Construction of infectious potato spindle tuber viroid cDNA clones

Dean E.Cress¹, Michael C.Kiefer³ and Robert A.Owens²

¹Tissue Culture and Molecular Genetics Laboratory, and ²Plant Virology Laboratory, US Department of Agriculture, Beltsville, MD 20705, and ³Department of Botany, University of Maryland, College Park, MD 20742, USA

Received 21 March 1983; Revised and Accepted 8 September 1983

ABSTRACT

Contiguous restriction fragments from two cloned partial-length potato spindle tuber viroid (PSTV) cDNAs were used to construct recombinant DNAs containing full-length monomeric and dimeric PSTV cDNA. When five different PSTV cDNA plasmids and RNA isolated from *E. coli* cells harboring these plasmids were tested for infectivity on tomato, plasmid DNAs containing PSTV cDNA dimers were infectious. RNA transcripts containing the sequence of PSTV from these plasmids were also infectious. The sequences of the viroid progeny and the cloned DNA were identical. *In vitro* mutagenesis of infectious PSTV cDNAs will allow systematic investigation of the role of specific sequences in viroid replication and pathogenesis.

INTRODUCTION

Viroids are the smallest autonomously replicating pathogenic agents known and cause transmissible diseases in several economically important crop plants (reviewed in 1). Determination of the nucleotide sequence of several viroids (2-9), and investigations of the physical-chemical properties of viroids (reviewed in 10) have provided a detailed model of viroid structure. All known viroids are small, unencapsidated, and covalently closed circular single-stranded RNA molecules with extensive secondary structure.

Knowledge of the mechanisms of viroid replication and symptom induction has accumulated more slowly than knowledge of their structure. Evidence that viroids do not code for proteins suggests that the biological properties of viroids are the consequence of direct viroid-host molecular interactions; hence viroids can be considered a minimal genetic and biological system. It has been postulated that disease induction might result from viroid interference with host gene expression (1). A hypothetical mechanism for such interference involves the disruption of host pre-messenger RNA processing (3,11,12)

One genetic approach to the analysis of viroid-host interaction has focused on nucleotide sequence comparisons of different viroids (3,5,6,7) and of strains of the same viroid differing in the degree of symptom severity

(4,8,9). Several regions that are strongly conserved among several different viroids have been identified, and strain-specific sequence differences in potato spindle tuber viroid (PSTV) appear to be quite limited. These regions within the PSTV molecule may be essential for replication and pathogenesis, respectively.

An alternate genetic approach to the study of viroid-host interaction would involve modifications of specific regions (sequences) of a single viroid molecule with subsequent bioassay to detect phenotypic variation. The development of recombinant DNA and related nucleic acid technologies has permitted application of "reverse genetics" to the analysis of a variety of DNA genomes (reviewed in 13). RNA genomes that have been copied into DNA can be studied in a similar fashion provided that the DNA is also biologically active.

We have previously reported the construction of recombinant plasmids containing DNA inserts complementary to PSTV (14). We now describe the construction of plasmids containing full-length PSTV cDNAs from these partial length cDNAs. Some of these recombinant DNAs, as well as RNA extracted from *E. coli* cells containing these plasmids, are infectious when inoculated onto tomato seedlings. Preliminary reports describing these results have been published (15,16).

MATERIALS AND METHODS

Plasmid DNA isolation and sequencing

Plasmid DNA was extracted (17) from chloramphenicol-amplified cultures and purified through two successive ethidium bromide-CsCl equilibrium gradients. Restriction enzyme digestions were performed according to instructions of the supplier (New England Biolabs* or Bethesda Research Labs). Conditions for the purification of restriction fragments, 5'-end labelling by incubation with T4 polynucleotide kinase (Boehringer Mannheim) and [γ - ^{32}P] ATP (Amersham), chemical sequencing reactions, and gel electrophoresis were as described by Maxam and Gilbert (18). All experiments were carried out in compliance with current NIH guidelines for recombinant DNA research.

