Construction and Use of Cloned cDNA Biotin and ^{32}P-Labeled Probes for the Detection of Papaya Mosaic Potexvirus RNA in Plants

B. P. Roy, M. G. AbouHaidar, T. L. Sit, and A. Alexander

Postdoctorate, associate professor, graduate student, and associate professor, Department of Botany, University of Toronto, Toronto, Ontario, Canada, M5S 1A1. Present address of last author: Departement de Biologie, Université de Moncton, New Brunswick, Canada.
Correspondence should be addressed to second author. Salaries and research support provided by a strategic grant from the Natural Sciences and Engineering Research Council of Canada.
Accepted for publication 12 June 1988.

ABSTRACT

Roy, B. P., AbouHaidar, M. G., Sit, T. L., and Alexander, A. 1988. Construction and use of cloned cDNA Biotin and ^{32}P-labeled probes for the detection of papaya mosaic potexvirus RNA in plants. Phytopathology 78:1425-1429.

Complementary DNA to papaya mosaic potexvirus RNA (PMV RNA) was synthesized by reverse transcription, cloned into pUC 18, and amplified in *Escherichia coli*. RNA probes were prepared by random-primed labeling (using either biotin-7-dATP or ^{32}P-dATP) from plasmids containing inserts representing approximately 97% of the viral genome. Partially purified total RNAs from healthy and infected plants were spotted onto nitrocellulose filters and hybridized to biotin- and ^{32}P-labeled probes. Different blocking and stringent washing conditions for cDNA and cloned PMV DNA probes resulted in the reduction of the nonspecific signals with cDNA probes and even total elimination with cloned DNA probes. The minimum detection level of PMV RNA using biotinylated probes made from cloned DNA or complementary DNA to viral RNAs was about 50 pg. Glyoxalation or formylation of total plant RNAs before spotting onto nitrocellulose filters increased by several fold the sensitivity of detection. The detection limit for the biotin-labeled probes was similar to ^{32}P-labeled ones and comparable to the enzyme-linked immunosorbent assay (ELISA).

The detection of virus infection in plants has traditionally involved either biological disease and/or immunological methods (13,27). With the advent of recombinant DNA methodology, nucleic acid hybridization techniques have been applied to detect viruses and viroids using ^{32}P-labeled DNA probes (3,4,14,15–17,20,23,28). The molecular hybridization technique has the advantage of detecting the viral genome in all of its forms (single- or double-stranded, encapsidated or not), thereby providing a highly specific and sensitive method permitting early detection.

The goal of our research has been to exploit recombinant DNA techniques in order to synthesize nonradioactive cDNA probes and to develop an assay method that would be specific, sensitive, reliable, and less time consuming than presently used methods for the detection of viruses and viroids. The general approach has focused on probes labeled with biotin. Biotin- and photobiotin-labeled hybridization probes have been described for several DNA and RNA viruses and viroids (9,12,18,19).

The papaya mosaic potexvirus (PMV) contains a single-stranded plus-sense RNA of 2×10^6 daltons (10,26). In this paper, we describe the cloning of about 97% of the PMV genome starting at the 3′ end of the RNA, and we have used cloned DNA as well as cDNA directly as probes in dot-blot hybridization assays to detect viral RNA in infected plants. Here we demonstrate that biotin-based probes are specific, and their sensitivity and specificity are comparable to ^{32}P-labeled probes.

MATERIALS AND METHODS

Purification of papaya mosaic virus and viral RNA. The virus was propagated in papaya plants (*Carica papaya* L.). The virus and its RNA were extracted and purified as described by AbouHaidar and Bancroft (2).

Extraction, isolation, and purification of total plant RNA. Young papaya leaves from healthy and PMV infected plants were extracted essentially by the method of Palukaitis (21). Healthy and infected leaves (1–1.5 g) were ground to a powder with a chilled mortar and pestle. During grinding, diethylpyrocarbonate (30 μl) was directly added to the frozen leaves to prevent degradaton of RNA by ribonucleases. The powder was homogenized with 3 ml of 0.2 M Tris-HCl, 0.4 M LiCl, 25 mM ethylene diaminetetraactic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 0.1 M sodium acetate, pH 9.0, buffer.

Proteins in the leaf extract were removed by extraction with phenol saturated with 0.1 M Tris-HCl, pH 8.0, buffer. The residual phenol was removed from the aqueous phase by two chloroform extractions and centrifugation (10,000 *g* for 4 min). The RNAs were precipitated with 2.5 volumes of 95% ethanol at −20 C. Precipitated RNA samples were washed with 70% ethanol, vacuum-dried, and resuspended in 10 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Absorbance values at 260 and 280 nm were used to estimate the concentration and purity of total RNA. RNA samples were analyzed by electrophoresis in 1% agarose gels at 30 mA (constant current) for 45 min in 1 × TBE buffer (0.1 M Tris-HCl, 0.1 M boric acid, and 2 mM EDTA, pH 8.3). Gels were pre-electrophoresed for 30 min before sample loading.

