Hop stunt viroid: molecular cloning and nucleotide sequence of the complete cDNA copy

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ABSTRACT

The complete cDNA of hop stunt viroid (HSV) has been cloned by the method of Okayama and Berg (Mol.Cell.Biol.2,161-170.(1982)) and the complete nucleotide sequence has been established. The covalently closed circular single-stranded HSV RNA consists of 297 nucleotides. The secondary structure predicted for HSV contains 67% of its residues base-paired. The native HSV can possess an extended rod-like structure characteristic of viroids previously established. The central region of the native HSV has a similar structure to the conserved region found in all viroids sequenced so far except for avocado sunblotch viroid. The sequence homologous to the 5'-end of Ula RNA is also found in the sequence of HSV but not in the central conserved region.

INTRODUCTION

Viroids are small single-stranded circular RNA molecules which replicate and cause several infectious disease in cultivated plants (1). The complete molecular structures of several viroids have been established including potato spindle tuber viroid (PSTV, ref.2), chrysanthemum stunt viroid (CSV, ref.3,4), citrus exocortis viroid (CEV, ref.4,5), avocado sunblotch viroid (ASBV, ref.6) and coconut cadang-cadang viroid (CCCV, ref.7). These RNAs range in size from 246 to 371 residues and form extended rod-like structure with a high degree of intramolecular base-pairing.

Stunt disease of hop (*Humulus lupulus*, L.) was first described in Japan in 1970 (8), and aetiological agent of the disease was found to be a viroid (9, 10). We recently purified the hop stunt viroid (HSV) and have shown that HSV possesses biochemical properties similar to other viroids identified so far. HSV is a single infectious RNA species whose size is 290-300 nucleotides in length (11).

The detection of viroid is not so easy in general, because viroid lacks antigenic protein coat characteristic of virus. Bioassay even on suitable test plants takes time and is not so reliable. Detection by acrylamide gel electrophoresis is laborious. One better method developed (12) is the

hybridization of radioactive viroid cDNA with RNA extracts from plants. Cloned viroid cDNA is useful not only for the diagnosis of viroid disease but also for studying the replication mechanism. Regeneration in vitro or in vivo of biologically active viroid RNA from cloned cDNA would enable us to study thoroughly viroid replication and pathogenicity.

We report here molecular cloning of full-length cDNA of HSV and the complete nucleotide sequence.

MATERIALS AND METHODS

Enzymes.

PolyA polymerase was prepared from E. coli B/r (13). AMV reverse transcriptase was kindly supplied from Dr. Ishihama, Kyoto University. E. coli DNA polymerase I, E. coli RNase H and terminal deoxynucleotidyl transferase were purchased from BRL. E. coli DNA ligase was obtained from New England BioLabs, and restriction enzymes, T4 polynucleotide kinase and E. coli alkaline phosphatase were from Takara Shuzo Co., Kyoto.

Purification of HSV.

HSV RNA was purified from infected cucumber leaves (Cucumis sativus L. cv. Suuyo) as described (11). Final purification involved two cycles of polyacrylamide gel electrophoresis under nondenaturing condition and yielded a mixture of circular and linear HSV ($\text{HSV}_{\text{C+L}}$). To isolate circular (HSV_{C}) and linear molecules (HSV_{L}) the second electrophoresis was performed under denaturing condition (14).

Preparation of LiCl soluble nucleic acids fraction.

Total nucleic acids from HSV-infected or healthy cucumber leaves were prepared by direct phenol extraction (11). After removal of polysaccharides by 2-methoxyethanol extraction nucleic acids were precipitated with cetyltrimethylammonium bromide. LiCl soluble nucleic acids were obtained by adding an equal volume of 4M LiCl to solutions containing total nucleic acids.

Synthesis of HSV cDNA.

