Differential Hybridization with Cloned cDNA Sequences for Detecting a Specific Isolate of Citrus Tristeza Virus

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ABSTRACT

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Two plasmids with cDNA inserts representing different regions of the citrus tristeza virus (CTV) genome were used to compare five biologically distinct isolates of CTV. One of the plasmids (D2) hybridized strongly with all of the isolates. The other plasmid (D1) hybridized strongly with four of the isolates. A fifth isolate, which caused severe symptoms, hybridized weakly, if at all, with D1. Similar results were obtained using total plant RNA, RNA extracted from purified and partially purified virus preparations, or dsRNA preparations. Virion RNA of two CTV isolates, HT and VT, used as probes for hybridization with restriction patterns of the D1 CTV clone revealed DNA fragments that carry strain specific sequences.

Additional key words: closterovirus.

Citrus tristeza virus (CTV), a closterovirus, is the most economically important citrus virus in the world (5). Several aphid species transmit CTV in a semipersistent manner (3). In some citrus areas where CTV is not widespread, such as in Israel, the virus may be controlled effectively by the early diagnosis and eradication of CTV-infected trees. However, in Florida about 90% of the citrus budwood propagated on sour orange rootstock is infected with CTV (12). Most of the CTV strains in Florida are mild and do not cause decline, but occasionally infection by severe CTV strains causes considerable economic loss due to decline of trees on sour orange rootstock (5). Also, in 1983 it became apparent in some Florida citrus nurseries that some of the budwood source trees had become infected with severe CTV strains. Infected budwood propagated on sensitive sour orange rootstock produced severely dwarfed trees.

Several serological assays can detect CTV in plants, but these assays cannot differentiate CTV strains (9). Currently, the biological properties of CTV isolates are determined by indexing on a series of indicator plants (23), which requires considerable time and greenhouse space. There is a need, especially in Florida, for a rapid diagnostic test to differentiate CTV strains. In this article, we describe a differential hybridization method for screening CTV isolates with cloned cDNA from two different regions of the CTV genome. These clones were further analyzed by restriction endonuclease cleavage and hybridization with the genomic RNAs of two virus isolates.

MATERIALS AND METHODS

CTV isolates and plant material. Five isolates of CTV from Florida were used in this study. The T-4 isolate causes strong veinclearing, stunting, and stem-pitting in Mexican lime seedlings (*Citrus aurantifolia* Swingle), no visible decline on sweet orange (*C. sinensis* (L.) Osbeck) on sour orange rootstocks (*C. aurantium* L.) and no seedling yellows (SY) symptoms on sour orange or Eureka lemon (*C. limon* (L.) Burm. f.) seedlings (10). Isolate T-3, originally described by Grant and Higgins (14), also causes severe symptoms on Mexican lime, a severe decline of sweet orange on sour orange rootstock, and SY symptoms on Eureka lemon and sour orange seedlings. The T-36 isolate was collected from sweet orange trees on sour orange rootstock affected by quick decline near Winter Garden, FL (11). The T-36 isolate produces severe veinclearing, stunting, and stem-pitting on Mexican lime, mild SY symptoms on Eureka lemon and sour orange seedlings, and quick decline of sweet orange trees on sour orange rootstock. The T-26 and T-30 isolates produce very mild symptoms and little stunting on Mexican lime, do not produce SY symptoms on indicator plants, and do not cause decline of trees on sour orange rootstock.

The Israeli isolates, HT and VT, have been previously described (17). The HT isolate produces severe symptoms on Mexican lime and does not induce SY. The VT isolate produces moderate symptoms on Mexican lime and SY symptoms on sour orange and Eureka lemon.

Each of the five Florida isolates was graft transmitted to *C. excelsa* Webster, Duncan grapefruit (*C. paradisi* Macf.), citron (*C. medica* L. seeding clone 'Arizona 861', *C. hystrix* DC, Mexican lime, and Lab sweet orange. Several additional field selections of CTV, which varied in their biological activities, were transmitted to *C. excelsa* and citron. The plants were grown in a partially shaded, air-cooled glasshouse where temperatures ranged from 21 to 30 C.

