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An Extra Nucleotide in the Consensus Catalytic Core of a Viroid Hammerhead Ribozyme

IMPLICATIONS FOR THE DESIGN OF MORE EFFICIENT RIBOZYMES*

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Marcos De la Peña‡ and Ricardo Flores§

From the Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia, Avenida de los Naranjos s/n, Valencia 46022, Spain

Hammerhead ribozymes catalyze self-cleavage of oligomeric RNAs generated in replication of certain viroid and viroid-like RNAs. Previous studies have defined a catalytic core conserved in most natural hammerheads, but it is still unknown why some present deviations from the consensus. We have addressed this issue in chrysanthemum chlorotic mottle viroid (CChMVd), whose (+) hammerhead has an extra A (A10) between the conserved A9 and the quasi-conserved G10.1. Effects of insertions at this position on hammerhead kinetics have not hitherto been examined. A10 caused a moderate decrease of the *trans*-cleaving rate constant with respect to the CChMVd (+) hammerhead without this residue, whereas A10→C and A10→G substitutions had major detrimental effects, likely because they favor catalytically inactive foldings. By contrast, A10→U substitution induced a 3–4-fold increase of the rate constant, providing an explanation for the extra U10 present in two natural hammerheads. Because A10 also occupies a singular and indispensable position in the global CChMVd conformation, as revealed by bioassays, these results show that some hammerheads deviate from the consensus due to the involvement of certain residues in critical function(s) other than self-cleavage. Incorporation of the extra U10 into a model hammerhead also caused a similar increase in the rate constant, providing data for a deeper understanding of the hammerhead structural requirements and for designing more efficient ribozymes.

Viroids, subviral circular RNAs of 247–401 nucleotides (nt),[1] are the smallest autonomous replicons (1, 2). This minimal size imposes severe restrictions onto viroid genomes to accommodate a series of functions critical to their life cycle which include host selection, long distance and cell-to-cell movement, and targeting to specific subcellular organelles (nuclei or chloroplasts) where they replicate and accumulate. All these functions must result from the direct interaction of the viroid RNA, or some of its replicative intermediates, with cellular factors because the available evidence indicates that viroids do not code for proteins (3–5). Genetic information, therefore, must be extremely compressed and even overlapping in viroids.

Viroids replicate through a rolling-circle mechanism in which the infecting most abundant monomeric circular RNA, assumed by convention to have the (+) polarity, is successively transcribed into oligomeric (−) and (+) strands that are then excised into the linear monomeric forms and circularized to produce the progeny (6, 7). This is an RNA-based mechanism (8), and depending on whether or not the (−) oligomeric intermediates are cleaved and ligated to their corresponding monomeric circular counterparts, which then serve as the initial template for the second half of the cycle, the mechanism is considered to be symmetric or asymmetric, respectively (9). Due to the lack of messenger activity of viroid RNAs, the whole replication process should be in principle catalyzed by host enzymes. However, in avocado sunblotch viroid (10, 11), peach latent mosaic viroid, PLMVd (12), and chrysanthemum chlorotic mottle viroid, CChMVd (13), which together form the family Avsunviroidae (14), the cleavage step is autocatalytic and mediated by hammerhead structures that can be adopted by the strands of both polarities. Consequently, these three viroids are considered to replicate following the symmetric rolling-circle mechanism. In line with this view, the monomeric (−) circular RNA has been identified in avocado sunblotch viroid-infected avocado (15–17) and in PLMVd-infected peach (18). The rest of 25 viroid species, which make up the family Pospiviroidae (2), are assumed to follow the asymmetric rolling-circle mechanism because the oligomeric forms are the predominant (−) strands accumulating in tissues infected by representative members of this family, whereas the monomeric (−) circular RNA has not been identified (9, 19, 20). Cleavage of the oligomeric (+) RNA intermediates in family Pospiviroidae is generally believed to require a host ribonuclease (21, 22), although the possibility that the cleavage step is RNA-catalyzed in all cases has been also advanced (23).

The hammerhead ribozyme is a small RNA motif able to self-cleave at a specific phosphodiester bond in the presence of a divalent metal ion, generally Mg^{2+}, and under mild temperature and pH conditions, producing 2′,3′-cyclic phosphate and 5′-hydroxyl termini (11, 24, 25). Structural dissection of the 23 natural hammerhead structures reported so far (for a review, see Ref. 26) shows a central core composed of 11 strictly conserved nucleotides flanked by three double-helix regions (I, II, and III) with loose sequence requirements except positions 10.1 and 11.1, which in most cases form a G-C pair, and positions 15.2 and 16.2, which in most cases form a C-G pair (Fig. 1*A*). Site-directed mutagenesis has revealed that the conserved res-

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§ To whom correspondence should be addressed. Tel.: 34-96-3877861; Fax: 34-96-3877859; E-mail: rflores@ibmcp.upv.es.

[1] The abbreviations used are: nt, nucleotide(s); PLMVd, peach latent mosaic viroid; CChMVd, chrysanthemum chlorotic mottle viroid; sLTSV, satellite RNA of lucerne transient streak virus; sArMV, satellite RNA of Arabis mosaic virus; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

idues play a critical role in determining the rate constant of cleavage (27), and analysis by x-ray crystallography has uncovered a complex array of noncanonical interactions between the residues forming the central core (28, 29), prominent among which are three non-Watson-Crick pairs (involving A9 and G12, G8 and A13, and U7 and A14) that extend helix II (Fig. 1*A*). However, deviations from the consensus hammerhead core have been observed in some natural hammerhead structures. This is the case of the CChMVd (+) hammerhead structure, which is peculiar in having an extra A inserted between the strictly conserved A9 and the highly conserved G10.1 residues (13). These two residues are contiguous in all known natural hammerhead structures except in those of (+) strands of satellite RNAs of lucerne transient streak virus (sLTSV) (25) and *Arabis* mosaic virus (sArMV) (30), in which an extra U exists at the same position (Fig. 1*B*), and in those of (+) strand of satellite RNA of cereal yellow dwarf virus-RPV (31, 32) and (−) strand of the carnation small viroid-like RNA (33), in which the extra residue is a C. In the two latter hammerhead structures, the extra C is accompanied by an extra A inserted between the highly conserved C11.1 and the strictly conserved G12 residues.

