Specific RNA self-cleavage in coconut cadang cadang viroid: Potential for a role in rolling circle replication

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ABSTRACT
The rolling circle replication of the small, single-stranded viroid RNAs requires a specific processing reaction to produce monomeric RNAs that are ligated into the final circular form. For avocado sunblotch viroid, peach latent mosaic viroid, and chrysanthemum chlorotic mottle viroid, the hammerhead self-cleavage reaction is considered to provide this processing reaction. We have searched for a similar type of reaction in the 246-nt coconut cadang cadang viroid, the smallest viroid of the 24-member potato spindle tuber viroid (PSTV) group. RNA transcripts prepared from the cloned central or C domain of this viroid self-cleaved specifically after denaturation with methylmercuric hydroxide followed by incubation in the presence of spermidine but in the absence of added magnesium ions. The unique cleavage site was located in the bottom strand of the C domain within a potential hairpin structure that is conserved within members of all three subgroups of the PSTV group of viroids.

Keywords: catalytic RNA; ribozyme; rolling circle replication; viroid processing

INTRODUCTION
Viroids, the smallest known plant pathogenic RNAs, are considered to replicate via a rolling circle mechanism in vivo (Branch & Robertson, 1984). Such a mechanism requires a specific cleavage reaction for the accurate processing of the multimeric viroid copies into monomeric forms, which are then ligated to become the single-stranded circular progeny found in vivo (reviewed in Symons, 1992).

In the case of the three members in the avocado sunblotch viroid (ASBV) group, ASBV, peach latent mosaic viroid (PLMV), and chrysanthemum chlorotic mottle viroid (CChMV), self-processing via the hammerhead structure has been demonstrated in vitro for both the plus and minus RNAs (Hutchins et al., 1986; Hernández & Flores, 1992; Navarro & Flores, 1997). In order to identify in vivo replicative intermediates of ASBV, the structures of a series of RNAs extracted from ASBV-infected tissue were characterized (Darós et al. 1994). The 5’ termini of the (+)- or (−)-strand RNAs were identical to those produced in the in vitro self-cleavage reaction of (+) and (−) dimeric ASBV RNAs, thus supporting the in vivo functioning of the hammerhead structures. The (+) CChMV hammerhead structure also appears to be active in vivo, because the 5’ terminus of the linear (+) CChMV RNA isolated from infected tissue is that predicted by the corresponding hammerhead ribozyme (Navarro & Flores, 1997). Mutagenesis experiments involving the infectivity of cDNA clones of the 324-nt plant viroid-like, satellite RNA, or virusoid of lucerne transient streak virus have provided strong evidence for a role for the hammerhead self-cleavage reaction in vivo (Sheldon & Symons, 1993).

All viroids other than ASBV, PLMV, and CChMV belong to the potato spindle tuber viroid (PSTV) group, of which there are now 24 members (Martinezsoriano et al., 1996; Flores et al., 1997). Generally, attempts to find self-cleavage reactions in one or two viroids within this group have been unsuccessful, which has lead to a belief that the processing required during rolling circle replication could be protein-catalyzed, and a number of studies have been based on that premise (Tsagris et al., 1987; Steger et al., 1992; Baumstark & Riesner, 1995). In contrast, we consider that, as for ASBV, PLMV, and CChMV, self-cleavage reactions will also be involved in the replication of the PSTV group of viroids and preliminary supporting evidence is provided here.

The only report so far of a potential self-processing reaction in members of the much larger PSTV group is that of Robertson et al. (1985). They found that the
incubation of a dimeric transcript of PSTV under conditions used for group I splicing reactions led to approximately 1–5% cleavage to produce linear monomeric viroid RNA, as well as two other products of approximately 190 and 210 nt. The results placed the cleavage site between residues 250 and 270, which is in the bottom strand of the central conserved domain (C) of PSTV (Fig. 1A). In a less direct in vivo approach, mutagenesis studies on infectious longer-than-unit-length RNA transcripts of cDNA clones of the 371-nt citrus exocortis viroid (CEV) identified a potential in vivo processing site in the upper strand of the C domain (Visvader et al., 1985) that corresponded to G₉₅–G₉₉ of the PSTV sequence. However, a more comprehensive study of RNA transcripts prepared from cDNA clones of the 371-nt CEV at seven different sites within the CEV molecule showed that the requirement for infectivity appeared to be the ability of the monomeric viroid transcripts to show that the requirement for infectivity appeared to be the ability of the monomeric viroid transcripts to form a short double-stranded region of viroid and vector sequences at the junction of the two termini, and not to any specific site within the viroid molecule (Rakowski & Symons, 1994).

