Characterization of the RNA Species of Citrus Variegation Virus with Complementary DNA Clones[1]

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ABSTRACT. Citrus variegation virus (CVV) is an ilarvirus with a tripartite genome. The RNA of CVV was used to prepare a library of 76 complementary DNA (cDNA) clones. The clones were confirmed to be viral specific by hybridization analysis and were sized by restriction digest analyses. One clone of approximately 1750 bases was complementary (Northern blot analysis) to RNA 3 and RNA 4 of the CVV genome. Another clone of over 800 bases was shown to be complementary to the RNA 2 of CVV. This clone also hybridized with RNA 4a, which may be a subgenomic product of RNA 2. These clones were used as probes to characterize the homology between two isolates of CVV and one isolate of citrus leaf rugose virus.
Index words. ilarviruses, citrus leaf rugose virus.

Infectious variegation used to be considered as part of the psorosis disease complex (16). After citrus variegation virus (CVV) was purified and characterized, it was determined to be a member of the ilarvirus group (7). Citrus leaf rugose virus (CLRV) and CVV are serologically related viruses which cause diseases that are distinct from those caused by the psorosis viruses (7, 8).

The ilarviruses and the alfalfa mosaic virus (AlMV) group have been proposed as one genus of the family of tripartite viruses, the Tricornaviridae (15). Several members of the ilarvirus and AlMV groups have been well characterized. The complete tripartite genome of the AlMV (strain 425) has been sequenced. The RNA 1 and 2 each contain a single open reading frame (3, 4). The RNA 3 of AlMV and tobacco streak virus (TSV) have been sequenced and each contains two open reading frames (1, 5), but only the 5′ proximate coding region is translated. The RNA 4 is a subgenomic messenger RNA for the coat protein and is homologous to the 3′ proximal end of RNA 3. The proteins translated from RNAs 1-4 are designated P1-4, respectively.

The genome of CVV consists of three single-stranded messenger sense RNAs, which are encapsidated separately in icosahedral particles and are designated RNA 1, 2, and 3 in order of decreasing size. Two additional RNA species, designated RNA 4 and 4a, are present in viral RNA preparations (10). The RNA 4a has not been reported in CLRV or the other ilarviruses. The CVV RNAs 1, 2, and 3 require either the RNA 4 + 4a fraction or coat protein to be infectious (10). The coat proteins of other ilarviruses or AlMV can be interchanged with the coat protein of CVV to cause a mixture of the RNA 1, 2, and 3 species to be infectious (9, 11). The coat proteins of AlMV and TSV are interchangeable despite the lack of homology between the coat proteins (5). The coat protein binds preferentially to sequences in the 3′ terminal region of the RNA species, and this binding has been hypothesized to be required for recognition of viral RNAs by the viral replicase (12).

This is the first report on cDNA cloning of CVV RNA. Two cDNA probes were used to compare homology of the RNA species. These probes were also used to test their efficacy in detecting a second isolate of CVV and CLRV.

MATERIALS AND METHODS

Virus purification. The viral isolates, designated CVV-1 and CLRV and described by Garnsey (7, 8), were maintained in greenhouse cultivated RMA-861 Etrog citron plants. The

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isolate CVV-FM causes severe symptoms in citrus. The virions were purified by a method described by Garnsey (8) and modified to include two cycles of calcium phosphate gel clarification of the crude extract.

Materials used for cDNA cloning and analysis of the clones. Restriction enzymes, DNA polymerase I, polyadenine polymerase, terminal deoxynucleotidyl transferase, T-4 ligase, phenol, RNA molecular weight standards, lambda RF-DNA, and phiX 174 Hae III fragments were all from Bethesda Research Laboratories, Inc. (Gaithersburg, MD 20877). Deoxyribonucleotides, ribonucleotides, and 3-indolyl-B-D-galactosidase were obtained from Boehringer Mannheim (Indianapolis, IN 46250). The *Escherichia coli* strain JM 83 and the plasmid pUC-19 were described by Viera and Messing (16). Reverse transcriptase was from Seikaguki (St. Petersburg, FL 33702). The radioisotope $^{[32]}$p deoxycytosine triphosphate (dCTP) was from Amersham (Arlington Heights, IL 60005). Nitrocellulose membranes and NA-45 DEAE cellulose membranes were from Schleicher and Schuell (Keene, NH 03431).