Construction of complete PSTV cDNAs

As depicted in Figure 1A, equimolar amounts of the contiguous AvaII-HaeIII restriction fragments from clones pDC-29 and pDC-22 were incubated at 16 $^{\circ}$ C with a low T4 DNA ligase concentration to promote ligation at the AvaII cohesive ends and then were digested with HaeIII. The linear ligation and digestion product was purified by polyacrylamide gel electrophoresis and ligated to [5'- ^{32}P] labelled synthetic decanucleotide linkers (Collaborative

Research) encoding the HindIII recognition site. Following digestion with HindIII, the mixture was fractionated by electrophoresis in a 5% polyacrylamide gel, and the desired ligation product was identified by autoradiography and eluted. The fragment was ligated to HindIII-digested, dephosphorylated pBR322 DNA, and the recombinant molecules were transformed into *E. coli* C600 ($r_K^- m_K^+$). Clones carrying the inserted DNA were selected on the basis of their ampicillin-resistant, tetracycline-sensitive phenotype. Restriction digestions with HindIII, HaeIII, and BamHI were used to identify the structure of the inserts and their orientation in the plasmid vector.

The 365 bp HindIII fragment was subsequently cloned into the HindIII site of plasmid pGL101H, a vector derived from pBR322 by the insertion of a 95 bp fragment containing the lac uv5 promoter (Gail Lauer, personal communication).

Cloning and sequencing on bacteriophage M13mp9

The 365 bp HindIII fragment containing the full-length PSTV cDNA was ligated to HindIII-digested, dephosphorylated M13mp9 replicative form DNA (19). The ligation mix was used to transfect *E. coli* JM103, and recombinant plaques were identified by plating cells in the presence of 1 mM isopropyl-thio-galactoside and 0.004% 5-bromo-4-chloro-3-indolyl- β -D-galactoside (20). Single- and double-stranded phage DNAs with PSTV cDNA inserted in both orientations were isolated as described (21). DNA sequencing by the dideoxy chain termination method (22) was performed using the synthetic primer TCCCAGTCACGACGT.

Construction of PSTV cDNA dimers

The 359 bp fragment released by HaeIII digestion of the DNAs described above was purified by polyacrylamide gel electrophoresis, incubated with T4 DNA ligase, and digested with BamHI. The resulting 359 bp fragment was gel purified, inserted into the BamHI site of pBR322, transformed into *E. coli* C600, and recombinants were identified as ampicillin-resistant, tetracycline-sensitive colonies. The presence of monomeric or head-to-tail dimeric PSTV cDNA inserts in both orientations was determined by digestions with BamHI and SmaI.

Isolation and analysis of RNA from *E. coli*

E. coli strains containing PSTV recombinant plasmids were grown to mid-log phase in the presence of the appropriate antibiotic. Cultures were poured onto frozen buffer containing 10 mM Tris-HCl (pH 7.5)-5 mM MgCl₂-50 mM NaN₃, and the cells were collected by centrifugation after the resulting slurry had thawed. Cell pellets were resuspended in cold azide buffer and incubated with 150 μ g/ml lysozyme for 2 minutes at 25°C. The resulting spheroplasts were lysed by

addition of SDS to 1%, and the lysate was extracted three times with phenol-chloroform (1:1) and twice with diethyl ether before addition of one-quarter volume of 10 M LiCl. DNA was sheared by passing the lysate through a 21 gauge syringe needle during the first phenol-chloroform extraction. Nucleic acids soluble in 2 M LiCl were recovered by ethanol precipitation, redissolved in 10 mM Tris-HCl(pH 7.5)-2mM MgCl₂ (final concentration = 2 mg/ml), and incubated for 30 minutes at 37°C with 50 ug/ml pancreatic DNase. Following successive phenol/chloroform and ether extractions the RNA was recovered by ethanol precipitation and subjected to a second incubation with pancreatic DNase. Glyoxal-denatured RNAs were fractionated on 1.5% agarose gels (23) and transferred to nitrocellulose (24) before hybridization analysis of PSTV-related RNAs (25).

Bioassay and RNA extraction from infected tomato

Bioassay of recombinant DNAs or RNAs isolated from *E. coli* was performed using tomato seedlings (*Lycopersicon esculentum* Mill., cv. Rutgers) according to a published protocol (26). Ten microliters of a DNA (0.2-1.0 mg/ml) or RNA (0.6-3.0 mg/ml) solution in 20 mM Na phosphate, pH 7.0 were inoculated per plant. All plants were monitored visually for appearance of the characteristic symptoms of PSTV infection and by nucleic acid spot hybridization analysis of extracted leaf sap for the presence of PSTV-related RNA (27). Control plants inoculated with 20 mM Na phosphate, pH 7.0 were included in each experiment.