Complementary (cDNA) synthesis and molecular cloning. The first-strand synthesis of PMV cDNA was carried out using oligo dT(12-18) as primer (1). cDNA was precipitated with ethanol in the presence of 2 M ammonium acetate and redissolved in 100 μl of TE buffer followed by phenol extraction.

Second-strand synthesis was performed as described by Gubler and Hoffman (11). The double-stranded cDNA was treated with 1 μg of RNase A in 10 mM Tris-HCl buffer, pH 7.5, phenol extracted and ethanol precipitated in the presence of 2 M ammonium acetate. Gaps in the DNA were filled in with DNA polymerase I (Klenow fragment) as follows: the DNA precipitate was dissolved in 100 μl of 7 mM Tris-HCl, 50 mM NaCl, 7 mM $MgCl_2$ buffer, pH 7.5, containing a dNTP mix (50 μM each of dATP, dCTP, dGTP, and dTTP and 1 unit of DNA polymerase I, Klenow fragment). The mixture was incubated for 30 min at 37 C, extracted with phenol, and precipitated with ethanol.

dC and dG tailing. pUC 18 plasmid was linearized with restriction endonuclease *Pst*I and tailed with dG (5–15 nucleotides), and double-stranded cDNA was tailed with dC (5–15 nucleotides) using terminal deoxynucleotidyl transferase (TDT) as previously described (1). Transformation of *Escherichia coli* and analysis of cDNA clones were as follows: dG-tailed pUC 18 vector (fivefold molar excess) and tailed cDNA were annealed and used to transform *E. coli* cells (JM101) by the calcium chloride method (16). Plates containing X-Gal, IPTG, and ampicillin were used to

©1988 The American Phytopathological Society

select clones. Small-scale preparations of plasmid DNA were made by the alkaline lysis method (5). Plasmids with large inserts were selected for further studies. The identity of clones was determined by Southern hybridization (24) after digestion with *Pst*I to liberate the cDNA inserts.

Preparation of biotinylated and ^{32}P-labeled probes. The DNA probes used for this study were either biotinylated or radiolabeled by a modification of the procedure of Feinberg and Vogelstein (7). Plasmids containing cDNA inserts were first linearized with *Bam*HI and then used as templates for the labeling reaction. The reaction was carried out by annealing template DNA (1–2 μg) and random hexamer oligodeoxyribonucleotides (6.25 μg) (from Pharmacia). The annealed mixture was then either biotinylated (approximately 100 μM of biotin-7-dATP from BRL) or radiolabeled (50 μCi ^{32}P-dATP, >3000 Ci/mmol from ICN) in appropriate buffer in the presence of DNA polymerase I (Klenow fragment). The resultant mixtures were incubated for 30 min at room temperature, then at 37 C for 1 hr, followed by a further incubation for 2 hr after addition of 0.2–0.4 mM dATP. The reaction was terminated by the addition of 20 mM EDTA and TE. Probes were purified through a spin-down column of Sephadex G-50 (16) and stored at −70 C.

Complementary DNA to PMV RNA was synthesized using random hexamer fragments (see above) as primers for cDNA synthesis (22). Reaction mixtures in a final volume of 100 μl contained: 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 1 mM each of dGTP, dCTP, dTTP, and 150 μCi of α-^{32}P-dATP (>3,000 Ci/mmol) or 60 μM biotin-7-dATP.

PMV RNA (5 μg) and random primer (25 μg) were boiled for 4 min at 100 C and quenched in ice. Reverse transcriptase (35 units) was added to the reaction mixture and incubated at 42 C for 2 hr followed by a further incubation of 2 hr after addition of 2 μl dATP (10 mM). The reaction was terminated by addition of 20 mM EDTA and TE. Unincorporated ^{32}P-dATP or biotinylated dATP was removed by Sephadex G-50 spin-down column chromatography.

Spotting and hybridization conditions. RNA samples from healthy and infected plants were denatured with either 7.4% formaldehyde (29) or 8% glyoxal (25) for 5 min at 60 C before spotting onto nitrocellulose filters. PMV RNA used as a control was denatured by glyoxalation, formylation, or boiling at 100 C followed by quick cooling. Samples consisting of 1 μg of total RNA from infected or healthy plants or pure PMV RNA were diluted serially in distilled water and 1 μl was spotted onto nitrocellulose filters. The filter had been pretreated with distilled water, equilibrated with 20 × SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). The spots were either allowed to dry at room temperature or dried under a heat lamp, and the filters were then baked for 2 hr at 80 C under vacuum.

Each filter was then inserted into a sealable plastic bag to which prehybridization solution was added as indicated below. All experiments dealing with biotinylated probes were carried out in the dark. The incubation temperature, the buffer composition, and the washing conditions were different for the DNA and cDNA probes. All probes were denatured by boiling for 3 min and chilling on ice before hybridization.

