Volume 12 Number 8 1984 Nucleic Acids Research

Nucleotide sequence of cucumber pale fruit viroid: homology to hop stunt viroid

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Received 13 March 1984; Accepted 30 March 1984

ABSTRACT

Double stranded cDNA of cucumber pale fruit viroid (CPFV) has been cloned by the method of Okayama and Berg (Mol.Cell.Biol.2,161-170 (1982)) and the complete nucleotide sequence was established. The covalently closed circular molecules of single-stranded CPFV RNA consists of 303 nucleotides. The nucleotide sequence of CPFV was compared with the previously established sequence of hop stunt viroid (HSV), which consists of 297 nucleotides (Ohno et al. Nucleic Acid Res.11,6185-6197 (1983)). CPFV differs from HSV in the nucleotide sequence at 16 positions which include 8 exchanges, 7 insertions and 1 deletion. Both viroids share about 95% sequence homology. Considering the pathogenic properties of both viroids together, it is concluded that CPFV is a cucumber isolate of HSV.

INTRODUCTION

Viroid are infectious low molecular weight RNA species causing serious disease on higher plants (1). Eight viroids have been sequenced so far and shown to form highly base-paired unique rod-like secondary structures. Comparative sequence analysis of these viroids shows that potato spindle tuber viroid (PSTV) (2), citrus exocortis viroid (CEV) (3), chrysanthemum stunt viroid (CSV) (3,4), tomato planta macho viroid (TPMV) (5), and tomato apical stunt viroid (TASV) (5), belonging to "PSTV group", share 60-80% sequence homology (6). On the other hand, coconut cadang-cadang viroid (CCCV) (7), avocado sunblotch viroid (ASBV) (8) and hop stunt viroid (HSV) (9) are distantly related to "PSTV group".

Cucumber pale fruit viroid (CPFV) and HSV were found to be the causal agents of cucumber pale fruit disease (10) and hop stunt disease (11-13), respectively. They were found independently in different host plants, cucumber (Cucumis

sativus L.) and hop (Humulus lupulus L.), in the separated countries. Recently, we have reported that although both viroids were not distinguishable by their pathogenic properties (14), they could separate each other by polyacrylamide gel electrophoresis and CPFV was larger than HSV by only several nucleotides (15).

In this paper, we describe molecular cloning of cDNA of CPFV and the complete nucleotide sequence. HSV and CPFV share very high sequence homology.

MATERIALS AND METHODS

Enzymes.

PolyA polymerase was prepared from E.coli B/r (16). AMV reverse transcriptase was purchaced from Seikagaku Kogyo Co., E.coli DNA polymerase I, E.coli RNase H and terminaldeoxynucleotidyl transferase were purchased from BRL. E.coli DNA ligase was obtained from New England Biolabs. Restriction enzymes, T4 polynucleotide kinase and E.coli alkaline phosphatase were from Takara Shuzo Co., Kyoto.

Purification of CPFV.

CPFV, kindly provided by Dr.H.L.Sänger (Max-Planck Inst.), was maintained and propagated in cucubmber plants (C.sativus cv. Sūyō). Cucumber pale fruit viroid RNA was purified by the method described by Uyeda et al. (17). For the final steps of the purification, CPFV RNA was separated from cellular RNAs by 15% polyacrylamide gel electrophoresis under non-denaturing condition. CPFV RNA which contained both circular and linear molecules, was eluted from the gel, absorbed to and eluted from DEAE-cellulose (Whatman DE-32) to remove gel contaminants and used for dot blot hybridization analysis and molecular cloning.

Dot blot hybridization.

Sequence homology between HSV and CPFV was tested preliminary by dot blot hybridization analysis (18). ^{32}P-labelled pHSV-A60 (9), with a specific activity of 10^7 cpm/μg DNA, was prepared by nick-translation (19) and used as prove for hybridization.

Molecular cloning of CPFV.

The purified CPFV RNA was treated with E. coli alkaline

phosphatase to remove 3'-terminal phosphate residues from linear molecules, polyadenylated with polyA polymerase, fractionated by oligo (dT) cellulose affinity chromatography to recover polyadenylated CPFV linear molecules, and CPFV cDNA copies were cloned by the method of Okayama and Berg (20) with some modifications as described by Ohno et al (19). Ampicillin-resistant E. coli (HB 101) transformants were screened by in situ colony hybridization using ^{32}P-CPFV RNA fragments which were prepared as described by Ohno et al (9).

DNA sequencing.

Plasmid DNA was purified by the method of Katz et al. (21)with slight modification. Restriction fragments were prepared using restriction enzymes Eco RI, Saw3A I, Pst I, Xho I, Bam HI for pCP-55, and Hind III, Xho I, Pst I for pCP-61 and pCP-103. DNA sequencing was performed by the method of Maxam and Gilbert (22) with a slight modification.

RESULTS

Cross-hybridization between HSV and CPFV by dot blot hubridization.

