Relationships Between Strains of Tobacco Mosaic Virus and Other Selected Plant Viruses

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ABSTRACT


Sixteen strains of tobacco mosaic virus (TMV) and 17 strains from nine other plant virus groups were compared for nucleotide sequence homology by RNA-complementary DNA hybridization. The two subgroups of TMV having capsid proteins that are the most alike were found to have approximately 15% of their genomes in common, but no sequence homology between the other subgroups could be detected by this test. Differences were not detected between the nucleotide sequences of strains within a TMV subgroup, nor was sequence homology found among three comoviruses or between two potyviruses.

Many viral isolates from diseased plant tissues have been classified as strains of tobacco mosaic virus (TMV) because they share morphological properties and exhibit serological cross-reactivity (38). A number of studies have compared and categorized these strains on the basis of biological, physical, and chemical attributes related to similarities and differences of the capsid proteins (7,8,10,18,29,31,33,34,38,39). By these criteria, the strains fall into natural subgroups with few or no differences detectable between strains within a subgroup except for symptomatology. Easily detectable differences exist between these subgroups. The amino acid sequences of the capsid proteins of five TMV strains have been determined (30,40) and they clearly resemble each other. The proteins of U1 and dahlemense differ from each other in 18% of their amino acid positions; the U2 strain differs from both of these by 30% and the amino acids of cowpea and Holmes ribgrass differ from these and each other in 60% of the positions. Yet, their nucleic acids show no homology in competition-hybridization tests (37,42). Rabbit and mouse globins differ to about the same extent as U1 and dahlemense capsid protein, but in this case, considerable homology has been detected between their genes (12).

To explore this phenomenon further, the method of hybridization of RNA to complementary DNA (cDNA) was employed to analyze the relationships between TMV and other viral genomes.

MATERIALS AND METHODS

Virus strains. Strains of several plant viruses were obtained from a number of sources. Strains of TMV (U1, U2, U6, dahlemense, and cowpea) in addition to tobacco etch virus (TEV), broom mosaic virus (BMV), and tobacco rattle virus (TRV), are maintained in this laboratory for ongoing studies. All other strains were obtained as either viral inoculum, isolated virus, or purified RNA. C. A. Knight of the University of California, Berkeley, provided the Berkeley isolate of cucumber virus 4 (BCV-4), the Japanese isolate of cucumber virus 3 (JCV-3), the Czech isolate of cucumber virus 4 (CCV-4), the yellow and green tomato atypical mosaic viruses (YTAMV and GTAMV), yellow aucuba mosaic virus (YA), Holmes' ribgrass (HRG), orchid virus 06-67, and J14D1. A. O. Lana of the University of Massachusetts donated the isolate of TMV in an infected leaf.

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TMV strains U1, U2, U6, Ash, and dahlemense were grown in Nicotiana tabacum cv. Samsun. The doleicose strain was multiplied in cowpeas (Vigna sinensis). The cowpea strain of TMV was maintained in kidney beans (Phaseolus vulgaris). The different strains of TMV were purified from infected tissue by the method of Hari et al (13) or by an older technique of alternating polyethylene glycol (PEG) precipitations and low speed centrifugations (14).

Two members of the potyvirus group were included in this study. TEV, from our laboratory, and potato virus Y (PYV), supplied as infected tissue by A. O. Jackson, Purdue University, were both grown in N. tabacum cv. Xanthi n.e. They were purified by PEG precipitation and CsCl centrifugation (13).

Potato virus X, the only member of the potexvirus group that was tested, was obtained as infected tissue from Stan Pierpoint, Rothamsted Experimental Station, Harpenden, England. It was grown in N. tabacum cv. Xanthi n.c. and purified by alternating PEG precipitation and low speed centrifugations.

The CAM isolate of TRV was used in this study as a representative of the tobravirus group. It was maintained in the tobacco hybrid Christie (5) and purified by the method of Lister and Bracker (22).

Three strains of the comoviruses were investigated in this study. Cowpea mosaic virus strains SB (CPMV-SB) and DG (CPMV-DG) and broadbean true mosaic virus (EAMV) were obtained as purified virus preparations from George Bruening, University of California, Davis.

