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Determination of the sequence homology between the four RNA species of cucumber mosaic virus by hybridization analysis with complementary DNA

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ABSTRACT

The method of Taylor et al., (11) has been used to transcribe complementary DNA probes from the four major RNA species of cucumber mosaic virus (RNAs 1 - 4 in order of decreasing molecular weight). Analysis of the kinetics of hybridization of these probes in homologous and heterologous complementary DNA-RNA hybridization reactions has shown that the sequence of the smallest RNA (RNA 4), which contains the coat protein gene, is present within RNA 3. RNAs 1 and 2 are unique RNA molecules while each has a region of approximately 300 nucleotides in common with RNA 4.

INTRODUCTION

Cucumber mosaic virus (CMV) contains a functionally divided genome since the largest three of the four major RNA species found in purified virions (molecular weights 1.35, 1.16, 0.85 and 0.35 x 10^6, designated RNAs 1 to 4, respectively) are needed for infectivity (1-3). RNA 4 contains the coat protein gene as does RNA 3 which also codes for another protein of unknown function (3-5). The number of genes present on RNA 1 and RNA 2 is not known but there may be only one on each (5). Many of the molecular properties of CMV are found in other multicomponent viruses, such as brome mosaic virus (BMV) and alfalfa mosaic virus (AMV) (6-8). In addition it is possible to construct viable pseudorecombinant viruses by taking RNAs 1 and 2 from one virus and co-infecting with RNA 3 of another (3,9). The RNA 4 in progeny virions is almost certainly derived from RNA 3 and not from any RNA 4 added in the inoculum (8,10).

It is obviously of considerable importance to investigate

the primary structure of the RNAs of these multicomponent viruses. Such information is necessary for the proper classification of these viruses and for understanding the molecular basis of their replication. We describe here the preparation of complementary DNA (cDNA) to each of the four CMV RNAs by the simple and very effective method of Taylor et al., (11) and the use of this cDNA in hybridization studies with RNA to determine the sequence homology between the CMV RNA species. This approach is both sensitive and powerful and is directly applicable to other viruses with two or more RNA components. It is also applicable to the determination of the sequence relationship between RNA species present in different strains of the same virus.

MATERIALS AND METHODS

CMV (Q strain) was purified and the viral RNA isolated as described (1). CMV RNA was first fractionated in the native state by electrophoresis on a 2.5% polyacrylamide aqueous slab gel (1,5) and the four main bands, located by brief staining with 0.05% toluidine blue and destaining in water, were eluted by phenol-sodium dodecyl sulphate extraction (1,5) and recovered from the aqueous phase by ethanol precipitation. Each RNA was denatured by heating in formamide and then electrophoresed under the denaturing conditions of 4% polyacryamide-formamide tube gels (1,4,5); this step was taken to remove any nicked or aggregated RNA species from the main components. RNA bands were located and eluted as above and contaminating polyacrylamide was removed by centrifugation of the RNA on a linear 10-40% sucrose gradient (4,5). Peak fractions were collected, the RNA precipitated with ethanol and used for the preparation of cDNA transcripts. Later the simpler and more effective technique of polyacrylamide slab gel electrophoresis combined with electrophoretic elution was utilised (12).

^{32}P-cDNA to each RNA was prepared essentially by the methods of Taylor et al., (11) and Kemp (13). Reaction mixtures of 50 µl contained 50 mM Tris-HCl, pH 8.3, 8 mM dithioerythritol, 8 mM $MgCl_2$, 0.67 mM dATP, dGTP and dTTP,

0.1 mM α-^{32}P-dCTP (14) (specific activity 10^{10} cpm/μmole), 100 μg/ml actinomycin D, 1 μg CMV RNA, 125 μg salmon sperm primer DNA (11) and 10 units of avian myeloblastosis virus reverse transcriptase. Incubation was for 2.0 hours at 37°C. The reaction was stopped by the addition of 150 μl of 0.75% SDS and 300 μl of 0.5 N NaOH. After incubation overnight at room temperature to hydrolyse the RNA template, the cDNA was separated from the low molecular weight material by passage through a 1.5 x 15 cm column of Sephadex G-50 in 0.1 M NH_4HCO_3. The ^{32}P-cDNA was recovered by freeze-drying after the addition of excess triethylamine.

