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# The RNA of both polarities of the peach latent mosaic viroid self-cleaves *in vitro* solely by single hammerhead structures

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## ABSTRACT

**Hammerhead self-cleavage of dimeric, monomeric, truncated and mutated transcripts derived from both polarities of the peach latent mosaic viroid (PLMVd) were characterized. In contrast to some results previously published for a very close sequence variant (see ref. 1), these RNAs exhibit a virtually identical self-cleavage during transcription and after purification. By self-cleavage of dimeric transcripts with normal and mutated hammerhead domains and by complementation experiments, we show that the cleavage reactions involve only single hammerhead structures. This observation contrasts with the case of avocado sunblotch viroid (ASBVd), the other self-cleaving viroid, whose mechanism involves mostly double hammerhead structures, whereas single hammerhead cleavage is associated with viroid-like plant satellite RNAs. The difference in stability between the native secondary structures adopted by viroids and the autocatalytic structures, including the hammerhead motif, governs the efficiency of the self-cleavage reaction. The transition between these conformers is the limiting step in catalysis and is related exclusively to the left arm region of PLMVd secondary structure, which includes the hammerhead sequences. Most of the mutations between the variant we used and the sequence variant previously published are located in this left arm region, which may explain to a great extent the differences in their cleavage efficiency. No interactions with long-range sequences contributing to the autocatalytic tertiary structure were revealed in these experiments.**

## INTRODUCTION

Viroids are small single-stranded circular RNAs that infect higher plants, causing diseases in crop species and important economic losses in agriculture (2,3). It was proposed that viroids replicate in a DNA-independent manner by a rolling circle mechanism which involves synthesis of multimeric strands that have to be cleaved into monomeric fragments before being circularized in order to yield the progeny (4). Viroid-like plant satellite RNAs (also named virusoids) and two viroids (ASBVd and PLMVd) undergo specific self-cleavage of multimeric strands into monomeric strands with their autocatalytic 'hammerhead' or 'hairpin' structures (3). The molecular mechanism of these autocatalytic domains has been extensively studied using minimal structures (3,5). That cellular factors are also involved in the processing of multimeric strands cannot be excluded. For example, ribonuclease T1 was shown to cleave and ligate *in vitro* more than one unit length potato spindle tuber viroid (PSTVd) transcripts (6), which are not known to possess any autocatalytic sequences. The self-cleavage is probably an ancestral reaction and the cleavage involving cellular factors would derive from this primitive mechanism (7).

The ability of viroids that possess autocatalytic sequences to self-cleave appears to depend on the capacity of the RNA to adopt a conformation different from their most stable native structure. hammerhead self-cleavage occurs at single or double hammerhead structures, depending on the ability of the sequence to form stable stems surrounding the catalytic site, especially stem III (Fig. 1A and B; see refs 3 and 8). For example, the plus strand of ASBVd requires the association of two hammerhead structures to stabilize the catalytic core for self-cleavage to occur during and after *in vitro* transcription (8,9). In contrast, the minus strand of ASBVd also self-cleaves at double hammerhead structures during transcription, but mostly at single hammerheads after gel purification (9). In addition, a transcript of satellite 2 DNA from the newt self-cleaves by alternative single and double hammerhead modes (10). Unlike viroids, satellite RNAs, whose hammerheads contain stable stem IIIs, are likely to undergo intramolecular self-cleavage at single structures, as shown for the satellite RNA of lucerne transient streak virus (vLTSV), which performs self-cleavage uniquely at single hammerhead structures (8).

The peach latent mosaic viroid (PLMVd) is the causal agent of PLM disease (12). Like the viroid-like satellite RNAs, the hammerhead structures of both polarities from PLMVd have a stable stem III (Fig. 1C and D), suggesting that self-cleavage occurs via single structures. Hernandez and Flores (1) have shown that self-cleavage occurs efficiently during transcription from a monomeric PLMVd construction, but that after

