MOLECULAR AND CELLULAR BIOLOGY, Dec. 1991, p. 6109–6115
0270-7306/91/126109-07$02.00/0

Vol. 11, No. 12

Newt Satellite 2 Transcripts Self-Cleave by Using an Extended Hammerhead Structure

LIL M. PABÓN-PEÑA,[1] YI ZHANG,[2] AND LLOYD M. EPSTEIN[1,2]*

Department of Biological Science[1] and Institute of Molecular Biophysics,[2] Florida State University, Tallahassee, Florida 32306-3050

Received 16 July 1991/Accepted 9 September 1991

Synthetic transcripts of satellite 2 DNA from newts undergo self-catalyzed, site-specific cleavage in vitro. Cleavage occurs within a domain that is similar to the hammerhead domain used by a number of self-cleaving, infectious plant RNAs. The newt hammerhead has a potentially unstable structure due to a stem composed of two base pairs and a 2-nucleotide loop, and unlike other hammerheads that have been studied, it cannot cleave as an isolated unit. Here we show that cleavage by a single newt hammerhead requires additional satellite 2 sequences flanking both ends of the hammerhead domain. We also present a structural model of a truncated satellite 2 transcript which is capable of cleavage. The structure includes an internally looped extension to one of the conserved stems of the hammerhead. By in vitro mutagenesis, the identities of each of the five nucleotides composing one of the internal loops were shown to be critical for cleavage. Additional evidence that the extension stimulates self-cleavage in a manner other than by simply stabilizing the hammerhead is presented.

RNAs with the ability to undergo self-catalyzed, site-specific cleavage are widespread. The discovery of these and other RNAs with catalytic activities has helped to develop the theory that RNA preceded DNA and protein during early evolution (3). Catalytic RNAs have also been studied for their potential application as molecular tools for modifying RNA in vitro and in vivo (see references 4, 7, and 26 for examples). Studies of the mechanisms of RNA catalysis and the distribution of catalytic RNAs in nature are therefore of interest on both theoretical and practical grounds.

Defined by the sequence and structural elements required for activity, a variety of strategies for self-cleavage, most of which are found in single, representative RNAs, have evolved (6, 13, 17, 20, 27, 32). One strategy, however, is found in a number of plant virusoid, viroid, and viral satellite RNAs which are believed to use self-cleavage to process multimeric replication intermediates (30). A similar strategy has also been found in an animal system and is believed to be involved in the maturation of transcripts of satellite 2 DNA in the newt, *Notophthalmus viridescens* (8, 9). Approximately 100,000 copies of satellite 2 are organized into clusters of tandem repeats which are dispersed throughout each of the newt's 11 chromosomes (10). Repeat-length, strand-specific satellite 2 transcripts have been found in every newt tissue examined. Interestingly, the permutations of these transcripts differ in ovarian and nonovarian tissues, and only the nonovarian transcripts have a permutation compatible with the self-cleavage reaction described below (8). The function of these satellite 2 transcripts and the significance of the tissue-specific permutational difference remain to be determined.

Self-cleavage of satellite 2 transcripts has been studied by using transcripts prepared in vitro from a dimeric clone of satellite 2 DNA (9). In a reaction that requires Mg^{2+} and generates products with 5′-hydroxyl and 2′,3′-cyclic phosphate groups, these transcripts self-cleave at one site in each satellite 2 repeat. The site is in a region that has many of the conserved sequence and structural elements of the "hammerhead" domain required for cleavage of the infectious plant RNAs (Fig. 1A). Synthetic RNAs consisting of nothing more than the conserved features of the hammerhead are capable of cleavage (18, 31), demonstrating that the hammerhead possesses full catalytic activity. A synthetic RNA corresponding to a newtlike hammerhead domain, however, was incapable of cleavage in a monomolecular reaction (14). The inactivity of the isolated newt hammerhead was probably due to its unstable stem III consisting of two base pairs connected by a 2-nucleotide loop (Fig. 1B), since a 3- or 4-nucleotide insertion in the loop of stem III restored the ability of this RNA to cleave (28). To explain self-cleavage by unmodified satellite 2 transcripts, a double-hammerhead structure which results from the stable interaction between two separate cleavage domains was proposed. More recently, we demonstrated that while some satellite 2 transcripts do utilize interdomain interactions for cleavage, transcripts with other permutations of the same primary sequence cleave by using single-domain structures (11).

Here we show that single-domain cleavage by newt satellite 2 transcripts is dependent on sequences external to the conserved hammerhead domain. We also present a structural model for a truncated satellite 2 transcript that is capable of cleavage. This structure includes an internally looped extension to stem I of the hammerhead, and the identity of each nucleotide within the loop was found to be important for activity. It has previously been shown that sequences outside self-cleaving domains can reduce cleavage efficiency by inducing alternative, inactive RNA conformations (1, 15). The present paper includes the first description of sequences that can interact with a hammerhead to exert a positive effect on self-cleavage.

MATERIALS AND METHODS

Materials. T4 DNA ligase, T4 DNA kinase, and SP6 RNA polymerase were from Promega Corporation, exonuclease *Bal* 31 and T4 DNA polymerase were from Bethesda Research Laboratories, [α-^{32}P]UTP and [γ-^{32}P]GTP were from New England Nuclear, and restriction enzymes were from

* Corresponding author.