A more direct approach to search for self-cleavage in the PSTV-group viroids is to follow the techniques that have been used successfully in the discovery of the hammerhead self-cleavage in ASBV and the plant viroid-like, satellite RNAs or virusoids (Hutchins et al., 1986; Forster & Symons, 1987a, 1987b; Forster et al., 1987, 1990). For the work described here, we chose the 246-nt coconut cadang cadang viroid (CCCV), the smallest member of the PSTV group. Central to our approach has been the consideration that the lack of success in detecting self-cleavage of long multimeric transcripts of CCCV in our earlier unpublished and unsuccessful efforts has been due to our inability to fold the RNAs into active self-cleaving conformations. This conclusion was based on our earlier work on the self-cleavage of the 324-nt viroid-like satellite RNA or virusoid of lucerne transient streak virus, where RNA transcripts prepared from cDNA clones required a specific denaturation and refolding step to convert at least some of the RNA into active self-cleavage structures (Forster & Symons, 1987a, 1987b).

Relevant here also is the self-cleavage of hepatitis delta RNA, where, for optimal self-cleavage activity, the minimal length of the RNA is 84 nt 3’ to the cleavage site; sequences beyond +84 often strongly inhibit the self-cleavage due to their interactions with the catalytic sequence (Wu et al., 1989; Perrotta & Been, 1990; Tanner, 1995). Potential inhibitory elements could also exist in the PSTV-group viroids. By concentrating only on the C domain of CCCV, the most conservative and prospective region for a specific self-cleavage (Symons, 1997), the negative effects of any possible inhibitory elements could be minimized so that the chances of detecting self-cleavage could be enhanced. We report here specific self-cleavage in the bottom C domain strand of CCCV and discuss its potential as the in vivo processing site.

**RESULTS**

In the domain model of the rod-like structure of the PSTV-group viroids (Fig. 1A; Keese & Symons, 1985; Keese et al., 1988), the C domain has long been considered as a highly prospective region for a specific processing event to occur (Symons, 1997). This is because, first, the C domain includes the most conservative sequences that form the core basis for subdivision of the PSTV group of viroids into three subgroups (Koltunow & Rezaian, 1989). Second, the top and bottom strands of the C domain contain inverted repeat sequences and have the potential to form hairpin stem-loop structures (Fig. 1B,C). Furthermore, UV covalent crosslinking was found between G₉₉ and U₂₅₀ of PSTV in the C domain (Branch et al., 1985; see Fig. 1B,C), which indicates a structural juxtaposition of these two residues for the crosslinking to occur. This structure could also be essential for the viroid processing, because similar crosslinking was also found in the viroid-like domain of the self-cleavable hepatitis delta RNA (Branch et al., 1989), and a ribozyme structure is required for the replication of hepatitis delta RNA (Jeng et al., 1996).

For the proposed work, two cDNA plasmids were prepared from PCR-amplified DNA fragments of the top and bottom strands of the C domain, plus some flanking sequences from the P and V domains (see Materials and Methods). Both plasmids carried a T7 promoter sequence and an insert of head-to-tail-joined C domain sequences in a different order (Fig. 2). Linearization of the plasmids with appropriate enzymes generated templates for preparation of various RNA transcripts (Fig. 2B) to top C domain (TEL) or bottom C domain (BSL), as well as top–bottom-joined C domain (TEBL) or bottom–top-joined C domain (BSTL). In order to maximize our chances of obtaining active self-cleavage conformations in these transcripts, we tried a large number of denaturation and refolding approaches, as well as a range of cations.

