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Facilitator oligonucleotides increase ribozyme RNA binding to full-length RNA substrates in vitro

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Abstract **Primer extension arrest (PEA) studies have demonstrated that antisense oligonucleotides (β112C, β114C), which lie upstream of a ribozyme targeted to β-amyloid peptide precursor (βAPP) mRNA, but not sense oligonucleotides (β112S, β116S) or a scrambled oligonucleotide, β116M, affect ribozyme-mediated cleavage in vitro. Substrate dissociation experiments revealed that the ribozyme binding site in this mRNA was masked; PEA kinetics showed the association of the ribozyme and substrate was enhanced by antisense oligonucleotide binding. These studies suggest that masked ribozyme cleavage sites that may occur in disease-causing mRNAs can be targeted for degradation using "facilitator" oligonucleotides.**

Key words: Ribozyme; Facilitator oligonucleotide; βAPP mRNA

1. Introduction

Catalytic RNAs have been found in a large number of diverse organisms. Examples have been found in plants, animals, eubacteria, viruses and fungi [1–5]. Minimal catalytic motifs have been deduced for each of the seven types of known catalytic RNA, and *trans*-cleaving variants based on these motifs have been constructed [6–8]. Such molecules may potentially be useful therapeutically as antiviral agents or in attenuating or repairing mutant cellular messages that cause genetic diseases [9,10].

Subtle primary, secondary and tertiary structure interactions often lead to large differences in the cleavage rates of a particular catalytic motif [2,11–13]. In particular, we and others have shown that the hammerhead ribozyme cleavage rate is often substantially less for full-length substrate RNAs than for shorter substrates [14–16]. In order to generate ribozymes for therapeutic applications it is essential that they cleave full-length messages well. Several methods have been devised to improve ribozyme efficiencies. One such strategy, oligonucleotide-mediated or facilitated ribozyme cleavage, has been shown to enhance ribozyme catalysis and promote ribozyme cleavage at lower concentrations of magnesium in vitro [15–17]. The studies presented in this paper address how oligonucleotides facilitate ribozyme-mediated degradation of full-length messages in vitro.

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Abbreviations: βAPP, Alzheimer amyloid peptide precursor; βAPP_{695} or βAPP_{751}, alternatively spliced variants of βAPP mRNA; PEA, primer extension arrest

2. Materials and methods

2.1. Synthesis of oligonucleotides

Sense, antisense and scrambled oligodeoxynucleotides were synthesized by cyanoethyl phosphoramidite chemistry on an Applied Biosystems 380B DNA synthesizer [18]. Following cleavage from the solid support, samples were ethanol precipitated, washed with 70% ethanol and resuspended in sterile water. Aliquots were labeled with [γ^{32}P]ATP [19] and resolved on denaturing polyacrylamide gels to check for homogeneity.

2.2. Substrate dissociation kinetics

Ribozyme cleavage reactions were performed as described previously [20–22]. Substrate dissociation rates were measured by partitioning experiments [23] with minor modifications. Briefly, separate solutions of ribozyme core RNA, ^{32}P-radiolabeled substrate RNA and unlabeled substrate RNA were heat denatured at 95°C for 1 min and then preincubated at 37°C in 10 mM $MgCl_2$. In a typical experiment, two identical reaction mixtures were prepared containing ^{32}P-radiolabeled substrate RNA and a fifteen-fold excess of ribozyme core RNA. This ensured 10–20% substrate cleavage occurred. Following this, a ten-fold excess of unlabeled substrate RNA over ribozyme core RNA was added to one of the reaction mixtures and the incubation was continued. At the indicated times, 5-μl aliquots were withdrawn and the reaction stopped by adding 2.5 μl of 10 M urea. The resulting mixtures were quick frozen on dry-ice until they were used. RNA cleavage products were separated from unreacted substrate RNA by electrophoresis through 10% polyacrylamide/7M urea gels. Detection by autoradiography and quantification by scanning densitometry were performed as previously described [19]. No ^{32}P-radiolabeled substrate cleavage was detected in control reactions in which it was pre-diluted with the same excess of unlabeled substrate RNA that was used in the chase reaction. Values for k_{-1} were determined from the difference between the slopes of ln(FracS^*) versus time plots of cleavage in the presence or absence of competitor substrate RNA [24].