Virus purification. Partially purified CTV was prepared by pulverizing 0.5–1.0 g of tender bark tissue with liquid nitrogen in a mortar, then thawing and homogenizing the powder in five volumes of 0.05 M Tris-Cl buffer, pH 8.0. The homogenate was centrifuged for 10 min at 10,000 *g*. The volume of the supernatant was measured and 1/4 volume of a solution of 30% polyethylene glycol-8,000 (PEG) and 0.6 M NaCl was added. The tubes were sealed with Parafilm and shaken overnight at 4 C. The PEG precipitate was collected by centrifugation for 10 min at 10,000 *g* and resuspended in 400 μl of 0.05 M Tris-Cl buffer, pH 8.0.

Purified CTV was prepared as described by Lee et al (16).

Extraction of RNA. The RNA was extracted from partially purified CTV or from purified virus preparations using phenol/chloroform (1:1) as previously described (19). Total RNA was extracted from healthy and CTV-infected tissue by pulverizing tissue in liquid nitrogen and extracting in 2× GPS buffer (2× GPS

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= 0.2 M glycine, 0.1 M Na_2PO_4, and 0.6 M NaCl, pH 9.6) with phenol/chloroform as described by Baksh et al (2). The aqueous phase was removed after centrifugation for 10 min at 10,000 *g* and 2 1/2 volume ethanol was added to precipitate the nucleic acids.

Extraction of double-stranded RNAs. Double-stranded (ds) RNA was extracted from 4-g lots of tender bark and leaf tissue after the tissue was pulverized in liquid nitrogen. The CF-11 cellulose column procedure of Dodds and Bar-Joseph (7) was used, except extraction was in 2× GPS buffer.

Gel electrophoresis and blotting. The RNA was electrophoresed in a denaturing formamide/formaldehyde agarose gel and blotted onto nitrocellulose membranes as described by Fellous et al (8).

The dsRNA was fractionated on 5% polyacrylamide gels as described by Rosner et al (18). The gels were stained with ethidium bromide (18) and electroblotted onto diazophenylthioether (DPT)-paper (Schleicher & Schuell, Keene, NH), which was converted from 2-aminophenylthioether (APT)-paper to the diazo form immediately before use (1). The slab gels (1.5 mm thick) were soaked for 45 min in 50 mM NaOH plus 10 mM NaCl, 45 min in 0.1 M Tris-Cl buffer, pH 7.5, and 1 hr in 20× SSC buffer before electroblotting. The electrophoretic transfers employed a Bio-Rad Transblot apparatus (Bio-Rad, Richmond, CA) for 3 hr at 4 C at 32 V using TBE (90 mM Tris-borate, 0.2 mM EDTA, pH 8.3) buffer.

Dots were prepared by applying RNA samples (2–5 μl) onto a nitrocellulose membrane presoaked in 20× SSC buffer. After spotting, the paper was air-dried and then baked for 6 hr at 75–80 C.

Hybridization conditions. The D1 and D2 plasmids with cDNA inserts of about 2,200 and 800 BP, respectively, have been previously described (19). The plasmids were labelled with ^{32}P by nick translation (13) and hybridizations were done in 50% formamide, 4× SSC, 4× Denhardt solution, 50 mM sodium phosphate buffer, pH 6.5, containing 250 μg/ml salmon sperm DNA (6,17,18,22) at 42 C.