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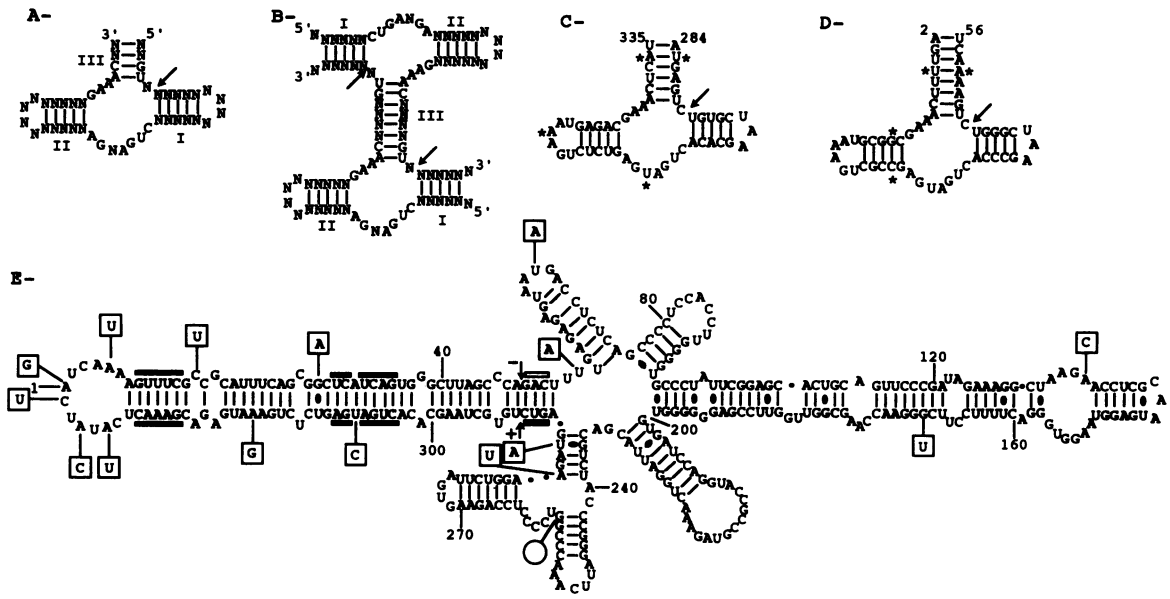


**Figure 1.** Proposed secondary structures. (**A** and **B**) single and double hammerhead consensus secondary structures. Arrows indicate the cleavage sites. (**C** and **D**) single hammerhead structures proposed for both plus and minus polarities of PLMVd. *Position of nucleotide differences between the sequence variants characterized. (**E**) The lowest free energy secondary structure proposed for the PLMVd sequence used in the present study. The secondary structure was determined using version 2.0 of the MFold program based on Zuker (12). Nucleotides in the sequence variant reported by Hernandez and Flores (1) that differ from the variant used here are in boxes and the additional G is indicated by a circle. Hammerhead consensus sequences are indicated by an open bar for minus polarity and by a closed bar for plus polarity.

purification, self-cleavage occurred at a reduced level, on both strands. Consequently, they proposed that the hammerhead structure is more easily adopted during transcription than after complete synthesis. Using a PLMVd sequence variant (Fig. 1E), we studied several features of transcript self-cleavage in order to clearly establish the mode of the hammerhead mechanism and to identify the features regulating the efficiency of the reaction. Unlike several studies using minimal structures, we used a complete viroid sequence, because the results obtained with the entire genome more accurately reflect the *in vivo* replication process.

## MATERIALS AND METHODS

### Reagents

Restriction enzymes, DNase I (RNase-free), calf intestinal alkaline phosphatase, T4 DNA ligase, RNA guard, the T7 sequencing kit and Sephadex G-50 were purchased from Pharmacia. *Taq* DNA polymerase, T3 polymerase and T7 RNA polymerase were from Promega (Fisher Scientific). [$\alpha$-$^{32}$P]UTP (3000 Ci/mmol) and [$\alpha$-$^{35}$S]dATP (1000 Ci/mmol) were from Amersham Canada.

### PLMVd plasmids

The pPL5 clone was a generous gift from Dr Ricardo Flores. The pPL5 clone is a recombinant plasmid derived from pBluescript II KS (+/–) (Stratagene), which contains at the *Pst*I site of the polylinker a monomeric *Pst*I insert of PLMVd initialy cloned in pSPT 18 (Fig. 2; 1). The pPL5 insert is a sequence variant of that used by Hernandez and Flores (1) in their original PLMVd self-cleavage characterization. For the pPD1 clone, the PLMVd insert of pPL5 clone was digested by *Pst*I and gel purified on a 1% agarose gel. Dimeric fragments resulting from the ligation (1:1 ratio) of *Pst*I monomeric fragments were ligated into the dephosphorylated pBluescript II KS (+/–) vector digested with *Pst*I. Both monomeric and dimeric inserts have been sequenced in both directions by the dideoxynucleotide chain termination method using the T7 sequencing kit (Pharmacia). Sequencing of several clones confirmed the presence of an additional G at position 258.

### *In vitro* transcription and RNA purification

For preparation of complete plus strands, clones were digested with *Bam*HI and transcribed with T3 RNA polymerase (see Fig. 2). To obtain complete minus strands, the clones were digested with *Eco*RI and transcribed with T7 RNA polymerase. Partial sequences of both polarities have been prepared using various restriction enzymes (see Table 2). Radioactive transcripts were prepared by incubating 5 µg linearized DNA template overnight at 37°C with 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.01 mM UTP, 40 µCi [$\alpha$-$^{32}$P]UTP, 40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 34 U RNA guard and 120 U T3 or T7 RNA polymerase in a volume of 100 µl. The mixtures were pre-incubated for 10 min at 37°C before adding RNA polymerase. After transcription, the mixtures were incubated for 10 min at 37°C with 30 U DNase I and 4 µl 0.5 M EDTA, pH 8.0, and then extracted with phenol–chloroform. For evaluation of self-cleavage efficiency, 0.5 vol stop buffer (0.3% bromophenol blue and xylene cyanol, 10 mM EDTA, pH 7.5, 97.5% deionized formamide) was added to transcriptional aliquots, denatured for 2 min at 65°C and analyzed on a 5% polyacrylamide gel in 100 mM Tris–borate, pH 8.3, 1 mM EDTA, 7 M urea. The fraction of cleaved molecules was determined by analysis of dried gels with a PhosphorImager (Molecular Dynamics). For purification, the radioactive transcripts were

