Molecular Cloning of Potato Leaf Roll Virus Complementary DNA

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ABSTRACT

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Potato leaf roll virus (PLRV) was purified from infected leaves of *Physalis floridana*. The viral RNA was poly(A)-tailed and used to synthesize double-stranded cDNA and then cloned into the *Pst*I site of the plasmid pUC9 using oligo(dG)-oligo(dC) tailing methodology. Three initial overlapping clones were selected and used as the source of leftward and rightward probes in colony hybridization experiments to identify additional cloned PLRV cDNAs by "plasmid walking." Three PLRV clones containing cDNA inserts of 3.3, 2.3, and 1.2 kilobase pairs (kbp) were identified. Restriction endonuclease and Southern-blot hybridization analyses indicated that these cDNAs formed an overlapping physical map representing a majority of the viral genome (6 kbp). Each clone was verified to contain viral cDNA by dot-blot hybridization to PLRV RNA and total RNA isolated from PLRV-infected *P. floridana* leaves. The composite 5′ to 3′ polarity of PLRV cDNA was established by the use of an M13 strand-specific hybridization probe and overlapping restriction endonuclease sites.

Additional keyword: luteovirus.

Potato leaf roll (PLR) is one of the most important viral diseases of potato (2,25,27). In addition to potato leaf roll virus (PLRV), beet western yellows virus (BWYV) also is known to be involved in the etiology of PLR in North America (8,9) and Tasmania (10) but not Scotland (1) or Israel (18). Surveys of North American PLR-symptomatic potato plants using the enzyme-linked immunosorbent assay (ELISA) indicate that 32–65% are dually infected with PLRV and BWYV (11,34,41).

PLRV and BWYV are classified as luteoviruses, the type member of which is barley yellow dwarf virus (19). This plant virus group includes the following characteristics: one molecule of positive-sense, single-stranded RNA (2×10^6 mol. wt.), one coat polypeptide (24,000 mol. wt.), icosahedral virions (25-nm diameter), replication confined to phloem tissue of infected plants, uninoculability by sap, and persistent transmission by aphid vectors.

The restriction of luteoviruses to phloem tissue of infected plants limits the quantity of purified virions and viral RNA available for basic biological studies; therefore, the genome structure and replication strategy of this group of plant viruses is poorly understood (7). In vitro translation of PLRV RNA has produced a major 71 Kdal polypeptide but no product corresponding in size to coat protein (20). It also has been demonstrated that PLRV RNA is nonpolyadenylated and covalently attached to a small protein (20).

To facilitate studies on the basic biology of PLRV, we have cloned DNA complementary to PLRV RNA. In this paper we describe the cloning, physical mapping, and 5′ to 3′ polarity of 6 kilobase pairs (kbp) of complementary DNA (cDNA), which represents a majority of the viral genome.

MATERIALS AND METHODS

Virus isolate. PLRV-infected potato (*Solanum tuberosum* L. 'Russet Burbank') seed tubers were provided by N. S. Wright, Agriculture Canada. This viral isolate has been employed in an ultrastructural study of virus-infected potato leaf midveins and designated strain 4 based on symptoms expressed by the indicator plant *Physalis floridana* Rydb. (33). Virus was transmitted from potato using the aphid *Myzus persicae* (Sulzer) and maintained in this host by serial aphid transmissions. The isolate in *P. floridana* was checked for contaminating BWYV by aphid transmission to *Capsella bursa-pastoris* (L.) Medic. and serological testing (8). The latter was conducted by J. E. Duffus, U.S. Department of Agriculture. These results confirmed that the PLRV isolate was not contaminated with BWYV (data not shown).

Virus purification and viral RNA isolation. Virus-infected leaves of *P. floridana* were harvested 4–6 wk postinoculation and stored at −80 C. Virus was purified by the methods of Takanami and Kubo (38). Viral RNA was isolated using proteinase K (500 μg/ml) and sodium decyl sulfate (SDS) (0.5%) as described by Pullin et al (26). Reaction mixtures were incubated 12 hr, followed by multiple extractions with phenol:chloroform:isoamyl alcohol (25:24:1). The final aqueous phase was adjusted to 200 mM sodium acetate, pH 5.0, followed by addition of 2.5 vol ethanol and −20 C storage overnight. RNA was recovered by centrifugation and suspended in sterile distilled water. The concentration of RNA was estimated spectrophotometrically using an extinction coefficient of 25 $(mg/ml)^{-1}$ cm^{-1} at 260 nm. RNA quality was assayed by electrophoresing samples in 40 mM of Tris-acetate, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA) at 80 V for 1 hr in mini-agarose gels using RNA isolated from purified tobacco mosaic virus (TMV) (12) as a marker. Contaminating DNA was removed from PLRV RNA preparations by DNase I treatment for 30 min at 37 C in reaction mixtures containing 5 mM Tris-Cl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 1,000 units/ml of ribonuclease inhibitor (Promega Biotec, Madison, WI), and 20 μg/ml of RNase-free DNase I (Bethesda Research Laboratories, Inc., Gaithersburg, MD). Reactions were terminated by the addition of EDTA and SDS to final concentrations of 10 mM and 0.5%, respectively. Following two extractions with phenol:chloroform:isoamyl alcohol (25:24:1), RNA was recovered by ethanol/sodium acetate precipitation and stored in sterile distilled water at −80 C.