The conditions for hybrid formation were essentially those of Kemp (13). Freeze-dried cDNA was dissolved in the appropriate volume of hybridization buffer (0.18 M NaCl, 0.01 M Tris-HCl, pH 7.0, 1 mM EDTA, 0.05% SDS) to approximately 2000 cpm/μl. To 40 μl of the appropriately diluted RNA solution was added 1 μl ^{32}P-cDNA in a siliconised test tube. After thorough mixing, the solution was transferred to a siliconised capillary tube (100 μl) which was then sealed at both ends. The capillary tubes were immersed in boiling water for 5 min and then incubated at 60°C for the appropriate length of time. Hybridization was terminated by chilling the capillaries on ice.

The formation of DNA.RNA hybrids was assayed with the single strand specific nuclease S1 of Aspergillus oryzae isolated from Taka-diastase powder (Parke Davis and Co). The purification method of Vogt (15), up to and including the DEAE-cellulose fractionation step, was used.

The sealed capillary tubes were opened with the aid of a glass cutter and the contents added to 400 μl S1 assay buffer (30 mM sodium acetate pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol) containing 40 μg/ml denatured calf thymus DNA. Two samples, each of 200 μl, were taken and to one was added 2.0 units S1 nuclease, the other serving as a control. Both samples were then incubated at 45°C for 30 min. Digestion was terminated by the addition of 1.0 ml 10% TCA and then bovine serum albumin (75 μg) was added as a carrier. After 30 min at 0°C the TCA precipitates were collected onto GF/A

filters, washed four times with 5% TCA and twice with 5 ml ether. The hybrids retained by the filters were then counted by liquid scintillation spectrometry. The percent hybrid formation was calculated from a comparison of the duplicates incubated either in the presence or absence of the S1 enzyme.

Melting profiles of the cDNA.RNA hybrids were performed as follows. Homologous and heterologous hybridization reaction mixtures were set up as previously described and the reactions allowed to proceed to a Rot of 1.0 to ensure complete hybrid formation. Capillary tubes containing the hybrid mixtures were then heated for 5.0 min at the designated temperature (range 40°C - 100°C), chilled on ice and the percent hybrid formation was determined as described above.

For hybridization of unlabelled cDNAs to radioiodinated template RNAs, total CMV RNAs were radioiodinated (16) and individual ^{125}I-RNA species isolated by polyacrylamide slab gel electrophoresis and electrophoretic elution (12). Each ^{125}I-RNA was diluted to 2000 cpm/µl and 1 µl added to serially diluted unlabelled cDNA (in excess). The unlabelled cDNA was isolated after being transcribed from the same template RNA from which the ^{125}I-RNA was prepared. Hybrid formation was performed as previously described and the percent hybrid formation assayed using ribonucleases T1 and A (19). For the ribonuclease assays hybrids were diluted into 500 µl of 30 mM sodium acetate, 0.3 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, pH 4.6, containing 20 µg/ml E. coli tRNA. Duplicate samples (each of 200 µl) were incubated with or without ribonucleases T1 and A (10 units and 1.72 µg, respectively) at 37°C for 30 min. Undigested RNA.DNA hybrids were TCA precipitated, filtered, washed and the percent hybrid remaining calculated as described above.

Total plant leaf RNA was isolated by an adaption of the phenol extraction method of Ingle and Burns (17).

RESULTS AND DISCUSSION

Preparation and Characterization of cDNA

It was considered essential to rigorously purify the four CMV RNA species prior to the preparation of cDNA. This

was carried out by a three step procedure described in Materials and Methods. After the first step of a 2.5% polyacrylamide aqueous slab gel in which the RNA was electrophoresed in its native state, the four isolated RNA species were analysed under the denaturing conditions of 98% formamide-4% polyacrylamide tube gels. The stained gel patterns obtained (Fig. 1A) showed that RNAs 1, 2 and 3 were significantly contaminated with other RNA species while RNA 4 was substantially pure. After further purification on 4% polyacrylamide tube gels run in 98% formamide and a final purification by centrifugation on sucrose gradients to remove contaminating polyacrylamide, the RNAs were again analysed on formamide tube gels. It can be seen (Fig. 1B) that each RNA appeared to be uncontaminated on the basis of size by other RNA species.

