Cucumber Mosaic Virus-Associated RNA 5

V. Extensive Nucleotide Sequence Homology among CARNA 5 Preparations of Different CMV Strains

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The RNA components from purified preparations of five strains of cucumber mosaic virus (CMV) and from peanut stunt virus (PSV) were used to compete in hybridization experiments between $^{3}$H-labeled CMV-associated RNA 5 (CARNA 5) and double-stranded (ds) CARNA 5 from CMV-S infections. All CARNA 5 preparations competed almost fully with $^{3}$H]CARNA 5 for the minus strands in dsCARNA 5. In contrast, PSV-associated RNA 5 (PARN 5) did not compete at all. Apparently the five CARNA 5s have an extensive if not complete nucleotide sequence homology and could represent one and the same satellite RNA associated with different CMV strains. PARN 5, although associated with another cucumovirus, has no sequence homology with CARNA 5 and evidently is a different molecule. Thus, the ability of different cucumoviruses to support production of specific types of RNA 5 molecules could probably be used in estimating the degree of their strain relationship.

In preceding publications (1-3), we demonstrated that cucumber mosaic virus (CMV)-associated RNA 5 (CARNA 5) is present in five CMV strains of widely different geographic origin. Recently, we found CARNA 5 in eight CMV isolates from different regions in the U. S. (unpublished work). CARNA 5 from each of the above strains or isolates electrophoresed at the same rate in 4% polyacrylamide gels. When CARNA 5 was used as the inoculum in mixtures with the genomic RNAs of CMV-S, or any CMV which was capable of supporting CARNA 5, it incited tomato necrosis (4) and was readily multiplied in tobacco (2, 3). We have recently also identified a fifth major RNA component in peanut stunt virus (PSV), another member of the cucumovirus group (5). This PSV-associated RNA 5 (PARN 5) is slightly larger than CARNA 5, and its replication was not supported by the genomic RNAs of CMV (J. M. Kaper, M. E. Tousignant, J. R. Diaz-Ruiz, and S. Tolin, manuscript in preparation).

Here we present the results of competition hybridization experiments which examine the degree of nucleotide sequence homology between the CARNA 5 of CMV-S (South Africa) and CARNA 5 preparations from CMV strains D (France), R (France), Com (El Salvador), and WT (Wisconsin) and PARN 5 from PSV-V (Virginia).

The origin and conditions of propagation of CMV strain S, D, R, and Com have been described (2, 3). CMV-WT is a 1973 isolate from a tobacco field in Vernon County, Wisconsin, received from R. W. Fulton (University of Wisconsin, Madison). After the second passage in tobacco (Nicotiana tabacum L. cv. xanthi nc) (2), CMV-WT contained 36% CARNA 5 (unpublished work). PSV-V (isolate 74-23) was received from S. Tolin (Virginia Polytechnic Institute, Blacksburg, Virginia) in 1974. It was used to support the large-scale multiplication in tobacco of PARN 5 obtained from a later isolate of PSV-V (76-69).
All viruses except CMV-R were propagated in tobacco in order to have maximal production of the fifth RNA. CMV-R was propagated in squash (*Cucurbita pepo* L. cv. Caserta Bush), where it also produces large quantities of CARNA 5 (2). All viruses were purified with one uniform method (2) and yielded total RNA preparations from which CARNA 5 and PARNA 5 were fractionated as previously described (1). Double-stranded (ds) CARNA 5 and 3H-labeled CARNA 5 were prepared from CMV-S infections in tobacco (6). Polyacrylamide electrophoresis patterns showing the degree of purity of all CARNA 5 preparations and PARNA 5 used in this work are given in Fig. 1; that of dsCARNA 5 was published previously (6). In the mixed electrophoresis of [3H]CARNA 5 and unlabeled CARNA 5 from CMV-S (Fig. 1A), the gel was first scanned at 260 nm (7) and then sliced with a device made from stacked razor blades set 1 mm apart. The polyacrylamide slices were then digested according to the method of Hellung-Larsen (8) and counted.