DNA probes. The composition of the prehybridization solution was as follows: 50% deionized formamide, 5 × Denhardt's solution, 5 × SSPE (20 mM Na_2 EDTA, 0.2 M NaH_2PO_4, 1.5 M NaCl, pH 7.0), 0.1% SDS, 100 μg/ml of denatured herring sperm DNA.

The DNA probe (^{32}P- or biotin-labeled) in 0.5 ml of prehybridization solution was injected into the plastic bags containing the prehybridized filters. These were incubated for 20 hr at 42 C. After hybridization the filters were washed at room temperature twice for 10 min each time with 2 × (SSC) containing 0.1% SDS; then washed at 65 C for 45 min each in 1 × SSC/0.1% SDS; 0.5 × SSC/0.1% SDS, and 0.1 × SSC/0.1% SDS, respectively.

cDNA probes. The filters were prehybridized overnight at 65 C in a solution that did not contain any formamide but contained: 4 ×Denhardt's solution; 3 × SSC; 10 mM EDTA, and 250 μg/ml of denatured and sheared herring sperm DNA. The cDNA probes (^{32}P- or biotin-labeled) were added to the nitrocellulose filter and hybridization was carried out overnight at 65 C. The filters were then washed as follows: twice with 2 × SSC/0.5% SDS for 5 min each at room temperature; twice with 2 × SSC/0.5 SDS for 5 min each at 50 C; twice with 0.1 × SSC/0.5% SDS at 50 C for 15 min each.

Detection of PMV RNA by dot-blot hybridization. For ^{32}P-labeled DNA and cDNA probes, the detection was carried out by autoradiography using Kodak X-ray (XAR-5) films. Exposure time varied from overnight to several days.

For biotinylated DNA probes, PMV RNA was detected by the method of Chan et al (6) using a "Blugene" kit (BRL) with some modifications. To block nonspecific binding sites the filter was incubated for 90 min at room temperature with the following blocking buffer: 100 mM NaCl, 100 mM Tris-HCl, 3 mM $MgCl_2$, and 0.5% Tween 20, pH 7.5.

Streptavidin-alkaline phosphatase (1 mg/ml) was diluted to a concentration of 1 μg/ml and 1 ml of diluted enzyme solution per 10 cm^2 filter was used for enzyme incubation. The incubation buffer was similar to the blocking buffer except that 0.05% Tween 20 was used. Incubation time was 30 min, which is sufficient to allow filters to become completely saturated at room temperature.

The filters were extensively washed at room temperature with blocking buffer and preequilibrated before color development in 0.1 M Tris-HCl (pH 9.0), 0.1 M NaCl, and 50 mM $MgCl_2$ buffer.

In early trials, the biotinylated cDNA probes gave false positive results. It was determined that an extensive post-hybridization washing was required. The following procedure gave satisfactory results: Nonspecific binding sites were blocked by incubating the filter at room temperature for 30 min in: 1 × PBS, 2% bovine serum albumin (BSA), 0.05% Triton X-100, 5 mM EDTA buffer. The enzyme complex (from Enzo) was diluted in 1 × PBS, containing 5 mM EDTA, 0.5% Triton X-100, 0.1% BSA, and incubated for about 2 hr at room temperature. The filters were washed at least five times (5 min each), in 10 mM potassium phosphate buffer, pH 6.5, containing 0.5 M NaCl, 0.5% Triton X-100, 1 mM EDTA, 2% BSA. The filters were preequilibrated in a developing buffer containing 100 mM Tris-HCl, pH 8.8, 100 mM NaCl, and 5 mM $MgCl_2$.

Colorimetric detection. The method was adapted from one provided by Gibco, BRL, Canada. Filters (preequilibrated in buffer) were incubated in dye solution. To each 7.5 ml of developing buffer were added 33 μl NBT (nitroblue tetrazolium at 75 mg/ml in 70% dimethyl formamide) and 25 μl BCIP (5-bromo-4-chloro-3-indolylphosphate at 50 mg/ml in dimethyl formamide); this quantity was used for 100 cm^2 filter. The incubation of the filter and dye was carried out in the dark for a maximum of 3 hr or until background color began to develop. The reaction was terminated by addition of 20 mM Tris-HCl buffer, pH 7.5, 0.5 mM Na_2EDTA. Filters were stored dry.

RESULTS

Cloning of the PMV cDNA. Several PMV cDNA clones were obtained, and their viral origin was determined by Southern hybridization using single-stranded cDNA probes generated by random primers annealed to viral RNA. Plasmids A99, CT21, and H3 were selected for further studies. Plasmids A99 and CT21 contain approximately 3-kb inserts, which were further identified as containing the 3′ noncoding region, the 3′ poly(A) tail, and the capsid protein gene (1). Plasmid H3 contains an insert of approximately 4.6 kb. The restriction map of the viral cDNA inserts is shown in Figure 1. These three inserts represent about 97% of the genome of PMV. These plasmids were used as probes for dot-blot hybridization experiments employing total RNA from healthy and virus-infected plants.