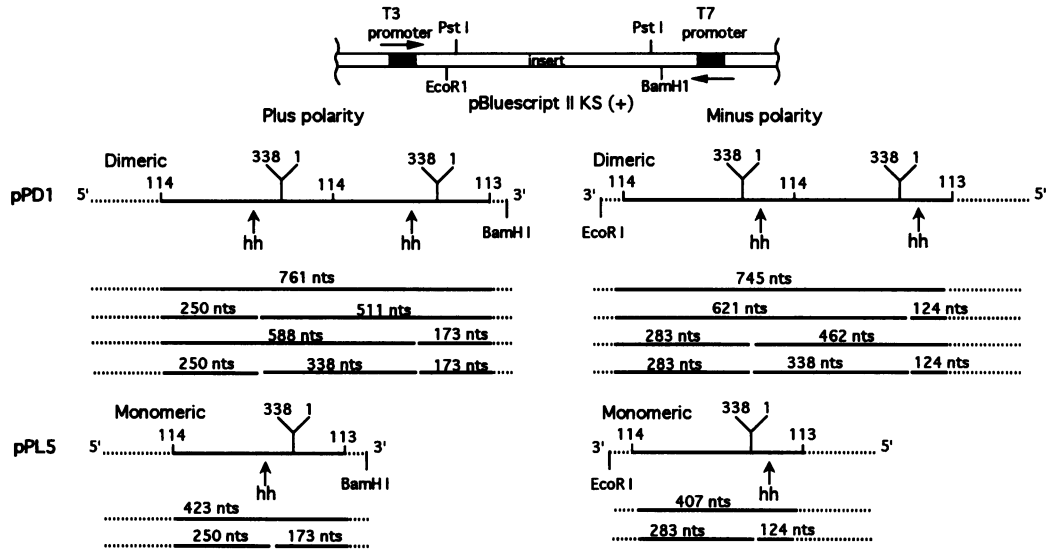



**Figure 2.** PLMVd plasmid constructs and transcriptional products. Monomeric and dimeric constructs are reported in Materials and Methods. Full lines indicate sequence from PLMVd and dashed lines sequence from the vector. Arrows denoted by hh are for hammerhead cleavage sites and the length of each fragment in nucleotides (nt) is indicated.

**Table 1.** Self-cleavage efficiency of dimeric transcripts prepared by PCR amplification from the pPDI construction

| | plus polarity | | minus polarity | |
|---|---|---|---|---|
| | GAAAC | GAAC | GAAAC | GAAC |
| 5'↓ 3' | 62/60 | 63/67 | 51/54 | 56/53 |
| 5' ↓3' | 89/93 | -/- | 88/91 | -/- |
| 5'↓ ↓3' | 56/53 | -/- | 47/43 | -/- |
| 5'↓ | /65 | /62 | /53 | /54 |
| ↓3' | /91 | /- | /87 | /- |

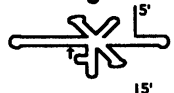
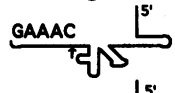
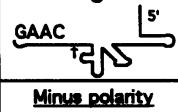
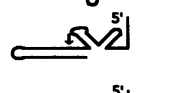
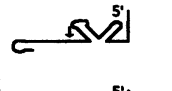
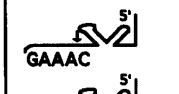
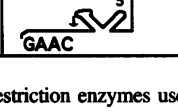
Percentages apply to the hammerhead cleavage sites identified by arrows in the schematic representation of dimers. Percentages are for during transcription (numerator) and after gel purification (denominator). The GAAAAC and GAAC columns are for the wild-type and mutated 3′ hammerhead sequence; the 5′ hammerhead is a wild-type in all cases. – Cleavage was not detected at the hammerhead sites marked by pointed arrows. The first three rows in each of the four full dimeric sequences and either during transcription or after purification are from quantification of bands in a single gel track, so the percentages of cleavage in the cases of a single cleavage come from the addition of the percentage in that case and the one where two cleavages occurred. The last two rows in all cases are values that correspond to purified transcripts that had cleaved at one of their sites during transcription. A blank is when the cleavage percentage does not apply.

precipitated before gel separation. Transcripts were detected by autoradiography and were then excised, eluted, precipitated, passed twice through Sephadex G-50 spun columns, lyophilized and conserved at –70°C. For preparation of non-radioactive transcripts, the procedure was identical to that for the radioactive transcripts except that the concentration of UTP was 0.5 mM and the radioactive UTP was omitted from the pool. Products from the self-cleavage reactions were purified on 5% PAGE (7 M urea), detected by UV shadowing, extracted and their concentration determined from their absorption in aqueous solution at 260 nm.