Successive three-fold dilutions of purified CPFV RNA (10, 3.3, 1.1, 0.4, 0.12, 0.04 ng) in 1 µl H_2O were bloted onto nitrocellulose paper. The dot blot hybridization was performed using ^{32}P-labelled pHSV-A60 which contains full length cDNA of HSV RNA as probe. As shown in Fig. 1, ^{32}P-labelled pHSV-A60

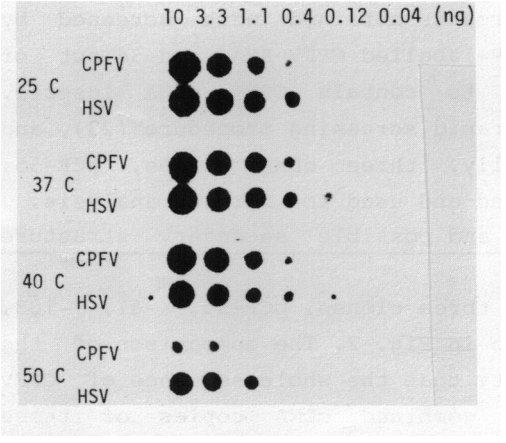

Fig. 1. Autoradiograph of a dot blot hybridization of CPFV RNA and HSV RNA probed with nick-translated pHSV A-60 containing the complete cDNA to HSV RNA. After hybridization, blots were washed at 25, 37, 40 and 50 C.

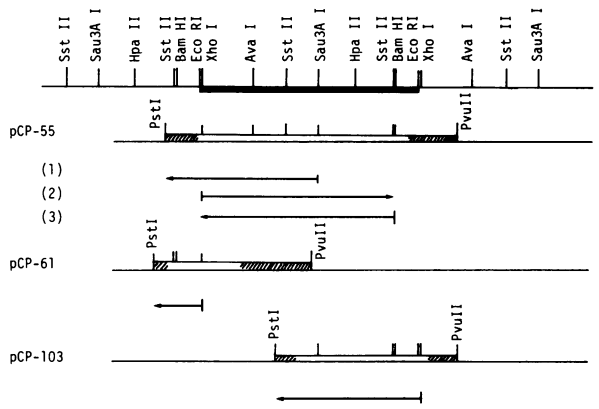

Fig. 2. Restriction maps of the cloned cDNA copies of CPFV and the strategy for DNA sequencing. The map of supposed tandemly repeated CPFV sequence is shown at the top. The unit length of CPFV is shown by a thick line as one example. Open horizontal bars show the cloned cDNA region including dG-dC and dA-dT tails (hatched area). The direction and extent of sequence determination are indicated by arrows. Virtical bars of the arrows indicate the labeled 5' ends of sequenced fragments.

hybridized not only with the homologous CPFV RNA and the hybrids were stable at 37 C, 40 C and 42 C. A large amount of the hybrids, especially of the heterologous hybrids, was dissociated by washing at 50 C. The result suggested that the nucleotide sequences of HSV and CPFV are highly homologous but not identical. Neither the same amount of PSTV RNA nor 100 ng of low molecular weight RNAs from healthy cucumber plants formed hybrids (data were not shown).

Molecular cloning of CPFV cDNA.

Ampicillin-resistant transformants obtained were screened by colony hybridization with ^{32}P-labelled CPFV RNA, and 34 out of 214 transformants were shown to contain CPFV cDNA inserts. Plasmid DNA was analyzed by rapid screening procedure (23), and the restriction map. Finally, three cDNA clones, pCP-55, pCP-61, pCP-103, were selected and used for further analysis.

Complete nucleotide sequence and possible secondary structure of CPFV.

The restriction maps of three clones, pCP-55, -61, -103, used for sequencing are shown in Fig. 2. The comparison of the maps indicates the possibility that the whole sequence of CPFV unit length is integrated in combined cDNA copies of these

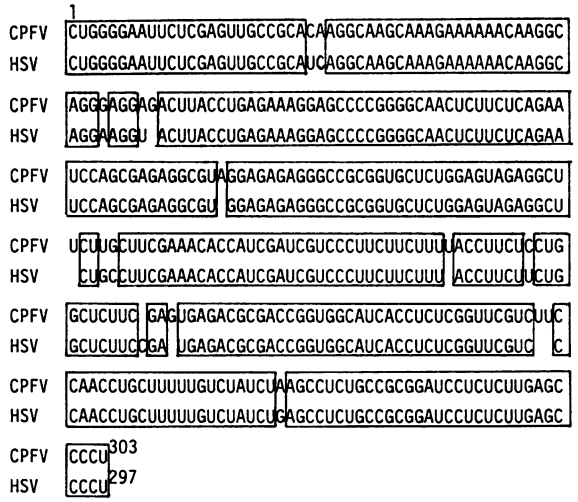

Fig. 3. An alignment of the sequences of CPFV and HSV. The residues of CPFV sequence common to those of HSV are boxed.

three clones. The assembly of the sequences of these cDNA inserts could construct the circular CPFV RNA molecule consisting of 303 nucleotides (Fig. 3). No sequence heterogeniety was found in these three cNDA inserts. CPFV differs from HSV in the nucleotide sequence at 16 positions which include 8 exchanges, 7 insertions and 1 deletion and both viroids share about 95% sequence homology. A possible secondary model for the native CPFV RNA was constructed using the computer program described by Zuker and Stiegler (24). The result indicated, as shown in Fig. 4., that CPFV can form rod-like conformation with extensive base-pairing characteristic to the other viroids.