Poly(A) tailing of viral RNA. Homopolymer tailing of RNA with adenosine 5′-triphosphate (ATP) was based on the methods of Sippel (35). RNA was heat denatured at 68 C for 3 min and immediately quenched in ice before the reaction. Reaction mixtures (75 μl) containing 12 μg of heat-denatured RNA (6 picomoles), 20 units/ml of *Escherichia coli* poly(A) polymerase (Bethesda Research Laboratories), 100 mM Tris-Cl, pH 8.3, 250 mM NaCl, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 10 mM dithiothreitol, 100 μg/ml of bovine serum albumin (BSA), 500 units/ml of ribonuclease inhibitor, and 0.2 mM ATP were incubated at 37 C for 15 min. Reactions were terminated by the addition of EDTA and SDS to final concentrations of 40 mM and 0.4%, respectively. The mixture was adjusted to 200 μl with TNE (10 mM Tris-Cl, pH 7.4, 100mM NaCl, 1 mM EDTA), followed by two extractions with phenol:chloroform:isoamyl alcohol (25:24:1). One-half volume 7.5 M ammonium acetate was added to the final aqueous phase followed by the addition of 2.5 vol ethanol and −20 C storage overnight. These conditions resulted in the addition of approximately 30 adenosine 5′-monophosphate residues per RNA molecule in a test assay using 12 μg of TMV RNA (approximately 6 picomoles) and 0.2 mM ATP plus 50 μCi [alpha-^{32}P]ATP (590 Ci/mmol) (ICN Biomedicals) based on standard liquid scintillation counting methods.

Double-stranded cDNA synthesis. Poly(A)-tailed RNA (in water) was heat denatured at 68 C for 3 min and immediately quenched on ice before first-strand cDNA synthesis. Reaction mixtures (110 μl) contained 100 mM Tris-Cl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dATP, dGTP, and dTTP, 0.5 mM dCTP, 4 mM sodium pyrophosphate, 30 μg/ml of oligo$(dT)_{12-18}$, 1,000 units/ml of ribonuclease inhibitor, 80–100 μg/ml of poly(A)-tailed RNA, 300 units/ml of AMV Reverse Transcriptase-XL (Life Sciences, Inc., Greenwich, CT) and 55 μCi of lyophilized [^{3}H]dCTP (22.6 Ci/mmol) (Amersham Corp., Arlington Heights, IL). Ten microliters of this mixture was transferred to a second tube containing 20 μCi of lyophilized [alpha-^{32}P]dCTP (600 Ci/mmol) (ICN Biomedicals). This pilot mixture was processed to prepare ^{32}P-labeled cDNA for assaying first-strand cDNA length by alkaline gel electrophoresis (17). Both tubes were incubated at room temperature for 2 min, followed by 42 C for 1 hr. The preparative reaction (100 μl) was terminated by the addition of EDTA to a final concentration of 20 mM, followed by extraction with phenol and phenol:chloroform:isoamyl alcohol (25:24:1). Unincorporated nucleotides were removed by Sephadex G-50 spun-column chromatography, and the cDNA/RNA hybrids were recovered by ethanol/ammonium acetate precipitation. The pilot mixture was adjusted to 20 mM EDTA in a final volume of 100 μl, followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) with 100 μg of tRNA carrier and ethanol/ammonium acetate precipitation. Standard liquid scintillation counting methods were used to estimate the yield of first-strand ^{3}H-cDNA.

Second-strand cDNA synthesis followed the protocol of Gubler and Hoffman (13) using 0.27 μg of first-strand ^{3}H-cDNA (equivalent to 0.54 μg of cDNA:RNA hybrids). *E. coli* DNA ligase and B-NAD were omitted. Molecular weight markers for alkaline gel electrophoresis were prepared by end-labeling *Hin*dIII fragments of lambda DNA with ^{32}P using T4 DNA polymerase (17).

Double-stranded cDNA tailing and cloning. Homopolymer tailing of double-stranded cDNA with dCTP was conducted in 25-μl reactions containing 100 mM potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM of dithiothreitol, 22 ng of double-stranded cDNA, and 130 units/ml of terminal deoxynucleotidyl transferase (Bethesda Research Laboratories). Four different reactions were conducted using 10, 25, 50, and 100 M dCTP. Reactions were incubated at 37 C for 30 min and terminated by the addition of EDTA to a final concentration of 25 mM. Ten micrograms of tRNA carrier was added, followed by ethanol/ammonium acetate precipitation. After overnight storage at −20 C, dC-tailed cDNA was recovered by centrifugation and the ethanol/ammonium acetate precipitation was repeated.

Double-stranded, dC-tailed cDNA (approximately 22 ng) was directly suspended in a 25-μl annealing mixture containing 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1 mM of EDTA, and 270 ng of *Pst*I-cleaved, dG-tailed pUC9 (P. L. Biochemicals, Milwaukee, WI). Mixtures were incubated at 65 C for 2 hr, then cooled slowly to room temperature for 2 hr. One-third of the annealing mixture was used to transform 200 μl of *E. coli* JM109 cells as described by Yanisch-Perron et al (40).