Isolation of viral RNA and polyadenylation of the viral RNA. The RNA was extracted from purified virus by treatment with 0.1% sodium dodecyl sulfate (SDS) in 0.05 M Tris, pH 8.0, for 5 min at 60 C, followed by phenol, phenol:chloroform and chloroform extration. Ammonium acetate was added to a final concentration of 2.5 M and two volumes of absolute ethanol were added to the RNA preparation before precipitating the RNA by centrifugation (13). The pellet of CVV-RNA was rinsed with 70% ethanol and dried. The RNA was resuspended in sterile water and the quantity estimated by spectrophotometric analysis.

The RNA was polyadenylated using the enzyme poly A polymerase to catalyze a 3′ tailing reaction following the protocol of Devos, *et al.* (6). The reaction mixture was subjected to phenol:chloroform extraction and the RNA was recovered by ethanol precipitation.

Synthesis and cloning of cDNA. The hybrid cDNA: RNA cloning procedure described by Cann, *et al.* (2) was used as the basis for the construction of a cDNA library of CVV RNA. The vector, pUC-19, was cut with the Pst I restriction enzyme and tailed at the 3′ termini with deoxygunaidine using the enzyme terminal deoxynucleotidyl transferase to catalyze the reaction. The reaction was terminated by the addition of EDTA and the reaction mixture was subjected to phenol:chloroform treatment and ethanol precipitation.

The cDNA synthesis was primed by the addition of oligodeoxythymidylic acids (oligo $(dT)_{12\text{-}18}$) to 2-4 μg of polyadenylate CTV RNA. The reaction was terminated by the addition of EDTA and the reaction mixture was phenol: chloroform extracted. The unincorporated nucleotides were removed by spin column chromatography (13) using Bio-rad P60 agarose as the column matrix gel, and the fractions containing incorporated isotope were ethanol precipitated. The cDNA: RNA hybrids were then tailed with deoxycytosine using the enzyme terminal deoxynucleotidyl transferase to catalyze the reaction. The procedures used to stop the reaction and remove the unincorporated nucleotides were repeated. The cDNA: RNA hybrids preparations were resuspended with water and annealed to the deoxyguanidine tailed pUC-19 using the conditions described by Cann. *et al.* (2). The cDNA:RNA hybrids annealed to pUC-19 were used to transform cells of *Escherichia coli* strain JM 83 which had been made competent using a calcium chloride treatment. The bacterial cells were grown on Luria-B medium containing ampicillin at 50 μg/ml and 3-indolyl-β-D-galactosidase at 40 μg/ml. The resultant white and light blue colonies were verified to contain cDNA inserts of CVV RNA

by colony hybridization with [32]P deoxycytosine triphosphate labeled first-strand cDNA transcribed from randomly primed CVV RNA.

Analysis of the cDNA clones. The plasmids of the CVV-cDNA clones were purified using alkali purification and the DNA transferred to nitrocellulose membranes using procedures described by Maniatis, *et al.* (13). Selected cDNA inserts were recovered from the agarose gel with NA-45 cellulose papers. These cDNA inserts were [32]P labeled by nick translation reactions (13). The labeled inserts were used as probes for the detection of either total CVV RNA or for CVV RNA which had been glyoxalated and separated into its RNA species by agarose gel electrophoresis and transferred to nitrocellulose filters. The hybridization solution contained sodium chloride and sodium citrate (6X SSC), 5X Denhardt's solution, and salmon sperm (13). The filters were rinsed for 5 min at room temperature and then for 15 min at either 50 C or 68 C in 2X SSC and 0.5% SDS. The final rinse was for 15 min at either 50 C or 68 C in 0.5X SSC and 0.5% SDS.

RESULTS

The species of CVV RNA were estimated to be 3520, 2990, 2350, 1120, and 890 bases in size for the RNA 1, 2, 3, 4, and 4a, respectively (fig. 1). These are equivalent to 1196, 1015, 800, 380, and 304 kDa, respectively. The molecular weights of the CLRV RNA species were nearly identical to their CVV RNA counterparts. No 4a species was detected in the CLRV preparations.