RNA sequencing

Two methods were used to determine the nucleotide sequence of PSTV prepared from bioassay plants inoculated with either recombinant DNA or a severe strain of PSTV. The sequence of selected regions was determined by partial enzymatic cleavage after preliminary fragmentation of purified PSTV with RNase U2 and 5'-terminal labelling by incubation with [γ -³²P]ATP and T4 polynucleotide kinase (5). Complete RNA sequences were determined by base-specific modification and cleavage (18) of PSTV cDNAs transcribed from 2 M LiCl-soluble RNA (28). 5'-Terminal labelled restriction fragments complementary to PSTV nucleotides 105-146 and 266-288 were used as primers for cDNA synthesis.

RESULTS

Construction of full-length and dimeric PSTV cDNA

Some time ago we reported the construction of recombinant DNAs containing double-stranded PSTV cDNAs (14). Restriction site mapping of the largest insert, pDC-29 (460 bp), suggested that most of the PSTV sequence had been

cloned but that a portion contained an altered restriction pattern. Subsequent nucleotide sequence analysis of pDC-29 has revealed that the 337 bp PSTV cDNA sequence between positions 196 and 173 is identical to that predicted by the RNA sequence data of Gross et al. (2) (data not shown). The sequence following position 196 in the cDNA is an inverted terminal duplication of PSTV sequences between positions 96 and 173. The site of the strand switch from position 196 to 96 is adjacent to a 4 bp CCCG sequence at positions 199 to 196 that is complementary to a 4 bp GGGC sequence at positions 92- 95. The additional common SmaI-HpaII-AvaI site is found in the inverted terminal repeat.

This duplication and rearrangement of PSTV cDNA sequences in clone pDC-29 is similar to that observed in other cDNA clones (29). The region immediately preceding the site of sequence rearrangement, nucleotides 186-196 of the PSTV template, is uridine-rich. The resulting unstable dA-U base-pairing during cDNA synthesis could have permitted dissociation of the nascent cDNA strand, its fold-back to the complementary GGGC sequence at nucleotides 92-95, and synthesis of a sequence complementary to the original nascent cDNA strand.

Restriction analysis of the insert from a second clone, pDC-22, suggested that it contained cDNA sequences not present in pDC-29. Partial nucleotide sequence analysis of the pDC-22 insert showed that the PSTV cDNA sequence between positions 146 to 341 was identical to that predicted by the RNA sequence (data not shown). Thus, clone pDC-22 contained the 22 bp PSTV cDNA sequence between positions 174 to 195 that was absent from pDC-29. Since the partial-length cDNA sequences in these two clones were overlapping and possessed common restriction sites, it was possible to construct full-length cDNA inserts by ligation of contiguous fragments.

Figure 1A presents the strategy used to construct clones containing full-length double-stranded PSTV cDNA. The 285 bp AvaII-HaeIII fragment from pDC-29 was ligated at the AvaII site to the contiguous 74 bp AvaII-HaeIII fragment from pDC-22. HindIII oligodeoxynucleotide linkers were added to the HaeIII blunt ends of this linear ligation and, following digestion with HindIII, this fragment was cloned in the HindIII site of pBR322. Because use of this particular HindIII decanucleotide linker reconstructed the terminal HaeIII sites of the PSTV insert, digestion of a majority of the clones with HaeIII released a 359 bp fragment, a full-length double-stranded PSTV cDNA. The 365 bp HindIII fragment was subsequently subcloned into the HindIII site of plasmid pGL101H, immediately downstream from the lac uv5 promoter sequence. Such a construction theoretically permits transcription of the PSTV cDNA sequences under the control of an efficient promoter.

A **B**

Figure 1. Construction of full-length double-stranded PSTV cDNAs.