Physical mapping of cloned PLRV cDNA. Transformants were screened for the size of cDNA inserts by isolating plasmids from small-scale bacterial cultures (2.5 ml) (14) followed by restriction endonuclease digestion with *Pst*I and agarose gel electrophoresis. Molecular weight markers used were *Hin*dIII restriction fragments of Lambda DNA and *Hae*III restriction fragments of ϕX174 replicative form DNA (Bethesda Research Laboratories). Recombinant plasmids containing inserts greater than 1,000 bp were isolated from large-scale bacterial cultures and physically mapped with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*cII, *Hin*dIII, *Pst*I, *Sal*I, *Sma*I, and *Xho*I (Bethesda Research Laboratories) using a combination of single and double digestions. The presence of overlapping restriction fragments in different recombinant plasmids was confirmed by Southern-blot hybridization analysis (37).

Identification of additional PLRV clones. Leftward and rightward probes derived from three initial overlapping clones were used in colony hybridization experiments (17) to identify, by partial sequence overlap, plasmids containing adjacent cDNA sequences ("plasmid walking"). Probes were isolated by restriction endonuclease digestion followed by electroelution (36). ^{32}P-labeled probes were prepared by nick-translation (28) and employed in colony hybridization experiments, using 2×10^5 cpm/ml. Hybridization-positive clones were physically mapped and confirmed to be homologous by Southern-blot hybridization analysis as described previously. Clones were verified to contain PLRV cDNA sequences by preparing ^{32}P-labeled recombinant plasmids by nick-translation followed by hybridization to dot-blots of PLRV RNA and RNA isolated from leaves of PLRV-infected *P. floridana* plants. TMV RNA and healthy leaf RNA were used as controls.

Dot-blot hybridization. RNA was adjusted to a final volume of 100 μl in water, mixed with 300 μl of 20× SSC:formaldehyde (1:1) and incubated at 65 C for 15 min, followed by dot-blotting (16) to nitrocellulose using a filter manifold (Schleicher & Schuell, Inc., Keene, NH) (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Prehybridizations were conducted for 12 hr at 42 C in buffer containing 50% formamide, 5× SSPE, 0.1% SDS, 100 μg/ml of sonicated, denatured, salmon sperm DNA, and 0.2% each of Ficoll, polyvinylpyrrolidone, and BSA (1× SSPE is 0.18 M NaCl, 0.010 M sodium phosphate, pH 7.7, 0.001 M EDTA). Hybridization was for 24 hr at 42 C in buffer containing 50% formamide, 5× SSPE, 0.1% SDS, 100 μg/ml of sonicated, denatured, salmon sperm DNA, 0.04% each of Ficoll, polyvinylpyrrolidone, and BSA, and 0.5×10^6 cpm/ml of ^{32}P-labeled DNA. Blots were washed with three changes of 2× SSC and 0.1% SDS for 10 min each at room temperature followed by four changes of 0.1× SSC and 0.1% SDS for 15 min each at 55 C. Dried blots were exposed to X-ray film at room temperature without an intensifying screen.

Production of M13 hybridization probes. PLRV cDNA was subcloned, in both orientations, into M13 double-stranded DNA (23). Single-stranded cDNA hybridization probes were synthesized based on the protocol of Hu and Messing (15): 100 ng of single-stranded DNA was mixed with 4 ng of hybridization probe primer (New England BioLabs, Boston, MA) and 1.5 μl 10× DNA polymerase buffer (70 mM Tris-Cl, pH 7.4, 500 mM NaCl, and 70 mM $MgCl_2$) in a final volume of 10 μl. Primer and DNA were annealed by heating at 55 C for 5 min, then cooled slowly to room temperature for 1 hr. The mixture was adjusted to 20 μl by the addition of 4 μl 10 μCi/μl of [alpha-^{32}P]dATP (800 Ci/mmol) (New England Nuclear, Boston, MA), 2 μl of 100 mM dithiothreitol, 2 μl of 0.5 mM dNTP (dCTP, dGTP, dTTP), and 2 μl 0.5 units/ml of large-fragment DNA polymerase I (U.S. Biochemical Corp., Cleveland, OH). The reaction was incubated at

15 C for 1 hr and terminated by the addition of 2 μl of 250 mM EDTA, pH 8.0. After the addition of 100 μl of TNE and extraction once with phenol:chloroform:isoamyl alcohol (25:24:1), unincorporated nucleotides were removed by Sephadex G-50 spun-column chromatography. Probe-specific activities were 1–2 $\times 10^8$ cpm/μg.

