Alternative modes of self-cleavage by newt satellite 2 transcripts

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ABSTRACT

Synthetic transcripts of satellite 2 DNA from the newt undergo self-catalyzed cleavage *in vitro*. In this report we present evidence that there are at least two distinct modes of satellite 2 transcript self-cleavage. In one mode, a single cleavage domain folds into a structure which cleaves at a slow rate. This structure may be analogous to, or a variant of, the 'hammerhead' structure believed to be active during the self-cleavage of a number of infectious plant RNAs. In an alternative mode, multiple cleavage domains interact to cleave at an enhanced rate. The permutation of the repeated satellite 2 sequence determines which of these modes of cleavage will predominate, presumably by influencing the overall conformation of the RNA. We present a model for the self-processing of multimeric satellite 2 transcripts which incorporates both of these modes of self-cleavage.

INTRODUCTION

Satellite 2 is a 330 base pair, tandemly repeated sequence in the genome of the newt, *Notophthalmus viridescens* (1). Repeat length transcripts homologous to satellite 2 DNA have been found in every newt tissue examined, although their function is not known. It is also not understood how monomeric transcripts are produced from tandemly repeated DNA, although the demonstration that synthetic satellite 2 transcripts undergo site-specific, self-catalyzed cleavage *in vitro* suggests that self-processing of multimeric primary transcripts may occur *in vivo* (2). In fact, while the ends of satellite 2 transcripts in the ovary are different than those produced by *in vitro* cleavage (2), transcripts in non-ovarian tissues such as the liver have the same end structures as those on the *in vitro* cleavage products (S. Coats and L.M. Epstein, unpublished results).

The *in vitro* reaction requires magnesium, generates products with 5'-hydroxyl and 2',3'-cyclic phosphate groups, and therefore resembles the self-cleavage of certain plant viroid, virusoid, and viral satellite RNAs (3). Self-cleavage of these RNAs is believed to be responsible for the processing of multimeric replication intermediates. These RNAs, as well as the newt satellite 2 transcripts, have a domain surrounding the cleavage site that is highly conserved in primary sequence and

potential secondary structure (4,5), and which is capable of self-cleavage as an isolated unit (6–8). The proposed structure of this domain has been termed the 'hammerhead,' and is shown in Figure 1a.

Figure 1b illustrates that while the newt satellite 2 cleavage site can be folded into a hammerhead structure, it has a potentially unstable stem III which consists of two base pairs connected by a two nucleotide hairpin loop. An alternative structure, the double hammerhead (Fig. 1c), was proposed for the processing of multimeric satellite 2 transcripts, as well as for the processing of the plus and minus strands of the avocado sunblotch viroid which also have stems with questionable stability (9). In the double-hammerhead, two single hammerheads, formed by the interaction of two separate cleavage sites, are connected by an extended, and theoretically more stable stem III. This model received support when synthetic RNAs, consisting of single newt-like cleavage site domains, were shown to cleave in a concentration-dependent manner (9). The higher concentrations were apparently required to promote formation of inter-molecular structures such as the double-hammerhead.

In this report we present evidence that, in contrast to the above studies, individual satellite 2 cleavage site domains are capable of self-cleavage when in the context of complete, monomeric, satellite 2 transcripts. The additional satellite 2 sequences either stabilize the hammerhead, or result in an alternative structure active in cleavage. Cleavage utilizing single domains also occurs in certain greater than unit length transcripts which have two cleavage domains in the same molecule. However, transcripts with different permutations of the satellite 2 sequence can utilize inter-domain interactions to cleave at an enhanced rate. The interaction involved in this mode of cleavage could be the formation of a double-hammerhead structure. A model is presented for the progressive processing of multimeric satellite 2 transcripts *in vivo* which incorporates both of these modes of self-cleavage.

METHODS AND MATERIALS

DNA clones

All clones used in this analysis (Fig. 2) were derived from the genomic satellite 2 dimer clone pSP6D6 (2). pSP6D6 consists of two similar, but not identical tandem repeats of satellite 2

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Figure 1. Hammerhead structures. a) Consensus sequence and proposed structure of the 'hammerhead' domain required for self-cleavage of viroid, virusoid and viral satellite RNAs. b) The potential hammerhead structure of the first cleavage site in transcripts from the newt satellite 2 dimer clone pSP6D6. c) The intermolecular double-hammerhead structure proposed to be active in the cleavage of synthetic, 40 nucleotide newt-like cleavage domains (9). Structures are represented using the conventions of Forster and Symons (5), where stems are labeled I to III, arrows denote cleavage sites, and conserved nucleotides are boxed. In (b), the ov subscripts denote the nucleotides found in ovarian transcripts at the indicated positions.