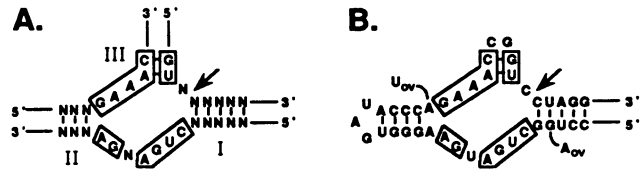

FIG. 1. Hammerhead structures. (A) Consensus sequence and proposed secondary structure of the hammerhead domain required for self-cleavage of viroid, virusoid, and viral satellite RNAs. The structure is represented by using the conventions of Forster and Symons (16), in which stems are labeled I to III, conserved nucleotides are boxed, and the arrow denotes the site of cleavage. (B) The potential hammerhead structure of newt satellite 2 transcripts. The sequence shown is derived from the genomic satellite 2 clone, pGM1. The "ov" subscripts denote nucleotides found in ovarian transcripts at the indicated positions (10).

Promega Corporation, Bethesda Research Laboratories, and New England Biolabs.

Clones. pGM1, a 333-nucleotide monomer of satellite 2 in the plasmid vector pGEM 3Zf−, was previously subcloned from the satellite 2 dimer clone, pSP6D6 (11). 5′-deletion clones pG47 and pG36, which have 47 and 36 satellite 2 nucleotides upstream from the cleavage site, were also prepared previously from pSP6D6 by using the exonuclease *Bal* 31. pGES1 was constructed by first digesting pG36 DNA at the *Sty*I site located at the 3′ end of the hammerhead domain and at the *Eco*RI site in the pGEM polylinker. The excised DNA was replaced with annealed, complementary oligonucleotides with the sequences 5′-CTAGGCCAAGCT TCG-3′ and 5′-AATTCGAAGCTTGGC-3′. When annealed, these oligonucleotides form *Sty*I and *Eco*RI half sites on their ends and an internal *Hin*dIII site. When the resulting construct, pGES1, is digested with *Eco*RI and transcribed with SP6 RNA polymerase, the transcribed *Hin*dIII sequence is complementary to the 5′ end of the transcript, since there is a *Hin*dIII site just downstream of the SP6 promoter. The result is an extended region of complementarity between the 5′ and 3′ ends of the transcript.

pG36 SLI, pG47 SLI, and pGES1 SLI were constructed by site-specific mutagenesis with single-stranded uracil-containing DNA templates (19) and the variant oligonucleotide primer 5′-GATACCCAGAAACCTTCGGGTCCTAGG-3′. Transcripts from the mutated clones had the sequence 5′-UUCG-3′ inserted in the loop of stem III of the hammerhead structure.

Clones containing other mutations in the hammerhead domain or in the internal loop of the stem I extension were prepared by site-specific mutagenesis with appropriate primers. The sequence of the transcribed portion of all clones used in this analysis was verified by dideoxynucleotide sequencing (25).

In vitro transcription reactions. Transcripts were named by indicating the clone from which they were derived followed by a slash and the restriction enzyme used to linearize the DNA template prior to in vitro transcription with SP6 RNA polymerase. Transcription reaction mixtures (10 μl) consisted of 1 to 2 μg of linear template; 40 mM Tris (pH 7.5); 6 mM $MgCl_2$; 5 mM spermidine; 10 mM dithiothreitol; 250 μM (each) ATP, CTP, and GTP; 5 μM UTP; 10 μCi of [α-^{32}P] UTP; and 10 U of SP6 RNA polymerase. To produce 5′-labeled transcripts, the ATP, UTP, and CTP concentrations were raised to 500 μM, the GTP concentration was lowered to 100 μM, and 15 μCi of [γ-^{32}P]GTP was used as the radioactive nucleotide. This labeled the 5′ end of the transcripts, since G is the first nucleotide incorporated by SP6 RNA polymerase and only the 5′ nucleotide maintains its γ-phosphate. After incubation at 37°C for 60 min, unincorporated label was removed by chromatography through Sephadex G-50. Full-length transcripts were purified from polyacrylamide gels as described previously (9).

In vitro cleavage reactions. Transcripts in 1 mM EDTA, pH 8.0, were heated at 80°C for 5 min, quick-cooled on ice water, and combined with 2 volumes of prewarmed buffer to initiate self-cleavage. Self-cleavage reaction mixtures consisted of 0.002 to 0.004 ng of transcripts per μl, 133 mM morpholineethanesulfonic acid (MES) (pH 6.9), 30 mM $MgCl_2$, 10 mM NaCl, and 0.3 mM EDTA. Six-microliter reaction mixtures were incubated at 42°C for the indicated times, and reactions were stopped by the addition of 6 μl of gel loading buffer (80 mM EDTA, 95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue). Cleavage products were separated on denaturing polyacrylamide gels, and dried gels were quantified with a Betascope 603 Blot Analyzer (Betagen Corp.). First-order rate constants (k) were determined from plots of the log of the fraction of transcripts remaining versus time, using the relation $k = -2.3 \times$ slope. Rate constants were standardized to rate constants determined from parallel reactions of wild-type transcripts (k_{rel}). Half-lives were derived from the relation $t_{1/2} = 0.693/k$.