## Synthesis of deletion mutants

Truncated transcripts were synthetized from PCR-prepared templates where the control hammerhead GAAAC sequence was either conserved or mutated to GAAC, following the approach described previously for ASBVd self-cleavage studies (8,9). For the plus strand, both pPL5 and pPD1 plasmids were linearized with *Bam*HI and the viroid sequence was PCR amplified with a sense oligonucleotide of 17 nt corresponding to the T3 promoter and an antisense oligonucleotide of 23 (5′-GATATGA**GTTC**G-TCTCATTTCAG-3′, deletion) or 24 nt (5′-GATATGA**GTTTC**-GTCTCATTTCAG-3′, control) corresponding to positions 315–338 of PLMVd (see Fig. 1). PCR mixtures were 1 µg DNA, 5 pmol each oligonucleotide, 10 µl Perkin-Elmer 10× buffer (500 mM Tris–HCl, pH 8.3, 500 mM KCl), 2.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase in a total volume of 100 µl. The amplification cycle (94°C for 2 min, 30°C for 1 min, heated by 1°C/3 s up to 72°C, 72°C for 1 min) was repeated only five times in order not to restrict the PCR product to the shortest product from the pPD1 template, which includes only one hammerhead sequence. After phenol–chloroform extraction and isopronanol precipitation, PCR reactions were gel purified on a 2% agarose gel and the bands of 313 (control) and 312 (deletion) (from pPL5 and pPD1), 651 (control) and 650 nt (deletion) (from pPD1) were isolated, extracted, ethanol precipitated and rinsed, dried and then *in vitro* transcribed as described above. The same approach was used for the minus strand of pPL5 and pPD1 previously linearized with *Eco*RI. The sense oligonucleotides of 26 (5′-CATCAAAA**GTT**-**C**GCCGCATTTCAGCG-3′, deletion) or 27 nt (5′-CATCAAA-**AGTTTC**GCCGCATTTCAGCG-3′, control) covered the positions 338–24, while the antisense oligonucleotide corresponds to the T7 promoter (5′-TAATACGACTCACTATA-3′).

**Table 2.** Percentage self-cleavage efficiency of truncated transcripts during transcription and after purification

| Rest. enz. | Secondary structure | Transcription | Purification |
|---|---|---|---|
| | Plus polarity | | |
| BamH I |  | 69 | 61 |
| Sty I |  | 68 | 65 |
| * |  | 94 | 95 |
| * |  | — | — |
| | Minus polarity | | |
| EcoR I |  | 54 | 52 |
| Kpn I |  | 51 | 54 |
| Mbo II |  | 54 | 49 |
| Alw26 I |  | 84 | 88 |
| * |  | 85 | 94 |
| * |  | — | — |

The names of restriction enzymes used are reported. *When the templates were prepared by PCR amplification, GAAAC or GAAC idenfity the hammerhead sequence. A schematic secondary structure of the truncated transcripts, based on the secondary structure presented in Figure 1E, is given to allow localization of their 3′ extremity. – Undetectable level of self-cleavage →; indicates the hammerhead cleavage site.

Amplification conditions were similar to those described above and the products of 173 and 511 nt for the control and 172 and 510 nt for the mutant were isolated.

### *In vitro* self-cleavage of purified transcripts

Prior to self-cleavage incubation, the samples (25 000–100 000 c.p.m.) were heated in 1 mM EDTA, pH 6.0, at 100°C for 1 min and snap-cooled on ice for 1 min. Self-cleavage of purified transcripts was initiated by adding the reaction buffer to a final concentration of 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EDTA in a total volume of 10 µl and then samples were incubated for 15 min at 37°C. The reactions were stopped by addition of 0.5 vol stop buffer, kept on ice, denatured for 2 min at 65°C, purified on 5% PAGE (7 M urea) and the dried gels were analyzed by PhosphorImager. Variations to this protocol in some experiments are indicated below.

## RESULTS

### Monomeric and dimeric transcripts self-cleave similarly

The pPL5 and pPD1 clones included an insert of monomeric and dimeric PLMVd sequence respectively, as shown in Figure 2. After linearization, these constructions allowed the synthesis of either plus or minus transcripts according to whether T3 or T7 RNA polymerase was used. During *in vitro* transcription, RNA of both plus and minus polarities self-cleaved efficiently (Fig. 3A), indicating that hammerhead structures were adopted. Once purified, complete transcripts, as well as the intermediate products from dimeric transcripts, which self-cleaved at a unique site during transcription showed autocatalytic cleavage at the same level as monomeric and dimeric transcripts (Fig. 3B and C). If $Mg^{2+}$ or snap-cooling or both were omitted (Fig. 3B and C, lanes 1 and 5), self-cleavage could not be detected. Both monomeric and dimeric plus polarity transcripts displayed a slightly greater self-cleavage than did the minus polarity transcripts (Fig. 3); 60–70% of plus transcripts self-cleaved, while for minus transcripts cleavage was 50–55%. The total cleavage at one site in a dimeric transcript was obtained by addition of the percentage of product resulting from cleavage at this site only and that from the cleavage which occurred at both sites. Furthermore, 5′ and 3′ self-cleavage appear to be virtually identical within a dimeric transcript, suggesting that the location of the hammerhead domain, as well as the sequences derived from the vector, do not influence the reaction efficiency.

