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Infectivity and *in vitro* mutagenesis of monomeric cDNA clones of citrus exocortis viroid indicates the site of processing of viroid precursors

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ABSTRACT

Monomeric cDNA clones of citrus exocortis viroid (CEV) were constructed in the plasmid vector pSP6-4 and the infectivity of the clones plus in vitro-synthesized RNA transcripts determined by inoculation onto tomato seedlings. Infectivity was dependent on the site of the viroid molecule used for cloning and the orientation of the cDNA insert. Only the plus BamHI cDNA clone was infectious and produced progeny viroid with wild-type sequence at the region corresponding to the BamHI cloning site. Infectivity correlated with the terminal repetition of 11 nucleotides of viroid sequence, 5'GGATCCCCGGG 3', in the vector adjacent to the insert. The 11-nucleotide sequence lies within the highly conserved central region of viroids. Site-directed mutagenesis of a single nucleotide in the repeat at the 5'-end of the CEV insert to 5' GGATCCCC(T,A)GG 3' gave two point mutants. The two mutant CEV inserts, when excised from the vector, were not infectious. However, plasmid DNA and RNA transcripts from non-excised mutant CEV inserts were infectious. The progeny of one of these clones was examined and contained wild-type sequence. It was concluded that in vivo processing of longer-than-unit-length CEV occurs at one of three adjacent sites in the 11 nucleotide sequence and that the G nucleotide at position 97 is important for viroid replication.

INTRODUCTION

Viroids are the smallest known pathogens and consist of single-stranded circular RNA molecules existing in rod-like structures (1-3). We are using two approaches to investigate the relationship between viroid structure and function: 1) the sequence determination of isolates of citrus exocortis viroid (CEV) which differ in pathogenicity (4,5), and 2) the construction of viroid mutants for infectivity studies. The second approach was shown to be feasible by Cress et al. (6) who found that tandem dimeric cDNA clones of potato spindle tuber viroid (PSTV) in the plasmid vector pBR322 were infectious and produced viroid progeny with an identical sequence to the viroid cDNA. Since their monomeric PSTV clones were not infectious, Cress et al. (6) considered that dimeric viroid cDNA clones in the plant act as templates for the production of longer-than-unit-length plus viroid RNAs which are

then processed at two sites to give linear viroid molecules of unit length. These linear molecules can then be circularized by a plant RNA ligase (7) and enter the normal pathway of viroid replication. This is consistent with the finding of longer-than-unit-length plus and minus forms of viroid RNAs in infected tissue (8-11).

Infectivity studies with RNA transcripts derived from monomeric and dimeric plus and minus clones of hop stunt viroid (HSV) in the plasmid vector pGL101 showed that only plus dimeric RNAs were infectious (12). Monomeric inserts excised from clones of HSV were also infectious (13). It was proposed that ligation of the monomeric DNA inserts occurs in the plant to give circular or multimeric viroid cDNA which can be transcribed to produce longer-than-unit-length RNA capable of entering the viroid replication cycle (13).

We report here the construction of infectious and non-infectious full-length monomeric cDNA clones of CEV in the plasmid vector pSP6-4. Infectivity correlated with the repetition of several nucleotides of viroid sequence in the vector adjacent to the 3'end of the viroid insert which allows the synthesis of longer-than-unit-length CEV RNA transcripts. The results support the findings of Tabler and Sänger (14) where infectivity of a monomeric PSTV cDNA clone was attributed to the presence of the same repeated nucleotides. We have postulated that processing occurs in the 11 nucleotide repeat sequence. Results from *in vitro* mutagenesis of a single nucleotide in one of the terminal repeats indicated that *in vivo* processing of longer-than-unit-length CEV occurs at one of three adjacent sites in the 11 nucleotide sequence. Structural models are proposed for the region around the putative processing site in the CEV molecule.

