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In vitro and *in vivo* self-cleavage of a viroid RNA with a mutation in the hammerhead catalytic pocket

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ABSTRACT

Peach latent mosaic viroid (PLMVd) can adopt hammerhead structures in both polarity strands. In the course of a study on the variability of this viroid a natural sequence variant has been characterized in which the hammerhead structure of the plus polarity strand has the sequence CCGA instead of the conserved uridine turn motif CUGA present in the catalytic pocket of all natural hammerhead structures. The viroid RNA containing this mutant hammerhead structure, but not those with the two other possible substitutions, U→A and U→G, in the same position of the catalytic pocket, showed significant self-cleavage activity during *in vitro* transcription. Moreover, the corresponding full-length PLMVd cDNA was infectious and the mutation was retained in a fraction of the viroid progeny. These results indicate that the sequence flexibility of the hammerhead structure, acting *in vitro* and *in vivo*, is higher than anticipated and provide relevant data for a deeper insight into the catalytic mechanism of this class of ribozymes and into the structure of the uridine turn motif.

INTRODUCTION

Hammerhead ribozymes have been found in a group of small plant RNAs, including three viroids and several satellite RNAs (1–5), in the RNA form of a carnation retroviroid-like element (6), as well as in a small transcript from the newt (7). Hammerhead structures mediate *in vitro* self-cleavage *in cis* of the RNAs in which they are naturally found (8,9) and in some instances there is strong evidence indicating that they are also operative *in vivo*. This is the case for the three known viroids with hammerhead ribozymes, avocado sunblotch viroid (ASBVd) (10), peach latent mosaic viroid (PLMVd) (11) and the recently characterized chrysanthemum chlorotic mottle viroid (CChMVd) (5). Evidence for *in vivo* function comes from three observations: (i) the 5′-termini of two sub- and supra-genomic plus ASBVd RNAs and of one sub-genomic minus ASBVd RNA isolated from infected avocado tissue are identical to those produced in the corresponding *in vitro* self-cleavage reactions (12); (ii) the 5′-terminus of the monomeric plus CChMVd RNA isolated from infected chrysanthemum tissue is also identical to that produced in the *in vitro* self-cleavage reaction (5); (iii) the nucleotide changes found in PLMVd clones in the regions of both hammerhead structures do not affect their stabilities because when they are found in helices II and III the substitutions are compensatory or because they are located in loops (11; Ambrós *et al.*, unpublished data). Hammerhead structures have also been engineered to act *in trans*, an aspect with major biotechnological implications (13,14).

Considerable interest has been devoted to an attempt to understand the catalytic mechanism through which hammerhead ribozymes function. The crystal structures recently solved for two ribozymes of this class (15,16) have assigned specific roles to the 11 core nucleotides of the single-stranded central loop, providing some clues as to why they are strictly conserved in all natural hammerhead structures. Prominent in this core is the uridine turn motif, the tetranucleotide CUGA, which together with the active site nucleotide forms the catalytic pocket. The sequence of this motif, originally found in the anticodon loop of a tRNA (17), is preserved in all natural hammerhead structures (2,4–7,11) and any change introduced by site-directed mutagenesis essentially abolishes *in vitro* self-cleavage in a hammerhead ribozyme acting *in trans* (18). Moreover, *in vitro* selection experiments have shown that active ribozymes selected from a pool of ribonucleotides completely randomized at all positions of the central core contain the CUGA sequence forming the uridine turn (19,20). Here we report that a natural sequence variant of PLMVd with a mutation in the catalytic pocket of the plus hammerhead structure shows significant self-cleavage not only *in vitro* but also *in vivo*. To the best of our knowledge this is the first study on *in vivo* functioning of a mutant hammerhead structure of this class.

MATERIALS AND METHODS

cDNA synthesis and cloning

PLMVd cDNA clones were obtained from a severe isolate (D168) by RT-PCR. First-strand cDNA was synthesized on purified circular forms of the viroid with primer RF-43, 5′-d(CTGGATCACACCCCCCTCGGAACCAACCGCT)-3′, and avian myeloblastosis virus (AMV) reverse transcriptase as indicated (21). For synthesis of second-strand cDNA an aliquot (1/20) of this preparation was PCR-amplified with primers RF-43