Time courses of self-cleavage for purified transcripts were performed (Fig. 4). Under the conditions we used, self-cleavage occurred almost immediately for dimeric transcripts; after 15 s most of the substrate which could self-cleave had done so (Fig. 4). Similar results were obtained at concentrations ranging from 0.2 to 45 nM. Almost no additional transcripts reacted when incubations were carried out for from 15 to 600 s, and even after 1 h of incubation the level of self-cleavage remained unchanged. Similar results with time courses for monomeric transcripts from pPL5 and intermediate transcripts with one hammerhead site from pPD1 were also observed (data not shown). In addition, the preliminary snap-cooling step was characterized for several transcripts and showed similar results. For example, the experiments using the plus polarity monomeric RNA showed that the denaturation must be at 90°C or higher to observe efficient self-cleavage. If a second cycle of snap-cooling and incubation at 37°C was performed, self-cleavage increased in the same proportion as during the first cycle, while after three cycles almost all the transcripts cleaved, indicating that the population is fully productive (Fig. 4C). Thus all transcripts are reactive and the limiting step appears to be catalytic core formation, not the cleavage step.

### Only single hammerhead structures are involved in self-cleavage of PLMVd

To investigate specifically the single versus double hammerhead modes of self-cleavage, we prepared truncated monomeric RNAs of both polarities from pPL5 by PCR followed by *in vitro* transcription, with the hammerhead GAAAC sequence either conserved or mutated to GAAC, according to the strategy reported for ASBVd self-cleavage studies (8,9). For both polarity transcripts, self-cleavage was productive during *in vitro* transcription and after purification when the GAAAC sequence was conserved, while self-cleavage was not detectable when it was mutated to GAAC (Fig. 5, lanes 1 and 2). These results indicate the absolute requirement for the highly conserved GAAAC sequence for self-cleavage, as reported previously (8) (levels of self-cleavage of monomeric transcripts prepared from pPL5 will

