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Studies on the primary and secondary structure of potato spindle tuber viroid: products of digestion with ribonuclease A and ribonuclease T_1, and modification with bisulfite

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ABSTRACT

Potato spindle tuber viroid (PSTV), a small infectious RNA, has been completely digested with RNase T_1 and RNase A, and the resulting oligonucleotides have been sequenced using 5'-terminal ^{32}P-labelling with γ-^{32}P ATP and T4 polynucleotide kinase, fingerprinting and controlled nuclease P_1 digestion. Modified nucleotides have not been detected in 5'-positions of these oligonucleotides. PSTV consists of about 359 nucleotides and contains a remarkable stretch of 18 purines, mainly adenosines; there is no AUG initiation triplet present. The established oligonucleotide sequences preclude a perfect intramolecular base complementarity within the covalently closed viroid circle. Therefore, the rigid, rod-like native secondary structure of PSTV, as seen in the electron microscope, must be based on a defective rather than on a homogeneous RNA helix. The detailed analysis of the bisulfite-catalized modification of cytidine to uridine in PSTV revealed a higher reactivity for the majority of the cytidines than would be expected for a perfect helix. Since only cytidines in single-stranded regions are known to be fully reactive, this finding provides additional evidence for defects in the helical secondary structure of PSTV.

INTRODUCTION

Viroids are a novel and unique class of pathogens presently known only in higher plants. The viroids identified until now are the potato spindle tuber viroid (PSTV) (1), the citrus exocortis viroid (CEV) (2,3), the chrysanthemum stunt viroid (CSV) (4,5), the cucumber pale fruit viroid (CPFV) (6), the chrysanthemum chlorotic mottle viroid (CCMV) (7), the coconut cadang-cadang viroid (CCCV) (8,9), and recently the hop stunt viroid (HSV) (10).

In contrast to conventional viruses, viroids exist as uncoated infectious RNA molecules with a molecular weight of about 120.000.

We have recently presented physical, electron microscopical and biochemical evidence that viroids represent also a new class of nucleic acids: they are single-stranded, covalently closed circular RNA molecules existing as highly base-paired rod-like structures (11).

Circularity of viroids has later been confirmed by another group (12), who found circular molecules among a majority of linears in their preparations (13). However, there was only evidence from electron microscopy, which, even if done under denaturing conditions, does not allow to distinguish reliably between covalently closed rings or hairpin structures with short and very stable base pairing at the open ends.

In a previous study we demonstrated by comparative oligonucleotide fingerprints that the viroids causing spindle tuber disease of potato, the exocortis disease of citrus and the stunt disease of chrysanthemum are individual viroid "species" (14). In this report, we present the first detailed study on the sequences of the complete RNase A and RNase T_1 oligonucleotides of the potato spindle tuber viroid (PSTV) as obtained by ^{32}P -labelling in vitro. In addition, we provide evidence for a unique secondary structure of this viroid on the basis of its oligonucleotide sequences and its reactivity against bisulfite. This study is the first step towards our aim to establish the primary and secondary structure of viroids.

MATERIALS

RNase T_1 was from Sankyo Co., Tokyo; RNase A and alkaline phosphatase from E. coli were from Boehringer, Mannheim. Nuclease P_1 (15) from Penicillium citrinum was from PL Biochemicals, Inc., Milwaukee. Cellulose acetate strips for ionophoresis were from Schleicher and Schüll, Dassel, DEAE-cellulose paper "DE-81" from Whatman. 20 x 40 cm DEAE-cellulose thinlayer plates were bought from Macherey, Nagel Co., Düren, 20 x 20 cm cellulose thinlayer plates from Merck, Darmstadt.

γ-^{32}P ATP was prepared according to Glynn and Chappell (16) using carrier-free ^{32}P phosphoric acid from New England Nuclear Corp., Na_2ATP and enzymes from Boehringer, Mannheim;

γ-^{32}P ATP was purified by column chromatography on DEAE-cellulose using a triethylammonium bicarbonate buffer gradient

and had a specific activity of about 100 Ci/mmol.- Polynucleotide kinase from T4 phage-infected E.coli was prepared as indicated earlier (14).

METHODS

Origin and Propagation of the Potato Spindle Tuber Viroid (PSTV).

The isolate of PSTV, originally provided by Dr. T.O. Diener, Beltsville, in the tomato (Lycopersicum esculentum) cultivar "Rutgers" (1), was propagated in the bush tomato cultivar "Rentita". Successful viroid infection and replication was indicated by the characteristic symptoms of stunting and epinasty in the host plant. The appearance of an extra band of viroid RNA absent in RNA preparations from healthy control plants was verified in stained polyacrylamide gels as shown earlier (11).

Isolation of PSTV

The viroid was purified from total nucleic acids isolated from systemically infected leaf tissue using described methods (11, 14, 17).

Digestion, in vitro Labelling and Fingerprinting of PSTV.