(a). Ligation of specific fragments from overlapping, partial-length cDNAs followed by addition of synthetic oligonucleotide linkers yields a full-length cDNA whose termini are derived from the unique HaeIII cleavage site (PSTV nucleotides 145-148). The inner circle represents the 359 nucleotide sequence of PSTV (2); positions of 5 unique restriction sites in double-stranded PSTV cDNA are shown. The concentric curves indicate the portions of the complete PSTV cDNA sequence present in partial-length clones pDC-29 and pDC-22. Heavy portions of these curves indicate the contiguous AvaII-HaeIII fragments used for ligation. (b). Schematic representations of recombinant plasmids depict the orientation of the PSTV cDNA inserts with respect to the adjacent plasmid promoters. PSTV cDNA sequences are shown as heavy solid lines with the vertical marks indicating their HaeIII termini (left) or BamHI termini (right), and the arrows indicate the orientation of the PSTV DNA sequence. Plasmid DNA sequences are shown as broken lines.

Dimeric PSTV cDNA inserts were of particular interest in light of the evidence for multimeric viroid replicative intermediates (see DISCUSSION section). To facilitate construction of cDNA dimers, it was desirable to obtain PSTV cDNA with cohesive termini. Therefore, a permuted form of the full-length PSTV cDNA insert having BamHI termini was constructed as outlined in MATERIALS AND METHODS. Clones with monomeric and head-to-tail dimeric inserts were obtained. Figure 1B depicts the structures of various recombinant plasmids containing PSTV cDNA inserts.

Figure 2. Analysis of 1M glyoxal-denatured, PSTV-related RNAs isolated from *E. coli* C600 cells harboring recombinant plasmids. (a & a') DNA size standards prepared by BamHI digestion of pVB-6 DNA and a pVB-6 derivative whose central BamHI site has been removed by HSO₃ mutagenesis. (b & b') 0.25 ug total cellular RNA isolated from PSTV-infected tomato leaf tissue. (c-h & c'-h') 25 ug 2M LiCl-soluble RNA isolated from *E. coli* clones harboring pBR322 (c & c'), pGL101H-2 (d & d'), pGL101H-3 (e & e'), pVB-6 (f & f' and g & g'), and pVB-8 (h & h'). RNAs in lanes g & g' were incubated with 40 ug/ml pancreatic RNase before denaturation and electrophoresis. Strand-specific hybridization probes (25) were complementary to nucleotides 105-146 of PSTV or nucleotides 147-183 of cPSTV.

Viroid-related transcripts in *E. coli*

Because the PSTV cDNA sequences in our recombinant plasmids were inserted downstream from either the tet (pBR322) or lac (pGL101H) promoter (Figure 1B), it was important to determine if stable PSTV-related RNAs were produced in the *E. coli* strains harboring the various recombinant plasmids described above. The polarity of any such transcripts can be established by hybridization of RNAs extracted from exponentially-growing *E. coli* cultures with strand-specific probes. Figure 2 presents the results of such an analysis of glyoxal-denatured RNA that has been fractionated by agarose gel electrophoresis and transferred to nitrocellulose.

Each of the four *E. coli* clones tested contained PSTV-related transcripts that were heterogeneous in size but of only one polarity. Susceptibility to pancreatic RNase digestion (compare lanes f and g) proved that the hybridizing

material was RNA. Apparent discrete transcripts between 359 and 718 nucleotides in length are visible in all four RNA preparations analyzed, and the transcripts from the dimeric pVB-6 cDNA were noticeably longer than those from the monomeric pGL101H-2 cDNA (compare lanes d and f). Transcripts having the polarity of PSTV (lanes d and f) were more abundant than transcripts of the opposite polarity (cPSTV, lanes e' and h'). *E. coli* clones harboring the pGL101H plasmids contained a higher concentration of PSTV-related RNA than the pBR322 recombinants (compare lanes d and f or lanes e and h). The structure of these RNAs is currently under investigation.

Infectivity analysis

After constructing a series of recombinant plasmids containing full-length monomeric and dimeric PSTV cDNA inserts that can be transcribed in *E. coli* to give possible full-length transcripts of defined polarity, we initiated a series of experiments to determine whether the double-stranded plasmid DNAs or the RNAs extracted from *E. coli* were infectious when inoculated onto tomato seedlings. Infectivity was assayed by the appearance of the characteristic disease symptoms (stunting and epinasty) and by nucleic acid spot hybridization analysis (25) of leaf sap prepared from the bioassay plants.

Table 1 summarizes the results obtained with five different PSTV cDNA clones and their corresponding RNA preparations. DNA infectivity was consistently observed for the dimeric cDNA clones pVB-6 and pVB-8, while no evidence was obtained for infectivity of any of the monomeric cDNA clones. Disease symptoms were almost always observed when positive hybridization results were obtained, indicating that the infection usually produced high titers of viroid progeny. PSTV could not be detected in the buffer-inoculated control plants included in each experiment.

