COMPLEMENTARY DNA CLONING AND EXPRESSION OF THE PAPAYA RINGSPOT VIRUS SEQUENCES ENCODING CAPSID PROTEIN AND A NUCLEAR INCLUSION-LIKE PROTEIN

By

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In memory of my father, Robert C. Nagel.
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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

COMPLEMENTARY DNA CLONING AND EXPRESSION OF THE PAPAYA RINGSPOT VIRUS SEQUENCES ENCODING CAPSID PROTEIN AND A NUCLEAR INCLUSION-LIKE PROTEIN

By

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Papaya ringspot virus type W (PRSV-W), formerly watermelon mosaic virus 1, is a member of the potyvirus group of plant viruses. The potyviral genome consists of a single plus-stranded RNA, about 10,000 nucleotide residues in molecular size, with a small protein attached to the 5' end and a poly (A) tail on the 3' end. In this research, two complementary DNA (cDNA) cloning methods were used to clone portions of the PRSV-W genome. Poly (T) was used to prime cDNA synthesis in both procedures. In the Heidecker and Messing procedure (Nucl. Acids Res. 11,4891-4906) the vector was pUC9 and the viral RNA was copied into double-stranded DNA before transforming Escherichia coli JM83. In the Cann et al. procedure (Nucl. Acids Res. 11,1267-1281) an RNA:cDNA hybrid inserted into pUC19 was used to transform E. coli JM83. The Cann method was more efficient than the Heidecker and Messing procedure, both in the number of
clones produced, and in the length of time required to complete the procedure. The clones produced by either procedure represented approximately the same area of the genome.

Three cDNA clones that expressed viral gene products in *E. coli* were characterized. The expressed polypeptides were fusion products with the amino terminus of the α-donor fragment of B-galactosidase. Clones Wl-77 and W2-1 were 1480 and 630 base pairs (bp) in size and expressed fusion products with apparent molecular weights of 40,000 and 14,000, respectively, which were serologically related to PRSV capsid protein. Clone Wl-18 was 1330 bp in size and produced a 52K product that was serologically related to a 54K nuclear inclusion protein of tobacco etch virus. The sequences encoding the capsid and 57K nuclear inclusion-like proteins of PRSV-W were physically mapped to adjacent positions through Southern blot analyses of clones Wl-77 and Wl-18 and are presumed to be located at the 3' end of the viral genome.
CHAPTER 1
INTRODUCTION

Papaya ringspot virus is a potyvirus which causes destructive diseases of papaya and cucurbits. The virus limits the production of papaya in Caribbean countries, Florida, Hawaii, India, South America, and Taiwan (Yeh et al., 1984; and references therein) and causes serious losses in cucurbit crops worldwide (Purcifull et al., 1984). The strain of PRSV used in this study was type W, which until 1984, was known as watermelon mosaic virus 1 (Purcifull et al., 1984). The type W strain differs from the prototype, PRSV-P, only in one host range characteristic; type W does not infect papaya (Purcifull et al., 1984). In Florida PRSV-W overwinters in the wild cucurbit hosts, Melothria pendula and Momordica charantia, and is transmitted nonpersistently by many species of aphids (Adlerz, 1972; Purcifull et al., 1984).

The PRSV particle is a long flexuous rod approximately 780 nm in length and 12 nm in width. The coat is constructed of identical, repeating subunits each with a molecular weight of 36,000 (36K) (Purcifull et al., 1984). The potyviral genome consists of a single, plus-stranded RNA, ca. 10,000 nucleotide residues (10kb) in size with a genome-linked protein on the 5' end (Hari, 1981) and a poly (A) tail on the 3' end (Hari et al., 1979). At least seven proteins are thought to be encoded by the genome (de Mejia
et al., 1985b). Two of the proteins of PRSV aggregate separately in the cytoplasm of host plant cells to form cylindrical inclusions (Christie and Edwardson, 1977) and amorphous inclusions (de Mejia et al., 1985a). The cylindrical inclusions are composed of 70K protein subunits which array themselves to form 'pinwheel' structures attached to the plasma membrane, frequently over plasmodesmata (Christie, personal communication). Later in infection, the cylindrical inclusions usually move away from the plasmalemma and aggregate near the nucleus (Christie and Edwardson, 1977). The amorphous inclusions consist of aggregations of a 51K protein subunit along with associated, uncharacterized RNA (de Mejia et al., 1985a). The 51K protein is the first and only nonstructural protein of potyviruses to which a function has been ascribed; it appears to be a helper factor which enables potyviruses to be transmitted by aphids (de Mejia et al., 1985b).

Certain potyviruses, such as tobacco etch virus (TEV), form a third type of inclusion in the nucleus of host plant cells. These nuclear inclusions are composed of an equimolar mixture of two viral proteins, a 49K protein subunit and a 54K protein subunit (Knutsen et al., 1974). Other potyviruses, such as PRSV, that are not known to form nuclear inclusions in vivo have been demonstrated to form products serologically related to the 49K and 54K proteins of TEV during in vitro translations (de Mejia et al., 1985b; Dougherty and Hiebert, 1980). It is probable that these
protein subunits are also formed in infected host cells but do not aggregate in the nucleus to form structures apparent by light microscopy.

Over 25 potyviruses have been translated in vitro (Hiebert, unpublished) and their products have been immunoprecipitated with various antisera including those to the capsid, cylindrical inclusions, amorphous inclusions, and the 2 nuclear inclusion proteins. Through analysis of the immunoprecipitated products, gene maps have been proposed to locate the protein coding regions on the viral genome (Dougherty and Hiebert, 1980; Hellman et al., 1980; Vance and Beachy, 1984; Xiong, 1985; de Mejia et al., 1985b). Each of these gene maps differs to some degree; for example, Hellman et al. (1980) and Vance and Beachy (1984) report that the capsid protein coding region is located near the 5' end of the genome while others define its position at the 3' end (Dougherty and Hiebert, 1980; Xiong, 1985; de Mejia et al., 1985b). At the present time the potyviral gene map is controversial. The map which was used as a reference in this study is by Xiong (1985), and when adapted for the molecular weights of the PRSV proteins is 5' end; 60K 'unknown' protein; 51K amorphous inclusion protein; 40K 'unknown' protein; 70K cylindrical inclusion protein; 49K nuclear inclusion protein; 57K nuclear inclusion protein; 36K capsid protein; 3' end.