**Specific cleavage of C domain transcripts**

TEBL and BSTL occurs when the RNAs are denatured by methylmercuric hydroxide before the addition of spermidine

Among all reagents we have tried for denaturation, methylmercuric hydroxide was the most successful. This chemical is an effective denaturant for nucleic acids by interacting with the imino NH bonds of uridine and guanosine residues of nucleic acids (Simpson, 1964; Gruenwedel & Davidson, 1966; Bailey & Davidson, 1976). The binding of methylmercury to nucleic acids is re-
versible by reaction with a strong reducing agent, such as DTT or 2-mercaptoethanol (Bailey & Davidson, 1976).

TEBL and BSTL, the *cis* C domain transcripts (see Fig. 2B, the bottom forms), were denatured by methylmercuric hydroxide at a concentration of 10 mM at room temperature for 20 min. Cleavage buffer and reagents were then added followed by excess DTT (see details in Materials and Methods). The final cleavage reaction

![Diagram](image-url)
mixture contained 40 mM HEPES, pH 8.0, 5 mM spermidine, 0.01% SDS, and 16% of glycerol (standard cleavage reaction), and the mixture was incubated at 50°C for 1 h. Polyacrylamide–urea gel electrophoresis analysis of the reaction mixture produced two major products, 59P and 39P (Fig 3A, lane 5, labeled sdg). These two products from the TEBL and BSTL transcripts had a total length equivalent to the full-length transcript (FL), indicating that they resulted from a single cleavage in the RNA. The difference in radioactivity between the two products was partly due to the difference in the number of 32P-UMP residues in each of the fragments (for TEBL, 16 × U in 5'P and 6 × U in 3'P; for BSTL, 9 × U in 5'P and 15 × U in 3'P) and presumably some loss of the small 24-nt 3'P of TEBL during ethanol precipitation. These products were also weakly detected when SDS and glycerol were omitted from the cleavage buffer (Fig. 3A,B, lane 4, labeled s; bands were clearly seen in the original autoradiograph), but not when spermidine was absent (Fig. 3A,B, lane 6, labeled dg). Hence, it appears that both TEBL and BSTL RNAs may cleave in a similar manner and that spermidine is an essential co-factor.

In an initial experiment, spermidine was used at a concentration of 2, 5, or 10 mM in the cleavage reaction. Because no difference of self-cleavage of TEBL and BSTL was found, 5 mM was chosen as the standard concentration for spermidine in all other self-cleavage experiments.

Two minor cleavage products, 5'X and 3'X (Fig. 3B, sdg, lane 5), which also had a total length equivalent to the full-length BSTL, were found to always accompany the two cleavage products from BSTL. Apart from the difference in UMP content between the two fragments, the radioactivity detected in the shorter product, 5'X, appeared lower than it should be. However, quantita-

FIGURE 2. In vitro synthesis of RNAs of the C domain of CCCV. A: Schematic diagram of the C domain and flanking sequences of the P and V domains in the rod-like form (vertical dashed lines show the boundaries of the C domain). Nucleotides shown at each end are those involved in a restriction site in the sequence or later generated during cloning. Straight lines represent the base paired regions; bulged areas are unpaired single-stranded regions. Position of each end nucleotide in the viroid sequence is indicated and length of each strand is given at the right. B: Diagram of the C domain DNA templates and T7 RNA polymerase products generated by transcription of linearized templates with different enzymes. Arrows on top of the templates indicate orientations of RNA transcription and end nucleotides of the cloned strands are given underneath. Inserted restriction enzyme sequences are shown in full in the transcripts where the nonviroid sequences are in lower case.
major products

5 and 39 phorImager in some other experiments showed that

tive analysis of the cleavage products using a Phos-

Cleavage of the cis C domain transcripts TEBL and BSTL

FIGURE 3. Cleavage of the cis C domain transcripts TEBL and BSTL. Cleavage products were analyzed on a denaturing 8% polyacryl-

amide gel containing 7 M urea followed by autoradiography. Purified 32P-UMP-labeled transcripts of (A) top–bottom-joined C domain