After transformation of *E. coli* with the cDNA:RNA hybrid annealed with the pUC-19 plasmids, 76 of the 105 white and light blue colonies were positive in colony hybridization tests. The average length of the cDNA inserts for 27 clones was approximately 500 bases. Four of the cDNA inserts were approximately 1750 bases in length. One of these four clones (CVV-34) hybridized with RNA 3 and

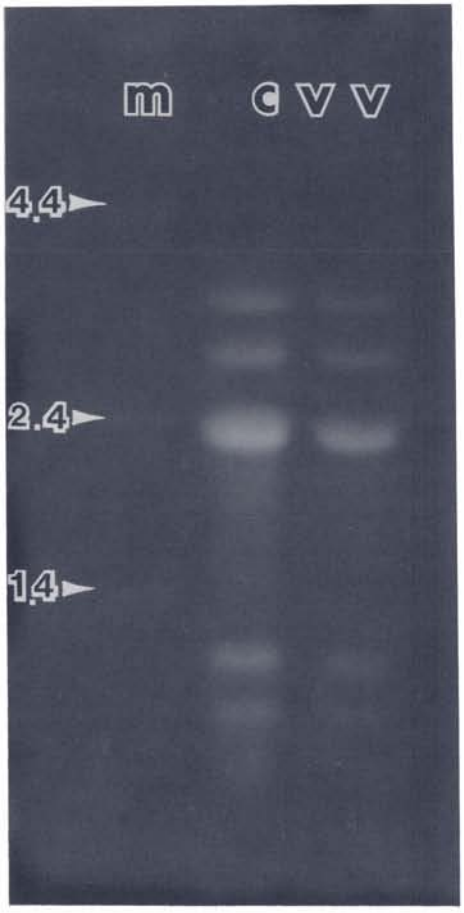

Fig. 1. Separation of RNAs 1, 2, 3, 4 and 4a after glyoxalation and electrophoresis on agarose gels. Ethidium bromide was used to stain the RNAs. The lane m contains single-stranded RNA standards of 4.4, 2.4, and 1.4 kilobases which are indicated with arrows from top to bottom, respectively. RNAs extracted from the CVV-1 isolate are in concentrations of 3 μg and 1 μg per lane (quantified by optical density) in the middle and left lanes, respectively.

RNA 4 of CVV in Northern blot analysis at 68 C (fig. 2). These four clones were similar in size and had common restriction sites. A series of restriction digests for the clone CVV-34 analyzed on agarose gels are shown in fig. 3. The two fragments of 1100 and 650 bases that result when the plasmid was cut with Hind III and Bam HI restriction endonucleases were purified using agarose gel electrophoresis and NA-45 cellulose papers. Both the fragments were nick