2.3. Primer extension arrest

Ribozyme cleavage reactions with full-length RNA substrates were performed as described previously [15]. Briefly, 100 nM of βAPP_{695} or βAPP_{751} mRNA and 400 nM of active or inactive ribozyme core RNAs were separately heat denatured at 95°C for 1 min and then preincubated at 37°C in 10 mM $MgCl_2$ for 5 min. Reactions, in a total volume of 12 μl, were initiated by combining the substrate, core ribozyme RNA solutions and, where appropriate, facilitator oligonucleotides together. Incubation was allowed to continue at 37°C. Four-microliter aliquots of the reaction mixtures were withdrawn at various times subsequently and quenched by ethanol precipitation in the presence of 2 μg of glycogen carrier. The resulting populations of RNAs and oligonucleotides were recovered by centrifugation; the pellets were washed with 70% ice-cold ethanol and then redissolved in 6.2 μl of H_2O.

Primer extension arrest (PEA) [25,26] of cleaved or uncleaved full-length βAPP_{695} or βAPP_{751} mRNA in the presence or absence of 10 μM facilitator oligonucleotides was performed as described previously [15]. Two picomoles of primer was incubated with each set of ribozyme reaction samples in 8 μl of 9 mM Tris-HCl pH 7.0, 63 mM NaCl for 1 min at 90°C. After slow cooling at room temperature for 10 min, 2 μl of 30 mM $MgCl_2$, 25 mM DTT and 3 μCi [α^{32}P]dCTP (3000 Ci/mmol) was added. The resultant hybridization mixture in 2.75 μl aliquots was added to 2.5 μl extension buffer (34 mM Tris-HCl pH 7.0, 50 mM NaCl, 5 mM DTT, 250 μM dATP, 250 μM dGTP, 250 μM dTTP and 10 μM dCTP). The mixture was incubated

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for 30 min at 42°C with 1.25 units of AMV reverse transcriptase. Subsequently, 1 μl of a chase solution (2 mM dATP, 2 mM dGTP, 2 mM dTTP, 2 mM dCTP in extension buffer) was added and incubation continued for an additional 15 min at 42°C. Reactions were stopped by adding 5 μl of 10 M urea, 0.3% xylene cyanole, 0.3% bromphenol blue; the resulting samples were stored at −100°C until they were used. PEA products were resolved on 10% polyacrylamide (19:1 polyacrylamide/bisacrylamide) 7M urea sequencing gels and quantified by scanning densitometry [23].

3. Results

3.1. The βAPP-133 ribozyme binding site is masked by substrate secondary structure

We have previously shown that *trans*-acting hammerhead ribozymes targeted to the first naturally occurring cleavage site in βAPP mRNA, codon 47, cleave both full-length and short forms of βAPP RNA in vitro, and specifically reduce steady-state βAPP levels in vivo [13,15,20–22,27]. However, the rate of full-length RNA substrate cleavage was 25-fold less than that observed with kinetically well behaved short model RNA substrates [15,23]. Preliminary evidence suggested that this reduction might be caused by higher order RNA effects. To determine whether association of the core ribozyme RNA and $βAPP_{695}$ or $βAPP_{751}$ mRNA was hindered, the rate of substrate dissociation of the β133 *trans*-acting ribozyme, Fig. 1, was examined by pulse-chase experiments using short and full length substrate competitor RNAs. Fig. 2 shows the results of a typical experiment. It is quite clear from these data that excess unlabeled β133 substrate RNA completely inhibited ^{32}P-radiolabeled β133 substrate cleavage. This indicates that substrate dissociation for this short RNA is fast relative to the cleavage step. In contrast, excess full-length $βAPP_{751}$ RNA was unable to effectively compete ^{32}P-radiolabeled substrate cleavage. This result suggests that binding of the ^{32}P-radiolabeled β133 substrate to the core ribozyme is much stronger than that of the full-length substrate. Since both the short and full-length substrate RNAs contain identical ribozyme binding sites, the inability of the full-length form to compete ^{32}P-radiolabeled β133 substrate RNA supports the hypothesis that the site is masked by higher order structure.