5 µg PSTV were digested with 0.6 units RNase T_1 or 0.05 µg RNase A in 17 µl 60 mM Tris-HCl, pH 8.0 for 3 hours at 37° C. The digest was treated with 0.002 units of alkaline phosphatase from E.coli for 2 hours at 37° C; the phosphatase was then inactivated with nitrilotriacetic acid as described by Simsek et al.(18). From this digest (20 µl), 5 µl were used for in vitro 5'-end group labelling of the fragments according to Simsek et al. (18).- Fingerprints of in vitro 5'-labelled RNase digests were obtained by a combination of high voltage electrophoresis (5000 V) on cellulose acetate strips at pH 3.5, and homochromatography on DEAE-cellulose thinlayer plates in the second dimension (14), followed by autoradiography (10 to 30 min).

Elution of 5'- ^{32}P-Oligonucleotides from the DEAE Plates.

The DEAE-cellulose was collected by scraping the plate with the open end of a glass tube (3 x 50 mm) having the cotton wool filled tip on the other end fixed to a vacuum tube. Urea and salt were removed from the DEAE-cellulose by elution with 2 ml ethanol. Labelled oligonucleotides were eluted from the DEAE-cellulose with 300 µl 2 M triethylammonium bicarbonate buffer,

pH 8.5. This buffer was removed by evaporation over phosphopentoxide for 18 h.

Nucleotide Sequence Determination. All 5'-^{32}P-labelled oligonucleotides were degraded by controlled digestion with nuclease P_1. The incubation mixture contained 1 - 5 A_{260} units of carrier RNA from the homomix, 50 ng nuclease P_1 per A_{260} unit of RNA, and 5000 - 50.000 cpm 5'-^{32}P-labelled oligonucleotide in 10 µl 50 mM ammonium acetate, pH 5.3; incubation was at 20° C. After 0, 5, 10 and 20 min, aliquots were pipetted onto 1 µl 25 mM Na_2EDTA, and put into a boiling waterbath for 4 min. This digest was evaporated to dryness and redissolved in 3 µl water. Aliquots of this digest were used for sequence determination (a) by DEAE-paper electrophoresis in pyridin-acetate (pH 3.5) and in 7% formic acid (19), and (b) by the mobility shift method, i.e., a combination of ionophoresis on cellulose acetate strips at pH 3.5 in the first dimenstion and homochromatography on DEAE-cellulose thinlayer plates in the second dimension (14). Autoradiography was for 2 to 20 days.

5'-End group analysis was performed by complete nuclease P_1 digestion of a 1000 cpm aliquot of each 5'-labelled oligonucleotide. The incubation mixture was as described above, however, the reaction was for 2 h at 37° C. The digest was evaporated and dissolved in 3 µl of a marker solution containing about 0.05 A_{260} units of each of the four authentic 5'-mononucleotides pA, pC, pU and pG. Two different solvents were used to identify the 5'-^{32}P-nucleotides by cellulose thinlayer chromatography; solvent A: isobutyric acid - conc. ammonia - water, pH 4.3 (577 : 38 : 385; v : v : v); solvent B: t-butanol - conc. hydrochloric acid - water (7 : 1.5 : 1.5; v : v : v). The authentic 5'-nucleotides were visualized in 254 nm UV light; the 5'-^{32}P-nucleotides were located by autoradiography overnight.

Bisulfite - Catalyzed Modification of Cytidine to Uridine in PSTV. This reaction was performed mainly as described by Goddard and Maden (20). 0.6 A_{260} units of PSTV in 500 µl 3 M sodium bisulfite pH 5.9 were incubated for (a) 26 h / 5° C in presence of 20 mM $MgCl_2$, (b) 26 h / 25° C without $MgCl_2$, and (c) 96 h / 25° C without $MgCl_2$. The modified PSTV was obtained after a series of dialyses (20). This material was used for RNase A

and RNase T_1 digestion, 5'-^{32}P-labelling *in vitro*, fingerprinting and sequence analysis as described above for unmodified viroid. The oligonucleotide AUCCCCG (T-26, Table 1) was modified to AUUUUUG as described above for PSTV except that excess bisulfite was removed from the oligonucleotide by biogel P-2 chromatography.

RESULTS

Fingerprints and Sequence Analysis of Oligonucleotides Present in Complete RNase T_1 and RNase A Digests of PSTV.

Fig. 1 shows the fingerprints of complete RNase T_1 (Fig. 1A) and RNase A (Fig. 1B) digests of PSTV. The corresponding nucleotide sequences and molar yields of these 5'-labelled oligonucleotides are presented in Table 1. The sequences listed in this table were determined by (a) complete nuclease P_1 digestion for 5'-end group analysis, (b) partial digestion with nuclease P_1 followed by onedimensional DEAE-cellulose paper ionophoresis and by twodimensional ionophoresis / homochromatography (21, 22, 23). As an example for the latter method, Figs. 2A and 2B show the sequence analyses of fragments T-37 and T-26 from Fig. 1A. It is evident that the mobility shifts shown here, together with 5'-end group analyses and sequence analyses using onedimensional DEAE-cellulose paper electrophoresis (not shown), give the information needed to establish the oligonucleotide sequences. The separation of coinciding oligonucleotides which is needed for quantitation and sequence analysis was achieved by running the first dimension on 100 cm cellulose acetate strips, whereby the marker dye, xylene cyanol, was allowed to move 50 cm.

