precipitated three times with ethyl alcohol in the cold. It was resuspended in TEM buffer, made to final optical density $(OD)_{260}$ per ml of 0.05, and stored in 0.2-ml portions at −30 C until use.

Preparation of D-TRV. D-TRV and D-PEBV cultures were originally obtained by transfer of single lesions produced by highly dilute inocula of long particles alone. This "proteinless" long RNA was propagated continuously in the corresponding host plants. Highly infectious and partly purified preparations were prepared by homogenizing batches of 50 g of infected leaves in the cold in 200 ml of TEM buffer containing 20 mg of bentonite per ml. The homogenate was clarified for 10 min at 10,000 × *g*, and the resulting supernatant was stirred with 5 mg of bentonite per ml for 5 min. Upon clarification, D-TRV was precipitated from the solution with ethyl alcohol, resuspended in TEM buffer, diluted to produce about 100 local lesions per leaf, and stored in portions at −30 C until use.

Infectivity assay. All infectivity assays and inoculations were performed with micro-amounts of inoculum which were kept in an ice bath during the inoculation procedure. Small droplets (ca. 0.05 ml) of the corresponding solutions were placed on inverted glass spatulas with Pasteur pipettes. The spatulas were tilted and the hanging droplet was inoculated on leaves of tobacco (*N. tabacum* L. var. *Xanthi-nc*) and bean plants (*Phaseolus vulgaris* L. var. *Prelude*) dusted with a mixture of four parts of carborundum and one part of bentonite (w/w). For each preparation, 10 leaves of both test plants were used, and the inoculated leaves were rinsed immediately with running tap water. The plants were kept at about 20 C, and the local lesions produced were counted 7 days after inoculation. In some series, *Chenopodium amaranticolor* Coste & Reyn. was included as a test plant.

Differentiation procedure for C-TRV and D-TRV lesions. The nature of the virus (C-TRV or D-TRV) synthetized in local lesions was determined in infectivity tests after these lesions had been subjected to the modified pretreatment of Lister (13), as previously described (21). This procedure clearly differentiates C-TRV lesions from D-TRV lesions. In contrast to particulate and stable C-TRV, "free" infectious long RNA in D-TRV lesions is usually inactivated completely after freezing and thawing followed by incubation of the homogenate at room temperature. Accordingly, when pretreated lesions produced up to nine lesions per leaf, they were D-TRV lesions; when they produced more lesions (usually about 50 to 100), they were C-TRV lesions in which complementation had taken place.

Complementation tests. For the complementation experiments, the corresponding D-TRV inocula and the solutions of short RNA were used alone or in combination. Equal volumes (0.5 ml) of the components alone with buffer, or of both components together, were thoroughly mixed and immediately inoculated onto 10 tobacco leaves. For all combinations with PEBV, bean leaves were also used. The infectivity of the final D-TRV inoculum was about 50 lesions per leaf, which greatly increased the chances for possible complementation. The final concentration of short RNA was 0.025 OD_{260} per ml. To determine the relative numbers of D-TRV and C-TRV lesions produced by these inocula, 50 of the resulting local lesions were randomly selected and individually tested by the differentiation procedure.

Serology. Antisera for C-TRV were prepared and agar-gel diffusion tests were performed as previously described (21). Absorbed antisera were obtained by adding the corresponding antigens in increments until there was no more precipitation. Before use, the absorbed antisera were centrifuged for 2 hr at 140,000 × *g* to pellet any nonadsorbed virus.

Electron microscopy. Mounts and measurement were made as previously described (21). For plotting the frequency distributions, arbitrary length categories of 5 nm were adopted and about 600 particles were measured for each isolate.

RESULTS

Properties and genetic markers of virus systems. When the five viruses used were freshly prepared and centrifuged in sucrose gradients under the conditions described, all of them produced two separate and well-defined bands, which is in agreement with previous work (8, 9). The population of the top band comprises noninfectious short particles, the population of the bottom band consists of infectious long particles (*see* Fig. 2). The differences between the isolates are most pronounced in the lengths of their short particles and less so in the lengths of their long ones. However, the occurrence of additional particle populations producing bands has often been reported (9, 23, 24). I found that such additional particle populations may arise from subsequent aggregation or breakage of either or both of the long and short particles. This occurs to a varying extent upon aging of purified virus preparations or upon the use of certain buffers for certain isolates. For this reason, phosphate and borate buffers were abandoned and replaced by TEM buffer (H. L.