MATERIALS AND METHODS

Construction of full-length cDNA clones

The construction of a full-length cDNA copy of the CEV isolate, CEV-A (nucleotides 90-89; ref. 15), in plasmid pBR322 using the single BamHI site of CEV has been described (ref.4; Fig.1). The CEV-A insert was recloned into the BamI site of the bacteriophage vector M13mp93 and the plasmid vector pSP6-4. The synthesis of full-length double-stranded cDNA of another CEV isolate, CEV-J, and the blunt-ended ligation of this cDNA into the SmaI site of the phage vector M13mp9 has been described (ref.5; Fig.1). This CEV isolate was found to contain at least 9 sequence variants (5). The cDNA clones of two of these variants, CEV-Jd (nucleotides 200-199) and CEV-Jh

(nucleotides 199-198) were restricted on either side of the insert with SalI and EcoRI, and the inserts were ligated into pSP6-4 which had also been restricted with SalI and EcoRI. We define an SP6-4 clone containing an insert in the plus orientation as one which produces RNA transcripts containing plus viroid sequence. Plasmid DNA was extracted from E. coli cultures and purified by ethidium bromide-CsCl equilibrium density gradient centrifugation (17).

Site-directed mutagenesis

A plus full-length cDNA clone of CEV-A in the BamHI site of phage M13mp93 was mutated at position 97 of the CEV-A sequence using a mixture of three 17-nucleotide primers, 5' dGGTTTCCC(A,G,T)GGGGATCC 3' (BRESA Pty. Ltd., Adelaide), each containing a single internal mismatch to the wild-type G97. Procedures were essentially as described (18) with the exception that the alkaline sucrose density gradient centrifugation step was replaced by cleavage of the DNA with BamHI after the primer-extension reaction; the insert was then isolated by polyacrylamide gel electrophoresis and cloned into BamHI-cut M13mp93 vector. Single-stranded phage DNA, isolated from individual plaques, was screened (18) and sequenced by the dideoxynucleotide chain termination method (19).

Infectivity experiments

RNA transcripts were prepared with SP6 RNA polymerase (BRESA Pty. Ltd., Adelaide) from the pSP6-4 clones (16) after cleavage at the EcoRI site downstream from the inserts. Initiation of transcription was from 34 or 35 nucleotides upstream from the inserts, while termination of full-length transcripts was either 18 or 11 nucleotides downstream from the inserts, depending on the clone transcribed. All transcription reactions were monitored by gel electrophoresis. For infectivity, plasmid DNA (20-40 µg), Bam HI-excised inserts (about 0.1 µg), SP6 RNA transcripts (about 0.2 µg) purified from plasmid DNA by polyacrylamide gel electrophoresis, or an unpurified mixture of plasmid DNA (3-4 µg) and RNA transcripts in 50 µl of 0.1 M potassium phosphate, pH 6.8, containing 0.1 volume of 37 mg/ml bentonite suspension was inoculated per tomato seedling (Lycopersicon esculentum cv. Gross Lisse). Plants were grown as described (4) and monitored for epinasty and stunting, symptoms characteristic of CEV infection. Nucleic acid extracts of all plants were prepared 4-6 weeks after inoculation and examined by Northern hybridization analysis (11) using either polyacrylamide or agarose gels.

Purification and sequencing of CEV

CEV was purified from partially purified nucleic acid extracts (20) of tomato leaves harvested 4 weeks after inoculation. Circular CEV was sequenced by the dideoxynucleotide chain termination method (19,21) using a CEV-specific oligonucleotide, 5' dCGAAAGGAAGGAGACGAGCTCCTG 3' (BRESA Pty. Ltd., Adelaide) as primer.

RESULTS

Infectivity of Monomeric CEV cDNA Clones

Full-length, double-stranded cDNAs of three sequence variants of CEV were originally cloned in the plasmid vector pBR322 or the phage vector M13mp93 (4,5). For the work described here, the cDNA inserts were recloned into the plasmid vector pSP6-4 (16) which allows the preparation for infectivity studies of large quantities of full-length RNA transcripts (of either plus or minus orientation) using phage SP6 RNA polymerase. The two sites of cloning in the CEV molecule are given in Fig. 1.