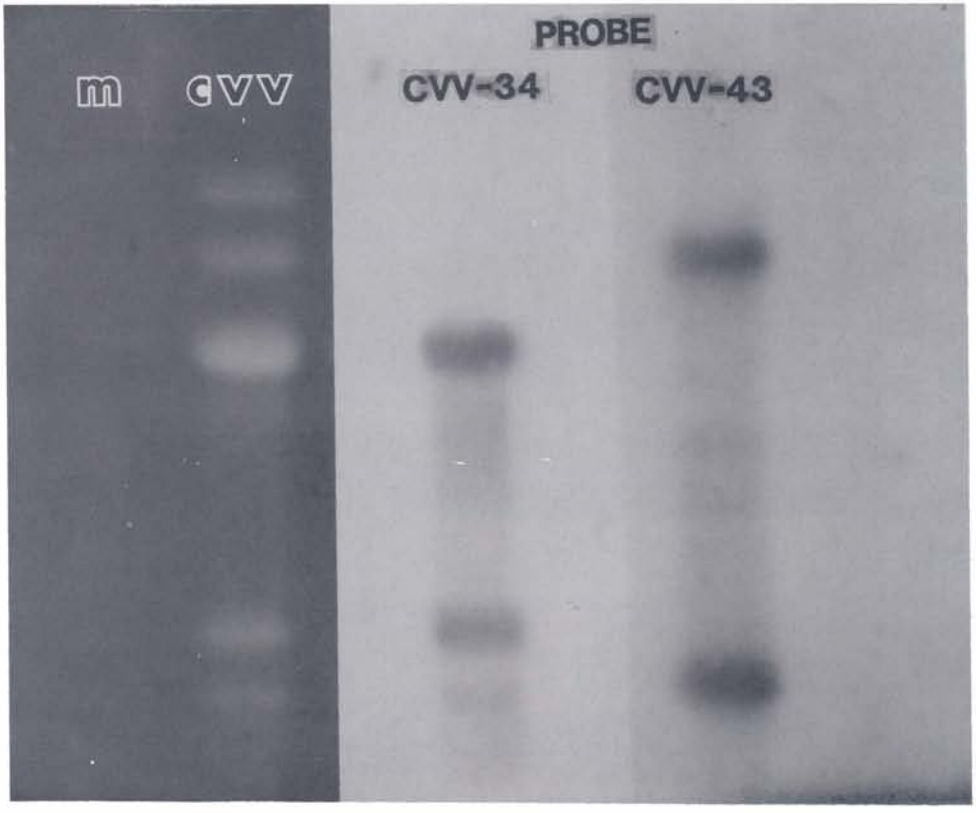

Fig. 2. Separation of CVV-1 RNAs after glyoxalation and electrophoresis on an agarose gel. The lane CVV contains the CVV-1 RNAs stained with ethidium bromide. Lanes CVV-34 and CVV-43 are autoradiograms of CVV-1 RNAs which were transferred to nylon membranes and hybridized at 68 C with $^{[32]}$p-labelled clones CVV-34 and CVV-43. Probe CVV-34 hybridized with RNAs 3 and 4, and probe CVV-43 hybridized with RNAs 2 and 4a.

translated using $^{[32]}$P dCTP as a label and they hybridized to both the RNA 3 and RNA 4 in Northern blot analysis at 68 C. Using these clones to screen a second library of CVV clones, the 650 base fragment hybridized with 18 clones and the 1100 base fragment hybridized with 7 of the clones.

Another CVV clone, CVV-43, with an insert of about 800 bases. hybridized with RNA 2 of CVV in Northern blot analysis at 68 C. The clone CVV-43 also hybridized with a fifth species of RNA that is smaller than RNA 4 (fig. 2). No internal restriction site has been determined for this clone.

The nick translated clones CVV-34 and CVV-43 were used as probes to detect the RNA of the CVV-FM isolate and CLRV using conditions that were less stringent than those used in detection of the RNA of the homologous strain CVV-1. The hybridization of these probes and the washing of the filters were done at 50 C. Even when the filters were overexposed, no hybridization with CVV-FM or CLRV could be detected (fig. 4).

DISCUSSION

The DNA: RNA hybrid method of cDNA as described by Cann, *et al.* has been used successfully to clone viruses which contain polyadenine sequences on their 3′ termini (2, 14). Because CVV RNAs lack 3′ polyadenine sequences, the CVV RNA was polyadenylated *in vitro*, which permitted oligo dT to be used