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and RF-44, 5′-d(TGTGATCCAGGTACCGCCGTAGAAACT)-3′, and 2.5 U cloned *Pfu* DNA polymerase using the buffer suggested by the producer (Stratagene) for maximal fidelity: 20 mM Tris–HCl, pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM NH_4SO_4, 0.1% Triton X-100, 100 µg/ml nuclease-free BSA and 400 µM each of the four NTPs. Primers RF-43 and RF-44 are complementary and identical to positions 208–178 and 199–225 respectively of the PLMVd reference sequence (11) and overlap a *Sau*3A restriction site located in a domain of the molecule with a very limited sequence variability (Ambrós *et al.*, unpublished data). The PCR cycling profile (30 cycles) was 94°C for 40 s, 60°C for 30 s and 72°C for 2 min, with a final extension step at 72°C for 10 min. PCR products were separated by PAGE and the DNA of the expected length was digested with *Sau*3A and cloned in pBluescript II KS+ linearized with *Bam*HI. Sequences of the inserts were determined by chain terminating inhibitors (22) and T7 DNA polymerase. Two recombinant plasmids were selected for further studies: pGDS23, with a full-length PLMVd insert differing from the reference sequence (11) in 23 positions which do not include the 11 core nucleotides conserved in all natural hammerhead structures; pGDS59, with an insert identical to that of pGDS23 except for the change U4→C in the uridine turn of the PLMVd plus hammerhead structure.

Site-directed mutagenesis

The protocol reported previously (23) was followed with minor modifications. Recombinant plasmid pGDS23 (5 ng) was PCR-amplified with 250 ng each of the phosphorylated primers RF-103, 5′-d(TCACTCAAAAGTTTCGCCGT)-3′, and RF-106, 5′-d(CAAGAGTTCGTCTCATTTC)-3′. RF-103 and RF-106 are homologous and complementary respectively to two adjacent regions of the pGDS23 insert except in a position of RF-106 which was deleted to change the conserved motif GAAAC of the PLMVd plus hammerhead structure into GAAC. Additionally, pGDS23 was also PCR-amplified with the phosphorylated primers RF-126, 5′-d(GAAATGAGACGAAACTCTTGTCACTC)-3′, and RF-127, 5′-d(AGAGACTCATC**C/T**GTGTGCTTAGCA)-3′. RF-126 and RF-127 are homologous and complementary respectively to two adjacent regions of the pGDS23 insert except in a position of RF-127 which was degenerated (residues in bold) to change the conserved motif CUGA of the PLMVd plus hammerhead structure into CAGA or CGGA. PCR amplifications were catalyzed by 2.5 U *Pfu* DNA polymerase using the buffer indicated above. The PCR cycling profile, designed to allow annealing of the primer with the mismatch and amplification of the complete plasmid, consisted of: a hot start of 94°C for 2 min and 72°C for 3 min (with the enzyme added at this stage), followed by three cycles of 94°C for 40 s, 37°C for 30 s and 72°C for 5 min and 25 additional cycles of 94°C for 40 s, 50°C for 30 s and 72°C for 5 min, with a final extension step at 72°C for 10 min. PCR products were separated by agarose (0.8%) gel electrophoresis and the DNA of the expected length was eluted and ligated with 8 U T4 DNA ligase. After transformation the inserts of the new plasmids (pGDS23-1, pGDS23-2 and pGDS23-3) were sequenced to confirm that the expected mutations (GAAAC→GAAC, U4→A and U4→G respectively) were the only changes introduced.

In vitro transcription

Recombinant plasmids with PLMVd full-length inserts in appropriate orientations were digested with *Eco*RI and transcribed with T7 RNA polymerase to obtain the plus polarity strands. Transcription led to products containing the complete PLMVd sequence plus two 5′- and 3′-tails of 47 and 22 nt respectively from the vector. Transcription reactions contained 50 ng/µl linearized plasmid, 1 U/µl T7 RNA polymerase, 40 mM Tris–HCl, pH 8, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 1–2 U/µl human placental ribonuclease inhibitor, 1 mM each GTP, CTP and UTP and 0.1 mM ATP plus 0.5 µCi/µl [α-^{32}P]ATP. After incubation at 37°C for 1 h the template was digested with RNase-free DNase and the transcripts were separated in 1× TBE polyacrylamide gels (5%) containing 8 M urea plus 40% formamide (to improve separation of RNAs with high secondary structure). The gels were dried and scanned with a bioimage analyzer (Fuji BAS1500).

