Cloning of Four Plant Viruses from Small Quantities of Double-Stranded RNA

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ABSTRACT

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The cloning of less than microgram amounts of dsRNA of four plant viruses, cucumber mosaic virus (CMV), plum pox virus (PPV), raspberry leaf spot virus (RLSV), and strawberry mild yellow-edge virus (SMYEV), by using three methods of denaturation was accomplished. PPV and RLSV clones were up to 1,600 bp in length while the largest insert of SMYEV was 3,000 bp. Denaturation methods used were: boiling for 5 min; incubation in 90% dimethyl sulfoxide (DMSO) at 65 C for 30 min; and incubation at room temperature in 20 mM methylmercuric hydroxide (MeHg) for 10 min. The first strand and second strand synthesis were basically that of Gubler and Hoffman with the omission of mung bean nuclease digestion. After second strand synthesis, the DNA was size-fractionated on a Sepharose CL-4B column, C-tailed, annealed to G-tailed pBR322/pUC9, and *Escherichia coli* DH1/DH5a cells transformed. No differences in length of cDNA and size of inserts was observed when heat- and DMSO-denaturation were used. However, use of MeHg yielded greater incorporation of ^{32}P-dATP into first strand when compared to the boiling method of denaturation.

Molecular cloning of representative viruses of several groups of plant viruses has been carried out during the past decade. One objective of cloning plant viruses has been the improvement of virus detection and diagnosis. The greatest success in detection has been with viroids that are not detectable by serological methods. Moreover, detection of mycoplasmalike organisms has been demonstrated recently with cDNA probes (14). With viruses, group-, virus- and strain-specific differentiations have been made that were not possible with conventional serological techniques (21). As templates for cDNA synthesis, single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), or DNA are usually extracted from purified virus preparations in relatively pure form and in rather large amounts (greater than 50 μg). RNA cloning is generally done by reverse transcription into complementary DNA (cDNA) followed by insertion into plasmid or lambda vectors and transformation of strains of *Escherichia coli* (19). These strategies rely on the purification of virus particles from infected plants. However, there are many recalcitrant viruses or virus isolates that cannot be purified by current methods and, therefore, the standard nucleic acid templates are not accessible for their cloning. It is these viruses for which there are no available antisera that alternate methods of detection and diagnosis are needed. For several of these viruses, the application of dsRNA extraction techniques (6) from herbaceous or woody hosts has permitted the detection of virus replicative nucleic acids (dsRNAs) that can serve as templates for cDNA synthesis. Molecular cloning of dsRNA is a well-established method but has only been reported from large amounts of viral genomic dsRNA purified from virus particles (5,7,9,13,22). The first successful attempt of cloning the genome of a dsRNA plant virus was that of Asamizu et al (2) for wound tumor virus. Recently, the cloning of genomic dsRNA from beet cryptic virus was reported (1).

The objectives of this study were the production of cDNA clones generated from less than microgram quantities of dsRNA templates purified from virus-infected plants. Different dsRNA denaturing techniques before cDNA synthesis were investigated for their utility in obtaining large cDNAs. Because relatively large amounts of pure cucumber mosaic virus (CMV) dsRNA can be extracted readily, it was included in our studies as a model system.

MATERIALS AND METHODS

Virus isolates and maintenance. The plum pox potyvirus isolates PPV-AT and PPV-NAT (18) were maintained in *Nicotiana clevelandii* Gray. The Q strain of cucumber mosaic cucumovirus (CMV-Q) was obtained from R. Symons (University of Adelaide) and propagated in *Nicotiana tabacum* var. *xanthi*. The strawberry mild yellow-edge virus isolate was MY-18 described by Martin and Converse (20) and grafted into *Rubus rosifolius* Smith. The aphid-borne virus from raspberry was described by Kurppa and Martin (15) and maintained in raspberry selection SCRI 1/B/11 since it was first observed in field plots in 1982. This virus was previously designated raspberry leaf spot virus (RLSV), based on dsRNA analysis (15). Although we are not certain that our virus isolate is the same as the one described in Britain, we are retaining this designation.