The primary objective of this research was to develop a system to clone portions of a potyviral genome and obtain
the transcription and translation of these portions in *Escherichia coli*. By serologically identifying the polypeptides expressed in *E. coli* and correlating them with specific areas of the viral genome, the gene map may then be verified. This approach was taken by Daubert et al. (1982) to map the location of the capsid protein gene of cauliflower mosaic virus on the viral genome.

Also through such an expression system, polypeptides could potentially be obtained for the two 'unknown' coding regions of the potyviral genome. The products of these coding regions are not known to aggregate in host cells and have not been purified or used to produce antisera; their existence has been demonstrated only in *in vitro* translation products. Saito et al. (1984) cloned cDNA fragments coding for parts of two tobacco mosaic virus nonstructural proteins and expressed the sequences in *E. coli*. These nonstructural proteins are normally extremely difficult to isolate from infected plants, but by expressing the proteins in *E. coli*, sufficient amounts were obtained for characterization and antibody production. Other examples of plant viral proteins expressed in *E. coli* include the capsid protein of satellite tobacco necrosis virus (Van Emmlo., et al., 1984) and the capsid protein of brome mosaic virus (Miglietta and Kaesberg, 1984).
CHAPTER 2
COMPLEMENTARY DNA CLONING AND EXPRESSION OF THE CAPSID AND A NUCLEAR INCLUSION-LIKE PROTEIN OF PAPAYA RINGSPOT VIRUS IN E. COLI

Introduction

The Heidecker and Messing procedure (1983) was selected for the cloning of PRSV-W because the procedure was reported to be very efficient and yield a high proportion of full-length clones. In theory approximately one-sixth of the cDNA clones produced by this procedure should also express protein products for the cloned inserts in E. coli (Helfman et al. 1983; Heidecker and Messing, 1983). Finally, because cDNA synthesis is primed by a poly (T) tail that is covalently attached to the pUC9 plasmid, it was assumed that the resultant cDNA clones would have originated from the poly (A) tail on the 3' end of the viral RNA. Such a factor would have facilitated future mapping studies by providing a known starting point.

Materials and Methods

Materials

Restriction enzymes and the Klenow fragment of DNA polymerase I were from Bethesda Research Laboratories, Inc. (BRL, Gaithersburg, MD 20877). Reverse transcriptase was from the Seikaguki Company (St. Petersburg, FL 33702). [\(^{35}\)S] Methionine, \(^{14}\)C-labeled molecular weight standards, and
$^{125}$I-labeled protein A were from Amersham (Arlington Heights, IL 60005). *Escherichia coli* JM83, pUC8, and pUC9 were from D.C. Loschke. Antisera to three strains of PRSV: type W, type P, and type T were from D.E. Purcifull, D. Gonsalves, and L. Quiot-Douine, respectively.

Papaya ringspot virus type W (PRSV-W), formerly watermelon mosaic virus-1 (Purcifull et al., 1984), was propagated in pumpkin plants (*Cucurbita pepo* L. 'Small Sugar') and purified 2.5 wk after inoculation by the method of Purcifull et al. (1984). RNA was extracted from purified virus by treatment for 5 min at 60°C with 2.0% sodium dodecyl sulfate (SDS), 2 mM ethylenediamine tetraacetic acid (EDTA), and 200 mM ammonium carbonate, pH 9 (Brakke and Van Pelt, 1970a), and isolated on sucrose log-linear gradients (Brakke and Van Pelt, 1970b). The RNA was then precipitated with ethanol, centrifuged, and resuspended in a small volume of sterile, distilled water. The phenol extraction of the RNA was similar to that described by Maniatis et al. (1982) except that after the phenol and phenol/chloroform extractions, the organic phases were removed with a capillary tube, leaving the aqueous phase and interface. Only the aqueous phase, containing the RNA, was retained after the final chloroform extraction. The combined organic phases were reextracted according to Maniatis et al. (1982).
cDNA Synthesis and Molecular Cloning

Complementary (c) DNA clones of PRSV-W were prepared by the method of Heidecker and Messing (1983). This involved 1) cutting the plasmid, pUC9, with the restriction endonuclease, Pst I; 2) tailing the cut plasmid with 40-60 deoxythymidylate residues; 3) adding the viral RNA and using the poly (T) tail of the plasmid as a primer for first strand cDNA synthesis; 4) tailing the first strand of cDNA with dGTP; 5) denaturing and purifying the plasmid with attached cDNA by centrifugation through alkaline sucrose gradients; 6) reannealing the size-fractionated plasmid with attached cDNA to dCTP-tailed plasmid; and 7) filling in the single-stranded area with the Klenow fragment of DNA polymerase I (see Appendix A).

Escherichia coli JM83 cells were transformed by a calcium chloride shock treatment and plated onto L plates containing 50 ug/ml ampicillin and the indicator 5-bromo-4-chloro-3-indoly1-B-D-galactoside (Xgal) at 40 ug/ml (Maniatis et al., 1982; see Appendix A). Insertional inactivation of the B-galactosidase gene of pUC9 (Viera and Messing, 1982) was used as a color marker to select bacterial colonies containing pUC9 with a cDNA insert. The presence of PRSV-W sequences in white colonies was verified by colony hybridization of cloned DNA with $^{32}$P-dCTP labeled first-strand cDNA of PRSV-W RNA (Maniatis et al., 1982). Because Close et al. (1983) reported that not all insertions into the B-galactosidase fragment of pUC vectors result in white colonies, a portion of the blue and light
blue colonies were also tested for PRSV sequences. Insert size was determined by restriction endonuclease digestion of plasmid DNA and agarose gel electrophoresis. Molecular weight markers were lambda DNA (supplied by D. Pring) and \( \phi X 174 \) RF DNA (BRL), digested with Hind III and Hae III, respectively.

**Detection of Expressed Viral Proteins**

Bacterial cells from 12 to 16 hr cultures grown in L broth (Maniatis et al., 1982) were collected by centrifugation at 5,000xg, and lysed in 1/10 volume (original cell suspension) of Laemmli dissociation buffer (Laemmli, 1970). The lysed cells were centrifuged at 27,000xg for 30 min, and the supernatant was retained. Gel electrophoresis, transfer to nitrocellulose sheets, and detection of proteins with antibody (Western blots) were performed as described by de Mejia et al. (1985a). Agar gel (Ouchterlony) immunodiffusion tests were conducted as described by Purcifull and Batchelor (1977). The immunodiffusion medium contained 0.8% Noble agar, 0.5% SDS, and 1% sodium azide (Purcifull and Batchelor, 1977).