Three strains of the type member of the nepovirus group, tobacco ringspot virus (TRSV), were tested in this study. WS-TRSV was obtained as purified virus from I. R. Schneider, Beltsville Agricultural Research Center. An isolate from New Jersey (NJ-TRSV) and an isolate from tomatoes (TOM-TRSV) were obtained as infected tissue from A. F. Murant, Scottish Horticultural Research Institute, Invergowrie, Scotland. NJ-TRSV and TOM-TRSV were grown in N. rustica and purified by alternating PEG precipitation and low-speed centrifugation.

BMV of the bromovirus group was maintained in barley (Hordeum vulgare) and was purified as described (1).

Besides the type member, turnip yellow mosaic virus (23), three other viruses of the tymovirus group were included in this study. Eggplant mosaic virus, okra mosaic virus, desmodium yellow mosaic virus, and the type member were gifts of R. E. F. Matthews, University of Auckland, New Zealand. These viruses were received as purified preparations.

Freeze-dried alfalfa mosaic virus (AMV) RNA was a gift of L. van Vloten-Doting, the State University of Leiden, The Netherlands, as was tobacco streak virus of the Ilarivirus group.

Purification of viral RNA. The RNA of strains of CPMV were
Hybridization reactions between $^{125}$I RNA and $^3$H cDNA. Reactions in which labeled RNAs were annealed to labeled cDNAs were similar to those described by Kummert and Kettman (21). Two to six nanograms of RNA in 24 µl of hybridization buffer were incubated for 70 hr at 68°C with cDNA (2-25 ng) in 0.5-ml conical polypropylene tubes. Hybridization reactions were terminated on ice. The amount of $^{125}$I RNA formed in a double stranded structure with homologous cDNA was assessed by determining the proportion of total $^{125}$I that had become resistant to ribonuclease (35). Radioactivity was determined in a scintillation gamma system. Each experiment included controls without cDNA and undigested controls. The percent of hybridization was calculated by dividing the ribonuclease-resistant counts by the total counts.

RESULTS

Hybridization kinetics. The hybridization kinetics of each of the cDNA probes to an excess of unlabeled homologous RNA are shown in Fig. 1. Each point is an average of at least three separate determinations. Three to six percent of the cDNA probes were S1 nuclease resistant even at zero Rot. Several attempts to reduce this background radioactivity were unsuccessful. The Rot$_{50}$ value obtained for the TMV strains under our experimental conditions was $1.5 \times 10^{-7}$ mol/sec/L, whereas for T4 and CP it was greater, very nearly $5 \times 10^{-7}$ mol/sec/L.

$^{3}$H hybridization experiments. The results of experiments in which a fixed quantity of $^{3}$H-labeled template RNA was annealed with increasing amounts of homologous $^3$H-labeled cDNA are presented in Fig. 2. The shape of these titration curves, as well as the fact that essentially all of the $^{125}$I became resistant to ribonuclease in the presence of excess cDNA, indicates that virtually all of the sequences in each RNA template were represented in $^{3}$H cDNA (15, 20, 21).

Excess RNA-cDNA hybridizations. Sixteen strains of TMV and 17 virus strains representing nine other RNA plant viruses were compared for nucleotide sequence homology by excess RNA-cDNA hybridization with cDNAs transcribed from four strains of TMV (U1, U2, dahlemense, and BCV-4), T4, and CP. The extent of homology between an RNA and a cDNA probe was measured by determining the amount of radiolabeled cDNA that formed an S1 nuclease-resistant, double stranded structure with the test RNA. The hybridization reaction between heterologous strains was driven to a Rot (0.4 mol/sec/L) at which the homologous reaction was complete, using the data shown in Fig. 1. The results of these tests are presented in Table 1.

Five strains of TMV (U1, YA, J14D1, 06-67, and Ash) unite with U1 cDNA to approximately the same extent as does U1 RNA, indicating extensive homology between these strains and the type strain. These results were confirmed by determining the kinetics of hybridization between these strains and U1 cDNA. The kinetics of reaction between the RNA of each of these strains and U1 cDNA were found to be the same as that of the homologous reaction (Fig. 1A). Three other strains of TMV (dahlemense, YTAM, and HRG) reacted with U1 cDNA to an intermediate extent, indicating a partial sequence homology between these RNAs and U1 RNA.

