Molecular cloning of cDNA to soybean mosaic virus RNA

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LITERATURE REVIEW

Biology of the Potyvirus Group

Brandes and Wetter (1959) first proposed the establishment of 12 groups of plant viruses with elongated particles based on their modal length and certain other characteristics (e.g., diameter, shape, thermal inactivation, and mode of transmission). The potyvirus group contained 14 definite members, including soybean mosaic virus. Soybean mosaic virus (SMV) is a member of the largest group of plant viruses, the potyvirus group. The group was named for the type member, potato virus Y (PVY). Soybean mosaic virus was first reported as an infectious agent in Connecticut in 1916 (Clinton, 1916). In 1971 (Harrison et al., 1971) the name potyvirus was first used to designate the group, which had become a more precisely defined group. The first report of the International Committee of Nomenclature of Viruses (ICNV) (Wildy, 1971) described the group with 12 definite and 13 possible members. Since that time more members have been added (Edwardson, 1974; Fenner, 1976; Matthews, 1979, 1982; van Regenmortel, 1982); presently there are 48 definite members and 56 possible members.

It is now generally agreed that in order to classify a virus isolate in the PVY group, it must have particles

which are flexuous rods and be able to induce cylindrical "pinwheel" inclusions in the cytoplasm of infected cells (Matthews, 1982). Potyvirus members have flexuous rod-shaped particles between 680 and 900 nm in length and about 12 nm in diameter (Francki, 1985). Most potyviruses have fairly restricted host ranges, are transmitted non-persistently by aphids and in some cases through seeds. They can also be transmitted by mechanical inoculation.

Morphological studies of potyviral particles have not generated much detail in fine structure; however, some studies have shown particles with narrow central canals (Hollings and Brunt, 1981). Optical diffraction studies (Varma et al., 1968) have indicated that the protein subunits (i.e., coat protein molecules) are arranged in a helix with a pitch of 3.3 to 3.5 nm. The reported variation of the modal length of particles is believed to be dependent upon the presence or absence of divalent metal cations present in the suspending medium (Moghal and Francki, 1981; Govier and Woods, 1971).

Polyacrylamide gel electrophoresis studies on dissociated coat proteins of potyviruses have indicated that each virion is constructed from a single species of coat protein; minor faster migrating proteins have been observed but are believed to be degradation products (Hill and Benner, 1980b; Moghal and Francki, 1976) or may be due

to variation in the disulfide-bridge patterns of the protein (Hill and Benner, 1980b). The molecular weight of the potyviral coat protein ranges from 32,000 to 40,000 (Edwardson, 1974; Gough and Shukla, 1981; Hiebert and McDonald, 1973); these variations are thought to be mainly due to different methods used by different investigators (Francki, 1985). True differences between viruses and virus strains may exist (Gough and Shukla, 1981).

The molecular weight of potyviral RNA has been estimated to be, using polyacrylamide gels and sedimentation rates, about 2.9 to 3.5 x 10^6 (10 kilobases) (Brakke and van Pelt, 1970b; Hari et al., 1979; Hill and Benner, 1976; Pring and Langenberg, 1972). The RNA has been shown to have a poly A sequence at the 3' end (Hari et al., 1979; Hellmann et al., 1980; Vance and Beachy, 1984b); the length of the sequence has been estimated to vary between 20 to 120 bases, but is believed that the majority of molecules have 20 to 40 residues. The 5' end has been shown to have a VpG protein covalently linked but the size of the protein appears to be variable.

Cell-free translation of potyviral RNA has been used extensively in recent years; these studies have revealed significant information regarding gene expression and genomic structure. Dougherty and Hiebert (1980a,b,c) used rabbit reticulocyte lysates to translate tobacco etch and

pepper mottle virus RNA. Translation of both RNAs resulted in the formation of a complex set of polyproteins. Using immunoprecipitation studies, two distinct mechanisms for the formation of coat protein in the cell-free system were indicated. Monospecific antisera to tobacco etch virus coat protein was immunoreactive to several high molecular weight (MW) (about 85,000) translation products along with coat protein (molecular weight, 30,000). Tobacco etch virus (TEV) was believed to initially express a large polyprotein precursor, which was processed to yield individual gene products, including the coat protein. Only the coat protein translation product (molecular weight, 33,000) of pepper mottle virus (PeMV) RNA was immunoreactive with monospecific antisera to PeMV coat protein, indicating the synthesis of discrete translation products with little or no synthesis of polyproteins. Immunoprecipitation studies, performed with the products formed by cell-free translation, allowed Dougherty and Hiebert to devise the following genetic map for the potyvirus: 5 'end - 78-87,000 MW protein gene - 49,000 MW nuclear inclusion protein gene - 41-50,000 MW protein gene - 68-70,000 MW cylindrical inclusion gene - 54-56,000 MW nuclear inclusion gene - 30-33,000 MW coat protein gene - 3' end.

Koziel et al. (1980) used a wheat germ extract for

translating tobacco etch virus RNA and found only a single translation product; it was postulated that these results differed from those obtained by Dougherty and Hiebert because of different salt concentrations or simply a different translation system.

Tobacco mottling vein virus (TVMV) RNA was translated (Hellmann et al., 1980) in rabbit reticulocyte lysates and produced a wide range of polypeptides (molecular weight 20,000 to 100,000); the predominant species (P75) had a molecular weight of 75,000. At high ionic strength, only P75 was synthesized while at low ionic strength only peptides 52,000 daltons and lower were synthesized. Immunoprecipitation of polypeptides with antibody to whole virus precipitated many polypeptides, including P75, which suggested that they contain the coat protein sequence. This work was continued (Hellmann et al., 1983) with more extensive immunoprecipitation studies using antisera against TVMV coat protein, cylindrical inclusion, and helper component along with antisera against the TEV 49,000 and 54-56,000 MW nuclear inclusion proteins. Each of the five antisera precipitated a distinct pattern of polypeptides which were shown to define five different non-overlapping regions of the TVMV genome. These results indicated that the helper component protein may be encoded by the potyviral genome.

Vance and Beachy (1984a) used both rabbit reticulocyte lysates and wheat germ extracts for the cell-free translation of soybean mosaic virus RNA. Both systems produced the same ten to twelve polypeptides. Immunoprecipitation with antisera against SMV coat protein indicated that six protein products were related to the coat protein and one of the proteins was shown to comigrate with coat protein during electrophoresis. Antisera against SMV cytoplasmic inclusion protein and TEV nuclear inclusion protein precipitated two classes of antigenically distinct polypeptides. By labeling translation products with [^{35}S]methionine or with [^{35}S]methionyl tRNA (which labels only the amino-terminus of translation products), it was determined that the putative coat protein and a translation product related to the nuclear inclusion protein were generated by proteolytic processing. From their data Vance and Beachy concluded that the coat protein gene may be located at the 5' end of the genome, which would be contrary to the results obtained with other potyviruses.

Yeh and Gonsalves (1984) used rabbit reticulocyte lysates to translate papaya ringspot virus (PRV) RNA. Translation of the RNA resulted in synthesis of more than 20 polypeptides ranging in molecular weight from 26,000 to 220,000. Antiserum to PRV coat protein was immunoreactive to a subset of these polypeptides including a 36,000 MW

protein that comigrated with PRV coat protein during electrophoresis. Antiserum to PRV cylindrical inclusion protein was immunoreactive with another subset of polypeptides including 70,000, 108,000, 205,000, and 220,000 MW proteins as the major precipitates. The 70,000 MW protein comigrated with authenic cylindrical inclusion protein; the 205,000 and 220,000 MW proteins were related to both coat and sytoplasmic inclusion proteins. Antiserum to PRV amorphous inclusion protein was immunoreactive to a unique subset of polypeptides with a 112,000 MW protein as the major precipitate; the 51,000, 65,000, and 86,000 MW proteins were the minor precipitates. The 51,000 MW protein comigrated with authentic amorphous inclusion protein. When translation of PRV RNA was done without the reducing agent, dithiothreitol, a major protein product of molecular weight 330,000 was observed. Immunoprecipitation studies indicated that this protein was related to the presumed coat, cytoplasmic inclusion, and amorphous inclusion proteins. The presence of this polyprotein and its possible precursor relationship to other polypeptides suggested that proteolytic processing may be involved in the translation of PRV RNA.

Translation of potyviral RNA <u>in vivo</u> has not been studied in much detail. Full length genomic SMV RNA has been shown (Vance and Beachy, 1984b) to be, in infected

soybean tissue, associated with the polyribosome fraction, indicating translation of genomic length RNAs only. Otal and Hari (1983) detected both subgenomic and genomic length TEV RNA in the total RNA from infected tobacco plant cells. Dougherty (1983) also detected subgenomic and genomic length RNAs in total RNA from infected plants, but attributed the smaller RNAs to electrophoretic anomalies. Both of the studies with TEV did not determine, however, whether the viral RNAs detected were being translated in infected cells. Valverde et al. (1986) detected ,from infected tissue, double stranded RNA (presumedly the replicative form) for TEV and SMV, while possible subgenomic double stranded RNAs were detected for PeMV, PVY, turnip mosaic virus (TuMV), and dasheen mosaic virus (DMV). These subgenomic RNAs were believed not to be artifacts. The striking difference in banding patterns of double stranded RNAs for TEV and PeMV may related to the different translation strategies presumedly used by these potyviruses.

Synthesis of complementary DNA (cDNA) to potyviral RNA has advanced the conclusions made about genomic structure and gene expression using cell-free translation systems. Allison et al. (1986) determined the nucleotide sequence of the 3' terminal 2324 nucleotides of TEV RNA by sequencing DNA complementary to this region. Analysis of

the sequence revealed an open reading frame of 2135 nucleotides and an untranslated region of 189 nucleotides adjacent to the 3' terminal polyadenylate region. The sequence began with an open reading frame, which suggested that the start signal for translation was located upstream of the cloned sequence. This open reading frame could be translated into a protein with a molecular mass of at least 80,830 daltons. This appears to confirm the observation made with cell-free translation that a polyprotein is synthesized from TEV RNA. The primary sequence of amino acids at the amino-terminus of tobacco etch coat protein was determined by amino acid sequencing of purified coat protein. The sequence of the first 20 amino acids from the N-terminus was identical to amino acids -263 through -244 predicted from the nucleotide sequence. The total amino acid composition between tobacco etch coat protein and the amino acid compostion predicted by the nucleotide sequence were shown to be in close agreement.

Dougherty et al. (1985) sequenced cDNA complementary to the 3' terminal 1481 nucleotides of pepper mottle virus RNA. Analysis of the nucleotide sequence revealed a single large open reading frame of 990 nucleotides on the corresponding positive-sense RNA. The open reading frame began with a start signal for translation and was terminated by a single stop signal located 333 nucleotides

from the 3' terminal polyadenylate region. This open reading frame would encode for a 37,669 dalton protein. This data tends to support the observation made with cell-free translation that a discrete coat protein is synthesized from PeMV RNA. The primary sequence of amino acids at the amino-terminus of pepper mottle coat protein was determined by amino acid sequencing of purified coat protein. The sequence of the first 21 amino acid residues of the amino-terminus was shown to be identical to the nucleotide predicted sequence of amino acids 64 through 84 of the large open reading frame. A protein with a primary sequence of amino acids 64 through 330 of this open reading frame would have a molecular weight of 30,120 daltons, which is in the range of the value obtained for coat protein as determined by polyacrylamide electrophoresis.