Sänger, *unpublished data*). The properties of such experimentally produced populations of particles which may form additional bands have not been studied as yet.

The genetic markers of the virus systems are (i) the lengths of the particles, (ii) the morphology of the local lesions on test plants, and (iii) the specificity of the viral coat protein. Particle length measurements (Table 1) suggested that the subcultured isolates had long and short particle modal lengths within acceptable variation (10%) of previously published values (9). The lengths of these particles are determined by the lengths of the corresponding RNA; therefore, they are reflected in the particle-length distributions of the corresponding isolates. The histograms of two natural isolates, C-GER and C-USA, are shown in Fig. 1a and b.

The morphology of the local lesions on tobacco (*see* Table 1) is determined solely by infectious long RNA and appears not to be influenced by the presence of the short particles. It is, therefore, a suitable marker for the type of long RNA present in the inoculum. The most obvious and valuable differences in lesion morphology exist between the TRV isolates and PEBV-N on bean leaves (Table 1). Here, all TRV isolates produce pinpoint lesions on bean, whereas large ring-type lesions are produced by D-PEBV-N as well as by C-PEBV-N. With this isolate, no difference in symptoms between both forms was observed as described for another isolate, PEBV-B (14, 15). On tobacco leaves, the isolate USA can be clearly distinguished from all other isolates by its large and fully necrotic lesions. The differences in lesion morphology on tobacco between these isolates are somewhat influenced by the age and the physiological condition of leaves and by temperature. Selection of homogeneous plant material and control of temperature (20 ± 1 C) were found to guarantee good reproducibility of the primary symptoms.

The D-TRV cultures used in these experiments correspond to the so-called "unstable isolates" of TRV (4, 13). The infectious entity of D-TRV as extracted with bentonite is largely identical to the single-stranded long RNA extracted from long virus particles with the aid of phenol and occurs in infected tissue as free infectious RNA (H. L. Sänger, *unpublished data*). It produces persistent viral infections, and its primary infections on tobacco are morphologically indistinguishable from C-TRV lesions. The secondary infections of D-TRV differ significantly; they are much more necrotic and severe than the mild symptoms of systemic C-TRV infections. However, this difference cannot be used as a marker because secondary D-TRV infections, if they develop at all, do so extremely slowly and erratically. Particulate virus is distributed within the plants quite rapidly through the conducting elements of the vascular system and produces

TABLE 1. *Sources and properties of TRV, including PEBV*

Virus isolate	Source	Predominant particle length (nm)		Lesion morphology on		Supplied by
		Short	Long	Bean[a]	Tobacco[a]	
GER	Hessia, Germany	70	180	Pinpoint necrotic spots	Necrotic rings	Local isolate (21)
USA	Oregon, U.S.A.	105	195	Pinpoint necrotic spots	Fully necrotic spots	T. C. Allen, Jr., Corvallis, Oregon, U.S.A. (1, 2)
CAM	Campinas, Brazil	52	195	Pinpoint necrotic spots	Chlorotic rings	B. D. Harrison ex A. S. Costa, Campinas, Brazil (9)
BEL	Suffolk, England	90	185	Pinpoint necrotic spots	Fully necrotic spots	B. D. Harrison ex K. M. Smith, Cambridge, England (9)
PEBV-N	The Netherlands	105	210	Large necrotic rings	Faint chlorotic rings	L. Bos, Wageningen, Netherland (3)

[a] *P. vulgaris* L. var. *Prelude* and *N. tabacum* L. var. *Xanthi-nc*.

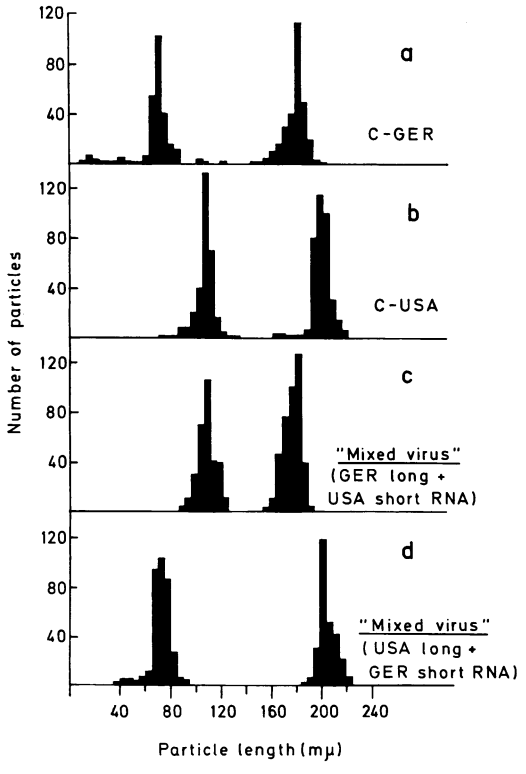

FIG. 1. *Particle-length distributions of two naturally occurring and two experimentally produced and mixed TRV systems.*