Infectivity from the *E. coli* RNA preparations was observed for pVB-6, which produces transcripts with the polarity of PSTV, but not for pVB-8, which produces transcripts with the polarity of cPSTV. Incubation (30 minutes at 37°C in the presence of 10 mM Tris-HCl(pH 7.5)-2 mM MgCl₂) with 40 µg/ml pancreatic RNase destroyed the infectivity of RNA prepared from *E. coli* containing plasmid pVB-6, but incubation with 50 µg/ml pancreatic DNase had no effect (data not shown).

The only instance where RNA transcribed from a monomeric cDNA clone appeared to be infectious was pGL101H-2, a clone whose transcripts also have the polarity of PSTV. In this case only 1 of 5 inoculated plants in a single experiment contained PSTV progeny. The significance of this low level of infectivity awaits further analysis.

Table 1. Infectivities of cloned PSTV cDNAs

Clones	Length	Transcript Polarities	Infectivity	
			DNA	RNA
pDC-29	336 nuc.	both (unstable)	not done	0/1
pGL101H-3	359 nuc. monomer	cPSTV	0/1	not done
pGL101H-2	"	PSTV	0/2	1/2 [1/5]
pVB-1	"	cPSTV	0/4	0/1
pVB-2	"	PSTV	0/4	0/2
pVB-8	718 nuc. dimer	cPSTV	1/1 [5/10]	0/2
pVB-6	"	PSTV	8/10 [1/5-9/10]	6/7 [1/6-9/10]

Infectivity values: Trial where infectivity was detected/total trials
 [] = Plants testing positive by hybridization/inoculated plants

Inoculum concentrations: 200-1000 µg/ml plasmid DNA
 600-3000 µg/ml E. coli 2M LiCl-soluble RNA

Because the procedure used to isolate supercoiled plasmid DNAs from E. coli does not remove all traces of RNA contamination (data not shown), it was necessary to determine if the infectivity observed with plasmid DNA could be due to the presence of low levels of a highly infectious RNA species. To test this possibility pVB-6 DNA was digested with EcoRI plus SalI, enzymes which cleave the plasmid vector sequences outside the PSTV cDNA insert. A portion of this digest was then incubated with 0.3N NaOH under conditions that would degrade any contaminating RNA, and renatured to recover the double-stranded EcoRI-SalI fragment containing the dimeric PSTV cDNA. The data presented in Table 2 show that the infectivity was not significantly affected by alkaline hydrolysis, indicating the double-stranded DNA is itself infectious. This conclusion is further supported by experiments in which incubation with pancreatic RNase had no effect on infectivity of DNA (Table 2). Digestion with

Table 2. Inactivation of infectious PSTV cDNAs

DNA	Transcript Polarities	Infectivity	
None		0/6	
pVB-6: EcoRI + SalI	PSTV	5/6	4/6
EcoRI + SalI alkaline hydrolysis renaturation		2/6	3/6
pVB-6: Untreated	PSTV	4/6	
Pancreatic + T1 RNase		4/6	
BamHI + HaeIII		0/6	
pVB-8: Untreated	cPSTV	3/6	
Pancreatic + T1 RNase		3/6	
BamHI + HaeIII		0/6	

Infectivity values: Plants testing positive by hybridization/inoculated plants

Inoculum concentration: 1000 µg/ml plasmid DNA

Pancreatic RNase digestion: 2 hours at 37°C in presence of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) containing 10 µg/ml pancreatic RNase and 250 units/ml T1 RNase.

Restriction digestion: 2 hours at 37°C in presence of 35 mM Tris-acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DDT, and 100 µg/ml BSA (pH 7.9) containing 600 units/ml placental RNase inhibitor and 0.5-1.0 unit restriction enzymes/ug DNA

Alkaline hydrolysis: 14-16 hours at 37°C in presence of 0.3N NaOH

BamHI plus HaeIII leaves no intact PSTV cDNA and completely destroyed the infectivity of pVB-6 and pVB-8 DNAs.