Hybridization results with biotinylated probes for the detection of PMV RNA. Dot-blot hybridization results for the total RNA from healthy and from infected plants are shown in Figure 2. When biotinylated cDNA probes were used, the color intensity faded with dilution; in undiluted extracts (1 μg of total RNA per spot) the color was equally intense with samples from healthy and from

infected plants (Fig. 2A). However, with progressive dilutions (1:10 to 1:100) the spots corresponding to healthy plant extracts became much less visible than those corresponding to infected plant extracts. As little as 20 pg of equivalent PMV RNA from total plant RNAs can be detected (compare i in lane 3 to d in lane 2) (Fig. 2A). Moreover, when extracts from infected plants were serially diluted (Fig. 2B and C), spotted onto nitrocellulose filters, and hybridized with biotinylated DNA probes prepared from cloned cDNA to PMV-RNA, the color could be detected at a dilution of 1:128 or even 1:256, which corresponds to the equivalent of 0.5 ng of PMV RNA (compare h in lane 7 to e in lane 9, Fig. 2B). Under the same hybridization conditions, cloned PMV cDNA can be easily detected at 4–10 pg per spot (Fig. 2B, lane 4). However, as much as 0.5 ng per spot of pure PMV RNA is required for minimum detection (lane 9, Fig 2B).

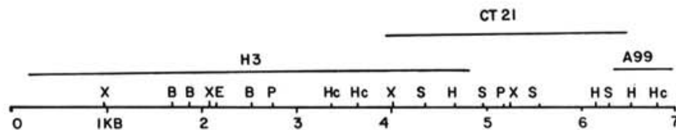

Fig. 1. Partial restriction map of papaya mosaic potexvirus (PMV) cDNA clones representing about 97% of genomic RNA. Three overlapping clones A99, CT21, and H3 (starting at the 3′ end of PMV RNA) are shown. Restriction endonuclease sites for *Bam*HI (B), *Eco*RI (E), *Hin*cII (Hc), *Hin*dIII (H), *Pst*I (P), *Sst*I (S), and *Xba*I (X) are mapped. Sizes are in kilobases (KB).

When biotinylated probes prepared with random primers from plasmids A99, CT21, and H3 were used for hybridizations, only samples from infected plants, and not from healthy plants, hybridized with these probes (Fig. 2B and C).

Hybridization with ^{32}P-labeled probes. Figure 3 shows the results obtained with ^{32}P-labeled probes. PMV-RNA was detected in extracts from infected leaves diluted 128-fold (i.e., containing approximately 7.5 ng of total cellular RNA) (Fig. 3B, lane 4). Purified PMV RNA can be detected at 0.2 ng (Fig. 3B, lane 6). The control DNA (A99, CT21 and H3) can be detected at 4 pg (Fig. 3A). The sensitivity of detection with ^{32}P-labeled probes could be further improved by longer exposures (3–5 days) of X-ray films (data not shown).

Effect of glyoxalation and formylation. The effect of glyoxal and formaldehyde treatments on RNA detection by the dot-blot assay was studied. Glyoxalated and non-glyoxalated RNA samples from healthy and infected plants as well as PMV RNA control were compared. Generally, an increase of several magnitudes in sensitivity was noticeable with glyoxalation. Biotinylated and ^{32}P-labeled probes showed a similar increase (i.e., compare lanes 7 and 8 in Fig. 2B and lanes 4 and 5 in Fig. 3B). The sensitivity of detection was also increased with PMV RNA controls (Fig. 2B and 3B). The effect of formaldehyde on RNA samples seems to be similar to that of glyoxal (Fig. 2C and 3C). However, some nonspecific hybridization was noticeable with ^{32}P-labeled probes only when formylated samples were used (lane 9, Fig. 3C). Glyoxal treatment produced results that were more reproducible than formaldehyde treatment. The lowest amount of glyoxalated PMV RNA detected with biotinylated probes was approximately 50 pg