Isolation of RNA from plant leaves. Total cellular RNA was isolated from leaf samples of *P. floridana* using a modification of the protocol of Okita and Greene (24). Leaf samples (2 g) were ground to a powder in liquid nitrogen and immediately homogenized using a polytron (Brinkmann Instruments Co., Westbury, NY) for 30–45 sec at medium speed in 20 ml of ice-cold 5.0 M guanidine-HCl, 10 mM Tris-Cl, pH 8.5, 5 mM EGTA, 0.1% laurylsarcosine, 100 mM 2-mercaptoethanol, and 0.1% antifoam A. After clarification by centrifugation at 25,000 *g* for 10 min, the supernatant was collected and one-tenth volume of 3.0 M sodium acetate, pH 5.5, was added followed by an equal volume of isopropanol. After −20 C storage overnight, the pellet was collected by centrifugation and washed with 80% ethanol. After suspension in 20 ml of TNE containing 250 μg/ml of proteinase K and 0.5% laurylsarcosine, the mixture was incubated at 37 C for 4 hr followed by two extractions each of phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1) followed by isopropanol/sodium acetate precipitation. The resulting pellet consisted of RNA, DNA, and polysaccharide. Samples were DNase I-treated in 400 μl of reaction mixtures containing approximately 5 A_{260nm} units/ml of total nucleic acid and 50 μg/ml of macaloid-treated DNase I (17) as described above but omitting ribonuclease inhibitor and dithiothreitol. Excess polysaccharide was removed by suspending RNA pellets in 500 μl of 100 mM sodium acetate, pH 5.5, followed by precipitation with one-half volume of 1% cetyltrimethylammonium bromide (3). The precipitate was triturated in 400 μl of water followed by the addition of one-tenth volume of 3.0 M sodium acetate, pH 5.5, and 2.5 volume ethanol. After −20 C storage, the final RNA pellet was recovered by centrifugation, suspended in sterile distilled water, and stored at −80 C.

RESULTS

Viral RNA isolation. Neutral agarose gel electrophoresis of RNA isolated from purified PLRV preparations showed that heterogeneously sized DNA was present (Fig. 1). This DNA was removed by DNase I treatment. As shown in Figure 1, PLRV RNA migrated slightly faster than TMV RNA (2.05×10^6 dal), which is consistent with the estimated molecular weight values of $1.85–2.0 \times 10^6$ reported for PLRV RNA (21,30,39).

cDNA synthesis, cloning, and physical mapping. The yield of ^{3}H-labeled first-strand cDNA corresponded to a 5% copying efficiency of the poly(A)-tailed RNA template. Alkaline gel

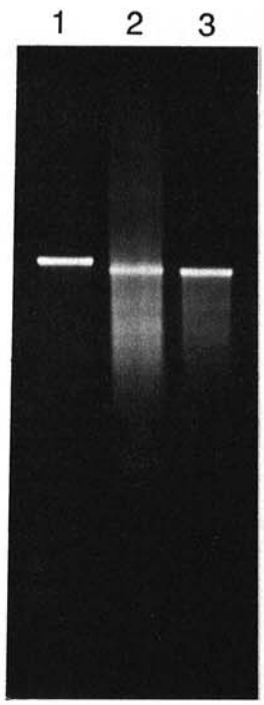

Fig. 1. Agarose gel electrophoresis of potato leaf roll virus (PLRV) RNA. Samples were electrophoresed in a 1% agarose mini-gel at 80 V for 1 hr in 40 mM Tris-acetate, pH 8.0, 1 mM of ethylenediaminetetraacetic acid containing 0.5 μg/ml ethidium bromide, and the ultraviolet-fluorescent bands were photographed. Samples (approximately 0.5 μg) consist of tobacco mosaic virus RNA (lane 1), nucleic acid extracted from sucrose density gradient-purified PLRV (lane 2), and PLRV RNA isolated after DNase I-treatment of a preparation corresponding to lane 2 (lane 3).

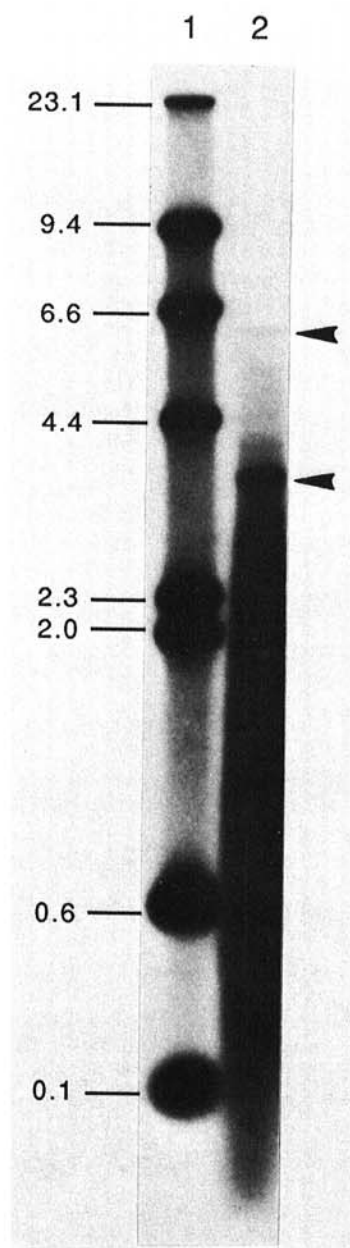

Fig. 2 Alkaline-agarose gel electrophoresis of ^{32}P-labeled viral cDNA. First-strand cDNA was labeled with ^{32}P in a 10-μl pilot reaction, separated by electrophoresis in a 1% alkaline-agarose gel, and autoradiographed (lane 2). *Hind*III-digested lambda DNA fragments end-labeled with ^{32}P using T4 DNA polymerase were used as molecular weight markers (lane 1). The fragment sizes of lambda DNAs are shown in kilobases (kb) on the left. The arrows indicate (in descending order) first-strand potato leaf roll virus cDNA transcripts of 6 and 3.5 kb.

electrophoresis of ^{32}P-labeled first-strand cDNA from the pilot reaction resulted in a smear of radioactivity, indicating the synthesis of a heterogeneously sized population of first-strand cDNAs. A major transcript of 3.5 kilobases (kb) was observed in this mixture, suggesting the presence of a strong stop sequence for reverse transcriptase (Fig. 2). As shown in Figure 2, a small percentage of transcripts were 6 kb in size, which is consistent with a full-length cDNA corresponding in size to PLRV RNA. The yield of second-strand cDNA was not determined, and conversion of the cDNA:RNA hybrid to double-stranded cDNA was assumed to be complete (13).

