Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5302–5306, September 1980
Cell Biology

Molecular cloning and characterization of potato spindle tuber viroid cDNA sequences

(viroids/recombinant DNA/complementary RNA)

ROBERT A. OWENS* AND DEAN E. CRESS†

*Plant Virology Laboratory, Plant Protection Institute and †Cell Culture and Nitrogen Fixation Laboratory, Plant Physiology Institute, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Beltsville, Maryland 20705

Communicated by Theodor O. Diener, June 9, 1980

ABSTRACT **Double-stranded cDNA has been synthesized from a polyadenylylated potato spindle tuber viroid (PSTV) template and inserted in the *Pst* I endonuclease site of plasmid pBR322 by using the oligo(dC)-oligo(dG)-tailing procedure. Tetracycline-resistant ampicillin-sensitive transformants contained sequences complementary to PSTV [^{32}P]cDNA, and one recombinant clone (pDC-29) contains a 460-base-pair insert. This cloned double-stranded PSTV cDNA contains the cleavage sites for six restriction endonucleases predicted by the published primary sequence of PSTV as well as one additional site each for *Ava* I, *Hae* III, *Hpa* II, and *Sma* I. The additional *Ava* I, *Hpa* II, and *Sma* I sites are explained by the presence of a second C-C-C-G-G-G sequence in the cloned double-stranded cDNA. The largest fragment released by *Hae* III digestion contains approximately 360 base pairs. These results suggest that we have cloned almost the entire sequence of PSTV, but the sequence cloned differs slightly from that published. Hybridization probes derived from pDC-29 insert have allowed detection and preliminary characterization of RNA molecules having the same size as PSTV but the opposite polarity. This RNA is present during PSTV replication in infected tomato cells.**

Viroids are distinct RNA species of relatively low molecular weight ($1.1–1.3 \times 10^5$) and unique structure that cause several important diseases of cultivated plants (1). Viroids are the smallest known agents of infectious disease and replicate autonomously despite their small size and lack of a protective capsid. Considerable progress has been made during the past several years in establishing the primary and secondary structure of viroids. The complete 359-nucleotide primary sequence of potato spindle tuber viroid (PSTV) has been determined, and the covalently closed circular RNA forms an extended rod-like structure characterized by a series of double helical sections and internal loops (2). Progress toward establishing the mechanism of viroid replication and possible mechanisms of pathogenesis has been much slower.

Two groups have synthesized complementary DNA (cDNA) for viroids and have pointed out the potential applications of such probes to studies of the site(s), relative rate, and molecular mechanism of viroid replication (3, 4). An ideal viroid cDNA preparation would contain homogeneous full-length copies of the viroid template used for cDNA synthesis. We report here on the molecular cloning and characterization of double-stranded PSTV cDNA sequences. Our results indicate that we have cloned nearly the entire sequence of PSTV and that the cloned DNA can be used to detect RNA sequences having the same or opposite polarity as PSTV. These RNA sequences are present during PSTV replication in infected tomato cells.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Purification of PSTV. The Beltsville strain of PSTV was purified from infected tomato tissue (*Lycopersicon esculentum* Mill., cv. Rutgers) (5). Final purification involved two cycles of polyacrylamide gel electrophoresis and yielded an approximately equimolar mixture of circular and linear PSTV molecules (6–8).

Synthesis of Double-Stranded cDNA. PSTV was treated with 0.025 unit of *Escherichia coli* alkaline phosphatase (electrophoretically purified, Worthington‡) per μg of RNA to remove 3′-terminal phosphate residues from linear molecules (9). Poly(A) was added by incubation with poly(A) polymerase (ATP:polynucleotide adenylyltransferase) from maize seedlings (10), and the polyadenylylated PSTV was recovered by phenol/chloroform extraction and ethanol precipitation.