(TEBL) and (B) bottom–top-joined C domain (BSTL) were denatured by methylmercuric hydroxide then subjected to self-cleavage under

standard conditions described in Materials and Methods (lanes 1– 6

and 8). Lane 1 (c), control reaction in 40 mM HEPES buffer, pH 8,

without any additives. Lanes 2–6, reactions in HEPES buffer with

10 mM MgCl2 (m, lane 2), 5 mM spermidine and 10 mM MgCl2 (sm, lane 3), 5 mM spermidine (s, lane 4), 5 mM spermidine and 0.01%

SDS plus 16% glycerol (sdg, lane 5), 0.01% SDS and 16% glycerol

dg, lane 6), 5 mM spermine and 0.01% SDS plus 16% glycerol (ndg,

lane 8). RNA sample in lane 7 (hsdg) was heated at 95 °C for 1 min

in 0.1 mM EDTA, snap-cooled on ice, then subjected to cleavage

reaction in the same buffer mixture as that for the sgd sample in

lane 5. FL, full-length transcript. 5′P and 3′P, 5′- and 3′-end products

from self-cleaveage (see Fig. 4 for characterization of these products).
5′X and 3′X, minor cleavage products of BSTL. Reference lengths

(nt) of markers are also shown (M).

tive analysis of the cleavage products using a Phos-

PhosphorImager in some other experiments showed that

5′X and 3′X had a similar molar ratio to that of the

major products, 5′P and 3′P. No matching minor
cleavage products were found in the TEBL cleavage

(Fig. 3A, sgd, lane 5).

Interestingly, no cleavage of either TEBL or BSTL

was found when the cleavage buffer contained 10 mM

MgCl2 (Fig. 3A,B, lane 2, labeled m), even in the pres-

eence of spermidine (Fig. 3A,B, lane 3, labeled sm). The

addition of magnesium to the reaction mixture contain-

ning the optimal combination of contents that showed

the best cleavage result as seen in lane 5 (sgd) in

Figure 3 also resulted in no cleavage (data not shown).

Thus, the cleavage of the two cis C domain transcripts

appears to occur in a way different from many known

types of self-cleavage, such as hammerhead, hairpin,

and HDV ribozymes, which require divalent cations to

assist their cleavage (Scott & Klug, 1996).

Considering the size and charge difference between

spermidine and magnesium, a further experiment to

replace spermidine in the cleavage buffer with sper-

amine, a larger molecule with one more positive charge,

was performed under the standard conditions described

above. Cleavage of the two cis transcripts occurred

(Fig. 3A,B, lane 8, labeled ndg), but to a lesser extent

compared with spermidine (Fig. 3A,B, sgd, lanes 5).

To assess the importance of denaturation with methyl-

mercuric hydroxide for this self-cleaveage, other meth-

ods tried for denaturing and refolding the RNA transcripts

and the results obtained are summarized in Table 1.

With the same combination of contents in the cleavage

buffer, a trace of self-cleavage occurred on prior dena-

turation using 5 M guanidine thiocyanate (GTC), but

only when the GTC was diluted to 0.5 M prior to incu-

bation in the standard cleavage buffer. The likely ex-

planation is that the RNAs denatured by 5 M GTC could

not refold into a detectable level of active conforma-

tions until the GTC concentration was reduced to 0.5 M

or lower. No self-cleavage was detected when using

other methods for denaturation and refolding the RNAs

(Table 1; see also Fig. 3A,B, hsgd, lane 7 for results

from the heating–snap-cooling denaturation). Overall,

the results indicated that a proper prior denaturation

and refolding of the RNAs was a crucial factor for the

self-cleavage to occur and that the methylmercury

denaturation–refolding method appeared to be the most

appropriate.

Self-cleavage occurs at a unique site

in the bottom C domain strand

When TEBL and BSTL transcripts were 5′-32P labeled

and then subjected to cleavage under the standard

conditions, only one major cleavage fragment was de-

tected. This corresponded in size to the 5′-end product

(5′P), the longer fragment of the two cleavage products

in TEBL (Fig. 4, lane 1, labeled 5′-C) and to the shorter

fragment in BSTL (Fig. 4, lane 6, 5′-C; the band was

faint but proved to be 5′P by a control sample, U′-C in

lane 7). When the transcripts were first subjected to
Self-cleavage in coconut cadang cadang viroid

TABLE 1. Methods of denaturing and renaturing C domain transcripts for detection of self-cleavage.