DNA. The first repeat in pSP6D6 was excised with *Sau3A* and inserted into the *Bam*HI site of the plasmid pSP6-4 to generate the intermediate construct pD6M1. The insert and some flanking vector nucleotides were excised by digestion with *Hind*III and *Eco*RI and inserted into *Hind*III/*Eco*RI digested pGEM-3Zf(-) (Promega Corporation) to generate pGM1. pGM1M2A is identical to pGM1 except that the conserved GAAAC sequence adjacent to the cleavage site (see Fig. 1) was changed to GAAC by site-specific mutagenesis using a variant oligonucleotide primer (10). The dimer clone pGD was constructed by combining two, identical *Sau3A* inserts from pGM1 into a single clone. The mutant dimers (pGD1M, pGD2M, pGD1,2M) were constructed in a similar manner using the appropriate combinations of inserts from pGM1 and pGM1M2A. The compositions of these and all other clones used in this analysis were verified by dideoxynucleotide sequencing (11).

pG47:331 was generated during a Bal31 deletion analysis as follows. Deletions from the *Hind*III site in pD6M1 (5' end of sense strand) were blunt-ended with T4 DNA polymerase, ligated to *Hind*III linkers, and digested with *Hind*III and *Eco*RI. The resulting fragments were inserted into a pGEM-3Zf(-) vector with a 7 nucleotide deletion in the SP6 promoter region. Transcripts from the resulting constructs have only 8 vector derived nucleotides on their 5' ends (shown in expanded form below pG47:331 in Fig. 2). Bal31 generated deletions from the *Hind*III site in pSP6D6 (3' end of sense strand) were blunt-ended, ligated to *Eco*RI linkers, and digested with *Sty*I and *Eco*RI. These fragments were ligated to *Sty*I/*Eco*RI digested 5' deletions, to generate a series of clones with various combinations of 5'- and 3'-end deletions, pG47:331 being one of them. Mutant derivatives of pG47:331 were prepared by site-specific mutagenesis, and confirmed by dideoxynucleotide sequencing.

In vitro transcription

DNAs were linearized with *Eco*RI prior to transcription with SP6 RNA polymerase. Transcriptions were performed as described previously (2), except that the UTP concentration was lowered to 5 μ M for the production of transcripts labeled with [α -³²P]UTP, and raised to 1.25 mM for the production of

Figure 2. SP6 RNA polymerase transcripts of the genomic satellite 2 dimer clone, pSP6D6, and its derivatives. Restriction sites used in cloning are shown below the transcripts for convenience. The two, non-identical satellite 2 repeats in pSP6D6 are represented by open and closed rectangles. pGM1 is represented by an open rectangle because it was derived from the first repeat in pSP6D6. pGD was formed by dimerizing the insert in pGM1; thus it has two, identical satellite 2 repeats, both represented by open rectangles. pG47:331 was derived from an internal segment of pSP6D6, and therefore has segments from both repeats. Stippled rectangles indicate the locations of the conserved 'hammerhead' domains, and the arrows indicate the sites of cleavage. The expected sizes of cleavage products are given above the transcripts; the numbers in brackets represent vector derived nucleotides at the ends of the transcripts. The sequence of the deleted promoter region in pG47:331 is shown below the transcript. R, *Eco*RI; S, *Sau3A*; St, *Sty*I; H, *Hind*III.

greater amounts of unlabeled transcripts. Full length transcripts were purified by electroelution from acrylamide gel slices as previously described (2). The amounts of labeled transcripts used in individual reactions were determined from their specific activities; the amounts of unlabeled transcripts were determined from their optical density.

In vitro cleavage reactions

Precipitated, gel-purified labeled and unlabeled transcripts were resuspended in 1 mM EDTA, pH 8.0, and distributed individually or in combination to 1.5 ml microtubes. Unless otherwise indicated, transcripts were heated at 80°C for 5 minutes, quick cooled on ice water for 20 seconds, and combined with 2 volumes of prewarmed buffer to initiate self-cleavage. Self-cleavage was performed in a 6 μ l reaction volume of 133 mM MES, pH 7.0, 30 mM MgCl₂, 10 mM NaCl, and 0.3 mM EDTA at 42°C. Reactions were terminated by adding 6 μ l of stop buffer (20 mM EDTA, 95% formamide), and electrophoresed on 7.5% polyacrylamide, 7 M urea gels. Dried gels were quantified using a Betascope 603 Blot Analyzer (Betagen Corporation).