Hellmann et al. (1986) determined the genomic structure of tobacco vein mottling virus by hybrid-arrest translation, using cloned single stranded cDNA probes followed by immunoprecipitation of translation products separated by electrophoresis. The genetic map devised from this data was: 5' end - 25,000 MW protein gene - 53,000 MW helper component protein gene - 50,000 MW protein gene - 70,000 MW cylindrical inclusion protein gene - 52,000 MW nuclear inclusion protein gene - 56,000 MW nuclear inclusion protein gene - 32,000 MW coat protein

gene - 3' end.

Allison et al. (1986) recently determined the complete nucleotide sequence of tobacco etch virus genomic RNA by sequencing complementary DNA. Analysis of the TEV nucleotide sequence revealed a single open reading frame which could presumedly translate a large molecular weight polyprotein. The TEV large nuclear inclusion protein (54,000 daltons) was tentatively assigned a replicase function from this sequence data when compared to other viral replicases.

Complementary DNA (cDNA) Cloning

With the methods initially used for cDNA cloning (Maniatis et al., 1982; Rabbitts, 1976; Rougeon et al., 1975), the yield of recombinant DNAs that had full length sequences was low. The cDNA clones synthesized were useful as hybridization probes, but they could not direct the synthesis of complete proteins after their introduction into bacterial or mammalian cells via the appropriate expression vectors.

The enzyme used in these cDNA synthesis' was a RNA-directed DNA polymerase (reverse transcriptase) (Verma, 1977) derived from Avian Myeloblastosis Virus (AMV). AMV reverse transcriptase consists of two subunits, alpha

(molecular mass of 62,000 daltons) and beta (molecular mass of 95,000). The alpha subunit has been shown to possess both DNA polymerase and RNase H activities (Verma, 1977). The beta subunit is thought to be a larger precursor of the alpha subunit; the most stable form of the enzyme is the alpha,beta complex.

Reverse transcriptase (RT) requires a primer in order to initiate polymerase activities. There are two kinds of primers that are commonly used: oligodeoxythymidylate [oligo (dT)], which is used to anneal with RNAs possessing a polyadenylated 3' end; the alternative is to use an oligonucleotide which will anneal to a complementary sequence within the body of the RNA template. RNA templates which possess polyadenylated 3' ends include eukaryotic messenger RNAs and many viruses that infect eukaryotic cells. This poly A tail has been exploited as the basis for selecting and purifying such RNAs by using the technique of oligo (dT)-cellulose chromatography (Aviv and Leder, 1972).

Another RT that has been very recently used for the synthesis of cDNA is a cloned version derived from Moloney-murine leukemia virus (M-MLV). This RT (which is a single polypeptide with a molecular mass of 80,000 daltons) may be a superior alternative to AMV RT because it is missing the DNA endonuclease activity associated

with AMV RT in addition to a reduced RNase H activity in reaction conditions optimal for the copying of an RNA template. It has been shown (Gerard, 1985) that there was, in general, a higher efficiency of full length cDNAs synthesized using the cloned M-MLV RT than compared with AMV RT.

The quality of the reverse transcriptase, the condition of the RNA template, and the reaction conditions can all influence the length of cDNA produced (Manaitis et al., 1982). An important factor in the synthesis of long cDNAs is the quality of reverse transcriptase used in the reaction. Contaminating RNases can decrease the efficiency of full length cDNA synthesis and their effects can be eliminated by additional purification of the enzyme or by the addition of potent inhibitors of RNase, such as vanadyl-ribonucleoside complexes or RNasin (de Martynoff et al., 1980; Maniatis et al., 1982; Puskas et al., 1982). The addition of sodium pyrophosphate or ribonucleoside triphosphates (Kacian et al., 1972) will increase efficiency of reverse transcription by protecting the RNA template from RNases.

Other parameters such as monovalent and divalent cation concentrations, deoxynucleotide triphosphate concentrations, temperature, and pH can all have an effect on the optimal conditions for cDNA synthesis (Buell et al.,

1978; Efstratiadis et al., 1976; Retzel et al., 1980). The ratio of reverse transcriptase to the RNA is also important in optimizing the yield of full length cDNA (Maniatis et al., 1982). The optimum conditions for cDNA synthesis varies as to the RNA that is being used as a template.

Following synthesis of the first strand cDNA the RNA of the cDNA:RNA hybrid can then be destroyed by either alkaline hydrolysis or by RNase H, which is an RNase that destroys RNA in a DNA:RNA hybrid. The 3' end of single stranded cDNAs, for unknown reasons, is capable of forming a hairpin structure which can act as a primer for synthesis of a second cDNA strand by either Escherichia coli DNA polymerase I (Pol I) or by reverse transcriptase. The mechanism by which these structures are generated, and the actual structure of the hairpin loops have not been studied in detail. Second strand synthesis using the Klenow fragment of Pol I, which lacks 5'-3' exonuclease activity, can also be used in succession with Pol I. This is done since either Pol I or reverse transcriptase may pause or stop at different sequences, so partially synthesized second strands produced by one enzyme may be completed by the other.

Once this has been completed the two cDNA strands are covalently joined by the hairpin loop. By using the

strand-specific nuclease S1, the loop may be cleaved yielding single stranded ends. The resulting end termini may be repaired with the Klenow fragment of E. coli DNA polymerase I, which increases the efficiency of cloning. The resulting duplex DNA is then either fractionated according to size (the largest molecules being inserted into the cloning vector of choice) or a wide range of various sizes of double stranded DNA is cloned to generate a cDNA library.

The most commonly used cloning vectors for cloning cDNA have been bacterial plasmids. The plasmids that have been used in cDNA cloning are, in general, genetically engineered derivatives of naturally occurring drug resistance plasmids. In general, these vectors are of low molecular weight, confer a selectable phenotype, and have single sites for a large number of restriction endonucleases which are preferably in genes with an easily scorable phenotype. The use of plasmid vectors becomes unadvantageous when the cDNA to be cloned is larger than 10 kb in length; with larger insert DNA the efficiency of transformation is decreased.

Two methods have been used to link the double stranded cDNA to the plasmid vector. One involves the addition of complementary homopolymer tails (Deng and Wu, 1981) to the double stranded cDNA and to the plasmid DNA,

which will then allow the two to join by hydrogen bonding between the two complementary homopolymeric tails. This yields open circular moleules, which are capable of transforming E. coli. A second method involves the addition of synthetic linkers to the ends of the double stranded cDNA. After digesting with the appropriate restriction enzyme, the cDNA molecules are inserted into plasmid DNA that has been cleaved with a compatible restriction enzyme.

The hybrid molecule is then transformed into Escherichia coli, the most commonly used host. One of the first plasmid vectors used, and one that is still commomly used, is pBR322 (Maniatis et al., 1982). This plasmid contains the ampicillin resistance and tetracycline resistance genes from plasmids RSF 2124 and pSC101 respectively, combined with the replication elements of pMB1, a Col E1-like plasmid. Double stranded cDNA is generally inserted into one of the two resistance genes and the other gene is used as the selectable marker for the plasmid. Transformed cells are first selected for those containing plasmid and then selection for plasmids containing insert DNA. The pUC series of plasmids (Vieira and Messing, 1982; Yannisch-Perron et al., 1985) have recently become popular cloning vectors. These plasmids contain the pBR322 ampicillin resistance gene, the pBR322

origin of replication, and a portion of the lac Z gene of E. coli. The lac Z gene contains a polylinker sequence of unique restriction endonuclease sites identical to those in the multiple cloning sites of the replicative form of bacteriophage M13. Double stranded cDNA is usually inserted into this multiple cloning site, resulting in an inactive (beta-galactosidase) gene product. The ampicillin resistance gene is used as the selectable marker for the plasmid.

To confirm that the cloned DNA is complementary to the RNA of interest, Southern blot analysis (Southern, 1975) is used. Restriction enzyme digestion of the recombinant plasmid is done to release the cloned DNA fragment and these fragments are separated by electrophoresis. The DNA is then transferred to nitrocellulose. Using single stranded, radiolabeled cDNA as a probe, hybridization of the probe to the cloned DNA fragment, under the proper stringency conditions, would indicate complementary nucleic acid sequences. Northern blot analysis can also be done by tranferring the RNA of interest to nitrocellulose, using the cloned DNA fragment as a probe. This is the classical scheme used to clone and analyze cDNA; many improvements have been made in recent years to make the whole cloning process more efficient.

A major criticism of the classic procedure focused on

the use of, when priming from the 3' polyadenylate tail for first strand synthesis, the hairpin loop as a primer for second strand synthesis and the use of S1 nuclease to destroy the hairpin. Second strand synthesis is at best a poorly controlled step (Okayama and Berg, 1982) that utilizes a fairly inefficient primer, the hairpin loop; the loop may be variable in both size and location (Efstratiadis et al, 1976). S1 nuclease digestion is a difficult reaction to control and can result in major loss of the 5' terminal sequences of the RNA template (Okayama and Berg, 1982; Gubler and Hoffman, 1983). Several methods have been developed to obviate the need for S1 nuclease digestion.

Land et al. (1981) increased the chances of cloning full length cDNA by tailing the first strand cDNA with deoxycytidine (dC) tails using the enzyme terminal deoxynucleotidyl transferase (TdT); second strand synthesis was then primed using oligodeoxyguanylate (oligo-dG). The resulting double stranded cDNA was once again tailed with dC tails and was annealed to the cloning vector, which had been cut with a restriction enzyme and tailed with complementary dG tails.

Another method for cDNA cloning developed by Okayama and Berg (1982) uses a plasmid vector which serves as the primer for both first and second strand cDNA synthesis.

Using rabbit reticulocyte mRNA, about 10 % of the 100,000 plasmid-cDNA recombinants obtained per microgram of mRNA contained a complete alpha or beta globin mRNA sequence, and at least 30 to 50 % contained the entire globin coding regions. Other features of this procedure are that the full length, or nearly full length, reverse transcripts are preferentially converted to duplex cDNAs. The nick translation repair of the cDNA:mRNA hybrid, mediated by E. coli RNase H, Pol I, and DNA ligase is used to synthesize the second cDNA strand.

Gubler and Hoffman (1983) developed a method whereby the classical first strand synthesis of cDNA was combined with the Okayama and Berg method of second strand synthesis. In this method the first strand cDNA is primed by the addition of oligo (dT) to the reverse transcription mixture; second trand synthesis is accomplished by nick translation repair of the cDNA:mRNA hybrid as performed in the Okayama and Berg procedure. Cloning efficiencies have been reported as being as high as 1 million recombinants per ug of mRNA. This method has been able to produce full length transcripts to bovine preproenkephalin mRNA, which is 1300 nucleotides long.