3.2. Mechanism of facilitator action in vitro

Primer extension arrest (PEA) studies have shown that short "facilitator" antisense deoxyoligonucleotides targeted upstream of the ribozyme binding site, Table 1, enhanced the rate of ribozyme-mediated cleavage of full-length $βAPP_{751}$ mRNA but had no effect upon the cleavage of model substrate RNAs in vitro. The magnitude of this enhancement depended on both the length of the deoxyoligonucleotide and its position with respect to the 3′ end of the ribozyme [15]. We now demonstrate that facilitator oligonucleotides elicit their effect on *trans*-ribozyme cleavage by enhancing ribozyme core RNA binding to its target sequence.

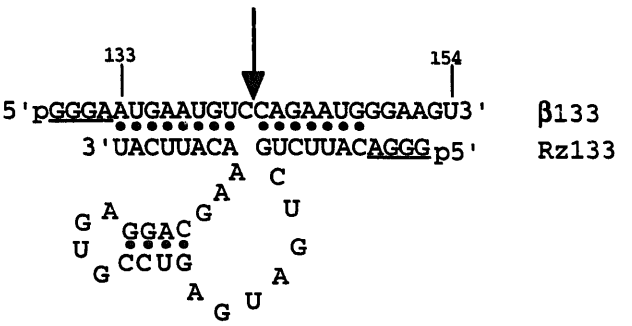

Fig. 1. β133 *trans*-acting hammerhead ribozyme composed of RNA substrate strands β133, or $βAPP_{751}$ (not shown) and core ribozyme RNA, Rz133. Numbering is based upon the $βAPP_{695}$ cDNA sequence [33]. The arrow marks the site of substrate cleavage.

PEA of full-length $βAPP_{751}$ or $βAPP_{695}$ mRNA ribozyme cleavage reactions, in the presence and absence of facilitator oligonucleotides, was used to determine this. PEA relies on the fact that reverse transcription can be paused or stopped in structured regions of RNA, or at modified nucleotides, or at sites of RNA cleavage [[15] and references therein]. In the population of cleaved and uncleaved forms of βAPP mRNA that exist in a typical ribozyme cleavage reaction a number of specific PEA products have been observed. Primer extension to the ribozyme cleavage site results in a 60 base labeled cDNA. If the sample contains uncleaved βAPP mRNA, a 225 base labeled cDNA corresponding to extension to the end of the message is also observed [15]. In addition, a 74 base labeled cDNA arising from extension to the 5′ end of the facilitator oligonucleotide might be expected in reactions containing uncleaved βAPP mRNA, providing the facilitator oligonucleotide binds tightly enough at the extension temperature (42°C). An experiment confirming this prediction is shown in Fig. 3A. At both 1 and 2 h, in the presence of 10 μM β112C or β114C, specific arrests for the full-length message (not shown), the ribozyme cleaved message, and the facilitator 5′ end are observed (lanes 3 and 4); the latter arrest does not occur in the absence of the facilitators (lanes 1 and 2). In parallel experiments using a mutationally inactive ribozyme RNA [15,20,22], β112C and β114C were also found to produce full-length and facilitator-arrested cDNAs but, as expected, not the ribozyme cleavage site-arrested cDNA (not shown). The specificity of facilitator binding was further demonstrated in the experiment shown in Fig. 3B comparing

Table 1
Facilitator oligonucleotides

Facilitator[a]	Sequence	Target region[b]
β112C	5′ACACCGTCTGACTTGTA3′	112–128
β114C	5′ACCGTCTGACTTGTA3′	114–128
β116M	5′CGAGACTGATGTA3′	116–128
β116S	5′GCAGACTGAACAT3′	116–128
β112S	5′TGTGGCAGACTGAACAT3′	112–128
β121C	5′GACTTGTACGTGT3′	121–133

[a]Oligonucleotide nomenclature: βXC = antisense facilitator oligonucleotide; βXS = sense strand oligonucleotide; βXM = scrambled oligonucleotide. X refers to the position of the oligonucleotide relative to the 5′ end of βAPP mRNA.
[b]Numbering based on the start of the βAPP mRNA coding sequence.