RESULTS

Cleavage of monomeric satellite 2 transcripts is not concentration-dependent

Previous studies on isolated satellite 2-like cleavage site domains suggested that self-cleavage was dependent upon interactions between more than one domain (9). We first wanted to determine if similar interactions were required for cleavage of intact, monomeric satellite 2 transcripts. Theoretically, these interactions could occur intra-molecularly in multimeric transcripts, or inter-molecularly between separate, monomeric transcripts. In the latter case, the second order rate equation predicts that the relative rate

Figure 3. Effect of concentration on self-cleavage of monomeric pGM1 transcripts. a) 7.5% acrylamide, 7M urea gel of a 10,000-fold concentration range of pGM1 transcripts after self-cleavage for the indicated times. Transcripts were heated at 80°C for 5 min quick cooled on ice just prior to cleavage. Each reaction contained 0.004 ng/μl of 32 P-labeled transcripts and the required amount of unlabeled transcripts to attain the indicated, final concentrations. The transcripts and expected cleavage products are represented diagrammatically to the left using the conventions described in Figure 2. T, 5', and 3' represent full length transcript, 5' cleavage product, and 3' cleavage product, respectively. b) The gel in (a) was quantified as described in the Methods and Materials, and the time course of cleavage for the 40, 0.4 and 0.004 ng/μl transcripts was plotted.

Figure 4. Self-cleavage of mutant pGM1M2A transcripts. a) Time course of self-cleavage of a 10,000-fold concentration range of pGM1M2A transcripts. These transcripts differ from pGM1 transcripts by a single deletion of an A residue in the conserved GAAAC segment of the cleavage domain. Transcripts were heated for 5 min at 80°C and quick cooled on ice prior to self-cleavage. b) Time course of cleavage of trace amounts of radioactively labeled mutant pGM1M2A transcripts (0.004 ng/μl) in the presence of unlabeled, wildtype, pGM1 transcripts. The concentration of the pGM1 transcripts ranged from 0 to 40 ng/μl. Mixtures of transcripts were heated prior to cleavage as in (a). c) Time course of cleavage of pGM1M2A transcripts, at a concentration of 0.004 ng/μl, after heating at 80°C for 0, 2, 5, and 10 min in the presence of 40 ng/μl of unlabeled pGM1 transcripts.

of cleavage would be directly proportional to the transcript concentration. The effect of concentration on self-cleavage was investigated for transcripts of the monomer clone, pGM1, which consists of the first repeat of the original dimer clone pSP6D6 (Fig. 2). Trace amounts of labeled transcripts (0.004 ng/μl final concentration) were mixed with increasing concentrations of unlabeled transcripts, heated to 80°C for 5 minutes, quick cooled on ice, and subjected to self-cleavage conditions for the times indicated in Figure 3a. At all concentrations, there was a steady increase with time in the appearance of the 216 nucleotide 5' and 174 nucleotide 3' products of cleavage. The percent cleavage at each time point was quantified and plotted in Figure 3b. While cleavage is stimulated at higher concentrations, the effect is minimal with only an approximate 25% increase in the relative cleavage rate (ng labeled transcript/μl min) during the initial stages of the reaction over the 10,000-fold concentration range. These results are incompatible with the second order rate equation which

predicts a 10,000-fold increase in the relative cleavage rate over the same concentration range. Either inter-molecular interactions are not required for cleavage, or these interactions occur near-maximally at even the lowest concentration we tested.

Inter-molecular interactions between monomeric transcripts are not favored

In order to better characterize inter-molecular satellite 2 transcript interactions and determine their potential contribution to the self-cleavage reaction, we developed a system where self-cleavage of monomer-sized transcripts was dependent upon such interactions. The conserved GAAAC near the cleavage site in pGM1 was mutated to GAAC by site-directed mutagenesis to produce the clone pGM1M2A. In previous studies using dimeric transcripts of the avocado sunblotch viroid (9), a domain caring this mutation cleaved only when complexed with a wildtype domain, presumably through the formation of a double-