Sixty-nine bacterial transformants were initially obtained and replica plated to nitrocellulose followed by hybridization to first-strand cDNA prepared to poly(A)-tailed PLRV RNA. Hybridization-positive transformants were screened for the size of cDNA inserts by agarose gel electrophoresis of *Pst*I-digested DNA (data not shown). Clones pPLRV4-15, -28, and -53 containing inserts of 1.2, 1.9, and 1.5 kbp, respectively, were selected for analysis. Physical mapping and Southern-blot hybridization analysis indicated that these clones formed an overlapping map (data not shown). Additional cDNA was cloned, and approximately 400 ampicillin-resistant, B-galactosidase-negative transformants, including the initial clones, were screened by conducting a series of colony hybridization experiments. The purpose of these experiments was to identify cDNAs in the leftward and rightward direction from the overlapping pattern of clones pPLRV4-15, -28, and -53.

Four different colony hybridization experiments were conducted corresponding to the use of hybridization probes A, B, C, and D as shown in Figure 3. Clone pPLRV4-323 was selected by hybridization to probe B and probe C. Two additional clones, pPLRV4-173 and pPLRV4-228, were selected based on hybridizations to probes D and A, respectively (Fig. 3). Clones pPLRV4-173, -228, and -323 contained cDNA inserts of 3.3, 2.3, and 1.2 kbp, respectively. These clones were physically mapped and aligned with clones pPLRV4-15, -28, and -53 based on common restriction endonuclease sites to produce an overlapping pattern of six partial cDNAs (Fig. 3). This alignment was confirmed by Southern-blot hybridization analysis.

cDNA 323 was isolated by digesting pPLRV4-323 with *Hin*dIII and *Eco*RI followed by agarose gel electrophoresis and electroelution. Recombinant plasmids were restriction endonuclease-digested, Southern-blotted, and hybridized to ^{32}P-labeled, nick-translated cDNA 323. The results of this analysis confirmed the alignment of partial cDNAs. As predicted from this alignment (Fig. 3) and as shown in Figure 4B, ^{32}P-labeled cDNA 323 did not hybridize to cDNA 15 (lane 3) and did hybridize to the 670-bp *Eco*RI fragment of pPLRV4-28 (lane 4), the 630-bp and 370-bp *Bam*HI fragments of pPLRV4-53 (lane 5), the 220-bp *Eco*RI fragment of pPLRV4-173 (lane 6), and the 590-bp and 370-bp *Bam*HI fragments of pPLRV4-228 (lane 7).

Hybridization of cloned cDNA to PLRV RNA. To verify that clones pPLRV4-173, -228, and -323 contained viral sequences, plasmid DNA was ^{32}P-labeled by nick-translation and hybridized