Single-stranded PSTV cDNA was synthesized by incubating 6 μg of polyadenylylated PSTV with 114 units of reverse transcriptase (obtained from J. W. Beard, Life Sciences, St. Petersburg, FL) in a 50-μl reaction mixture containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 0.04% Triton X-100, $p(dT)_{12-18}$ at 1.4 A_{260} unit/ml, and 1 mM each of dATP, dCTP, dGTP, and dTTP. After a 2-hr incubation at 37°C, the mixture was heated at 100°C for 3 min, quenched at 0°C, and added to 50 μl of solution containing 180 mM potassium phosphate (pH 7.0), 10 mM $MgCl_2$, 20 mM dithiothreitol, 600 μM each of dATP, dCTP, dGTP, and dTTP, bovine serum albumin at 100 μg/ml, and 15 units of *E. coli* DNA polymerase I (Boehringer Mannheim). Second-strand DNA synthesis was allowed to proceed for 1 hr at 37°C before the double-stranded cDNA was recovered by ethanol precipitation. Non-base-paired regions were removed from the double-stranded PSTV cDNA by a 1-hr incubation at 37°C with S1 nuclease at 200 units/ml in the presence of cucumber mosaic virus RNA carrier at 10 μg/ml double-stranded and 120 μg/ml single-stranded. S1-digested double-stranded PSTV cDNA was recovered by phenol/chloroform extraction and ethanol precipitation.

Single-stranded PSTV [^{32}P]cDNA was synthesized as described above except that the reaction mixture contained 60 μM dCTP [52 Ci/mmol (1 Ci = 3.7×10^{10} becquerels)], and the cDNA synthesis reaction mixture was not heated before isolation of the cDNA by alkaline hydrolysis and ethanol precipitation.

Construction of Hybrid Plasmids. Oligo(dG) and oligo(dC) tails were added to *Pst* I-cleaved pBR322 plasmid DNA and

Abbreviations: PSTV, potato spindle tuber viroid; DBM paper, diazobenzyloxymethyl paper; bp, base pair(s).

‡ Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

S1-digested double-stranded PSTV cDNA, respectively, by incubation with calf thymus terminal deoxynucleotidyltransferase [P-L Biochemicals] in the presence of 1 mM Co^{2+} (11). Oligo(dC)-tailed double-stranded PSTV cDNA and oligo(dG)-tailed pBR322 DNA were combined at a ratio of 1:10 (wt/wt), recovered by phenol/chloroform extraction and ethanol precipitation, and annealed before transformation (12).

Transformation and Identification of Recombinant Clones. *E. coli* C600 (r_k^- m_k^+) was transformed by a modification of the Mn^{2+}, Ca^{2+} transfection protocol of Enea *et al.* (13) under P1–EK1 containment conditions as required by the then current National Institutes of Health Guidelines for Recombinant DNA Research. Tetracycline-resistant ampicillin-sensitive transformants were screened by a modified colony hybridization procedure (14) using PSTV [^{32}P]cDNA as probe.

Isolation of Plasmid DNA. Chloramphenicol-amplified (15) cultures were lysed with Triton X-100 (16), and ribonuclease-treated cleared lysates were extracted with phenol/chloroform and precipitated with ethanol. Supercoiled DNA was isolated by two successive CsCl/ethidium bromide centrifugations (17).

Isolation, Sizing, and Characterization of Cloned DNAs. All DNA fragments were prepared and analyzed by electrophoresis in 5% polyacrylamide gels containing Tris/borate/EDTA buffer (18). DNA fragments were transferred to diazobenzyloxymethyl (DBM) paper before hybridization with PSTV [^{32}P]cDNA (19). Double-stranded restriction fragments were labeled at their 5′ termini with [γ-^{32}P]ATP by using phage T4 polynucleotide kinase (20). Restriction endonuclease digestions were performed under the conditions recommended by the enzyme suppliers (New England BioLabs, Beverly, MA, or Bethesda Research Laboratories, Rockville, MD). Autoradiography was done with Kodak X-Omat film and Du Pont Cronex Lightning-Plus intensifying screens at −70°C.