Figure 5. Self-cleavage of dimeric transcripts with wildtype and mutant cleavage domains. **a)** Wildtype or mutant dimeric transcripts were heated at 80°C for 5 min, quick cooled on ice, and cleaved for the indicated times at low concentrations (0.004 ng/μl). The reactions were electrophoresed on a 7.5% acrylamide, 7M urea gel. The diagram illustrates the expected cleavage products of the full length transcripts (T). These include the partial products P1 and P2, the internal monomer M separating the two cleavage sites, and the 5'- and 3'-end fragments. pGD is the wildtype transcript; the other constructs are deleted for an A residue in the conserved GAAAC segment of either their first (pGD1M), their second (pGD2M), or both (pGD1,2M) cleavage domains. **b)** The gel in (a) was quantified and cleavage at the first site for the wildtype and single mutant constructs was calculated as the % of the total radioactivity found in P2, M and the 5' fragment, after correcting M for the loss of its 3' end by cleavage at the second site.

hammerhead structure where the mutant GAAC and wildtype GAAAC sequences were provided *in trans* between the two cleavage sites (see Fig. 1c). We reasoned that the same mutation in the single cleavage domain in pGM1M2A transcripts should restrict cleavage of these transcripts to that resulting from inter-molecular interactions with wildtype transcripts.

We first performed a time course of cleavage of a 10,000-fold concentration range of pGM1M2A transcripts to test the effect of this mutation (Fig. 4a). As expected, there was no detectable cleavage at the lower concentrations, but at a concentration of 40 ng/μl, 5% of the transcripts were cleaved after 5 hours. Thus, under conditions that promote inter-molecular complex formation, structures are formed that are slightly less sensitive to the GAAC mutation than structures formed at low concentrations.

If, as exemplified in the double-hammerhead model, the structures formed at high concentrations involved a productive association between the cleavage site from one molecule and the GAAAC motif from another molecule, cleavage of the mutant monomer should be stimulated by interaction with a wildtype monomer. Labeled, mutant pGM1M2A transcripts were subjected to self-cleavage after heating for 5 minutes either alone or in the presence of an equal amount, a 10-fold excess, or a 10,000-fold excess of unlabeled wildtype pGM1 transcripts (Fig. 4b). The enhanced cleavage of the mutant transcripts in the presence of increasing amounts of wildtype transcripts suggests that productive, inter-molecular structures can be formed. However, even at the highest concentration of wildtype transcripts (40 ng/μl), only 30% of the mutant transcripts were cleaved after 5 hours, and the initial rate of cleavage was an order of magnitude lower than for cleavage of wildtype transcripts at low concentrations (Fig. 3b).

It is possible that the sub-optimal cleavage of the mutant transcripts in the above experiment was due to the presence of preformed mutant complexes which inhibited the formation of

productive complexes. To examine this possibility, labeled pGM1M2A transcripts and a 10,000-fold excess of unlabeled, wildtype pGM1 transcripts were combined and subjected to differential heat treatments prior to self-cleavage. Figure 4c shows that there were only minor differences in the initial rates and overall extents of mutant transcript cleavage, even between samples that were heated for 10 minutes at 80°C and samples that were not subjected to any heat treatment. Thus, we found no evidence for the extensive occurrence of preformed, mutant complexes which would have been differentially disrupted by the heat treatments. The results of this and the preceding experiments indicate that either productive complexes are not readily formed between monomeric, satellite 2 transcripts, or cleavage by a hybrid complex is not efficient. Evidence is presented below that under different circumstances, domains with the same GAAC mutation can be cleaved very efficiently by interaction with wildtype domains. We conclude that inter-molecular complexes between monomers are not favored, although they can form during prolonged incubation. Wildtype transcripts must utilize an alternative strategy, presumably involving single domain structures, to account for their increased cleavage efficiency over the mutant transcripts which depend upon unfavorable inter-molecular interactions for cleavage.