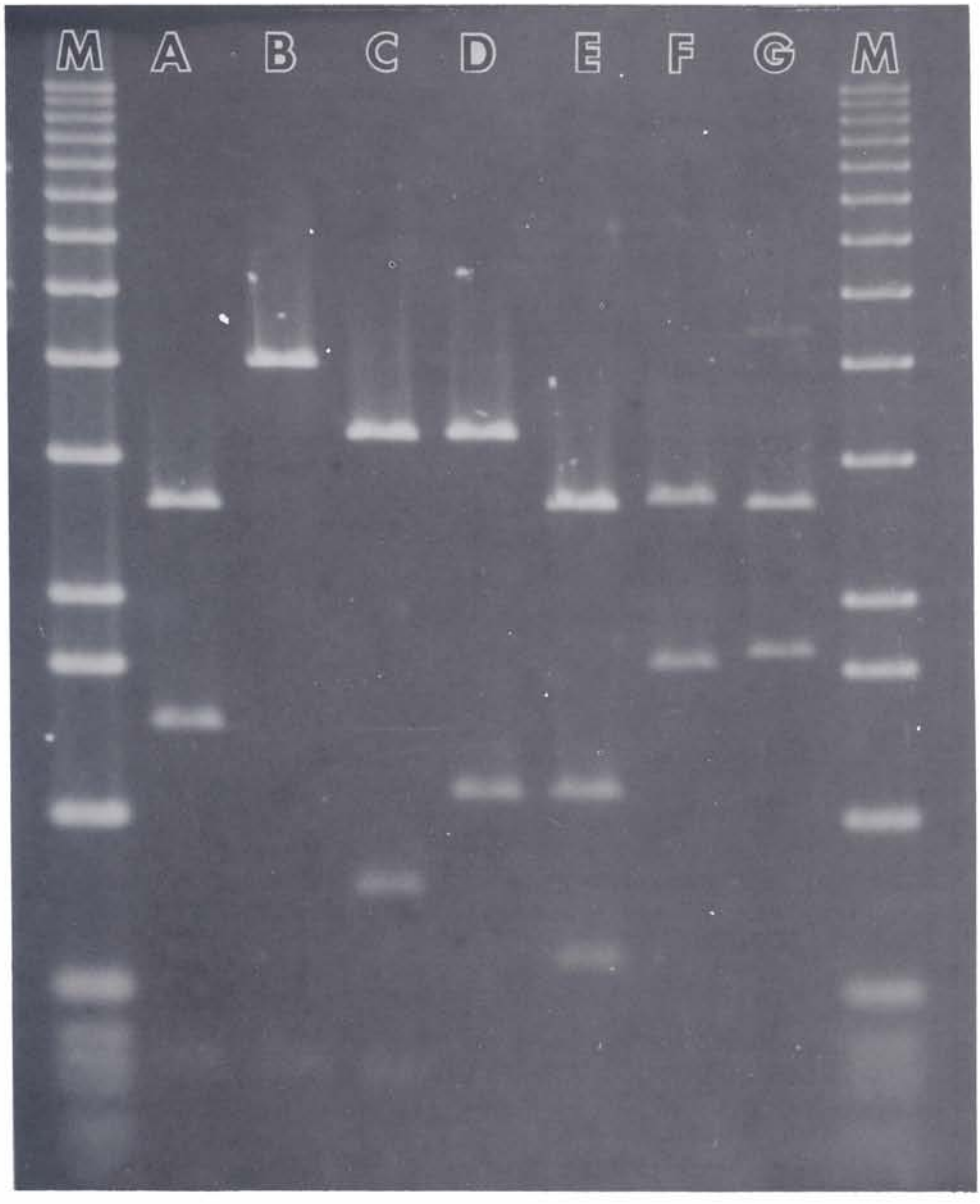

Fig. 3. Agarose gel separated restriction digests of the cDNA clone CVV-34 stained with ethidium bromide. Lanes M are markers, lane A is a digest with the restriction enzymes Hind III and Eco RI, lane B Eco RI, lane C Eco RI and Bam HI, lane D Bam HI, lane E Hind III and Bam HI, lane F Pst I, and lane G Hind III and Sst I. The 2700 base fragment in lanes A, E, F, and G is the pUC-19 vector fragment. The cDNA clone is in the vector pUC-19 and was inserted at the Pst I site. The Hind III restriction site in the polylinker region is adjacent to the 3′ terminus.

as the primer for the first strand cDNA synthesis. The polyadenine polymerase reaction requires the presence of a free 3′ hydroxyl group. Since the 3′ termini of ilarviruses are known to fold into a secondary structure resembling tRNAs, it was not known if CVV RNA could be polyadenylated. The success of the CVV cloning showed that cloning methods that rely on the presence of a polyadenine 3′ termini can be adapted to ss-RNA viruses that normally lack a polyadenine tail.