Enzyme-linked immunosorbent assay (ELISA). The double antibody sandwich ELISA procedure as described by Garnsey et al (9) for CTV was used. Antisera were prepared against whole, unfixed CTV isolate T-36. The IgG fraction was collected using a protein A affinity column (15). Aliquots of PEG precipitated CTV preparations were diluted 1/100 in 0.05 M Tris-Cl buffer, pH 8.0, and were loaded in flat-bottomed Immulon II plates that had been previously coated with 1 μg/ml of CTV IgG. A dilution series of known amounts of a highly purified CTV isolate, T-3, was loaded onto each plate to establish a standard curve. CTV concentrations in preparations partially purified with PEG were estimated from the standard curve. The substrate, p-nitrophenyl phosphate (Sigma, St. Louis, MO), concentration was 1 mg/ml in substrate buffer (19). The plates were read after 30 min on a Bio-Tek EIA plate reader (Bio-Tek, Burlington, VT) at 405 nm.

Characterization of cloned cDNA sequences. End-labelling of virus RNA with ^{32}P was after the procedure of Rosner et al (18). Cleavage of plasmid DNA with the restriction endonucleases, Hae III, Alu I, Hinf, Hpa II, and Pst I, was as instructed by the producer (New England Biolabs, Beverly, MA). The methods employed for agarose gel electrophoresis and Southern blots were according to Southern (20).

RESULTS

Detection of CTV by hybridization. Citrus tristeza virus nucleic acid was detected in extracts from infected plants by hybridization with cloned cDNA sequences. Aliquots of the total RNA and dsRNA fractions from plants infected with each of the five biologically defined Florida CTV isolates were spotted onto nitrocellulose membranes and hybridized with the two cloned cDNA sequences, D1 and D2. Probe D2 hybridized with the dsRNA and total RNA extracted from plant tissue infected with all five Florida CTV isolates, but not with similar RNAs extracted from healthy tissue (Fig. 1). The weak hybridization of total RNA from T-4 is not typical. Although the D1 probe hybridized well with four of the five CTV isolates (T-3, T-4, T-26, and T-30), it consistently hybridized weakly, if at all, with the fifth isolate, T-36 (Fig. 1).

The RNA of the five biologically defined isolates extracted from partially purified preparations was also analyzed. Aliquots from the resuspended PEG-precipitated pellets before RNA extraction were subsequently diluted and virus concentrations were estimated by ELISA. RNA from isolate T-36 (spots 4 and 15 in Fig. 2) did not

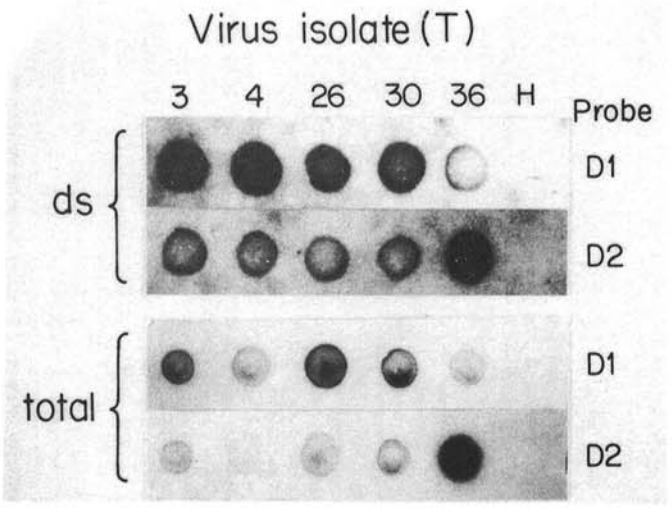

Fig. 1. Dot hybridization of cloned cDNA sequences to RNA extracted from healthy and citrus tristeza virus (CTV) infected *Citrus excelsa* bark tissue. Double-stranded (ds) RNA and total RNA extractions were made from healthy bark tissue and from bark tissue infected with CTV isolates T-3, T-4, T-26, T-30, and T-36. The RNA was dotted onto nitrocellulose and hybridization was carried out with either D1 or D2 plasmids as indicated.