A mixture of $\text{HSV}_{\text{C+L}}$ and HSV_{L} , which was heated in H_2O at 70° for 5 min and chilled in dry ice-ethanol, was treated with 0.05 U of E. coli alkaline phosphatase per μg of RNA in a reaction mixture containing 50 mM Tris HCl (pH 7.9) and 0.1 mM MgCl_2 to remove 3'-terminal phosphate residues from linear molecules. After incubation at 37° for 1 hr the HSV was recovered by phenol extraction and ethanol precipitation. Poly A was added to HSV RNA as described (15) in a reaction mixture (650 μl) containing 4.5 μg of HSV and

65 μ l of poly A polymerase. Prior to polyadenylation, HSV RNA was heated and quenched as above. The reaction was carried out at 37° for 6 min. Polyadenylated HSV molecules were recovered by oligo (dT) cellulose affinity chromatography (16). The reaction mixture (20 μ l) for cDNA synthesis contained 50 mM Tris HCl (pH 7.9 at 42°), 10 mM MgCl₂, 10 mM DTT, 30 mM KCl, 1 mM each dATP, dTTP, dGTP, and [α -³²P]dCTP (2000 cpm/pmol), 2.5–5.0 μ g/ml of polyadenylated HSV_L, 5 μ g/ml of oligo(dT)_{12–18}, 4 mM sodium pyrophosphate, and 100 U/ml of reverse transcriptase. Prior to the reaction HSV RNA in H₂O was heated and quenched as above. cDNA synthesis was initiated by the addition of reverse transcriptase and continued at 42° for 1 hr. The reaction was stopped with 2 μ l of 0.5 M EDTA(pH 8.5). The cDNA was precipitated with ethanol, dissolved in 20 μ l of formamide(90%)-NaOH(30 mM)-EDTA(1 mM) solution, and analyzed by polyacrylamide gel electrophoresis under denaturing condition.

Molecular cloning of cDNA.

HSV cDNA copies were cloned by the method of Okayama and Berg (17) with some modifications. The vector-primer and the linker DNA were prepared from the plasmids p3-2-1 and pX as described (17-19). cDNA was synthesized as described above except that the reaction mixture (80 μ l) contained 1 mM [³H]dCTP (0.625 μ Ci/n mol), 25 μ g/ml of vector-primer instead of [α -³²P]dCTP and oligo (dT). Plasmid-cDNA:HSV RNA hybrid obtained was dissolved in 40 μ l of 140 mM sodium cacodylate - 30 mM Tris HCl(pH 6.8) buffer containing 1 mM CoCl₂, 0.1 mM DTT, 67 μ M [α -³²P] dCTP(4500 cpm/pmol), and 20 U of terminal deoxynucleotidyl transferase were added. The reaction was carried out at 37° for 9 min to add about 15 residues of dCMP per end. After cleavage at the unique Hind III site of the vector-primer, the vector-cDNA : HSV RNA derivatives, with a Hind III cohesive end and an oligo(dC) tail at the respective termini, was cyclized by *E. coli* DNA ligase (20 U/ml) using a linker DNA segment. The RNA strand of the insert was replaced by DNA by repair synthesis and nick-translation using *E. coli* DNA ligase (10 U/ml), *E. coli* RNase H (8 U/ml) and *E. coli* DNA polymerase I (40 U/ml) as described (17,19). Transformation of *E. coli* K-12 (strain HB101) was carried out as described (17).

Screening of recombinant clones.

Ampicillin-resistant *E. coli* transformants were screened for the presence of HSV cDNA by *in situ* colony hybridization (20). ³²P-HSV RNA fragments for the probe were prepared as follows. Purified HSV_C molecules (76 μ g/ml) were cleaved by incubating in 25 mM glycine-NaOH buffer (pH 9.0),

5 mM MgCl_2 at 37° for 3 hr (14). The 5' ends of the fragments were labeled in a reaction mixture (20 μl) containing 50 mM Tris HCl (pH 7.9 at 37°), 10 mM MgCl_2 , 5 mM DTT, 2.5 μM [$\gamma\text{-}^{32}\text{P}$] ATP (1000 Ci/mmol), 50 $\mu\text{g}/\text{ml}$ HSV fragments and 150 U/ml T4 polynucleotide kinase. After incubation at 37° for 30 min, the labeled RNA fragments were collected by ethanol precipitation.

Polyacrylamide gel electrophoresis and Northern blot hybridization.