The uncorrected molar yields given in Table 1 are the result of Cerenkov counting of spots from several independent fingerprints of PSTV. For these calculations, the molar yields of the oligonucleotides A-12 (AAAC) and T-19 (AAAAG), respectively, were defined to be 1.0, because short oligonucleotides with several adenosines at the 5'-end are practically quantitatively phosphorylated. Low molar ratios, according to our experience, occur in the case of guanosine rich and/or long oligonucleotides. The corrected molar ratios in Table 1 were obtained by cross-checking the oligonucleotide sequences from the RNase A

spot	A	a	b	spot	B	a	b
T-1	CG	6.4	6	A-1	AC	6.7	7
T-2	UG	7.3	7	A-2	GC	13.1	13
T-3	AG	8.0	8	A-3	AU	1.0	1
T-4	CCG	2.0	2	A-4	GU	5.9	6
T-5	CUG	1.2	1	A-5	AAC	0.9	1
T-6	UCG	1.2	1	A-6	AGC	1.7	2
T-7	UUG	1.0	1	A-7	GAC	2.0	2
T-8	CAG	2.7	3	A-8	GGC	3.1	4
T-9	ACG	0.9	1	A-9	AAU	1.0	1
T-10	AAG	2.1	2	A-10	AGU	1.4	1
T-11	CCCG	1.2	1	A-11	GGU	4.2	5
T-12	CUCG	1.1	1	A-12	AAAC	1.0	1
T-13	ACAG	0.9	1	A-13	GAAC	0.8	1
T-14	CUUCG	2.2	2	A-14	GAGC	0.8	1
T-15	CCCAG	0.8	1	A-15	GGGU	0.6	1
T-16	UUUAG	1.1	1	A-16	GAAAC	0.7	1
T-17	AACCG	1.7	2	A-17	AGGAC	0.7	1
T-18	AACUG	0.8	1	A-18	GAAGC	0.5	1
T-19	AAAAG	1.0	1	A-19	GGAAC	1.4	2
T-20	CUCCCG	1.0	1	A-20	GGAGC	0.7	1
T-21	CCCUUG	0.7	1	A-21	GGGGC	0.3	1
T-22	UUCCUG	0.9	1	A-22	AGGGU	0.6	1
T-23	CUUCAG	0.9	1	A-23	GAGAAC	0.6	1
T-24	AAACAG	0.8	1	A-24	GGAAAC	0.6	1
T-25	UUCCUCG	0.7	1	A-25	AGGAGU	0.5	1
T-26	AUCCCCG	0.8	1	A-26	AGGGAU	0.5	1
T-27	AAACCUG	1.0	1	A-27	GAGGGU	0.4	1
T-28	AAAAAAG	1.1	1	A-28	GGGGAGU	0.2	1
T-29	ACCUCCUG	0.8	1	A-29	GGGGAAAC	0.3	1
T-30	CAAAAAAG	1.0	1	A-30	GGAGGAGC	0.2	1
T-31	CCCCCUUUG	0.9	1	A-31	AAAAAAGGAC	0.6	1
T-32	UAAUUCCCG	0.8	1	A-32	AGAAAAGAAAA-AAGAAGGC	0.1	1
T-33	AAACAACUG	0.9	1				
T-34	UCCUUCCUCG	0.8	1				
T-35	UUCACACCUG	0.7	1				
T-36	CUACUACCCG	0.7	1				
T-37	ACCACCCCUCG	0.8	1				
T-38	AACUAAACUCG	0.6	1				
T-39	CUUUUUCUCUA-UCUUACUUG	0.7	1				
T-40	UUUUCACCCUU-CCUUUCUUCG	0.6	1				

Table 1. Oligonucleotide sequences and molar yields from fingerprints of RNase T_1 (A) and RNase A (B) digests of PSTV. Uncorrected molar yields (a) were determined by Cerenkov counting of spots from several independent fingerprints; the molar yields of the oligonucleotides T-19 and A-12, respectively, were defined to be 1.0. Corrected molar ratios (b) were calculated as described in Results. There is yet a discrepancy, possibly in the number of dinucleotides, since only 43 RNase T_1 oligonucleotides end on PyrG, whereas 45 RNase A oligonucleotides begin with G.