<table>
<thead>
<tr>
<th>Method</th>
<th>Denaturation</th>
<th>Renaturation</th>
<th>Incubation</th>
<th>Cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylmercuric hydroxide</td>
<td>10 mM, 22 °C, 30 min</td>
<td>Excess DTT after adding buffer and additives for cleavage</td>
<td>50 °C, 1 h</td>
<td>+</td>
</tr>
<tr>
<td>Guanidine thiocyanate</td>
<td>5 M, 37 °C, 20 min</td>
<td>Dilute to 0.5 M with buffer and additives for cleavage</td>
<td>37 °C, 1 h</td>
<td>Trace</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>10 or 50 or 100 mM, 22 or 25 °C, 10–30 min</td>
<td>Neutralize to desired pH with buffer and additives for cleavage</td>
<td>50 °C, 1 h</td>
<td>–</td>
</tr>
<tr>
<td>Heating–snap-cooling</td>
<td>95 °C, 1 min, in water or 0.1 mM EDTA</td>
<td>Snap-cool on ice, then add ice-cold buffer and additives for cleavage</td>
<td>50 °C, 1 h</td>
<td>–</td>
</tr>
<tr>
<td>Formamide</td>
<td>9, 13, or 18 M, 80 °C, 2 min</td>
<td>Snap-cool on ice, then add ice-cold buffer and additives for cleavage</td>
<td>56 °C, 1 h</td>
<td>–</td>
</tr>
</tbody>
</table>

*a Denaturation was also tried at 0 °C, 37 °C, or 50 °C, respectively, but no better cleavage result was found.

b No cleavage was detected when the guanidine thiocyanate concentration was higher than 0.5 M in the cleavage buffer.

*c Denaturation was also tried at 0 °C, 65 °C, or 50 °C followed by snap-cooling or gradually cooling to 50 °C.

Cleavage of C domain transcripts in trans

When separate top (TEL) and bottom (BSL) C domain transcripts (see Fig. 2B) were 5′-32P labeled and subjected to cleavage under the standard conditions after methylmercuric hydroxide denaturation, no cleavage of TEL was found, either without (Fig. 6, T* lane 1) or with addition of nonlabeled BSL (Fig. 6, T*+B, lane 2). A trace cleavage of BSL was detected (5′P) in the absence of TEL (Fig. 6, B*, lane 3) and a higher extent of the BSL cleavage was found when nonlabeled TEL was present (Fig. 6, B*+T, lane 4). The 5′-end cleavage fragment of BSL co-migrated with the 5′-end cleavage fragment of BSTL, the bottom–top cis C domain transcript (Fig. 6, 5′-labeled BSTL, 5′BSTL in lane 5 and uniformly labeled BSTL, U5BSTL in lane 6). This indicated that the cleavage site of the trans transcript was the same as that found in the cis cleavage.

Self-cleavage occurs as soon as an active structure of RNA is formed

Despite our efforts to increase the yield of the self-cleavage fragments from the cis transcripts by altering the incubation conditions, little variation in the extent of cleavage was obtained. For example, after the denaturation–renaturing step, cleavage of the TEBL or BSTL transcript at 50 °C increased on incubation up to 60 min and then plateaued (Fig. 7). About 1% of both transcripts cleaved without the 50 °C incubation (see Fig. 7, 0 time) and in the short time between addition of DTT, snap-cooling, and the start of ethanol precipitation of the RNA. Hence, about one quarter of the total cleavage had occurred in a very short time before the 50 °C incubation started. After a second round of de-
The extent of self-cleavage in a number of experiments varied from 0.4% to 9% using the standard conditions, indicating that optimal and reproducible self-cleavage conditions have yet to be determined. However, a preliminary analysis showed that the variation appears to be RNA concentration dependent, indicating possible bimolecular interactions as found for the double hammerhead self-cleavage structures of ASBV (Forster et al., 1988).