Later CMV RNA purification was performed using slab polyacrylamide gel electrophoresis and electrophoretic elution of the RNA species (12). This latter method proved identical or superior in its ability to prepare intact, purified RNA species. It should be emphasized that great care is needed in

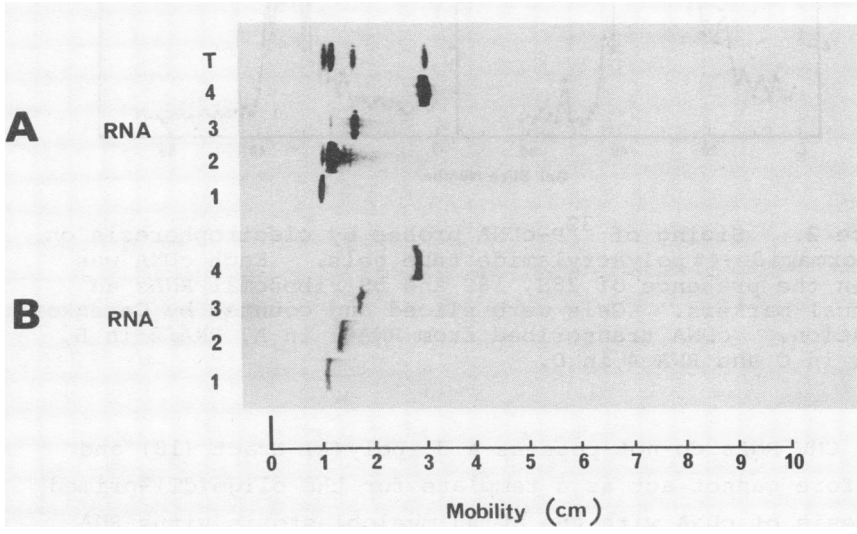

Figure 1. Electrophoresis of the individually purified CMV RNAs on 98% formamide-4% acrylamide tube gels. A, RNAs 1 - 4 after slab gel electrophoresis; O indicates the origin of the gels and T is a gel of unfractionated CMV RNA. B, RNAs 1 - 4 after final purification steps.

the isolation and purification of CMV RNA. In one preparation of CMV RNA, we found extensive contamination of RNA 2 by RNA 1 even though both species were essentially pure on the basis of size as determined by formamide gel electrophoresis. Since we believe that this degradation of RNA 1 to the same size class as RNA 2 occurred on storage of the purified virus, we now routinely isolate the viral RNA immediately the virus is purified.

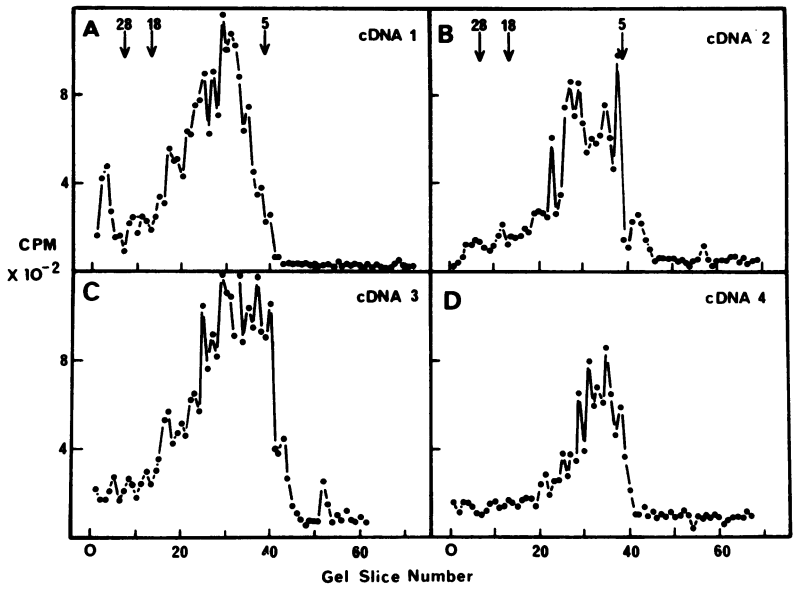

Figure 2. Sizing of ^{32}P-cDNA probes by electrophoresis on 98% formamide-4% polyacrylamide tube gels. Each cDNA was run in the presence of 28S, 18S and 5S ribosomal RNAs as internal markers. Gels were sliced and counted by Cerenkov radiation. cDNA transcribed from RNA 1 in A, RNA 2 in B, RNA 3 in C and RNA 4 in D.

CMV RNAs do not possess a 3'-poly(A) tract (18) and therefore cannot act as a template for the oligo(dT)-primed synthesis of cDNA with the avian myeloblastosis virus RNA dependent-DNA polymerase (reverse transcriptase). However, ^{32}P-cDNA to each of the four rigorously purified CMV RNAs was prepared by the method of Taylor et al., (11). This technique utilises random oligonucleotides, generated by

DNAase I digestion of salmon sperm DNA, as internal primers for the reverse transcriptase catalysed reaction. Both template and primer were essential for the transcription of cDNA from the four CMV RNAs (results not shown). Electrophoretic analysis of the four CMV cDNAs under the denaturing conditions of formamide-polyacrylamide tube gels (Fig. 2) indicated a broad size range of transcripts of each RNA, with an average chain length of about 400-500 nucleotides.