Only the GTAM strain of TMV reacts with U2 cDNA. GTAM RNA combines with U2 cDNA to the same extent as U2 RNA and with the same kinetics (Fig. 1B), indicating extensive homology between these two strains.

The results of experiments in which dahlemense cDNA was used as probe indicate that dahlemense, YTAM, and HRG are closely related. The three strains also exhibit the same hybridization kinetics as dahlemense cDNA (Fig. 1C). Six other strains of TMV (U1, U6, YA, 06-67, Ash, and J14D1) react with dahlemense DNA to a limited extent, indicating partial sequence homology between these strains and dahlemense RNA.

CCV-4 RNA protects the BCV-4 probe as well as does BCV-4 RNA. CCV-4 RNA and BCV-4 RNA also hybridize to the BCV-4 cDNA with the same reaction kinetics (Fig. 1D). This indicates that the two strains are closely related. Other test strains, including another TMV cucumber strain (JCV-3), do not react with BCV-4 cDNA.
A complete lack of sequence similarity was found between the SB strain of CPMV and any other virus strain employed in this study, including two other comoviruses (CPMV-DG and EAMV).

No test strain was found to react with TEV cDNA except its homologous RNA. This indicates that the TEV genome has a sequence that differs from all other strains tested, including another member of the potyvirus group, PVY.

As expected, no homology could be detected between any of the TMV strain cDNAs and the RNAs of other viruses tested, including TRV, potato virus X, members of the tymovirus group, members of the potyvirus and nepovirus groups, BMV, AMV, and tobacco streak virus. The same proved to be true for the cDNAs prepared to CPMV-SB and TEV RNAs.

**DISCUSSION**

Previous assessment of TMV strain genome homology employing competition-hybridization analysis (37) demonstrated that TMV strains fall into subgroups already established by criteria other than genome homology. Within a subgroup, strains were indistinguishable, whereas between subgroups no homology could be detected. Similar all-or-nothing types of results from competition experiments have been reported for several groups of animal (24,32,41) and plant (26,28) viruses. The present reanalysis of TMV strain relatedness confirms the observation that members of a subgroup share a high degree of sequence homology. The data also confirm the increased sensitivity of the excess RNA-cDNA hybridization technique (11), which revealed a limited amount of homology (15%) between the U1 and dahlemense subgroups. Such homology was not seen by the competition-hybridization method.

In addition to the subgroups previously observed (characterized by strains U1, U2, and dahlemense), another subgroup, containing strains BCV-4 and CCV-4, was revealed by the BCV-4 cDNA probe. Cucumber green mottle mosaic virus (16) is probably also a member of this subgroup and, thus, strains in the BCV-4 subgroup show no homology with the legume strains of TMV (listed in Table 1 as dolicoes and cowpea strains), despite the fact that both the cucumber and legume strains, in contrast to other strains, have their encapsidation recognition site located within the capsid protein coding sequence (25).

The discovery of partial sequence homology (approximately 15%) between U1 and dahlemense complements capsid amino acid sequence data (40) that indicate that of all subgroups examined, U1 and dahlemense are most alike, differing in only 18% of their 154 amino acid positions.

The coding sequences for U1 and dahlemense capsid proteins may differ by at least 36% and thus probably do not exhibit homology in the applied tests. This presumption derives from the analysis of divergent but related protein pairs (17), which showed that in most cases coding sequence nucleotide substitutions were from 2 to 3.4 times more frequent than amino acid replacements in the specified protein. Thus, more highly conserved regions may exist in the 95% of genome that is not capsid coding than in the region that is. Indeed, the 60-70-nucleotide, 5' terminal fragments of U1 and dahlemense differ only very little from each other, in contrast to the comparable U2 fragment, which differs extensively from these two (19).

We consider the apparent total homology between HRG and the dahlemense subgroup and its partial homology to the U1 subgroup an exceptional result. The amino acid sequence of the HRG capsid protein differs from those of U1 and dahlemense by 60%. For this reason, we suspect that a contamination of our HRG stock yielded anomalous data.

The virtual lack of sequence homology between most subgroups of TMV observed with both hybridization techniques raises the question of the relationship between these viruses. If they

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**Fig. 1.** Hybridization of \(^3^H\) cDNA to homologous RNA. A, U1; B, U2; C, dahlemense; D, Berkeley isolate of cucumber virus 4; E, cowpea mosaic virus strain SB; F, tobacco etch virus. Rot is the concentration of the unlabeled RNA in moles of nucleotides per liter times the reaction time in seconds.