The infectivity results obtained from the inoculation of tomato seedlings with four different wild-type CEV cDNA clones and/or their RNA transcripts are shown in Table 1. A mixture of plasmid DNA and RNA transcripts was generally used for infectivity studies because RNA was found to be more infectious than DNA (unpublished results). Plasmid DNA and the plus RNA

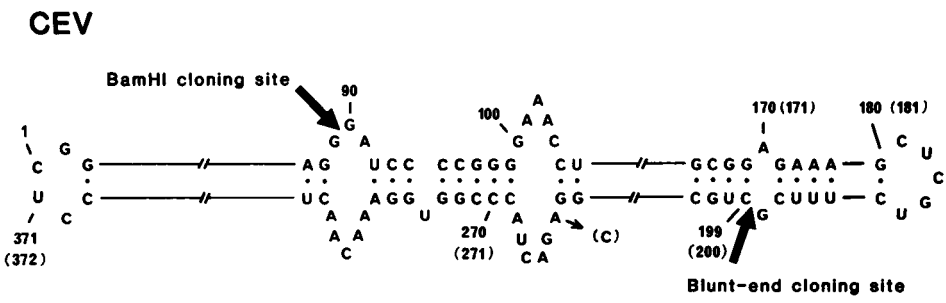

Figure 1: The two sites of the CEV molecule used in the construction of full-length cDNA clones. The BamHI site used for the cloning of CEV-A (between nucleotides 89 and 90) is in the highly conserved central region of viroids. The blunt-ended cloning site used for the cloning of two sequence variants of CEV-J (CEV-Jd and CEV-Jh) is near the right-hand end loop. The site lies between nucleotides 199 and 200 of CEV-Jd and nucleotides 198 and 199 of CEV-Jh. Nucleotide positions of the CEV molecules are indicated; those that differ in CEV-Jd are in parentheses. An A occurs at position 263 in variants CEV-A and CEV-Jh while a C, shown in parenthesis, occurs in CEV-Jd.

transcripts of CEV-Jd and CEV-Jh, in which the cloning site is near the right-hand end of the rod-like structure (Fig. 1), were not infectious. This is consistent with the lack of infectivity of monomeric clones of PSTV (6,15) and HSV (10). However, some monomeric PSTV cDNA clones (14) and RNA derived from E. coli cells transformed with a monomeric PSTV clone (6) were

TABLE 1: Infectivity of Monomeric CEV cDNA Clones and Excised Inserts

Full-length insert (nucleotide positions)[a]	Orientation of insert in pSP6-4 vector	Nucleic acid used for infection[b]	Infectivity[c] Experiment number 1	2	3	4
Wild-type clones						
CEV-Jd (200-199)	+	DNA + RNA	0/4	0/4		
CEV-Jh (199-198)	+	DNA + RNA	0/4	0/4		
CEV-A (90-89)	-	DNA + RNA	0/5	0/5		
	DNA	0/4				
	RNA	0/3				
CEV-A (90-89)	+	DNA + RNA	4/4	5/5		
	DNA	2/4	1/4			
	RNA	5/6	3/6			
Mutant clones						
CEV-A/A97 (90-89)	+	DNA + RNA	5/5	4/4		
CEV-A/U97 (90-98)	+	DNA + RNA	4/5	5/5	4/10	7/10
Wild type insert						
CEV-A (90-89)		cDNA insert	7/10	5/6		
Mutant insert						
CEV-A/A97 (90-89)		cDNA insert	0/5	0/5		
CEV-A/U97 (90-98)		cDNA insert	0/5	0/5	0/5	

[a] Refer to Fig. 1 for cloning sites.
[b] Amounts of nucleic acid used are given in Materials and Methods.
[c] Infectivity is shown as the number of tomato plants with symptoms over the number of inoculated plants. Nucleic acid extracts were prepared from leaves pooled from the plants of each experiment, and the infectivity results were confirmed by Northern hybridization analysis (11). The size of the progeny RNA was always indistinguishable from that of CEV-A. Mock-inoculated control plants from all experiments (not shown) were symptomless and uninfected by Northern hybridization analysis.