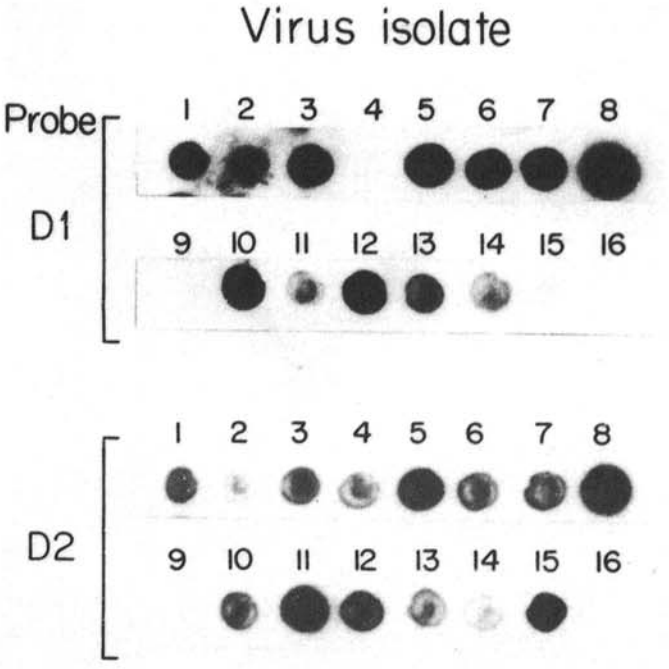

Fig. 2. The RNA was extracted from the partially purified preparations from CTV-infected and healthy bark, dotted onto nitrocellulose, and hybridization carried out with either the D1 or D2 plasmid clones. Spots 1–11 were bark of *Citrus hystrix*, spots 12–14 were from bark of grapefruit, and 15 and 16 were from bark of *C. excelsa*. The quantities of CTV represented in each spot was estimated by ELISA and are as follows: spot 1, 10.2 μg T-3 isolate; spot 2, 5.0 μg T-4; spot 3, 8.2 μg T-26; spot 4, 12.9 μg T-36; spot 5, 17.2 μg T-3; spot 6, 11.0 μg T-30; spot 7, 12.9 μg T-30; spot 8, 17.3 μg T-3; spot 9, healthy tissue; spot 10, 4.2 μg T-30; spot 11, 30.7 μg T-36; spot 12, 12.8 μg T-30; spot 13, 12.4 μg T-30; spot 14, 2.4 μg T-30; spot 15, 5.0 μg T-36; and spot 16, healthy tissue.

hybridize with D1 at concentrations of 12.9 and 5.0 μg CTV per spot, whereas spot 11 (Fig. 2), which contained 30.7 μg CTV per spot did hybridize weakly with probe D1. The amount of CTV per spot for the other isolates ranged from 2.4 to 17.3 μg per spot (Fig. 2). Isolate T-4 hybridized only weakly with D2 probe (Fig. 2, spot 2), but this differential hybridization did not occur in other experiments using partially purified preparations.

Fractionation of virus RNA in a denaturing gel. The size of the genomic RNAs of the various isolates after partial purification with PEG was compared by denaturing agarose gel electrophoresis. Nitrocellulose blots of this gel were hybridized with the cDNA clones. The genomic RNAs of the five isolates were essentially identical in size (Fig. 3). The RNA of CTV isolate T-36 hybridized much more weakly with the D1 probe (Fig. 3A) than with the D2 probe (Fig. 3B). This pattern was even more pronounced when equal amounts of concentrated RNA from purified virus particles was hybridized with D1 and D2 probes after electrophoresis and blotting (Fig. 3C). The D2 probe hybridized more strongly with the RNA from purified virus and produced a more diffused and broader band, whereas the D1 probe hybridized with a narrower, sharper band.

dsRNA from CTV infected plants. The dsRNA extracted from plants infected with the five CTV isolates gave somewhat different patterns after electrophoretic fractionation in a polyacrylamide gel (Fig. 4A). All isolates contained the putative full-length replicative form of about 13×10^6 MW (7). Several smaller sizes of dsRNA were also observed and the banding patterns varied with the isolate.