RNA samples were fractionated by electrophoresis in a 10% polyacrylamide slab gel under nondenaturing condition or in a 5% polyacrylamide gel under fully denaturing condition as described (14). Fractionated RNA samples were transferred to DBM-paper for hybridization (21). A buffer mixture (50% formamide, 0.9 M NaCl, 50 mM Na phosphate buffer (pH 7.0), 5 mM EDTA, 0.1% SDS, 100 $\mu\text{g}/\text{ml}$ yeast tRNA and 0.1% each of BSA, Ficoll and polyvinyl pyrrolidone) was used for the prehybridization reaction (16 hr at 42°). The hybridization was carried out at 42° for 24 hr in the same buffer mixture except that the concentrations of BSA, Ficoll and polyvinyl pyrrolidone were decreased to 0.02% each and ^{32}P -labeled DNA probe was added at 5×10^5 cpm/ml. ^{32}P -labeled DNA with a specific activity of 5×10^7 cpm/ μg was prepared by nick-translation (22).

DNA sequencing.

Plasmid DNA was purified by the method of Katz *et al.* (23) with slight modification. Restriction fragments were prepared using restriction enzymes EcoRI, HhaI, HinfI, HapII, TaqI for pHSV-A48, BamHI, HindIII, AccI, PvuII, PstI for pHSV-A60 and Sau3AI, PstI, HinfI, PvuII for pHSV-C39. DNA sequencing was performed by the method of Maxam and Gilbert (24) with slight modification (25).

RESULTS

Synthesis of HSV cDNA.

HSV RNA used is a mixture of single-stranded circular and linear form, the length of which was estimated to be 290-300 nucleotides (11). There is, however, no information of the primary structure. In order to synthesize faithful and full-length cDNA copies we used polyadenylated linear HSV molecules as templates. Purified HSV_{C+L} and HSV_L, after treated with bacterial alkaline phosphatase, were polyadenylated at the 3' end using *E. coli* poly(A) polymerase. The condition used here was that determined in preliminary experiments to add on the average 60 residues of AMP to the 3' end of TMV RNA (15). The sizes of polyadenylated HSV obtained were in fact estimated to be mainly over 500 bases using single-stranded DNA

Fig. 1. Analysis of polyadenylated HSV RNA (lane a) and HSV cDNA (lane b) by polyacrylamide gel electrophoresis.³ Samples of ³²P-labeled HSV RNA added with ³H-poly A and ³²P-labeled HSV cDNA primed with oligo (dT) were analyzed by 5% polyacrylamide gel electrophoresis under denaturing condition and visualized by fluorography and autoradiography, respectively. HaeIII-cleaved M13mp7 single-stranded DNA fragments were used as size markers to give the scale on the right. The position of HSV_L RNA is shown.

fragments as size markers (Fig. 1a) and the length of poly(A) added were much longer than expected.

The polyadenylated HSV RNA could be used as a template for oligo (dT) primed cDNA synthesis. Fig. 1b shows the size of cDNA produced. In addition to cDNAs with various sizes corresponding to those of the template polyadenylated HSV, a distinct prominent band of about 300 bases in length, the size of HSV, can be seen.

Molecular cloning of HSV cDNA.

For cloning the cDNA copies of HSV RNA we used the method of Okayama and Berg (17). The method has several advantages over other conventional ones. cDNA synthesis was primed by the poly (dT) tails of vector-primer DNA and the direction of insert should be constant. The second strand cDNA synthesis was primed by the linker DNA having poly dG tail which anneals to the poly dC-tail added to the 3' end of the first strand cDNA of vector-cDNA : template RNA derivatives. This enables the full- or nearly full- length cDNA to be converted preferentially to duplex cDNA and to be cloned. The duplex cDNA was synthesized by nick-translation repair of the cDNA: RNA hybrid. This decreases the possibility that the second-strand synthesis does not proceed completely.

Fig. 2. Northern blot hybridization of RNA samples with ³²P-labeled cloned HSVcDNA. 0.6 µg and 0.5 µg of purified HSV (lane 1 and 2), 200 µg and 20 µg of LiCl soluble nucleic acids from HSV-infected cucumber leaves (lane 3, 4), 20 µg of LiCl soluble fraction from healthy cucumber leaves (lane 5) and 0.5 µg of purified CEV (lane 6) were subjected to 10% polyacrylamide gel electrophoresis under nondenaturing condition. After staining with EtBr (left), RNA samples were transferred to DBM-paper and hybridized with the ³²P-labeled pHSV-C39 fragments (right). The positions of HSV and CEV are shown by arrows.

Ampicillin-resistant transformants were screened by colony hybridization with ³²P-labeled HSV, rapid screening procedure (26) and comparison of the restriction maps. Three cDNA clones, pHSV-A48, -A60 and -C39 were selected for further analysis.