Additional evidence for the infectivity of the recombinant DNAs was provided by direct RNA sequence analysis of the viroid progeny extracted from the bioassay plants. Because the RNA sequence of the PSTV strain used as template for cDNA cloning has been shown to differ from that of the severe strain of PSTV which we now routinely propagate (J. Odell, personal communication), sequence analysis can be used to determine whether or not PSTV progeny are derived from the recombinant DNA inoculum.

Fragment patterns in partial RNase U2 digests of severe strain PSTV and PSTV purified from tomatoes inoculated with pVB-6 DNA were consistent with the

Figure 3. Partial RNA sequence of PSTV isolated from tomatoes inoculated with pVB-6 DNA. Base-specific cleavages were produced by RNases T1(G), U2(A), Phy M(A+U), and extracellular RNase from *Bacillus cereus*(C+U) as described by Haseloff and Symons (5). (PSTV) Sequence of a severe strain of PSTV routinely propagated in our laboratory and originally obtained from M. Zaitlin. (pVB-6) Sequence of PSTV isolated from a tomato plant inoculated with pVB-6 DNA. The lower and upper arrows mark nucleotides 44 and 53 of the PSTV sequence (2). The actual nucleotide sequences for the two viroid RNAs are shown in bold letters outside the autoradiogram. NE: controls incubated in the absence of ribonuclease. Unlabelled lanes contain RNA heated for 10 minutes at 90 C in the presence of 0.05M $\text{HCO}_3^-/\text{CO}_3^{2-}$ buffer (pH 9).

expected sequence differences (data not shown). Fragments containing the region of interest were sequenced by base-specific partial enzymatic cleavage (5). Figure 3 shows that the PSTV isolated from plants inoculated with pVB-6 contains the GAGCAGAAAG sequence (PSTV nucleotides 44-45) found in the cloned cDNA rather than the GACAAGAAAG sequence found in the PSTV severe strain which we now propagate.

The complete nucleotide sequence of the PSTV progeny was also determined by base-specific modification and cleavage of $[5'-^{32}\text{P}]$ labelled cDNAs as

described in MATERIALS AND METHODS. It was identical to the sequence of the cloned PSTV DNA (data not shown). Particular attention in sequence analysis was devoted to PSTV sequences between nucleotides 43-53, 116-124, and 305-317, regions previously shown (4, 9, 30) to vary in different naturally-occurring PSTV strains.

DISCUSSION

We have shown that recombinant DNAs containing head-to-tail PSTV cDNA dimers are infectious. Inoculation of tomato seedlings with recombinant DNA results in the appearance of PSTV progeny with the RNA sequence predicted by the sequence of the cloned PSTV cDNA. *E. coli* cells harboring certain recombinant plasmids also contain infectious viroid RNA transcripts. Plant viroids, therefore, join two single-stranded RNA viruses, bacteriophage Q β (31) and poliovirus (32), as the third example where a cloned cDNA copy of an RNA genome is able to generate infectious RNA progeny after transfection into a host cell. Although it is not known in detail how these cDNAs initiate the infection process, there appear to be significant differences among the three systems. Explanations for these differences may be important to our current efforts to use infectious viroid cDNAs to study viroid structure-function relationships.

Whereas intact recombinant DNAs containing monomeric Q β poliovirus cDNA inserts are infectious, only DNAs containing a head-to-tail PSTV cDNA dimer were infectious in our studies (Table 1). Primary transcription of the Q β or poliovirus cDNAs by host DNA-dependent RNA polymerase could initiate within either plasmid sequences or the adjacent homopolymeric "tails", but our full-length PSTV cDNA clones do not contain homopolymeric "tails". Neither Q β phage production (31) nor PSTV replication (Tables 1 and 2) depend upon orientation of the cDNA insert, arguing against the use of a promoter in the plasmid vector. These two observations, an apparent requirement that the PSTV cDNA be present as a dimer and infectivity that is independent of insert orientation, suggest that either the DNA template for PSTV replication is generated by DNA recombination in vivo or that a longer than unit-length PSTV transcript is required to initiate the infection process.