The observation that natural selection has allowed an extra residue between positions A9 and G10.1 in a significant number of natural hammerhead structures is intriguing and raises the question of whether this could provide some adaptive advantage to the corresponding RNAs. A plausible explanation is that the extra residue might be also involved in determining a functional property other than self-cleavage and, on this basis, be preserved. Here we have put this hypothesis to the test using the CChMVd/chrysanthemum system, which is very suitable for this purpose because recombinant plasmids containing dimeric head-to-tail viroid cDNA inserts, or *in vitro* transcripts thereof, are infectious and incite symptoms in a relatively short time (12–15 days) (13). Moreover, although an extra residue between positions 9 and 10.1 is compatible with extensive self-cleavage during *in vitro* transcription and after purification (13, 25, 34), a kinetic analysis of the effects of mutations in this particular position on the corresponding rate constants of cleavage is lacking, despite the ample biochemical and biophysical analyses to which the hammerhead ribozyme has been subjected (35, 36). We have addressed this second issue with a *trans*-acting hammerhead structure derived from the CChMVd (+) RNA and then by extending the analysis to a well known model hammerhead structure. Our results show that the nature of the extra residue between positions A9 and G10.1 has profound effects on viroid infectivity and on hammerhead-mediated RNA cleavage, leading in some cases to a significant increase in the catalytic efficiency of the ribozyme.

EXPERIMENTAL PROCEDURES

cDNA Synthesis, Cloning, and Sequencing—Circular forms of the CChMVd, purified by two consecutive PAGE steps, were reverse transcribed and PCR-amplified with primers RF-146 (complementary to nt 133–108) and RF-147 (homologous to nt 134–159 of the CM5 reference sequence of CChMVd (Ref. 37; see also Fig. 2)). Reverse transcription, PCR amplification, and cloning were performed as described previously (13). Inserts were sequenced with an ABI Prism DNA apparatus (PerkinElmer Life Sciences).

Site-directed Mutagenesis of CChMVd—The protocol reported previously (38) was followed with minor modifications. The recombinant plasmid pCM5 (5 ng), containing a monomeric insert of the CChMVd reference sequence (13), was PCR-amplified with *Pfu* DNA polymerase and 500 ng each of the phosphorylated primers RF-142 (5′-CATGGATC**V**TCATCAGGACACACCGAC-3′), complementary to nucleotides 11–35 of the CM5 sequence (except the residue in bold that was degenerated to change the A27, corresponding to A10 in the CChMVd plus hammerhead, into C, G, or U), and RF-134 (5′-ACAGGATCGAAACCTCTTCCAGTT-3′), homologous to nucleotides 36–59 (Fig. 2). Plasmid pCM5 was also PCR-amplified with the phosphorylated primers RF-133 (5′-CATGGATCTCATCAGGACACACCGAC-3′), complementary to nucleotides 11–35 of the CM5 sequence (except in the position corresponding to A27 that was deleted) and RF-134. The PCR products were electrophoretically separated in 1% agarose gels, and those of plasmid length were eluted and circularized with T4 DNA ligase. After transformation, the inserts of the new plasmids, pCM5-C10, pCM5-G10, pCM5-U10, and pCM5-Δ10, were sequenced to confirm that only the expected mutations had been introduced. From these constructs, plasmids pCM5d, pCM5d-C10, pCM5d-G10, pCM5d-U10, and pCM5d-Δ10, containing the corresponding head-to-tail dimeric inserts, were generated following standard protocols.

Infectivity Bioassays and Detection of Viroid RNA—Chrysanthemum (*Dendranthema grandiflora* Tzvelez, cv. "Bonnie Jean") was propagated in growth chambers (13). Plants were mechanically inoculated either with the recombinant plasmids pCM5d, pCM5d-C10, pCM5d-G10, pCM5d-U10, and pCM5d-Δ10 (2 μg of plasmid/plant) or with their monomeric CChMVd RNAs (0.1 μg of RNA/plant) resulting from self-cleavage during *in vitro* transcription. CChMVd replication in the inoculated plants was analyzed by dot-blot hybridization following extraction of leaves with buffer-saturated phenol and chromatography on nonionic cellulose (CF11, Whatman) (37).

Self-cleavage during in Vitro Transcription of CChMVd RNAs—The recombinant plasmids pCM5, pCM5-C10, pCM5-G10, pCM5-U10, and pCM5-Δ10 were linearized with *Bam*HI and *in vitro* transcribed with T3 RNA polymerase (39). The primary transcripts and their self-cleavage products were separated by PAGE in 5% gels containing 8 M urea and 40% formamide that were quantitatively scanned with a bioimage analyzer (Fuji BAS1500).

Synthesis of Ribozymes and Substrates—Ribozymes with the sequence of the CChMVd (+) hammerhead from positions 14 to 53 of the CM5 reference sequence (Fig. 2) and mutants thereof at position 27, which corresponds to position A10 of the hammerhead (Fig. 4), were synthesized by *in vitro* transcription of *Xba*I-linearized plasmids containing these sequences, immediately preceded and followed by the T7 promotor and the *Xba*I site, respectively. Transcription reactions (50 μl) contained 40 mM Tris-HCl, pH 8, 6 mM $MgCl_2$, 2 mM spermine, 10 mM dithiothreitol, 2 mM each of ATP, CTP, GTP and UTP, 2 units/μl human placental ribonuclease inhibitor, 20 ng/μl plasmid DNA, and 4 units/μl T7 RNA polymerase. After incubation at 37 °C for 1 h, transcription products were separated by PAGE in 15% denaturing gels and those with the expected length were eluted, recovered by ethanol precipitation, and resuspended in 50 mM Tris-HCl, pH 7.5. The model hammerhead ribozyme HH8 (Fig. 6) and its mutants at position 10 of the hammerhead were also prepared by *in vitro* transcription following the same protocol. Substrate RNAs (5′-AAGAGGUCGGCACC-3′) and (5′-GAAUGUCGGUCG-3′) for the CChMVd (+) and the HH8 hammerheads, respectively (Figs. 4 and 6), were obtained by chemical synthesis using 2′-orthoester protection (Dharmacon Research, Boulder, CO) and sequentially deprotected with 0.2 M acetic acid and Tris-HCl, pH 8.7. After purification by PAGE in 20% denaturing gels, the substrate RNAs were eluted and labeled at their 5′ termini using [α-^{32}P]ATP (Amersham Pharmacia Biotech, 3000 Ci/mmol) and T4 polynucleotide kinase (40).