RESULTS

Analysis of transcripts from truncated DNA templates. pGM1/RI transcripts contain a single repeat of satellite 2 and 53 vector-derived nucleotides (see Fig. 2A for a diagram of this and other transcripts used in this analysis). Despite their theoretically unstable hammerhead domain, these transcripts cleave in the presence of Mg^{2+} to generate two products of 216 and 175 nucleotides (Fig. 2B) (11). To determine whether this ability required nucleotides outside the hammerhead domain, we tested the activity of transcripts produced from truncated DNA templates. Templates were deleted at the 5′ ends by using the exonuclease *Bal* 31 and at the 3′ ends by digesting with restriction enzymes that recognized sites within the satellite 2 sequence prior to transcriptions.

pG47/*Fok*I transcripts have only 11 and 16 satellite 2 nucleotides 5′ and 3′ to the hammerhead domain, respectively, and were competent for cleavage (Fig. 2C). A comparison of cleavage by pGM1/RI and pG47/*Fok*I transcripts is shown graphically in Fig. 2D. The half-lives determined from these plots were 145 min for pGM1/RI transcripts and 190 min for pG47/*Fok*I transcripts. While half-lives differed by as much as 30% for the same transcripts in independent experiments, pG47/*Fok*I transcripts consistently cleaved at a slightly reduced rate compared to pGM1/RI transcripts.

The activity of pG47/*Fok*I transcripts is dependent on the sequences flanking both ends of the hammerhead domain. This was illustrated when transcripts without the additional 5′-end sequence (pG36/*Fok*I), the 3′-end sequence (pG47/*Sty*I), or both the 5′- and 3′-end sequences (pG36/*Sty*I) failed to undergo detectable self-cleavage after 3 h (Fig. 2C).

Analysis of 3′-truncated RNAs. We employed a modification of the terminal-RNA-deletion analysis used by Forster and Symons (15) to further delimit the sequences on the 3′ side of the hammerhead domain required for self-cleavage. pG47/*Rsa*I transcripts, which have the same 5′ end but which have 33 more nucleotides on their 3′ end than do pG47/*Fok*I transcripts, were 5′ end labeled and subjected to limited alkaline hydrolysis (see Fig. 2A for the location of the