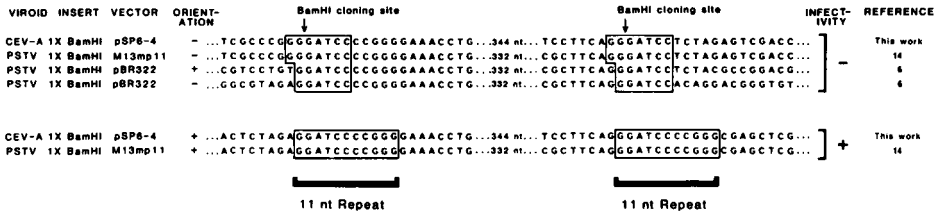

Figure 2: Correlation between sequences around the cloning site of monomeric viroid cDNA clones in plasmid and phage vectors and the infectivities of these clones and/or their RNA transcripts. The BamHI restriction sites used for the construction of the clones are shown. Sequences between the cloning sites were derived from the viroid insert while those on either side of the cloning sites were derived from the vector. cDNA clones were used for infectivity experiments with PSTV clones while a mixture of plasmid DNA and *in vitro*-synthesized RNA transcripts were used in the case of CEV-A clones. For DNA clones containing an insert in the minus orientation, the complementary sequences are shown. All clones contain sequences which can give rise to longer-than-unit-length viroid sequence in RNA transcripts; the duplicated nucleotides in these sequences are boxed.

poorly infectious. As infection was not reported to occur in more than a single plant, or confirmed by Northern hybridization analysis, further studies are required to confirm these results.

For the cDNA clones of CEV-A in pSP6-4, in which the BamHI cloning site (Fig. 1) is in the conserved central region of viroids (8), the plus, but not the minus, clone and transcripts were infectious (Table 1). This difference in infectivity was not due to the polarity of the pSP6-4 transcripts because infectivity studies with the plasmid DNAs alone gave similar results (Table 1).

It is unlikely that the difference in infectivity between the CEV-J and CEV-A plus clones was due to the difference in sequence between the cloned sequence variants because they show extensive sequence homology (5). The site of cloning was therefore considered to account for the difference in infectivity, while the infectivity results with the CEV-A clones showed that infectivity was also dependent on the orientation of the cloned insert (Table 1). Since monomeric BamHI clones of PSTV in both orientations in the plasmid pBR322 were non-infectious on tomato (6) and PSTV shares extensive sequence homology with CEV (15), this suggested that infectivity was also dependent on the vector.

Analysis of the vector sequences adjacent to the BamHI sites of the monomeric CEV and PSTV clones in the vectors pSP6-4 and pBR322 (Fig. 2) showed that 6,7 or 11 nucleotides of viroid sequence are repeated. Hence

these clones all contain longer-than-unit-length viroid sequence; the 6 to 11 nucleotide terminal repeats in these viroid sequences are boxed in Fig. 2. A correlation can be made between infectivity and the number of repeated nucleotides of viroid sequence in the vector on the 3' side of the insert. The non-infectious clones have 5 repeated nucleotides (GATCC) whereas the infectious CEV clone has 10 (GATCCCCGGG), suggesting that the 5 extra repeated nucleotides of viroid sequence, CCGGG, account for the difference in infectivity. This is consistent with the findings of Tabler and Sänger (14) where the infectivity of a monomeric BamHI cDNA clone of PSTV in the phage vector M13mp11 correlated with the presence of the same five repeated nucleotides of vector sequence. Nucleotides occurring outside the 11 nucleotide repeats may also be important for the infectivity of the monomeric cDNA clones.

The infectivity of all tested dimeric cDNA clones of viroids (6,10,12, 14) also supports this correlation since they all contain an 11 nucleotide repeat, either GGATCCCCGGG (Fig. 2) or GGAGCCCCGGG (in HSV). Deletion mutagenesis of dimeric clones of HSV in the plasmid pGL101 has shown that a repetition of only 65 nucleotides of HSV was sufficient for infectivity (T. Meshi, personal communication). This 65 nucleotide terminal repeat contains the sequence, GGAGCCCCGGG, with 10 of the 11 nucleotides repeated in CEV.

Partial Sequencing of Progeny Viroid indicated that in vivo Processing of CEV Precursors occurs at the 11 Nucleotide Terminal Repeats

Twenty tomato seedlings were inoculated with a nucleic acid extract prepared from tomatoes infected with the CEV-A plus clone and transcripts from experiment 1 (Table 1). Sequencing of the progeny viroid across the region corresponding to the BamHI cloning site (nucleotides 74-116), using a 24-nucleotide primer that hybridized at the right-hand end loop of the native structure (nucleotides 175-198 of CEV-A), revealed that the sequence in this region was the same as wild-type CEV-A. To explain the absence of any vector sequence in this region of the viroid progeny we propose that processing of the RNA transcripts occurred at the same site within each of the 11-nucleotide repeats at the ends of the viroid insert (i.e. at one of the 12 phosphodiester linkages), thereby generating a unit-length viroid which then entered the normal replication pathway.