Interactions between two sites in dimeric transcripts are not required for cleavage

A system similar to that used to study cleavage of the monomeric transcripts was then used to study potential interactions between two cleavage site domains when present in the same, dimer sized transcript. We used the same GAAC mutation to determine if the critical GAAAC sequence was necessary for cleavage of the adjacent or opposite sites in the dimers. The inserts from pGM1 and pGM1M2A were combined to produce dimeric clones with different combinations of wildtype and mutant cleavage site

Figure 6. Self-cleavage of transcripts from pG47:331 and its mutant derivatives. a) Transcripts were heated at 80°C for 5 min, quick cooled on ice, self-cleaved for the indicated times at low concentrations (0.004 ng/μl), and electrophoresed on a 7.5% acrylamide, 7M urea gel. The full length transcripts (T), their partial cleavage products P1 and P2, and the excised monomer (M) are illustrated to the left. The 55 nucleotide 5'- and 7 nucleotide 3'-end fragments were run off the gel. pG47:331 is the wildtype construct, the other constructs are deleted for an A residue in the conserved GAAAC segment in either or both of their cleavage domains. b) Time course of cleavage of the first site in wildtype and mutant transcripts. The gel in (a) was quantified and cleavage at the first site was calculated as the % of the total radioactivity found in P2 and M after all cleavage products were corrected for the loss of their 5'- and/or 3'-end fragments. c) Time course of cleavage of the second site in the wildtype and mutant transcripts. Second-site cleavage was determined as in (b), except that after correction for the loss of the end fragments, cleavage was calculated as the % of the total of P2 and M. d) Time course of cleavage of purified partial cleavage products P1 and P2. Partial products P1 and P2, generated at low but detectable levels during the transcription of wildtype clone pG47:331, were purified from acrylamide gel slices. The purified products were heated to 80°C for 5 min, quick cooled on ice, and cleaved prior to cleavage at a concentration of 0.004 ng/μl.

domains. As illustrated in Figure 5a, cleavage of the wildtype dimer, pGD, generated the 216 nucleotide 5'- and 174 nucleotide 3'-end fragments, as well as a 333 nucleotide internally derived monomeric fragment M. In addition, the partial products P1 and P2, resulting from cleavage at only the second or first site, respectively, were generated early in the reaction, but were then lost as cleavage neared completion.

As seen with the monomer transcripts, the conserved GAAAC sequence was required for cleavage of the dimer transcripts. This was illustrated when both domains were mutated (pGD1,2M), and cleavage at both sites was completely inhibited. However, cleavage at both sites was restored in either of the single-site mutants (pGD1M and pGD2M). This is evident from the generation of the monomer (M) and both 5'- and 3'-end fragments from transcripts of either of these constructs, and indicates that the critical GAAAC sequence can be provided by either domain for cleavage at either site. We conclude that cleavage can occur with or without inter-domain interactions. An alternative explanation involving inter-molecular interactions between dimer transcripts is unlikely at these low concentrations (0.004 ng/μl).

The relative importance of inter-domain interactions for cleavage can be inferred by comparing cleavage of each individual site in the wildtype and mutant constructs. Fig 5b shows the time course of cleavage of the first site in transcripts from the various constructs. When both sites were wildtype (pGD), the first site cleaved with similar kinetics as the single site in monomeric

pGM1 transcripts (compare to Fig. 3b). Cleavage of the first site in the dimer was inhibited when either domain was mutated, but the inhibition was greater when the first domain itself was mutant (pGD1M). An analysis of cleavage of the second site indicates that it also was most severely affected by a mutation in its own domain (data not shown). While it is not possible from this data to determine the actual contributions of single and inter-domain interactions to the cleavage of wildtype transcripts where both modes of cleavage are available, it is clear that the domain surrounding a cleavage site is more important for cleavage than the opposite domain.

Enhanced cleavage by inter-domain interactions in a permuted transcript

During a deletion analysis of the dimer clone pSP6D6 (see Discussion), a construct was formed that produces transcripts with remarkable self-cleaving abilities. This construct, pG47:331, has 47 satellite 2 nucleotides on the 5' side and 331 satellite 2 nucleotides on the 3' side of the first cleavage site in the parent clone pSP6D6 (Fig. 2). The satellite 2 sequence ends precisely at the second site of cleavage in pSP6D6, and is followed by the EcoRI linker used to clone the deletion. The linker nucleotides apparently restore activity to the second cleavage domain, since transcripts from this construct undergo rapid cleavage at both sites. Figure 6a shows the products of self-cleavage of pG47:331 transcripts at low concentrations. The 55 nucleotide 5'-end and

Figure 7. Potential double-hammerhead structures formed between the cleavage domains of pG47:331 transcripts. a) Potential double-hammerhead structure formed between the first and second domain in pG47:331 transcripts. The lower case letters represent EcoRI linker derived nucleotides on the 3' end of these transcripts. b) Potential partial double-hammerhead structure formed in pG47:331 partial cleavage product P1. P < represents the 2',3' cyclic phosphate generated by self-cleavage.