**DISCUSSION**

**A new type of self-cleavage reaction**

The results reported above have demonstrated a specific self-cleavage of in vitro-synthesized RNAs containing the C domain of CCCV. The cleavage occurs when the RNAs are appropriately denatured and refolded in the presence of spermidine but not added Mg$^{2+}$. Products resulting from this cleavage have a 5'-hydroxyl and possibly a 2', 3'-cyclic phosphate that blocks the 3' end from being labeled with 5'-32P-pCp using T4 RNA ligase. The 3'-terminal 2', 3'-cyclic phosphate is consistent with all in vitro self-cleavage reactions of other small plant pathogenic RNAs (Symons, 1992; Scott & Klug, 1996). However, the sequences around the self-cleavage site cannot form into the well-characterized hammerhead, hairpin, or pseudoknot structures of other self-cleaving RNAs (Symons, 1994), indicating that a new type of active tertiary structure is involved.

The findings that RNA transcripts of the plant viroid-like satellite RNA or virusoid of lucerne transient streak virus only self-cleave upon addition of Mg$^{2+}$ after heat denaturation and snap-cooling and assembly of the reaction mixture on ice (Forster & Symons, 1987a; Sheldon et al., 1990) led to the concept of active and inactive self-cleavage conformations within a population of RNA molecules of the same sequence. Our results have provided further evidence to support this concept. The observation that only a small percentage of self-cleavage occurred in the TEBL and BSTL RNA transcripts under our standard conditions indicates that these RNAs folded into multiple conformations that included a small fraction active for self-cleavage.

Furthermore, Nakaya et al. (1995) found 11 highly stable structures of the CCCV molecule at almost the same low level of free energy by RNA secondary structure prediction using a highly parallel computer. This is consistent with our conclusion that different structural forms may exist in an RNA population. Our observation that the cleavage of TEBL and BSTL only occurs in the presence of spermidine and absence of added Mg$^{2+}$ indicates that Mg$^{2+}$ contributes to the formation of inactive structures.

The minor self-cleavage products 5'X and 3'X (see Figs. 3, 4) consistently accompanied the dominant self-

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Characterization of the self-cleavage products of TEBL and BSTL. Cleavage products were analyzed on a denaturing 8% polyacrylamide gel containing 7 M urea followed by autoradiography. All cleavage reactions were performed under the standard conditions (see Materials and Methods). Lanes 1 and 6, cleavage of 5’-labeled RNAs (5'-C). Cleavage of uniformly labeled BSTL (U*-C, lane 7) is used as a control. Lanes 2–3 (TEBL) and lanes 4–5 (BSTL), nonradioactively labeled RNAs were cleaved and subjected to 5' end labeling without prior dephosphorylation (C-5’, lanes 2, 4) or after dephosphorylation with alkaline phosphatase (C-D-5’, lanes 3, 5). Uncleaved full-length transcript (FL), 5’ and 3’ cleavage products (5’P and 3’P), as well as the minor cleavage products (5’X and 3’X) are indicated by arrows. Reference lengths (nt) of markers are given at left side of the figure.

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The minor self-cleavage products 5’X and 3’X (see Figs. 3, 4) consistently accompanied the dominant self-
cleavage of the BSTL transcript, but were not found in the TEBL transcript. If self-cleavage is involved in viroid processing in vivo, the cleavage site found here in both TEBL and BSTL is likely to be the most prospective site, whereas the minor site would represent a labile site under some conditions.

**Potential self-cleavage site in other members of PSTV-group viroids**

The self-cleavage site of TEBL and BSTL is located at the bottom strand between the highly conserved central sequence (core sequence) and the left-hand side inverted repeat sequence (Fig. 8A); this site is four nucleotides away from the 3' end of the region in PSTV (residues 250 and 270, corresponding to residues 167–187 in the CCCV sequence, see Fig. 5B), where the cleavage site is proposed by Robertson et al. (1985). Secondary structures of this region in either the rod-like structure or a potential alternative conformation are conserved in all members of the PSTV subgroup (Fig. 8B). Hence, it is feasible that self-cleavage at a site corresponding to that of TEBL and BSTL will be found in other members of this subgroup.