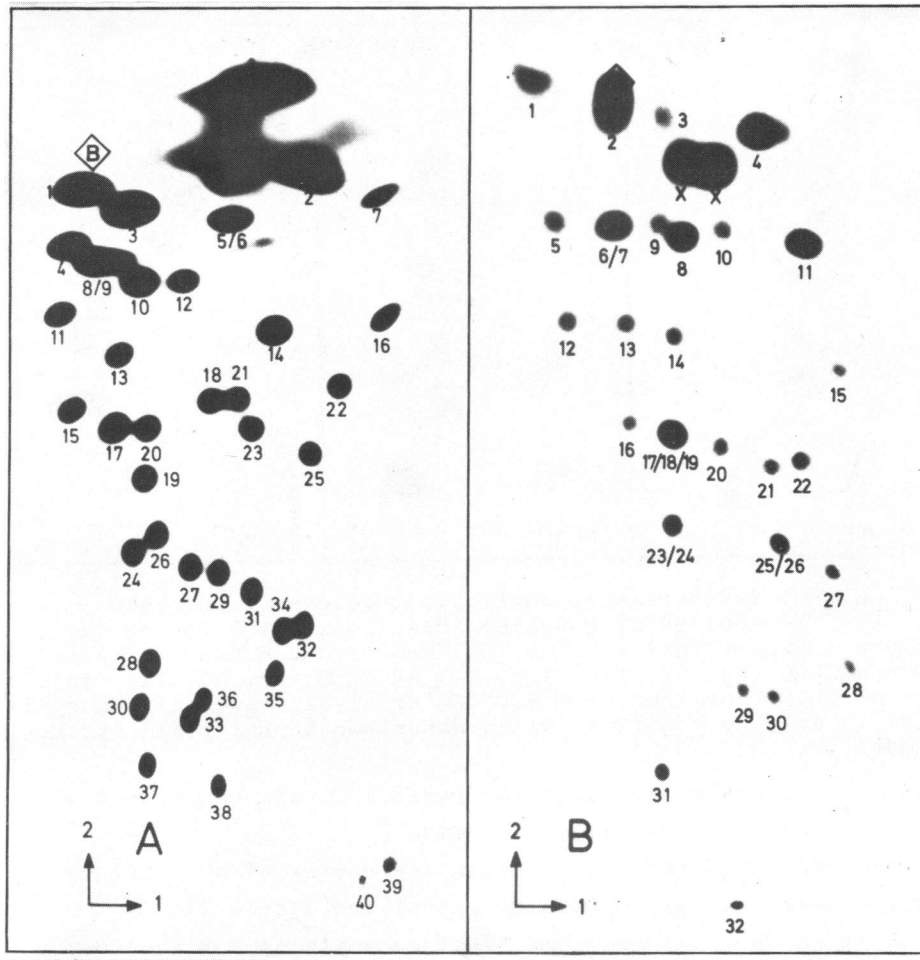

Fig. 1. Autoradiograms of 5'-^{32}P-labelled oligonucleotides obtained from RNase T_1 (A) and RNase A (B) digests of PSTV. First dimension: ionophoresis on cellulose acetate strips, pH 3.5; second dimension: homochromatography in 30 mM KOH "homomix" on DEAE-cellulose thinlayer plates. The sequences and molar yields of the oligonucleotides are compiled in Table 1. X = unidentified nucleoside 3',5'-diphosphates; B = position of the blue marker dye, xylene cyanol. In Fig. 1B the xylene cyanol marker coincides with oligonucleotide 2.

fingerprint with those from the RNase T_1 fingerprint, and vice versa. For example, the uncorrected molar ratio for the oligonucleotide A-32 is 0.1 and hence it could represent a contaminating impurity. However, its correctd molar ratio has to be 1.0, since the RNase T_1 oligonucleotides T-19 and