When the dsRNAs were electrophoresed on a denaturing agarose gel, blotted, and then hybridized with D2 probe, several RNA bands in addition to the high molecular weight replicative form were seen (Fig. 4C). Two RNA bands unique to T-36 were present (see arrows in Fig. 4C). When the dsRNAs electrophoresed in a nondenaturing 5% polyacrylamide gel, as in Fig. 4A, were electroblotted to DPT paper and then hybridized with the D2 probe, the area corresponding to the full-length replicative form RNA was identified (Fig. 4B). However, the smaller "subgenomic" dsRNAs were not recognized by the probe, except possibly in one instance with T-3 (Fig. 4B).

Restriction-hybridization analysis of the D1 plasmid clone. The D1 clone hybridized strongly with some CTV isolates and weakly with others (Figs. 1–3) (17). To locate strain specific regions on the D1 insert, the recombinant D1 plasmid was cleaved with multiple site restriction endonucleases (Fig. 5A) and the digest patterns were hybridized with RNAs of the VT and HT tristeza isolates as probes (Fig. 5B and C, respectively). The Pst I restriction endonuclease cleaved on both sides of the D1 insert and at a single site within it, resulting in two fragments of about 4×10^6 and 2.1×10^6 MW, respectively (Fig. 5A, lane 5). However, only the larger fragment hybridized with VT (Fig. 5B, lane 5) and HT (Fig. 5C, lane 5) RNA probes. The HT-RNA probe hybridized weakly with only one DNA fragment in each restriction endonuclease lane (Fig. 5C), whereas the VT-RNA specifically recognized several more DNA fragments (Fig. 5B) and the hybridization in general was much stronger.

DISCUSSION

A library of cloned DNA sequences complementary to the genomic RNA of a severe SY strain of CTV was previously prepared (19) using calf-thymus DNA as a random primer for cDNA synthesis (21). The collection of cloned virus sequences thus obtained presumably cover the whole virus genome.

CTV strains are numerous and differ greatly in biological activity (3–5,14). These differences in biological activity must reflect differences in base sequences of the CTV genome. Analysis of the viral nucleic acids should reveal such differences. Previously, we have demonstrated the applicability of using cloned cDNA probes for detecting CTV (17). In the present report, unrelated