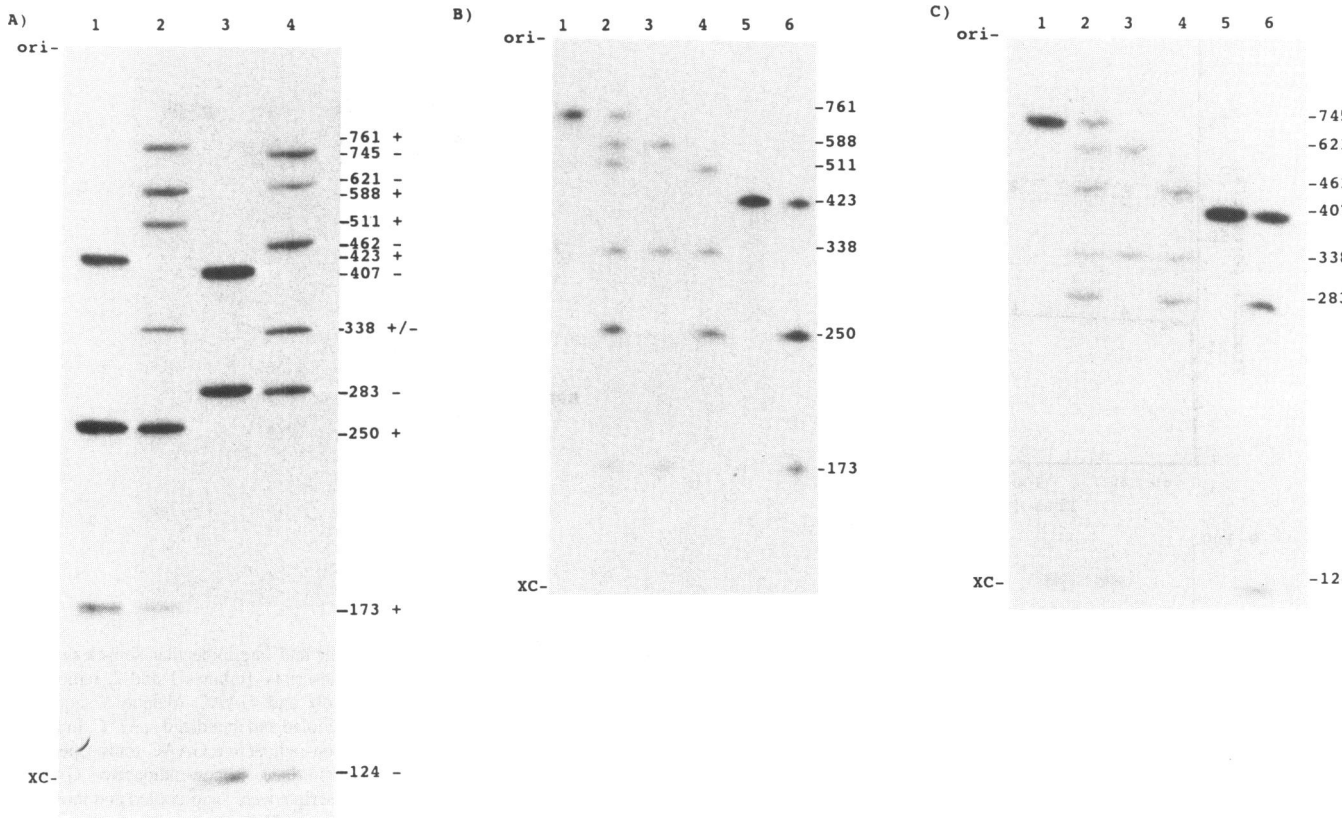



**Figure 3.** Evaluation of self-cleavage efficiency. (**A**) During *in vitro* transcription. Lanes 1 and 2 are monomeric and dimeric plus polarity transcripts and lanes 3 and 4 monomeric and dimeric minus polarity transcripts. The length of the products is indicated in the margin with the symbol specifying the polarity. (**B** and **C**) Self-cleavage of purified plus and minus polarity transcripts respectively. Lanes 1 and 2, full dimeric transcripts; lane 3, dimeric transcripts with a 3′ hammerhead sequence; lane 4, dimeric transcripts with a 5′ hammerhead sequence; lanes 5 and 6 the monomeric transcripts. In lanes 1 and 5, snap-cooling and $MgCl_2$ were omitted. ori denotes origin of migration and XC xylene cyanol.

be analyzed further with the content of Table 2). To investigate whether self-cleavage could occur *in trans* at double hammerhead structures, complementation experiments were performed. When radioactive GAAAC transcripts and non-radioactive GAAC transcripts were snap-cooled and incubated together, only slightly less cleavage was detected as compared with the GAAAC transcripts alone (Fig. 5, lane 3 versus 1). These results indicate that intramolecular folding is largely favored compared with the intermolecular process. Conversely, when a mixture of radioactive GAAC transcripts and non-radioactive GAAAC transcripts were incubated together, only small amounts of self-cleavage were detected, indicating once again that the reaction is largely intramolecular (Fig. 5, lane 4).

The same primers as above were used for PCR amplification with the pPD1 dimeric construct under conditions that allowed synthesis of products of both polarities starting from the promoter and truncated either after the first or the second hammerhead site (see Materials and Methods). The smallest templates are identical to the amplification products from pPL5 and exhibit similar self-cleavage during transcription, as well as after isolation (data not shown). The largest amplification products allowed transcription of RNA that possessed two hammerhead structures. The transcripts of both polarities having the conserved GAAAC sequence at both sites (control) self-cleaved at both sites, while those that were mutated to GAAC at the 3′ hammerhead sequence self-cleaved only at the 5′ site during transcription and after isolation (Table 1) (levels of self-cleavage of dimeric transcripts prepared from pPD1 will be analyzed further). If double hammerhead structures were involved in self-cleavage, the GAAC mutated transcripts would have allowed self-cleavage at the 3′ site by the action of a non-neighbouring GAAAC 'catalytic' strand on the 3′ site mutated 'substrate strand'. Hence, the results from PCR-mutated transcripts provide strong evidence for the involvement of only single hammerhead structures in self-cleavage of PLMVd transcripts of both polarities.

## Self-cleavage of truncated transcripts

To investigate the importance of secondary structure and the possibility of interactions with long-range sequences influencing the self-cleavage reaction, we prepared various transcripts from pPL5 previously digested with different restriction enzymes and evaluated their autocatalytic efficiency during transcription and after purification (Table 2). Self-cleavage efficiency was virtually identical for the transcripts truncated in a region corresponding either to the right arm or the central region of the PLMVd (plus polarity, *Bam*HI, *Sty*I; minus polarity, *Eco*RI, *Kpn*I). The transcripts of minus polarity synthesized after template linearization with *Mbo*II, which cuts at the right extremity of the left arm region, self-cleaved at the same level as the full-length *Eco*RI transcripts. Hence, under the reaction conditions we used, the central and the right arm regions did not influence self-cleavage