Intact recombinant DNAs containing monomeric polyoma (33,34), SV-40 (35), and cauliflower mosaic virus (CaMV) (36,37) DNA inserts were non-infectious, while DNAs containing head-to-tail multimers (or partial multimers) are infectious. Excision of monomeric circular viral DNAs would allow replication to proceed by exactly the same mechanism used by conventional virus particles,

but neither healthy nor PSTV-infected tomato tissue contains DNA sequences complementary to PSTV (38,39). Such a fundamental difference in the nature of viroid and DNA virus replication intermediates could be reflected in differences in the specific infectivities of their cloned DNAs. Comparison of the amount of DNA required to infect approximately 1/3 of the inoculated plants and the relative genome size for PSTV (1.4 ug and 359 bp) and CaMV (0.15 ug and 8,024 bp, Table 2 in reference 37) indicates that plasmids containing PSTV cDNA dimers are approximately 200-fold less infectious than plasmids containing CaMV dimers.

The second potential explanation, a requirement for a longer than unit-length PSTV transcript that undergoes cleavage and ligation to circular PSTV monomers in the tomato host cell, seems more likely at this time. Viroid replication involves RNA-RNA replicative intermediates (40, 14), and it is not immediately apparent why possible excision of a circular double-stranded PSTV cDNA template should facilitate infectivity. Viroid replication appears to proceed via a "rolling circle" mechanism (25,41), and multimeric forms of both infectious PSTV and its complementary strand can be found in PSTV-infected cells. Head-to-tail dimers of PSTV cDNA provide a recombinant DNA template that allows synthesis of multimeric PSTV transcripts and their subsequent cleavage and ligation to form circular PSTV RNA.

Further characterization of both infectious and non-infectious PSTV transcripts from E. coli may clarify the precise requirements for cDNA infectivity. The apparent low level of infectivity observed with E. coli transcripts from a monomeric cDNA clone, pGL101H-2, suggests that RNAs containing non-duplicated PSTV sequences can also be replicated in the host cell (Table 1). We are now examining the size and structure of the infectious PSTV RNA(s) present in the various E. coli RNA preparations in order to clarify this important point.

One immediate goal in our further studies with these infectious PSTV cDNAs will be to determine the effect of in vitro mutagenesis (13) of specific regions of the PSTV sequence upon viroid replication and/or pathogenesis. Sequence comparisons of PSTV strains which differ dramatically in symptom severity (30) indicate that sequences near positions 45, 120 and 315 may be involved in regulating symptom expression. The extensive homology between PSTV nucleotides 258-282 and the 5' end of eucaryotic U1 RNA suggests that this region may also be important for viroid function (3,11,12). Construction of modified PSTV cDNAs will permit systematic investigation of the role of these and other sequences in viroid replication and pathogenesis.

ACKNOWLEDGEMENTS

This work was supported in part by USDA Competitive Research Grant No. 59-2246-1-1-719-0. The work was performed at the Beltsville Agricultural Research Center under a cooperative agreement with the Department of Botany, University of Maryland, College Park, Maryland (Project Director P.M. Bottino). We thank T.O. Diener and G.E. Bruening for numerous suggestions and discussions, B.L. Smith and S.M. Thompson for technical assistance, L. Vodkin and P. Rhodes for aid in dideoxy sequencing with M13mp9, and C. Collmer and J.L. Zimmerman for constructive criticism of the manuscript.

* Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

REFERENCES

1. Diener, T.O. (1979) Viroids and Viroid Diseases. Wiley, New York.
2. Gross, H.J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H., and Sanger, H.L. (1978) *Nature* 273, 203-208.
3. Gross, H.J., Krupp, G., Domdey, H., Raba, M., Jank, P., Lossow, C., Alberty, H., Ramm, K. and Sanger, H.L. (1982) *Eur. J. Biochem.* 121, 249-257.
4. Gross, H.J., Liebl, U., Alberty, H., Krupp, G., Domdey, H., Ramm, K., and Sanger, H.L. (1981) *Bioscience Reports* 1, 235-241.
5. Haseloff, J. and Symons, R.H. (1981) *Nucleic Acids Res.* 9, 2741-2752.
6. Visvader, J.E., Gould, A.R., Bruening, G.E., and Symons, R.H. (1982) *FEBS Letters* 137, 288-292.
7. Symons, R.H. (1981) *Nucleic Acids Res.* 9, 6527-6537.
8. Haseloff, J., Mohamed, N.A., and Symons, R.H. (1982) *Nature* 299, 316-321.
9. van Wezenbeek, P., Vos, P., van Boom, J. and van Kammen, A. (1982) *Nucleic Acids Res.* 10, 794-7957.
10. Gross, H.J. and Riesner, D. (1980) *Angewandte Chemie (English edition)* 19, 231-243.
11. Diener, T.O. (1981) *Proc. Natl. Acad. Sci. USA* 78, 5014-5015.
12. Dickson, E. (1981) *Virology* 115, 215-221.
13. Shortle, D., DiMiao, D. and Nathans, D. (1981) *Ann. Rev. Genet.* 15, 265-294.
14. Owens, R.A. and Cress, D.E. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5302-5306.
15. Cress, D.E. and Owens, R.A. (1981) *DNA* 1, 90 (abstract).
16. Owens, R.A. and Cress, D.E. (1982) *Proceedings of the 12th International Congress on Biochemistry*, p. 170 (abstract).
17. Holmes, D.S. and Quigley, M. (1981) *Anal. Biochem.* 114, 193-197.
18. Maxam, A.M. and Gilbert, W. (1980) In: *Methods in Enzymology* (L. Grossman and K. Moldave, eds.) 65(1) 499-560. Academic Press, New York.
19. Messing, J. and Viera, J. (1982) *Gene* 19, 269-276.
20. Messing, J., Gronenborn B., Muller-Hill, b. and Hofschneider, P. (1977) *Proc. Natl. Acad. Sci. USA* 74, 3642-3546.