Cleavage Kinetics—Single-turnover experiments with excess ribozyme (covering a range from 100 to 1000 nM in different experiments in order to assure saturating conditions) and trace ^{32}P-labeled substrate (less than 1 nM) were used to determine the rate constant of cleavage (41). Cleavage reactions were carried out in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ at 25 °C as described previously (42). The ribozyme and substrate were first annealed in 50 mM Tris-HCl, pH 7.5, by heating at 95 °C for 1 min and slowly cooling down to 25 °C for 15 min. Reactions were initiated by adding $MgCl_2$ to a final concentration of 10 mM. Aliquots were removed at appropriate time intervals and quenched with a 5-fold excess of stop solution (8 M urea, 50% formamide, 50 mM EDTA, 0.1% xylene cyanol, and bromphenol blue dyes) at 0 °C. Substrate and product from each time point were separated by PAGE in 20% denaturing gels. The fraction of product at different times F was determined by radioactivity quantitation of the corresponding gel bands with a bioimage analyzer and fitted to the equation $F = F_\infty (1 - e^{-kt})$, where F_∞ is the fraction of product at the end point of the reaction and k the first order rate constant of cleavage (k_{cat}). In the case of the HH8 hammerhead, cleavage rates were also measured under multiple-turnover conditions (43) using at least six different substrate concentrations, ranging from 50 to 1000 nM, in excess over those of the ribozyme (from 10 to 40 nM depending on the catalytic activity of the ribozyme). Reactions were initiated by mixing at 25 °C the ribozyme (15 μl) and the substrate (15 μl) previously heated at 95 °C for 1 min in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$. Aliquots were removed as before,

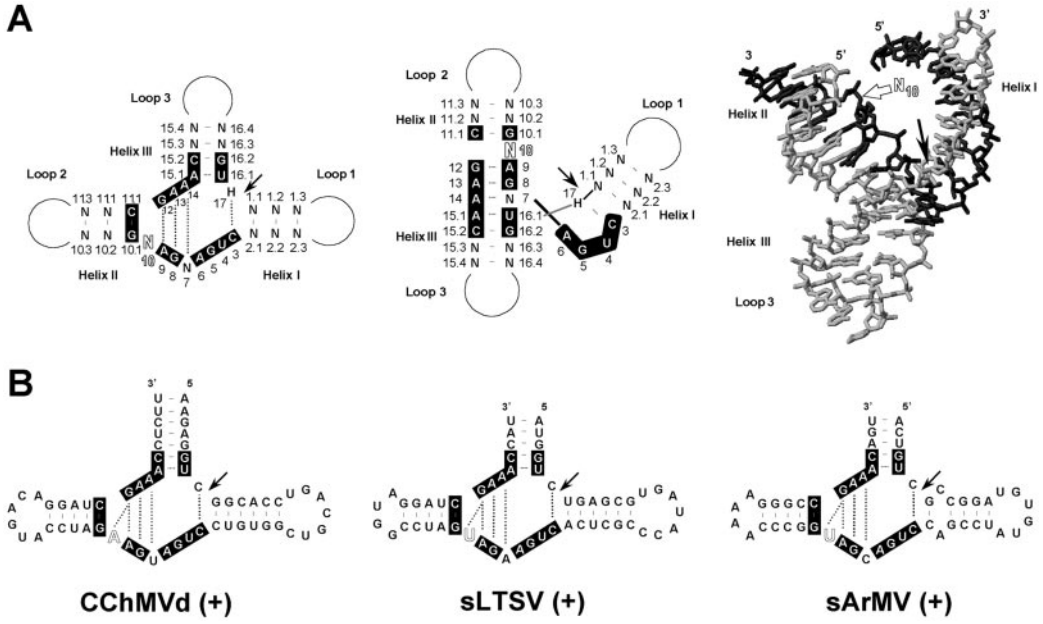


FIG. 1. **Representations of the hammerhead structure.** *A*, schematic illustration showing the strictly or highly conserved residues in natural hammerheads in a *black background* (*H* = A, U, or C; *N* = any residue). *Numbering* is based on the standard criterion for the consensus hammerhead (54), with the exception of the position between the highly conserved residues A9 and G10.1, which was not previously considered and is referred here as *10* (*outlined font*). Canonical and noncanonical pairs are indicated by *continuous* and *dashed lines*, respectively (*left*). *Center*, an alternative schematic representation based on the three-dimensional structure derived from x-ray crystallography (28, 29). *Right*, a more detailed spatial view of the complex of a hammerhead ribozyme (*gray*) and its substrate (*black*) in the I/II format (29). *B*, structure of three natural hammerhead structures containing an extra residue at position 10 that is an A in the CChMVd (+) RNA (*left*) and a U in the sLTSV and sArMV (+) RNAs (*center* and *right*, respectively). The alternative interactions that the extra residue could potentially form with G12 are indicated. *Arrows* indicate self-cleavage sites.

and data were fitted to Eadie-Hofstee plots to obtain the values for k_{cat} and K_m. The errors reported for kinetic parameters were obtained from triplicate experiments with different preparations of RNA.

RESULTS

The Role in Infectivity of the Extra Residue Found in the Catalytic Core of the CChMVd (+) Hammerhead Structure—Hereafter, we will refer to the position occupied by the extra A in the CChMVd (+) hammerhead structure, and by any other residue in this or in other hammerhead structures, as position number 10 considering that it is located between the strictly conserved A9 and the highly conserved G10.1 in the consensus hammerhead structure (Figs. 1 and 2). The extra A10 of the CChMVd (+) hammerhead structure also holds a special place in the branched secondary structure of lowest free energy predicted for the (+) strand of this viroid (position A27 in the genomic reference sequence of CChMVd), connecting two helices of a cruciform domain (Fig. 2). It is worth noting that the *in vivo* significance of this proposed branched conformation, which is inactive for self-cleavage, is strongly supported by the analysis of the sequence heterogeneity found in more than 100 natural CChMVd variants, because the observed changes are located in loops or when affecting a base pair the substitutions are compensatory, and also because no variability has been observed at this particular position A27 (13, 37).[2] In the interest of simplicity and to avoid any confusion that would result from referring to the same residue with two numbers, 10 in the CChMVd (+) hammerhead and 27 in the genomic reference sequence, we will only use the first number.