In Vitro Mutagenesis of CEV cDNA Clones localizes the Processing Site at One of Three Phosphodiester Linkages

Since the repeated GGATCCCCGGG sequence correlates with infectivity

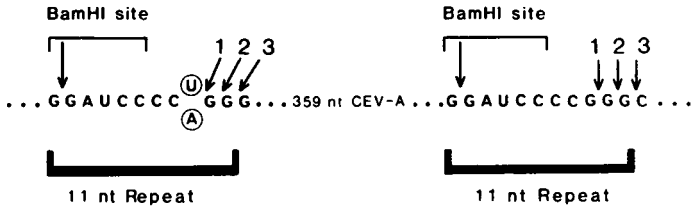

Figure 3: Potential in vivo processing sites of RNA transcripts derived from two point mutant clones of CEV-A. The circled nucleotides represent the point mutations introduced into CEV-A at position 97, resulting in CEV-A/U97 and CEV-A/A97. The BamHI cloning sites and adjacent sequences are shown. The 11-nucleotide repeat sequence that correlates with infectivity and the proposed sites of processing at positions 1, 2, or 3 are indicated.

whereas the repeated BamHI site, GGATCC, does not (Figs. 2,3), nucleotides in the repeated CCGGG may be essential for processing. Point mutants in the 11-nucleotide repeat at the 5'-end of the CEV insert in phage M13mp93 DNA were constructed by site-directed mutagenesis of the CCGGG of the CEV-A insert to either CCTGG or CCAGG (Fig. 3). The sequencing and screening of single-stranded phage DNA indicated that mutants were produced at about 25% frequency. As expected, these mutations resulted in the complete loss of one of the two AvaI sites present in wild-type CEV clones. From two mutant clones, full-length BamHI inserts called CEV-A/U97 (where G97 was mutated to U) and CEV-A/A97 (where G97 was mutated to A) were isolated and recloned into pSP6-4.

Infectivity studies with plasmid DNA and in vitro synthesized RNA transcripts of CEV-A/U97 and CEV-A/A97 in pSP6-4 showed that both point mutants were infectious (Table 1). Progeny viroid was purified from tomato seedlings which had been inoculated with a nucleic acid extract from tomatoes infected with the CEV-A/U97 clone and transcripts (experiment 1, Table 1). The sequence across the region of the BamHI cloning site (nucleotides 74-116) was the same as wild-type CEV-A. To rule out the possibility of contamination of the CEV-A/U97 clone with a wild-type CEV-A clone, one colony containing the CEV-A/U97 plasmid was streaked three times. Plasmid DNA was prepared from a culture grown from a single colony after streaking and was restricted with AvaI. This resulted in the complete loss of one of the two AvaI sites which was expected for the mutated cDNA clone. Plasmid DNA and RNA transcripts prepared from this clone were used for two infectivity experiments (Table 1, experiments 3 and 4). Once again, the plasmid plus transcripts were infectious and the progeny RNA from the infected

plants had wild-type sequence at nucleotides 74-116.

One explanation for the generation of wild-type sequence is that reversion from T or U to G occurred during *in vitro* transcription or transcription in the plant. However, a BamHI cDNA clone of CEV-A containing a point mutation at residue 351 (G→U), which lies in the left-hand region of the native structure (Fig. 1), was not infectious (unpublished data).

A second, and more likely, explanation is that processing of the RNA transcripts occurred at one of the three phosphodiester linkages on the 3' side of the point mutation (i.e. at positions marked by 1,2, or 3 in Fig. 3). This would generate a full-length wild-type viroid molecule, since the point mutation lies on the 5' side of the putative 5' processing site(s). Thus, our results indicate that the repeated CCGGG which is present in the infectious BamHI clone of CEV-A may correlate with infectivity because it contains the viroid processing site. Each of the three processing sites would result in the processing of a GpG dinucleotide in longer-than-unit-length viroid RNA. This dinucleotide commonly spans the splice junction sites of mRNA precursors (22).