To ensure that each cDNA was representative of the whole RNA template from which it was transcribed, four unlabelled cDNAs were prepared and hybridized in excess to their homologous purified ^{125}I-RNAs (see Materials and Methods). The formation of cDNA.RNA hybrids was monitored by resistance of the ^{125}I-RNA to ribonuclease A plus T1 digestion (19). The hybridization kinetics (Fig. 3) showed smooth, sharp transitions to essentially 100% protection of each of the four ^{125}I-RNAs. This more or less complete hybridization and the absence of discrete plateaus in the hybridization curves indicated that the cDNAs represented the entire template and were not grossly enriched for one or several regions of their template.

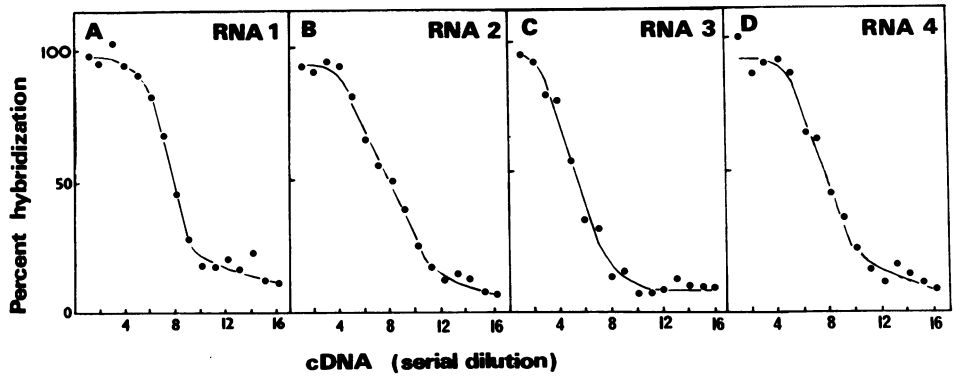

Figure 3. Formation of unlabelled cDNA-^{125}I-RNA hybrids as determined by resistance of the ^{125}I-RNA to ribonuclease A plus T1 digestion. A, hybrid formation between ^{125}I-RNA 1 and cDNA 1; B, ^{125}I-RNA 2 and cDNA 2; C, ^{125}I-RNA 3 and cDNA 3; D, ^{125}I-RNA 4 and cDNA 4. Hybridization was at 60°C for 96 hours, this gave a Dot of 3.5 - 7.0 mol s l^{-1} for undiluted cDNA.

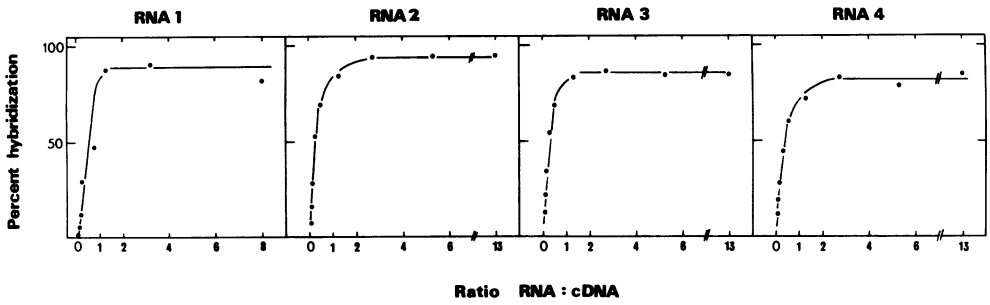

Figure 4. Titration of ^{32}P-cDNA transcribed from CMV RNAs 1 - 4 with their corresponding RNAs. For each determination of the percent ^{32}P-cDNA in hybrid form, 4 ng of ^{32}P-cDNA was hybridized to varying amounts of its corresponding RNA to a Dot of 0.5 mol s l^{-1}. Hybridization conditions and S1 nuclease assays were as described in Materials and Methods.

Although the ^{32}P-cDNAs transcribed from each species of RNA were representative of the entire nucleotide sequence of that RNA, it was important to show that each sequence was equally represented in the cDNA population; i.e., no enrichment for a particular sequence had occurred. This was achieved by assaying the percent of each ^{32}P-cDNA which hybridized to its corresponding RNA at varying mass ratios of RNA:cDNA (22). The results in Fig. 4 show that at least 85% of each cDNA hybridized to its corresponding RNA while the shape of the titration curves indicated that virtually all of the sequences in each RNA were more or less equally represented in ^{32}P-cDNA (22).