To determine whether the extra A10 plays any role in infectivity, chrysanthemum plants were inoculated with recombinant plasmids containing dimeric tandem inserts of CChMVd cDNA with all possible mutations at this position introduced by site-directed mutagenesis. Ten days later, only those control plants inoculated with the plasmid containing the wild-type CChMVd cDNA (pCM5d-A10) developed the characteristic symptoms of the chlorotic mottle disease. All plants inoculated with plasmids containing the substitutions A10→C and A10→U (pCM5d-C10 and pCM5d-U10, respectively) showed symptoms 15–20 days after inoculation, but only two of the four plants inoculated with pCM5d-G10, containing the substitution A10→G, displayed the typical symptoms 25 days after inoculation. Interestingly, none of the plants inoculated with the plasmid not containing A10 (pCM5d-Δ10) developed symptoms during the observation period (up to 3 months). Analysis by dot-blot hybridization confirmed that the inoculated plants showing symptoms were indeed infected, whereas no signal was observed in those remaining symptomless (data not shown). These experiments were repeated twice with similar results. When the inoculations were performed with the monomeric CChMVd RNAs resulting from self-cleavage during *in vitro* transcription of the dimeric cDNA inserts, symptoms induced by RNAs with the substitutions A10→C, A10→U, and A10→G, appeared with only a short delay (1–2 days) with respect to those induced by the wild-type RNA, but again none of the plants inoculated with the RNA without A10 developed symptoms, and dot-blot hybridization confirmed that they had not been infected. Reverse transcription-PCR amplifications of viroid progenies from the infected chrysanthemum plants and sequencing of the resulting full-length clones (from 5 to 9 for each construct) revealed that the three substitutions at position 10 had reverted to the original A10 in all cases. Altogether, these results demonstrate that the A10 residue is indispensable for infectivity. CChMVd sequences with substitutions at this position are less infectious, most likely because they have to revert to the wild type, whereas the reversion does not occur when this residue is deleted. The higher infectivity of CChMVd RNAs when compared with their cDNAs is not surprising considering that the latter must be recognized and transcribed by

[2] M. De la Peña and R. Flores, unpublished data.

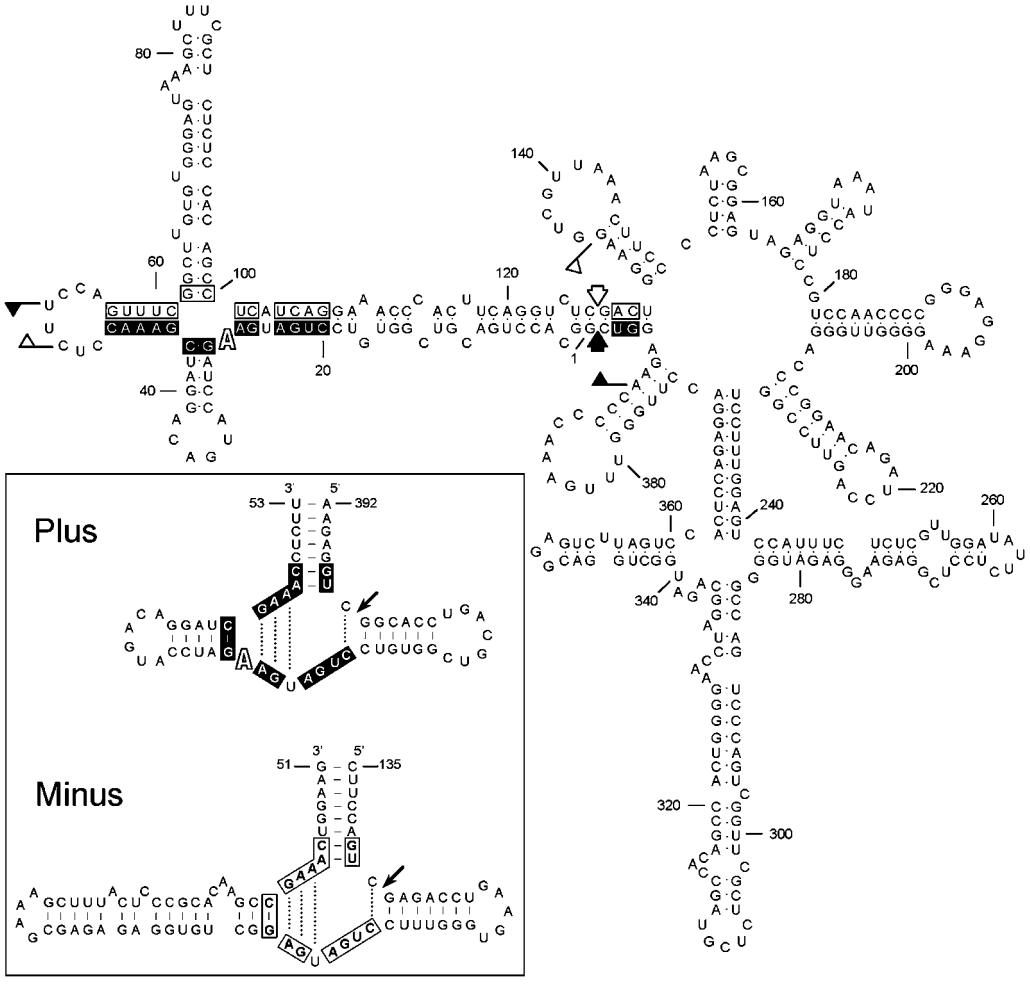


FIG. 2. **Predicted secondary structure of lowest free energy of the CChMVd plus RNA (reference variant CM5, adapted from Ref. 13 with minor modifications).** The existence of a pseudoknot between the residues around positions 220 and 260 has been suggested by comparison with the situation found in PLMVd (55). Plus and minus self-cleavage domains are delimited by *flags*, residues conserved in most natural hammerhead structures are *boxed*, and the self-cleavage sites are indicated by *arrows*. *Black* and *white backgrounds* in *flags*, *boxes*, and *arrows* refer to plus and minus polarities, respectively. *Inset*, hammerhead structures of the plus and minus strands of CChMVd. Residues conserved in most natural hammerhead structures are on *black* and *white backgrounds* in the plus and minus polarities, respectively, and the self-cleavage sites are denoted by *arrows*. *Outlined fonts* indicate the position of the extra A in the CChMVd secondary structure and in its plus hammerhead structure. Other details are as described in the legend to Fig. 1.

a host RNA polymerase before entering into the standard RNA-RNA replication cycle.