Synthesis of partial length fragments of PLMVd

PLMVd-cDNA fragments were PCR-amplified using 0.5 µg each primers RF-89, 5′-d(TAATACGACTCACTATA**G**GAAAGAGTCTGTGCTAAGC)-3′, and RF-88, 5′-d(CAAGAGTTTCGTCTCATTTC)-3′, previously phosphorylated. Primers RF-89 and RF-88 are homologous and complementary to sequences delimiting the 5′- and 3′-borders of the PLMVd plus hammerhead structure respectively, except for the sequence underlined in primer RF-89, which is the T7 RNA polymerase promoter, and the G in bold in this same primer, which is an extra residue. Aliquots of 10 ng of the three recombinant plasmids containing full-length PLMVd inserts with the wild-type plus hammerhead structure (pGDS23) or its mutated versions (pGDS23-1 and pGDS59) were used as templates. Other components of the PCR reactions and the PCR cycling profile were as indicated above. PCR products of the expected length were cloned into the *Sma*I site of pUC18 and the sequence and orientation of the inserts were confirmed by sequencing. Transcription from these constructs linearized with *Bam*HI was analyzed in 1× TBE polyacrylamide gels (10%) containing 8 M urea. Primary transcripts contained the sequences of the corresponding hammerhead structures (Fig. 1) plus two short 5′- and 3′-tails, GGA and GGGGGAUC respectively.

RNA self-cleavage

Self-cleavage of the purified transcripts was performed at 40°C for 1 h in 50 mM Tris–HCl, pH 8, 5 mM $MgCl_2$ and 0.5 mM EDTA. Prior to incubation the samples were heated in 1 mM EDTA, pH 6, at 100°C for 3 min and snap cooled on dry ice/methanol. Products from the self-cleavage reactions were separated by denaturing PAGE as indicated above.

Hammerhead self-cleavage kinetics

Intramolecular cleavage rates were measured during *in vitro* transcription of the partial length PLMVd RNAs having only the sequences of the wild-type and the mutated U4→C hammerhead structures. For this purpose a procedure reported previously (24) with some modifications was followed. Reactions were carried out at 37°C in a transcription buffer containing 50 ng/µl linearized plasmid, 2 U/µl T7 RNA polymerase, 40 mM Tris–HCl, pH 7, 6 mM $MgCl_2$, 50 mM DTT, 10 mM spermidine,

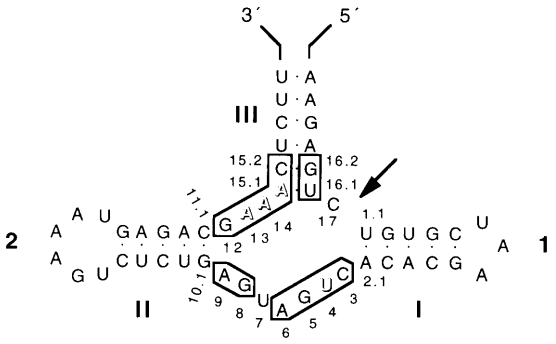

Figure 1. Wild-type hammerhead structure of the plus PLMVd strand represented as proposed previously (42). The conserved nucleotides present in all natural hammerhead structures, with two exceptions in which the C-G base pair formed in helix III by residues 15.2 and 16.2 is substituted by a U-A base pair (4,43), are boxed. Core residues in the central loop affected by mutations are shown in outlined fonts. The arrow indicates the predicted self-cleavage site.

2 U/µl human placental ribonuclease inhibitor, 0.5 mg/ml RNase-free bovine serum albumin, 0.1% Triton X-100, 1.5 mM each ATP, CTP, GTP and UTP plus 0.5 µCi/µl [α-^{32}P]ATP. Aliquots were removed at appropriate times and quenched with a 5-fold excess of stop solution (9 M urea in 50 mM EDTA). Reaction rates were measured by determining the extent of self-cleavage at various times from denaturing PAGE separation and quantitation as indicated above. The data were fitted to the equation derived previously (24) using a least squares method. Each calculated rate constant, determined by plotting the logarithm of the uncleaved fraction versus time, was the average value of at least three independent experiments.