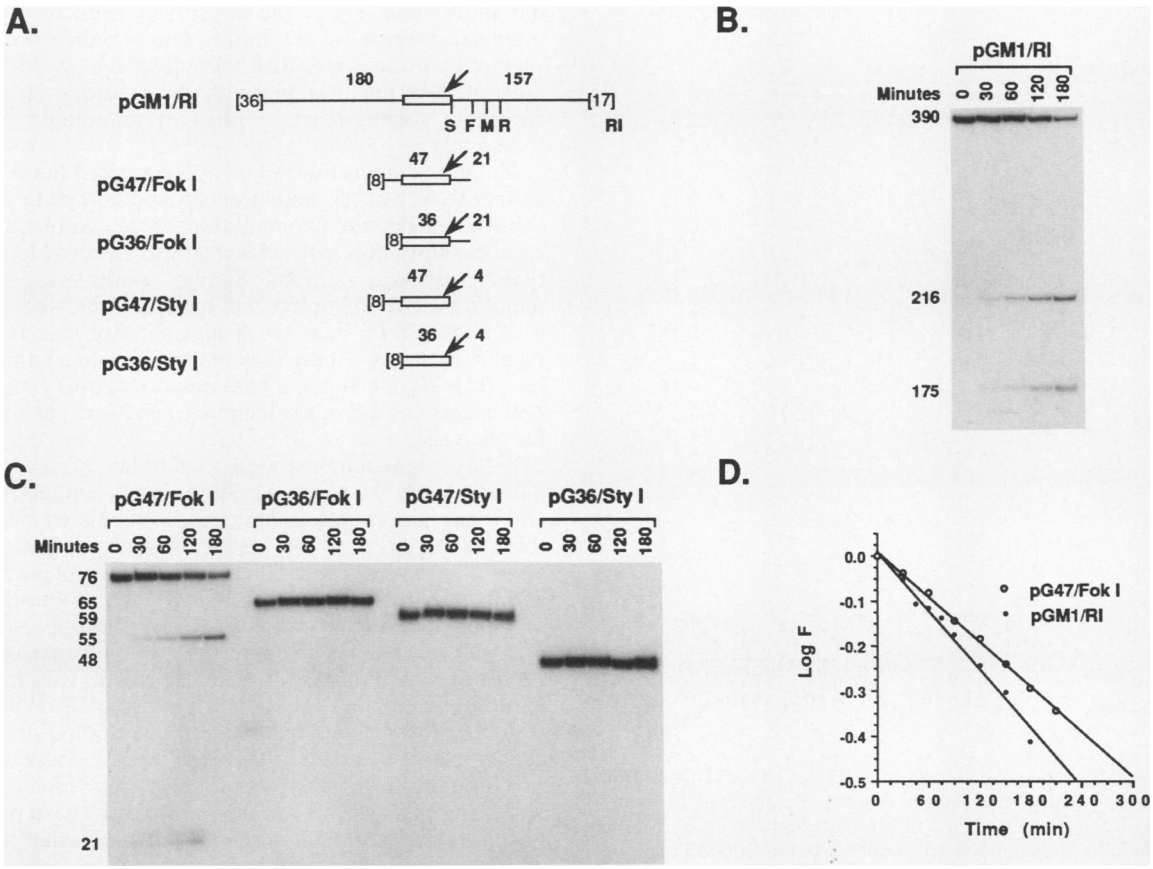

FIG. 2. Self-cleavage of satellite 2 transcripts derived from truncated DNA templates. (A) Diagrammatic representation of the SP6 RNA polymerase transcripts used in this analysis. Numbers above the transcripts represent the number of satellite 2 nucleotides on each side of the cleavage site, numbers in brackets represent vector-derived nucleotides at the ends of the transcripts, the open boxes denote the conserved hammerhead domains, and the arrows point to the sites of cleavage. Restriction enzyme sites used to linearize the templates prior to transcription are shown below the pGM1/RI transcript for convenience. S, *Sty*I; F, *Fok*I; M, *Mbo*II; R, *Rsa*I; RI, *Eco*RI. (B) Radiolabeled pGM1/RI transcripts were subjected to self-cleavage conditions for the indicated times, and the products were visualized by autoradiography after separation on a 7.5% polyacrylamide–7 M urea gel. Sizes (in nucleotides) of the full-length transcripts and the products of self-cleavage are indicated to the left. (C) A 10% polyacrylamide–7 M urea gel of the self-cleavage products of the indicated truncated transcripts. (D) pGM1/RI and pG47/*Fok*I transcripts were subjected to self-cleavage for an extended series of time points. The data were quantified and plotted as described in Methods and Materials. Log F is the logarithm of the decimal fraction of transcripts remaining after each time point.

*Rsa*I site). Electrophoresis then produced a ladder of products with identical 5′ ends (by virtue of their being radioactively labeled) but each progressively shorter by 1 nucleotide on its 3′ end. Starting with the fragment that corresponded to the pG47/*Fok*I transcript, sets of three bands were purified from the gel and subjected to self-cleavage conditions for 0 and 6 h. Figure 3A shows the results of this analysis and the nucleotide sequence of the hydrolysis products as determined by parallel sets of enzymatic RNA sequencing reactions (data not shown). The cleavage efficiencies for each set of fragments are shown above the postulated secondary structure for pG47/*Fok*I transcripts in Fig. 3B. Transcripts deleted to position +6 from the cleavage site and therefore ending at the 3′ end of the hammerhead domain failed to undergo detectable self-cleavage. Transcripts with additional 3′ satellite 2 nucleotides were competent for cleavage, and cleavage nearly doubled with each additional set of 3 3′ nucleotides. At position +18, the 70.5% cleavage after 6 h was comparable to the cleavage of full-length, pG47/*Fok*I transcripts (74.0%).

Additional satellite 2 sequences do not simply stabilize the hammerhead structure. After we examined the potential structure for pG47/*Fok*I transcripts (Fig. 3B), it seemed possible that the nucleotides flanking the hammerhead domain promoted cleavage by forming an extension to stem I and thereby stabilizing the hammerhead structure. To test this, we prepared transcripts with a 14-bp, nonhomologous substitution for the wild-type stem I (pGES1) (Fig. 4A). These transcripts showed no detectable cleavage after 5 h. We tentatively concluded that it was not sufficient to simply stabilize stem I to confer activity to the satellite 2 hammerhead.

An alternative explanation for the failure of pGES1 transcripts to cleave, however, was that the nonhomologous extension resulted in a stable conformation that inhibited the formation of an active hammerhead. To eliminate this possibility, we showed that the nonhomologous extension did not interfere with the activity of a hammerhead that was competent for cleavage. For this experiment, pG36/*Sty*I transcripts, which correspond to isolated satellite 2 hammerhead domains, were activated for cleavage by inserting 4 nucleotides in the stem III loop (pG36 SLI/*Sty*I). Consistent

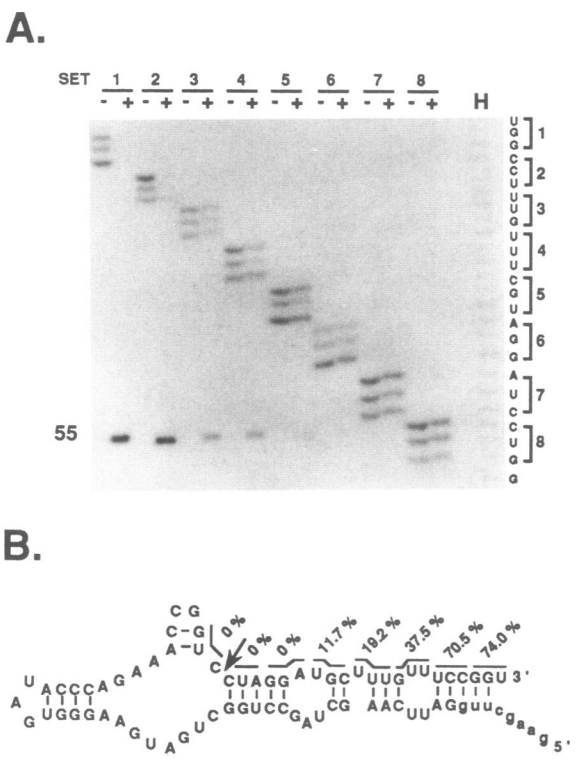

FIG. 3. Self-cleavage of 3′-truncated RNAs. (A) pG47/*Rsa*I transcripts were 5′ end labeled, gel purified, and subjected to limited alkaline hydrolysis as described in Methods and Materials. Sets of three hydrolysis fragments were purified from a second polyacrylamide gel and either subjected to self-cleavage conditions for 6 h (+) or not subjected to self-cleavage (−). The products were separated on a 10% polyacrylamide–7 M urea gel. Since the transcripts were labeled at their 5′ ends, only the 5′-cleavage product is visible on the autoradiogram, and this product is the same (55 nucleotides) for each of the hydrolysis fragments which differ in the length of their 3′ ends. The last lane (H) is a sample of the hydrolysis of pG47/*Rsa*I transcripts, and the 3′ nucleotide of each hydrolysis fragment, as determined by parallel enzymatic RNA sequencing reactions (data not shown), is indicated to the right. (B) Cleavage percentages were determined for each set of three hydrolysis fragments in panel A and are indicated above a potential secondary structure for the pG47/*Fok*I transcripts. Lowercase letters represent vector-derived nucleotides. The arrow points to the site of cleavage. The extended stem I of this structure was generated by the RNA Fold program of Zuker and Stiegler (34).