Heidecker and Messing (1983) have developed an efficient full length cDNA cloning procedure in which the 3' polyadenylated RNA is annealed to linearized and oligo

(dT) tailed plasmid vector DNA (which primes synthesis of the first cDNA strand). Oligo (dG) tails are then added to the cDNA-plasmid molecule, which are subsequently centrifuged through an alkaline sucrose gradient. Denatured, oligo (dC) tailed plasmid DNA is added (in excess) and conditions are adjusted to favor circulation by the complementary homoplymer tails. These circular molecules have a free 3' hydroxyl on the oligo (dC) tail which primes second strand synthesis using Pol I.

Applications of cDNA to Plant Virology

cDNA to plant viral RNA can have many possible applications: a) elucidation of genomic structure and gene expression; b) rapid diagnosis of viral disease; c) development of viral gene vectors; d) determination of taxonomic relationships between virus strains based on sequence homology.

The usefulness of cDNA in determining genomic structure and gene expression of the potato virus Y group has already been shown. These techniques have been used successfully with other plant virus groups, including the comovirus and bromovirus groups. Site-directed mutagenesis has been used by Bujarski et al. (1986) of cloned cDNA to brome mosaic virus (BMV) RNA for the

elucidation of the function of the 3' terminal tRNA-like sturcture of BMV RNA in viral replication and aminoacylation. These studies indicated both of these activities are regulated by this structure (in vivo and in vitro) and that the two activities act independently of each other.

There have been a number of reports on the use of nucleic acid probes for the detection of plant viruses and viroids (Owens and Diener, 1981; Maule et al., 1983; Baulcombe et al., 1984; Barker et al., 1985). In a recent report by Waterhouse et al. (1986) hybridization probes were developed to be used for general diagnosis of infection of barley yellow dwarf virus (BYDV). Because BYDV is an economically important RNA plant virus there is a need for a widely available, rapid, standardized, and sensitive assay. Cloned genome fragments from two serotypes of BYDV (RPV and PAV) were used as hybridization probes and were shown to have little or no homology between each other. The probes were shown to detect as little as 1 ng of virus in sap extracts, which was at least as sensitive as the results obtained in parallel ELISA tests using polyclonal antisera against RPV and PAV. The probes used were radioactively labeled and this may, in some instances, preclude its use as a routine diagnostic probe; however, with the advent of biotinylated DNA probes that are ap-

proaching the sensitivity of the radioactive probe (Chan et al., 1985) this problem will most likely be alleviated. These types of hybridization probes can also be used for identifying sequence homologies between viruses to determine taxonomic relationships, for use in crop or seed surveys, or for quarantine purposes. Baulcombe et al. (1984) has demonstrated that this type of assay could be used to screen large numbers of potatoes when indexing for potato virus X (PVX) in breeding programs.

Cloned cDNA to the entire genome of an RNA plant virus can be useful in the development of a gene vector for use by plant breeders for the construction of genetically modified plants. French et al. (1986) have done some basic work in this area. Using cDNA to the entire RNA genome of brome mosaic virus (BMV) (Ahlquist and Janda, 1984), a multicomponent virus, they removed from RNA 3 the coat protein gene and inserted the gene encoding for chloramphenicol transacetylase (CAT). Testing done with this engineered virus indicates expression of the CAT gene in barley protoplasts. The stability of an inserted gene within a RNA genome has been questioned (Van Vloten-Doting et al., 1985) because of the high mutation rate of RNA genomes (Holland et al., 1982), but field studies indicate stable phenotypic traits are faithfully inherited

(Siegel, 1985).

cDNA hybridization analyses have been used to investigate sequence homologies between the genomes of diverse viruses including cucumoviruses (Gonda and Symons, 1978), tobamoviruses (Palukaitis and Symons, 1980), and potyviruses (Abu-Samah and Randles, 1981). The data generated from these analyses have supported, with few exceptions, the taxonomic determinations made by other criteria such as serology. However, serology is based on the properties of the surface of the viral coat protein which represents only a small part of the information encoded by the viral genome (with the potyvirus group, about one-tenth of the coding capacity of the potyviral RNA is used to encode coat protein). Since the surface of the coat protein is the antigen, only a few amino acids are involved. Thus. less than 1 % of the genetic information of the virus is used in a serological assay. Nucleic acid hybridization analyses would greatly increase the precision of the taxonomic relationship between questionable members of a virus group by comparing larger amounts of the viral genome. Relationships between virus groups can also be determined. Ahlquist et al. (1985) have found sequence homology among non-structural proteins of tobacco mosaic virus, alfalfa mosaic virus, brome

mosaic virus, and Sinbis virus. There is the suggestion that these viruses may be evolutionarily related.

MATERIALS AND METHODS

Virus Propagation and Purification

Virus

Soybean mosaic virus (SMV) isolate Ia 75-16-1 was used in this study. The isolate was supplied by J. H. Hill (Iowa State University, Ames, IA).

Virus propagation

Glycine max (L.) Merr. cv. Williams was mechanically inoculated with SMV on the primary leaves, before the formation of trifoliate leaves. Inoculation was done by first dusting plants with carborundum (600 mesh); then the leaves of infected plants were ground, using a mortar and pestle, in about 10 ml of 0.01 M potassium phosphate buffer ,pH 7.0 (0.01 M potassium phosphate dibasic was titrated against 0.01 M potassium phosphate monobasic to pH 7.0). The volume was adjusted to about 15 milliliters (ml) with buffer and was rubbed gently onto leaves using the thumb and index finger. Infected plants were harvested about one month later.

Virus purification

Systemically infected plants were cut down and weighed (generally, about 2 kg of infected tissue was used

in a purification); the plant tissue was then ground, using a commercial Waring blender, in 0.5 M sodium-potassium phosphate buffer ,pH 7.6 (made by titrating 0.5 M sodium phosphate dibasic against 0.5 M potassium phosphate monobasic), containing 1% (v/v) mercaptoethanol. The product was then filtered thru two layers of cheesecloth; to the resulting liquid (foam, when present, was allowed to settle for about 20 minutes before continuing) a chloroform:butanol (1:1) mixture was added to 8 % (v/v) and stirred for 30 minutes. The mixture was then centrifuged in a Sorvall refrigerated centrifuge (7000 rpm for 20 minutes in a GSA rotor). All centrifugations in the purification procedure were done at 4 C in precooled rotors. The supernatant was collected by filtering thru glass wool and then the volume was measured. Polyethylene glycol (PEG, molecular weight = 6000, Fisher Scientific Co., Fair Lawn, NJ) was added to 6% (w/v) and sodium chloride was added to a concentration of 0.3 M; the mixture was stirred until the PEG had dissolved and then it was allowed to stand for 1 hour. This was then centrifuged at 8000 rpm for 20 minutes in a GSA rotor. The supernatant was discarded and the pellets were resuspended overnight by shaking at 4 C in urea buffer (0.05 M sodium-potassium phosphate, pH 7.6, 0.5 M deionized urea, 0.1% (v/v) mercaptoethanol).

For further clarification, the resuspended pellets were centrifuged at 5000 rpm for 10 minutes in a SS-34 rotor. The supernatant was removed and the pellet was extracted in urea buffer and centrifuged again. The supernatants were combined and were then centrifuged at 45,000 rpm for 1.25 hours in a Beckman type 45 ti rotor. The supernatant was discarded and the pellets were resuspended, using a glass Dounce homogenizer (Arthur H. Thomas Company, Philadelphia, PA), in urea buffer. This suspension was then centrifuged at 5000 rpm for 10 minutes in a SS-34 rotor with the pellet being resuspended in urea buffer, centrifuged again, and the supernatants from both centrifugations combined. The combined supernatant was layered onto 9 ml of 30% sucrose in urea buffer and centrifuged at 28,000 rpm for 3 hours in a Beckman type 30 rotor. The pellets were resuspended in urea buffer, using a glass homogenizer. Birefringence, which would suggest the presence of virus, was detected by shaking the resuspended virus solution between two crossed polarizing lenses and the light patterns were observed.

Two cycles of cesium chloride centrifugation were then performed for further purification in a Beckman type SW 50.1 rotor. Each centrifugation was at 42,000 rpm for 18 hours; following centrifugation, the virus band was removed from the gradient by puncturing the side of the

tube with a syringe followed by dialysis of purified virions was performed overnight at 4 C against 0.05 M borate buffer, pH 8.0. Virus concentration was estimated spectrophotometrically by using an extinction coefficient at 260 nm of 2.4 (Purcifull, 1966). Yields were approximately 25 mg per kg of infected tissue. Extracted virus was stored at 4 C.

Purification of Viral RNA

Preparation of reagents and materials

All reagents were prepared from reagent grade 1 water (distilled-deionized water). The water was obtained by passing distilled water through a Super-Q Ultra Pure water system (Millipore Corp., Bedford MA) containing activated charcoal, which will remove organic ions, and a mixed bed ion-exchange resin, for the removal of inorganic ions. The chemical reagents used were of the highest obtainable grade and, if available, were nuclease free.

All Eppendorf tubes (USA/Scientific Plastics Inc., Ocala, FL) and pipettemen tips (Rainin Instrument Co., Woburn, MA) used were autoclaved for 30 minutes at 121 C. Glassware used during the manipulation of ribonucleic acids were baked overnight at 200 C.

RNA purification protocol

The method used for purification of SMV RNA was a slight modification of the procedure used by Vance and Beachy (1984a). To 1 mg of purified SMV virions was added SDS (sodium dodecyl sulfate) to 1% and EDTA to 20 mM; this was then incubated at 65 C for 5 minutes. Protease (type XIV, Sigma Chemical Co., St. Louis, MO) was then added to 1 mg/ml, from a stock solution of 0.1 g/ml in 200 mM sodium phosphate ,pH 7.5 (made by titrating 200 mM sodium phosphate dibasic with 200 mM sodium phosphate monobasic), which had been predigested for 10 minutes at room temperature, and this mixture was incubated for 10 minutes at 37 C. The solution was then made 1.5 % with SDS and incubated at 55 C for an additional 10 minutes. NaCl was added to a concentration of 250 mM.

RNA was extracted with 2 volumes of redistilled phenol. Extractions were performed by vortexing mixtures until an emulsion formed, followed by centrifugation for 1 minute in an Eppendorf microfuge. The phenol phase was reextracted with 1 ml of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The aqueous phases were then extracted with 2 volumes of phenol:chloroform (1:1) followed by extraction with 2 volumes of chloroform. RNA was precipitated by adding 2 volumes of 95% ethanol and storing at -20 C for at least 30 minutes. Centrifugation of precipitated RNA

was for 30 minutes at 4 C in an Eppendorf microfuge. The resulting pellet was washed first in 70% ethanol and then dried using a Savant speed vac concentrator (Savant Instruments, Hicksville, NY); the pellet was then resuspended in sterile distilled-deioninzed water and stored at -20 C (RNA was found, in general, to be degraded by ribonucleases after about 7 days of storage following purification). RNA yields were determined by using an extinction coefficient of 25 and RNA purity was assessed by using a 260 nm/280 nm optical density ratio of 2.0. Yields of RNA isolated ranged from 50 to 99% (5 % of the SMV virion is RNA). The optical density ratios were generally around 1.7.