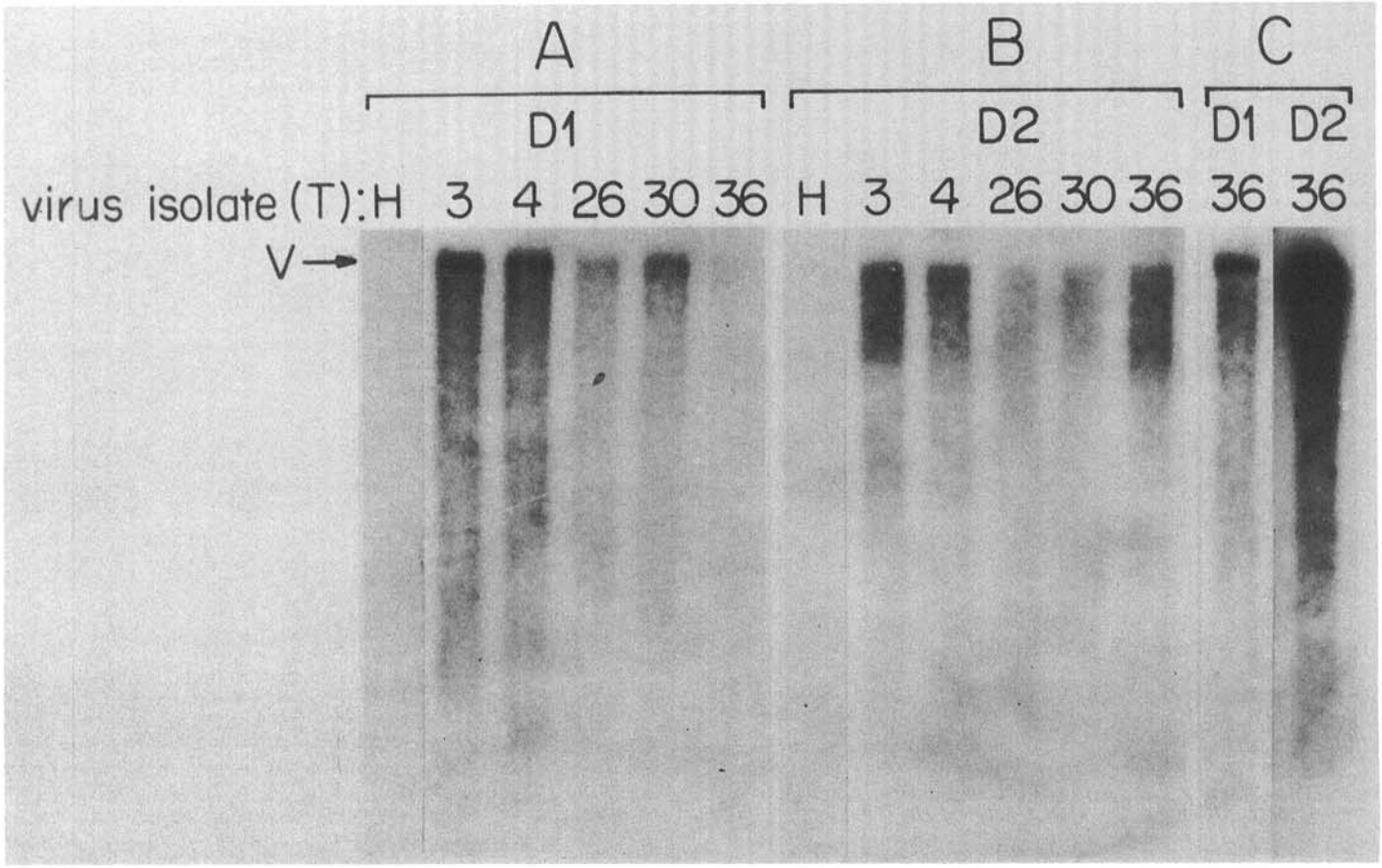

Fig. 3. Fractionation of RNA from partially purified and purified preparations of CTV in a denaturing formamide/formaldehyde agarose gel. After electrophoresis the RNAs were blotted to nitrocellulose and hybridized with either the D1 probe (**A**) or the D2 probe (**B**). Similar blots of RNA extracted from purified virus particles of the T-36 isolate are shown in **C**.

cloned cDNA sequences hybridized differentially with the RNA of a severe Florida isolate, T-36. This T-36 isolate could be distinguished from other isolates by nucleic acid hybridization. These results indicate relatively long stretches of base sequence divergence among the CTV isolates, as minor shifts in the nucleotide sequence in the virus genomic RNA would be indistinguishable if hybridized with a long cDNA sequence. Although it will be useful in future work with cross protection to differentiate the severe T-36 Florida isolate from other Florida CTV isolates, more importantly these results demonstrate that it is possible to differentiate CTV strains by hybridization. Its application for the identification of more CTV isolates will require the analysis of other cloned cDNA sequences derived from different parts of the virus genome and/or from other virus isolates.