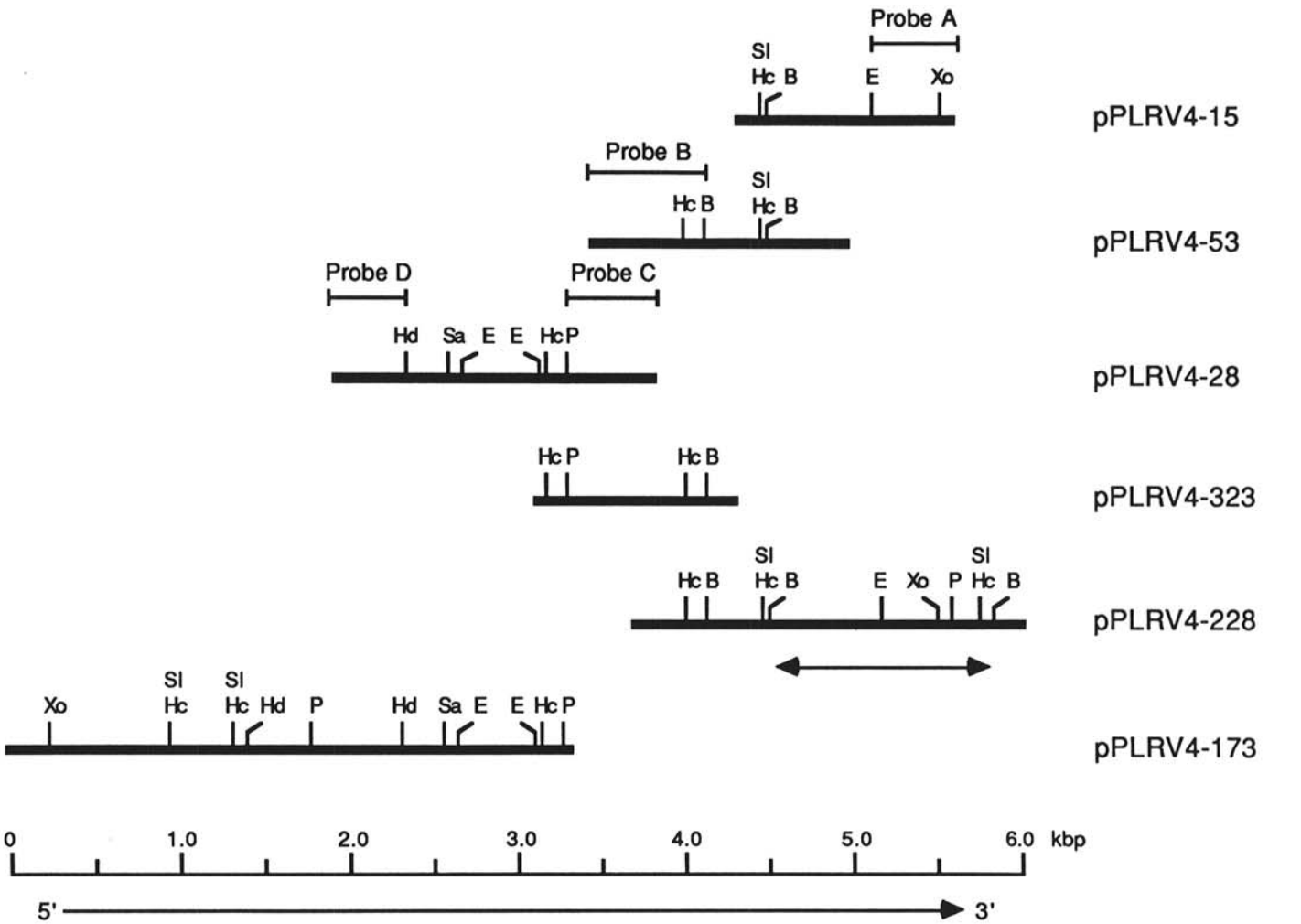

Fig. 3. Physical maps and 5′ to 3′ polarity of potato leaf roll virus (PLRV) cDNA. Probes A, B, C, and D were ^{32}P-labeled by nick-translation and used in four separate colony hybridization ("plasmid walking") experiments to identify clones pPLRV4-173, -228, and -323 from a library of 400 transformants. The 5′ to 3′ polarity was established by subcloning the 1.3-kilobase-pair *Bam*HI subfragment of pPLRV4-228 (double arrow) into the bacteriophage M13 followed by the production of strand-specific hybridization probes and a test for viral RNA complementarity as outlined in Figure 6. Restriction endonuclease sites identified are *Bam*HI (B), *Eco*RI (E), *Hin*cII (Hc), *Hin*dIII (Hd), *Pst*I (P), *Sal*I (Sl), *Sma*I (Sa), and *Xho*I (Xo). Clones are bordered by oligo(dG)-oligo(dC) tails and *Pst*I sites (not shown).