RESULTS AND DISCUSSION

In vitro self-cleavage of full-length PLMVd RNA containing a hammerhead structure with a substitution in the catalytic pocket

In the progress of a study on the sequence variability of PLMVd we have cloned and sequenced a large number of sequence variants of this viroid (25). In one of the PLMVd variants we observed the change U4→C in the catalytic pocket of the plus polarity hammerhead structure (Fig. 1). Contrary to our initial expectations, in spite of this substitution 35–45% of the corresponding full-length RNA self-cleaved *in vitro* during transcription, whereas the same RNA with the wild-type hammerhead structure self-cleaved to 85–87% (Fig. 2A). No detectable self-cleavage was observed when the conserved GAAAC motif of the wild-type hammerhead structure was mutated to GAAC by site-directed mutagenesis (Fig. 2A), in agreement with previous results showing that this deletion destroys the catalytic activity of ASBVd and PLMVd hammerhead ribozymes (26,27).

Primer extension experiments confirmed that self-cleavage occurred at the predicted site in the RNA containing the hammerhead structure with the change U4→C in the catalytic pocket (Fig. 2B). On the other hand, when the purified complete transcripts were denatured by heating and incubated under standard self-cleavage conditions, 6–10% of both the wild-type RNA and the RNA with the substitution U4→C in the catalytic pocket self-cleaved, whereas RNA with the deletion in the

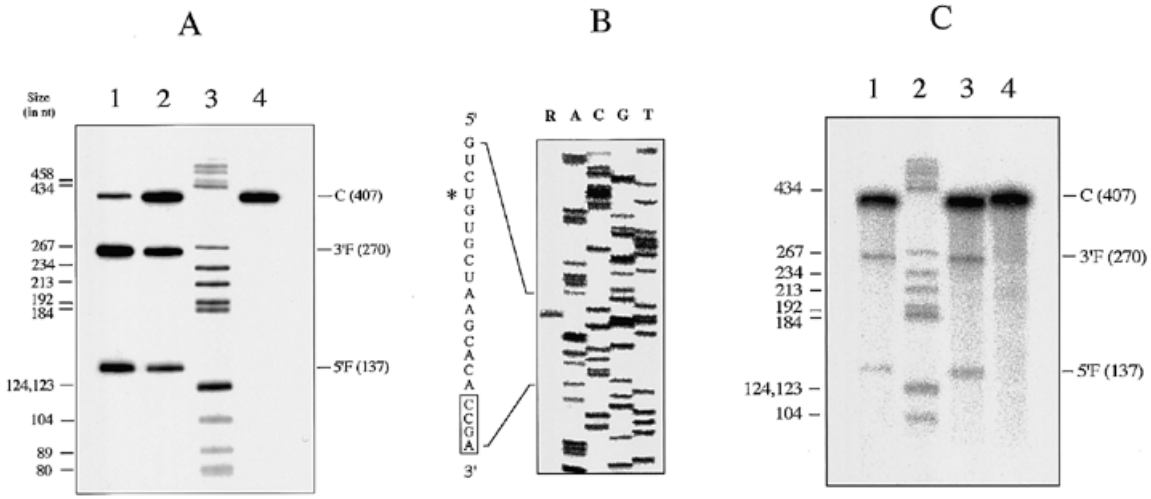

Figure 2. Effects of changes in conserved residues of the plus hammerhead structure of PLMVd on the *in vitro* self-cleavage of full-length viroid strands. (**A**) Analysis by denaturing PAGE (5%) and autoradiography of self-cleavage during transcription of plus PLMVd RNAs with the wild-type sequence (lane 1) and with mutations U4→C (lane 2) and GAAAC→GAAC (lane 4) in their hammerhead structures. DNA markers with their sizes in nucleotides indicated on the left are shown in lane 3. The positions and sizes of the complete transcripts C and of the self-cleavage fragments 5′F and 3′F are indicated on the right. (**B**) Determination of the self-cleavage site of plus PLMVd RNA with the mutation U4→C in its hammerhead structure. The electrophoretic mobility in denaturing PAGE of the cDNA synthesized by reverse transcription with primer RF-43 of the purified plus 3′F fragment resulting from self-cleavage (lane R) was compared with the ladders obtained by sequencing the monomeric PLMVd cDNA clone with dideoxynucleotides and the same primer (lanes A, C, G and T refer to the template which was copied and therefore the 3′-end is at the bottom of the gel). The nucleotide in the 3′ position with respect to the self-cleavage site is indicated by a star and the tetranucleotide containing the mutation U4→C is boxed. (**C**) Analysis by denaturing PAGE and autoradiography of self-cleavage of purified plus PLMVd RNAs. Prior to self-cleavage complete PLMVd transcripts with the wild-type sequence (lane 1) and with mutations U4→C (lane 3) and GAAAC→GAAC (lane 4) in their hammerhead structures were eluted from a preparative gel as in (A) and after heat denaturation and snap cooling on ice they were incubated under standard self-cleavage conditions. DNA markers are shown in lane 2. Other details are as in (A).