with previous studies (28), these transcripts cleaved with near-wild-type activity (Fig. 4B). This activated newt hammerhead was then combined with the nonhomologous stem I extension (pGES1 SLI) (Fig. 4C). In this case, the nonhomologous extension resulted in a more than 10-fold increase in the single-order rate constant, probably by stimulating rather than interfering with hammerhead formation. The failure of the same extension to promote cleavage by the unaltered satellite 2 hammerhead domain (pGES1 transcripts shown in Fig. 4A) indicates that the wild-type stem I extension in pG47/*Fok*I transcripts promotes cleavage in a manner other than by simply stabilizing the hammerhead structure.

Mutational analysis of hammerhead domain. It was possible, although unlikely, that the pG47/*Fok*I transcripts adopted a structure other than the hammerhead yet cleaved at the site used by the hammerhead. We performed a mutational analysis of the conserved features of the hammerhead domain to determine whether they were in fact required for cleavage. The mutations that were tested are illustrated in Fig. 5 along with the resulting cleavage rate constants standardized to rates of unmutated pG47/*Fok*I transcripts.

Mutations to position +3 or −33 disrupted the central base pair in stem I of the hammerhead and completely inhibited cleavage. When the two mutations were combined to form a compensatory base pair, cleavage was restored to near-wild-type levels (k_{rel} = 0.60). Similar results were seen with mutations that disrupted the base pair between positions −12 and −22 in stem II. Again, activity was restored by combining the two individual mutations into a compensatory base pair (k_{rel} = 0.58). These results strongly suggest that both stems I and II of the hammerhead form and are required for cleavage.

It has been demonstrated that many of the conserved nucleotides in the single-stranded region connecting stems I and II are important for hammerhead-mediated cleavage and that the G at position −28 is essential (22–24, 29). In agreement with these findings, cleavage of pG47/*Fok*I transcripts was completely inhibited by each of the three possible nucleotide substitutions at this position.

The A residues at positions −11 and −23 are opposed to each other at the base of stem II. In the vast majority of naturally occurring RNAs which are known or believed to self-cleave by using hammerhead structures, stem II is closed with a base pair at this location (2). Surprisingly, the two mutations to pG47/*Fok*I transcripts (A-11/U-23 and U-11/A-23) that resulted in the formation of base pairs at this position totally inhibited cleavage, and other mutations which did not generate a base pair also inhibited cleavage. Only one combination of nucleotides that was tested was not inhibitory (A-11/C-23; k_{rel} = 1.18). Thus, while the results above indicate that stem I and stem II of the hammerhead do form, there is an unexplained requirement in stem II not found in other hammerheads that have been analyzed.

Mutations within the internal loop of the extension to stem I. The failure of the nonhomologous stem to support cleavage in pGES1 transcripts (Fig. 4A) indicated that the wild-type extension had a sequence or structural motif essential for cleavage. To define the essential features of the extension, we performed a mutational analysis on the internal loop nearest the cleavage site. The mutations that were tested and the relative cleavage rates of transcripts with these mutations are shown in Fig. 6. Surprisingly, the actual identity of each nucleotide in the loop was important, and most changes to any of the 5 nucleotides in the loop completely inhibited cleavage. Even the most permissive mutations resulted in a >90% loss in activity.

The stem I extension increases cleavage efficiency of a stable hammerhead. The ability of the stem I extension to promote cleavage by the theoretically unstable satellite 2 hammerhead prompted us to determine what effect the extension would have on a hammerhead that was capable of cleavage without the extension. For this purpose, we prepared pG47 SLI/*Fok*I transcripts which have both the stem I extension and the stable stem III insertion. As shown in Fig. 7, more than 60% of these transcripts cleaved within 1 min. Transcripts remaining after 1 min were most likely in inactive conformations (28). The slow second phase of the reaction would thus correspond to the slow reorganization of these transcripts into active conformations. The first-order rate constant for the initial phase of the reaction, estimated from the 30-s time point, was more than 2 orders of magnitude