DsRNA extraction. DsRNA extraction was as described previously (15,16). To eliminate dsRNA that was also present in healthy *Rubus* plants and achieve further purity, RLSV and SMYEV samples were separated on low melting agarose gels and reextracted as described (19). The CMV dsRNA concentration was determined spectrophotometrically with an extinction coefficient of 25 $(mg/ml)^{-1}$ cm^{-1} at A_{260nm}. A_{260nm} determinations were not done for PPV, RLSV, and SMYEV dsRNA because of low yields and absorbance-interference from plant components. Concentrations of these dsRNAs were estimated from ethidium bromide-stained bands after agarose gel electrophoresis.

Denaturing of dsRNAs. Denaturation of approximately 500 ng of PPV dsRNA and primer-annealing were done with heat or dimethyl sulfoxide (DMSO). Heat denaturation was carried out as follows; 48.7 μl of sterile distilled H_2O containing the dsRNA, 8.75 μg of random primers (approximately 20 bp of salmon sperm DNA) and 0.15 μg of oligo$(dT)_{12-18}$ (BRL) were boiled for 5 min, cooled on ice, and the reverse transcriptase mixture added immediately. For denaturation in DMSO, 500 ng of dsRNA, 8.75 μg of random primers and 0.15 μg of oligo$(dT)_{12-18}$ (BRL) were incubated in 90% DMSO for 30 min at 65 C, cooled on ice, adjusted to 150 mM NaCl and ethanol precipitated.

Approximately 500 ng of CMV dsRNA were denatured by heat as described above or with methylmercuric hydroxide (MeHg; Serva). For denaturation with MeHg, 500 ng of CMV dsRNA plus random primers in a total volume of 7 μl was denatured by adding 7 μl of 40 mM MeHg and the mixture incubated for

10 min at room temperature. The mixture was then frozen in liquid nitrogen and the reverse transcriptase mixture added as soon as the denatured dsRNA-primer mix was removed from liquid nitrogen. Estimated amounts of 500 ng of RLSV and SMYEV dsRNA were denatured using MeHg.

Random primed cDNA synthesis from denatured dsRNA. The reverse transcriptase mixture (RTM) for first strand synthesis (11) calculated for a final reaction volume of 100 μl contained 50 mM Tris-Cl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 10 mM DTT, 0.3 mM dATP, 1 mM dGTP, dCTP, dTTP each, 1.5 μg of actinomycin D (only used with PPV) 50 μCi of a^{32}P-dATP (3,000 Ci/mmol; Amersham, DuPont, ICN), 30 U of RNasin (Boehringer, BRL), and 37.5 U of AMV reverse transcriptase (Boehringer, Life Science). Depending on denaturation strategy, 100 μl minus denaturation volume of RTM were added to the denatured dsRNA template. The mixture was incubated for 5 min at room temperature, 10 min at 37 C, and 15 min at 42 C. After adding another 37.5 U of AMV reverse transcriptase incubation at 42 C was continued for another 30 min. The reaction was stopped by adding 5 μl of 0.5 M EDTA, pH 8.0, followed by a phenol/chloroform and a chloroform extraction. First strand cDNA was precipitated with an equal volume of 4 M ammonium acetate and 2.5 volume (v/v) ethanol. The dry cDNA pellet was redissolved in 100 μl of second strand reaction mixture containing 20 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 10 mM ammonium sulfate, 100 mM KCl, 0.15 mM NAD (only used with PPV), 0.1 mM each of dNTPs, 25 U of *E. coli* DNA polymerase I (BRL), 4 U of *E. coli* DNA Ligase (Boehringer) (only used with PPV) and 1.14 U of RNAase H (BRL). After incubating the mixture for 1 hr at 12 C and 1 hr at 22 C the reaction was stopped by adding 5 μl of 0.5 M EDTA, pH 8.0, and 1 vol (v/v) phenol/chloroform. After centrifugation, the supernatant was fractionated on a Sepharose CL-4B column that was loaded in a Pasteur pipette and equilibrated with 10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, and 10 mM NaCl. Using the equilibration buffer, cDNA was eluted in 100-μl aliquots and counts per minute were measured. The first four cDNA-containing fractions were combined and cDNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volume (v/v) of ethanol.