Isolation, Fractionation, and Analysis of PSTV-Related RNAs. Total RNA was isolated from PSTV-infected tomato tissue and fractionated with LiCl (5). RNA samples were fractionated on 5% acrylamide gels containing Tris/borate/EDTA buffer and 8 M urea at 55°C (8). Samples were heated for 2 min at 100°C in 50% (vol/vol) deionized formamide, quenched, and diluted with an equal volume of 9 M urea/1:5-diluted Tris/borate/EDTA buffer immediately before electrophoresis. RNA transfer and dextran sulfate-accelerated hybridization with either PSTV [^{32}P]cDNA or 5′-^{32}P-labeled recombinant DNA were as described (19) except that the buffer contained 40% formamide/180 mM NaCl/15 mM sodium cacodylate (pH 7.0)/0.3 mM EDTA, and the incubation temperature was 50°C.

RESULTS

Synthesis of Double-Stranded PSTV cDNA. Synthesis of full-length PSTV cDNA was essential to our efforts to clone the entire sequence of PSTV. Several possible approaches were investigated in preliminary experiments, and polyadenylylation of linear PSTV molecules with the poly(A) polymerase from maize prior to oligo(dT)-primed cDNA synthesis proved most suitable. Fig. 1 (lanes 1, 2, and 3) compares the average sizes of cDNA synthesized by this procedure and cDNA synthesized by random priming (3). A distinct band of full-length cDNA is present in the oligo(dT)-primed cDNA preparations and migrates slightly slower than the linear PSTV marker. A number of discrete cDNA size classes are visible; the most prominent band contains about 180 nucleotides.

The short hairpin loops present at the 3′-termini of single-stranded cDNA were used to prime the synthesis of the second

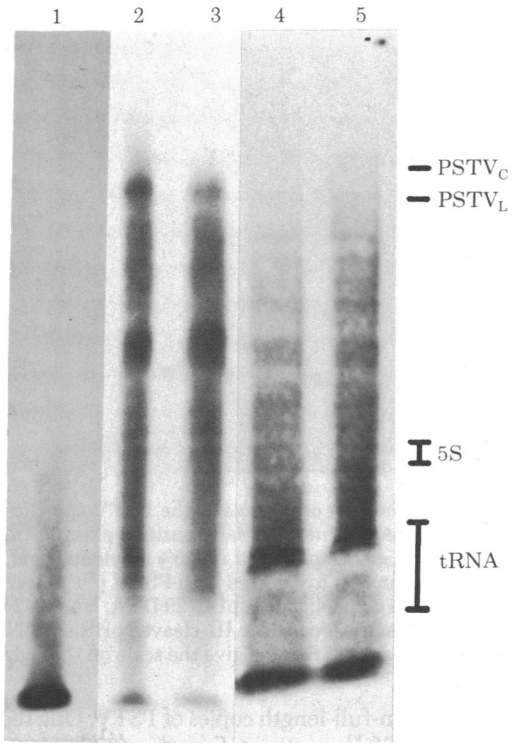

FIG. 1. Analysis of PSTV cDNA preparations by polyacrylamide gel electrophoresis. Samples of ^{3}H-labeled PSTV cDNA and S1-digested double-stranded cDNA (^{3}H label present in first DNA strand synthesized) were analyzed by electrophoresis in the presence of 8 M urea at 55°C and visualized by fluorography (21). Lane 1, random oligodeoxynucleotide-primed PSTV cDNA; lanes 2 and 3, oligo(dT)-primed PSTV cDNA synthesized in the presence of actinomycin D at 20 μg/ml (lane 2) or 4 mM sodium pyrophosphate (lane 3); lanes 4 and 5, double-stranded PSTV cDNA digested with S1 nuclease at either 40 units/ml (lane 4) or 8 units/ml (lane 5) before electrophoresis. The positions of circular and linear molecules of PSTV are indicated by PSTV$_C$ and PSTV$_L$, respectively.

DNA strand by *E. coli* DNA polymerase I after the PSTV template and the newly synthesized cDNA strands were separated by heat denaturation (22). The electrophoretic profiles of oligo(dT)-primed PSTV cDNA and S1-digested double-stranded PSTV cDNA are quite similar (Fig. 1, compare lanes 2–5). A notable difference between S1-digested double-stranded PSTV cDNA and oligo(dT)-primed PSTV cDNA is the marked reduction of the distinct band migrating slightly more slowly than the linear PSTV marker in the S1-digested preparation.