21. Heidecker, G., Messing, J. and Gronenborn, B. (1980) *Gene* 10, 69-73.
22. Messing, J., Crea, R. and Seeburg, P.H. (1981) *Nucleic Acids Res.* 9, 309-321.
23. Carmichael, G.G. and McMaster, G.K. (1980) In: *Methods in Enzymology* (L. Grossman and K. Moldave, eds.) 65(I), 380-391. Academic Press, New York.
24. Smith, G.E. and Summers, M.D. (1980) *Anal. Biochem.* 109, 123-129.
25. Owens, R.A. and Diener, T.O. (1982) *Proc. Natl. Acad. Sci. USA* 79, 113-117.
26. Diener, T.O., Hadidi, A. and Owens, R.A. (1977) In: *Methods in Virology* (K. Maramorosch and H. Koprowski, eds.) 6, 185-217.
27. Owens, R.A. and Diener, T.O. (1981) *Science* 213, 670-672.
28. Ghosh, P.K., Reddy, V.B., Piatak, M., Lebowitz, P. and Weissmann, S.M. (1980) In: *Methods in Enzymology* (L. Grossman and K. Moldave, eds.) 65(I), 580-595. Academic Press, New York.
29. Fagan, J.B., Pastan, I. and deCrombrughe, B. (1980) *Nucleic Acids Res.* 8, 3055-3064.
30. Sanger, H.L. (1982) In: *Encyclopedia of Plant Physiology New Series* (B. Parthier and D. Boulter, eds.) 14B, 368-454. Springer-Verlag, Berlin.
31. Taniguchi, T., Palmieri, M. and Weissmann, C. (1978) *Nature* 274, 223-228.
32. Racaniello, V.R. and Baltimore, D. (1981) *Science* 214, 916-919.
33. Chan, H.W., Israel, M.A., Garon, C.F., Rowe, W.P. and Martin, M.A. (1979) *Science* 203, 887-892.
34. Fried, M., Kieln, B., Murray, K., Greenaway, P., Tooze, J., Boll, W. and Weissmann, C. (1979) *Nature* 279, 811-816.
35. Schaffner, W. (1980) *Proc. Natl. Acad. Sci. USA* 7, 2163-2167.
36. Howell, S.H., Walker, L.L. and Walden, R.M. (1981) *Nature* 293, 483-486.
37. Lebeurier, G., Hirth, L., Hohn, B. and Hohn T. (1982) *Proc. Natl. Acad. Sci. USA* 79, 2932-2936.
38. Branch, A.D. and Dickson, E. (1980) *Virology* 104, 10-26.
39. Hadidi, A., Cress, D.E., and Diener, T.O. (1981) *Proc. Natl. Acad. Sci. USA* 78, 6932-6935.
40. Grill, L.K. and Semancik, J.S. (1978) *Proc. Natl. Acad. Sci. USA* 5, 896-900.
41. Branch, A.D., Robertson, H.D. and Dickson, E. (1981) *Proc. Natl. Acad. Sci. USA* 78, 6381-6385.