systemic infections within about 2 weeks. The free infectious RNA of D-TRV, however, invades tobacco plants by spreading from cell to cell, especially in the vascular parenchyma, and the resulting partly systemic infection may take 3 to 4 months to develop.

Properties of the components. To prevent possible contamination in the complementation tests, initial experiments were concerned with the purification of the components to be used and the determination of their properties.

The absence of homologous short particles or their RNA in preparations of long RNA was assured by using inocula from continuous cultures of D-TRV. Neither short particles nor short RNA was detected in D-TRV cultures. Moreover, their absence was reflected by the persistence of the defectiveness of D-TRV. After 20 passages, not a single D-TRV culture had reverted back to particulate C-TRV.

The fractions of short particles, on the other hand, were effectively freed from long particles by four serial centrifugations in sucrose gradients under the conditions described. The yield was only about 2 to 5% refined short particles in the final preparations. The infectivity of these fractions ranged between 5 and 10 local lesions per tobacco leaf at a concentration of 1.0 OD_{260}. The short RNA to be used in the complementation usually produced no lesions at a concentration of 0.025 OD_{260}, and appeared, therefore, virtually free from long RNA (Table 2).

Homologous complementation. The interaction between the components of the same isolate may be called homologous complementation. Complementation experiments with components from the same isolate were carried out to test the complementing activity of the components to be used later in heterologous tests. The results of a series of homologous tests and of the corresponding controls are shown in Table 3. All of the local lesions produced by D-TRV plus buffer were also D-TRV lesions. Because of the complete absence of homologous short RNA in these inocula, particulate C-TRV could evidently not be produced. When, on the other hand, mixtures of D-TRV and its homologous short RNA were inoculated, particulate C-TRV was synthetized in more than 50% of all the local lesions formed. The genetic markers of five experimentally produced isolates were investigated for each combination. It was found that all of them were indistinguishable from the parent culture in particle distribution, lesion morphology, and serological behavior (Table 3). These results reinforce those of previous reports (5, 13, 15) and substantiate the participation of short RNA in coat protein production for both particles.

Heterologous complementation. It has been proposed that short RNA might carry the information for the coat protein of the C-TRV particles (5, 13, 15). This hypothesis was tested by

TABLE 2. *Infectivity of long and short RNA as used for complementation*

Virus isolate	Infectivity[a]	
	Free infectious long RNA[b]	Short RNA[c] (0.025 OD_{260} units/ ml)
GER	42	0
USA	54	0
CAM	61	0
BEL	38	1
PEBV-N	47[d]	0[d]

[a] Number of lesions per leaf calculated from 10 tobacco leaves.

[b] Extracted from continuous D-TRV cultures with bentonite; partly purified and concentrated by ethyl alcohol precipitation.

[c] Extracted from highly purified short particles.

[d] Number of lesions per leaf calculated from 10 bean leaves.

TABLE 3. *Complementation between homologous long and short RNA of different tobacco rattle viruses*

Inoculum (v/v)[a]		Result of complementation	
Source of long RNA	Source of short RNA (or buffer)	Lesions containing C-TRV[b]	Nature of C-TRV progeny[c]
		%	
D-GER	Buffer	0	
	C-GER	64	C-GER
D-CAM	Buffer	0	
	C-CAM	72	C-CAM
D-USA	Buffer	0	
	C-USA	54	C-USA
D-BEL	Buffer	0	
	C-BEL	66	C-BEL
D-PEBV-N	Buffer	0	
	C-PEBV-N	52	C-PEBV-N

[a] For infectivity of the component solutions, see Table 2.

[b] Based on the test of 50 randomly selected local lesions after the differentiation procedure.