7 nucleotide 3'-end fragments were run off the gel in order to better resolve the larger cleavage products. Based on the appearance of partial product P2 and excised monomer M, cleavage of the first site was nearly complete within 15 minutes. This rapid rate of cleavage was not seen with transcripts from a series of other clones with the same 5' end but lacking part of the second cleavage domain (data not shown). It therefore seemed possible that the second domain was involved in the rapid cleavage of the first site, and that the particular permutation of this transcript promoted favorable inter-domain interactions.

To investigate this possibility, the GAAAC in either the first, the second, or both cleavage domains was mutated to GAAC. When the first domain was mutated (Fig 6a, pG47:331-1M), neither P1 nor M were produced, indicating that cleavage at the second site was inhibited. Cleavage at the first site, however, was not affected, as illustrated in Figure 6b where the time course of cleavage is plotted for the first site in wildtype and mutant transcripts. These results differ from the results of the dimer analysis, and indicate that with this particular transcript, cleavage of the first site relies almost exclusively on the GAAAC sequence from the second domain. The initial rate of first site cleavage in pG47:331-1M was 20-fold greater than for the same site in the monomer transcripts (Fig. 3b). As alluded to above, these results demonstrate that a domain bearing the GAAC mutation can be very efficiently cleaved by interaction with a wildtype domain.

Further evidence for a strong, inter-domain interaction was obtained by mutating the second domain (Fig. 6a, pG47:331-2M). This mutation reduced cleavage at the first site to less than 5% after 5 hours, but did not inhibit cleavage at the second site, as judged by the formation of product P1. Figure 6c illustrates that cleavage of the second site was actually increased relative to cleavage of the same site in wildtype transcripts. As explained below, this increase is most likely a secondary effect of the inhibition of first site cleavage by this mutation.

Differential ability of partial products to undergo subsequent cleavage

The accumulation of P2 during the cleavage of unmutated pG47:331 (Fig. 6a) suggests that if cleavage occurs initially at the first site, the product P2 is either inert, or cleaves slowly to generate monomer (M). To determine if P2 can undergo subsequent cleavage, radiolabeled P2 fragments were purified

from an acrylamide gel and subjected to self-cleavage conditions. Figure 6d illustrates that even though the P2 fragments have intact second cleavage sites, they are unable to cleave. P2 is therefore not an intermediate in the production of M. This also explains the increase in second site cleavage when the second domain was mutated. The mutation inhibited first site cleavage and the production of P2, and therefore insured that the second site was always available for cleavage.

These results suggest that the monomer M is formed either by rapid, simultaneous cleavage at both sites in the full length transcript, or by using P1 as an intermediate. Figure 6d shows that gel-purified P1 is capable of self-cleavage, and that cleavage is nearly complete in 15 minutes. This rapid rate of cleavage accounts for the lack of accumulation of P1 during cleavage of the wildtype transcripts. Furthermore, the ability P1 to undergo rapid cleavage, even though it lacks part of the second cleavage domain, has important implications concerning progressive processing of multimeric transcripts and will be discussed below.

DISCUSSION

Evidence was presented that newt satellite 2 transcripts can cleave without interactions between cleavage site domains. This evidence includes the ability of monomeric transcripts to cleave independently of concentration, the demonstration that interactions between monomeric transcripts are not favored, and the demonstration that in dimeric transcripts, mutations predominantly affect cleavage within the mutant domain itself. These results contrast with previous studies where small RNAs designed after the cleavage domain of ovarian satellite 2 transcripts depended upon inter-domain interactions for cleavage (9). One possible explanation for this discrepancy is that there are sequence differences in the cleavage domains used in this and the previous study (compare Fig. 1b to 1c). Two of the sequence differences result from differences between the first cleavage site in the genomic clone pSP6D6, from which the clones used in the present analysis were derived, and the satellite 2 transcripts sequenced in total ovary RNA (denoted by ov subscripts in Fig. 1b). These differences are possible because the ovarian transcripts are believed to be transcribed from a subset of genomic satellite 2 DNA repeats not represented by pSP6D6. However, these nucleotide substitutions do not account for the ability of the monomeric transcripts to cleave at low concentrations. By *in vitro* mutagenesis, we substituted the ovarian nucleotides at these two positions into the monomer clone pGM1, and transcripts from this modified clone cleaved comparably to pGM1 transcripts at low concentrations (Y.Zhang and L.M.Epstein, unpublished results).