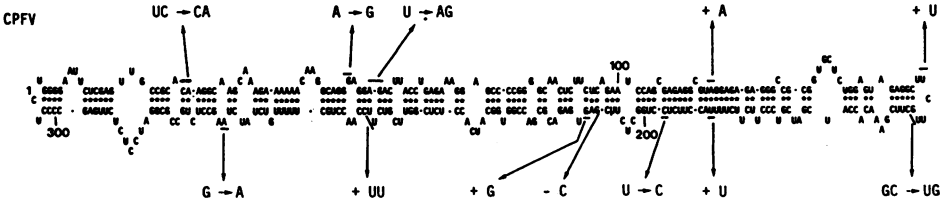

Fig. 4. Secondary structures of CPFV predicted from the nucleotide sequences. It is shown to provide maximum sequence and structural homology with the proposed secondary structures of other viriods sequenced. The numbering of residues follows the convention established for PSTV.

Table 1. Comparison of chain length, sequence homology and nucleotide difference among individual viroids and viroid isolates (or strains).

	numbers of nucleotides	sequence homology[1)] with PSTV	among isolates	numbers of nucleotides changed among isolates (or strains) exchange	insertion	deletion	References
PSTV Type	359	100%	100%	—	—	—	(2)
Mild	359		99	2	1	1	(6)
Severe	359		99	4	0	0	(6)
TPMV	360	83					(5)
TASV	360	73					(5)
CEV A	371		100	—	—	—	(25)
AM	371		99	4	0	0	
C	371	73	99	4	0	0	
DE 25	371		99	4	0	0	
DE 26	371		93	15	6	6	
CSV E	354	73	100	—	—	—	(3)
A	356	69	97	6	4	2	(4)
HSV	297	55	100	—	—	—	(9)
CPFV	303	55	95	8	7	1	
ASBV	247	18					(8)
CCCV RNA 1f	246	11					(7)

1). Sequence homology was calculated from sequences aligned for maximum homology ; (numbers of nucleotide homologous between two viroids)/(total numbers of nucleotide of viroid compared with PSTV or Type isolate).

DISCUSSION

The complete nucleotide sequence of CPFV was established. The covalently closed single-stranded circular molecule of CPFV RNA consists of 303 nucleotides, which are larger than HSV by 6 nucleotides. The result is well agreed with our previous finding that CPFV was larger than CPFV by several nucleotides (15).

The nucleotide sequences of eight viroids, PSTV (2,6), CEV (3, 25, 26), CSV (3,4), CCCV (7), ASBV (8), HSV (9), TPMV (5) and TASV (5) has been reported. The differences in nucleotide sequence among these individual viroids and viroid isolates (or strains) were summarized, and the nucleotide sequence of CPFV determined in this experiment was compared with those of HSV and other viroids (Table 1). Among individual viroids, CEV, CSV, TPMV and TASV, which belong to "PSTV group", share 69-83% sequence homology with PSTV Type strain, and each viroid consists of different number of nucleotide from PSTV (6). On the other hand, the viroid isolates (or strains), such as 3 strains of PSTV, 5 isolates of CEV and 2 isolates of CSV share 99, 93-99 and 97% sequence homology respectively. Total numbers of nucleotides are the same among PSTV strains and CEV isolates, though there are some base exchanges, insertions or

deletions. On the other hand, total numbers of nucleotides of English isolate and Australian isolate of CSV, 354 and 356 respectively, are different, and the differences in nucleotides are 6 exchanges, 4 insertions and 2 deletions.

In this paper, our result indicates that the total number of CPFV nucleotides is 303, which include 8 exchanges, 7 insertions and 1 deletion from HSV nucleotide sequence and both viroids share about 95% sequence homology. As we have reported previously, HSV and CPFV were not distinguishable in their pathogenic properties (14). Both HSV and CPFV dveloped stunting, vein-clearing and leaf curling on cucurbitaceous plants including cucumber, caused latent infection to tomato, and did not infect Gynura. The finding of high sequence homology between HSV and CPFV strongly supports the similarlity of biological nature of both viroids. We concluded, thereore, that CPFV is a cucumber isolate of HSV.

ACKNOWLEDGMENT

We are grateful to Dr.K.Fujinaga and Dr.Y.Shimizu (Institute for Cancer. Sapporo Medical college) for their useful advices, and Dr.H.L.Sänger (Max-Planck Inst.) for supplying CPFV.

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