For immunoprecipitations, bacterial cells were labeled with \(^{35}\)S-methionine. In these experiments bacterial cultures were grown in potassium morpholinopropane sulfonate (MOPS) medium (Neidhardt et al., 1974) supplemented with 2% dehydrated methionine assay medium (Difco, Detroit, MI 48232) and 10 uCi/ml \(^{35}\)S-methionine. The cells were incubated for 8 to 16 hr at 37°C with shaking, and lysates
were prepared as described above.

Immunoprecipitations were performed by incubating for 1 hr at room temperature 100 ul of [35S]-methionine-labeled bacterial lysate with 100 ul of antiserum and 400 ul of 0.5% Nonidet P-40 in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 0.02% sodium azide (NET buffer) containing 1 mg/ml ovalbumin, and 2 mM methionine (Kessler, 1975). One hundred microliters of a 10% Staphylococcus aureus protein A solution was then added, and the incubation was continued for 20 min. The mixture was washed three times in 0.05% NP-40 NET buffer and resuspended in 40 ul of Laemmli dissociation buffer. Before electrophoresis in SDS-permeated polyacrylamide gels (SDS-PAGE) the samples were heated in a boiling water bath for 5 min. Products were visualized by fluorography (Bonner and Laskey, 1974).

Nick translations were performed as described by Rigby et al. (1977). Southern blots were as described by Maniatis et al. (1982).

Results

DNA Analysis of Expression Clones

Three of the six resulting cDNA clones expressed viral proteins and were characterized. Clone W1-77 contained a 1480 base pair (bp) insert and clone W2-1 contained a 630 bp insert. Each insert could be excised from the plasmid by cutting on both sides of the insert, in the polylinker region of pUC9, with Bam HI and Hind III (Figs. 2-1, 2-2). Eco RI cut at a site in the polylinker region and also at
sites within the inserts of Wl-77 and W2-1, producing fragments of approximately 580 and 3600 bp for Wl-77, and 550 and 2780 bp for W2-1. Clone Wl-18 contained a 1330 bp insert with an internal Hind III site (Fig. 2-2). When Hind III alone was used to restrict Wl-18, fragments of 450 and 3580 bp were observed. Bam HI cut only in the polylinker region of pUC9; it did not cut the Wl-18 insert.

From Southern blotting analyses, clone Wl-18 was found to overlap with Wl-77 but not W2-1. When the 880 bp Bam HI to Hind III fragment of Wl-18 was isolated, nick translated, and used as a probe, only self homology was observed (Fig. 2-3). When the 450 bp fragment of Wl-18 was used as a probe, it hybridized to itself and the Wl-77 insert, but not to the W2-1 insert (Fig. 2-4). The Wl-77 insert, when used as a probe, hybridized to itself, the W2-1 insert, and only the 450 bp insert of Wl-18 (Fig. 2-5).

The orientation of Wl-77 in pUC9 is shown in Figure 2-1a. The direction of transcription and translation in Figure 2-1 is counterclockwise, in pUC9 from the Hind III site to the Eco RI site. The cDNA insert of clone Wl-18 was transferred from pUC9 into pUC8 to orient the insert in the proper direction for transcription and translation (Fig. 2-1b).

Identification of Expressed Proteins

Clones which expressed protein were identified by serological testing. Similar serological results were observed in Western blots and immunoprecipitation tests of
protein extracts for each expression clone (Figs. 2-6, 2-7). Clone Wl-77 produced predominately a polypeptide of Mr 40,000 (40K) that reacted with PRSV capsid antiserum (Fig. 2-6, lane 2; Fig. 2-7, lane 2). When the insert of clone Wl-18 was in pUC9, no reactions were observed in either Western blots or in immunoprecipitations. However, when the Wl-18 insert was in pUC9 a 52K polypeptide that serologically reacted with an antiserum to TEV 54K nuclear inclusion protein was formed (Fig. 2-6, lane 8; Fig. 2-7, lane 11). A number of extra bands were observed with immunoprecipitation tests with the TEV 54K protein antiserum (Fig. 2-7, lanes 3, 7, and 11), and with the Western blots (Fig. 2-6) with either serum. These bands were assumed to be reactions of the antisera with certain unidentified bacterial proteins and occurred regardless of whether the bacteria contained pUC9 or any of the clones. Clone W2-1 produced a 14K polypeptide antigenically related to PRSV capsid protein (Fig. 2-6, lane 3).