Confirmation of a HSV cDNA clone by Northern blot hybridization.

It was confirmed by Northern blot hybridization that the selected recombinant contains the sequence of HSV and not any unrelated sequences contaminated in HSV preparation. To eliminate the possibility that poly dT-tail included in a recombinant plasmid hybridizes to polyA-containing RNA in RNA samples, the Bam HI-Hind III fragment containing a cDNA sequence of 280 bp (residues 296-278) derived from clone pHSV-C39 (see below) was used as the hybridization probe. Fig. 2 shows that purified HSV RNA and only the

Fig. 3. Restriction maps of the cloned cDNA copies of HSV and the strategy for DNA sequencing. The map of supposed tandemly repeated HSV sequence is shown at the top. The unit length of HSV 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 the arrows. Vertical bars of the arrows indicate the labeled 5' ends of sequenced fragments.

corresponding RNA fraction in LiCl-soluble nucleic acids samples from HSV-infected cucumber leaves hybridize with the probe but RNA samples from healthy cucumber does not at all. Purified CEV did not hybridize under the condition used. These results indicate that a clone pHSV-C39 contains the cDNA specific to HSV sequence.

Complete nucleotide sequence of cloned HSV cDNA.

The restriction maps of the three clones, pHSV-A48, -A60, and -C39 are shown in Fig. 3. The comparison of the maps indicates the possibility that the whole sequence of HSV unit length is integrated in combined cDNA copies of these three clones.

The nucleotide sequence of HSV cDNA insert was determined using these three clones. The cDNA inserts of pHSV-A48, -A60 and -C39 were 295, 297 and 286 bases, respectively. The assembly of the sequences of three cDNA inserts could construct the circular HSV RNA molecules and pHSV-A60 was found to include the complete sequence of the 297 residues of HSV (Fig. 4). The cDNA sequences of the other two clones are included in that of pHSV-A60 and completely agreed with each other except one base substitution. The T residue at position 260 of pHSV-A60 and -C39 is replaced by A residue in the sequence of pHSV-A48. We don't know for the present whether the heterogeneity found resulted from the polymorphism of the HSV or from the

Fig. 4. Circular representation of the 297-nucleotide covalently closed circular RNA sequence of HSV (inner circle) and cDNA inserts in the recombinant plasmids indicated (outer circle). Restriction endonuclease cleavage sites present in double-stranded cDNA are marked. An and Gn show dA and dG tails in the recombinants. Numbers show the residues of the 5' and the 3' end of cDNA inserted in each plasmid (see Fig. 5).

artefact in the process of the cloning.

Secondary structure of HSV.

A possible secondary structure model for the native HSV RNA was constructed using the computer program described by Zuker and Stiegler (27). It is shown to provide maximum sequence and structural homology with the proposed secondary structures of other viroids (Fig. 5). The native HSV RNA will possess extensive regions of intramolecular base-pairing and can form rod-like native structure similar to other viroids. The properties of the proposed structure are summarized in Table 1 together with those of the published structures of other viroids. The HSV native molecule will possess

Fig. 5. Secondary structure of HSV predicted from the nucleotide sequence. It is shown to provide maximum sequence and structural homology with the proposed secondary structures of other viroids sequenced. The numbering of residues follows the convention established for PSTV (2).

TABLE 1

Properties of predicted secondary structures for HSV
and other viroids sequenced.

Viroid	No. of residues	Residues base paired (%)	No. of base pairs				G:C base pairs as % of total	Reference
			A:U	G:C	G:U	total		
CCCV 1 (fast)	246	65	19	55	6	80	69	7
ASBV	247	67	43	28	12	83	34	6
HSV	297	67	29	64	7	100	64	This work
CSV	356	70	44	64	16	124	52	3
	354	68	41	67	12	120	56	4
PSTV	359	70	37	73	16	126	58	3
CEV	371	67	34	72	18	124	58	5
	371	66	37	74	11	122	61	4

the highest proportions of G : C base-pairing next to CCCV among all viroids whose sequences have been determined so far.

Possible translation products of HSV and the complement.