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developed from a common ancestor, one might expect to find intermediate viruses that hybridize partially to several subgroups. However, divergence of sequences to the extent that homologies cannot be detected by available methods has apparently occurred in the plant viruses. In the present study, comoviruses and potyviruses, like most of the TMV groups, exhibited no sequence similarity. The RNAs of comoviruses EAMV and CPMV-DG were found to have no sequences in common with CPMV-SB RNA. Although the three viruses exhibit some serological cross-reactivity (2, 4), they can be considered to be different comoviruses rather than strains of a single virus. Likewise, in the potyvirus group, PVY RNA would not hybridize to TEV cDNA.

Others have made similar observations. Eighteen isolates of cucumber mosaic virus were found to form three subgroups, the members of which had RNAs that were totally homologous within a subgroup but without similarity between subgroups (28). Turnip yellow mosaic virus was compared to two other tymoviruses and was found by RNA-cDNA hybridization to have a distinct sequence (21). Four strains of AMV were shown to be completely homologous (3), as were five strains of barley stripe mosaic virus (26).

An evolutionary scheme to explain the formation of discrete subgroups is difficult to imagine. Because all of the nucleotide sequences of the members of a subgroup are nearly alike and because a continuum of sequences between subgroups is not observed, one is led to the conclusion that a subgroup nucleotide sequence is evolutionarily successful. A possible mechanism for the development of such a pattern might involve the cross-protection phenomenon. This is an observation that a plant infected with one virus is protected from the effects of a secondary infection with a closely related virus. This phenomenon might keep mutation to an alternate successful sequence from replicating to any large extent but nonetheless allow it to be retained in the infection. Only when the overall sequence has changed extensively is the "new" virus able to assert itself and then, possibly, only in a host in which it has an advantage over the parental sequence. It, in turn, would express the same control over mutations of its own sequence.

This and other studies show that a more sensible statement concerning the evolutionary relationships between these viruses must await further biochemical evidence, including complete sequencing of the viral genomes.

During the final stages of preparation of this manuscript, after the experimental work had been completed, a paper appeared (27) in which the nucleotide sequences of 13 tobamovirus RNAs were compared by essentially the same method we use. The results, where comparable, are in principle the same. A number of differences exist, however. Paikaitis and Symons found the RNAs of strains CV-3 and CV-4 to have about 50% homology, whereas we detected no homology. We found a tobamovirus originally isolated from orchids (06-67) to have extensive homology with the U1

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**Fig. 2.** Hybridization of different amounts of $^3$H cDNA with a constant amount of $^{125}$I homologous RNA. Two to six nanograms of $^{125}$I RNA was annealed with 2-20 ng of homologous cDNA for 30 hr at 68 °C. A, U1; B, U2; C, dahlemense; D, Berkeley isolate of cucumber virus 4; E, cowpea mosaic virus strain SB; F, tobacco etch virus.
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<th>Unlabeled RNA from</th>
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<th>(^\text{Dahlmense experiment})</th>
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*Thirty to 40 pg of \(^\text{H}\) cDNA was annealed to 800 pg of the listed unlabeled RNAs. The data not in parentheses are the SI-nuclease-resistant cpm expressed as a percentage of the homologous-reaction SI-nuclease-resistant cpm.*

*SI nuclease-resistant cpm in the reaction without RNA. These have been subtracted from the other results before calculation of the percentages.*

*SI nuclease-resistant cpm obtained in the homologous reaction.*

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strain, whereas Palukaitis and Symons found no such homology between an orchid-derived strain and U1. In both of these instances, the strains in the two laboratories bearing similar designations may not, in fact, be the same. Palukaitis and Symons observed small differences in homology between some of the strains within a group with the aid of S1 nuclease conditions of two different stringencies. We may have observed a similar phenomenon (for example, in the hybridization data of TMV strains U6 and U6-67 with the TMV U1 cDNA probe in Table 1). However, we are reluctant to interpret our data in this manner because of the limited number of repetitions and the occasional apparent hybridization percentages greater than 100.

LITERATURE CITED