In Ouchterlony immunodiffusion tests the expressed protein of Wl-77 reacted homologously with antiserum to
Fig. 2-1. Locations of cDNA inserts in pUC plasmids: a. W1-77 in pUC9. b. W1-18 in pUC8. Transcription and translation proceed counterclockwise.
Fig. 2-2. Restriction analysis and Southern blotting of DNA from clones Wl-77, W2-1, and Wl-18. Lanes contain: 1. pUC9, 2. Wl-77, 3. W2-1, 4. Wl-18 DNA cut with Bam HI and Hind III and electrophoresed on a 1.2% agarose gel. Panel A, gel stained with ethidium bromide. Panel B in an autoradiograph of the DNA transferred onto nitrocellulose and hybridized with a $^{32}$P-labeled probe consisting of randomly primed first-strand cDNA of PRSV-W.
Fig. 2-3. Restriction analysis and Southern blotting of DNA from clones Wl-77, W2-1, and Wl-18. Lanes contain: 1. pUC9, 2. Wl-77, 3. W2-1, 4. Wl-18 DNA cut with Bam HI and Hind III and electrophoresed on a 1.2% agarose gel. Panel A, gel stained with ethidium bromide. Panel B is an autoradiograph of the DNA transferred onto nitrocellulose and hybridized with a [$^{32}$P]-labeled probes consisting of nick translated 880 bp fragment of Wl-18.
Fig. 2-4. Restriction analysis and Southern blotting of DNA from clones Wl-77, W2-1, and Wl-18. Lanes contain: 1. pUC9, 2. Wl-77, 3. W2-1, 4. Wl-18 DNA cut with Bam HI and Hind III and electrophoresed on a 1.2% agarose gel. Panel A, gel stained with ethidium bromide. Panel B is an autoradiograph of the DNA transferred onto nitrocellulose and hybridized with a $[^{32}\text{P}]$-labeled probe consisting of nick translated 450 bp fragment of Wl-18.
Fig. 2-5. Restriction analysis and Southern blotting of DNA from clones WL-77, W2-1, and WL-18. Lanes contain: 1. pUC9, 2. WL-77, 3. W2-1, 4. WL-18 DNA cut with Bam HI and Hind III and electrophoresed on a 1.2% agarose gel. Panel A, gel stained with ethidium bromide. Panel B is an autoradiograph of the DNA transferred onto nitrocellulose and hybridized with a $^{32}$P-labeled probe consisting of nick translated 1480 insert of WL-77.
Fig. 2-6. Western blot of lysates from E. coli JM83 containing: pUC9 (lanes 1 and 5), clone W1-77 (lanes 2 and 6), clone W2-1 (lanes 3 and 7), and clone W1-18 (lanes 4 and 8). The proteins were analysed by electrophoresis in an SDS-permeated polyacrylamide gel, electrophoretically blotted onto nitrocellulose, incubated with an antiserum to PRSV-P capsid protein (lanes 1-4), or an antiserum to TEV 54K nuclear inclusion protein (lanes 5-8), then incubated with $^{125}$I-labeled protein A and autoradiographed. Lane 9 contains protein molecular weight markers. Arrows identify the estimated sizes of the immunoreactive proteins.
Fig. 2-7. Immunoprecipitations of $^{35}$S methionine labeled E. coli fusion proteins related to PRSV capsid protein and TEV 54K nuclear inclusion protein. The proteins were analysed on an SDS-permeated 7.5 to 15% gradient polyacrylamide gel which was processed by fluorography. Lanes 1, 2, 3, and 4 contain lysate from JM83 containing clone Wl-77; lanes 5, 6, 7, and 8 contain lysate from JM83 containing pUC9, and lanes 9, 10, and 11 contain lysate from JM83 containing clone Wl-18. Lysates in lanes 2, 6, and 10 were immunoprecipitated with antiserum to PRSV-P capsid. Lysates in lanes 3, 7, and 11 were immunoprecipitated with antiserum to TEV 54K nuclear inclusion protein. Lysates in lanes 4 and 8 were immunoprecipitated with pre-immune serum. Arrows identify the locations of the major immunoreactive proteins.
Fig. 2-8. Serological relationships of capsid protein from clone WL-77, PRSV-W, and PRSV-T. Central wells contain: P = antiserum to PRSV-P capsid, G = antiserum to PRSV-T, N = pre-immune serum. Peripheral wells contain: c = lysate from JM83 containing clone WL-77, g = extract from pumpkin plants infected with PRSV-T, p = extract from pumpkin plants infected with PRSV-W, b = lysate from JM83 containing pUC9, and h = extract from healthy pumpkin plants.
either PRSV-W virions or PRSV-P capsid protein (Fig. 2-8), which is consistent with previous reports that PRSV-P and PRSV-W are serologically indistinguishable in immunodiffusion tests (Purcifull et al., 1984, and references therein). The protein expressed by W1-77 reacted heterologously with an antiserum to virions of PRSV-T, a strain serologically distinct from PRSV-P or PRSV-W (Quiot-Douine et al., 1985). No reactions were evident with pre-immune sera or with crude extracts of bacteria containing only pUC9. The expressed protein from clone W2-1 did not produce a visible reaction in these tests. Apparently either the protein was not in a great enough concentration for a precipitin formation, or perhaps the antisera used contained antibodies predominately to the amino end of the capsid protein.

Discussion

Two clones of PRSV-W were identified as expressing polypeptides that are antigenically related to PRSV capsid protein, and one clone was demonstrated to produce a polypeptide antigenically related to a nuclear inclusion protein of TEV. The expressed proteins are fusion products because 27 bp exist between the translational start site of the B-galactosidase fragment and the Pst I site into which the cDNA was inserted (Viera and Messing, 1982). Therefore, the first 9 amino acids of each of the expressed proteins are from the amino terminus of the α-donor of B-galactosidase. The plasmid lac promoter of pUC9 is constitutive for B-galactosidase when the E. coli JM83 host
is used, and so no inducer was necessary for the expression of the fusion proteins. Sufficient amounts of the fusion protein of clone Wl-77 were synthesized that the crude lysates of bacterial cells could be used in Ouchterlony immunodiffusion tests.

It was demonstrated with Southern blot analyses that clones Wl-77 and Wl-18 overlap, and therefore concluded that the two sequences encoding the capsid and 57K nuclear inclusion-like proteins are adjacent to one another on the viral genome. However, their positions on the viral genome were not established in this study. The location of the capsid coding region has been identified for two other potyviruses, pepper mottle virus (Dougherty et al., 1985) and TEV (Allison et al., 1985) at the 3' end of the viral RNA. It seems probable, therefore, that the capsid coding region of PRSV is also located at the 3' end of the viral RNA. The proposed locations of clones Wl-18 and Wl-77 in reference to the PRSV-W gene map are illustrated in Figure 2-9.

The cDNA insert in Wl-77 was 1480 bp long and after subtracting 88 bp for the poly (A) sequence (sequence data not shown) and a presumed 3' non-coding region of 100 to 300 bp, it could theoretically code for a protein in the range of 40K to 48K. The predominant antigenic polypeptide found in either Western blots or in immunoprecipitations of Wl-77 protein was approximately 40K. The amino terminus of B-galactosidase would account for approximately 1K and so
the bacterially expressed protein was larger than the capsid protein from virions by 3K. The additional 3K may represent the carboxyl end of the 57K nuclear inclusion-like protein. This would imply that there is no recognized stop codon between the 57K nuclear inclusion coding region and the capsid protein coding region in the PRSV-W genome. The 57K nuclear inclusion coding region has been reported previously to lie adjacent to and in the same reading frame as the capsid protein coding region through analysis of in vitro translation 'read through' or polyprotein products (de Mejia et al., 1985b; Dougherty and Hiebert, 1980). We were not able to detect any reaction of the 40K protein with the antiserum to TEV 54K nuclear inclusion protein in immunoprecipitations or in Western blots. This is not surprising considering that such a small part of the nuclear inclusion protein is presumed to be present and that the antiserum is not homologous for PRSV.

After subtracting the same 188 to 388 bp to account for the poly (A) and 3' non-coding regions, clone W2-1 could theoretically code for a polypeptide of 9K to 16K. The observed 14K polypeptide serologically related to the capsid protein is within the predicted size range.