Construction and Identification of Recombinant DNA Clones. Inserting cDNA in the *Pst* I cleavage site of pBR322 by using the oligo(dG)-oligo(dC)-tailing procedure (23) has at least two advantages over other methods. Transformants containing PSTV cDNA inserts can be selected as tetracycline-resistant ampicillin-sensitive colonies. The cloned cDNA sequences can subsequently be removed from the recombinant plasmid by *Pst* I digestion.

Transformation of *E. coli* C600 with a 10:1 (wt/wt) mixture of *Pst* I-cleaved oligo(dG)-tailed pBR322 DNA and oligo(dC)-tailed double-stranded PSTV cDNA yielded a transformation frequency of approximately 2600 transformants per μg of pBR322 DNA. In comparison, intact supercoiled and *Pst* I-cleaved oligo(dG)-tailed pBR322 DNAs yielded 583,000 and 460 transformants per μg of DNA, respectively. Transformed clones were selected by antibiotic resistance and tested for the presence of PSTV-specific DNA sequences by hybridization with PSTV [^{32}P]cDNA. Fig. 2 shows that most of the clones

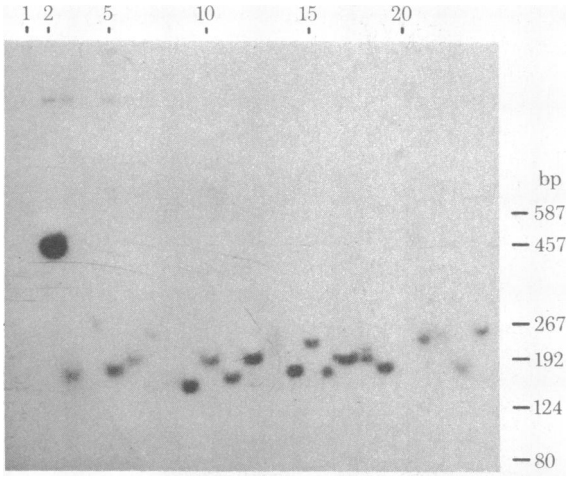

FIG. 2. Determination of the size of the PSTV-specific inserts present in recombinant clones. Partially purified plasmid DNA was digested with *Pst* I, electrophoresed in a 5% acrylamide gel, transferred to DBM paper, and hybridized with PSTV [^{32}P]cDNA. Lane 1, pBR322 DNA as a control; lane 2, pDC-29 DNA; lanes 3–24, DNA from other recombinant clones. *Hae* III-cleaved pBR322 DNA fragments were used as size markers to give the scale on the right (24).

contained less-than-full-length copies of PSTV. One recombinant clone, pDC-29 (lane 2), contained a 460-base pair (bp) PSTV-specific insert and was selected for further study.

Characterization of pDC-29 by Partial Restriction Mapping. Microgram quantities of the PSTV-specific insert from clone pDC-29 were digested with one of six restriction enzymes, and the 5′-termini of the resulting fragments were labeled with $^{32}PO_4$ by the T4 polynucleotide kinase-catalyzed exchange reaction (20). Polyacrylamide gel electrophoresis of the labeled fragments allows simultaneous determination of both the size of each resulting fragment and the fragment stoichiometry. Fig. 3 shows that the pDC-29 insert contains one *Alu* I, three *Ava* I, one *Bam*HI, two *Hae* III, three *Hpa* II, and two *Sma* I restriction sites. The two *Alu* I fragments contain 275 and 184 bp; the four Ava I fragments 174, 87–110 (two fragments), and 65 bp; the two *Bam*HI fragments 363 and 110 bp; the three *Hae* III fragments 363 and 53 bp (two fragments); the four *Hpa* II fragments 200, 95–110 (two fragments), and 69 bp; and the three *Sma* I fragments 267 and 104 bp (two fragments). Each *Alu* I and *Bam*HI fragment contains one *Hae* III site, but only the two smaller *Sma* I fragments are cleaved by *Hae* III in a double digestion (results not shown).