Oligo (dT)-cellulose chromatography

The procedure used for oligo (dT)-cellulose chromatography is essentially that described by Maniatis et al. (1982). Polyadenylated RNA was separated from nonpolyadenylated RNA using an oligo (dT)-cellulose column. The column was prepared in a sterile, siliconized Pasteur pipette with a glass wool plug at the bottom. One-hundred mg of oligo (dT)-cellulose (Sigma Chemical Co., St. Louis, MO) was equilibrated in sterile loading buffer (20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS). RNA in loading buffer was heated for 5 minutes

at 65 C, cooled to room temperature, and applied to the column. The to the column. The column was washed with 5-10 column volumes of loading buffer, followed by 3-4 column volumes of loading buffer containing 0.1 M NaCl. Each column volume was collected separately and 260 nm/280 nm optical density values were determined using a Uvicon 810 spectrophotometer (Kontron Electronics Inc., Redwood City, CA). To elute polyadenylated RNA, the column was washed with 3 column volumes of sterile 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.05% SDS.

Electrophoresis of RNA

The electrophoresis method used to determine integrity of the viral RNA is a slight modification of that described by McMaster and Carmichael (1977). RNA was denatured in freshly deionized 6 M Glyoxal, 50% dimethyl sulfoxide (DMSO) , 0.01 M sodium phosphate ,pH 7.0 (made by titrating 0.01 M sodium phosphate dibasic with 0.01 M sodium phosphate monobasic), for 1 hour at 50 C. The glyoxal was deionized by adding,and replacing, the ion-exchange resin Amberlite MB-3 (Sigma Chemical Co., St. Louis, MO) until the Amberlite did not change color from green to yellow, indicating deionization. Using a Hoefer model HE 99 submarine agarose gel unit (Hoefer Scientific Instruments, San Francisco, CA), a 1% horizontal agarose

gel (100 ml volume) was prepared in electrophoresis buffer (0.01 M sodium phosphate, pH 7.0); the agarose was autoclaved for 15 minutes before pouring the gel. A 1/5 volume of sterile loading buffer (50% glycerol, 0.01 M sodium phosphate, pH 7.0, 0.4% bromphenol blue) was added to the glyoxalated RNA; samples were loaded and electrophoresis was carried out at 4 volts/cm for 2 hours. The buffer was constantly recirculated during electrophoresis using a peristaltic pump; a neutral pH was required in order to maintain denatured RNA molecules.

Gels were stained with ethidium bromide (0.5 ug/ml in water) or acridine orange (30 ug/ml in water) for 30 minutes. The gel was photographed on a Fotodyne model 3-3000 UV transilluminator (Fotodyne Inc., New Berlin, WI) with Polaroid type 667 black and white film for 1 second at f 5.6, using a red filter. An alternative stain that was also used was Stains-all (Bio-Rad Ind., Richmond, CA).

Synthesis of cDNA Using M-MLV RT

M-MLV reverse transcriptase

The first method used to synthesize cDNA to SMV RNA was done by using Moloney-murine leukemia virus reverse transcriptase (M-MLV RT) (Bethesda Research Labs.,

Gaithersburg, MD).

M-MLV RT standard reaction conditions

The following reagents were dispensed into an autoclaved 1.5ml Eppendorf tube in an ice bath and in the following order:

1. Ten ul of 5X reverse transcription buffer (250 mM Tris-HCl, pH 7.5, 375 mM KCl, 50 mM dithiothreitol, Boehringer Mannheim Biochemicals [BMB], Indianapolis,IN, and 15 mM magnesium chloride) were added.

2. Twenty ul (1.0 ug) of SMV RNA in sterile distilled-deionized water were added.

3. Then, 2.5 ul of nucleotide solution [10 mM each of dATP (deoxyadenosine triphosphate), dCTP (deoxycytidine triphosphate), dGTP (deoxyguanosine triphosphate), and dTTP (deoxythymidine triphosphate) (Pharmacia Inc., Piscataway, NJ) in 10 mM Tris-HCl, pH 7.5] were added.

4. Five ul of oligo (dT)12-18 solution (100 ug/ml) (Pharmacia) were added.

5. Five ul of actinomycin D solution (500 ug/ml) (BMB) were added.

6. A volume representing 1.0 to 2.5 uCi of (alpha-32P) dATP (400 to 3,000 Ci/mmol) (Amersham Corp., Arlington Heights, IL) were added.

7. Sterile double-distilled water was added to 49

ul.

8. One ul of M-MLV RT (200 units) was added.

The total volume (50 ul) was mixed by gentle vortexing and the reaction mixture returned to the bottom of the tube by brief centrifugation. One ul was then immediately removed from the tube and placed on a glass filter fiber disc (Whatman 934-AH filter, Whatman Inc., Clifton, NJ). The tube was incubated for 1 hour at 37 C. After 1 hour, 10 ul of 0.2 M EDTA was added.

Analysis of First Strand cDNA Synthesis

TCA precipitation and liquid scintillation counting

Five ul of the reaction mixture were removed and put into an Eppendorf tube; the remaining portion of the reaction mixture was placed at -70 C. To the 5 ul sample 50 ul of yeast tRNA (1 mg/ml) were added, followed by the addition of 1 ml of 5 % trichloroacetic acid (TCA), 0.1 M sodium pyrophosphate. The mixture was inverted three times and then placed at 0 C for 1 hour. The mixture was then removed using a Pasteur pipette and placed onto a glass filter fibre disc (previously soaked in 5 % TCA, 0.1 M sodium pyrophosphate) which had been placed on a stainless steel precipitation apparatus (ICN Pharmaceuticals Inc., Irvine, CA). The tube was rinsed two times with 5 %

TCA, 0.1 M sodium pyrophosphate and the filter was washed three times with 5 ml of 5% TCA, 0.1 M sodium pyrophosphate. The filter was then removed with tweezers and placed into a liquid scintillation vial and 5 ml of liquid scintillation cocktail (2 parts toluene, 1 part triton X-100, 0.6 % PPO [2,5-diphenyloxazole], and 0.01 % POPOP [1,4 bis-{2-(5 phenyloxazolyl)}-benzene]) was added. The incorporation of radioactively labeled dATP was determined using a LKB Rack Beta liquid scintillation counter (LKB Wallac, Finland).

Neutral agarose electrophoresis

Using a Model H6 "Baby Gel", (BRL, Gaithersburg, MD) a 1% agarose gel was poured in TBE electrophoresis buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.5 M EDTA, pH 8.0). The electrophoresis buffer was made as a 5X stock solution as follows:

Tris base	54 g
boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20.0 ml
distilled-deionized water	980 ml

To cDNA samples (5000-10,000 cpm) to be analyzed, electrophoresis loading buffer (0.025 % bromophenol blue, 0.025 % xylene cyanol, 2.5 % Ficoll in dd-water) was added to 10 % (v/v) and the samples were then loaded onto the

gel. Hind III cut lambda DNA was used as a molecular weight standard. Electrophoresis was carried out at 1.8 volts/cm until the samples migrated into the gel; the voltage was then increased to 4.4 volts/cm for 1 to 2 hours (until the dye front reached the end of the gel). The lane containing the lambda molecular weight standards was cut away from the rest of the gel and stained separately in ethidium bromide (0.5 ug/ml in water) for 30 minutes.

Alkaline agarose electrophoresis

The procedure used was that described by Maniatis et al. (1982). Using the Model H6 "Baby Gel", a 1 % agarose gel was prepared in 50 mM NaCl, 1 mM EDTA. Once the gel was poured and solidified it was soaked in alkaline electrophoresis buffer (30 mM NaOH, 1 mM EDTA) for at least 30 minutes; the cDNA samples (5000-10,000 cpm) were loaded onto the gel with 10 % (v/v) alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2.5 % Ficoll, 0.025 % bromocresol green). Electrophoresis was carried out at 1.8 volts/cm until the samples entered the gel, then the voltage was increased to 3 volts/cm for 2 to 3 hours (until the dye front reached the end of the gel). Hind III digested lambda DNA was used as the molecular weight standard and the lane containing it was cut away from the

rest of the gel and stained with ethidium bromide.

Autoradiography

The portion of the gel from both neutral and alkaline agarose electrophoresis which contained radioactive samples were wrapped in Saran wrap; in a dark room the gel was taped to a sheet of XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) and placed into a Kodak X-ray exposure holder (Eastman Kodak). The holder was placed at -70 C for 12 to 24 hours. The film was developed in Kodak X-ray film developer for 4 minutes, transferred to a 3 % acetic acid stop bath for 30 seconds, and then transferred to Kodak rapid fixer for 3 minutes. The film was then placed in a cold running water bath for about 15 minutes.

Phenol and chloroform extractions

cDNA samples were extracted with equal volumes of redistilled phenol and chloroform. The chloroform was actually a 24:1 (v/v) mixture of chloroform and iso-amyl alcohol.

Ethanol precipitations

Ethanol precipitations were used not only to concentrate cDNA samples but were also used to separate unincorporated radioactive nucleotides from the cDNA. To the first strand synthesis mixture a 0.1 volume of sodium

acetate, pH 5.2, was added followed by the addition of 2 volumes of 95 % ethanol at room temperature. The tube was then gently mixed by inverting ten times and was then immediately centrifuged at 4 C in an Eppendorf microfuge for 30 minutes. The pellet was resuspended in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), sodium acetate was added to 0.1 volume, 2 volumes of 95 % ethanol at room temperature, and centrifuged again for 30 minutes at 4 C. The pellet was then washed with -20 C chilled 70 % ethanol and the pellet was then vacuum dried.

Amersham cDNA Synthesis Kit

Synthesis of double stranded cDNA was performed using the Amersham cDNA synthesis system (kit RPN.1256, Amersham Corp., Arlington Heights, IL). An instruction manual was provided with the kit and the directions were followed as prescribed.

AMV reverse transcriptase

Avian myeloblastosis virus reverse transcriptase (AMV RT) was the enzyme supplied with the kit.

Standard reaction conditions for cDNA synthesis

The following components provided with the kit were

dispensed into an autoclaved 1.5 ml Eppendorf tube in an ice bath and in the following order:

1. Four ul of 5x first strand synthesis reaction buffer (composition of buffer was proprietary information of Amersham Corp.) were added.