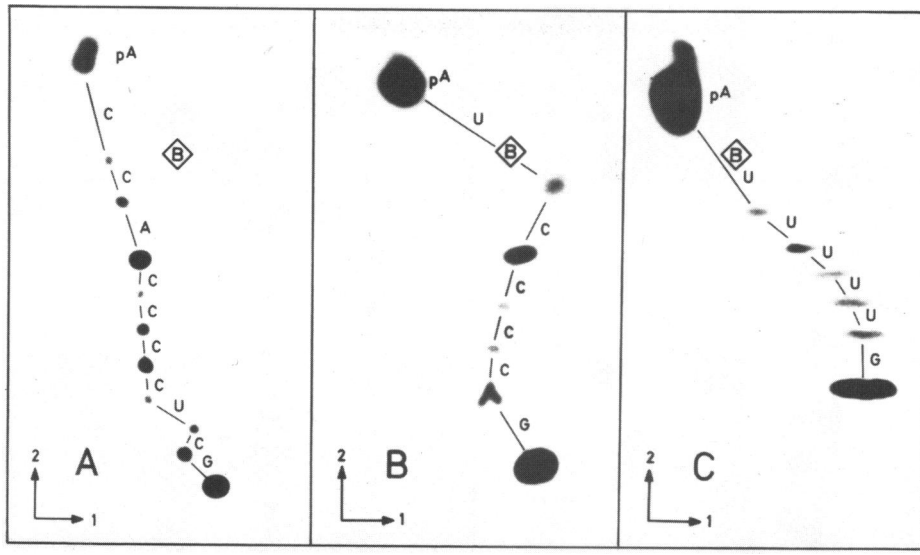

Fig. 2. Autoradiograms of controlled nuclease P_1 digests on 5'-^{32}P-labelled oligonucleotides. Separation was as in Figure 1 except that a 50 mM KOH "homomix" was used. A = ACCACCCCUCG (no. 37, Fig. 1A); B = AUCCCCG (no. 26, Fig. 1A); C = AUUUUUG (obtained from AUCCCCG by bisulfite-catalyzed cytidine to uridine modification as described in Materials and Methods).

T-28, each occuring in the molar ratio 1.0, are components exclusively of the A-32 oligonucleotide.

From the corrected molar ratios the number of nucleotides and the base composition can be calculated (Table 2). On the basis of these considerations, PSTV consists of 359 nucleotides corresponding to a molecular weight of 115,480; the A:U and G:C ratios being nearly 1.

Bisulfite-Catalyzed Modification of Reactive Cytidines to Uridines in PSTV.

Reversible addition of bisulfite to the 5,6-double bond of cytidine at slightly acidic pH causes deamination of this nucleoside to uridine (24, 25, 26). This modification is known to depend on the secondary structure of the RNA: cytidine is fully reactive in single-stranded regions, whereas it is resistant against modification in base-paired structures (20, 26).

Fig. 3 shows the fingerprints of PSTV after bisulfite treatment for 26 h at 5° C in presence of 20 mM $MgCl_2$ (3A and 3D), for 26 h at 25° C without $MgCl_2$ (3B and 3E), and for 96 h at

	N	% calculated from N
AMP	73	20.3
CMP	107	29.8
GMP	102	28.4
UMP	77	21.4
	359	

Table 2. Nucleotide composition of PSTV. N, the number of nucleotides was calculated from the oligonucleotides listed in Table 1.

25° C without $MgCl_2$ (3C and 3F). These fingerprints, if compared to those in Fig. 1, contain one group of oligonucleotides which remain in the same position as in Fig. 1. These oligonucleotides do not contain cytidine at all, or their cytidines are resistant against this modification. The other group are modified oligonucleotides generated by modification of reactive cytidines to uridines: As can be seen in Fig. 3, the modification of each cytidine to uridine causes a defined shift towards the right due to an increased electrophoretic mobility in the first dimension of the fingerprint. The results of the reactivity studies are summarized in Table 3, in which the oligonucleotides containing the most reactive and the most resistant cytidines are listed. The oligonucleotides not present in this Table do not contain cytidine or have an intermediate reactivity.

The modification conditions which we applied on PSTV are partly identical to those used for ribosomal 28S RNA (20). Compared with other nucleic acids, viroid RNA shows a far higher reactivity than expected for a rod-like, perfectly base-paired structure. Fig. 3 shows that, at least under the more stringent conditions, most of the oligonucleotides have an increased electrophoretic mobility in the first dimension of the fingerprints indicating a cytidine to uridine modification. The oligonucleotides, marked with an asterisk in Fig. 3, have been characterized by sequence analysis as described for other oligonucleotides in Fig. 2. The informations compiled from the sequence analyses of the modified and unmodified oligonucleotides have been summarized in schematic presentations, Fig. 4A and 4B. These diagrams display the correlation between the