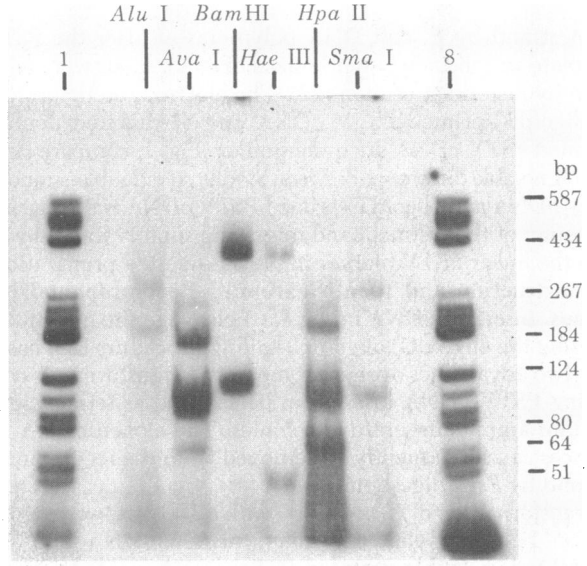

FIG. 3. Restriction analysis of the PSTV-specific insert present in recombinant clone pDC-29. Samples of purified insert DNA were digested with one of six restriction enzymes, and the resulting fragments were labeled at their 5′ termini by using the T4 polynucleotide kinase exchange reaction. The labeled DNA fragments were analyzed by electrophoresis in a 5% acrylamide gel under nondenaturing conditions. Lanes 1 and 8, *Hae* III-digested pBR322 DNA included as size markers; lanes 2–7, pDC-29 insert digests.

Fig. 4 compares the restriction map expected from the published sequence of the Beltsville strain of PSTV (Fig. 4*A*) with the map deduced for pDC-29 (Fig. 4*B*). Comparison of these maps reveals that the pDC-29 insert contains each of the cleavage sites for six different restriction endonucleases predicted by the published sequence of PSTV and that these sites show the expected spatial arrangement. The single *Bam*HI site has been placed near the 3′ terminus of the insert because each of the *Bam*HI and *Alu* I fragments contains a single *Hae* III site. If we assume that the nick in the PSTV template was on the 5′ side of the *Bam*HI site, the resulting cloned cDNA would have a *Bam*HI site near the 5′ terminus and both *Hae* III sites in the larger *Alu* I fragment. Because each *Alu* I fragment contains one *Hae* III site, this orientation cannot be correct. Preliminary sequencing data confirm the proposed orientation of the double-stranded PSTV cDNA insert in clone pDC-29.

The additional *Ava* I, *Hpa* II, *Hae* III, and *Sma* I sites have been arranged at the 5′ end of the insert (in the 184-bp *Alu* I fragment) at positions consistent with the observed fragment

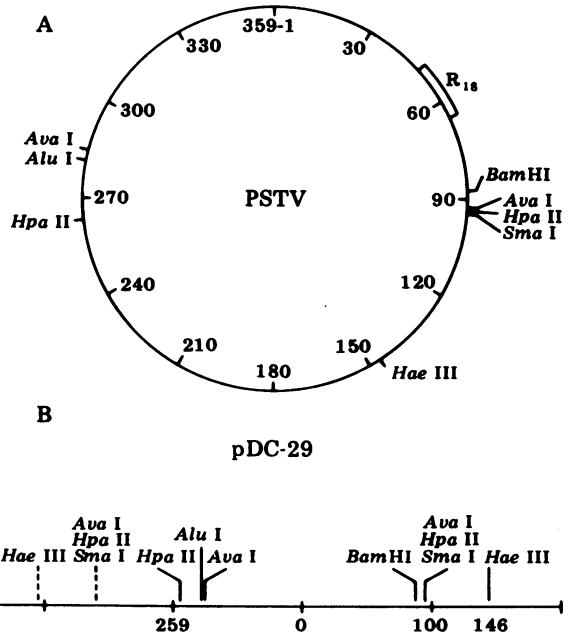

FIG. 4. Comparison of the predicted and observed restriction maps of double-stranded PSTV cDNA. (*A*) Circular representation of the 359-nucleotide covalently closed circular RNA sequence of PSTV (2). The sites where the six restriction endonucleases used in this investigation would cleave an exact double-stranded DNA copy of the PSTV RNA sequence are marked. R_{18} indicates the polypurine sequence present in several viroids (2) but of presently unknown function. (*B*) Linear representation of the 460-bp insert present in pDC-29. Observed restriction sites have been arranged as required by double restriction endonuclease digestions and preliminary DNA sequence data. Solid lines indicate restriction sites predicted by the RNA sequence, and broken lines indicate sites whose origin has not yet been established. The numbering system is that of Gross *et al.* (2).