[c] Based on sedimentation profile, particle length distribution, serology, and symptoms of five different C-TRV cultures selected for each combination.

heterologous complementation experiments. In such tests, the proteinless progeny of the long RNA (= D-TRV) of one isolate was inoculated together with the short RNA of another isolate, and the nature of the resulting progeny virus was determined. The key marker in this test was the specificity of the coat protein of the particulate virus produced, which was determined serologically. The results of a series of such experiments are summarized in Table 4. Complementation between heterologous components was limited to 2 out of the 20 possible combinations tested. Only the long and the short RNA of the isolates GER and USA, respectively, complemented each other to produce "mixed" systems of particulate C-TRV. There was no apparent sign of heterologous interaction producing particulate C-TRV in any other combination tested.

Analysis of the mixed viruses. Individual local lesions containing C-TRV were used to propagate and characterize the virus systems produced by heterologous complementation. Together with the parent viruses, they were analyzed for their properties and the genetic markers introduced by their components. The distribution of lengths of particles for the four relevant virus systems is shown in Fig. 1; their sedimentation profile, morphology of their lesions, and their types of coat protein are shown diagrammatically in Fig. 2. The two parent isolates, C-GER and C-USA, which resulted from homologous complementation, were well characterized by their specific markers (Fig. 1a and b; Fig. 2). However, when USA-long RNA was complemented with GER-short RNA, the resulting mixed C-TRV was composed of USA long (195 nm) and GER short (70 nm) particles (Fig. 1d). The local lesions produced by this mixed virus were fully necrotic spots, as determined by the USA-long RNA (Fig. 2). This experimentally produced isolate was serologically indistinguishable from the isolate

TABLE 4. *Complementation between heterologous long and short RNA of different tobacco rattle viruses*

Inoculum (v/v)[a]		Result of complementation	
Source of long RNA	Source of short RNA	Lesions containing C-TRV[b]	Nature of C-TRV progeny[c]
		%	
D-GER	C-CAM	0	
	C-USA	40	Mixed C-TRV
	C-BEL	0	
	C-PEBV-N	0	
D-CAM	C-GER	0	
	C-USA	0	
	C-BEL	0	
	C-PEBV-N	0	
D-USA	C-GER	32	Mixed C-TRV
	C-CAM	0	
	C-BEL	0	
	C-PEBV-N	0	
D-BEL	C-GER	0	
	C-CAM	0	
	C-USA	0	
	C-PEBV-N	0	
D-PEBV-N	C-GER	0	
	C-CAM	0	
	C-BEL	0	
	C-USA	0	

[a] For infectivity of the component solutions, see Table 2.

[b] Based on the test of 50 randomly selected local lesions after the differentiation procedure.

[c] Based on sedimentation profile, particle length distribution, symptoms, and serology of 10 different C-TRV cultures selected for each combination (for details, see Fig. 1 and 2).

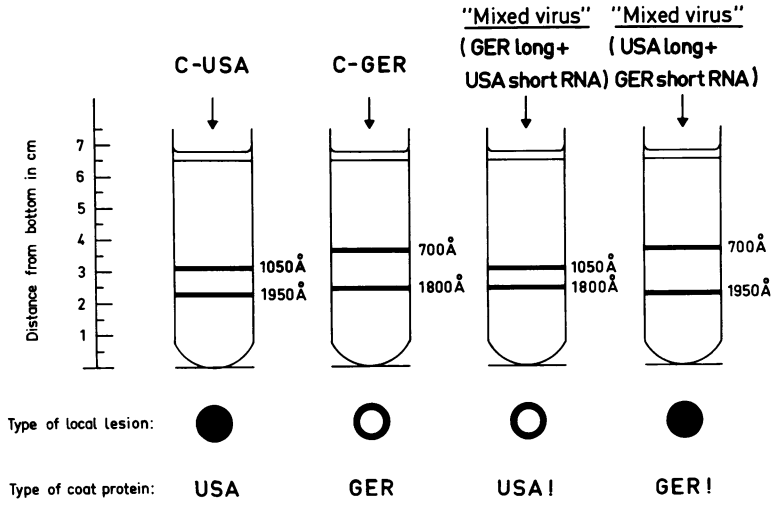

FIG. 2. *Sedimentation profiles, lesion morphology, and coat protein specificity of two naturally occurring and two experimentally produced and mixed TRV systems. The banding of the particle population is shown as it occurs in a 10 to 40% sucrose gradient [prepared in 0.1 M Tris hydrochloride buffer (pH 8.0) containing 0.01 M EDTA and 0.001 M mercaptoethanol] after 120 min at 24,000 rev/min in the tubes of an SW25.1 rotor. The type of local lesions shown is produced on leaves of Nicotiana tabacum L. var. Xanthi-nc.*