Competition hybridization was carried out with an improved procedure derived

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**Fig. 1.** Optical density (260 nm) scans and radioactivity profile of polyacrylamide gels (6%) after electrophoresis of RNA 5 preparations from different CMV strains and PSV. Running time, 4.5 hr at 4 mA/gel. (A) S-CARNA 5 + S-[3H]CARNA 5 (dote); (B) D-CARNA 5; (C) R-CARNA 5; (D) Com-CARNA 5; (E) WT-CARNA 5; (F) V-PARNA 5. The amounts of unlabeled RNA 5 applied were 12 µg; [3H]CARNA 5 was an amount equivalent to 3000 cpm.
from the one used previously (6). Tripli-
cicate samples of \([^{3}H]C\)ARNA 5 (0.097 \(\mu\)g),
dsCARNA 5 (0.194 \(\mu\)g), and competing
RNA 5 were each mixed at 0° in 40%
formamide and 1× SSC (SSC = 0.15 \(M\)
NaCl, 0.015 \(M\) Na-citrate, pH 7.2) in a total
volume of 0.1 ml. The molar ratios of
competitor/[\(^{3}H\)]CARNA 5 were 0, 0.9, 1.2,
3, 6, 9, and 12. In a separate experiment,
the same competing RNA samples were
also tested at about 100× \([^{3}H]C\)ARNA 5.
The mixtures were drawn into glass capil-
rary tubes, sealed, and heated in a water
bath at 100° for 2 min. Annealing pro-
ceeded by cooling the bath to 58° over a
period of 1 hr and was followed by an
incubation of 20 hr at 58°. The capillaries
were then opened, and the contents were
digested with RNase A (Worthington Bio-
chemical) at a final concentration of 50
\(\mu\)g/ml for 30 min at 37°. The samples were
spotted in their entirety on Whatman GF/
C filters and soaked in 10% trichloroacetic
acid (TCA)-0.04 \(M\) Na-pyrophosphate (PP)
for 10 min at 0°. The filters were then
washed twice with ice-cold 5% TCA-0.02
\(M\) PP, once with 70% ethanol, once with 95%
ethanol, and once with acetone and dried
under a heat lamp for 15 min. The radio-
activity in the dried filters was counted in
toluene-based scintillation cocktail in a
Searle Mark III liquid scintillation
counter. In the absence of competing
CARNA 5, this improved hybridization
procedure resulted in a radiolabeling effi-
ciency of 80% or better in our standard
homoduplex (\([^{3}H]C\)ARNA 5/dsCARNA 5
ratio of 1/2, w/w), whereas in the past no
better than 65-70% efficiency was ob-
tained. When \([^{3}H]C\)ARNA 5 was hybrid-
ized in the presence of increasing quanti-
ties of dsCARNA 5, and the fraction of
radioactivity fixed in the duplex (\(F\)) was
estimated at an infinite dsCARNA 5 con-
centration from the reciprocal plots (9), a
value of 0.64 instead of the previously
reported 0.47 (6) was found for \(F\) (results
not shown).

Table 1 shows that the CARNA 5 prep-
arations from different CMV strains com-
pe almost fully with CMV-S \([^{3}H]C\)ARNA 5
for duplex formation with the minus
strands of CMV-S dsCARNA 5. In con-
trast, PARNA 5 does not compete at all.
This indicates that the CARNA 5 of the
CMV strains tested have extensive nucleo-	tide sequence homologies (Table 1, last
column), whereas PARNA 5 has none.

Somewhat puzzling is the fact that none
of the CARNA 5 preparations was able to
compete for the full 100%. This was also
found in several experiments with the
homologous CMV-S CARNA 5. (The value
of \(f = 0.79\) listed in Table 1 represents
only one of five intercepts which yielded
an average of \(f = 0.85\) for CMV-S CARNA
5.) We have confirmed this incomplete
competition in a separate hybridization
experiment where each of the competing
RNAs was included in large excess over
\([^{3}H]C\)ARNA 5 (Table 2). Both sets of data
seem to suggest that competing CARNA 5
preparations are unable to prevent some
15% of the \([^{3}H]C\)ARNA 5 from combining
with the CARNA 5 minus strands in ds-
CARNA 5. We have performed control
experiments to determine whether there
was incomplete RNase digestion due to
the presence of relatively large quantities
of competing RNA, or whether small inter-
nally complementary nucleotide se-
quences, normally not detectable, might
have been stabilized. To this end, the
standard quantity of \([^{3}H]C\)ARNA 5 (0.097
\(\mu\)g with a radioactivity of about 2600 cpm)
was annealed with the same excess quan-
tities of competing CARNA 5 (Table 2) in
the absence of dsCARNA 5. The radioac-
tivities in such samples after RNase treat-
ment varied from 0 to 50 cpm over the
background. There is also the possibility
that the small amount of faster electro-
phoresing material in our \([^{3}H]C\)ARNA 5
preparation (Fig. 1A) represents RNA
which is unrelated to CARNA 5, but which
is able to bind to one of the strands of
dsCARNA 5. If this material is not present
in the competitor RNAs, it would explain
their inability to compete fully. Although
this seems unlikely, there are some differ-
TABLE 1
Hybridization of [3H]CARNA 5 and dsCARNA 5 from CMV-S in the Presence of Increasing Quantities of Competing RNA 5 from CMV Strains and PSV