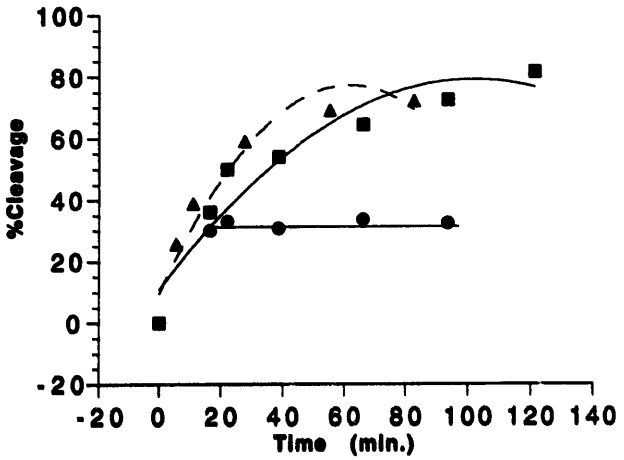

Fig. 2. β133 substrate dissociation kinetics. ^{32}P-radiolabeled β133 RNA's ability to dissociate from Rz-133 RNA was analyzed by competition with excess unlabeled β133 RNA (●), or unlabeled full length βAPP_{751} RNA (■) [15]. For comparison, a reaction in the absence of competitor RNA (▲) is also shown.

PEA products in the presence of β112C and β121C. Here, the 5 base difference between facilitator binding sites is clearly observed. Additional arrests corresponding to extension from each facilitator oligonucleotide to the end of uncleaved βAPP mRNA are also observed.

A 49 base cDNA was also observed in the presence of either β112C or β114C, and ribozyme core RNA (or inactive ribozyme core RNA) Fig. 3A; interestingly, the site of this arrest coincides with the 5′ end of the ribozyme core RNA. Since there are equal amounts of ribozyme RNA in reactions with and without the facilitator, the presence of an arrest only in reactions containing the ribozyme and the facilitators indicate that the facilitators are linked to its production. The simplest explanation of this is that facilitator binding to uncleaved βAPP mRNA enhances ribozyme RNA binding during primer extension. This hypothesis is supported by the observation that the intensities of the 49 base ribozyme-arrested cDNA are directly proportional to the intensities of the 5′ facilitator arrested cDNA over this time period (compare lanes 3 and 4). In addition, β121C which enhanced βAPP mRNA cleavage 3-fold less than β112C under these conditions also produced proportionately less of the 49 base cDNA, Fig. 3C. Finally, neither a sense cognate of β112C, β112S, or another sense oligonucleotide β116S, or a scrambled oligonucleotide control, β116M, that should hybridize poorly to βAPP mRNA under PEA conditions produced 5′ facilitator or 5′ ribozyme-arrested cDNAs, Table 2.

Another facilitator-enhanced arrest was also observed in Fig. 3A, lanes 3 and 4. This arrest occurs at G_{144} in βAPP mRNA and, like the arrest at the ribozyme 5′ end, its intensity depends on the length of the facilitator used. The relatively low concentration of radiolabeled dCTP in the PEA assay results in hypersensitive G-pausing [25]. Nonetheless, the G_{144} arrest was significantly enhanced in the presence of the facilitator compared to G_{148}, G_{149} and G_{150}. There are at least two explanations for this phenomenon. First, it is possible that G_{144} represents an alternative ribozyme cleavage site. While this is not without precedent [28], its existence was not detected in model reactions where other novel cleavage sites were exposed [29]. A more likely scenario for the origin of this arrest is that it too arises from βAPP mRNAs that still have the ribozyme bound. Since arrests are comprised of both kinetic pauses as well as absolute stops, it is plausible to believe that AMV reverse transcriptase might pause at this particular G located near the end of the 7 base ribozyme helix III. In contrast, unpaired G_{149} and G_{150}, and G_{148}, near the beginning of ribozyme helix III, should be less susceptible to reverse transcriptase pausing. Thus, these data also support the hypothesis that facilitator oligonucleotides enhance the association of the ribozyme with βAPP mRNA. We conclude therefore, that facilitator oligonucleotides enhance ribozyme-mediated full-length RNA cleavage in vitro by unmasking the ribozyme binding site.