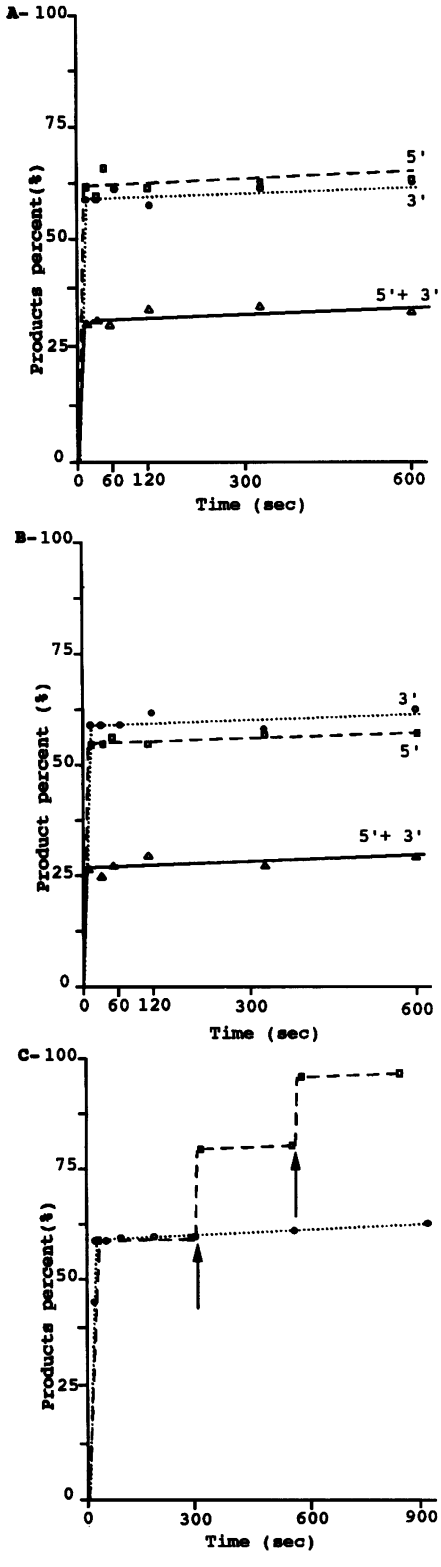



**Figure 4.** Self-cleavage time courses of 1 nM purified transcripts. (**A** and **B**) time courses of complete dimeric transcripts of plus and minus polarities respectively. Solid lines (—) are for self-cleavage occuring at both hammerhead sites, while dashed lines are for self-cleavage occuring at the 5′ site (----) and the 3′ site (····). (**C**) self-cleavage of plus polarity monomeric transcripts. Doted lines (·····) are for self-cleavage after a single snap-cooling at 90°C and dashed lines (-----) are for the case where a second and third snap-cooling (indicated by arrows) have been performed.

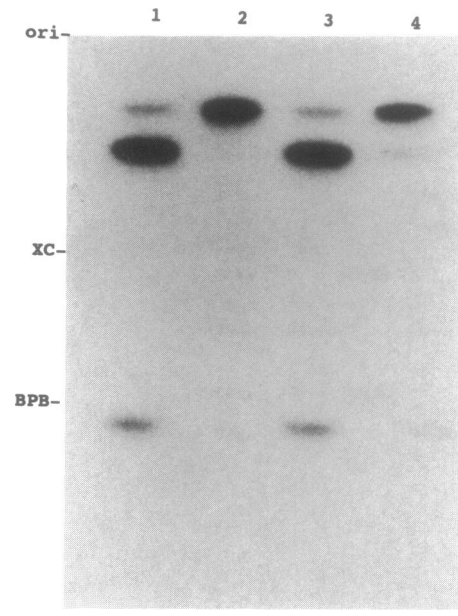



**Figure 5.** Evaluation of intramolecular and intermolecular self-cleavage of truncated monomeric plus polarity transcripts. In lanes 1 and 2, radioactive transcripts with the GAAAC (control) and GAAC (deletion) sequences respectively were independently snap-cooled and incubated at 37°C. In lane 3, radioactive GAAAC transcripts and non-radioactive GAAC transcripts were snap-cooled and incubated together. In lane 4, non-radioactive GAAAC transcripts and radioactive GAAC transcripts were snap-cooled and incubated together. ori denotes origin of migration and XC xylene cyanol.

efficiency. These results suggest that no important structures and sequences localized in these regions influence self-cleavage. In contrast, self-cleavage was largely improved with transcripts that allow full hammerhead sequence synthesis but are truncated near its extremity, thus preventing the synthesis of the opposite strand of the native structure (minus polarity, Alw26 I; both polarities, PCR-prepared templates). Hence, self-cleavage is likely hindered when adoption of the lowest free energy structure is accomplished. These results propose that the left arm region bearing the hammerhead sequences governs self-cleavage efficiency.

Efficiencies of self-cleavage of the transcripts prepared from the largest amplification products of the pPD1 dimeric construction led to similar conclusions (Table 1). In all these transcripts, the 5′ hammerhead sequences are intact (GAAAC), are not truncated near their extremity and display a similar self-cleavage to the full monomeric and dimeric transcripts of both polarities (see Fig. 3 and Table 2); 60–67% of plus transcript 5′ sites self-cleaved, while for minus transcripts cleavage was 51–56% (Table 1 and below). In comparison, the 3′ hammerhead sequence, which has the GAAAC sequence but is truncated near its extremity, thus preventing the synthesis of the opposite strand of the native structure, exhibits greater self-cleavage efficiency, like the corresponding monomeric transcripts; 89–93% of plus polarities self-cleaved, while for minus transcripts cleavage was 87–91% (Table 1). The improvement in self-cleavage at the 3′ hammerhead site has the effect of proportionally increasing the accumulation of one unit length PLMVd transcripts, which result from the cleavage of both hammerhead sequences. Together these results confirm that self-cleavage is likely hindered when adoption of the lowest free energy structure is realized and that

both 5′ and 3′ hammerhead sequences are independent in their autocatalytic action.