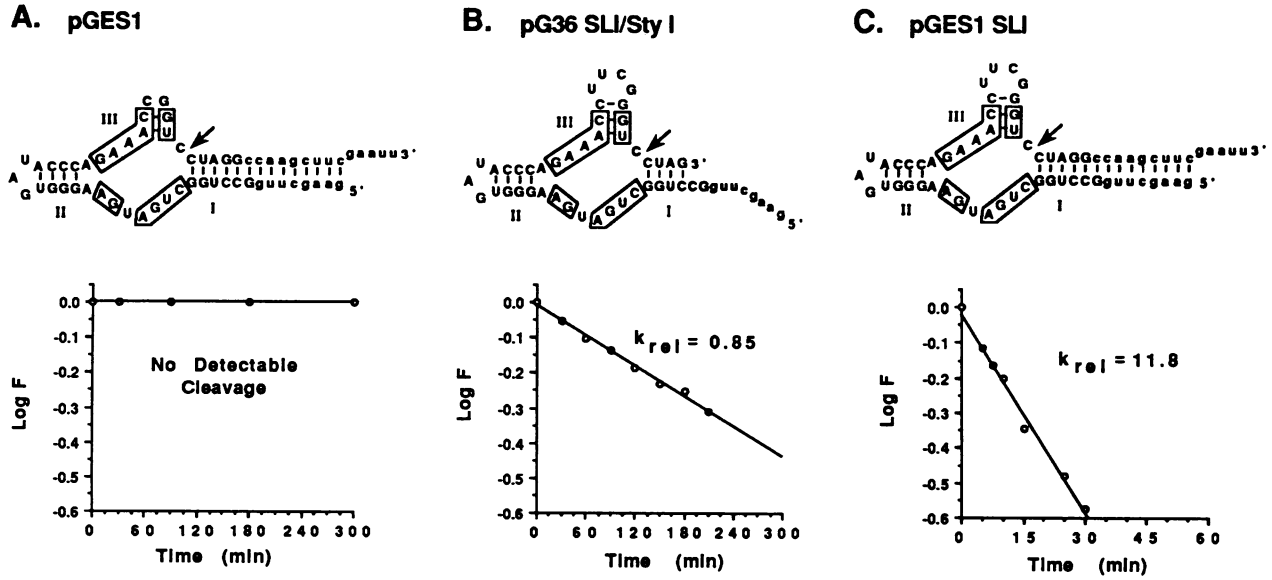

FIG. 4. Nonhomologous stem I does not confer activity to the satellite 2 hammerhead. (A) Time course of cleavage of pGES1 transcripts, in which the wild-type stem I is replaced with a nonhomologous, perfectly base-paired extension. (B) Time course of cleavage of pG36 SLI/*Sty*I transcripts which correspond to isolated satellite 2 hammerhead domains with a four nucleotide insertion in the loop of stem III. (C) Time course of cleavage of pGES1 SLI transcripts, which have both the nonhomologous stem I extension and the stem III loop insertion. Lowercase letters represent non-satellite 2 nucleotides. Log F is the logarithm of the decimal fraction of transcripts remaining after each time point. Each arrow points to the site of cleavage.

greater than that for transcripts with either the stable stem III insertion (pG36 SLI/*Sty*I) (Fig. 4B) or the stem I extension (pG47/*Fok*I) (Fig. 2D) alone. Additionally, the wild-type stem I extension was 20 times more effective than the nonhomologous extension in pGES1 SLI (Fig. 4C) in enhancing cleavage by a competent hammerhead.

DISCUSSION

Newt satellite 2 transcripts undergo self-catalyzed cleavage in vitro within a domain that has the conserved features of the hammerhead structure used by a number of self-cleaving plant RNAs (9). Unlike other hammerheads that have been studied, however, the satellite 2 hammerhead does not cleave as an isolated unit, probably because of its abbreviated and unstable stem III (28). In the present study, we demonstrated that self-cleavage of satellite 2 transcripts is dependent upon the satellite 2 sequences adjacent to the hammerhead domain. The additional sequences have the potential to form a looped extension to stem I of the hammerhead. The evidence that this extension is critical for cleavage was that transcripts without either the 5′ or the 3′ half of this extension failed to self-cleave, and a number of

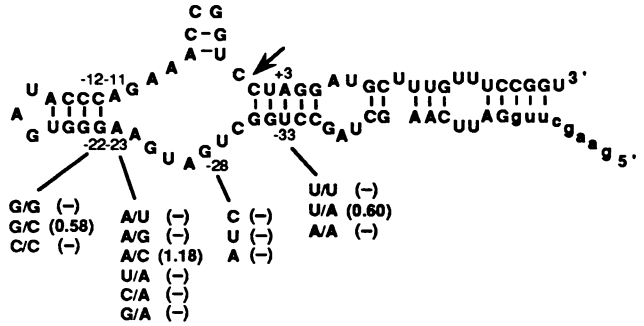

FIG. 5. Mutational analysis of the hammerhead domain in pG47/*Fok*I transcripts. The indicated nucleotides were changed at the DNA level by site-specific mutagenesis, and transcripts from the modified clones were tested for self-cleavage. Nucleotides are numbered upstream (–) or downstream (+) from the cleavage site. First-order rate constants were determined from a minimum of five time points showing less than 60% cleavage. The rate constants were normalized to the cleavage rates of parallel samples of wild-type pG47/*Fok*I transcripts (k_{rel}), shown in parentheses. –, no detectable cleavage after 2.5 h. The arrow points to the site of cleavage.

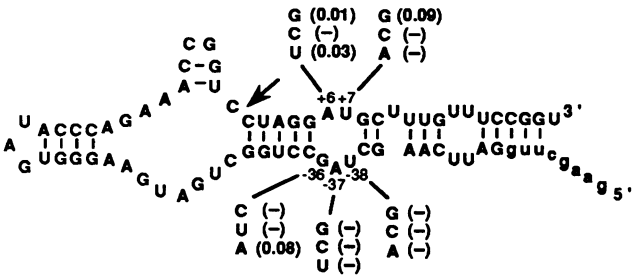

FIG. 6. Mutational analysis of the internal loop in the stem I extension nearest the cleavage site. The indicated nucleotides were changed at the DNA level by site-specific mutagenesis, and transcripts from the modified clones were tested for self-cleavage. First-order rate constants were determined as for Fig. 5, except that transcripts containing mutations in the upper part of the loop, which includes the recognition sequence for *Fok*I, were prepared from *Mbo*II-digested templates and compared with pG47/*Mbo*II transcripts. Because of the high proportional variability in the cleavage of transcripts with barely detectable activity, the k_{rel} values (in parentheses) are the averages from two or three (G+6 and G+7) independent experiments. –, no detectable cleavage after 2.5 h. The arrow points to the site of cleavage.