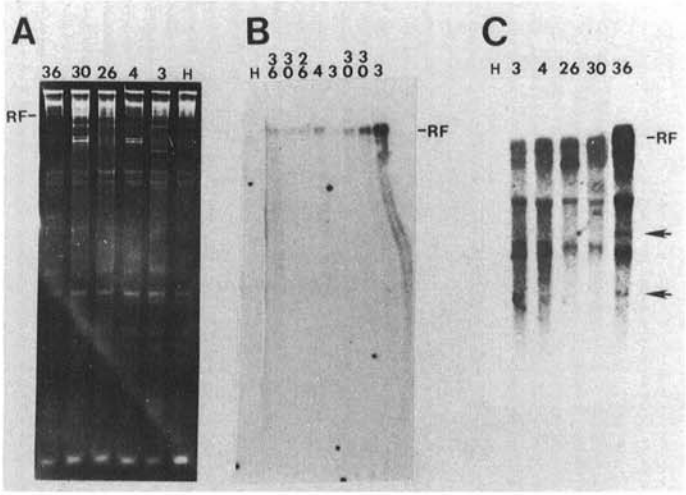

Fig. 4. Fractionation of dsRNAs extracted from healthy *Citrus excelsa* plant (H) and *C. excelsa* plants infected with CTV isolates T-3, T-4, T-26, T-30, and T-36. **A,** Patterns of dsRNAs after electrophoresis in a 5% polyacrylamide gel and stained with ethidium bromide. **B,** The hybridization pattern after electroblotting of the dsRNA to a DPT membrane and hybridization with D2 probe. **C,** Similar samples of dsRNAs after electrophoresis in a denaturing formamide/formaldehyde 1% agarose gel, blotting onto nitrocellulose, and hybridization with D2 probe. Arrows (C) indicate the location of RNAs present in T-36 that were not present in other isolates.

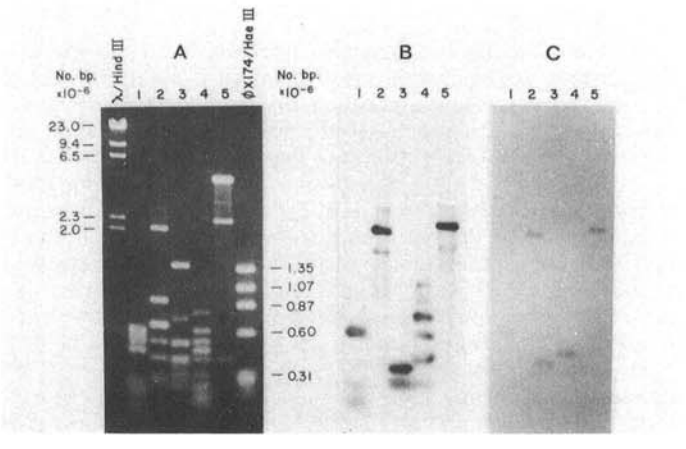

Fig. 5. Southern-blot hybridization of the D1 clone with RNAs of two CTV isolates. Purified DNA of the D1 clone was cleaved with the following restriction endonucleases: Hae III, Alu I, Hinf, Hpa II, and Pst I (lanes 1–5, respectively) and fractionated in an agarose gel together with DNA size markers of λ and φX174 DNA digests (**A**). Southern-blots of this gel were hybridized with 32P kinase labelled RNAs of either the VT (**B**) or HT (**C**) CTV isolates (17).

In a previous study, we showed that two of the Israeli CTV isolates (HT and MT) weakly hybridize with the D1 clone, whereas they strongly hybridize with an unrelated D2 clone (17). In the present study, we demonstrated hybridization of the HT and VT virus RNAs to specific fragments of the D1 clone (Fig. 5). This finding suggests that a strain specific probe could be constructed by subcloning such DNA fragments.

Differences in banding patterns from denaturing gels of dsRNA extracted from infected tissues (Fig. 4B) may also aid in strain diagnosis.

The size of the genomic RNA of all five Florida CTV isolates was the same, including isolate T-36 (Fig. 3A). In addition, no differences were found in the immunological properties of the various Florida isolates by ELISA or other serological tests and polyclonal antisera prepared to several different isolates of CTV (9, *unpublished results*).

The hybridization method proved more rapid and convenient than biological indexing to differentiate CTV strains. It is at least as sensitive as ELISA for detecting CTV in plants (17). The cDNA probes do not hybridize with samples from healthy plants. The hybridization method, therefore, can be applied to diagnose CTV.

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