In Vitro Self-cleavage of the Monomeric CChMVd (+) RNA and of Its Four Mutants at Position A10—To initially assess the effect of the extra A10 residue on the catalytic activity of the CChMVd (+) hammerhead structure, we determined the extent of self-cleavage of CChMVd (+) RNAs transcribed from five recombinant plasmids containing monomeric inserts with the wild-type CChMVd sequence (pCM5-A10), with the three possible substitutions at this position (pCM5-C10, pCM5-G10 and pCM5-U10) and with the A10 deleted (pCM5-Δ10). The extent to which the five RNAs self-cleaved during transcription ranged from ~55% (the wild-type RNA and those with the changes A10→U and A10→G) to 65% (the A10→C and A10→Δ RNAs) (Fig. 3). When the uncleaved monomeric transcripts were purified and incubated under standard self-cleavage conditions (39), the differences were even smaller with the extent of self-cleavage varying between 65 and 70% (data not shown). Although these experiments seemed to suggest that the extra A10 does not play a major role in the catalytic efficiency of the CChMVd (+) hammerhead structure, the extent of self-cleavage measured using full-length CChMVd (+) transcripts and only one reaction time was probably too rough an estimate for this aim. Therefore, we decided to re-examine this question using a more accurate approach.

trans-Cleavage Kinetics of the Minimal CChMVd (+) Hammerhead Structure and of Its Four Mutants at Position 10—Since the observed self-cleavage could be influenced by either vector or viroid sequences external to the hammerhead structure, or even by the cloning site of the viroid cDNA and by some of the components present in the *in vitro* transcription reaction, a kinetic analysis under protein-free conditions of the minimal CChMVd (+) hammerhead structure in the well known I/III *trans* format (35) (Fig. 4), was performed. The substrate was the same in all cases, whereas the ribozyme contained the wild-type sequence of CChMVd (+) hammerhead structure and the mutants A10→Δ, A10→C, A10→G, and A10→U. The cleavage rate constants for these hammerheads were determined under standard conditions (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, at 25 °C), using trace concentrations of substrate (<1 nM) and concentrations of ribozyme between 100 and 1000 nM (single-turnover kinetics).

The value obtained for the rate constant of the hammerhead ribozyme with the consensus core sequence (A10→Δ) was ~1 min^{-1} (Fig. 5), in full agreement with those reported under the same conditions for other hammerhead ribozymes with the same consensus core sequence (35). The wild-type ribozyme with the extra A10 exhibited a slightly decreased rate constant,

whereas those of the ribozymes with the changes A10→C and A10→G were considerably reduced (Fig. 5). These comparatively low values could result from the extra C10 forming a Watson-Crick pair with the conserved G12, impeding the noncanonical interaction formed by this residue with the conserved A9 in the active catalytic folding (28, 29), and the extra G10 favoring the adoption of an alternative (catalytically inactive) ribozyme-substrate complex involving the conserved or quasi-conserved residues at positions 4–9 of the consensus hammerhead structure (Fig. 4). Unexpectedly, the ribozyme with the A10→U substitution showed a rate constant ~3-fold higher than that corresponding to the hammerhead ribozyme with the consensus core sequence (Fig. 5), indicating that this substitution not only does not affect negatively self-cleavage but even favors it.

Effects of an Extra Residue at Position 10 on the trans-Cleavage Kinetics of a Model Hammerhead Ribozyme—There is the possibility that the results observed for the minimal CChMVd (+) hammerhead structure and its mutants, particularly the increase in the rate constant of the variant with the substitution A10→U, could be a peculiarity of this specific ribozyme. To address this question, the *trans*-cleavage rate constants were determined again but using a model hammerhead structure (HH8, format I/III) (Fig. 6), which earlier analyses have shown to exhibit a canonical kinetic behavior (Ref. 35, and references therein). Moreover, the HH8 hammerhead has helices I and II shorter than those of the CChMVd (+) hammerhead. The cleavage rate constants were determined under the same standard conditions indicated in the previous section and also with an excess of the ribozyme over the substrate (single-turnover kinetics). The rate constant obtained for the HH8 ribozyme with the consensus core sequence (without an extra residue at position 10) was 1.5 min^{-1} (Fig. 7), in consonance with the 1.4 min^{-1} value reported previously for this same hammerhead (42). The rate constant of the HH8 ribozyme with an extra A10 was slightly lower, whereas the reduction observed in the corresponding values for the HH8 ribozymes with an extra C10 or G10 was considerably more pronounced, specially in this latter case, which displayed some anomalous kinetic behavior (Fig. 7). Interestingly, the rate constant of the HH8 ribozyme containing an extra U10 was almost 4-fold higher than its HH8 counterpart with the consensus core sequence. Collectively, the results confirmed those obtained with the CChMVd (+) hammerhead structure and showed that an extra U10 has a clear beneficial effect on the catalytic efficiency of the hammerhead ribozyme.