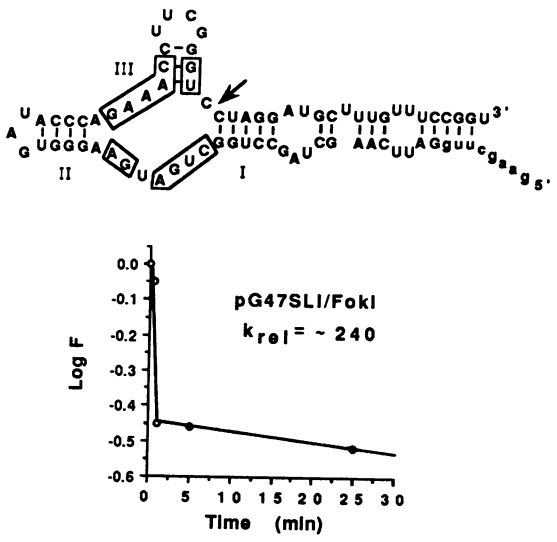

FIG. 7. Self-cleavage of pG47 SLI/*Fok*I transcripts. Time course of cleavage of pG47 SLI/*Fok*I transcripts which have the SLI insertion in the loop of stem III in association with the wild-type stem I extension. k_{rel} is the first-order rate constant standardized to the cleavage rate of parallel reactions of wild-type pG47/*Fok*I transcripts. The arrow points to the site of cleavage.

single-nucleotide substitutions within this extension completely abolished the transcripts' ability to self-cleave. Furthermore, a nonhomologous stem I extension did not enable the isolated satellite 2 hammerhead to cleave.

It is unlikely that cleavage of satellite 2 transcripts results from the formation of an active structure other than the hammerhead. Mutations designed to disrupt important features of the hammerhead had inhibitory effects on self-cleavage, and regions base paired in the hammerhead were demonstrated to be base paired in the satellite 2 structure by compensatory mutagenesis. The unusual requirement for specific nucleotides at the base of stem II might be a reflection of a novel interaction between the conserved hammerhead domain and the stem I extension. It should be noted that satellite 2 transcripts in the newt ovary do have a base pair at this position in stem II (Fig. 1). While this might explain the failure of these ovarian transcripts to cleave at their intact cleavage sites (8, 9), the presence of a base pair at this position does not necessarily preclude the occurrence of self-cleavage. We prepared monomeric transcripts which had this base pair and an A substituted for G-31 in stem I, the other difference in the hammerhead domain between ovarian transcripts and the cloned genomic repeat used in our studies. These transcripts cleaved at a rate comparable to that of unmodified transcripts (33). Further work is necessary to determine whether these results do in fact result from an interaction between stems I and II.

It is difficult to speculate on the action of the stem I extension, since the detailed mechanism of hammerhead-mediated self-cleavage is not known. The reaction involves the nucleophilic attack of the phosphodiester bond by the adjacent 2′-hydroxyl, but the role of the required divalent cation in this process is not clear. Mg^{2+} may be involved in stabilizing structural interactions, activating the 2′-hydroxyl for nucleophilic attack, or stabilizing the pentacoordinated phosphorus intermediate (5, 21, 23). The satellite 2 stem I extension could stimulate catalysis by providing one or more alternative coordination points for Mg^{2+} or, as suggested above, by interacting with available regions of the hammerhead domain to promote the formation of an active tertiary conformation.

A more trivial explanation for the effect of the stem I extension is that it merely allows the hammerhead structure to form, while all other transcripts tested have alternative, inactive conformations. Fedor and Uhlenbeck (12) demonstrated that while there were large differences in the efficiencies of hammerheads that differed in the identities and lengths of their base-paired stems, the actual rates of catalysis were the same. The differences in efficiency were attributed to differential abilities to form active conformers. We believe that it is unlikely that the present results can be explained in this manner. The nonhomologous extension which failed to promote cleavage of the wild-type satellite 2 hammerhead actually increased the rate of cleavage by an active hammerhead. In addition, this explanation would require each of 11 single-nucleotide substitutions in the internal loop of the stem I extension to result in alternative conformations that were so energetically favored that they totally excluded the formation of the active structure. The simplest hypothesis from these data is that the extension has a specific, positive effect on hammerhead formation or catalysis. To test this hypothesis, it will be necessary to split the reaction into a two-component (enzyme-substrate) system and perform a more detailed kinetic analysis. More information about the specific sequence or structural requirements for proper interaction between the hammerhead domain and the stem I extension is needed, however, before this can be successfully accomplished.

ACKNOWLEDGMENTS

This work was supported by NIH grant GM 38791 to L.M.E. and by a James R. Fisher fellowship from the American Cancer Society to L.M.P.

We thank Teresa Graham for her excellent technical assistance.