Hybridization of Purified CMV RNAs to CMV cDNAs

Hybridization kinetics of the four cDNAs to excess unlabelled CMV RNAs 1 - 4 are shown in Fig. 5A - D. As determined by resistance of the double-stranded hybrids to the single-strand specific nuclease S1, cDNA 1 hybridized completely to RNA 1 in a sharp transition with a mid-point ($Rot_{\frac{1}{2}}$) of 1.0 x 10^{-2} mol s l^{-1} (Fig. 5, Table 1). Likewise, the hybridizations of the other cDNAs to their homologous RNA (Fig. 5B - D) gave sharp transitions with each $Rot_{\frac{1}{2}}$ being internally consistent with the known complexity of each size class of CMV RNA; i.e., the $Rot_{\frac{1}{2}}$ values were proportional to

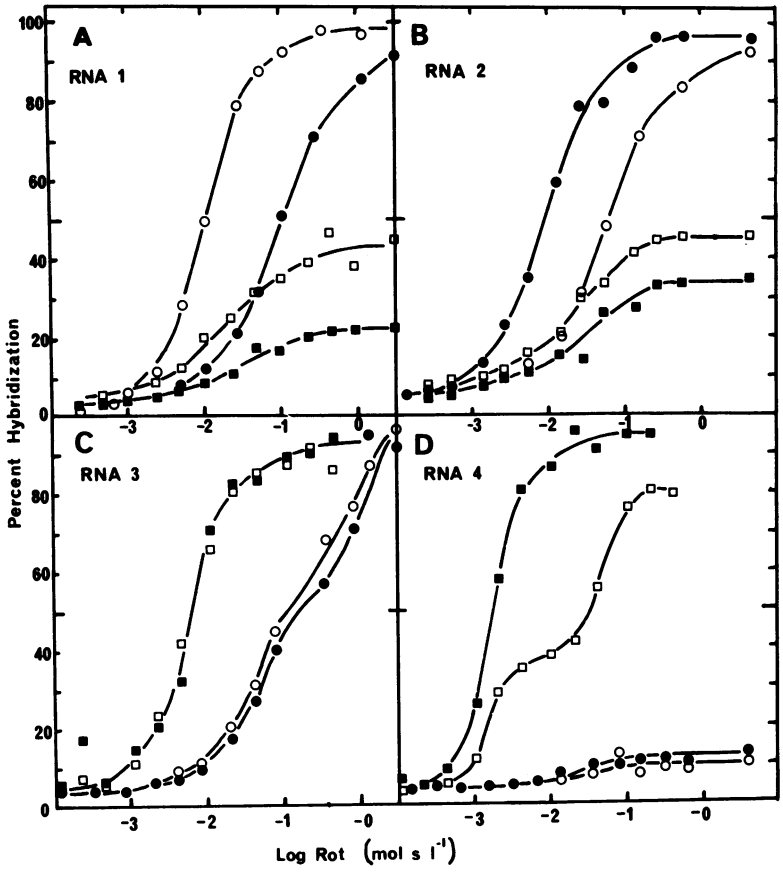

Figure 5. Hybridization of the four CMV RNAs to each of the four ^{32}P-cDNAs. A, cDNAs 1 - 4 vs. RNA 1; B, cDNAs 1 - 4 vs. RNA 2; C, cDNAs 1 - 4 vs. RNA 3; D, cDNAs 1 - 4 vs. RNA 4. The $Rot_{\frac{1}{2}}$ values and other data derived from this figure are given in Table 1. O, cDNA 1; ●, cDNA 2; □, cDNA 3; ■, cDNA 4.

the nucleotide complexity of each RNA (Table 1, right hand column; see also below). In addition, the lack of a rapidly annealing fraction indicates that no detectable reiterated sequences were present in any of the RNAs.