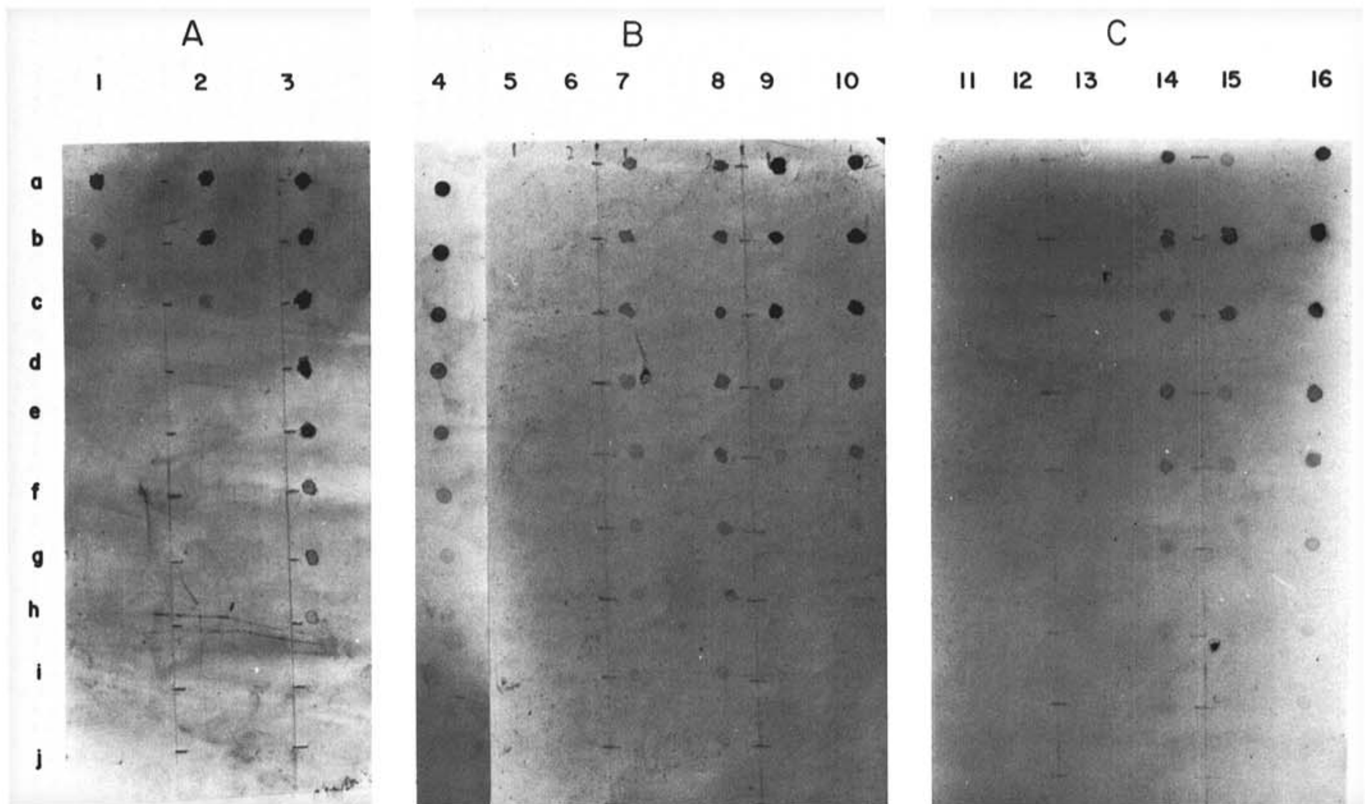

Fig. 2. Dot-blot hybridization for the detection of papaya mosaic potexvirus (PMV) RNA in total plant RNAs using biotinylated PMV cDNA probes. **A,** Biotinylated PMV cDNA probes prepared with random primers from PMV RNA. Total RNA extracted from (1) healthy and (2) infected plants and (3) heat denatured purified PMV RNA. Total plant RNAs or PMV RNA per spot: a = 1,000, b = 100, c = 10, d = 1, e = 0.5, f = 0.2, g = 0.1, h = 0.05, i = 0.02, and j = 0.01 ng, respectively. **B,** Biotinylated cloned PMV DNA probes were hybridized to glyoxalated and non-glyoxalated RNA samples. (4) Cloned PMV DNA control contained per spot: a = 100, b = 10, c = 1, d = 0.1 ng; e = 50, f = 20, g = 10, h = 4, i = 2, and j = 1 pg, respectively. Non-glyoxalated (5 and 7) and glyoxalated samples (6 and 8) from healthy (5 and 6) and infected (7 and 8) leaves, respectively. Amounts per spot of total plant RNAs: a = 1,000, b = 500, c = 250, d = 125, e = 62.5, f = 31.2, g = 15.6, h = 7.8, i = 3.9, and j = 1.9 ng, respectively. (9) Non-glyoxalated and (10) glyoxalated PMV RNA control, respectively. Amounts of PMV RNA are as in lane 3. **C,** Biotinylated DNA probes from cloned PMV DNA were hybridized to formylated (12 and 14) and non-formylated (11 and 13) RNA samples from healthy (11 and 12) and infected (13 and 14) leaves. Purified PMV RNA without (15) and with formylation (16). Amounts of PMV RNA control are as in (3). Sample 15a did not show a strong signal due either to lack of binding of RNA to filter or to an error in the amount of RNA spotted.

per spot (Fig. 2B, lane 10), and that, detected with ^{32}P-labeled probes, was approximately 20–50 pg (Fig. 3B, lane 7).