Infectivity Studies with Excised Monomeric Inserts derived from Two CEV cDNA Clones containing a Point Mutation

Double-stranded cDNA inserts were excised from a wild-type CEV-A clone and two mutated clones of CEV (CEV-A/U97 and CEV-A/A97) using BamHI and purified by polyacrylamide gel electrophoresis. The results of infectivity experiments with these inserts are given in Table 1. Inserts derived from the wild-type clone were highly infectious. Similar results have been demonstrated for monomeric inserts derived from HSV cDNA clones (13) and PSTV cDNA clones (14). However, the monomeric inserts derived from the CEV-A/U97 and CEV-A/A97 clones were not infectious; this is in direct contrast to the infectivity of the cDNA clones from which these inserts were derived (Table 1). These results demonstrate that nucleotide G97 is important for viroid replication. The mutation of G97 to C has not been tested.

Attempted In Vitro Processing of Dimeric CEV Transcripts

Infectivity studies and *in vitro* mutagenesis have allowed the prediction of the site of processing of longer-than-unit-length CEV transcripts *in vivo*. In view of the extensive self-processing of *in vitro*-synthesized dimeric RNA transcripts of ASBV (Hutchins, Rathjen, Forster and Symons, in preparation), the possibility that CEV may also self-process was investigated. Negligible, if any, processing occurred during the *in vitro* transcription of monomeric or dimeric plus clones of CEV whereas *in vitro*

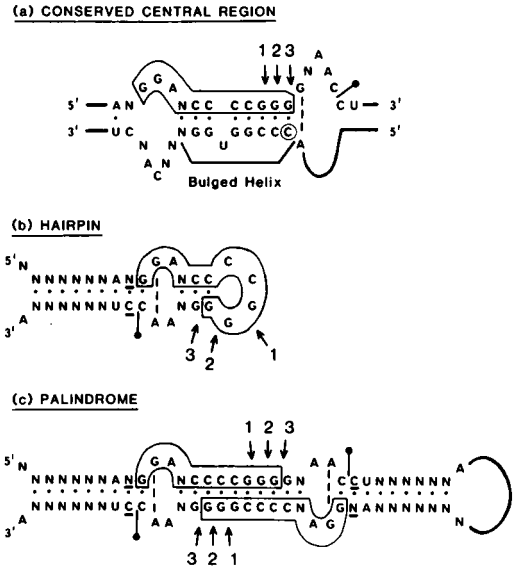

Figure 4: Conserved nucleotides and potential structures in the central region of all viroids except ASBV. N represents a non-conserved nucleotide. The 11-nucleotide sequence which correlates with infectivity is boxed. Our proposed site of processing is at positions 1,2, or 3 as indicated by arrows. In HSV, the circled C is not present and a U is inserted in the positions marked with a filled circle. In (b) and (c), this U could pair with an A which is represented by N leaving an unpaired C which is marked C. Potential A:G base pairs are joined by broken lines.

transcription of dimeric clones of ASBV resulted in almost quantitative processing at specific sites of the ASBV transcripts to produce a linear monomeric ASBV transcript (Hutchins et al., in preparation). CEV-A transcripts derived from cDNA clones in pSP6-4 containing plus dimeric HindIII inserts did not process when incubated under a variety of ionic conditions, including treatments known to induce self-processing of RNA molecules (Hutchins et al., in preparation, refs. 23-25). As ASBV shows no significant sequence homology with other viroids (26), the processing mechanisms of ASBV and other viroids may differ.

Robertson et al. (27) have recently reported the self-processing of in vitro-synthesized dimeric PSTV RNA transcripts. However, only 1 to 5% of the dimeric transcripts were cleaved, and cleavage occurred between nucleotides 250 and 270, which is outside the region of processing proposed from our in vivo data.