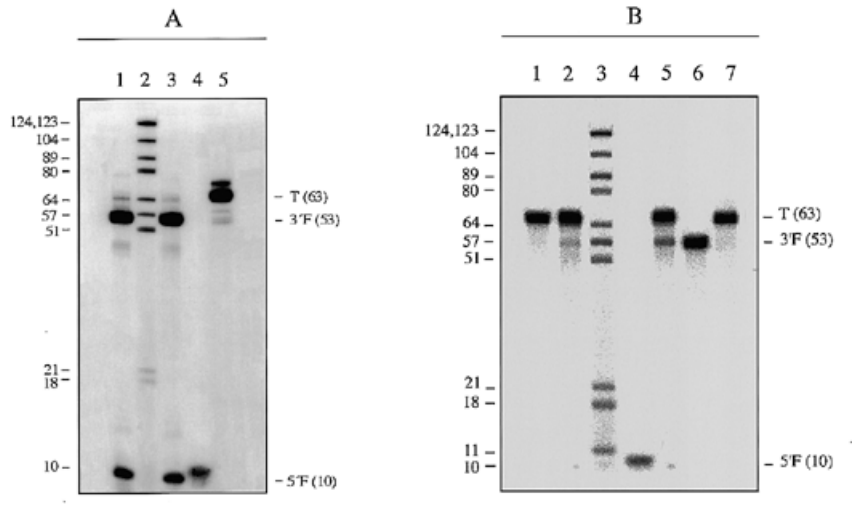

Figure 3. Effects of changes in conserved residues of the PLMVd plus hammerhead structure on *in vitro* self-cleavage of viroid strands having only the sequences of the self-cleaving domain plus two short 5′- and 3′-tails. (**A**) Analysis by denaturing PAGE (10%) and autoradiography of self-cleavage during transcription of plus PLMVd RNAs with the wild-type sequence (lane 1) and with mutations U4→C (lane 3) and GAAAC→GAAC (lane 5) in their hammerhead structures. DNA markers with their sizes in nucleotides indicated on the left are shown in lane 2 and a 10mer DNA marker in lane 4. The positions and sizes of the primary transcripts T and of the self-cleavage fragments 5′F and 3′F are indicated on the right. (**B**) Analysis by denaturing PAGE and autoradiography of self-cleavage of purified plus PLMVd RNAs. Prior to self-cleavage primary transcripts with the wild-type sequence (lane 2) or containing the mutations U4→C (lane 5) or GAAAC→GAAC (lane 1) in their hammerhead structures were eluted from a preparative gel as in (A) and after heat denaturation and snap cooling on ice they were incubated under standard self-cleavage conditions. DNA markers with their sizes in nucleotides indicated at the left are shown in lane 3 and a 10mer DNA marker in lane 4. RNA markers consisting of the complete transcript and the 3′F fragment eluted from preparative gels as in (A) are shown in lanes 7 and 6 respectively. Other details are as in (A).

conserved GAAAC motif of the wild-type hammerhead structure showed no detectable self-cleavage (Fig. 2C). The more efficient self-cleavage of the plus polarity strand of PLMVd during transcription than after purification is consistent with our previous results (11) and is probably due to the adoption of inactive conformations during RNA purification and/or during the heat and snap cooling treatment.

A partial length PLMVd RNA containing only the sequences of the hammerhead structure with U4→C in the catalytic pocket shows *in vitro* self-cleavage

To determine whether the self-cleavage observed here could be dependent on viroid sequences external to the hammerhead structure, constructs having only the sequences of the wild-type or of the two mutated variants of the PLMVd plus hammerhead structure were synthesized via PCR and cloned. During transcription 80–95% of both the wild-type RNA and RNA with the substitution U4→C in the catalytic pocket self-cleaved, whereas only 0–4% of RNA with the deletion in the conserved GAAAC motif of the wild-type hammerhead structure self-cleaved (Fig. 3A). Self-cleavage of the purified transcripts was reduced to 6–8% and 10–15% in the transcripts with the wild-type hammerhead structure and with the substitution U4→C respectively and to 0–1% in the transcript with the deletion GAAAC→GAAC in the hammerhead structure (Fig. 3B). Identification of the self-cleavage fragments was based on their electrophoretic mobilities (Fig. 3A), as well as on end-labeling experiments: when the [α-^{32}P]ATP in the transcription reactions was substituted by [γ-^{32}P]GTP only the full-length product and the 5′F fragment, with a 5′-terminal G in both cases, were labeled and, moreover, most of the label was in the 5′F fragment, confirming extensive self-cleavage (data not shown). Therefore, there is no need to invoke long distance interactions to explain self-cleavage activity of the ribozyme with the pyrimidine transition in the catalytic pocket because the sequences forming the hammerhead structure, by themselves, can account for the observed activity.