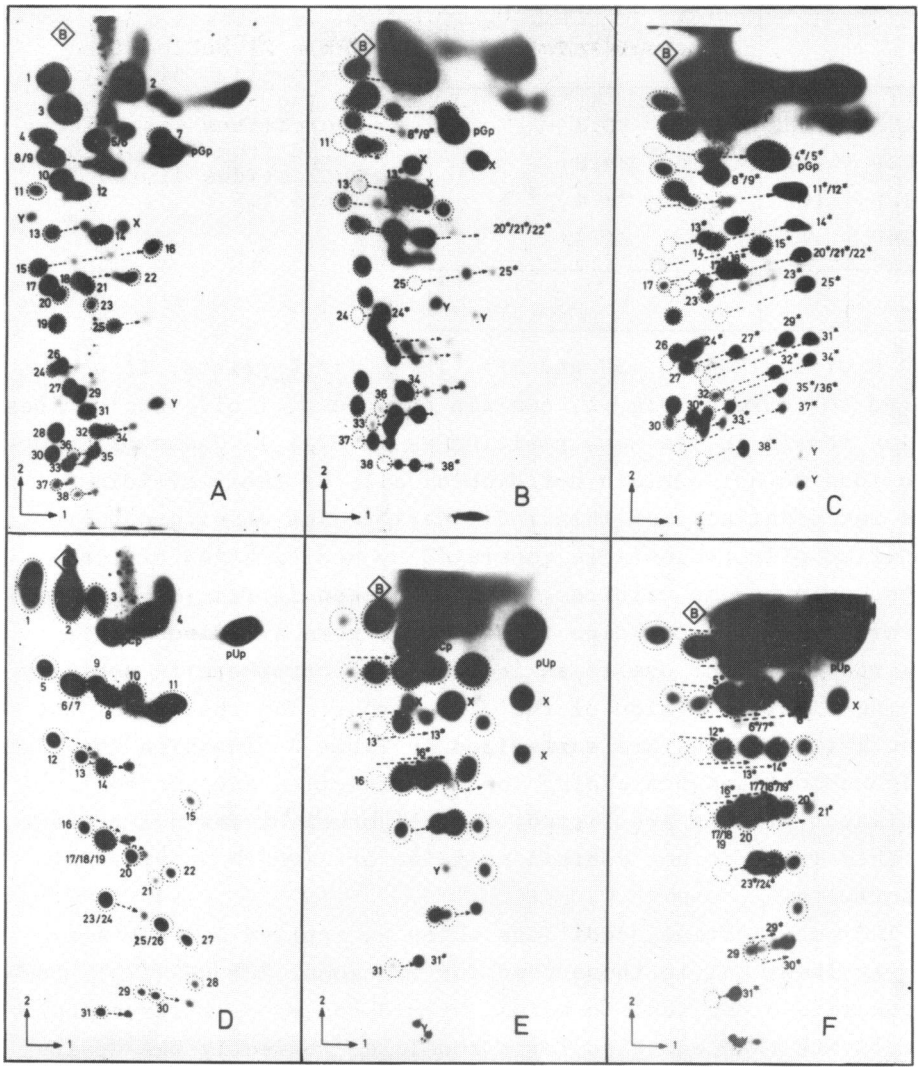

Fig. 3. Autoradiograms of 5'-^{32}P-labelled oligonucleotides obtained from RNase digestions of bisulfite-treated PSTV. A, D : Bisulfite treatment for 26 h at 5° C in 20 mM $MgCl_2$. B, E: Bisulfite treatment for 26 h at 25° C. C, F: Bisulfite treatment for 96 h at 25° C. A, B, C: RNase T_1 fingerprints; D, E, F: RNase A fingerprints. Dotted circles indicate the original position of the oligonucleotides before modification (Fig. 1). Dotted lines follow the mobility shifts caused by cytidine to uridine modification. Oligonucleotides with completed cytidine to uridine modification are marked with an asterisk. X: dinucleoside triphosphates and trinucleotide tetraphosphates (due to incomplete 3'-dephosphorylation); Y: unidentified. The results of this Figure are summarized in Table 3.

A		B	
T-11	(CCCG)	T-14	(CUUCG)
T-13	(ACAG)	T-17	(AACCG)
T-24	(AAACAG)	T-23	(CUUCAG)
T-25	(UUCCUCG)	T-26	(AUCCCCG)
T-33	(AAACAACUG)	T-27	(AAACCUG)
T-34	(UCCUUCCUCG)	T-30	(CAAAAAAG)
T-37	(ACCACCCCUCG)	T-32	(UAAUUCCCG)
T-38	(AACUAAACUCG)		
A-13	(GAAC)	A-17	(AGGAC)
A-16	(GAAAC)	A-18	(GAAGC)
A-31	(AAAAAAGGAC)	A-19	(GGAAC)
		A-20	(GGAGC)
		A-29	(GGGGAAAC)

Table 3. Oligonucleotides containing very bisulfite-sensitive (A) and very bisulfite-resistant (B) cytidines. This Table summarizes the results of the bisulfite modifications of PSTV (Fig. 3).