For all the transcripts studied, the efficiency of self-cleavage is relatively similar both after purification and during transcription (Tables 1 and 2), suggesting that snap-cooling is efficient for folding of the hammerhead catalytic core. We were anticipating a superior efficiency of self-cleavage during transcription than after purification, because the region including the hammerhead sequence is synthesized before its complementary strand, to which it base pairs, favoring the adoption of the native structure. However, in PLMVd, these regions are adjacent and separated by only a few nucleotides (9 nt in the plus polarity; 7 nt in the minus polarity). We suggest that as soon as the hammerhead sequence is synthesized, synthesis of the complementary strand is initiated and within a very short time the native secondary structure is adopted. Therefore, for PLMVd the transcriptional advantage for sequential production and folding of the hammerhead sequences is limited. To further investigate this hypothesis, we performed transcription of a sequence corresponding only to the left arm region (F. Bussière and J.-P. Perreault, unpublished data). Self-cleavage of the resulting transcripts was greatly improved (>95%) when, instead of only one ribonucleotide, two ribonucleotides at a concentration of 10 μM were included in the transcriptional mixture. Under these conditions, the polymerase elongation rate is reduced, probably allowing time for the hammerhead catalytic core to be formed and self-cleavage to occur before synthesis of the complementary sequence favors adoption of the native structure and prevents self-cleavage.

## DISCUSSION

The various experiments reported here lead us to conclude that RNAs of both polarities from PLMVd self-cleave at a single hammerhead structure during transcription and also after purification. The secondary structures of the autocatalytic conformation are less stable in terms of free energy than the native rod-like structure, which limits the reaction. As for the satellite RNA of vLTSV (13), the rate-limiting step for PLMVd self-cleavage is the conformational change from the native structure to the alternative structure, including the hammerhead catalytic core, and not the chemical cleavage step. In contrast, for several minimal hammerhead structures Long and Uhlenbeck (14) showed that the chemical cleavage step is rate limiting. This difference was expected, since minimal hammerheads are usually designed to assume mostly the catalytic structure in solution.

Eleven of the 15 nucleotide differences between the two PLMVd variants, including the one used in the present study and the one used by Hernandez and Flores (1), are in the left arm region, which is the most stable region of PLMVd. These nucleotide differences affect the native secondary structure locally (see Fig. 1E), but the free energies (ΔG) of the complete left arm region in its rod-like form of both polarities remains identical, –50 kcal/mol and –53 kcal/mol respectively for plus and minus polarities. Furthermore, these nucleotide differences do not affect the hammerhead secondary structures (Fig. 1C and D); hence, co-variation of base paired nucleotides is observed, which suggests selective pressure in favor of the self-cleavage activity. The free energies (ΔG) of the left arm secondary structures, including the hammerhead conformation for each variant of both polarities, has been estimated by considering two separate domains: the sequence forming the hammerhead structure and the balance of the sequence of the left arm. The estimated free energies (ΔG) of the left arm region in the hammerhead structure reveal that the variant used here is slighty more stable than the variant characterized by Hernandez and Flores (1). Consequently, the free energy difference (ΔΔG) of the native structure and that of the hammerhead structure is smaller (~20%) for the variant used in the present study. This difference could explain, at least partially, why the variant we used self-cleaved more efficiently after purification (>50%) than the variant used by Hernandez and Flores (~10% in the absence of formamide or snap-cooling on dry ice; 1). Furthermore, when we estimated the free energy of the structures adopted by the plus polarity hammerhead sequence of the variant used by Hernandez and Flores, the autocatalytic structure was not the most stable alternative structure, with a free energy of ~20% less. This difference may also account for the lower level of self-cleavage of their variant. Our results support the hypothesis that the efficiency of self-cleavage is governed by the stability of the structures, including the hammerhead motif, as compared with the native structure. However, these results do not explain all the differences in self-cleavage efficiency between variants of both polarities and do not account for the putative contribution of any tertiary interactions. Efficient adoption of alternative structures by viroids, including the hammerhead conformation, is of crucial importance for their replication, since autocatalytic cleavage releases monomeric copies from multimeric strands. Study of viroid conformational isomers is obviously important to understand related biological activities.

In contrast to ASBVd, PLMVd shows the involvement of only single hammerhead structures in the processing of multimeric strands. This characteristic was previously associated only with satellite RNAs. Our study indicates that the mode of self-cleavage is not a strict classification character differentiating between viroids and other related satellite RNAs. On the other hand, it supports the proposed phylogenetic position of PLMVd between ASBVd and vLTSV (1). ASBVd has been proposed as the evolutionary link between viroids and satellite RNAs in a monophylogenetic tree (15). PLMVd appears to have evolved from vLTSV and it has conserved its self-cleavage properties. Therefore PLMVd may be a unique case among viroids.

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