lengths. The presence of a common 104-bp fragment suggests that the new *Ava* I, *Hpa* II, and *Sma* I restriction sites may result from a single C-C-C-G-G-G sequence. Determination of the complete nucleotide sequence will be required to characterize this portion of the insert.

Labeled hybridization probes for RNA molecules having the same polarity as PSTV or the opposite polarity were prepared by labeling the 5′ termini of *Bam*HI-cleaved pDC-29 DNA (20) and cleaving the labeled DNA with *Pst* I. The resulting 110-bp and 363-bp fragments, composing the entire PSTV-specific insert in pDC-29, were purified by electrophoresis. The ^{32}P-labeled strand in the 110-bp *Bam*HI fragment has the same polarity as PSTV. Therefore, the 363-bp and 110-bp fragments can be used as hybridization probes for RNA molecules having the same or opposite polarity as PSTV, respectively. Fig. 5 shows that RNA molecules of both polarities can be detected in RNA isolated from PSTV-infected tomato.

Total RNA prepared from PSTV-infected tomato seedlings was fractionated by the same 2 M LiCl precipitation step used in purification of PSTV (5). In this way the distributions of PSTV and RNA molecules of opposite polarity within the total cellular RNA can be compared. Comparison of samples treated with ribonuclease before RNA·RNA hybridization to those treated after hybridization reveals the proportion of each type of RNA present in a double-stranded configuration after RNA isolation. Aliquots of each RNA fraction were analyzed by polyacrylamide gel electrophoresis under conditions that separate the circular and linear forms of PSTV. After electrophoresis, the RNA samples were transferred to DBM paper and hybridized with 5′-^{32}P-labeled DNA probes. Appropriate control experiments have shown that double-stranded RNA is stable under the ribonuclease digestion conditions used and is denatured by our sample denaturation protocol.