The hybridization kinetics of both cDNA 1 and cDNA 2 to RNA 1 showed single sharp transitions (Fig. 5A); however, the rate of hybridization of cDNA 2 to sequences complementary in the RNA 1 preparation was about 13 times slower than the rate of the homologous reaction (cDNA2.RNA2). Since the forma-

TABLE 1

Hybridization of ^{32}P-cDNA to CMV RNA

RNA (Molecular weight)	RNA species from which ^{32}P-cDNA was transcribed	% Hybridization	Number of Residues	$Rot_{\frac{1}{2}}$ (x 10^3)	$\frac{Rot_{\frac{1}{2}}}{\text{mol.wt. of RNA}}$ (x 10^9)
RNA 1	1	0 - 100	4,000	10	7.4
(1.35 x 10^6)	2	0 - 100		100	
	3	0 - 40	1,000	25	
	4	0 - 25	250	28	
RNA 2	1	0 - 100		50	
(1.16 x 10^6)	2	0 - 100	3,400	7.9	6.8
	3	0 - 45	1,100	25	
	4	0 - 35	350	25	
RNA 3	1	0 - 100	4,000	100	
(0.85 x 10^6)	2	0 - 100	3,400	100	
	3	0 - 100	2,500	5.6	6.6
	4	0 - 100	1,000	5.6	
RNA 4	1	0 - 7	280	28	
(0.35 x 10^6)	2	0 - 8	270	35	
	3	0 - 40	1,000	1.8	
		40 - 80		79	
	4	0 - 100	1,000	2.2	6.3
Avian globin mRNA (0.663 x 10^6)	Avian globin mRNA	0 - 100	1,950	4.8	7.2

The percent hybridization and $Rot_{\frac{1}{2}}$ values are from the data of Figure 4. The molecular weights of the CMV RNAs are from Peden and Symons (1) while a residue weight of 340 has been used for all calculations.

tion of hybrids continued to 100% at these higher Rot values, RNA 1 must contain additional sequences complementary to cDNA 2. From a comparison of the $Rot_{\frac{1}{2}}$ for the heterologous reaction (1.0×10^{-1}) to the homologous reaction (cDNA2.RNA2; 7.9×10^{-3} mol s l^{-1}) it can be calculated that RNA 1 was contaminated by 8% by weight with RNA 2. Thus the hybridization technique is very sensitive in detecting the type and amount of contaminating RNA. Similarly, from Fig. 5A, cDNA 3

and cDNA 4 were protected against S1 nuclease digestion by hybrid formation with RNA 1 to levels of 40 and 25% respectively. This indicates that 40% of the cDNA sequences transcribed from RNA 3 (equivalent to 1,000 nucleotides) and 25% of the cDNA sequences transcribed from RNA 4 (equivalent to 250 nucleotides) were homologous with RNA 1 (Table 1). As considered below, most of this apparent homology of RNA 3 with both RNAs 1 and 2 was due to contamination of RNA 3 by breakdown fragments derived from RNAs 1 and 2.

The hybridization of RNA 2 to cDNA 1 and cDNA 2 showed single, sharp transitions (Fig. 5B). Since the RNA 2.cDNA 1 hybridization went to completion, all RNA 1 sequences were present in RNA 2. However, a comparison of the $Rot_{\frac{1}{2}}$ values of RNA 1.cDNA 1 (10×10^{-3} mol s 1^{-1}) and RNA 2.cDNA 1 (50×10^{-3} mol s 1^{-1}) indicates that RNA 2 was contaminated to 20% by weight with sequences from RNA 1. Again, the plateau levels for the hybridization of RNA 2 against cDNA 3 and cDNA 4 (Fig. 5B) show that RNA 3 and RNA 4 had approximately 1,100 and 350 nucleotides, respectively, in common with RNA 2 (Table 1).

When RNA 3 was hybridized to either cDNA 3 or cDNA 4 (Fig. 5C), essentially complete hybridization occurred in both cases with sharp transitions and the same $Rot_{\frac{1}{2}}$ value, indicating that all RNA 4 sequences were present in RNA 3. RNA 3 exhibited complex hybridization kinetics with both cDNA 1 and cDNA 2 as shown by the inflections in the hybridization curves of Fig. 5C which suggest a composite curve of two or more reactions. The complete hybridization in each case indicates that RNA 3 was contaminated with all the sequences from both RNA 1 and RNA 2. An estimation of the % contamination by weight of RNA 3 with fragments of the same size class derived from RNA 1 and RNA 2 was obtained from a comparison of the $Rot_{\frac{1}{2}}$ values of each homologous reaction (cDNA 1.RNA 1 and cDNA 2.RNA 2) and the respective heterologous hybridizations with RNA 3 (Table 1). Hence, RNA 3 was contaminated with approximately 10% by weight of RNA 1 and 13% by weight of RNA 2 sequences.