4. Discussion

For therapeutic applications, *trans*-acting ribozymes must associate with a specific target mRNA to form a cleavage-competent structure. Higher order RNA interactions can have deleterious effects on the rate of ribozyme cleavage. Indeed, both viroids and virusoids have been shown to regulate *cis*-cleavage in this manner. For example, the transition between the native and autocatalytic conformers of peach latent mosaic viroid (PLMVd) is the rate-limiting step in ribozyme catalysis [30]. Similarly, additional sequences in helix II of the satellite RNA of barley yellow dwarf virus (sBYDV) form a thermodynamically favored pseudoknot that inhibits self-cleavage [12]. Mutations that disrupt the pseudoknot increase

Table 2
PEA intensities in the presence and absence of 10 μM facilitator oligonucleotides

Facilitator	Size[b]	Homology[b]	Relative PEA intensity[a]		
			3′ Facilitator	Cleavage site	5′ Ribozyme
—	NA[c]	NA	0	0.12	0
β112C	17	17/17	1	0.46	0.12
β114C	15	15/15	0.36	0.28	0.05
β116M	13	7/13	0	0.18	0
β116S	13	9/13	0	0.13	0
β112S	17	10/17	0	0.14	0

[a]PEA intensities were measured following 2 h cleavage reactions of full-length βAPP_{695} mRNA. The intensity of the β112C 5′ facilitator arrest was arbitrarily set to 1.0; all other values were normalized accordingly. The results are the mean values of 2–3 experiments for each oligonucleotide.

[b]Homology to βAPP_{695} or βAPP_{751} mRNA was determined using the FASTA algorithm from the Genetics Computer Group (GCG Inc., Madison, WI).

[c]NA, not applicable.