2. One ul of sodium pyrophosphate solution was added.

3. One ul of human placental ribonuclease inhibitor (25 units) was added.

4. Two ul of deoxynucleoside triphosphate mix (10 mM dATP, dGTP,and dTTP; 5 mM dCTP) were added.

5. One ul of Oligo dT (1 ug) were added.

6. Then, 0.5 ul of ^{32}P-dCTP (0.5 uCi) was added.

7. One ug of polyadenylated RNA was added.

8. Water (nuclease free) provided with kit was added to bring the final volume to 20 ul.

To this mixture, 1 ul (20 units) of AMV RT was added and the tube was incubated at 42 C for 1 hour. Following this incubation, the mixture was placed at 0 C. Two, 1 ul samples were removed for analysis of first strand synthesis by TCA precipitation and glyoxal denaturing gel electrophoresis using procedures described earlier.

To the first strand reaction mixture (containing cDNA/mRNA hybrids), the following components for second strand synthesis were added in the following order:

1. First, 37.5 ul of second strand synthesis buffer (composition of buffer was proprietary information of Amersham Corp.) were added.

2. Five ul of ^{32}P-dCTP (5 uCi) were added.

3. One ul of E. coli ribonuclease H (0.8 units) was added.

4. Then, 6.6 ul of E. coli DNA polymerase I (23 units) were added.

5. Water (nuclease free) provided with the kit was added to bring the final volume to 100 ul.

The mixture was incubated sequentially at 12 C for 1 hour (to partially hydrolyze the RNA) and 22 C for 1 hour (for second strand synthesis by DNA polymerase I using the nicked RNA as a primer; for total hydrolysis of the RNA template). Following these incubations the mixture was incubated at 70 C for 10 minutes to inactivate enzymatic activity. The tube was then placed on ice and 0.5 ul (2.0 units) of T4 DNA polymerase was added to remove any small 3' overhangs from the first strand cDNA; this was incubated at 37 C for 10 minutes. The reaction was terminated by the addition of 10 ul of 0.25 M EDTA, pH 8.0, and 10 ul of 10 % SDS. The double stranded cDNA was purified by phenol/chloroform extraction and ethanol precipitation as described earlier.

Homopolymer Tailing

Homopolymer tailing of double stranded cDNA

Double stranded cDNA synthesized with the Amersham kit was tailed with deoxycytidine triphosphate (dCTP) residues using terminal deoxynucleotydyl transferase (TdT) (Bethesda Research Laboratories) as follows in order:

1. The cDNA sample was resuspended in 20 ul of dd-water and placed in an ice bath.

2. Three ul of 10 mM deoxycytidine triphosphate (in sterile water) were added.

3. Six ul of 5X tailing buffer (500 mM potassium cacodylate, pH 7.2, 10 mM cobalt chloride, 1.0 mM dithiothreitol) were then added.

4. The tube was vortexed gently and centrifuged for 1 second to bring contents down to the bottom of the tube.

5. One ul of TdT (20 units) was added and the tube was then incubated at 37 C for 5 minutes.

6. The mixture was phenol/chloroform extracted once, then the cDNA was precipitated as described earlier for single stranded cDNA.

7. The tailed, double stranded cDNA was resuspended in 50 ul of annealing buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA). This was stored at -20 C until an-

nealed to the plasmid vector.

Homopolymer tailing of pUC 9

Plasmid pUC 9 was first digested with the restriction enzyme Hinc II (BRL) as follows. To 1 ug of pUC 9 in 10 ul of TE buffer, 1.2 ul of 10X restriction enzyme buffer (100 mM Tris-HCl, pH 8.0, 600 mM NaCl, 10 mM magnesium chloride, 10 mM dithiothreitol) and 2 ul (20 units) of restriction enzyme were added; incubation was at 37 C for 1 hour. The mixture was extracted once with an equal volume of phenol/chloroform (1:1). The DNA was precipitated once with 95 % ethanol, and pelleted as described earlier. The DNA pellet was resuspended in 10 ul of sterile distilled-deionized water. Confirmation of digested plasmid was done by loading a 1 ul sample onto a 1 % neutral agarose gel along with undigested pUC 9 as a control followed by electrophoresis as described earlier for neutral agarose gel electrophoresis. Once digestion was confirmed, a 3 ul sample was removed (about 300 ng of cut plasmid) and diluted to 20 ul with sterile dd-water. The procedure used for homopolymer tailing of Hinc II digested pUC 9 was the same used for tailing of the double stranded cDNA except that deoxyguanidine triphosphate (dGTP) was used.

Transformation of E. coli 71-18 with Recombinant pUC 9

Annealing of dC-tailed cDNA with dG-tailed pUC9

dG-tailed pUC 9 was added to the tailed cDNA to give a volume of 100 ul and was heated to 65 C for five minutes. The DNAs were allowed to anneal by incubating at 57 C for 1 to 2 hours. The reannealed DNAs were placed in and ice or placed at -20 C until ready for transformation.

Maintenance of E. coli 71-18

E. coli K-12 (strain 71-18) was maintained on M9 minimal salts agar (Maniatis et al., 1982). Agar plates were made as as described by Maniatis et al. (1982). Plates were kept at 4 C and fresh plates were inoculated monthly with old plates being discarded.

Transformation procedure

The transformation procedure used was performed as follows:

1. 1 ml of a 5 ml overnight culture of E.coli 71-18 grown in L broth (Maniatis et al., 1982) was transferred into a 250 ml side arm flask containing 100 ml of L broth. The cells were grown with shaking to an optical density at 600 nm of 0.4.

2. The cells were cooled on ice for 20 minutes. The cells were then pelleted by centrifuging in a Sorvall rerigerated centrifuge for 5 minutes in a SS-34 rotor at 5000 rpm at 4 C.

3. The cells were resuspended in 50 ml of ice cold 0.1 M magnesium chloride and repelleted immediately as before. The cell pellet was resuspended in 5 ml of ice cold 0.1 M calcium chloride.

4. The cells were left on ice for 30 minutes. The cells at this point gained competence for transformation.

5. Then, 0.2 ml of competent cells was added to annealed pUC 9-cDNA insert and was left on ice for 30 minutes. Competent cells were added to pUC 9 as a positive control; competent cells alone were used as a negative control.

6. The suspension was heat shocked at 42 C for 2 minutes then immediately returned to ice for a further 30 minutes.

7. One ml of L broth was added and the cells were then incubated at 37 C for 1 hour before plating onto tranformation plates, so that the ampicillin resistance gene on the transforming DNA had time to be expressed.

8. Then, 0.1 ml aliquots were plated onto L agar plates (Maniatis et al., 1982) containing 50 ug/ml each of ampicillin and MUG-gal (4-methylumbelliferyl-β-D-

galactoside).

9. The plates were incubated overnight at 37 C.

10. The next morning plates were inspected for ampicillin resistant colonies that did not produce a functional beta-galactosidase, which is encoded by the lac Z gene contained within pUC 9. The Hinc II restriction site is in a multiple cloning site that is within the lac Z gene of pUC 9. A non-functional beta-galactosidase was determined by inspecting plates with a Fotodyne model 3-3000 UV transilluminator; colonies that did not "glow" (i.e., did not utilize the galactose analog MUG-gal) were indicative of cells containing recombinant plasmid. An active beta-galactosidase enzymatically cleaves the MUG-gal molecule producing MUG which fluoresces when exposed to ultraviolet light. This, in general, indicates that no DNA was inserted into the multiple cloning site within the lac Z gene.

Analysis of Cloned cDNA

Rapid small scale isolation of plasmid DNA

Five ml of L broth containing 50 ug/ml each of ampicillin and MUG-gal were inoculated with one bacterial colony and incubated overnight at 37 C. The plasmid isolation procedure and the restriction enzyme digestions

were performed as described in the alkaline lysis method of Maniatis et al. (1982). The restriction enzymes used to cut plasmids were Eco RI, Hind III, and Bam HI. Neutral agarose gel electrophoresis was performed as previously stated, with the exception that 1.2 % agarose gels were used.

Southern Blot Analysis of Cloned cDNA

To confirm that the DNA that was cloned was complementary to the RNA template used in reverse transcription, Southern blot analysis (Jones, 1985) was performed.

Southern transfer of DNA to nitrocellulose

1. The ethidium bromide stained gel was transferred to a glass dish and 500 ml of denaturing solution (1.5 M NaCl, 0.5 M NaOH) were added. This was incubated with constant agitation for 1 hour at room temperature.

2. The denaturing solution was removed and replaced with 500 ml of neutralizing buffer (1 M Tris-HCl, pH 8.0). This was agitated, as in step 1, for 1 hour. The buffer was discarded and step 2 was repeated once.

3. During the second neutralization a clean glass tray with a glass plate bridging the sides was prepared. Three pieces of Whatman 3MM paper were cut so that they

were wider than the agarose gel and long enough to hang over the sides of the glass plate and serve as wicks. The wicks were saturated in 10X SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and placed on the glass plate so that both ends were well submerged in 10X SSC in the glass tray. A piece of nitrocellulose was cut out to the exact size of the agarose gel and hydrated in 10X SSC. All manipulations with nitrocellulose were performed with vinyl gloves on at all times.

4. After the second neutralization wash, the agarose gel was placed bottom side up on the wicks and any air bubbles between the gel and the wick were removed. The nitrocellulose sheet was then placed on top of the agarose and any bubbles between the gel and the nitrocellulose were removed. Four pieces of Whatman 3MM paper were cut out to the exact size of the gel, moistened with 10X SSC, and placed on top of the nitrocellulose; any air bubbles were removed. Microscope slides were the placed around the edges of the agarose so that no contact could occur between the top and bottom layers of Whatman paper (so that all buffer absorption occurs through the agarose). A three inch thick layer of brown paper towels (cut to the exact size of the gel) was placed on top of the Whatman paper. A 1 kg weight was then placed on top of the paper towels. The transfer was allowed to occur for at least 18

hours.

5. After the transfer was complete, the stack was disassembled and the position of the wells were marked using a soft lead pencil. The nitrocellulose was then turned over and the upper right-hand corner was trimmed off to provide orientation when the filter was developed. The membrane was then sandwiched between two pieces of clean Whatman 3MM paper and allowed to air dry. The membrane was then baked at 80 C for 2 hours in a vacuum oven.

Reagents for prehybridization and hybridization of Southern blot

Deionized formamide About 2.5 g of amberlite MB-3 (Sigma) were added to 50 ml of formamide and stirred for 30 minutes at room temperature. The resin was removed with a Buchner funnel. This was prepared and used on the same day.

Salmon sperm DNA DNA was added to dd-water to a concentration of 1 mg/ml. This solution was then forced back and forth thru an 18-gauge syringe needle 10 times. The solution was then boiled in a boiling water bath for 15 minutes and immediately chilled on ice until used. This was boiled the day it was used.

50X Denhardt's solution To 100 ml of double-

distilled water, 1 g of each of the following chemicals was added: bovine serum albumin (fraction V) (Sigma), polyvinylpyrollidone (Sigma), and Ficoll (400,000 dalton molecular weight) (Pharmacia).

Prehybridization of Southern blot

The membrane was placed into a Seal-A-Meal bag and add the following was added to the prehybridization mixture: 4.5 ml of deionized formamide, 3.5 ml of salmon sperm DNA, 1.0 ml of 50X Denhardt's solution, 0.5 ml of 20X SSC (3 M NaCl, 0.3 M sodium citrate), and 0.5 ml of 0.5 M sodium phosphate buffer (pH 7.0). The bag was sealed and prehybridized with constant agitation at 42 C for 4 hours.