Molecular cloning of cDNA. To remove any possible hairpin structures, the cDNA was treated for 45 min at 25 C with 15 U of mung bean nuclease (Pharmacia, BRL) (only used with PPV and CMV). After a phenol/chloroform and chloroform extraction and ethanol precipitation the cDNA was C-tailed (19) and either annealed to G-tailed pBR322 (BRL, with PPV) or pUC9 (Pharmacia). Competent *E. coli* DH1 cells (12) (only used with PPV) or DH5a cells purchased from BRL were transformed with recombinant plasmids. Transformants were plated on LB-ampicillin (100 μg/ml) plates containing 50 μg/ml Bluo-gal (not used with PPV).

Screening of recombinant DNAs. Recombinant plasmids were screened by colony filter hybridization (10) with ^{32}P-end-labeled single-stranded viral RNA with PPV (16) or first strand cDNA that was synthesized as described above but excluding any non-labeled dATP for RLSV and SMYEV. Colonies with strong signals in filter hybridization were further analyzed by rapid plasmid isolation (3) and digestion with various restriction enzymes. The presence of virus specific inserts was confirmed by Northern hybridizations (19) with selected clones to single-stranded (only used with PPV) or double-stranded virus RNA. Preparation of ^{32}P-labeled DNA probes from DNA templates was by oligo-labeling (8).

Electrophoresis and blotting of nucleic acids. cDNAs were separated on 1% agarose slab gels in the presence of a DNA marker that was stained with ethidium bromide (19). ^{32}P-labeled cDNA was visualized by exposing the dried gel to an X-ray film. RNA and dsRNA were separated on 1% agarose slab gels and nucleic acids stained with ethidium bromide. Denaturing agarose gels were either run as described (4) or in the presence of MeHg (19). Blotting and hybridizations were performed as described (19,24).