It is possible that these estimates of the level of contamination of RNA 3 by fragments of RNA 1 and RNA 2 may be too

low. For example, the RNA 3.cDNA 1 and RNA 3.cDNA 2 curves of Fig. 5C could each consist of two curves, one plateauing at about 50% with a $Rot_{\frac{1}{2}}$ of about 40 x 10^{-3} mol s l^{-1} and the other proceeding from 0 - 100% hybridization with a $Rot_{\frac{1}{2}}$ of roughly 400 x 10^{-3} mol s l^{-1}. If this is so, then the first curve indicates that about 50% of the sequences of RNA 1 and of RNA 2 each contaminated RNA 3 to the level of 20 - 25% by weight; i.e., 40 - 50% by weight of RNA 3 consisted of fragments derived from part of RNA 1 and RNA 2. The second curve would indicate contamination by all sequences from RNA 1 and RNA 2 but only to a level of about 2% by weight of RNA 3. These calculations indicate the difficulties inherent in attempting to determine an accurate level of contamination by a complex mixture of fragments, especially in this case where the contamination level is high. Hence, it is important not to try and overinterpret the data.

The hybridization of RNA 4 to both cDNA 3 and cDNA 4 (Fig. 5D) again support the conclusion that all RNA 4 sequences were present in RNA 3. The hybridization of cDNA 4 to RNA 4 showed a sharp transition with a $Rot_{\frac{1}{2}}$ of 2.2 x 10^{-3} mol s l^{-1} while that of cDNA 3 vs. RNA 4 showed two transitions. The $Rot_{\frac{1}{2}}$ of hybrids which plateaued at 40% was 1.8 x 10^{-3} mol s l^{-1}, indicating that 40% of RNA 3 sequences (1,000 nucleotides) were present in RNA 4; i.e., sequences equivalent to full length RNA 4. The second transition with a $Rot_{\frac{1}{2}}$ of 79 x 10^{-3} mol s l^{-1} appears to be due to contamination of RNA 4 to a level of 7% by weight with RNA 3 sequences not contained in RNA 4.

Essentially the same results as those obtained for RNA 3 and RNA 4 in Fig. 5 have been obtained with two other preparations of these RNAs purified by methods (5, 12) different to that reported in Materials and Methods. Thus, hybridization data (results not given) gave plateau levels of 30 - 40% for RNA 1.cDNA 3 and RNA 2.cDNA 3 and 30 - 33% for RNA 1.cDNA 4 and RNA 2.cDNA 4 (cf. Fig. 5A and B). Further, hybridizations of RNA 3.cDNA 1 and RNA 3.cDNA 2 gave smooth transitions with no inflections (cf. Fig. 5C).

Since hybridization data (Fig. 5C) indicated a total

contamination of RNA 3 by RNA 1 and RNA 2 fragments to at least 23% by weight (10% plus 13%), then the level of hybridization of cDNA 3 to RNA 1 and to RNA 2 (about 40%, Fig. 5A and B) is an overestimate. Since RNA 1 was contaminated by RNA 2 and vice versa (see below), a more reasonable estimate of the maximum plateau level would be 40% - 23% = 17%; i.e., a maximum of about 17% of RNA 3 sequences (430 nucleotides) were homologous with RNA 1 and with RNA 2. A level of this order is to be expected since RNA 4, which is completely contained within RNA 3, had approximately 250 - 350 nucleotides in common with both RNA 1 and RNA 2 (Table 1).

The extensive contamination of RNA 3 with fragments of both RNA 1 and RNA 2 of the same size class could have originated during virus assembly in the intact plant cell with breakdown fragments of RNA 1 and RNA 2 of the same size class as RNA 3 sometimes being incorporated in place of RNA 3. Another feasible alternative is that there is some specific degradation of viral RNA during purification of the virus; the RNA in purified virions is sensitive to added pancreatic ribonuclease in vitro (20).

RNA 4 was essentially pure with respect to contamination by fragments of RNA 1 and RNA 2. This was shown by the hybridization curves of Fig. 5D in which the RNA 4.cDNA 1 and RNA 4.cDNA 2 curves reached a plateau of 7 - 8% (Table 1). Since the reaction was taken to a Rot of 5 without further increase in the % hybridization, contamination by RNA 1 and RNA 2 was less than 0.2%. Hence, the plateau levels of RNA 1.cDNA 4 and RNA 2.cDNA 4 in Fig. 5A and B are essentially correct and require no correction for contamination. These results further confirm the earlier conclusion that both RNA 1 and RNA 2 have a 250 - 350 nucleotide homology with RNA 4.