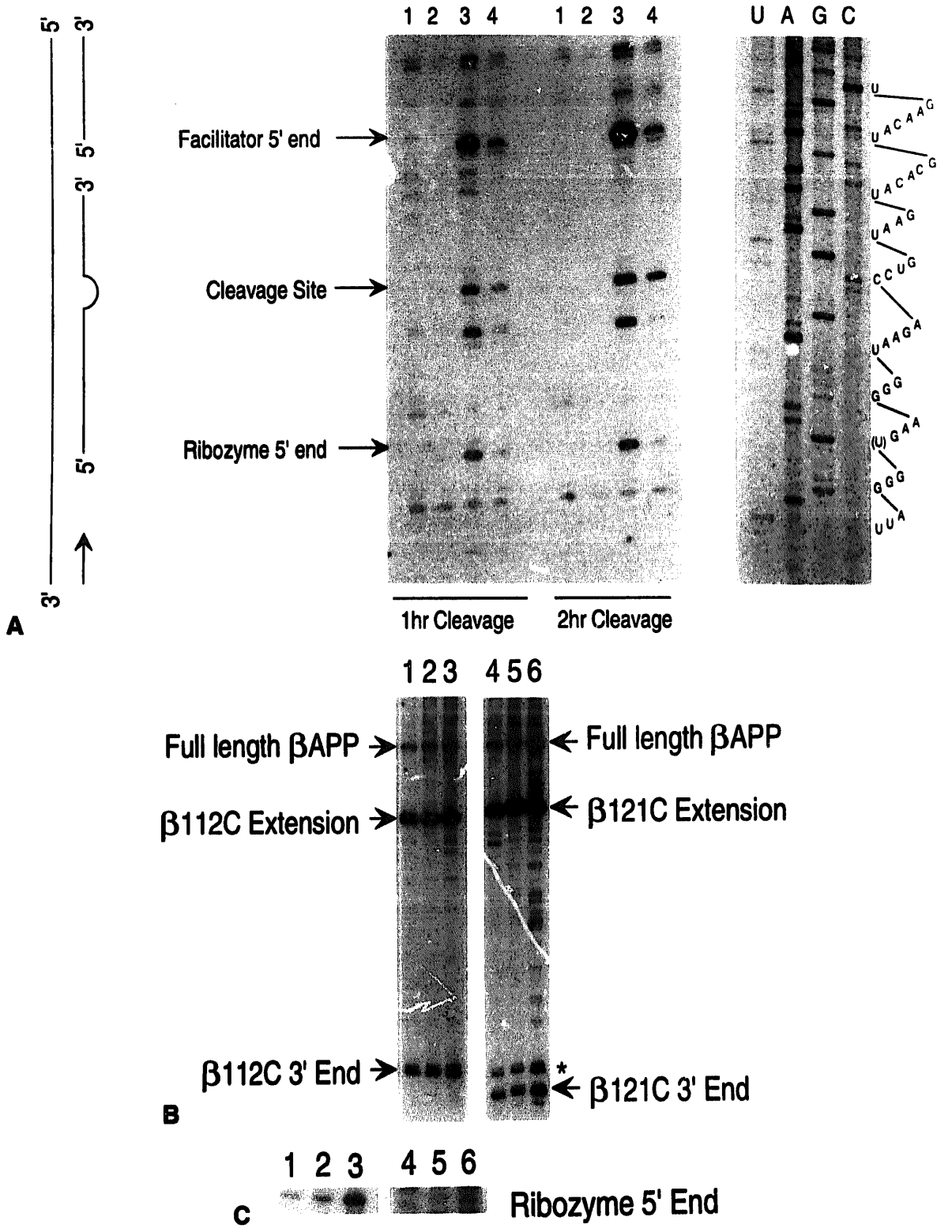

Fig. 3. Primer extension arrest products arising from βAPP_{751} mRNA cleavage reactions. (A) PEA of 1 and 2 h βAPP_{751} mRNA cleavage reactions in the absence of ribozyme (lane 1), in the presence of ribozyme (lane 2), in the presence of ribozyme and 10 μM β112C (lane 3), and in the presence of ribozyme and 10 μM β114C. The βAPP_{751} message, ribozyme core RNA, and facilitator oligonucleotide and their orientations are shown schematically on the left-hand side of the figure. The cDNA arising from reverse transcription is depicted by the arrow. Note that slightly more sample was loaded in the 2 h time point of sample 2 compared to the 1 h time point. (B) PEA of a 30–60 min time course of βAPP_{751} mRNA cleavage in the presence of ribozyme and 10 μM β112C (lanes 1–3) and in the presence of ribozyme and 10 μM β121C (lanes 4–6). Time points taken at 30, 40 and 60 min, respectively, are shown. The doublet bands arising from extension to the 5′ ends of β112C and β121C are due to reverse transcriptase stuttering [25]. *, β121C binding also produces a significant reverse transcriptase arrest within its binding sequence. (C) 5′ Ribozyme binding site region of (B).

self-cleavage several hundred fold but cause the satellite RNA to replicate poorly, indicating that rapid RNA cleavage is not essential for (sBYDV) replication [31].

Separation of ribozymes into *trans*-acting variants containing a substrate strand and an enzymatic core further compounds the effects of higher order RNA structure interactions because in these cases, both RNAs may fold into structures that inhibit self-cleavage [13,23,32]. The masking of a particular ribozyme cleavage site via secondary structure interactions is especially problematic if the site represents an ideal target such as a disease-causing mutation [22]. The data presented in Fig. 2 clearly show that codon 47 in βAPP mRNA is masked by higher order structure; therefore this site can be used as a model to explore strategies for enhancing ribozyme cleavage.

Facilitator deoxyoligonucleotides have been shown to enhance ribozyme cleavage in vitro [15,17]. Theoretically, enhancement could occur during ribozyme/substrate association, ribozyme/product dissociation or the chemical cleavage step. Previous work has delimited these possibilities to either association or dissociation [16]. In this study we employed facilitator oligonucleotides targeted upstream of a ribozyme designed to cleave βAPP mRNA at codon 47. The results, Fig. 3A and Table 2, demonstrate for the first time that facilitator oligonucleotides enhance the ribozyme binding step of the reaction. The ability to affect substrate binding rather than product dissociation should make facilitator oligonucleotides generally useful for targeting potentially interesting ribozyme cleavage sites that are masked by higher order RNA structure in vivo.

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