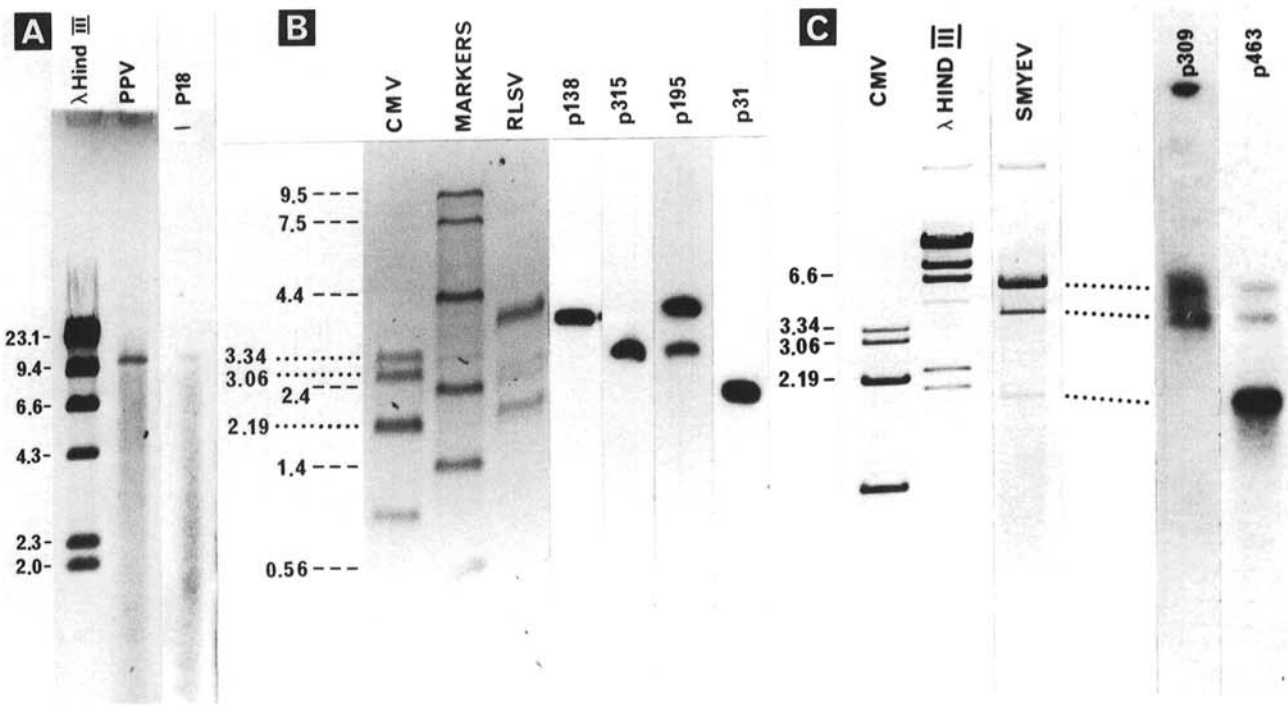

Fig. 1. Agarose gel (1%) analysis of double-stranded RNA. **A,** DsRNA of plum pox virus-AT isolate and *Hin*dIII cut lamba DNA as a molecular weight size marker separated on agarose gel (left two positions). A duplicate dsRNA sample was separated under denaturing conditions, blotted onto nitrocellulose and probed with ^{32}P-labeled cDNA clone pPPV-AT18 (p18). Molecular weights are given in kilobase pairs. **B,** DsRNAs (cucumber mosaic virus [CMV] and raspberry leaf spot virus [RLSV]) and RNA molecular weight size markers separated on agarose methylmercuric hydroxide gel and stained with ethidium bromide (left three positions). Four duplicates of RLSV dsRNA were blotted onto nylon membranes and probed with ^{32}P-labeled RLSV cDNA clones p138, p315, p195, and p31. Molecular weights are given in kilobase pairs. **C,** DsRNAs (CMV and strawberry mild yellow-edge virus [SMYEV]) and *Hin*dIII cut lambda DNA as a molecular weight size marker separated on agarose gel (left three positions). Two duplicate samples of SMYEV dsRNA were separated under denaturing conditions, blotted onto nylon membranes and probed with ^{32}P-labeled SMYEV cDNA clones p309, and p463. Molecular weights are given in kilobase pairs.

RESULTS

Purification of dsRNA templates. A single dsRNA of 9.8 kbp was purified from PPV-infected *N. clevelandii* (Fig. 1A). The dsRNA yield was approximately 500 ng per 20 g of leaf tissue. Approximately 500 ng of PPV dsRNA was used for cDNA synthesis without further purification. CMV dsRNA (Fig. 1B) was obtained in high purity at concentrations of 540 ng per gram of CMV-infected fresh leaf tissue of *N. tabacum*. RLSV dsRNA was extracted from virus-infected field material at estimated concentrations of 500 ng per 20 g of leaf tissue. SMYEV dsRNA was purified from infected leaf tissue of *R. rosifolius* with yields of approximately 300 ng per 20 g of leaf tissue. RLSV dsRNA bands 1, 2, and 3 (Fig. 1B), and SMYEV dsRNAs 1 and 2 (Fig. 1C) were used for cDNA cloning after reextraction from low melting agarose gels.

cDNA synthesis and molecular cloning. A total of 450 colonies were obtained following transformation of *E. coli* DH1 cells with PPV recombinant plasmids derived from either the heat-denatured or DMSO-denatured dsRNA. Five hundred- to 1,600-bp inserts were obtained from 145 plasmids that were chosen after colony filter hybridization with random primed PPV-NAT ssRNA as a probe and analyzed by restriction enzyme digestion. No differences in the length of inserts were observed between the clones resulting from either denaturation technique.