<table>
<thead>
<tr>
<th>Competing RNA 5 from</th>
<th>Radioactivities of duplexes and calculated f values*</th>
<th>Molar ratio, competitor/[3H]CARNA 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>CMV-S</td>
<td>1146</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(0.21)</td>
</tr>
<tr>
<td>CMV-D</td>
<td>1012</td>
<td>785</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(0.22)</td>
</tr>
<tr>
<td>CMV-R</td>
<td>1126</td>
<td>829</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(0.26)</td>
</tr>
<tr>
<td>CMV-Com</td>
<td>967</td>
<td>799</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(0.17)</td>
</tr>
<tr>
<td>CMV-WT</td>
<td>1148</td>
<td>871</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(0.24)</td>
</tr>
<tr>
<td>PSV-V</td>
<td>1068</td>
<td>1162</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(0)</td>
</tr>
</tbody>
</table>

* The radioactivities are expressed as counts per minute. The calculated f values are given in parentheses. f = fraction of [3H]CARNA 5 prevented from annealing to the minus strands of dsCARNA 5.

b The values this column were obtained from extrapolations in reciprocal plots of f versus competitor concentration (9) and represent the degree of nucleotide sequence homology with CMV-S [3H]CARNA 5.

TABLE 2
Hybridization of [3H]CARNA 5 and dsCARNA 5 from CMV-S in the Presence of Approximately A 100-fold Quantity of Competing RNA 5 of Different CMV Strains and PSV

<table>
<thead>
<tr>
<th>Competing RNA 5 from</th>
<th>Molar ratio, Competitor/[3H]CARNA 5</th>
<th>Radioactivity of duplex and f values*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1042 (0)</td>
</tr>
<tr>
<td>CMV-S</td>
<td>103</td>
<td>147 (0.86)</td>
</tr>
<tr>
<td>CMV-D</td>
<td>97</td>
<td>123 (0.88)</td>
</tr>
<tr>
<td>CMV-R</td>
<td>105</td>
<td>66 (0.94)</td>
</tr>
<tr>
<td>CMV-Com</td>
<td>106</td>
<td>232 (0.78)</td>
</tr>
<tr>
<td>CMV-WT</td>
<td>104</td>
<td>131 (0.87)</td>
</tr>
<tr>
<td>PSV-V</td>
<td>85</td>
<td>1088 (0)</td>
</tr>
</tbody>
</table>

* Radioactivity is expressed as counts per minute. The f values are given in parentheses. f = fraction of [3H]CARNA 5 prevented from annealing to the minus strands of dsCARNA 5. Due to large excess of competitor over [3H]CARNA 5, this value also approximates the degree of nucleotide sequence homology with CMV-S [3H]CARNA 5.

Nevertheless, the experiments presented in this communication seem to allow the conclusion that CARNA 5 molecules from different CMV strains have substantial if not complete nucleotide sequence homology. Lot et al. (11), who studied the oligonucleotide fingerprints of CMV-D CARNA 5, have also found few if any differences with the fingerprint of CMV-S CARNA 5 (K. E. Richards, personal communication). This and the fact that CARNA 5 can be readily interchanged among CMV helpers, while never failing to induce necrosis in tomato, suggest that it could be one and the same satellite-like molecule associated with different strains of CMV. That PARNA 5 has no sequence homology at all with leaf strips (10), produced and encapsidated from the third to fifth day after infection, whereas nonradioactive CARNA 5 is from virus synthesized in whole plants during the entire period between inoculation and leaf collection. This problem needs further investigation.
Carna 5 is no surprise in view of its size and the failure of PARNA 5 to be supported by CMV and CARKA 5 by PSV (J. M. Kaper, M. E. Tousignant, J. R. Diaz-Ruiz, and S. Tolin, manuscript in preparation). Although PSV is serologically related to CMV, the relationship is more distant than among CMV strains (12). There are also significant differences between PSV and CMV in host range and symptomatology (13) and in physical and chemical properties (7). Finally, there is no noticeable competition between CMV-RNA (devoid of CARKA 5) and [3H]PSV-RNA (devoid of PARNA 5) for complementary sequences in dsPSV-RNA (J. R. Diaz-Ruiz, personal communication). This indicates that there is little or no nucleotide sequence homology between the genomic RNAs of these two cucumoviruses. Thus, the ability of certain cucumoviruses to support the production of specific types of RNA 5 satellite molecules can probably be used as a genetic character in determining their strain relationships. In this respect, it would be interesting to estimate the degree of nucleotide sequence homology among the RNAs of CMV strains known to support CARKA 5 production and those that do not. At this time CMV-Ix [Ixora isolate from the Philippines (14)] is the only isolate we know which does not support CARKA 5 (2). This type of comparative study is in progress.

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REFERENCES