Previous observations have shown that the substitution U4→C in the catalytic pocket of a model intermolecular hammerhead structure results in an *in vitro* self-cleavage rate <0.7% of the wild-type hammerhead structure (18). A comparison with this situation was attempted by a procedure reported previously for determination of the intramolecular rates of hammerhead structures (24). However, when using the conditions recommended for this approach the extent of self-cleavage of both the wild-type hammerhead structure of PLMVd and of its U4→C mutant after 1 min was the same as after 1 h (data not shown), preventing any further kinetic characterization. In previous experiments with two intramolecular hammerhead structures, one derived from PLMVd (27) and the other from a virusoid (28), quantitative self-cleavage was also observed within 1 min, indicating that their intramolecular rates must be significantly higher than the values of 0.7 and 1.0 per min obtained for two model hammerhead structures acting *in trans* and *in cis* respectively (24). In an effort to estimate the intramolecular rates in the present case the magnesium concentration in the transcription buffer was reduced from 11 to 6 mM and the pH from 8 to 7, keeping the other components basically as reported (24). Under the new conditions we were able to determine a rate for the U4→C mutant hammerhead structure of PLMVd of 8% of its wild-type counterpart (the average actual rates were 0.24 and 3 per min respectively; see Fig. 4). Therefore, our data indicate that the PLMVd plus hammerhead structure can tolerate the U4→C change in the catalytic pocket to an extent ~10-fold higher than that reported previously (18). However, the two sets of data are not necessarily directly comparable considering that: (i) the two

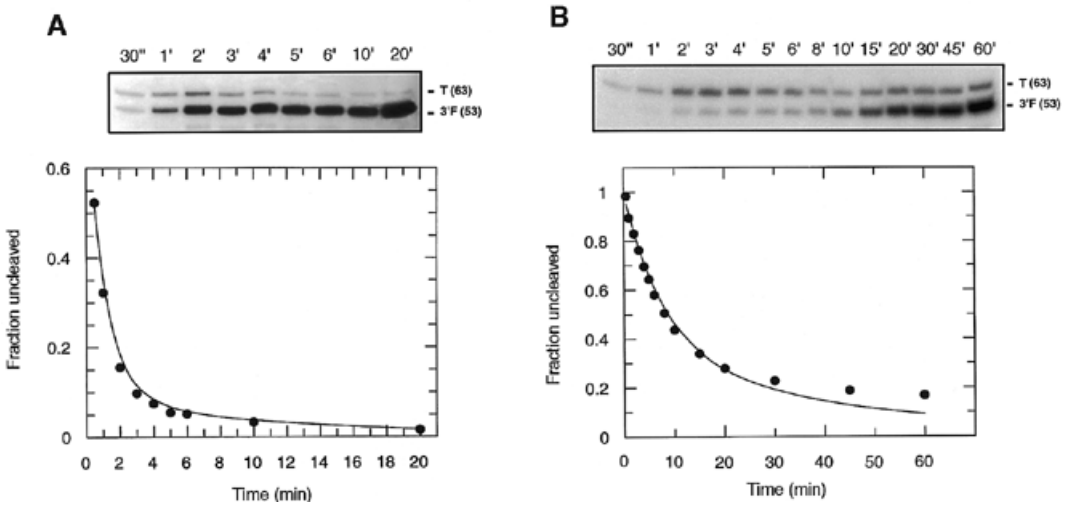

Figure 4. Intramolecular self-cleavage kinetics of two plus PLMVd hammerhead structures. Analysis by denaturing PAGE (10%) and autoradiography of *in vitro* self-cleavage during transcription of PLMVd RNAs having only the sequences of the wild-type (**A**) and the U4→C mutant (**B**) self-cleaving domains plus two short 5′- and 3′-tails. The positions and sizes of the primary transcripts T and of the 3′F self-cleavage fragments are indicated on the right of the upper panels. The data in the lower panels were fitted to the equation derived previously (24) by a least squares method.