To provide further support in this direction, the experiments were also performed under multiple-turnover conditions. Once again, the rate constant obtained for the HH8 ribozyme with the consensus core sequence (Table I) agreed well with the value reported previously for this ribozyme (43). On the other hand, the rate constants of the four HH8 ribozyme mutants at position 10 paralleled those obtained under single-turnover conditions (Table I). Differences in the K_m values were small between the HH8 ribozyme with the consensus core sequence and its mutants with an extra A10 or U10, whereas the ribozymes with an extra C10 and, particularly with an extra G10, displayed higher K_m values (Table I). However, the K_m differences were smaller than those observed between the cor-

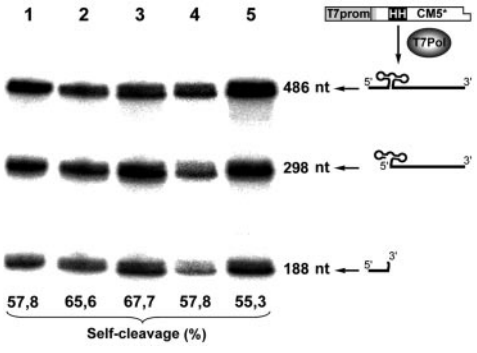


FIG. 3. **Self-cleavage during *in vitro* transcription of CChMVd (+) RNAs.** *Left*, analysis by denaturing PAGE (6%) of the wild-type containing an extra A10 in the hammerhead structure (*lane 1*) and of the mutant RNAs with this extra residue deleted (*lane 2*), and replaced by C, G, or U (*lanes 3–5*, respectively). The extension of self-cleavage was quantitated with a bioimage analyzer and is indicated *below* each *lane*. *Right*, a schematic representation of the recombinant plasmids serving as templates, and of the primary transcripts and the self-cleavage fragments resulting thereof with their size in nucleotides (in the case of *lane 2*, the primary transcript and the 3′ fragment are one residue shorter than indicated).

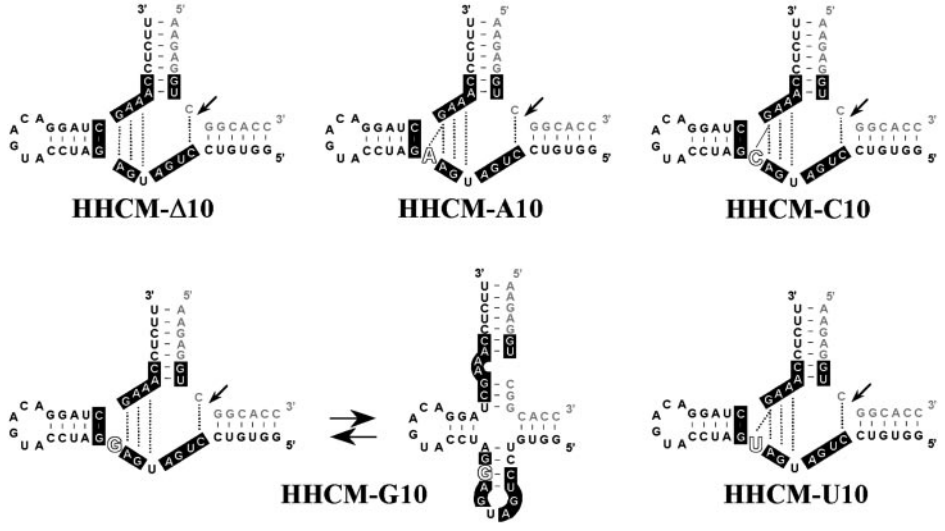
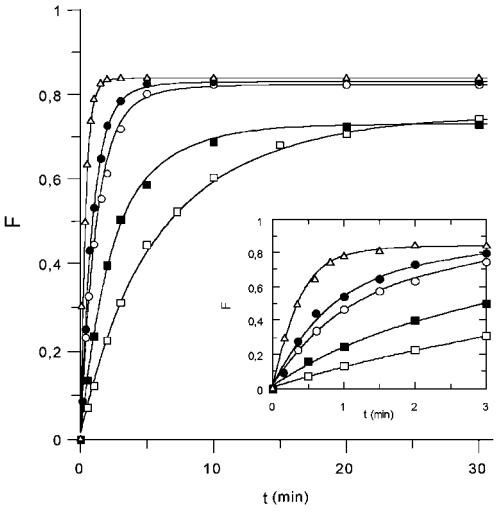


FIG. 4. **Ribozyme and substrate complexes derived from the CChMVd (+) hammerhead structure.** Ribozymes and substrates are in *black* and *gray letters*, respectively. Residues conserved in most natural hammerhead structures are on a *black background*, and the self-cleavage sites are denoted by *arrows*. *HHCM-A10* refers to the wild-type hammerhead structure containing an extra A10 (*outlined font*), and *HHCM-Δ10*, *HHCM-C10*, *HHCM-G10*, and *HHCM-U10* refer to the mutant forms with the extra A10 deleted and replaced by a C, G, or U residue (*outlined fonts*), respectively. The extra A10, C10, and U10 residues could potentially interact with G12, distort the catalytic core and reduce the corresponding rate constants. In the case of *HHCM-G10*, an alternative catalytically inactive complex is also presented. Other details as in the legend to Fig. 1.



	k_{cat} (min^{-1})	$\frac{k_{cat}}{k_{cat}(\text{HHCM-}\Delta10)}$
HHCM-Δ10 (●)	1.04 ± 0.12	(1)
HHCM-A10 (○)	0.80 ± 0.14	0.77
HHCM-C10 (■)	0.18 ± 0.02	0.17
HHCM-G10 (□)	0.39 ± 0.02	0.38
HHCM-U10 (△)	2.82 ± 0.63	2.71

FIG. 5. **Representative cleavage kinetics experiments using the hammerhead ribozymes derived from the CChMVd (+) hammerhead structure.** The experiments were performed under single-turnover conditions with a large excess of ribozyme (1000 nM) and trace amounts of substrate (less than1 nM). *HHCM-A10* refers to the wild-type hammerhead structure (containing an extra A10), and *HHCM-Δ10*, *HHCM-C10*, *HHCM-G10*, and *HHCM-U10* refer to the mutant forms with the extra A10 deleted and replaced by a C, G, or U residue, respectively. The fraction of product at different times (F) was determined by radioactivity quantitation of the corresponding gel bands with a bioimage analyzer and fitted to the equation $F = F_\infty (1 - e^{-kt})$, where F_∞ is the fraction of product at the end point of the reaction and k the first order rate constant of cleavage (k_{cat}). The *inset* displays the first 3 min for each experiment. The mean values obtained for the rate constants of the five hammerheads and the normalized values with respect to that of the HHCM-Δ10 hammerhead are shown at the *right*.