Hybridization of Southern blot

The probe used to determine whether the cloned DNA was complementary to the template RNA was the first strand cDNA synthesized using the Amersham cDNA synthesis kit. This cDNA was phenol:chloroform extracted and ethanol precipitated as described earlier and was then resuspended in sterile dd-water. The cDNA was fully denatured by boiling in a boiling water bath for fifteen minutes and immediately placed at 0 C. When cooled it was placed into the hybridization mixture, which consisted of the same

components in the prehybridization mixture plus the probe. After prehybridization, the prehybridization mixture was replaced with the hybridization mixture, the bag was sealed again, and was incubated at 42 C with constant agitation for 24 hours.

Post hybridization washes

The following post hybridization washes were performed:

1. The membrane was washed in 250 ml of 2X SSC, 0.1 % SDS for 2 minutes at room temperature. This step was repeated once.

2. The membrane was then washed in 250 ml of 0.2X SSC, 0.1 % SDS for 2 minutes at room temperature. This wash step was repeated once.

3. The membrane was finally washed in 250 ml of 0.16X SSC, 0.1 % SDS for 15 minutes at 50 C. This step was repeated once.

4. The membrane was rinsed briefly in 2X SSC, 0.1 % SDS at room temperature. The membrane was then placed on a clean piece of Whatman 3MM paper and allowed to air dry.

5. The membrane and paper was wrapped in Saran wrap and placed against a sheet of Kodak XAR-5 X-ray film.

This was placed at -70 C for 24 hours. The autoradiogram was developed as previously described.

RESULTS

Purification of Soybean Mosaic Virus

The yield of SMV from 2 kg of infected plants was 20 to 50 mg. The yields varied considerably, depending upon the time of year that purification was performed. Virus yields were comparable to those reported (Purcifull, 1966).

Purification of RNA

Attempts to obtain SMV RNA by the ammonium carbonate procedure (Brakke and Van Pelt, 1970b) were successful, but the RNA was shown to be degraded by nucleases as revealed by polyacrylamide gel electrophoresis (Hill and Benner, 1976). Consequently, different procedures of purification were performed: (1) ammonium carbonate disruption of virions followed by separation of RNA from viral coat protein using a NACS-52 Prepac column (Bethesda Research Laboratories, Gaithersburg, MD); (2) ammonium carbonate disruption followed by phenol/chloroform extration (Maniatis et al., 1982); (3) SDS/EDTA (1% SDS, 0.001 M EDTA) disruption followed immediately by phenol/chloroform extraction; (4) SDS/EDTA disruption followed by oligo (dT)-cellulose chromatography; (5) the

procedure Vance and Beachy (1984a). The first four procedures again led to RNA that was degraded and yields that were, in general, rather poor (i.e., under 20 %). The Vance and Beachy procedure yielded relatively undegraded RNA as determined by glyoxal denatured electrophoresis (Figure 1) with excellent yields (i.e., 50 to 99%).

Oligo (dT)-Cellulose Chromatography

Oligo (dT)-cellulose chromatography was a required step in the synthesis of cDNA to SMV RNA because previous evidence has indicated (Vance and Beachy, 1984b; Hari et al., 1979) that viruses in the PVY group of plant viruses possess RNAs that are both polyadenylated and non-polyadenylated when isolated from virions. The polyadenylated tail assured that degradation of the 3' end was not significant and also allowed for the use of oligo (dT) as a primer.

Results obtained from the application of SMV RNA onto the oligo (dT)-column, followed by washes of the column with buffers containing lower and no salt indicated the majority (over 50 %) of the RNA loaded onto the column was polyadenylated (Figure 2). A portion of the polyadenylated RNA was denatured with glyoxal and integrity of the RNA determined by electrophoresis in 1 % agarose. The

remaining portion was used in reverse transcription reactions.

The positive control used in determining the effectiveness of binding of polyadenylated RNA to the column was rabbit globin mRNA; negative controls used were E.coli rRNAs (Noller, 1984) and tobacco mosaic virus RNA (Goelet et al., 1982). Rabbit globin mRNA eluted off the column with the low salt wash indicating that the binding was not as great as that of polyadenylated SMV RNA. Both the negative controls were washed off the column with only loading buffer, indicating no binding to the column.

Synthesis of First Strand cDNA

The reverse transcription mixture used for first strand synthesis was made as described for use with the M-MLV RT and no attempt was made to alter the concentration of any of the components as recommemded by Bethesda Reasearch Laboratories [BRL], Gaithersburg, MD (Gary Gerard, personal communication). The positive control used for reverse transcription was a RNA ladder (BRL), which contained six polyadenylated RNAs ranging from 9.5 kb to 0.3 kb. Results from TCA precipitations indicated the presence of cDNA synthesis compared to the negative controls.

Determination of the length of the first strand cDNA was initially determined using neutral agarose gel electrophoresis (Figure 3). These results indicated apparent full length, or close to full length, cDNA to the RNA ladder except for the 0.3 kb RNA. Three major bands of cDNA to SMV RNA were synthesized: 0.5 kb, 1.4 kb, and 3.0 kb.

Confirmation of the size of cDNAs synthesized was made using alkaline agarose electrophoresis (Maniatis et al., 1982); however, results obtained indicated that only small cDNA fragments were being synthesized. Several attempts were made to determine whether the results obtained from this procedure were valid, all of which indicated synthesis of small cDNA fragments. Experiments were also done in which the RNA (RNAs of the RNA ladder in the cDNA:RNA hybrid were first hydrolyzed with 20 mM NaOH for various lengths of time (0,5,10, and 20 minutes) at temperatures of 25 C and 60 C. When these samples were loaded onto neutral agarose gels and electrophoresis was applied, the resulting autoradiograms indicated the same results at both temperatures and at all times except for 0 minutes. Various concentrations of NaOH were used (10 mM to 150 mM) at both of the previous temperatures, once again indicating synthesis of small cDNA fragments.

Synthesis of cDNA Using the Amersham Kit

Using non-denatured, polyadenylated SMV RNA first and second strand cDNAs were synthesized. TCA preciptation of cDNA products indicated synthesis of single and double stranded cDNA. The determination of the size of cDNAs synthesized was made using a 1 % glyoxal denaturing agarose gel. An autoradiogram of the first and second strand synthesis products is shown in Figure 4. The indicated length of the cDNA ranged from 500 to 700 bases. In order to increase the length of the cDNA synthesized, the RNA was treated in two ways: (1) SMV RNA was heated to 65 C for 5 minutes and quickly chilled on ice; then the RNA was added to the first strand synthesis mixture; (2) In a second experiment the RNA was added to the first strand synthesis mixture in the presence of 10 % DMSO. Results from the heat treated RNA increased the length of the cDNA synthesized from 500 to 700 bases, to a range of 600 to 2000 bases (Figure 5); the DMSO treated RNA yielded cDNA in a range from 400 to 600 bases (Figure 5).

Cloning of cDNA in *E. coli*

Transformation of the cDNA synthesized using SMV RNA

that was not denatured prior to reverse transcription resulted in frequencies of about 22 cfu (colony forming units) per ml of competent cells. Twelve colonies were selected for further analysis; six of these colonies were producing an active beta-galactosidase (lac Z +) and six colonies were not producing an active beta-galactosidase (lac Z -). Two of the lac Z + colonies contained cloned inserts as determined by rapid plasmid screens; all six of the lac Z - transformants appeared to contain insert DNA. One colony from each was analyzed further by restriction enzyme digestions and both contained an insert DNA of about 710 bases that was released by the double digestion of Eco RI and Hind III (Figure 6).

Transformation of the DMSO treated SMV RNAs resulted in transformation frequencies of about 20 cfu per ml of competent cells. Plasmid screens of 12 colonies (all active beta-galactosidase producers) from the untreated RNA showed only 3 of the 12 containing insert DNA. Restriction analysis of the three plasmids with inserts showed an insert DNA of about 500 bases. Transformation of the cDNA produced from heat treating the SMV RNA prior to reverse transcription yielded no transformants; this was most likely due to inefficient tailing of the cDNA and the plasmid vector by TdT.

Southern Blot Analysis of cDNA Clones

The cDNA clones analyzed were those synthesized to SMV RNA that was not denatured prior to reverse transcription. The DNA fragments shown in Figure 6 were transferred to nitrocellulose and were hybridized with a first strand cDNA probe. The Southern blot autoradiogram is shown in Figure 7. The autoradiogram confirms that the cloned DNA fragment is complementary to SMV RNA by the specific hybridization to the probe.

Figure 1. Electrophoresis of RNA thru a 1 % glyoxal denaturing agarose gel RNA was glyoxalated 1 h at 50 C prior to electrophoresis. Electrophoresis was at 4 V/cm for 2 h in 0.01 M sodium phosphate, pH 7.0. The buffer was constantly recirculated with a peristaltic pump. The gel was stained with ethidium bromide (0.5 ug/ml). Lane 1, SMV RNA (10 ug); lane 2, tobacco mosaic virus RNA (10 ug).


1
2

Figure 2. Fractionation of polyadenylated SMV RNA and non-polyadenylated SMV RNA by oligo (dT)-cellulose chromatography RNA samples were loaded onto the column in loading buffer (20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1 % SDS). The column volume was 1 ml; 1ml fractions were collected. The absorbance of each fraction was measured at 260 nm. Peak A, material not retained by the column; peak B, RNA eluted with lower ionic strength buffer (20 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 1 mM EDTA, 0.1 % SDS; peak C, RNA eluted with low ionic strength buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1 % SDS).