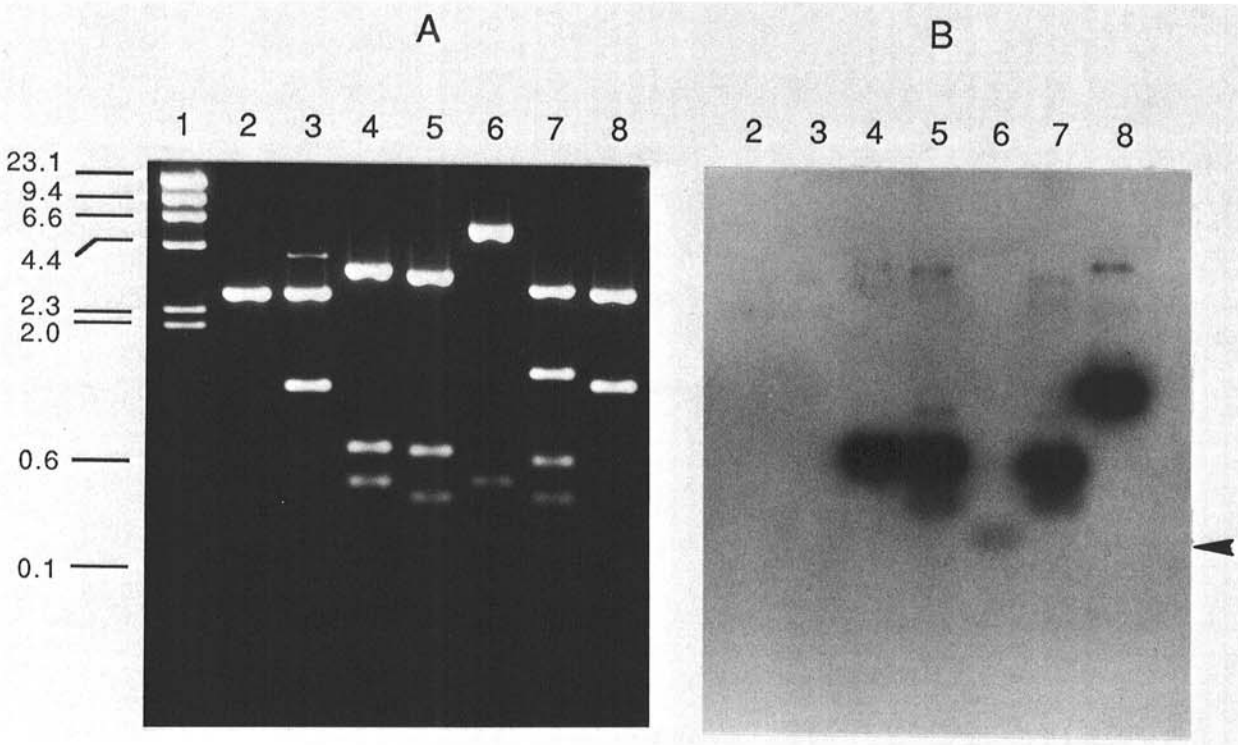

Fig. 4. Southern-blot hybridization analysis of cloned PLRV cDNA. DNA was electrophoresed in a 1.2% agarose gel for 8 hr at 80 V in 40 mM Tris-acetate, pH 8.0, 1 mM ethylenediaminetetraacetic acid containing 0.5 μg/ml ethidium bromide. The UV-fluorescent bands were photographed (**A**), then transferred to nitrocellulose, hybridized to ^{32}P-cDNA 323, and autoradiographed (**B**). Samples (0.3 μg) consist of *Pst*I-digested pUC9 (lane 2), *Pst*I-digested pPLRV4-15 (lane 3), *Eco*RI-digested pPLRV4-28 (lane 4), *Bam*HI-digested pPLRV4-53 (lane 5), *Eco*RI-digested pPLRV4-173 (lane 6), *Bam*HI-digested pPLRV4-228 (lane 7), and pPLRV4-323 double-digested with *Eco*RI and *Hin*dIII (lane 8). The arrow marks the position of a 220-bp fragment in lane 6 of **A** and **B**. Lane 1 contains *Hin*dIII-digested lambda DNA fragments. The fragment sizes of lambda DNAs are shown (in kilobase pairs) on the left. ^{32}P-labeled cDNA 323 was prepared by double-digestion of pPLRV4-323 with *Eco*RI and *Hin*dIII followed by agarose gel electrophoresis, electroelution, and nick-translation. This analysis verifies the alignment of partial cDNAs in Figure 3. See text for discussion.

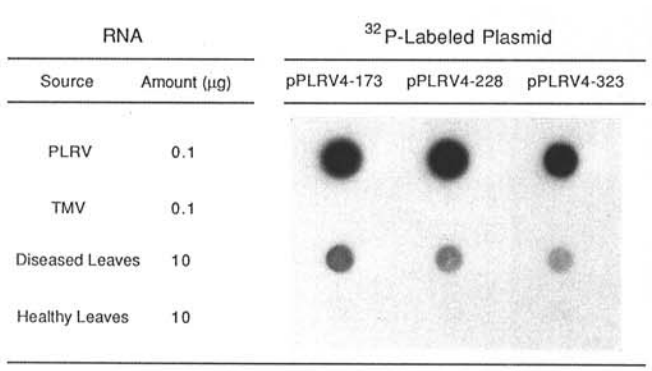

Fig. 5. Hybridization of cloned viral cDNA to potato leaf roll virus (PLRV) RNA. Plasmids were ^{32}P-labeled by nick-translation and hybridized separately to dot-blots of viral or leaf RNA. Leaf RNA samples were obtained from PLRV-infected *Physalis floridana* plants 2 wk after exposure to viruliferous aphids. Healthy leaf RNA and tobacco mosaic virus RNA served as controls.

to dot-blots of viral RNA and RNA extracted from diseased plant leaves. The results are shown in Figure 5. Each clone hybridized to PLRV RNA and total cellular RNA extracted from diseased leaves. Furthermore, the intensity of autoradiographic signals of diseased-leaf RNA extracts was correlated with the size of cDNA inserts: pPLRV4-173 > pPLRV4-228 > pPLRV4-323. There was no detectable hybridization to total cellular healthy leaf RNA or TMV RNA.

Determining the 5′ to 3′ polarity of cDNA. Strand-specific probes were produced by subcloning the 1.3-kbp *Bam*HI subfragment of cDNA 228 (Fig. 3) in both orientations into M13mp19 replicative form DNA. Orientations were confirmed by digesting subcloned replicative form DNA with *Pst*I, which cleaves the 1.3-kbp *Bam*HI subfragment of cDNA 228 asymmetrically (Fig. 3). Both orientations of single-stranded cDNA were isolated, followed by ^{32}P-probe synthesis and hybridization to dot-blots of RNA isolated from purified PLRV. As summarized in Figure 6, probe 2 differentially hybridized to viral RNA, indicating that the orientation of the *Pst*I site of cDNA 228 was 3′. Because the composite linear order of partial cDNAs was established by physical mapping and Southern-blot hybridization analyses (Figs. 3 and 4), the 5′ to 3′ orientation of cDNA 173, 228, and 323 was determined as 5′-cDNA 173 -cDNA 323 -cDNA 228 -3′ (Fig. 3). Data to support and confirm this orientation were obtained by DNA sequencing. Because single-stranded RNA was asymmetrically (3′) poly(A)-tailed before cDNA synthesis, it was expected that asymmetrically positioned, terminal poly(dA)-poly(dT) tails would be present in cloned double-stranded cDNA. Therefore, the leftward and rightward termini of cDNA 173, 228, and 323 were partially sequenced using alkaline-denatured plasmid templates (5). Asymmetrically positioned poly(dT) tails were identified for the rightward termini of cDNA 228 and 323 (Fig. 3), representing approximately 41 and 16 bases, respectively (data not shown). These data confirmed the 5′ to 3′ polarity established by the M13, strand-specific, hybridization probes. Homopolymer tails (dA or dT) were not identified for the leftward termini of cDNA 228 and 323 or for either terminus of cDNA 173. This latter observation suggests that the synthesis of cDNA 173 was primed internally, not involving poly(A).