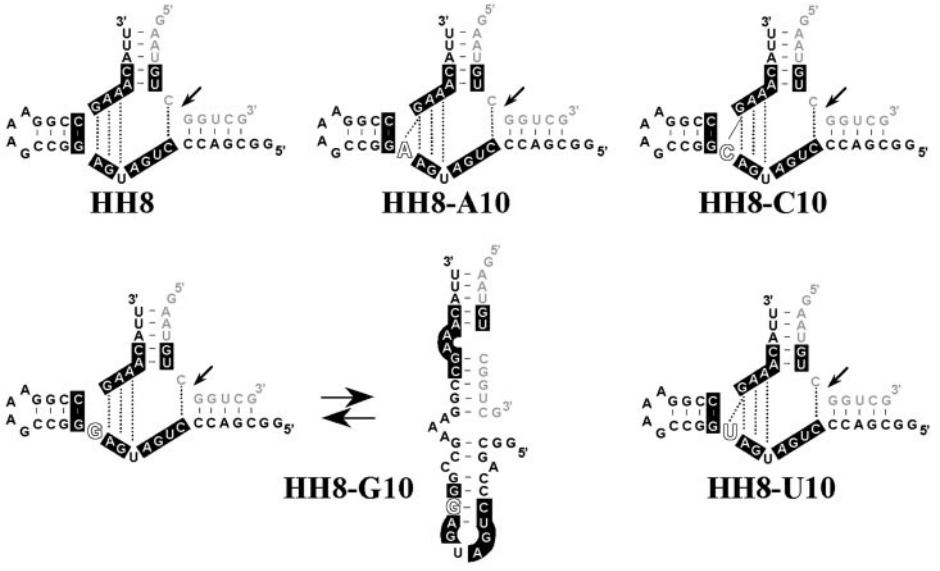


FIG. 6. **Ribozyme and substrate complexes derived from the HH8 hammerhead structure.** Ribozymes and substrates are in *black* and *gray letters*, respectively. Residues conserved in most natural hammerhead structures are on a *black background*, and the self-cleavage sites are denoted by *arrows*. *HH8* refers to the consensus hammerhead structure (without a residue at position 10), and *HH8-A10*, *HH8-C10*, *HH8-G10*, and *HH8-U10* refer to the mutant forms with an extra A10, C10, G10, and U10 residues (*outlined fonts*), respectively. The extra A10, C10, and U10 residues could potentially interact with G12, distort the catalytic core, and reduce the corresponding rate constants. In the case of *HH8-G10*, an alternative catalytically inactive complex is also presented. Other details are as described in the legend to Fig. 1.

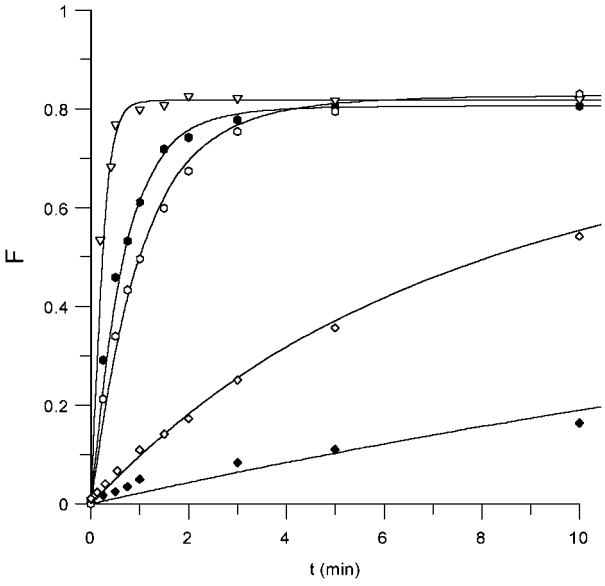
responding rate constants that, therefore, determine primarily the catalytic efficiency (k_{cat}/K_m).

DISCUSSION

The extremely small size of viroids, which within a genomic RNA of 246–401 nt must embody signals for the multiple functions they need to complete their replicative cycle, entails that these RNAs have been forced through evolution to explore a very limited sequence space to find economic solutions (in terms of nucleotides) to support such diverse functions. As a consequence of this high informational density, the involvement of certain regions within the viroid molecule in determining more than one function can be reasonably presumed. A situation of this kind is illustrated by CChMVd, in which a specific region (of only 1 nt residue) appears to be committed to more than one function. The (+) hammerhead structure of this viroid is exceptional in having an extra A (A10) inserted between the central core residues A9 and G10.1 (13). Because this extra residue is absent in most other natural hammerhead structures (26), and because there is direct evidence that the CChMVd (+) hammerhead structure is involved not only in the *in vitro* but also in the *in vivo* self-cleavage (13), the extra A10 can be presumed to be dispensable for self-cleavage but essential for another key function. Here we show that this particular region of the hammerhead structure tolerates some flexibility, an aspect relevant for the design of more efficient ribozymes.

Results from site-directed mutagenesis combined with bioassays in chrysanthemum showed a strict requirement for the presence of a residue at position 10 of the CChMV (+) hammerhead structure, corresponding to position 27 in the genomic reference sequence of CChMVd (Figs. 1 and 2). Infectivity was abolished when this residue was deleted, whereas the three possible substitutions at such a position resulted in infectious viroid RNAs, although plants showed a delay in symptom appearance and the resulting viroid progeny reverted to the wild-type A10. Why is there the preference for an A at position 10?

Crystallographic (28, 44) and biochemical (45) studies of the hammerhead ribozyme have identified a metal-binding site (P9 site) between the N_7 atom of G10.1 and the pro-R oxygen of the



	k_{cat} (min^{-1})	$\frac{k_{cat}}{k_{cat}(HH8)}$
HH8 (●)	1.48 ± 0.18	(1)
HH8-A10 (○)	0.95 ± 0.07	0.64
HH8-C10 (◇)	0.13 ± 0.02	0.09
HH8-G10 (◆)	0.04 ± 0.01	0.003
HH8-U10 (▽)	5.58 ± 0.85	3.8

FIG. 7. **Representative cleavage kinetics experiments using the hammerhead ribozymes derived from the HH8 hammerhead structure.** The experiments were performed under single-turnover conditions with a large excess of ribozyme (1000 nM) and trace amounts of substrate (less than 1 nM). *HH8* refers to the consensus hammerhead structure (without a residue at position 10), and *HH8-A10*, *HH8-C10*, *HH8-G10*, and *HH8-U10* refer to the mutant forms with an extra A10, C10, G10 and U10 residues, respectively (see Fig. 6). The fraction of product at different times (F) was determined by radioactivity quantitation of the corresponding gel bands with a bioimage analyzer and fitted to the equation $F = F_\infty (1 - e^{-kt})$, where F_∞ is the fraction of product at the end point of the reaction and k the first order rate constant of cleavage (k_{cat}). The extent of cleavage for the HH8-C10 hammerhead ribozyme eventually reached a value of 70%, whereas F_∞ for the HH8-G10 hammerhead ribozyme was ~30%. The mean values obtained for the rate constants of the five hammerheads and the normalized values with respect to that of the HH8 hammerhead are shown at the *right*.