hammerhead structures have different formats, since in the plus hammerhead structure of PLMVd helices I and II are closed by short loops 2 and 3 and there is no loop 3 closing helix III (Fig. 1), whereas in the hammerhead structure used in the experiments performed by Ruffner *et al*. (18) a short loop 3 closes helix III and loops 2 and 3 do not exist; (ii) the latter hammerhead structure acts *in trans* employing sub-saturating concentrations of the substrate as opposed to that from PLMVd, which acts *in cis*; (iii) reaction conditions were different in the two cases.

In this context it is also interesting to note that in a recent report on the reduction of catalysis in abasic variants of a model intermolecular hammerhead ribozyme the positions less affected of those forming the central single-stranded loop were U4 and U7 (29) and that when the U4 of another intermolecular hammerhead ribozyme was substituted by 4-thiouridine or 2-pyrimidinone ribonucleosides the catalytic activity was reduced but not abolished (30). Therefore, these observations are in line with the results here reported with an intramolecular natural hammerhead structure. In this respect it should be remarked that self-cleavage during *in vitro* transcription better reflects the *cis* functioning of hammerhead ribozymes in *in vivo* processing of the small RNAs in which they are contained.

Transversions of the U4 residue of the catalytic pocket destroy or significantly reduce ribozyme activity

To examine whether the substitutions U4→A and U4→G were also compatible with catalytic activity of the ribozyme recombinant plasmids containing PLMVd full-length inserts with these two changes were obtained by site-directed mutagenesis. As opposed to the case of the PLMVd RNA with the mutation U4→C in the catalytic pocket, no detectable self-cleavage was observed during *in vitro* transcription in the PLMVd RNAs with the substitution U4→A or U4→G (Fig. 5A). On the other hand, when the purified complete transcripts were denatured by heating and incubated under standard self-cleavage conditions, no self-cleavage was detected in the RNA with the change U4→A, whereas 15–25% of the RNA with the substitution U4→G self-cleaved (Fig. 5B). Therefore, it can be concluded from these results that the substitution U4→C in the catalytic pocket is the one preserving a major fraction of ribozyme activity, particularly during transcription.

The PLMVd hammerhead structure with the U4→C change in the catalytic pocket is also functional *in vivo*

Since we have previously observed that PLMVd cDNA inserts of exact monomeric length are infectious when inoculated into GF 305 peach seedlings (25), we wanted to know whether or not the PLMVd sequence variant with the substitution U4→C in the catalytic pocket was also infectious. In three different experiments 2/5, 8/15 and 3/5 of the inoculated plants became infected, a ratio similar to that obtained in an experiment with the wild-type PLMVd sequence variant, in which 13/20 of the inoculated plants became infected. No delay in symptom expression was observed between plants inoculated with either variant. When 11 PLMVd cDNA clones were obtained by RT-PCR from the viroid progeny extracted from one symptomatic plant resulting from infection with the PLMVd U4→C sequence variant the mutation was observed in two of them, whereas it had reverted to the wild-type in the other nine. Therefore, the substitution affecting the catalytic pocket is functional and is present in a fraction of the sequence variants of the progeny. Due to replication errors and selection pressures PLMVd accumulates *in vivo* as a population of closely related co-existing variants (25) which conform to what is known as a quasi-species (31). Similar situations have been observed previously in viral RNAs (32) as well as in other viroids (33–38). The fraction of PLMVd molecules with the substitution U4→C in the catalytic pocket is small, probably because it is outcompeted by the variants containing the wild-type hammerhead structure, which show more efficient self-cleavage. In fact, we have observed a correlation between *in vitro* self-cleavage of