DISCUSSION

Studies about the genome structure and expression strategy of luteoviruses, including PLRV, are not as advanced as those about other plant virus groups (6,7). This lack of progress is attributable to the localization of luteoviruses to phloem tissue of infected

plants (29) and the difficulty in obtaining adequate amounts of viral RNA for molecular biology studies. To circumvent limitations for such experiments, we cloned and physically mapped PLRV cDNAs, which represent a majority of the viral RNA genome.

Our observation that RNA isolated from purified PLRV was contaminated with DNA also has been reported by one other research group (21). These results indicate that degraded plant DNA copurifies with virus during sucrose density fractionation procedures. It is probable that this accounts for the erroneous reports by Sarkar (31,32) that the nucleic acid component of PLRV is DNA.

Our experimental approach of "plasmid walking" identified three partial cDNAs that form an overlapping physical map of 6 kbp (Fig. 3). Based on an estimated PLRV RNA molecular weight of $1.85–2.0 \times 10^6$ (21,30,39), we conclude that a majority of the viral RNA genome is represented by these cloned cDNAs. Clones were confirmed to contain viral sequences by dot-blot hybridization to PLRV RNA and total RNA extracted from diseased leaf tissue (Fig. 5).

In general, the linear cistron organization of monopartite RNA plant viruses appears to be characterized by a 5′-encoded viral replicase and a 3′-encoded viral coat protein gene (6,7). Therefore, as a preliminary step in functional mapping studies of the genome, we conducted an experiment to determine the 5′ to 3′ polarity of PLRV cDNA. We demonstrated that the internal *Pst*I site of clone pPLRV4-228 was 3′ orientated relative to viral RNA (Fig. 6). This result and the linear order of cDNA restriction endonuclease sites established the 5′ to 3′ polarity of PLRV cDNA clones as 5′-pPLRV4-173 - pPLRV4-323 - pPLRV4-228 - 3′ (Fig. 3).

The PLRV cDNA cloning results reported herein compare favorably with the results of other investigators who have used in vitro poly(A)-tailed, monopartite, plant viral RNAs. For example, Meshi et al (22) obtained three overlapping cDNA clones that represent 4 kbp or about two-thirds of the TMV RNA genome using poly(A)-tailed TMV RNA. A similar study conducted by Carrington and Morris (4) using poly(A)-tailed carnation mottle virus (CarMV) RNA produced two overlapping clones representing 3.9 kbp or a majority of the CarMV RNA genome.

The availability of cloned PLRV cDNA will facilitate investigations into the molecular biology of this important potato pathogen. Clones also will have utility for disease diagnosis.

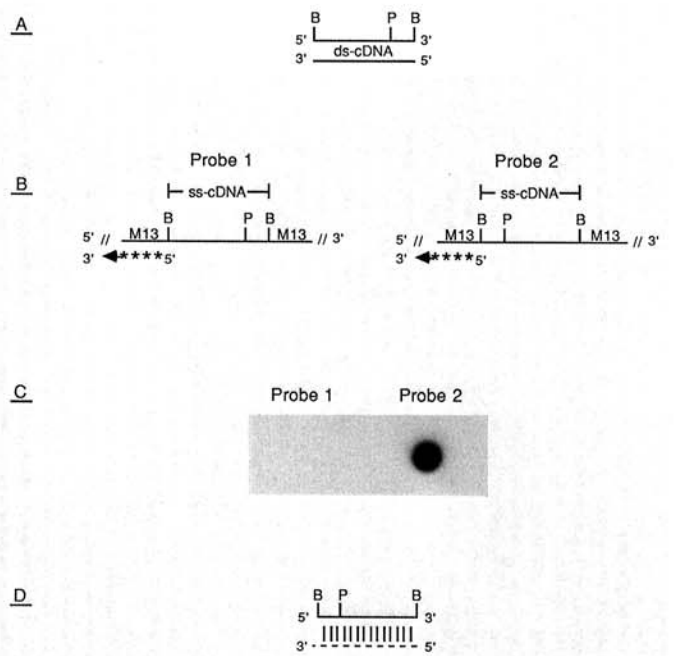

Fig. 6. Determination of 5′ to 3′ polarity of pPLRV4-228. The 1.3-kilobase-pair *Bam*HI subfragment of pPLRV4-228 (**A**), characterized by an asymmetric *Pst*I site, was subcloned in both orientations into M13mp19 replicative form DNA. Single-stranded DNA was isolated and ^{32}P-labeled (****) hybridization probes were synthesized (**B**). Probes were hybridized separately to dot-blots of potato leaf roll virus (PLRV) RNA (0.1 μg), followed by autoradiographic exposure (**C**). The differential hybridization of probe 2 (**C**) established that the *Pst*I site was 3′-orientated relative to PLRV RNA (- - -) (**D**). For clarity, M13 sequences are omitted from the Probe 2/PLRV RNA hybrid (**D**). Abbreviations used are *Bam*HI (B), *Pst*I (P), double-stranded (ds), and single-stranded (ss).

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