The low levels of hybridization which occurred with RNA 4.cDNA 1 and RNA 4.cDNA 2 (Fig. 5D) preclude an accurate estimation of the $Rot_{\frac{1}{2}}$ values. However, the values obtained (38×10^{-3} and 35×10^{-3} mol s l^{-1}, respectively) are appreciably higher than the expected value of 2.2×10^{-3} mol s l^{-1} (Table 1). This may be due to a combination of experimental error and the length of the ^{32}P-cDNA probe (400 - 500

residues, see above) being roughly twice that of the homologous length of RNA 1 and RNA 2 (250 - 350 residues) to which it hybridized. The latter situation would tend to slow the rate of hybridization (25).

The 250 - 350 nucleotide sequence homology between RNA 4 and RNA 1 and between RNA 4 and RNA 2 (Table 1) has been found with three different preparations of these RNAs. It is not possible from the results to determine if the 250 - 350 nucleotide sequence is the same in both RNAs 1 and 2 or if it exists in an uninterrupted stretch. However, it is possible that it may be present on the 3'-end of these RNAs since Bastin et al., (21) have shown that the 3'-terminal 160 residues of the four RNAs of the closely similar brome mosaic virus are practically identical.

Thermal denaturation of the cDNA.RNA hybrids showed that, under the conditions employed, hybrid formation was essentially specific as all hybrids melted with sharp thermal transitions and a high T_m (86°C - 88°C) (Fig. 6).

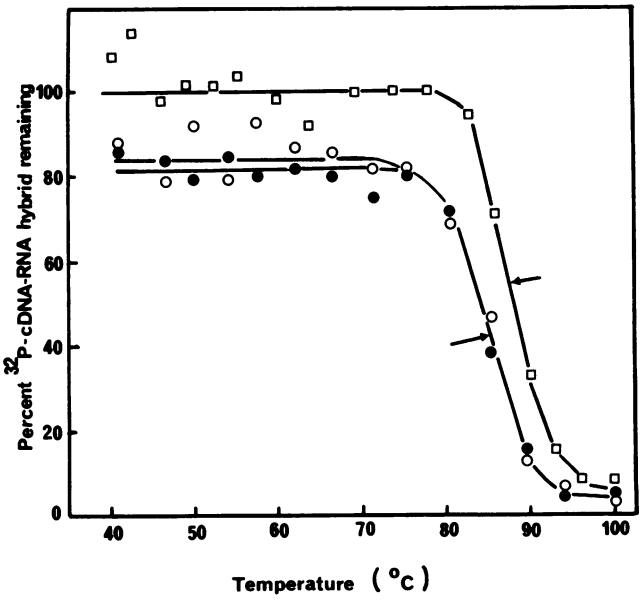

Figure 6. Thermal stability of CMV RNA.cDNA hybrids. Hybridizations and assays were as described in Materials and Methods. □ , cDNA 1 vs. RNA 1, T_m 88°C; ○ , cDNA 4 vs. RNA 4, T_m 86°C; ● , cDNA 4 vs. RNA 3, T_m 86°C. The arrows indicate the T_m values.

The CMV RNA species were not contaminated with host plant RNA since no hybridization above the background controls occurred when the four cDNAs were hybridized to total RNA extracted from tobacco and cucumber (host plants from which CMV was purified) to a Rot of 10^5 mol s l^{-1} (results not shown).

Sequence Complexity of CMV RNAs

The kinetics of hybridization of cDNA to excess unlabelled RNA are dependent on the sequence complexity of the RNA (22, 23). If the nucleotide sequences in each RNA are uniquely represented then the complexity should equal the physical molecular weight (22, 23). That this is the case for the four CMV RNAs was shown using avian globin mRNA (a gift of R.J. Crawford and J.R.E. Wells, ref. 24) as a kinetic hybridization standard. Hybridization to its homologous cDNA as in Table 1 gave a $Rot_{\frac{1}{2}}$ of 4.8×10^{-3} mol s l^{-1} which, when divided by the total molecular weight of avian globin mRNA (663,000 daltons; three separate RNA species each of 650 residues, ref. 24), gives a value of 7.2×10^{-9}. This is in very good agreement with the values of $6.3 - 7.4 \times 10^{-9}$ obtained for the four CMV RNAs and their homologous cDNAs (Table 1) and indicates that each RNA is a unique species.

In summary, therefore, we have shown that no significant levels of homology could be detected between RNA species 1 and 2, that RNA 4 is completely contained within RNA 3 and that sequences of 250 - 350 residues are common to both RNA 1 and RNA 4 and similarly between RNA 2 and RNA 4. In addition, there is a unique sequence for each of the RNAs which do not contain detectable reiterated sequences.

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