TABLE I

Kinetic parameters for cleavage under multiple turnover conditions of the consensus HH8 ribozyme and its four derived forms at position 10

Reactions were performed at 25 °C in 50 mM Tris-HCl (pH 7.5), 10 mM Mg^{2+}, and a range of substrate concentrations in excess over the ribozyme.

Hammerhead	k_{cat}	K_m	k_{cat}/K_m
	min^{-1}	nM	min^{-1} nM^{-1}
HH8	1.54 ± 0.07	54 ± 6	2.9×10^{-2}
HH8[a]	1.0	41	2.4×10^{-2}
HH8-A10	0.81 ± 0.14	69 ± 16	1.2×10^{-2}
HH8-C10	0.11 ± 0.02	139 ± 8	7.9×10^{-4}
HH8-G10	0.08 ± 0.02	261 ± 54	3.1×10^{-4}
HH8-U10	4.65 ± 0.98	57 ± 15	8.2×10^{-2}

[a] Ref. 43.

A9 phosphate (Fig. 1*A*). Such a P9 metal-binding site, despite being located 20 Å away from the scissile phosphodiester bond, has been directly implicated in catalysis (27, 45, 46), although a convincing mechanisms is still lacking (36, 47, 48). In the absence of crystallographic information for a hammerhead with an extra residue at position 10, the existence of the P9 metal binding can be assumed, although probably rearranged to preserve the catalytic activity. On the other hand, the two noncanonical interactions between G8 and A13, and A9 and G12, particularly the conformational switch of their sugar moieties that depends on the nature of the residues located 5′ to G8 and G12 (49), also play a crucial role in hammerhead catalysis (27). In principle, this conformational switch is not expected to be influenced by an extra A10 or U10, which are probably accommodated as a bulging residue not affecting the stacking of the adjacent base pairs formed by A9 and G12, and G10.1 and C11.1. However, this bulging residue could cause a bending of helix II and modify the angle between this helix with helix I and, as a consequence, the rate constant of cleavage. In contrast, the substitutions A10→C and A10→G have a marked deleterious effect on the cleavage rate constant (Fig. 5), most likely because they favor alternative conformations, involving conserved or quasi-conserved residues of the catalytic core, which are inactive for cleavage (Fig. 4). Therefore, from a strictly catalytic standpoint, the A10 and U10 alternatives seem permissible and their C10 and G10 counterparts unfavored.

The A10, in addition to forming part of the CChMVd (+) hammerhead, holds a unique location at the center of a cruciform domain within the CChMVd branched conformation (Fig. 2). This unpaired residue, which does not occur in a similar cruciform domain proposed in PLMVd (50), seems therefore peculiar to CChMVd and should force a distortion of the domain. If it is assumed that this distortion confers structural constraints determining critical interactions with other RNA regions and/or with host factors (needed for CChMVd replication, transport, or accumulation), it can be speculated that deletion of the extra A10 or substitution by a U, which has a smaller size and different chemical properties, would impair the interactions. A10, therefore, would appear as a compromise to cope with two distinct crucial functions. This interpretation is compounded by the structure of the two other natural hammerheads, those of the sLTSV (+) and sArMV (+) RNAs (25, 30), which also deviate from the consensus hammerhead in having only one extra residue at position 10. Interestingly, this extra residue is a U in both cases, as anticipated for an optimized self-cleavage assuming that the extra U does not cause detrimental side effect on any other critical function of the two RNAs. Furthermore, in the (+) and (−) hammerhead structures of satellite RNA of cereal yellow dwarf virus-RPV (31, 32) and carnation small viroid-like RNA (33), respectively, which contain an extra C10, this residue is accompanied by an extra A inserted between the highly conserved C11.1 and the strictly conserved G12. This may be regarded as an indication of the existence of a noncanonical interaction between the two extra residues, which would preclude the catalytic inactive conformation that the extra C10 by itself promotes, extending the helix formed by the base pair between G10.1 and C11.1, and the three non-Watson-Crick pairs involving A9 and G12, G8

and A13, and U7 and A14. These arguments, however, are based on extrapolating the situation observed *in vitro* (with a *trans*-acting ribozyme in protein-free conditions and high concentration of magnesium ions) to that *in vivo* (with a *cis*-acting ribozyme in an intracellular milieu with a low concentration of magnesium ions) and, therefore, should be considered with care. For example, maximum self-cleavage must not necessarily be the preferred alternative for the replication of a viroid or viroid-like RNA, because certain amounts of the circular forms are needed as templates.

In conclusion, the present results are pertinent to a better understanding of the structural requirements of the hammerhead ribozyme, which due to its simplicity has aroused much interest both as a model for the study of the reaction mechanisms operating in these catalysts and also as a biotechnological tool with great potential (see Ref. 51 for a series of reviews). Our data show that the region of the hammerhead ribozyme comprising the basis of helix II and the adjacent segment of the central core, gives some flexibility, which may be exploited to improve the catalytic efficiency. Previous experiments by *in vitro* selection have explored the sequence requirements in the part of this region between the G10.1 and C11.1 pair and the residues G12, A13, and A14 (52, 53), but not in the part between the G10.1 and C11.1 pair and residues A9 and G8. The results reported here show that the insertion of an extra U10 between A9 and G10.1 significantly increases (3–4-fold) the cleavage rate constant without affecting the K_m. Moreover, this increase is due to the extra U10 and not to other unknown peculiarities of the CChMVd hammerhead that might affect its catalytic properties, because a detailed kinetic analysis of the model hammerhead HH8 with the four possible insertions at position 10 revealed a behavior similar to that of their corresponding CChMVd counterparts. Therefore, insertion of a U10 offers a simple and general alternative to improve the catalytic efficiency of the hammerhead ribozyme.

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