Nonspecific reactions with crude plant extracts. When crude plant extracts from healthy and infected plants were treated with 1% SDS (But not phenol extracted) before spotting onto nitrocellulose filters, the hybridization with biotinylated probes resulted in a high degree of nonspecific (false positive) coloration for all samples from healthy and infected plants. However, ^{32}P-labeled probes were highly specific (data not shown).

On the assumption that the nonspecific binding of avidin-phosphatase is due to unknown substances (biotin or biotinlike substances) present in plant extracts, we purified the samples from crude plant extracts by chromatography in avidin-agarose columns. This treatment proved to be ineffective (data not shown). The plant extracts were also tested for their endogenous phosphatase activities, which might be responsible for the "false positive" or nonspecific coloration. Dot-blot filters were developed without the addition of the streptavidin-phosphatase. The results obtained indicated that crude plant extracts bound to nitrocellulose did not possess any endogenous phosphatase activities. On the other hand, strong positive reactions were obtained when extracts from healthy and from infected plants were hybridized without the presence of biotinylated probes (data not shown).

DISCUSSION

In this study, we have demonstrated that biotinylated cloned probes to PMV RNA are quite specific, for they did not hybridize with total RNA from healthy plants. In our early results, however, the biotinylated probes hybridized strongly, with extracts from healthy plants. Substances present in the crude extract might bind nonspecifically to biotinylated probes and give rise to false positive results. In an attempt to lower this nonspecific signal, we partially purified the extract. The method used was a combination of LiCl precipitation and phenol/chloroform extractions as described by Palukaitis (21). This treatment gave satisfactory results with cloned DNA probes, but the false positive signals persisted with cDNA probes. We recognized that the two probes differ in their optimal hybridizing and washing conditions. With the biotinylated cDNA probes, formamide was completely eliminated and, hence, the temperatures of hybridization and washings were 65 and 50 C, respectively. Low salt concentrations of 0.1 × SSC/0.5% SDS were also used for stringency requirements. This resulted in a reduction of false positive signals with the cDNA probe by about 10-fold (Fig. 2A); with cloned DNA probes the false positive signals were totally eliminated (Fig. 2B and C). It is possible that the nonspecificity of the cDNA probes may be attributed to contaminating host RNA in PMV-RNA. With DNA probes, formamide was used in hybridization fluid and the temperature was 42 C and washings at 65 C with low salt concentration (0.1 ×SSC/0.1% SDS).

The glyoxalation or formylation of RNA is an important step, for it can affect the binding of RNA to filters (25,29). Heat denaturation of RNA was found to increase the sensitivity of the assay used for potato spindle tuber viroid (PSTV) and citrus exocortis viroid (CEV) by five- to 10-fold (15). In the same study, denaturation with formamide or glyoxal did not affect the sensitivity. However, Flores (8) has shown that denaturation with formaldehyde dramatically increased the sensitivity of detection of citrus exocortis viroid in total nucleic acid preparation. From the results presented in this paper it is clear that denaturation with