When the WL-18 insert was in pUC9, no viral-specific product was made because the insert was oriented 3' to 5' with respect to the promoter. Transferring the insert into pUC8 made it 5' to 3' with respect to the promoter and allowed the transcription and translation of a polypeptide
antigenically related to the 54K nuclear inclusion protein of tobacco etch virus. Apparently the insert was already in the correct reading frame and no attempts to change the reading frame were necessary. The polypeptide expressed by clone Wl-18 was larger than is expected for its coding capacity. The 1330 bp insert could maximally code for a polypeptide of 49K; however, a protein of 52K was observed. One reason for this discrepancy could be that there is no stop codon in the cDNA insert, as already suggested for Wl-77, and transcription and translation continue into the B-galactosidase coding area. Indeed, the colony color of Wl-18 was light blue on indicator plates. The 52K protein is not large enough to contain both the predicted 49K nuclear inclusion polypeptide and the 16K B-galactosidase polypeptide fragment, so some post-translational cleavage might be occurring. Although the cleavage site is not known, it would seem likely that since B-galactosidase is a bacterial protein, it would be more resistant to degradation in E. coli than the nuclear inclusion protein. Therefore, the 52K protein may consist of 16K from B-galactosidase and 36K from the nuclear inclusion protein. The remaining 13K nuclear inclusion cleavage fragment, however, was not apparent in the Western blots or in the immuno-precipitations.
Fig. 2-9. Proposed locations of clones W1-18, W1-77, and W2-1 on the PRSV-W gene map. For discussion of the PRSV-W gene map, see Chapter 1.
CHAPTER 3
COMPLEMENTARY DNA CLONING OF PAPAYA RINGSPOT VIRUS RNA
BY AN RNA:CDNA HYBRID METHOD

Introduction

The RNA:cdNA hybrid cloning procedure by Cann et al. (1983) was selected as the second cloning method for PRSV-W. The authors reported that by using this technique with poliovirus RNA, which is structurally similar to potyviral RNA, they produced a set of clones with long, overlapping inserts which spanned the entire 7 Kb size of the poliovirus genome. The authors also observed that more of their clones represented the 5' half of the genome rather than the 3' half. Since clones of what is presumed to be the 3' end of the PRSV-W genome had already been produced by the Heidecker and Messing procedure (1983), this method was undertaken with the goal of obtaining clones with long cDNA inserts (>2000 bp) representing an area 5' to those areas previously cloned. Another reason this procedure was selected was because of its time efficiency; a minimal number of steps are involved and the entire procedure can be completed in two days.
Materials and Methods

Materials

Restriction enzymes, terminal transferase, deoxy nucleotides, and φX174 RF Hae III fragments were from Bethesda Research Laboratories (BRL, Gaithersburg, MD 20877). Reverse transcriptase was from Seikaguki (St. Petersburg, FL 33702). pUC19 and E. coli JM83 were from D.C. Loschke. Lambda molecular weight markers were from D.R. Pring.

Papaya ringspot virus type W (PRSV-W) was purified from inoculated Cucurbita pepo L. 'Small Sugar' by the procedure of Purcifull et al. (1984). RNA was extracted from purified virus (Brakke and Van Pelt, 1970a) and isolated on sucrose log-linear gradients (Brakke and Van Pelt, 1970b). The RNA was phenol extracted (Maniatis et al., 1982) before being used in the cloning procedure.

cDNA Synthesis and Molecular Cloning

The method of Cann et al. (1983) was used to produce cDNA clones of PRSV-W RNA. Complementary DNA synthesis was primed with oligo dT. After the first strand of cDNA was made, the RNA:cDNA hybrid was tailed on the 3' ends with dCTP. The pUC19 vector was cut with Pst I and tailed on the 3' ends with dGTP. The G-tailed vector and the C-tailed RNA:DNA hybrid were then combined and allowed to circularize (see Appendix B). Competent E. coli JM83 cells were transformed and plated onto L plates containing ampicillin at 50 ug/ul and the indicator 5-bromo-4chloro-3-indoly1-B-D-galactoside (Xgal) at 40 ug/ml (Maniatis et al., 1982; see
Appendix A). A schematic of the cloning procedure is shown in Figure 3-1. The resultant 113 white colonies and 19 randomly selected light blue colonies were tested for cDNA inserts by colony hybridizations (Maniatis et al., 1983; Close et al., 1983). The hybridization probe was randomly primed, $^{32}$P-labeled, first strand cDNA of PRSV-W.

Cultures of 81 white and 13 light blue colonies that were positive in the colony hybridizations for PRSV cDNA inserts were grown overnight in LB broth and the plasmid was purified by the method of Maniatis et al. (1982). Insert sizes were determined by digesting the plasmid DNAs with BamHI and Hind III and electrophoresing the samples on 1.2% agarose gels along with molecular markers consisting of lambda DNA Hind III and φX174 RF Hae III restriction fragments.

To determine if the cloned inserts represented the capsid or 57K nuclear inclusion coding regions of the viral genome, the plasmid DNAs were analyzed by Southern blot hybridizations. Duplicate nitrocellulose sheets were prepared by using sandwich blots to transfer the DNA from the agarose gels. One of the nitrocellulose sheets was hybridized with $^{32}$P-labeled nick-translated 1480 bp insert of clone W1-77, and the other was hybridized with $^{32}$P-labeled, nick-translated 880 bp insert of clone W1-18.

Results

Eighty-one of the 113 white and 13 of the 19 light blue colonies were positive in the colony hybridization tests.
Of these 94 colonies containing PRSV cDNA inserts, 8 had inserts between 1000 and 1300 bp, 10 contained inserts between 800 and 1000 bp, 21 contained inserts between 400 and 800 bp, and the remaining 55 colonies contained inserts under 400 bp in length.

All of the clones tested in Southern blot analyses hybridized to either clone Wl-77 or Wl-18. The Southern blots for 15 of the larger cDNA clones are shown in Figures 3-2 and 3-3. Seven of these clones appeared to be similar to clone Wl-18. They each contained a Hind III site within the cDNA insert; and after a double digestion with Hind III and Bam HI to excise the insert from the vector, the smaller fragment portion of each clone hybridized with the insert of Wl-77, and the larger fragment hybridized with the 880 bp fragment of Wl-18. With two of the clones, W4-27 and W4-56 (Figs. 3-2 and 3-3, lanes 2 and 13), the larger fragment hybridized to the 1480 bp fragment of Wl-77, and the smaller fragment hybridized to the 880 bp fragment of Wl-18. Clone W4-48 hybridized only to the 880 bp fragment of Wl-18 and did not contain a Hind III site. The remaining five clones also lacked an internal Hind III site and hybridized only to Wl-77.
Fig. 3-1. Schematic diagram of Cann et al. (1983) cDNA cloning procedure.
Clones W4-42 and W4-38 (Figs. 3-2 and 3-3, lanes 3 and 8) appeared to extend about 100 bp further towards the 5' end of the viral genome than did W1-18. Clones W4-27, W4-42, W4-43, and W4-48 were light-blue in color on indicator plates which may indicate that nuclear inclusion-like proteins were being expressed.