GER from which the short RNA was derived (Table 5). The combination of GER-long RNA plus USA-short RNA, on the other hand, produced mixed virus systems composed of GER long (180 nm) and USA short (105 nm) particles (Fig. 1c). Their local lesions resembled the necrotic rings as determined by GER-long RNA (Fig. 2). Serologically, however, these mixed viruses were indistinguishable from the isolate USA from which short RNA was taken for complementation (Table 5). These results clearly substantiate that the coat protein is specified by short RNA.

Complementation in the opposite direction. An additional series of experiments was carried out to test whether complementation could also be repeated in the opposite direction by using the heterologous species of RNA of the two mixed TRV isolates. The corresponding components were separated and purified, and their RNA was used to complement one of the original homologous components. When short GER-RNA and long GER-RNA, each obtained from the other mixed C-TRV, were inoculated together, an isolate was obtained which resembled the parent C-TRV-GER isolate in all its characteristics. Moreover, long USA-RNA and short USA-RNA obtained from the corresponding mixed TRV isolates complemented each other to produce an isolate identical to the original C-TRV-USA. This showed that it is possible to obtain the parent virus systems again by repeating complementation in the opposite direction. It can be concluded, therefore, that long and short RNA of the TRV system are replicated true to type, that they possess structural and functional autonomy despite their defectiveness, and that a direct genetic interaction (recombination) evidently does not occur between them.

Inability of short particles or short RNA to replicate. The short particles of TRV or their RNA are considered noninfectious because they are unable to produce local lesions or symptoms of systemic infections (8). Moreover, it has been postulated that short RNA is unable to replicate on its own and that it is dependent for its replication on infectious long RNA (5, 13, 15, 21). The possibility of inapparent replication of short RNA alone producing either short virus particles or short RNA has not yet been fully excluded. The following experiments were performed to answer this question. Purified short RNA of the GER-isolate was inoculated on four leaves of each of two young *Xanthi-nc* tobacco plants and on all leaves of four young *N. clevelandii* Gray plants; the latter is an excellent systemic host of TRV. The plants showed no signs of local or systemic infection after 2 weeks. Sap was prepared from the pooled half-leaves of the six plants. To detect the presumed virus particles, it was clarified and concentrated 200-fold by the centrifugation procedure used for purifying particular C-TRV. However, no infection was found when this preparation was tested on tobacco leaves and no tubular virus particles could be observed after examination with an electron microscope.

TABLE 5. *Cross-absorption tests with TRV antisera*[a]

Antiserum	Antigen used for absorption	Antigen			
				Mixed viruses	
		C-GER	C-USA	(GER long + USA short RNA)	(USA long + GER short RNA)
Anti-GER	C-GER	—	—	—	—
	C-USA	+	—	—	+
	Mixed TRV (GER long + USA short RNA)	+	—	—	+
	Mixed TRV (USA long + GER short RNA)	—	—	—	—
Anti-USA	C-GER	—	+	+	—
	C-USA	—	—	—	—
	Mixed TRV (GER long + USA short RNA)	—	—	—	—
	Mixed TRV (USA long + GER short RNA)	—	+	+	—

[a] Positive reaction (line of precipitation), +; no reaction, —.

Although no short virus particles were found, the possibility existed that short RNA might have been produced. Therefore, the total RNA was directly extracted from the other pooled half-leaves with phenol and concentrated 200-fold. Short RNA would be present in the phenol extract if its own replication were induced. Its presence could then be tested by the use of complementation tests. Accordingly, the RNA extract was inoculated simultaneously with its homologous infectious long GER-RNA which had been prepared from the corresponding continuous D-TRV-GER cultures. The latter was used at a concentration to produce about 100 local lesions per leaf in order to increase the chances for possible complementation. From the local lesions developing after inoculation with the mixture, 50 were randomly selected and individually tested for the occurrence of C-TRV infections. In addition, all of the remaining lesions were pooled, homogenized, and used for inoculation. All local lesions tested contained only D-TRV. The absence of any C-TRV infections indicates that there was no short RNA introduced into the complementation mixture. These results confirm the hypothesis that short RNA is unable to initiate its own replication and to produce particulate virus by itself and that its replication is dependent on a function(s) provided by replicating long RNA.