The core sequences of the apple scar skin viroid (ASSV) and coleus blumei viroid (CbV) subgroups are different from that of the PSTV subgroup (Fig. 8A), and the variations include the region corresponding to the TEBL and BSTL self-cleavage. However, at the secondary structure level, all three subgroups are very similar, and especially the potential hairpin stem-loop structures (Fig. 8B). This suggests that a specific tertiary structure of the C domain may be crucial for the self-cleavage corresponding to the TEBL and BSTL self-cleavage. Certain variations in the cleavage site sequence may be acceptable, as long as the confor-
formation of the active structure is not affected. Hence, there is a potential that self-cleavage may also happen in all members of the ASSV and CbV subgroups, although it has yet to be determined what and how a tertiary structure is involved in the self-cleavage of CCCV.

Of interest is that the position of the self-cleavage site of TEBL and BSTL in the rod-like structure is close to the site of UV crosslinking found in PSTV (G\textsubscript{98} and U\textsubscript{260}, corresponding to G\textsubscript{65} and U\textsubscript{177} in the CCCV sequence, Fig. 1B). The self-cleavage site in the hairpin ribozyme was also found to be close to a site of UV crosslinking in the two-dimensional structure (Butcher & Burke, 1994; Schmidt et al., 1996). Whether or not this close association is functionally relevant to self-cleavage is not known.

**Potential model for study of viroid processing in the PSTV-group**

If an RNA-catalyzed reaction is involved in the rolling circle replication of the PSTV-group viroids, the question arises as to why it is so difficult to reproduce such a reaction in vitro. It seems likely that the processing step occurs in vivo during the synthesis of longer-than-unit-length (+) RNA from the multimeric (−) RNA and that the environment must be right for the RNA to fold into a conformation that allows the specific cleavage. All evidence indicates that the replication of viroids depends entirely on host enzyme systems and that the host nuclear RNA polymerase II is one most likely responsible for in vivo synthesis of the PSTV-group viroids (Mühlbach & Sänger, 1979). Being a complex and multi-component enzyme, it could contribute to making the right folding environment for viroid processing.
Creating an appropriate in vitro environment similar to that in vivo for the viroid self-cleavage has proven to be difficult by our unsuccessful attempts in detecting self-cleavage in the PSTV group of viroids using longer-than-unit-length or multimeric molecules (unpubl. data). However, our success in identifying self-cleavage in our C domain transcripts TEBL and BSTL indicates that these C domain transcripts may well contain the essential elements of an active structure for the in vitro self-cleavage and, thus, similar simple models could be applied to the search of self-cleavage in other members of the PSTV group. This may then lead us toward a better understanding of the processing reaction.

MATERIALS AND METHODS

Construction of plasmids containing C domain of CCCV

Plasmids containing either a monomer or dimer insert of CCCV (constructed and kindly supplied by Dr J. McInnes) were used as templates for amplifying sequences encoding the top or bottom C domain strands of CCCV plus some flanking P and V domain sequences (see Fig. 1B) by PCR. All primers used in these reactions were synthesized on an Applied Biosystems 392 DNA synthesizer and are shown in Table 2. These primers were designed such that the final products generated by PCR would contain both of the C domain sequences plus the flanking P and V sequences joined head to tail, with a T7 promoter sequence at the 5' end and a created restriction site at the 3' end of either C domain sequence (see Fig. 2). These final PCR fragments were cloned into pUC18, between Hind III and Sal I for the top–bottom fragment or between Hind III and Xba I for the bottom–top fragment. Both cloned inserts were confirmed by conventional DNA sequence analysis.

RNA synthesis and purification

All RNAs used in this study were in vitro transcripts prepared using a method described by Gurevich et al. (1991) with slight modifications. With 1 μg of appropriately linearized plas-
mid DNA containing the C domain sequences (Fig. 2), a standard 50-μL reaction mixture contained 80 mM HEPES, pH 7.5, 12 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 3 mM each of the 4 NTPs (Pharmacia), 0.2 μg/μL RNasin (Promega), and 5 μg/μL T7 RNA polymerase. After incubation at 37 °C for 1 h, the samples were treated with RNase-free DNase (Promega) followed by phenol/chloroform extraction and ethanol precipitation. The RNAs were dissolved in 0.1 mM EDTA, pH 8.0, and two volumes of 95% formamide with 10 mM EDTA and 0.02% of xylene cyanol and bromophenol blue (formamide loading buffer) were added. Following heating at 80 °C for 2–3 min, samples were loaded onto an 8% polyacrylamide gel containing 7 M urea for electrophoresis. The desired RNAs were then isolated and eluted from the gel in 0.2% of xylene cyanol and bromophenol blue. Samples were heated at 80 °C for 2 min and snap-cooled on ice before loading onto a 7 M urea–polyacrylamide gel for electrophoresis. 