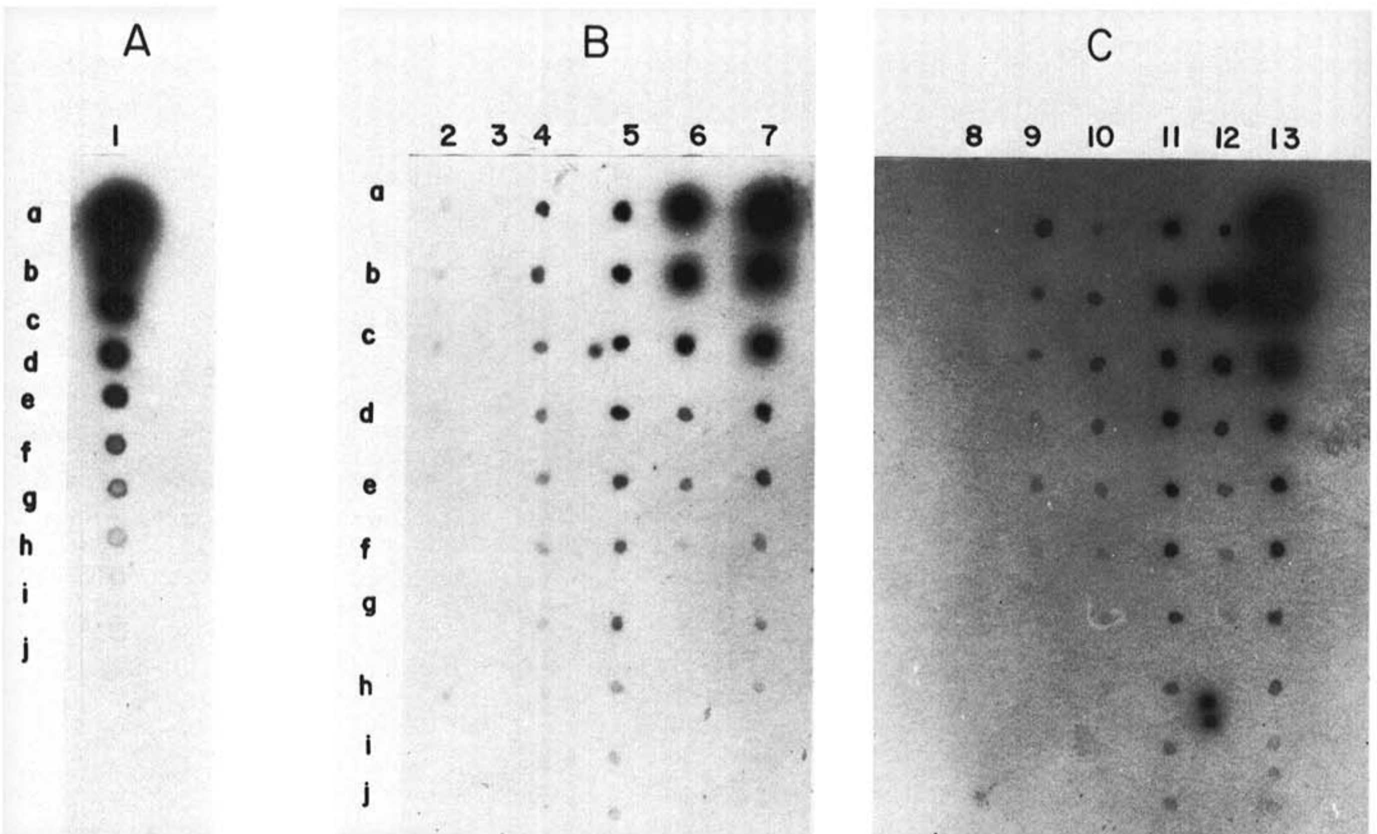

Fig. 3. Dot-blot hybridization for the detection of papaya mosaic potexvirus (PMV) RNA in total plant RNAs using ^{32}P-labeled cloned PMV DNA probes. **A,** Cloned PMV DNA (in pUC 18) used as a positive control. Amounts of total plasmid DNAs per spot: a = 100, b = 10, c = 1 ng; d = 100, c = 50, f = 20, g = 10, h = 4, i = 2 and f = 1 pg, respectively. **B,** Effect of glyoxalation on the detection of PMV RNA in total plant RNAs. Glyoxalated (3, 5, and 7) and non-glyoxalated (2, 4, and 6) RNAs from healthy plants (2 and 3), infected plants (4 and 5) and from purified PMV RNA (6 and 7), respectively. Amounts of total plant RNAs per spot: a = 1,000, b = 500, c = 250, d = 125, e = 62.5, f = 31.2, g = 15, h = 7.5, i = 3.75 and j = 1.87 ng, respectively. Those for purified PMV RNA (6 and 7) were: a = 1,000, b = 100, c = 10, d = 1, e = 0.5, f = 0.2, g = 0.1, h = 0.05, i = 0.02, and j = 0.01 ng, respectively. **C,** Effect of formylation on the detection of PMV RNA in total plant RNAs. Formylated (9, 11, and 13), and non-formylated (8, 10, and 12) RNA samples from healthy plants (8 and 9), infected plants (10 and 11), and purified PMV RNA (12 and 13). Amounts of RNA per spot were as in B.

glyoxal or formaldehyde increases the sensitivity by two- to fivefold. However, glyoxalation reactions were more successful in our hands.

A more quantitative assessment of the binding of total plant RNA to the filter may be possible by radiolabeling. Because of the large amount of cellular RNAs present with viral RNA, the saturation of nitrocellulose filters may prove to be possible. The binding of viral RNAs, which contain many secondary structures, to nitrocellulose filters may also be responsible for the relatively lower efficiency in the detection of viral RNA compared to viral DNA.

Overall the sensitivity of dot-blot hybridization using biotinylated or radioactive probes (^{32}P) is of the order of 50 or 20 pg of viral RNA extracted from infected plants. This amount of viral RNA corresponds to about 0.5–1 ng of virus (PMV contains 5% RNA). The sensitivity of this dot-blot hybridization is comparable to that of ELISA (enzyme-linked immunosorbent assay) where 1 ng of virus can be detected (28). With the method described in this paper, the limit of detection of pure PMV cloned DNA is about 4 pg (Fig. 2B) with biotinylated probes and even lower with radioactive probes.