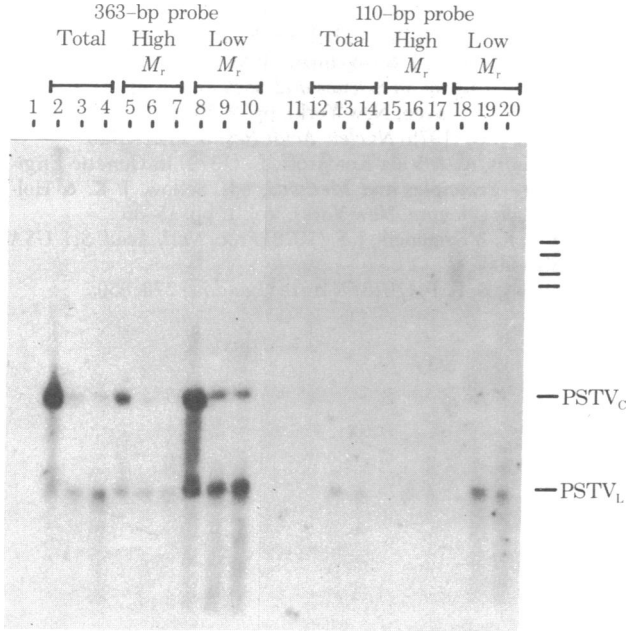

FIG. 5. Hybridization of ^{32}P-labeled pDC-29 insert DNA to PSTV and to RNA sequences of opposite polarity. Samples of RNA fractions prepared from PSTV-infected tomatoes (10 μg of untreated RNA or the RNase-resistant fraction from 40 μg of RNA that had been incubated for 30 min at 37°C with pancreatic RNase at 20 μg/ml and T1 RNase at 10 units/ml in the presence of 0.36 M NaCl) were fractionated by electrophoresis at 55°C in the presence of 8 M urea. After transfer to DBM paper, the RNA samples were hybridized with approximately 300,000 cpm of 5′-^{32}P-labeled *Bam*HI fragments from the pDC-29 insert (2–8 × 10^6 cpm/μg) and autoradiographed for 10 days. Each RNA fraction contains three samples; the first sample contains 10 μg of untreated RNA, and the second and third samples contain the RNase-resistant fraction from 40 μg of RNA treated with RNase before and after RNA·RNA hybridization (RNA concentration times incubation time = 100 mol of nucleotide/liter × sec), respectively. Lanes 1 and 11 contain 10 μg of low molecular weight RNA from cucumber mosaic virus-infected tomato as a control. Horizontal lines indicate positions of the four tomato rRNAs.

Fig. 5 (lanes 1–10) shows that, as expected, most of the PSTV present in a total RNA preparation is found in the low molecular weight RNA fraction and that most of the PSTV molecules in undigested preparations are circular. Ribonuclease digestion degrades most, but not all, PSTV molecules in all three fractions. RNA molecules with the opposite polarity can also be detected in all three RNA fractions (lanes 11–20). Like PSTV, the highest concentration of these molecules is found in the low molecular weight RNA fraction. Unlike PSTV, only one species of RNA with the opposite polarity is visible and migrates with linear molecules of PSTV. Most of this RNA appears to exist as ribonuclease-resistant duplexes after nucleic acid extraction; recovery is unaffected by RNA·RNA hybridization (compare lanes 13, 14, 19, and 20). In control experiments RNA preparations were heated to 100°C, quenched, and digested with ribonuclease without annealing. The RNA sequences with opposite polarity were destroyed by ribonuclease digestion immediately after heat denaturation, proving that the molecules are RNA.

DISCUSSION

Several procedures for cloning double-stranded cDNA are available and have been recently reviewed (25). Our final procedure differs in two respects from the procedures of most other laboratories: Removal of the PSTV RNA template by heating at 100°C (22) rather than by NaOH hydrolysis and incubation with *E. coli* DNA polymerase I at 37°C rather than at 15°C were required for efficient conversion of PSTV cDNA to S1-resistant double-stranded cDNA. The presence of relatively few full-length DNA molecules in both the single-stranded PSTV cDNA and S1-cleaved double-stranded PSTV cDNA preparations, the presence of numerous discrete bands of intermediate lengths, and the proportion of recombinant clones containing 180- to 200-bp inserts suggest that the extensive secondary structure of PSTV (2) inhibits the synthesis of full-length double-stranded PSTV cDNA (Figs. 1 and 2).

Comparison of the restriction map of the PSTV-specific insert present in clone pDC-29 with the restriction map predicted by the RNA sequence of PSTV (Fig. 4) suggests that pDC-29 will provide nearly full-length hybridization probes. It is clear that the pDC-29 insert contains a minimum of 245 of the 359 nucleotides in PSTV: i.e., the sequence between the *Hpa* II site at positions 262–265 and the *Hae* III site at positions 145–148. Preliminary sequencing data show that the PSTV-specific sequence has its 3′ terminus at position 172–173, a previously identified site of preferential nuclease cleavage (2). Therefore, clone pDC-29 appears to contain at least 269 of PSTV's 359 nucleotides.

Cloned double-stranded PSTV cDNA promises to be an extremely useful tool for investigating the mechanism of viroid replication. At the present time a RNA-directed mechanism for viroid replication appears most likely (1). The most convincing evidence for a RNA-directed mechanism is the demonstration that citrus exocortis viroid-infected *Gynura* plants contain RNA sequences capable of forming well-matched RNA·RNA duplexes with ^{125}I-labeled citrus exocortis viroid (26). Complementary RNA sequences involved in viroid replication must be complementary to the entire viroid sequence. A complete characterization of these complementary RNA sequences has not been published.

Fig. 5 demonstrates that cloned PSTV-specific DNA sequences containing a 5′-^{32}P label can be used to detect both PSTV and RNA molecules of opposite polarity in nucleic acids isolated from PSTV-infected tomatoes. This RNA of opposite polarity but the same mobility as linear PSTV appears to be present in much lower concentrations than PSTV. Its distribution within the total cellular RNA parallels that of PSTV.