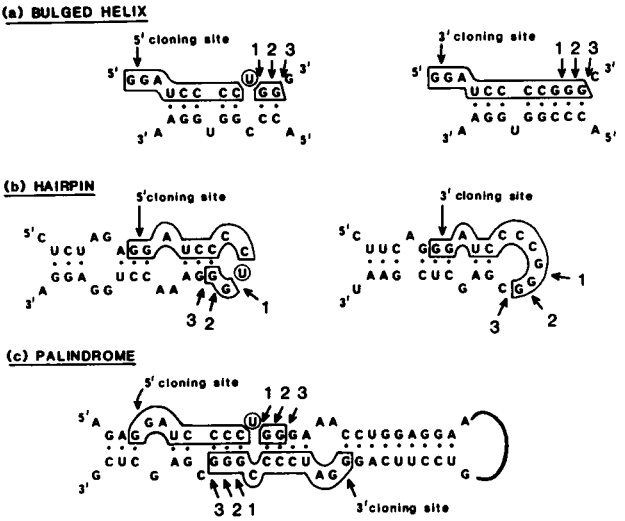

Figure 5: Structures resembling the bulged helix (a), hairpin (b) and palindrome (c) (see Fig. 4) that may form at the proposed 5'- and 3' - processing sites of the RNA transcripts of the mutated CEV clone, CEV-A/U97. The 5' and 3' cloning sites are indicated. The circled U represents the G to U point mutation in the 11-nucleotide repeat. The 11-nucleotide repeat that correlates with infectivity is boxed. Our proposed sites of processing at positions 1,2, or 3 are indicated by arrows.

DISCUSSION

The work described represents the first successful application of site-directed mutagenesis to the study of structure-function relationships in viroids. It has allowed the prediction of the site of processing *in vivo* of longer-than-unit-length CEV precursors and has shown the importance of nucleotide G97 in viroid replication.

The proposed region of processing in CEV is located in a region highly conserved in all viroids except ASBV (8,28) which has no significant homology with other viroids (26). There is strict conservation in these viroids of 10 of the 11 repeated nucleotides (Fig. 4a) and our results imply that a function of the conserved region is the processing of longer-than-unit-length viroid RNA to unit-length viroid. Nucleotide G97 is in the bulged helix present within this conserved region (Figs. 1,4a,5).

There are three conserved, potential structures, all of which contain the predicted processing site, in the central region (Fig. 4): a bulged helix (Fig. 4a; ref. 8), a stable hairpin containing helical segments of nine and three base pairs (Fig. 4b; refs. 29,30), and a partial palindrome

(Fig. 4c; ref. 25) which could base-pair with the same sequence in another part of the same longer-than-unit-length viroid precursor to give a structure containing adjacent helical segments of 9,10 and 9 base pairs (Fig. 4c). Interestingly, all structures in Fig. 4 have helices which terminate at one end with A and G nucleotides (joined by a dashed line). In view of the accumulating evidence that non-standard base pairs (i.e., base pairs other than G:C, A:T/U, and G:U) such as A:G, are present in RNA (31-33) and DNA (34), the presence of these base pairs in viroids must be considered. The G97 to U/A mutation in the conserved central region (Fig. 3) resulted in loss of infectivity of excised inserts (Table 1); this may be attributed to disruption of either base pairing in the bulged helix or palindrome structures of Fig. 4 or a recognition site present in one of the three structures in Fig. 4.

We suggest that one of the structures of Fig. 4 is a signal for *in vivo* processing. The hairpin is not present in the most thermodynamically stable structure of viroids (3,29,30), but the hairpin and palindrome structures may arise transiently during the replication cycle (27). It is interesting that both the hairpin and palindrome structures (Fig. 4b,c) are essentially conserved in minus viroid RNA. Hence, processing of longer-than-unit-length minus viroid RNA may also occur, as found for minus dimeric transcripts of ASBV *in vitro* (Hutchins *et al.*, in preparation).

The vector sequences adjacent to the 11-nucleotide terminal repeats of the infectious monomeric cDNA clones in Fig. 2 and of dimeric clones (not shown) were examined to determine whether the three proposed structures of the central conserved region (Fig. 4) could form in RNA transcripts derived from these clones. For example, structures resembling the bulged helix, hairpin and palindrome structures of Fig. 4 may form in the SP6 RNA transcripts from the CEV-A/U97 clone at the proposed 5'- and 3'-processing sites (Fig. 5), and they can also be drawn at the putative processing sites of the RNA transcripts from all other infectious clones (not shown). Mutagenesis of the vector sequence adjacent to the 11-nucleotide terminal repeats should allow the role, if any, of vector sequences in processing to be determined. It may also be possible to locate the precise site(s) of processing by generating infectious transcripts which have complete 5'-processing sites but have a 3'-end corresponding to each of the three proposed sites of processing.

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