Discussion

Many more clones were produced by the Cann et al. method (1983) than by the Heidecker and Messing method (1983) (94 vs 6 clones, respectively). The Cann method was also much less time consuming than the Heidecker and Messing procedure. Unfortunately, the goal of obtaining large clones from an area of the viral genome 5' to clone W1-18 was not met. Clones W4-42 and W4-38 did extend about 100 bp further than W1-18 toward the 5' end, but the remaining clones all represented an area of the genome previously cloned by the Heidecker and Messing procedure. Apparently the reaction conditions were not optimal and cDNA synthesis did not proceed past the 57K nuclear inclusion coding region.

It would seem advantageous in future cloning experiments to prime the cDNA reactions either with random primers or with a small segment from the 5' end of clone W4-42. Another approach would be to sequence the 5' end of W4-42 to find a unique restriction site. A synthetic oligonucleotide could then be prepared to the area immediately 3' to the restriction site and used to prime
the synthesis of new clones. The new clones could also later be joined to W4-42 through the unique restriction site to form longer clones.
CHAPTER 4
CONCLUSIONS

Two cloning procedures were used successfully to prepare cDNA clones of PRSV-W. The Cann et al. method (1983), however, involved fewer manipulations and more clones were produced. In the Heidecker and Messing procedure (1983), the addition of T-tails to the cloning vector can cause the appearance of long poly (A) sequences on cDNA clones which may have been primed by short, internal A-rich regions. Clones containing these long poly (A) regions may possibly be misidentified as representing the 3' end of the viral RNA.

Clone Wl-77 has a poly (A) sequence on one end approximately 88 bp in size. Wl-77 is assumed to represent the 3' end of the viral RNA, not because it contains poly (A), but because it causes the expression of a polypeptide serologically related to the capsid protein. The capsid protein coding regions of two other potyviruses have been located on the 3' end of the genome through direct comparisons of the amino acid sequences of the capsid proteins with the viral RNA sequences (Allison et al., 1985; Dougherty et al., 1985). The capsid protein coding regions of 23 potyviruses, including PRSV, have also been mapped to the 3' ends of the viral RNAs from analyses of products formed during in vitro translations (Hiebert, unpublished).

Clone Wl-18 was mapped to an internal location on the RNA, adjacent and 5' to clone Wl-77. It is unknown whether
this clone originated from a short internal A-rich region in the viral RNA or whether a recombinational event occurred during the cloning procedure to yield this internal clone. Sequencing studies should resolve the issue.

The expression of plant viral genes in bacteria has many potential uses. Bacteria can produce viral proteins that are not easily extracted from infected plants, and antisera produced to these proteins would not be contaminated with antibodies to plant antigens. For the production of such large amounts of proteins, however, vectors other than pUC8, 9, and 19 are recommended. Under the best conditions in this study, the expressed viral-specific proteins represented about 5% of the total soluble proteins (data not shown). Other expression vectors are available that express polypeptides for inserted sequences at levels of up to 30 to 50% of the total *E. coli* cellular protein (Amann et al., 1983; Masui et al., 1984; Schoner et al., 1985). The expression of the cloned inserts is tightly regulated with these vectors because the expressed products can be toxic to the bacterial host. In the pUC plasmids that were used in this study, the lac promoter is constitutive when the *E. coli* JM83 host is used, which may cause a selection pressure towards those clones that do not express the insert at a high level.
APPENDIX A
RECIPES FOR HEIDECKER AND MESSING CLONING PROCEDURE

Digestion of pUC9 DNA with PST I
20 ul pUC9 DNA (1 ug/ul)
110 ul H2O
15 ul BRL 10X Core buffer (500 mM Tris, pH 8.0, 100 mM MgCl2, 500 mM NaCl)
5.0 ul PST I (9 U/ul)
150 ul total
incubate 37 C, 90 min or longer
phenol extract, ethanol precipitate, resuspend in 20 ul H2O

C-tailing of pUC9
10 ul pUC9, cut with PST I
28.67 ul H2O
10.5 ul 5X tailing buffer (500 mM K-cacodylate pH 7.2, 10 mM CoCl2, 1 mM DTT)
1 ul 1.0 mM dCTP
0.33 ul 32P dCTP
2 ul terminal transferase (20 U)
52.5 total
incubate 37 C, 30 min.
phenol extract, ethanol precipitate 3 times
resuspend in 10 ul 10 mM Tris, pH 7.6, 10 mM NaCl, 1 mM EDTA
T-tailing of pUC9
10 ul pUC9, cut with PST I
10 ul 1 M K-cacodylate, pH 7.0
21 ul H2O
0.5 ul 0.1 M DTT
2 ul 1 mM dTTP
2 ul 32P dTTP (25 uCi)
1.5 ul terminal transferase (15 U)
5 ul 10 mM CoCl2
52 ul total
incubate 37 C, 30 min.
phenol extract, ethanol precipitate 3 times, warm to room
temperature before centrifuging.
resuspend in 10 ul 10 mM Tris pH 7.6, 10 mM NaCl, 1 mM EDTA

**cDNA Synthesis**

<table>
<thead>
<tr>
<th>Component</th>
<th>final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4 ul H2O</td>
<td></td>
</tr>
<tr>
<td>1 ul 12 mM dNTP's</td>
<td>800 uM</td>
</tr>
<tr>
<td>1 ul 1.05 M KCl</td>
<td>70 mM</td>
</tr>
<tr>
<td>1 ul 0.75 M Tris pH 8.2</td>
<td>50 mM</td>
</tr>
<tr>
<td>0.5 ul 300 mM MgCl2</td>
<td>10 mM</td>
</tr>
<tr>
<td>1 ul 30 mM DTT</td>
<td>2 mM</td>
</tr>
<tr>
<td>0.6 ul RNasin 25 U/ul</td>
<td>1 U/ul</td>
</tr>
<tr>
<td>0.5 ul 750 ug/ml actinomycin D</td>
<td>25 ug/ml</td>
</tr>
<tr>
<td>1 ul T-tailed pUC9 (1 ug/ul)</td>
<td>40 nM</td>
</tr>
<tr>
<td>1 ul PRSV-W RNA (5 ug/ul)</td>
<td>120 nM</td>
</tr>
<tr>
<td>1 ul reverse transcriptase (10 U/ul)</td>
<td>100 U/ml</td>
</tr>
<tr>
<td>15 ul total</td>
<td></td>
</tr>
</tbody>
</table>
incubate 37 C, 90 min.
phenol extract, ethanol precipitate 3 times, warm to room temp. before centrifuging, resuspend in 10 ul H$_{2}$O