REFERENCES

1. **Belinsky, M., and G. Dinter-Gottlieb.** 1991. Characterizing the self-cleavage of a 135 nucleotide ribozyme from genomic hepatitis delta virus. Prog. Clin. Biol. Res. **364**:265–274.
2. **Bruening, G.** 1989. Compilation of self-cleaving sequences from plant virus satellite RNAs and other sources. Methods Enzymol. **180**:546–558.
3. **Cech, T. R.** 1985. Self-splicing RNA: implications for evolution. Int. Rev. Cytol. **93**:3–22.
4. **Cotten, M., G. Schaffner, and M. L. Birnstiel.** 1989. Ribozyme, antisense RNA, and antisense DNA inhibition of U7 small nuclear ribonucleoprotein-mediated histone pre-mRNA processing in vitro. Mol. Cell. Biol. **9**:4479–4487.
5. **Dahm, S. C., and O. C. Uhlenbeck.** 1990. Characterization of deoxy- and ribo-containing oligonucleotide substrates in the hammerhead self-cleavage reaction. Biochimie **72**:819–823.
6. **Dange, V., R. B. Van Atta, and S. M. Hecht.** 1990. A Mn^{2+}-dependent ribozyme. Science **248**:585–588.
7. **Dzianott, A. M., and J. J. Bujarski.** 1989. Derivation of an infectious viral RNA by autolytic cleavage of in vitro transcribed viral cDNAs. Proc. Natl. Acad. Sci. USA **86**:4823–4827.
8. **Epstein, L. M., and S. R. Coats.** Gene, in press.
9. **Epstein, L. M., and J. G. Gall.** 1987. Self-cleaving transcripts of satellite DNA from the newt. Cell **48**:535–543.
10. **Epstein, L. M., K. A. Mahon, and J. G. Gall.** 1986. Transcription of a satellite DNA in the newt. J. Cell Biol. **103**:1137–1144.
11. **Epstein, L. M., and L. M. Pabon-Pena.** 1991. Alternative modes of self-cleavage by newt satellite 2 transcripts. Nucleic Acids Res. **19**:1699–1705.
12. **Fedor, M. J., and O. C. Uhlenbeck.** 1990. Substrate sequence effects on "hammerhead" RNA catalytic efficiency. Proc. Natl. Acad. Sci. USA **87**:1668–1672.

13. **Feldstein, P. A., J. M. Buzayan, and G. Bruening.** 1989. Two sequences participating in the autolytic processing of satellite tobacco ringspot virus complementary RNA. Gene **82**:53–61.
14. **Forster, A. C., C. Davies, C. C. Sheldon, A. C. Jeffries, and R. H. Symons.** 1988. Self-cleaving viroid and newt RNAs may only be active as dimers. Nature (London) **334**:265–267.
15. **Forster, A. C., and R. H. Symons.** 1987. Self-cleavage of virusoid RNA is performed by the proposed 55-nucleotide active site. Cell **50**:9–16.
16. **Forster, A. C., and R. H. Symons.** 1987. Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. Cell **49**:211–220.
17. **Hampel, A., R. Tritz, M. Hicks, and P. Cruz.** 1990. 'Hairpin' catalytic RNA model: evidence for helices and sequence requirement for substrate RNA. Nucleic Acids Res. **18**:299–304.
18. **Haseloff, J., and W. L. Gerlach.** 1988. Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature (London) **334**:585–591.
19. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
20. **Kuo, M. Y.-P., L. Sharmeen, G. Dinter-Gottlieb, and J. Taylor.** 1988. Characterization of self-cleaving RNA sequences on the genome and antigenome of human hepatitis delta virus. J. Virol. **62**:4439–4444.
21. **Mei, H.-Y., T. W. Kaaret, and T. C. Bruice.** 1989. A computational approach to the mechanism of self-cleavage of hammerhead RNA. Proc. Natl. Acad. Sci. USA **86**:9727–9731.
22. **Odai, O., H. Hiroaki, T. Sakata, T. Tanaka, and S. Uesugi.** 1990. The role of a conserved guanosine residue in the hammerhead-type RNA enzyme. FEBS Lett. **267**:150–152.
23. **Perreault, J.-P., D. Labuda, N. Usman, J.-H. Yang, and R. Cedergren.** 1991. Relationship between 2'-hydroxyls and magnesium binding in the hammerhead RNA domain: a model for ribozyme catalysis. Biochemistry **30**:4020–4025.
24. **Ruffner, D. E., G. D. Stormo, and O. C. Uhlenbeck.** 1990. Sequence requirements of the hammerhead RNA self-cleavage reaction. Biochemistry **29**:10695–10702.
25. **Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161–178.
26. **Sarver, N., E. M. Cantin, P. S. Chang, J. A. Zaia, P. A. Ladne, D. A. Stephens, and J. J. Rossi.** 1990. Ribozymes as potential anti-HIV-1 therapeutic agents. Science **247**:1222–1225.
27. **Saville, B. J., and R. A. Collins.** 1990. A site-specific self-cleavage reaction performed by a novel RNA in neurospora mitochondria. Cell **61**:685–696.
28. **Sheldon, C. C., and R. H. Symons.** 1989. RNA stem stability in the formation of a self-cleaving hammerhead structure. Nucleic Acids Res. **17**:5665–5677.
29. **Sheldon, C. C., and R. H. Symons.** 1989. Mutagenesis analysis of a self-cleaving RNA. Nucleic Acids Res. **17**:5679–5685.
30. **Symons, R. H.** 1989. Self-cleavage of RNA in the replication of small pathogens of plants and animals. Trends Biochem. Sci. **14**:445–450.
31. **Uhlenbeck, O. C.** 1987. A small catalytic oligoribonucleotide. Nature (London) **328**:596–600.
32. **Watson, N., M. Gurevitz, J. Ford, and D. Apirion.** 1984. Self cleavage of a precursor RNA from bacteriophage T4. J. Mol. Biol. **172**:301–323.
33. **Zhang, Y., and L. M. Epstein.** Unpublished data.
34. **Zucker, M., and P. Stiegler.** 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. **9**:133–148.