### TABLE 2. Synthetic oligonucleotides used in the construction of C domain clones of CCCV.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence*</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7PT+</td>
<td>5'-ACTCA CTATA GGGCT GCAGG AGAG</td>
<td>TEBL cloning</td>
</tr>
<tr>
<td>TEB-</td>
<td>5'-TCGAG GAGGC CCGAG CCGAG CCGAG</td>
<td>TEBL cloning</td>
</tr>
<tr>
<td>T7EB+</td>
<td>5'-ACTCA CTATA GGGAA TCCCG TCCCG CA T</td>
<td>TEBL &amp; BSTL cloning</td>
</tr>
<tr>
<td>BST-</td>
<td>5'-TCCTG TCGAC AGGTA AGAG</td>
<td>TEBL &amp; BSTL cloning</td>
</tr>
<tr>
<td>BST+</td>
<td>5'-TACCT GTCGA CAGGA GAGGC C</td>
<td>BSTL cloning</td>
</tr>
<tr>
<td>TEX-</td>
<td>5'-GGAGG TCTAG AATTC CCTTC CCAGA T</td>
<td>BSTL cloning</td>
</tr>
<tr>
<td>HIII-T7tag</td>
<td>5'-AGGCA CAAAT CCACT CACTA TA</td>
<td>5'-tag for TEBL &amp; BSTL</td>
</tr>
</tbody>
</table>

*Underlined sequences are restriction enzyme sites. Dotted underline indicates restriction site overlapped with another site.

The standard self-cleavage reaction started with RNA (0.1–0.5 μg/μL) denaturation in 10 mM methylmercuric hydroxide (Bailey & Davidson, 1976) at room temperature (−22 °C) for 10–20 min, then 1.25 volumes of buffer containing 80 mM HEPES, pH 8.0, 10 mM spermidine, 0.02% SDS, 32% glycerol were added; this reduced the concentration of methylmercuric hydroxide to 4 mM. After a total of 30 min at room temperature, DTT was added to a final concentration of 5–10 mM and the reaction mixture was immediately transferred to a heating block for 1-h incubation at 50 °C. The final reaction mixture contained 40 mM HEPES, pH 8.0, 5 mM spermidine, 0.01% SDS, 16% glycerol. Variations of self-cleavage conditions included 10 mM MgCl₂ or 5 mM spermidine. Control reactions were in 40 mM HEPES, pH 8.0, without any additives. All reactions were stopped by snap-cooling on ice and ethanol precipitation then dissolved in one volume of 0.1 mM EDTA and four volumes of formamide loading buffer (95% formamide, 10 mM EDTA, 0.02% of xylene cyanol and bromophenol blue). Samples were heated at 80 °C for 2 min and snap-cooled on ice before loading onto a 7 M urea–polyacrylamide gel for electrophoresis. 

### Quantification of cleavage products

³²P-labeled cleavage products were separated by PAGE as described above and detected by exposing to a Kodak phosphor screen. Each detected product was analyzed by a Storm 860 PhosphorImager (Molecular Dynamics) and quantitated with ImageQuaNT software (Molecular Dynamics). The amount of each product was expressed as percentage of the total RNA detected and calculated by signal strength from cleavage products over the total RNA.

### Secondary structure analysis

Viroid sequences were analyzed for secondary structures using the MFOLD program (Zuker, 1989) of a Macintosh version. The resulting suboptimal foldings with temperature dependence were compared to search for common features. Results were combined with manual drawing and used to generate the potential hairpin stem-loop structures.

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Self-cleavage in coconut cadang cadang viroid

TEBL and BSTL plasmids. We also thank Dr. N. Shirley and J. Li for synthesis of oligonucleotides.

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