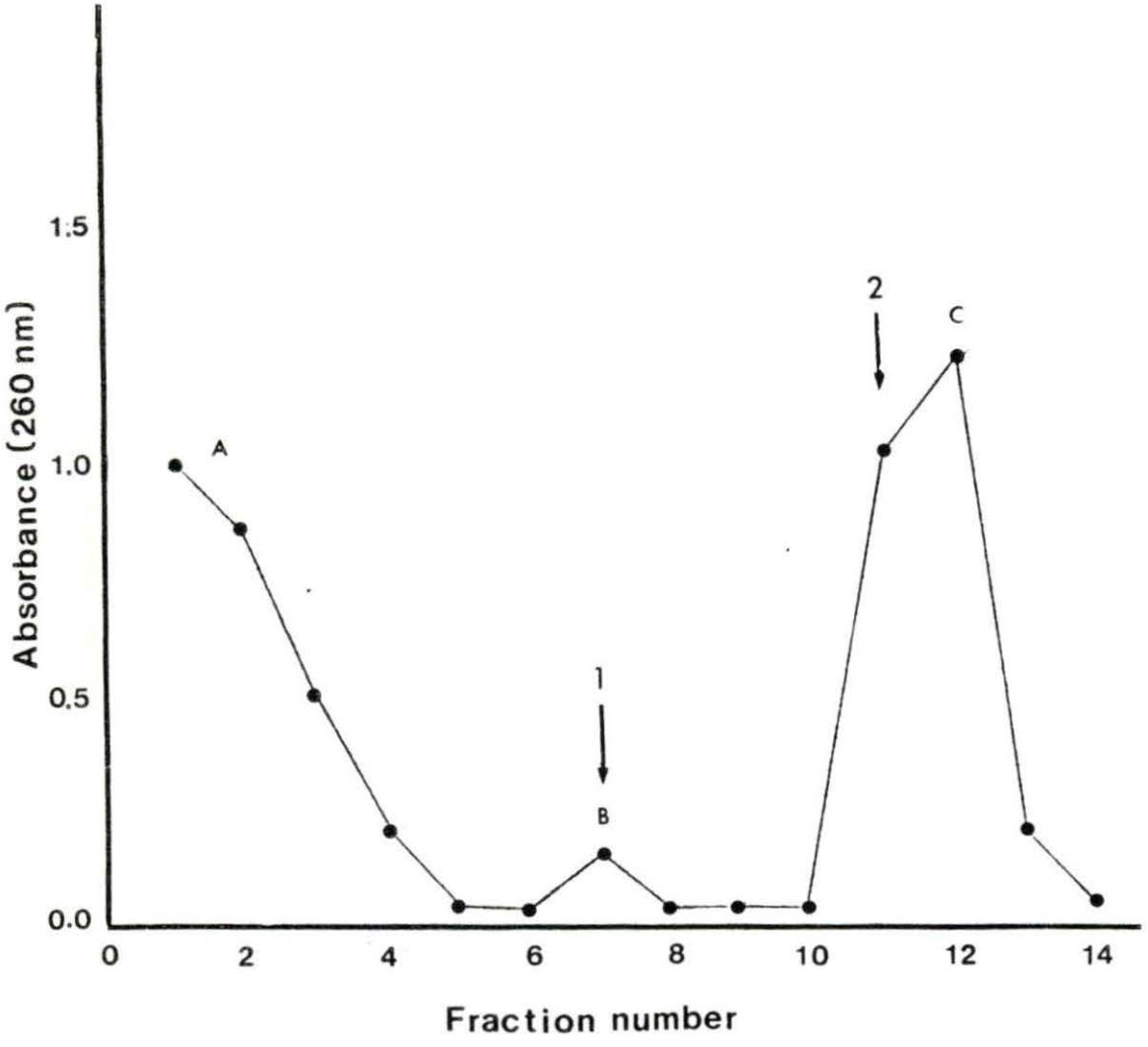
Absorbance (260 nm)
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
Fraction number
A
B
C
1
2

Figure 3. Autoradiogram of first strand ^{32}P-cDNA cDNA products were synthesized with Moloney-murine leukemia virus reverse transcriptase followed by electrophoresis thru a 1 % neutral agarose gel. Electrophoresis of samples (5000-10,000 cpm) was at 4.4 V/cm for 2 h in 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0. Lane 1, cDNA to RNA ladder; lane 2, cDNA to SMV RNA. Lengths of cDNA synthesized to the RNA ladder are indicated in kilobases.

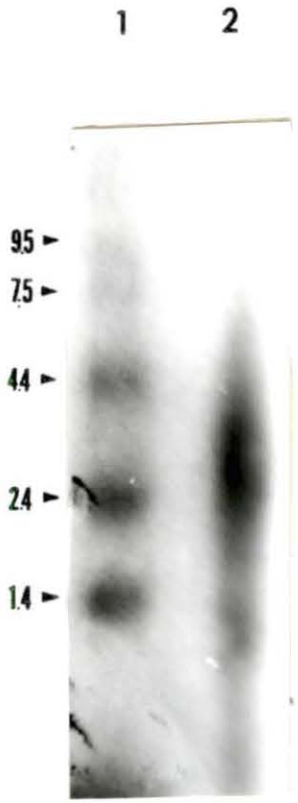
1
2
95
75
44
24
14

Figure 4. Autoradiogram of first strand ^{32}P-cDNA cDNA products were synthesized with Avian myleloblastosis virus reverse transcriptase (Amersham cDNA synthesis kit) followed by electrophoresis thru a 1 % glyoxal denaturing agarose gel. cDNA samples (5000-10,000 cpm) were glyoxalated for 1 h at 50 C prior to electrophoresis. Electrophoresis was at 4 V/cm for 2 h in 0.01 M sodium phosphate, pH 7.0. The buffer was constantly recirculated using a peristaltic pump. Hind III digested lambda DNA was used as a molecular size marker; this lane was separated from the remainder of the agarose gel and was stained with ethidium bromide. Lane 1, first strand cDNA to SMV RNA that was not denatured prior to reverse transcription.

L

Figure 5. Autoradiogram of first strand ^{32}P-cDNA cDNA products were synthesized with Avian myeloblastosis virus reverse transcriptase (Amersham cDNA synthesis kit) followed by electrophoresis thru a 1 % glyoxal denaturing agarose gel. cDNA samples (5000-10,000 cpm) were glyoxalated for 1 h at 50 C prior to electrophoresis. Electrophoresis was at 4 V/cm for 2 h in 0.01 M sodium phosphate, pH 7.0. The buffer was constantly recirculated using a peristaltic pump. Hind III digested lambda DNA was used as a molecular size marker; this lane was separated from the remainder of the agarose gel and was stained with ethidium bromide. Lane 1, first strand cDNA to SMV RNA heat denatured at 65 C for 5 m prior to reverse transcription; lane 2, double stranded cDNA to heat denatured SMV RNA; lane 3, first strand cDNA to SMV RNA denatured by the addition of DMSO to 10 % in the reverse transcription mixture; lane 4, double stranded cDNA to SMV RNA denatured with DMSO.


1
2
3
4

Figure 6. Electrophoresis of recombinant pUC 9 containing cloned cDNA to SMV RNA (pSMV-1) in a 1.2 % agarose gel Digestion of recombinant plasmid DNA (1 ug) for each sample was for 1 h at 37 C. Electrophoresis was at 4.4 V/cm for 3 h in 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0. Following electrophoresis, the agarose gel was stained with ethidium bromide. Lane 1, Hae III digested ϕX174 replicative form DNA; lane 2, undigested pSMV-1; lane 3, Eco RI/Hind III digested pSMV-1; lane 4, Eco RI digested pSMV-1; lane 5, Hind III digested pSMV-1; lane 6, Hind III digested lambda DNA. The lengths of the DNA size standards (lanes 1 and 6) are indicated in kilobases. The cloned cDNA was released from pUC 9 by the double digestion of pSMV-1 with Eco RI and Hind III.

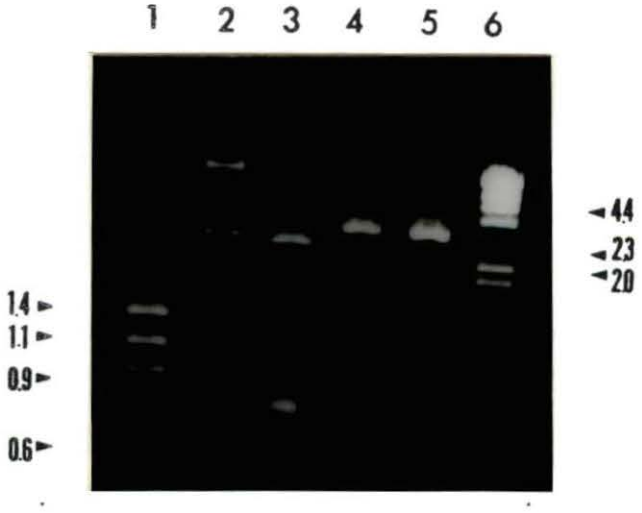
1
2
3
4
5
6
4.4
2.3
2.0
1.4
1.1
0.9
0.6

Figure 7. Southern blot analysis of pSMV-1 pSMV-1 was digested with restriction endonucleases and the DNA fragments separated by electrophoresis (Figure 6). The DNA in the gel was transferred to nitrocellulose and hybridized with a ^{32}P-labeled first strand cDNA to SMV RNA probe. Shown is the Southern blot autoradiogram. Lane 1, Hae III digested ϕX174 replicative form DNA; lane 2, undigested pSMV-1; lane 3, Eco RI/Hind III digested pSMV-1; lane 4, Eco RI digested pSMV-1; lane 5, Hind III digested pSMV-1; lane 6, Hind III digested lambda DNA.

1 2 3 4 5 6

DISCUSSION

The virus yields obtained from purification were comparable to those obtained by other investigators (J. H. Hill, Dept. of Plant Pathology, Seed and Weed Sciences, Iowa State University, personal communication). Potyviruses as a group, in general, attain a rather low titer in infected plant tissue and thus can make the purification process a very difficult task. Since only about 5 % of the virion is RNA (Hill and Benner, 1980b), the degree of difficulty in isolating intact viral RNA is increased.

Initial attempts to isolate intact, undegraded viral RNA were unsuccessful using the ammonium carbonate method of Brakke and Van Pelt (1970b) followed by separation of the coat protein molecules from the genomic RNA using linear-log sucrose gradients (Brakke and Van Pelt, 1970a). These results were puzzling at first because this procedure has been shown to work with other potyviruses (Hill and Benner, 1976) as well as with soybean mosaic virus (Hill and Benner, 1980b). One factor that is often overlooked in RNA isolation procedures is the amount of ribonucleases associated with any one investigator. It is a general belief that certain individuals possess more nucleases on their hands and body than others. One of the

first steps that was taken into account when critically evaluating the isolation procedure was this point. It was concluded that to diminish the possibility of nuclease contamination that vinyl gloves be worn during all RNA manipulations. Secondly, after evaluating the isolation procedure, it was concluded that this procedure would not be best suited for the needs of the planned research. In this procedure it was necessary to use about 50 to 60 mg of virus per isolation which would theoretically yield approximately 2.5 to 3.0 mg of RNA. It is a general observation that isolated RNA will remain undegraded, in even the most homogenoeous preparations, only about two weeks. Since the amount of RNA needed for one reverse transcription reaction is only 1 ug, this isolation procedure would obviously be an inefficient use of the viral RNA being isolated. The part of the isolation procedure that was very inefficient was the linear-log sucrose gradients because a large amount of degraded virus was required in order to attain a large enough yield of RNA to be detected in a density gradient tube. Another inefficient aspect of the isolation procedure were the poor yields obtained, generally around 30 % (J. H. Hill, Dept. of Plant Pathology, Seed and Weed Sciences, Iowa St. Univ., personal communication). At this point new procedures were tried to yield undegraded, intact viral RNA and

would give higher overall yields of RNA from lower starting quantities of purified virions.