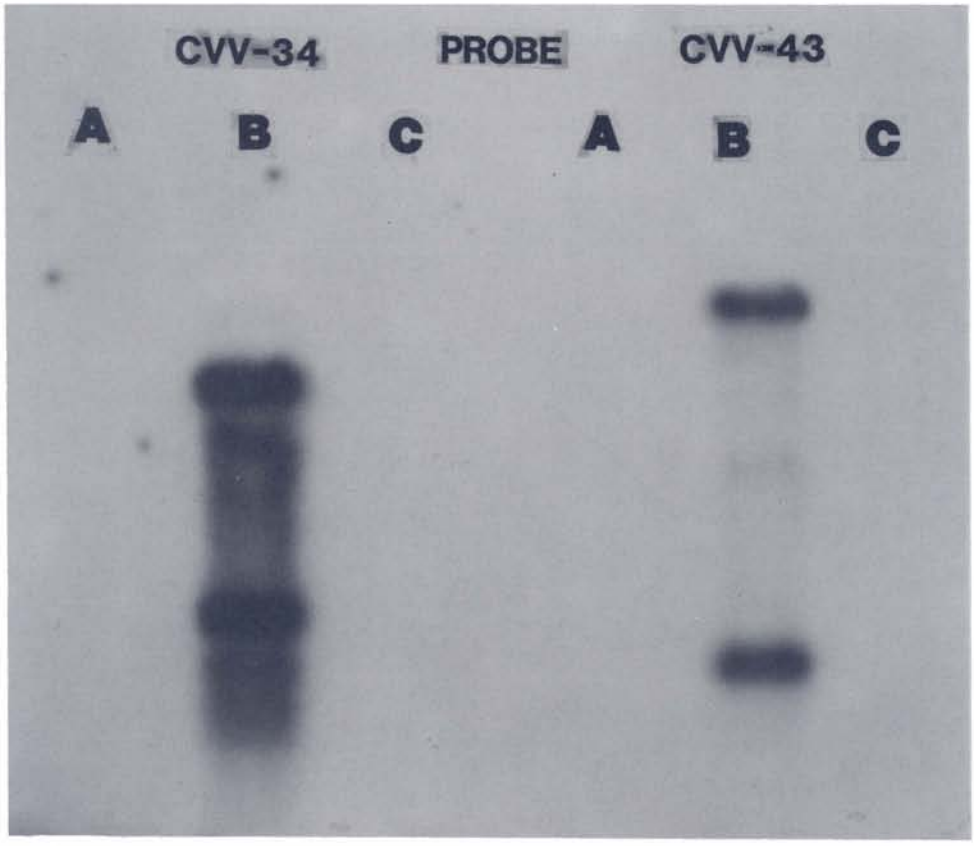

Fig. 4. Hybridization at 50 C of ^{32}P-labelled clones of CVV-34 (left) and CVV-43 (right) with Northern blots of RNAs from isolates CVV-FM, CVV-1, and CLRV, lanes A, B, and C, respectively. The autoradiogram was overexposed in an attempt to detect hybridization with CVV-FM isolate and CLRV.

The orientation of the CVV-34 clone was determined because both the 650 and 1100 base fragments hybridized with RNA 4. Since the RNA 4 size is estimated to be 1120 bases, the 650 base fragment must be the 3′ proximal region. The screening of the second library showed that three times as many clones hybridized with the 650 fragment as with the 1100 base fragment. The 3′ end of the RNA species would be expected to be in greater abundance because of the cDNA cloning method used.

The size estimates for the RNA species are approximately 10% higher than the previous estimates (10). The estimates in this report were made by denaturing the RNA with glyoxal and with glyoxylated ss-RNA standards. These estimates are similar to the sizes that are reported for other closely related viruses (1, 3, 4, 5). The RNA species that the clones complemented could easily be identified by hybridization analysis with CVV RNA which had been run on an agarose gel and transferred to a nitrocellulose filter. Using this method, the RNA 4a species was homologous to the RNA 2. While the 3′ end of the RNA species of AlMV are highly homologous (2), it is not likely that RNA 4a hybridized because of shared homology. When using lower stringency conditions (fig. 4) with the CVV-43 probe only RNA 2 and 4a were detected. It is possible that the RNa 4a is a subgenomic species, since it has the potential to encode a protein of more than 30 kDa.

The use of cDNA probes to detect viruses appears to be very specific. The viruses CVV-1, CVV-FM, and

CLRV are serologically related. Nevertheless, no homology between the viruses could be detected with the limited number of clones tested even when using less stringent conditions for hybridization and washing. This confirms that cDNA probes can be more specific for virus and strain identification than serology using polyclonal antisera.

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