LITERATURE CITED

1. AbouHaidar, M. G. 1988. Nucleotide sequence of the protein gene and 3′ non-coding region of papaya mosaic virus RNA. J. Gen. Virol. 69:219-226.
2. AbouHaidar, M. G., and Bancroft, J. B. 1978. The initiation of papaya mosaic assembly. Virology 90:54-59.
3. Barker, J. M., McInnes, J. L., Murphy, P. J., and Symons, R. H. 1985. Dot-blot procedure with [^{32}P]DNA probes for the sensitive detection of avocado sunblotch and other viroids in plants. J. Virol. Methods 10:87-98.
4. Baulcombe, D., Flavell, R. B., Boulton, R. E., and Jellis, G. J. 1984. The sensitivity and specificity of a rapid nucleic acid hybridization method for the detection of potato virus X in crude sap samples. Plant Pathol. 33:361-370.
5. Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.
6. Chan, V. T. W., Fleming, K. A., and McGee, J. O. D. 1985. Detection of subpicogram quantities of specific DNA sequences on blot hybridization with biotinylated probes. Nucleic Acids Res. 13:8083-8092.
7. Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonulease fragments to high-specific activity. Anal. Biochem. 132:6-13.
8. Flores, R. 1986. Detection of citrus exocortis viroid in crude extracts by dot-blot hybridization: Conditions for reducing spurious hybridization results and for enhancing the sensitivity of the technique. J. Virol. Methods 13:161-169.
9. Forster, A. C., McInness, J. L., Skingle, D. C., and Symons, R. H. 1985. Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, Photobiotin. Nucleic Acids Res. 13:745-761.
10. Francki, R. I. B., Milne, R. G., and Hatta, T. 1985. Atlas of Plant Viruses. Vol. II. CRC Press, Boca Raton, FL.
11. Gubler, U., and Hoffman, B. J. 1983. A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.
12. Habili, N., McInnes, J. L., and Symons, R. H. 1987. Nonradioactive photobiotin-labelled DNA probes for the routine diagnosis of barley yellow dwarf virus. J. Virol. Methods 16:224-238.
13. Hill, S. A. 1981. Methods in plant pathology part 2: Laboratory methods for diagnosing virus diseases of plants. Rev. Plant Pathol. 60:377-382.
14. Lakshman, D. K., Hiruki, C., Wu, X. N., and Leung, W. C. 1986. Use of phosphorus-32 RNA probes for the dot-hybridization detection of potato spindle tuber viroid. J. Virol. Methods 14:309-320.
15. MacQuaire, G., Monsion, M., Mouches, C., Candresse, T., and Dunez, J. 1984. Spot hybridization: Application to viroid identification. Ann. Virol. (Inst. Pasteur) 135E:219-230.
16. Maniatis, R., Fritsch, E., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor laboratory, Cold Spring Harbor, NY.
17. Maule, A. J., Hull, R., and Donson, J. 1983. The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. J. Virol. Methods 6:215-224.
18. McKeating, J. A., Al-Nakib, W., Greenaway, P. J. and Griffiths, P. D. 1985. Detection of cytomegalovirus by DNA-DNA hybridization employing probes labelled with 32-phosphorus or biotin. J. Virol. Methods 11:207-216.
19. Negro, F., Berninger, M., Chiaberge, E., Gugliotta, P., Bussolati, G., Actis, G. C., Rizetto, M, and Bonino, F. 1985. Detection of HBV-DNA by in situ hybridization using a biotin-labelled probe. J. Med. Virol. 15:373-382.
20. Owens, R. A., and Diener, T. O. 1984. Spot hybridization for the detection of viroids and viruses. Methods Virol. 3:173-187.
21. Palukaitis, P. 1984. Detection and characterization of subgenomic RNA in plant viruses. Methods Virol. 7:259-317.
22. Palukaitis, P. 1986. Preparation and use of cDNA probes for detection of viral genomes. Methods Enzymol. 118:723-742.
23. Schwinghamer, M. W., and Scott, G. R. 1986. Survey of New South Wales Australia potato crops for potato spindle tuber viroid with use of a phosphorus-32 DNA probe. Plant Dis. 70:774-776.
24. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Molec. Biol. 98:503-517.
25. Thomas, P. S. 1983. Hybridization of denatured RNA tranferred or dotted to nitrocellulose paper. Methods Enzymol. 100:255-266.
26. Tollin, P., Bancroft, J. B., Richardson, J. F., Payne, N. C., and Beveridge, T. J. 1979. Diffraction studies of papaya mosaic virus. Virology 98:503-517.
27. Torrence, L., and Jones, R. A. C. 1981. Recent developments in serological methods suited to use in routine testing for plant viruses. Plant Pathol. 30:1-24.
28. Varveri, C., Ravelonarandro, M., and Dunez, J. 1987. Construction and use of a cloned cDNA probe for the detection of plum pox virus in plants. Phytopathology 77:1221-1224.
29. White, B. A., and Bancroft, F. C. 1982. Cytoplasmic dot hybridization—simple analysis of relative mRNA levels in multiple small cell or tissue samples. J. Biol. Chem. 257:8569-8572.