Failure of unilateral complementation. In all cases in which heterologous complementation failed to produce a complete and biparticulate C-TRV system (Table 4, last column), the possibility of a unilateral interaction between the heterologous components existed. Under this condition, the short RNA might have been replicated with the help of the long one and, consequently, initiated coat protein synthesis. However, because of conformational differences, the coat protein synthetized might not have assembled with the heterologous long RNA, which then might have remained naked. One might assume that in this case the replicated short RNA would assemble with its homologous coat protein. Reasoning along these lines prompted the search for noninfectious short virus particles in all cases where stable (long) infectious virus particles could not be detected in the infectivity test (*see* Table 4). Sap was prepared from the pooled leaves of each series after the local lesions had been taken for infectivity tests. It was clarified, concentrated 200-fold by centrifugation, and examined with an electron microscope. In none of the combinations could any short virus particles alone be observed. This result indicates that unilateral complementation is rather unlikely to occur in heterologous systems.

DISCUSSION

The experiments described here reveal specific functions for RNA of the long and short particles of the TRV system. Noninfectious short RNA specifies the coat protein which assembles with long and short RNA of TRV to form the two characteristic particles of this system. Short RNA is evidently unable to induce its own replication, but it appears that replicating long RNA aids the replication of short RNA. One might assume that the RNA replicase induced by long RNA is also used to replicate short RNA. The complementing interaction described appears to exist in all TRV isolates investigated so far, which indicates a common function for long and short RNA of all TRV systems.

The complex participation in the process of viral replication by two characteristic components of a virion population has appeared in recent work with both plant and animal viruses. It is

known to occur in the system of tobacco necrosis virus, satellite virus (12), and it has recently been found in the multicomponent systems of alfalfa (lucerne) mosaic virus (26, 27) and cowpea mosaic virus (11). Comparable conditions are known in the complex "helper virus" systems of adenovirus-SV40-PARAvirus (19). Among all these viruses, TRV is unique because it is a system of two functionally defective and mutually complementing RNA species which appear to be specialized in early and late functions, respectively.

The function of short RNA was elucidated by heterologous complementation tests. This became possible when systems were found with components of suitable and contrasting genetic markers and complementation capacities in heterologous combinations. The two new viruses produced experimentally are mixed TRV systems, and the long particles of these mixed systems resemble phenotypically mixed virus because they are wrapped in a heterologous protein coat. Previous investigators also attempted heterologous complementation, but the actual function of the short RNA could not be delineated because the resulting progeny had no clearly identifying properties (5, 15, 25). The function of long RNA has been deduced by indirect and circumstantial evidence. Further work is necessary to substantiate this hypothesis by a more direct approach, if possible. The phylogenetic relationship between the two species of RNA cannot be assessed from the experiments described, and the possible interpretations (20, 21) are still purely speculative.

It is interesting that the size of long and short RNA molecules is several times that required for the two functions detected (15, 20, 21). It would appear, therefore, that several more viral functions might be found for the two species of RNA. There are, in fact, indications that the RNA of the short particles of PEBV may contain information that could affect symptomatology (14), but this evidently does not apply for short RNA of the two mixed TRV systems used in these investigations.

It was found that homologous complementation as it occurs in wild-type systems can be reproduced experimentally without difficulty. Complementation between heterologous components, however, could be demonstrated only experimentally between the RNA species of two of the five TRV systems tested. In all cases where heterologous complementation did not occur, only defective long RNA was found. The absence of short RNA or short virus particles in the progeny of such combinations indicates that short RNA was not replicated after mixed inoculation. Moreover, short RNA codes for the coat protein; therefore, it is very likely that the absence of short RNA is the factor which limits the production of particulate virus in these systems. It is postulated that the failure in complementation in certain combinations is primarily due to the inability of long RNA to aid replication of short RNA. It has been conjectured that RNA replicase induced by infectious long RNA is also used to replicate noninfectious short RNA (5, 21). Assuming that this is so and that the template specificity of this enzyme is controlled by a recognition mechanism similar to RNA replicases of bacteriophages Qβ and MS-2 (6, 7), the following prediction can be made. In all combinations in which complementation does occur, a partial sequence homology must exist between the corresponding long and short RNA. On the other hand, in all cases in which complementation does not operate, short RNA should be different from long RNA in the sites of recognition which must be reflected in a lesser degree of sequence homology. This prediction is open to experimental test.

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