**G-tailing reaction**

10 ul cDNA
4 ul 1 M K-cacodylate
1 ul 1 mM dGTP
1 ul 0.05 M DTT
2 ul 20 mM MnCl$_2$
1 ul terminal transferase (15 U/ul)
19 ul total

incubate 37 C, 15 min.
phenol extract, ethanol precipitate
resuspend in 50 ul 10 mM Tris, pH 7.6, 10 mM NaCl, 1 mM EDTA

**Alkaline Sucrose Gradient Centrifugation**

Recipe for 10 5-20% linear alkaline sucrose gradients with a 60% cushion

<table>
<thead>
<tr>
<th>Layer</th>
<th>g. sucrose</th>
<th>ml buffer*</th>
<th>amt. per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 5%</td>
<td>.5625</td>
<td>11.25</td>
<td>1.12</td>
</tr>
<tr>
<td>2. 10%</td>
<td>1.125</td>
<td>11.25</td>
<td>1.12</td>
</tr>
<tr>
<td>3. 15%</td>
<td>1.687</td>
<td>11.25</td>
<td>1.12</td>
</tr>
<tr>
<td>4. 20%</td>
<td>2.250</td>
<td>11.25</td>
<td>1.12</td>
</tr>
<tr>
<td>5. 60%</td>
<td>3.000</td>
<td>5.00</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*buffer: 0.2 M NaOH, 0.8 M NaCl, 1 mM EDTA

Add 50 ul of 5% sucrose layer to G-tailed cDNA. Layer on gradient. Centrifuge in 50.1 rotor at 36K, 4 C, for 17 hr. Fractionate gradient by puncturing bottom of tube and collecting 0.3 ml fractions. Measure amt. of radioactivity by Cerenkov radiation. Pool samples from bottom of tube that contain radioactivity, but do not use any fraction after the peak fraction.

Add 3 ul of C-tailed pUC9. Dialyse over night at 4 C against 3 changes of 10 mM Tris, pH 7.6, 10 mM NaCl, 1 mM EDTA. Add 25 ug/ml carrier tRNA and ethanol precipitate.
Resuspend in 50 ul 10 mM Tris, pH 7.6, 10 mM NaCl, 1mM EDTA.

### Reannealing Reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ul DNA</td>
<td>1-5 ul/ml</td>
</tr>
<tr>
<td>320 ul 100% formamide (deionized)</td>
<td>32%</td>
</tr>
<tr>
<td>20 ul 2.5 M NaCl</td>
<td>50 mM</td>
</tr>
<tr>
<td>10 ul 1 M Tris pH 8</td>
<td>10 mM</td>
</tr>
<tr>
<td>600 ul H₂O</td>
<td></td>
</tr>
<tr>
<td>1000 ul total</td>
<td></td>
</tr>
</tbody>
</table>

incubate 37 C for 24 hr. Dialyse overnight at 4 C against 100 mM NaCl, 10 mMTris, pH 8.0, 1 mM EDTA. Ethanolprecipitate, resuspend in 43.5 ul H₂O.

### Fill-in Reaction

<table>
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<th>Component</th>
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</tr>
</thead>
<tbody>
<tr>
<td>43.5 ul DNA</td>
<td></td>
</tr>
<tr>
<td>1 ul 2.5 M NaCl</td>
<td>50 mM</td>
</tr>
<tr>
<td>1 ul 1 M Tris pH 7.6</td>
<td>20 mM</td>
</tr>
<tr>
<td>1.67 ul 300 mM MgCl₂</td>
<td>10 mM</td>
</tr>
<tr>
<td>1 ul 0.05 M DTT</td>
<td>1 mM</td>
</tr>
<tr>
<td>1 ul 5 mM dNTP's</td>
<td>100 uM</td>
</tr>
<tr>
<td>0.8 ul Klenow Frag. DNA Pol. I</td>
<td>3 U</td>
</tr>
<tr>
<td>50 ul total</td>
<td></td>
</tr>
</tbody>
</table>

incubate 15 C for 60 min., then room temperature for 60 min. phenol extract, ethanol precipitate, resuspend in 50 ul 10 mM Tris pH 7.6, 10 mM NaCl, 1 mM EDTA.
Transformation Procedure

1. Inoculate 20 ml of L broth in a 100 ml flask with 1 ml of an overnight culture of E. coli JM83. Incubate culture at 37 C with vigorous shaking until reaching an OD_{550} of 0.5 (2 to 4 hr).

2. Pour the culture into a sterile capped centrifuge tube and cool on ice 10 min. Centrifuge the cell suspension at 6,000 rpm in an SW 34 rotor for 5 min at 4 C.

3. Decant the supernatant. Gently resuspend the pellet in 10 ml sterile, ice-cold CaCl_2, 10 mM Tris, pH 8.0. Incubate suspension on ice 15 min.

4. Centrifuge the cell suspension at 6,000 rpm for 5 min at 4 C.

5. Decant the supernatant. Resuspend pellet in 1.33 ml sterile, ice-cold 50 mM CaCl_2, 10 mM Tris, pH 8.0.

6. Aliquot 0.2 ml volumes into sterile prechilled tubes. Incubate on ice for 1 hr.

7. Add up to 100 ul of plasmid DNA in 10 mM Tris, pH 7.6, 10 mM NaCl, 1 mM EDTA. Incubate on ice 30 min.

8. Heat shock the cells by transferring tubes to a 42 C water bath for 2 min.

9. Add 1 ml of L broth to each tube and incubate at 37 C without shaking for 30 to 60 min.

10. Spread the transformed cells on to 10 or more L plates containing 50 ug/ul ampicillin and 40 ug/ml Xgal. Let plates dry, invert, and incubate at 37 C 12 to 16 hr.