Although there is no evidence for the *in vitro* and *in vivo* synthesis of viroid-specific proteins so far (28-30), we cannot completely exclude the possibility that subgenomic linear RNAs of viroids act as mRNAs to synthesize polypeptides not detected yet. The positive and the negative strand of HSV have 4 and 5 possible initiation codons, including AUG codons, respectively. There are, however, only two potential polypeptide products of 22 and 52 amino acid residues in length coded for by the positive strand; from residue 130-132(GUG) to 289-291(UGA) and from 220-222(GUG) to 289-291(UGA), respectively. Reading frames started from other possible initiation codons have no termination codon.

DISCUSSION

Viroids have structures with a high degree of intramolecular base pairing. It is suspected to be difficult to clone complete and faithful cDNA copies of viroid RNA having such unique configuration by conventional cDNA cloning procedure. Owens and Cress (31) reported for the first time molecular cloning of PSTV cDNA. The restriction map of the cloned cDNA did

Fig. 6. An alignment of the sequences of CCCV, HSV and PSTV. The residues of HSV sequence common to those of CCCV or PSTV are boxed.

not coincide in part with that expected from the PSTV RNA sequence and suggested that partial sequence was invertly repeated by artefact during cloning procedure. We could clone complete and nearly complete cDNA copies of HSV by the method of Okayama and Berg (17). The nucleotide sequences of three cDNA clones analyzed coincided with each other, except for one residue. This shows the method is reliable and does not cause the sequence rearrangement during cloning procedure.

The complete nucleotide sequence of the 297 residues of HSV was determined from the cloned cDNAs. It is reported that a trace amount of the negative strand RNA is detected in the viroid preparation (32). The coincidence of the sequences of three independent clones will eliminate the possibility that we cloned the negative strand of HSV. We cannot know the presence of modified nucleotides in the sequence of HSV by our method using

Fig. 7. The comparison of a central conserved region of HSV and PSTV. Boxed regions indicate those sequences common to PSTV, CSV, CEV and CCCV.

cdNA. The modified nucleotides are, however, not found in the sequences of viroids determined so far.

Fig. 6 shows the alignment of the nucleotide sequences of HSV, CCCV 1 (fast) (7) and PSTV (3). By inserting gaps in the sequences of HSV and CCCV for maximizing the homology with that of PSTV those viroids share several highly homologous regions. Of interest is it that the sites of gaps inserted coincide very well. The host plants and the area where each viroid disease was found are very different. So we assume these observed homologies will be the result of a convergent development towards a common functional requirement, although we cannot exclude that the homologies reflect a phylogenetic relationship.

Among the viroids PSTV, CSV, CEV and CCCV, there is a central region of the rod-like structures which is highly conserved in both sequence and structure (2-5, 7). One strand of the conserved base-paired region has a sequence homologous to small nuclear U1a RNA and the complementary sequence is possible to base-pair with the 5'-end of U1a RNA (4, 33). The proposed interaction between viroid complements and U1a RNA is postulated to reflect the origin of viroids from an intron ancestor (33) or as a basis for pathogenesis (4, 33). ASBV, however, does not share the common structure except for the sequence GAAAC (6). The corresponding region of HSV has also a similar sequence and structure but is not completely identical (Fig. 7). The common sequence GAAAC found in all viroids and virusoid (34) sequenced is not found in HSV. Especially HSV lacks the sequence homologous to the 5'-end of U1a RNA in the region. The sequence highly homologous to the 5'-end of U1a RNA, however, is found in another part, from residues 58 to 67 in HSV

Fig. 8. Homology of a part of HSV sequence with the 5'-terminal sequence of Ula RNA (a) and possible base-pairing between the HSV complementary sequence (cHSV) and Ula RNA (b). Nucleotide sequence corresponding to residue 55-84 of HSV and the complementary sequence are shown. The arrows point to a hypothetical splice junction.

(Fig. 8a). The complementary sequence of the corresponding region is possible to base-pair with the 5'-end of Ula RNA (Fig. 8b).

So far no viroid-encoded translation product has been found *in vitro* or *in vivo*, although all viroids including HSV can potentially encode translation products. All evidence indicates that the replication of viroids should rely entirely on host components and the nucleotide sequence and structure of viroid itself have some signals for replicatable RNA and some sequences for pathogenicity. We have now the complete sequence of HSV as a recombinant DNA clone. When biologically functional viroid RNA molecules are produced from the cloned cDNA, it will enable us to study thoroughly the sequence essential for replication, the function of the sequence homologous to the Ula RNA and the nature of pathogenicity by site-directed mutagenesis of DNA level.

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