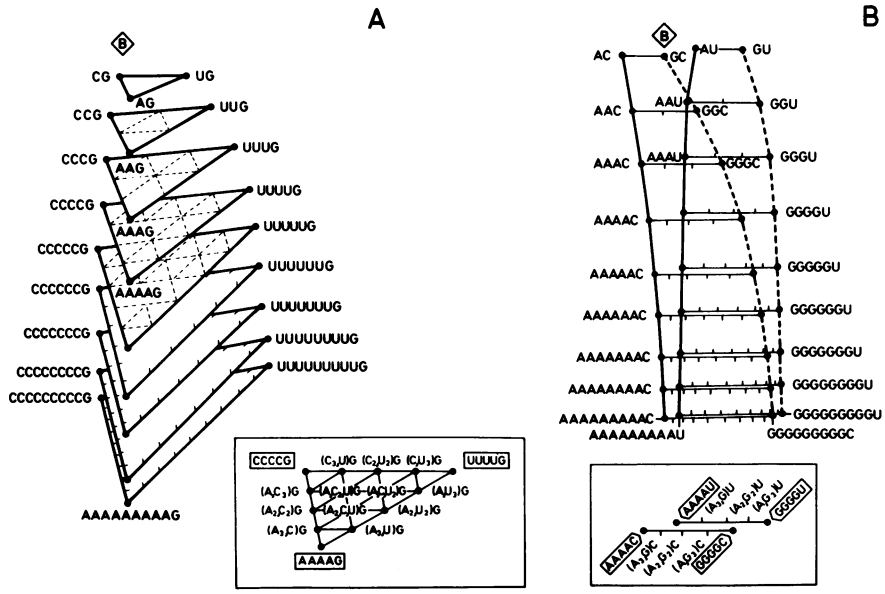

Fig. 4. Schematic correlation between the brutto nucleotide composition of any possible RNase T_1 (A) or RNase A (B) oligonucleotide and its position in homochromatography fingerprints as shown in Figs. 1, 2 and 3.
A detailed presentation of possible pentanucleotides is shown in boxes.

brutto nucleotide composition of any possible RNase T_1 or RNase A oligonucleotide and its location on the homochromatography fingerprint used in this work.

In contrast to the reactive majority, only very few oligonucleotides derive from bisulfite resistant structures of PSTV; these oligonucleotides are marked in Fig. 3 by numbers without asterisk (compare Fig. 1). Such an example for resistance against modification are the four cytidines in the oligonucleotide AUCCCCG (T-26; Fig. 1 and Fig. 3), the sequence analysis of which is shown in Fig. 2B. Our sequencing method, however, leaves open the possibility that in such a case the resistance against bisulfite may be due to the presence of four modified, resistant cytidines, e.g. 5-methylcytidines (m^5C). In this case, the isolated fragment AUCCCCG should be equally resistant against bisulfite. The result of this control experiment is shown in Fig. 2C. It is evident that bisulfite treatment of isolated AUCCCCG (Fig. 2B) leads to AUUUUUG as sequenced in Fig. 2C. Hence the striking bisulfite resistance of these four cytidines can only be explained on the basis of their location in a region of particularly stable secondary structure of PSTV. Bioassays and the subsequent search for an extra viroid band in RNA preparations from the corresponding plants have shown that bisulfite modified PSTV has lost infectivity because it is no more replicated in the inoculated tomato host plant.

DISCUSSION

Previous fingerprint studies of three plant viroids revealed their individuality, homogeneity and purity (14). In this report we present the first detailed study of the oligonucleotide sequences of a viroid, namely of PSTV. We have decided to apply postlabelling with γ-^{32}P ATP and polynucleotide 5'-kinase from T4 phage-infected E.coli, which has been successfully used for sequence studies of tRNAs, mRNAs and viral RNAs (18, 21, 22, 27, 28). Other approaches had to be excluded: In vivo labelling of viroid RNA is rather inefficient due to low specific activity of the labelled RNA; the use of in vitro labelling with radioactive iodine isotopes is very limited for the reasons detailed earlier (14). For sequencing we used nuclease P_1 (Fig. 2) since the oligonucleotides isolated from

homochromatography fingerprints contain several A_{260} units of carrier RNA. Due to its high specific activity only very low amounts of this enzyme are required in contrast to snake venom phosphodiesterase, which causes streaking in the first dimension, because it has to be applied in high concentrations.

From the established sequences and yields of the oligonucleotides of PSTV the following conclusions can be drawn: (a) no sequence heterogeneity was detected in the RNase A and RNase T1 oligonucleotides; (b) the different oligonucleotides are obtained in molar ratios if certain corrections by cross-checking are considered ("corrected" molar yields, Table 1); (c) the number of about 359 nucleotides for PSTV as obtained from the nucleotide composition in Table 2 corresponds to a molecular weight of about 115,500, corresponding to 119,500 in the ultracentrifuge after correction for Mg^{++} and Na^{+} according to Krakauer (29). This calculation is in fair agreement with earlier results (11). Only corrected molar yields have to be taken into account since in certain cases the uncorrected molar yields are too low either due to secondary degradation or due to low phosphorylation because of guanosine rich 5'-sequence or secondary structure of the oligonucleotide, i.e. selfcomplementarity or association of oligonucleotides. In those cases, for which it is not possible to cross-check corresponding RNase A and RNase T_1 fragments (e.g., GGGGC, A-21, Table 1), an uncorrected molar ratio of 0.3 indicates its presence at least once in the molecule since our viroid preparations are at least 99% pure (11). Thus even minor spots have to be considered unless they are identified by sequence analysis as secondary degradation products of complete oligonucleotides.

The 5'-end group analysis of the oligonucleotides listed in Table 1 did not provide any evidence for modified nucleotides in this position, and other methods will be needed to search for possible nucleoside modification. Interesting features of the viroid molecule are the absence of an AUG initiation triplet and the presence of a remarkable sequence of 18 purines, mainly adenosines, in a 19 nucleotide long RNase A fragment (A-32). Only one RNase A fragment (AGGGAU; A-26) can be con-

nected to a RNase T_1 fragment (AUCCCCG; T-26) to form the sequence AGGGAUCCCCG.