In an attempt to improve cDNA synthesis and the average length of inserts, DMSO denaturation was not further used but heat denaturation compared with MeHg denaturation using CMV dsRNA as template. With MeHg there was a 2.5-fold higher incorporation of ^{32}P after size fractionation of double-stranded DNA on a Sepharose CL-4B column and ethanol precipitation of the cDNA. Equal size distribution to full-length synthesis was obtained with MeHg denaturation while heat denaturation resulted in a higher portion of smaller cDNAs based on autoradiography of gel-separated cDNA after first strand synthesis. At least 50% of the dsDNA was lost when mung bean nuclease was used to give blunt ends after second-strand synthesis based on TCA precipitable counts. Because omitting this step did not result in fewer transformants, it was not used in cloning experiments with RLSV and SMYEV dsRNA. Of randomly picked transformants resulting from MeHg denaturation and heat denaturation, about 90 and 60%, respectively, contained inserts greater than 200 bp.

After size fractionation and ethanol precipitation of the second strand synthesis, with gel purified RLSV and SMYEV dsRNA and MeHg as denaturing agent, incorporation of ^{32}P was similar. A tenfold reduction in incorporation was observed when SMYEV dsRNA was not gel purified.

After colony filter hybridization of 350 white colonies of RLSV, 180 were analyzed by rapid plasmid isolation. Insert sizes of these clones ranged between 300 and 1,600 bp with an average length of 630 bp. Insert sizes of 110 SMYEV recombinant plasmids that were chosen after colony filter hybridization of 800 white colonies ranged between 400 and 3,000 bp. The average in length was 1,010 bp. The specificity of selected inserts was shown in Northern blot hybridizations (PPV, Fig. 1A; RLSV, Fig. 1B ; SMYEV, Fig. 1C).

DISCUSSION

The molecular cloning of four plant viruses by using dsRNAs that were extracted from virus-infected plants as templates for cDNA synthesis was accomplished. The method should have general utility for other plant viruses where only limited amounts of dsRNA template can be obtained.

Molecular cloning of viral dsRNA genomes was introduced by cloning of human reovirus and rotavirus (5,9,13) and bovine rotavirus (7). The described techniques differ in two major ways from that of single-stranded RNA cloning. The first is the necessity of denaturation of the dsRNA template before first strand synthesis, which was done by DMSO (5) or heat (7,13). The second is the alkali treatment of RNA-DNA hybrids after first strand synthesis and subsequent annealing of size fractionated plus and minus strand cDNA instead of synthesis of a second cDNA strand. Because these approaches included reextraction of cDNA from agarose gels, they require high amounts of template. In contrast to a minimum of 20 μg of template that was shown to be useful (7), we were able to generate clones from as little as 500 ng of template by applying second strand cDNA synthesis and only crude size fractionation on Sepharose CL-4B. Homopolymeric tailing of the second strand was successful without prior mung bean nuclease digestion to remove possible hairpin structures. Moreover, omitting this step prevented high losses of cDNA, which is critical when small quantities of nucleic acid must be concentrated by ethanol precipitations. It may also result in obtaining larger cDNA clones as reported for filamentous fungi (23).

Because reverse transcription of dsRNA is reported to be relatively inefficient when DMSO was used as denaturing agent of the template (9) we tried to improve first strand cDNA synthesis and length of clones by applying different denaturation techniques. Heat denaturation gave similar results when compared with DMSO. By using MeHg as denaturation agent, which was shown to be useful for first strand synthesis of yeast dsRNA (22), incorporation of ^{32}P was more than doubled and considerably larger cDNA strands were obtained compared with heat-denaturation when CMV dsRNA was tested. The purity of dsRNA had a crucial effect on the synthesis of first strand cDNA as was also reported for single strand mRNA cloning (23).

The generated PPV (17,18), RLSV, and SMYEV clones are being characterized and their application in virus detection is being tested.

In summary, when cloning of small amounts of dsRNA extracted from plant tissue is desired, we recommend a gel purification step, template denaturation with 20 mM MeHg, and first and second strand synthesis by standard methods, but without including a mung bean nuclease digestion before homopolymeric dCTP tailing.

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