In collaboration with P. J. Berger (Dept. of Microbiology, Iowa State University, Ames, IA), various other methods for the separation of coat protein from viral RNA were tried using the ammonium carbonate procedure for degrading the virion; these included the use of the NACS-52 Prepac column and phenol/chloroform extraction. Neither of these methods resulted in satisfactory results and in general were inefficient in the isolation of undegraded RNA as determined by glyoxal denaturing agarose gel electrophoresis. SDS/EDTA disruption of virions followed by phenol extraction resulted in slightly higher yields of RNA (about 20 %) as determined spectrophotometrically, but the RNA appeared to be degraded following electrophoresis. The method that finally gave good results was a procedure based essentially on the Vance and Beachy (1984a) procedure for isolating SMV RNA. The main feature of this procedure was the use of a protease during the isolation; with this one exception the procedure is basically equivalent to the SDS/EDTA procedure. The function of the protease in the isolation procedure was to degrade the coat protein molecules; this was important since during SDS disruption of the virions there may be a fairly high proportion of

coat protein subunits still attached to the RNA molecule. When this mixture was phenol/chloroform extracted, a large proportion of the RNA may have been carried into the non-aqueous phase along with the coat protein. The inclusion of the protease and the subsequent higher yields suggests this was the case. Preparations, after phenol/chloroform extraction and following ethanol precipitation, probably contained some proteinaceous material along with the isolated RNA; 260 nm/280 nm optical density ratios of an average preparation were about 1.7 where 2.0 indicates pure RNA. No attempts were made to characterize these proteins.

The resulting RNA isolation procedure had several desireable characteristics. One was that it was rapid; RNA could be isolated from intact virions in about 2 hours compared to the ammonium carbonate degradation/linear-log sucrose gradient centrifugation procedure which required about 2 days. Secondly, the percent yields obtained from this procedure allowed the use of very small quantities of purified virus to be degraded at one time (1 mg as compared to 50 mg). Since only a small quantity of virus was used in the degradation and phenol/chloroform extraction was used to separate nucleic acid from protein, allowed all mixtures to be contained in Eppendorf tubes. This was a very important variable when trying to eliminate the

introduction of nucleases into any of the working reagents or solutions.

The application of these RNA preparations to an oligo (dT)-cellulose column resulted in the fractionation of the RNA into two species: polyadenylated and non-polyadenylated RNA. This result has been observed previously with SMV (Vance and Beachy, 1984b) and with tobacco etch virus (Hari et al., 1979). The function or true existence of the non-polyadenylated RNAs within infected cells has not been determined, but it has been shown that non-polyadenylated tobacco etch RNA was infectious (Hari et al., 1979). The polyadenylated fractions that were eluted off the column were determined spectrophotometrically to be essentially free of any contaminating proteins. In some RNA preparations contaminating proteins were present and were being eluted off the column with changes in salt concentration; however, these proteins eluted off in different fractions than did the RNA.

The objective of this research was to synthesize and clone full length complementary DNA (cDNA) to soybean mosaic virus RNA. The choice of using Moloney murine leukemia virus reverse transcriptase was based on evidence (Gerard, 1985) that compared the efficiency of first strand synthesis between this enzyme and avian myeloblas-

tosis virus reverse transcriptase (AMV RT). The percent yields obtained with M-MLV RT were greater than that of AMV RT using rabbit globin mRNA and AMV 35S RNA. This cloned M-MLV RT is missing the RNase H activity that is associated with the AMV RT that has been implicated in lowering the yield and length of first strand cDNA (Gerard, 1985).

Initial analysis of this system with SMV RNA, and the RNA ladder indicated that full length cDNA was being synthesized for each of these RNAs. The basis of these conclusions were TCA precipitations and electrophoresis of first strand cDNA products thru a 1 % neutral agarose gel. The standard method of electrophoretic analysis of cDNA synthesis products is electrophoresis thru alkaline agarose gels; when this technique was used, the results indicated that the cDNA synthesized for each were very short fragments, probably less than 50 bases in length. Several experiments using this technique yielded similar results. The results from liquid scintillation analysis of these same first strand cDNA products, however, still indicated a significant amount of incorporation of radioactively labeled nucleotides. Attempts to make these cDNAs double stranded and the subsequent cloning into E. coli were unsuccessful.

Examination of Figure 3 would, considering the

results from alkaline agarose gel electrophoresis, indicate the bands seen are fragments of radioactively labeled cDNA still attached to their RNA templates. This appears to be the case with the RNA ladder cDNAs but does not totally explain the banding pattern seen in the SMV lane. It could be argued that the bands are the result of RNA that has been degraded into three specific sizes; this specific type of degradation, however, was never seen during the many RNA isolation procedures tried. SMV RNA that had been degraded by nucleases appeared in glyoxal denatured, agarose gels as a "smear" of a whole spectrum of various sized RNAs rather than a finite set of degraded RNA bands.

This system needs to be analyzed in greater detail than has been presented; there are still basic experiments that need to be performed before any conclusions can be made. If indeed the cDNA that was being synthesized by this system was short fragments, regardless of the RNA template, then this suggests a major problem with the enzyme functioning properly. An explanation for this occurring would be the cations required by the enzyme to function properly are at such non-optimal concentrations that the enzyme is crippled. Another question that needs to be addressed is whether alkaline agarose electrophoresis is the best system to analyze first strand

cDNA synthesis; glyoxal denaturing gels can also be used and it would be interesting to see these same results with another denaturing system.

The banding pattern of SMV cDNA seen in Figure 3 has been observed with tobacco mosaic virus (Meshi et al., 1982) and also with SMV (Roger N. Beachy, Dept. of Biology, Washington Univ, St. Louis, MO, personal communication). Two explanations given for the synthesis of several discrete cDNAs were: (1) there was a large amount of secondary structure in the RNA at these regions resulting in premature termination of transcription; (2) the reverse transcriptase was reading a pseudo-stop signal during transcription and this process was thus terminated prematurely. These facts tend to argue that fairly sizable cDNAs were being synthesized, but the process of synthesizing the second cDNA strand was destroying the first cDNA strand.

The results obtained with the Amersham cDNA synthesis kit indicated synthesis of cDNA to SMV RNA (non-denatured and denatured prior to cDNA synthesis) by TCA precipitation analysis. Synthesis of cDNA to SMV RNA was confirmed by electrophoresis of both single and double stranded cDNAs. Heat denaturation of SMV RNA before reverse transcription appeared to increase the size of cDNA synthesized whereas DMSO denaturation had no effect on the

size of the cDNA synthesized. It was not determined as to whether this system had been optimized for length of first strand cDNA synthesis.

Transformation of these cDNAs resulted in low frequencies of transformation, and in one case no transformation. This may be due to the fact that the homopolymer tails on either the plasmid or the cDNA were not of optimal length, resulting in low levels of transformation. Southern blot analysis confirmed that the cloned DNA was indeed complementary to SMV RNA.

None of the cDNAs that were cloned were full length clones. In order to obtain a full length cDNA clone to genomic SMV RNA it may be necessary to sequence the already cloned cDNAs, and chemically synthesize an oligonucleotide corresponding to the 5' end of this clone. This oligonucleotide would be used as a primer for reverse transcriptase, where cDNA synthesis would begin about 700 nucleotides from the 3' end of the viral genome. By repeating this process a set of cDNA clones could be made that would represent the whole genomic RNA. These clones could be annealed together with compatible restriction enzyme sites to yield a single cDNA to the entire SMV genome.

The presently cloned cDNA to SMV RNA can be used to answer interesting questions about the genomic structure

of the virus. From the cell-free translation results of Vance and Beachy (1984a), the coat protein gene was apparently located at the 5' end of the genome. Results obtained with other potyviruses indicate that the coat protein gene is located at the 3' end. This incongruity could be studied using these cDNA clones in three different experiments. One experiment would involve sequencing of the cDNA and comparing the nucleotide sequence to the nucleotide sequences of the 3' ends of other sequenced potyviruses. If sequence homology is found, as is the case with other potyviruses, this would suggest the coat protein gene is at the 3' end. Since the cDNA clone is about 710 bases long and it is believed that the 3' polyadenylated tail is generally about 20 to 40 bases long, and in other potyviruses there is a 200 base untranslated region at the very end, the clone could contain 400 to 500 bases of the coding region of the 3' terminal gene. The coat protein gene for other potyviruses is believed to be about 900 bases long, so this clone would be complementary to about half of the gene. This fact could be used in a second experiment where this cDNA is used in a hybrid arrested translation reaction with SMV RNA. If the coat protein gene is at the 3' end then the translation of SMV RNA would lead to a partial coat protein gene product. Western blot analysis of this gene

product using monoclonal antibodies to the SMV coat protein may be able to bind to this protein and confirm the location of the coat protein gene. A third experiment involves the use of cDNA clones in plasmids that express functional beta-galactosidase. Since the lac Z gene is being expressed then the cDNA is being transcribed and the RNA translated into a polypeptide. If the cDNA was inserted in the correct direction in association with the lac Z promoter and if the cDNA is in the correct reading frame then a partial coat protein gene product would be made. This could be detected by western blot analysis using monoclonal antibodies to the coat protein. If it turns out that the orientation or the reading is incorrect, both could be modified.

Recently, cross protection from tobacco mosaic virus was accomplished by the insertion into tobacco plants the coat protein gene (ds cDNA) of tobacco mosaic virus (Abel et al., 1986). Expression of the coat protein gene in tobacco plant cells protected them from infection when challenged with a virulent strain of tobacco mosaic virus. It is believed that the coat protein may coat the invading viral RNA, and thus not allowing the RNA to replicate. This explanation has not been proven and there are many theories as to how cross protection may occur. Since the research of this thesis may have cloned about

half of the coat protein gene of SMV, this cDNA if inserted into and expressed in soybean plant cells may provide some type of cross protection to SMV infection even though only part of the coat protein would be present in these cells.

SUMMARY

Complementary DNA (cDNA) to soybean mosaic virus (SMV) RNA was synthesized using two different reverse transcriptases. Using Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Bethesda Research Labs., Gaithersburg, MD), only short fragments of first strand cDNA were synthesized. There were indications that the reaction mixture may not have been optimized for first strand synthesis.

Avian myeloblastosis virus (AMV) reverse transcriptase included in a cDNA synthesis kit (RPN.1256, Amersham Corp., Arlington Heights, IL) was used to synthesize first and second cDNA to SMV RNA. Using non-denatured RNA, cDNAs approximately 600 bases were synthesized. The double stranded cDNA was cloned in E. coli strain 71-18 using pUC 9 as the plasmid vector. The longest cDNA cloned was determined by restriction enzyme analysis to be approximately 710 bases. Confirmation that the cDNA was complementary to SMV RNA was determined by Southern blot analysis of cloned cDNA, using single stranded cDNA as a probe.

In attempts to synthesize and clone longer cDNAs, SMV RNA was heat denatured prior to reverse transcription and also was added to a first strand synthesis reaction mix-

ture that was 10 % in DMSO. Heat treated SMV RNA yielded double stranded cDNAs ranging from 600 to 2000 bases; DMSO treated RNA yielded double stranded cDNAs of 600 bases. The cloning of these cDNAs did not produce larger cloned cDNAs that were obtained from non-denatured SMV RNA.

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