L broth: 10 g Tryptone
5 g Yeast Extract
5 g NaCl
bring volume up to 1 liter, autoclave

L plates: Add 15 g/1 Bacto Agar to L broth before autoclaving. After autoclaving, cool media to 55 C before adding 50 mg/1 ampicillin and 2 ml of a 20 mg/ml stock solution of Xgal in dimethyl formamide (40 mg/1 final concentration)
APPENDIX B

RECIPES FOR CANN ET AL. RNA:cDNA HYBRID CLONING PROCEDURE

G-tailing of pUC19

181 ul H2O
50 ul 5X BRL tailing buffer (500 mM K-cacodylate, pH 7.2, 10 mM CoCl2, 1 mM DTT)
15 ul pUC19 DNA cut with PST I (1 ug/ul)
1 ul 500 uM dGTP
3 ul terminal transferase (60 U)

250 ul total

incubate 37 C, 30 min.
phenol extract, ethanol precipitate
resuspend in 15 ul H2O

cDNA Synthesis

12.4 ul H2O
5 ul 1.0 M Tris pH 8.3
5 ul 2.0 M NaCl
1 ul 0.8 M MgCl2
1 ul 0.5 M DTT
4 ul R Nasin (25 U/ul)
0.5 ul oligo dT 12-18 (1 ug/ul)
2 ul PRSV RNA (1 ug/ul)
5 ul 10 mM dATP
5 ul 10 mM dTTP
5 ul 10 mM dGTP
1 ul 100 uM dCTP
0.5 ul $^{32}$P dCTP (5 uCi)

2.6 ul Reverse transcriptase (10-16 U/ul)

50 ul total

incubate 42 C, 15 min; add 2 ul 10 mM dCTP
incubate 42 C, 45 min; add 10 ul 0.2 M EDTA
incubate 65 C, 5 min
dilute 10 X with BRL NACS buffer C (0.5 M NaCl, 20 mM Tris, pH 7.2, 1 mM EDTA) and run column according to manufacturer's directions (BRL) elute sample with NACS buffer D (2.0 M NaCl, 20 mM Tris, pH 7.2, 1 mM EDTA).

C-tailing of cDNA

10 ul RNA:cDNA hybrid

28 ul H$_2$O

10 ul 5X tailing buffer

1 ul 100 ul uM dCTP

1 ul terminal transferase (40 U)

50 ul total

incubate 37 C, 15 min.
phenol extract, ethanol precipitate
resuspend in 15 ul H$_2$O

Reannealing Reaction

15 ul RNA:cDNA hybrid

170.5 ul H$_2$O

4 ul 0.5 M Tris pH 7.5, 10 mM EDTA

10 ul 2 M NaCl

0.5 ul G-tailed pUC19

200 ul total

incubate 65 C, 5 min, then incubate 45 C, 2 h
slow cool to room temp.

Transform competent E. coli JM83 cells as in Appendix A.
APPENDIX C
VARIOUS OTHER RECIPES

cDNA Synthesis for Hybridization Probes

25.5 ul H₂O
5 ul 10X cDNA buffer (500 mM Tris, pH 8.3, 80 mM MgCl₂, 250 mM NaCl)

2.5 ul 100 mM DTT
1.8 ul RNasin (25 U/ul)
1.3 ul actinomycin D (700 ug/ml)
3 ul 10 mM dATP
3 ul 10 mM dTTP
3 ul 10 mM dGTP
0.5 ul random primers (5 ug/ul)
2.0 ul PRSV-W RNA (1 ug/ul)
1.8 ul reverse transcriptase (10-16 U/ul)
0.6 ul³²P-dCTP (5 uCi)

50.0 ul total

incubate at 42 C, 30 min
add 0.5 ul 10 mM dCTP
incubate at 42 C, 60 min
add 3.0 ul 6 N NaOH
incubate at 56 C, 60 min.

Plasmid Purification

Grow cultures overnight in a 250 ml flask containing 40 ml L broth at 37 C with shaking. Cool culture in ice 30 min. Centrifuge 10K for 10 min. Decant supernatant.
Resuspend pellet in:

0.48 ml 25% sucrose, 50 mM Tris pH 8.0 (ST)

vortex

0.16 ml 10 mg/ml lysozyme in ST (make fresh)

incubate on ice 5 min

0.40 ml 0.2 M EDTA pH 8.0

incubate on ice 5 min

1.02 ml Triton lysis buffer

(1 ml 10% Triton X100, 5 ml 1.0 M Tris pH 8.0, 31.25 ml 0.2 M EDTA pH 8.0, 62.75 ml H2O)

incubate on ice 15 min, swirl occasionally

Centrifuge 15K, 30 min; collect supernatant

Phenol extract 2 times; ethanol precipitate; resuspend in 250 ul H2O.

Nick Translation

0.5 ul DNA (1 ug/ul)

5.0 ul 10X NTB (500 uM Tris pH 7.9, 50 uM MgCl2, 100 uM 2-mercaptoethanol)

34 ul H2O

5.0 ul 10X NTP (40 uM each dATP, dTTP, dGTP)

5.0 ul DNase I (1 ul of 50 ug/ml stock in 500 ul H2O)

0.5 ul 32P dCTP (5 uCi)

50 ul total

incubate 15 C, 4 h

add 2.5 ul 10% SDS, and 2 ul of 0.35% bromophenol blue

Run through a 1 ml Sephadex G-50 column with NTB buffer, collect first radioactive peak (when the bromophenol blue is about at the 80 cc mark on a 1 ml Tuberculin syringe)
LITERATURE CITED


BIOGRAPHICAL SKETCH

Julianne Nagel was born in Colver, Pennsylvania, on December 12, 1957, and grew up in Johnstown, PA. She graduated from Ferndale Area High School in 1975. She completed her undergraduate degree in horticulture at Pennsylvania State University, graduating in 1978 with the title of Student Marshall of the College of Agriculture. In 1979 she enrolled at the University of Florida, Plant Pathology Department, and obtained a Master of Science degree under the direction of Dr. F. W. Zettler in 1981. From 1981 until 1982 she was employed as a laboratory technician II for Dr. F.W. Zettler. She began her Ph.D. research in 1982 in the same department under the direction of Dr. E. Hiebert and completed her degree in 1985. She is currently a postdoctoral research associate with Dr. R. Shepherd in the Plant Pathology Department at the University of Kentucky.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ernest Hiebert, Chairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

L. Curtis Hannah
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David C. Loschke
Assistant Professor of Plant Pathology

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Daryl R. Pring
Professor of Plant Pathology
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Francis W. Zettler
Professor of Plant Pathology

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1985

Jack L. Fry
Dean, College of Agriculture

Dean, Graduate School