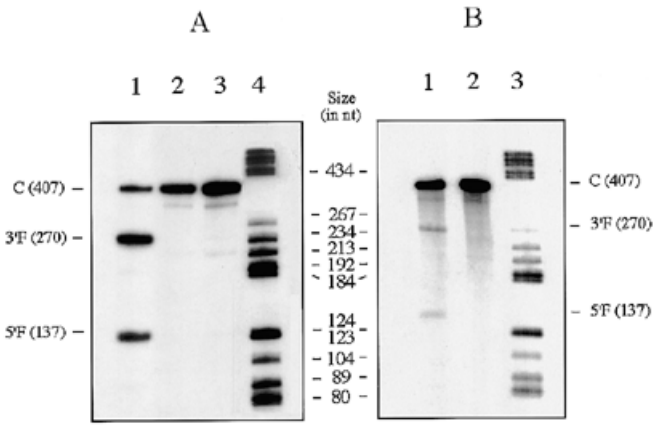

Figure 5. Effects of transversions in the U4 residue of the catalytic pocket of the plus PLMVd hammerhead structure on *in vitro* self-cleavage of full-length viroid strands. (**A**) Analysis by denaturing PAGE (5%) and autoradiography of self-cleavage during transcription of plus PLMVd RNAs with the wild-type sequence (lane 1) and with mutations U4→G (lane 2) and U4→A (lane 3) in their hammerhead structures. DNA markers with their sizes in nucleotides indicated on the right are shown in lane 4. The positions and sizes of the complete transcripts C and of the self-cleavage fragments 5′F and 3′F are indicated on the left. (**B**) Analysis by denaturing PAGE and autoradiography of self-cleavage of purified plus PLMVd RNAs. Prior to self-cleavage complete PLMVd transcripts with mutations U4→G (lane 1) and U4→A (lane 2) in their hammerhead structures were eluted from a preparative gel as in (A) and after heat denaturation and snap cooling on ice they were incubated under standard self-cleavage conditions. DNA markers are shown in lane 3. Other details are as in (A).

additional PLMVd mutants in other positions of the hammmerhead structure and their infectivity (Ambrós *et al.*, unpublished data). Preservation of the substitution U4→C in a fraction of the progeny molecules not only shows *in vivo* functioning of this hammerhead structure in the replicative cycle of the viroid and provides some information on the rate of mutation of the catalytic core to adjust to selective pressures, but also suggests that it may confer on the PLMVd quasi-species some advantage in another functional direction. In this context it should be noted that the small size of the viroid genome makes it likely that specific regions of the viroid molecule could be involved in determining more than one function.

Relevance of the mutation found in the catalytic pocket for our understanding of the hammerhead ribozyme and the uridine turn motif

Although the overall tertiary structures of the hammerhead ribozyme and of a catalytic intermediate thereof are known (15,16,39), there is still the need for a catalytic mechanism that could rationalize the crystallographic and enzymatic data (40), particularly considering that there is the possibility that the intermediate captured in the crystal may not accumulate appreciably in solution (39). The results reported here showing that a hammerhead structure with a pyrimidine transition in the catalytic pocket is active *in vitro* and *in vivo* provide further information on the enzymatic side that is pertinent for a better understanding of the structure of the hammerhead ribozyme and ultimately of the mechanism by which this ribozyme catalyzes specific bond cleavage. The crystallographic analysis has revealed a stacking interaction between the base of C17 and the exocyclic oxygen at position 2 of the U4 in the uridine turn, which makes a 3 Å aromatic–nπ stabilizing interaction (16). Since a change U4→C would allow a similar interaction, this may explain the activity of the hammerhead structure with this substitution as well as the lack of or reduced activity of the two other mutated hammerhead structures with the substitutions U4→A and U4→G in which the stabilizing interaction cannot be formed. However, additional interactions, including possible binding of the catalytic metal ion by U4 (16), must exist favoring the almost universal presence of a U4 in the catalytic pocket of the natural hammerhead structures.

On the other hand, the crystallographic data obtained for the uridine turn motif of a hammerhead structure indicate that the tertiary interactions that stabilize this domain include a hydrogen bond from N3 of U4 to the *pro*-S_P phosphate oxygen of U7, as well as a hydrogen bond between the 2′-hydroxyl of U4 and N7 of A6 (15). These interactions are consistent with a consensus sequence of UNR for the uridine turn, where N is any nucleotide, R is a purine and UNR is U4G5A6 in the hammerhead ribozyme (15). It is worth noting that only the change U4→C observed in the PLMVd hammerhead structure retaining a significant fraction of ribozymatic activity would allow both interactions. Moreover, the uridine turn motif has recently been predicted in the *Neurospora* VS ribozyme on the basis of the effects of site-directed mutagenesis of the conserved U and, interestingly, the substitution U→C was the change that least affected the rate of self-cleavage (41). Therefore, these results obtained with two very different ribozymes, the hammerhead and VS, indicate that some sequence variability can be tolerated in the U position of the uridine turn motif.

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