A further aspect to be discussed here is the possible intramolecular complementarity of the viroid RNA circle needed to form the stable rod-like structure derived from electron microscopy, analytical ultracentrifugation and from melting and temperature jump experiments (11, 30). An inspection of the oligonucleotides, however, precludes a long, perfectly helical region as a major structural element in PSTV. From the rod-like secondary structure and from the absence of extended base pairing we have to conclude that short unpaired loop regions are connected by short helices in a serial arrangement. This concept is corroborated by the unexpected, high reactivity of PSTV against the secondary structure-dependent, bisulfite-catalyzed modification of cytidines to uridines.
In fact, recent more quantitative analysis of the denaturation process of viroids (31) also shows that the high cooperativity and relatively low temperature of melting results from helical defects which are evidently distributed over the whole molecule.
In this work the unique secondary structure of a viroid, PSTV, has been discussed on the basis of established oligonucleotide sequences and secondary structure dependent modification. The establishment of the total nucleotide sequence of a viroid will be a significant step towards understanding a biological enigma.

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REFERENCES

1 Diener, T.O. (1971) Virology 45, 411-428
2 Sänger, H.L. (1972) Advances Biosci. 8, 103-116
3 Semancik, J.S. and Weathers, L.G. (1972) Nature New Biology 237, 242-244

4 Hollings, M. and Stone, O.M. (1973) Annals Appl. Biology 74, 333-348
5 Diener, T.O. and Lawson, R.H. (1973) Virology 51, 94-101
6 Van Dorst, H.J.M. and Peters, D. (1974) Netherlands J. Plant Pathol. 80, 85-96
7 Romaine, C.P. and Horst, R.K. (1975) Virology 64, 86-95
8 Randles, J.W. (1975) Phytopathology 65, 163-167
9 Randles, J.W., Rillo, E.P. and Diener, T.O. (1976) Virology 74, 128-139
10 Sasaki, M. and Shikata, E. (1977) Proc. Japan. Acad. 53, Ser. B., 109-112
11 Sänger, H.L., Klotz, G., Riesner, D., Gross, H.J. and Kleinschmidt, A.K. (1976) Proc. Natl. Acad. Sci. USA 73, 3852-3856
12 McClement, W.L. and Kaesberg, P. (1977) Virology 76, 477-484
13 Owens, R.A., Erbe, E., Hadidi, A., Steere, R.L. and Diener, T.O. (1977) Proc. Natl. Acad. Sci. USA 74, 3859-3863
14 Gross, H.J., Domdey, H. and Sänger, H.L. (1977) Nucleic Acids Res. 4, 2021-2028
15 Fujimoto, M., Kuninaka, A. and Yoshino, H. (1969) Agric. Biol. Chem. 33, 1517-1518
16 Glynn, I.M. and Chappell, J.B. (1963) Biochem. J. 90, 147-149
17 Singh, A. and Sänger, H.L. (1976) Phythopath. Z. 87, 143-160
18 Simsek, M., Ziegenmeyer, J., Heckman, J. and RajBhandary, U.L. (1973) Proc. Natl. Acad. Sci USA 70, 1041-1045
19 Sanger, F., Brownlee G.G. and Barrell, B.G. (1965) J. Mol. Biol. 13, 373-398
20 Goddard, J.P. and Maden, B.E.H. (1976) Nucleic Acids Res. 3, 431-440
21 Silberklang, M., Gillum, A.M. and RajBhandary, U.L. (1977) Nucleic Acids Res. 4, 4091-4108
22 Koper-Zwarthoff, E.C., Lockard, R.E., Alzner-DeWeerd, B., RajBhandary, U.L., and Bol, J.F. (1977) Proc. Natl. Acad. Sci. USA 74, 5504-5508

23 Alberty, H., Raba, M. and Gross, H.J. (1978) Nucleic Acids Res. 5, 425-434
24 Shapiro, R., Servis, R.E. and Welcher, M. (1970) J. Amer. Chem. Soc. 92, 422-424
25 Hayatsu, H., Wataya, Y., Kai, K. and Jida, S. (1970) Biochemistry 9, 2858-2864
26 Goddard, J.P. and Shulman, L.H. (1972) J. Biol. Chem. 247, 3864-3867
27 Szekely, M. and Sanger, F. (1969) J. Mol. Biol. 43, 607-617
28 Frisby, D. (1977) Nucleic Acids Res. 4, 2975-2996
29 Krakauer, H. (1972) Biopolymers 11, 811-828
30 Henco, K., Riesner, D. and Sänger, H.L. (1977) Nucleic Acids Res. 4, 177-194
31 Langowski, J., Henco, K., Riesner, D. and Sänger, H.L., (1978) submitted to Nucleic Acids Res.