We do not yet have any data concerning differences in the subcellular distribution of PSTV and RNA of opposite polarity. Because this RNA has the same mobility (and presumably molecular weight) as linear PSTV, these molecules may be the complete copy of PSTV required for an RNA-directed replication mechanism. Matthews has proposed that RNA of opposite polarity might function as mRNA in PSTV-infected tissue (27). If this were the case, however, we would not expect to isolate a large proportion of this RNA as ribonuclease-resistant duplexes. The presence of circular forms of RNA of opposite polarity to PSTV is uncertain. Further experimentation is clearly needed to establish the role of these RNA molecules of opposite polarity in PSTV replication, but the experimental systems and analytical techniques required are now available.

We thank T. O. Diener for his advice and encouragement throughout this investigation, R. J. Mans for generously providing purified maize poly(A) polymerase, R. Deich and B. Hausler for demonstrating the techniques for chemical determination of DNA nucleotide sequence, and G. Carpenter, K. Pessin, and B. Smith for technical assistance.

1. Diener, T. O. (1979) *Science* **205**, 859–866.
2. Gross, H. J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H. & Sanger, H. L. (1978) *Nature (London)* **273**, 203–208.
3. Owens, R. A. (1978) *Virology* **89**, 380–387.
4. Palukaitis, P. & Symons, R. H. (1978) *FEBS Lett.* **92**, 268–272.
5. Diener, T. O., Hadidi, A. & Owens, R. A. (1977) in *Methods in Virology*, eds. Maramorosch, K. & Koprowski, H. (Academic, New York), Vol. 6, pp. 185–217.
6. McClements, W. & Kaesberg, P. (1977) *Virology* **76**, 477–484.
7. Owens, R. A., Erbe, E., Hadidi, A., Steere, R. L. & Diener, T. O. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3859–3863.
8. Sanger, H. L., Ramm, K., Domdey, H., Gross, H. J., Henco, K. & Riesner, D. (1979) *FEBS Lett.* **99**, 117–122.
9. Simsek, M., Ziegenmeyer, J., Heckman, J. & RajBhandary, U. L. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1041–1045.
10. Mans, R. J. & Huff, N. J. (1975) *J. Biol. Chem.* **250**, 3672–3678.
11. Gordon, J. I., Burns, A. T. H., Christmann, J. L. & Deeley, R. G. (1978) *J. Biol. Chem.* **253**, 8629–8639.
12. Clarke, L. & Carbon, J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4361–4365.
13. Enea, V., Vovis, G. F. & Zinder, N. D. (1975) *J. Mol. Biol.* **96**, 495–509.
14. Grunstein, M. & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3961–3965.
15. Clewell, D. B. (1972) *J. Bacteriol.* **110**, 667–676.
16. Clewell, D. B. & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. USA* **62**, 1159–1166.
17. Radloff, R., Bauer, W. & Vinograd, J. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 1514–1522.
18. Maniatis, T., Jeffrey, A. & van de Sande, H. (1975) *Biochemistry* **17**, 3787–3794.
19. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3683–3687.
20. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
21. Lasky, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341.
22. Wickens, M. P., Buell, G. N. & Schimke, R. T. (1978) *J. Biol. Chem.* **253**, 2483–2495.
23. Boyer, H. W., Betlach, M., Bolivar, F., Rodriguez, R. L., Heyneker, H. L., Shine, J. & Goodman, H. M. (1977) in *Recombinant Molecules: Impact on Science and Society*, eds. Beers, R. F. & Bassett, E. G. (Raven, New York), pp. 9–20.
24. Sutcliffe, J. G. (1978) *Nucleic Acids Res.* **5**, 2721–2728.
25. Efstratiadis, A. & Villa-Komaroff, L. (1979) in *Genetic Engineering—Principles and Methods*, eds. Setlow, J. K. & Hollaender, A. (Plenum, New York), Vol. 1, pp. 15–36.
26. Grill, L. K. & Semancik, J. S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 896–900.
27. Matthews, R. E. F. (1978) *Nature (London)* **276**, 850.