Alternatively, the ability of satellite 2 transcripts to cleave using a single cleavage site domain may be dependent upon satellite 2 sequences external to the conserved 'hammerhead' domain. Support for this has been obtained from studies of truncated transcripts derived from *in vitro* deletions of pSP6D6. Transcripts consisting only of the 'hammerhead' domain did not cleave at low concentrations, but 11 additional satellite 2 nucleotides on the 5' end, and 33 on the 3' end, restored this ability (L.M.Pabón-Peña and L.M.Epstein, unpublished results). The additional nucleotides may help stabilize a hammerhead structure, or may result in an entirely different structure active in self-cleavage. At least four RNAs use self-cleaving structural motifs other than the hammerhead (12-17), and it is likely that further self-cleaving motifs will be found.

While monomeric and dimeric satellite 2 transcripts actively cleave using single domain structures, pG47:331 transcripts utilize inter-domain interactions to cleave at an enhanced rate. The two cleavage domains in these transcripts might associate to form a structure analogous to the previously proposed 'double-hammerhead' (Fig. 7a), which either forms more readily, or cleaves more efficiently, than the single domain structure. It is interesting to note that due to nucleotide substitutions between the ovarian satellite 2 transcripts and the two repeats in the original dimer clone pSP6D6, the upper and lower stem IIs of this potential double-hammerhead structure are missing one and two highly conserved base pairs (3), respectively. These modifications do not necessarily make the double-hammerhead an unlikely candidate for the cleavage of pG47:331 transcripts, however, since as pointed out by Sheldon and Symons (18), the hammerhead is a very flexible structure, and the absolute requirements for an active hammerhead have not yet been determined.

Cleavage at the first site in pG47:331 transcripts results in a partial product, P2, in which the second cleavage domain is intact, but which is nevertheless incapable of cleavage. Either the partial product folds into a conformation lacking a single-domain structure, or the nucleotide substitutions in the second domain completely inhibit cleavage by the single domain structure. It will be interesting to determine if these same substitutions, which do not inhibit cleavage by inter-domain interactions in the intact, pG47:331 transcripts, affect cleavage by the single-domain structure in pGM1 monomeric transcripts. Mutations which differentially inhibit cleavage by single and inter-domain structures would be strong evidence for fundamental differences in the nature of these structures.

If cleavage of pG47:331 transcripts initial occurs at the second site, partial product P1 is formed, which unlike P2, is capable of rapid cleavage at its remaining cleavage site. Cleavage of P1 at its intact first site must involve an inter-domain interaction, since P1 accumulates when the second domain is mutated, but not when the first domain itself is mutated (Fig. 6a). We propose that P1 cleaves using a 'partial double-hammerhead' structure formed between the first cleavage domain and the free, 3' end formed by cleavage (Fig. 7b). A similar structure was proposed to account for an increase in the rate of cleavage after an initial lag during the reaction of synthetic RNAs corresponding to newt-like hammerhead domains (19). This is the first instance, however, where such an interaction has been implicated in the cleavage of intact transcripts to generate monomeric units.

It is not known how these *in vitro* cleavage reactions relate to the formation of the repeat-length satellite 2 transcripts found in the newt. From these and other studies (5,19–22), it is clear that the same primary sequence, or permutations of that sequence, can display alternate conformations, which in turn determine how, and if, cleavage will occur. The permutation of the primary, *in vivo* satellite 2 transcript is not known. Furthermore, alternative strategies not displayed by these studies must be available during the formation of the satellite 2 transcripts found in the newt ovary, which have ends which differ from those formed by the *in vitro* cleavage reaction (2). It is possible that in this tissue, cellular factors modify how and where cleavage occurs.

For tissues such as the liver, however, where satellite 2 transcripts have the same ends as formed by *in vitro* cleavage, a pathway for the processing of primary transcripts can be envisioned. The multimeric precursor can cleave at a slow rate at any of its internal cleavage sites using either the proposed single

hammerhead or a modified single-domain structure. Once cleavage occurs at an internal site, a reactive end is formed which promotes cleavage at upstream sites at a fast rate by the formation of partial double-hammerheads. This model can be extended to explain the persistence of multimeric transcripts in tissues such as the liver. It is possible that dimers or larger multimers in which both ends were formed by cleavage fold into structures which do not promote further cleavage. This and other aspects of the model can be tested with the use of appropriately configured clones of satellite 2 DNA.

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