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Cleavage of full-length βAPP mRNA by hammerhead ribozymes

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ABSTRACT

The sequences surrounding the first 5′GUC3′ in the mRNA encoding the Alzheimer amyloid peptide precursor (βAPP) were used to construct a pair of trans-acting hammerhead ribozymes. Each ribozyme contained the conserved core bases of the hammerhead motif found in the positive strand of satellite RNA of tobacco ringspot virus [(+)sTRSV] and two stems, 7 and 8 bases long, complementary to the target, βAPP mRNA. However, one of the ribozyme cleaving strands was lengthened at its 3′ end to include the early splicing and polyadenylation signal sequences of SV40 viral RNA. This RNA, therefore, more closely mimics transcripts produced by RNA polymerase II from eucaryotic expression vectors *in vivo*. RNA, prepared by run-off transcription of cDNA oligonucleotide or plasmid constructs containing a T7 RNA polymerase promoter was used to characterize several properties of the cleavage reaction. In the presence of both ribozyme cleaving strands magnesium-ion dependent cleavage of a model 26 base βAPP substrate RNA or full-length βAPP-751 mRNA was observed at the hammerhead consensus cleavage site. Neither ribozyme was active against non-message homologs of βAPP mRNA, nor was cleavage detected when point mutations were made in the conserved core sequences. However, the k_{cat}/K_m at 37°C in 10 mM Mg^{+2} of the longer ribozyme was reduced twenty-fold when model and full-length substrates were compared. The use of short deoxyoligonucleotides (13 – 17 mers) that bind upstream of the ribozyme was found to enhance the rate of cleavage of the full-length but not βAPP model substrate RNAs. The rate of enhancement depended on both the length of the deoxyoligonucleotide used as well as its site of binding with respect to the ribozyme. These data demonstrate the utility of ribozymes to cleave target RNAs in a catalytic, site-specific fashion *in vitro*. Direct comparison of the efficiency of different ribozyme constructs and different modulating activities provide an experimental strategy for designing more effective ribozymes for therapeutic purposes.

INTRODUCTION

One of the hallmarks of Alzheimers disease (AD) pathology is the existence of extracellular senile neuritic plaques in certain regions of the brain (1, 2). The major proteinaceous component of isolated plaque cores, the so-called A4 or β-peptide (3), is a proteolytic fragment of a much larger protein, the Alzheimer amyloid peptide precursor (βAPP). Recent studies indicate that the β-peptide is formed at low levels through a normal processing pathway (4, 5). The majority of βAPP in cultured cells however, is not processed into β-peptide.

Several point mutations in the βAPP gene have been found in pedigrees of familial Alzheimers disease (FAD) victims (6, 7, 8) and other brain diseases characterized by β-peptide amyloidosis (9, 10). Since they map to residues adjacent to the amino or carboxyl terminal residues of the β-peptide these mutations may affect the rate of its production by affecting the utilization of processing pathways or the stability of amyloidogenic fragments or by increasing the stability of the precursor mRNA (11). These data are consistent with the hypothesis that overproduction of the β-peptide through either normal or aberrant processing pathways result in the neurodegenerative pathology observed in AD brains. Recently, direct experimental confirmation of this hypothesis has been observed in cultured cells that express a βAPP cDNA bearing the double point mutation found in a Swedish FAD family (12).

The role, if any of βAPP in normal cellular metabolism is not well defined. Based on structural considerations βAPP has been classified as an integral membrane protein (13) that may be involved in cell adhesion or cell-cell interactions (14, 15). Oltersdorf *et al* have shown that the extracellular portion of one of the alternatively spliced variants of βAPP, βAPP-751, is identical to protease nexin-2 (16). Therefore, βAPP may play a role in protease inhibition *in vivo*. This same form was also found to have mitogenic activity toward Swiss 3T3 cells (17).

We are interested in the use of targeted mRNA degradation by trans-acting catalytic ribozymes as a means of assessing protein function and, where applicable, as potential therapeutic reagents. Since its function is unknown and it has been implicated in the etiology of one of the world's burgeoning health care problems βAPP offers an ideal system to test the feasibility of this approach. In this report we examine several parameters involved in the successful use of targeted mRNA degradation reagents, namely

(a) the effect of vector-derived sequences on the ability of the ribozyme to fold into a cleavage-competent structure, (b) the effect(s) of mRNA higher order structure on the ability of a ribozyme to cleave its target and (c) the use of short deoxyoligonucleotides binding to adjacent sites on the target mRNA to facilitate ribozyme cleavage.

METHODS

βAPP ribozyme

The βAPP-141 trans-acting hammerhead ribozyme composed of a substrate RNA strand, βAPP-141 mRNA, and another RNA containing the conserved core residues of the hammerhead motif (18) were prepared by *in vitro* transcription from cDNA or plasmid constructs. The substrate RNAs used in this study were derived either from cDNA model substrate cassettes (19, 20) or from full-length βAPP-751 mRNA (21). Similarly, the RNAs containing the conserved hammerhead core sequences, APPrbz-141$_s$ and APPrbz141$_l$ RNAs, were derived from the plasmid pMAMneo-Ribozyme (19), linearized with either *XhoI* or *BamHI*. APPrbz-Dead$_l$ RNA was transcribed from plasmid pMAMneo-Dead, which was prepared by exchanging the 64 bp wild-type SalI/XhoI cassette of pMAMneo-Ribozyme with a cassette containing mutations in two of the conserved core residues of the ribozyme.

In vitro RNA transcription reactions

RNA was generated by runoff transcription with T7 RNA polymerase in the presence or absence of [α^{32}P]-CTP from cDNA oligonucleotide cassettes, or linearized plasmid DNA (20, 21). The resulting labeled RNAs were purified on 10% acrylamide (19:1), 7M urea gels (22). Following electrophoresis, the gels were fixed and urea removed by incubation in 5% methanol, 5% glacial acetic acid. RNA bands were visualized by autoradiography and eluted from the gel in 500 μl of 0.5 M NH_4OAc, 1 mM EDTA (23). The eluted RNAs were ethanol precipitated and the resulting pellets reconstituted in DEPC treated sterile water. ^{32}P-labeled RNA recoveries were measured by preciptating aliquots of the purified reaction mixture in 5% TCA and determining filter bound radioactivity by liquid scintillation; unlabeled RNAs were quantified spectrophotometrically.

Facilitator deoxyoligonucleotides

Facilitator deoxyoligonucleotides, Figure 1, were prepared on an Applied Systems 380B Synthesizer and purified as previously described (24).

In vitro ribozyme reactions

Labeled βAPP-141 RNA transcripts were annealed with various amounts of labeled APPrbz-141 RNA in 75 mM Tris−HCl pH 7.5, 1.5 mM EDTA, at 95°C for 1 min, snap-cooled on ice and subsequently incubated for 16 hr at 37°C after the addition of 10 mM $MgCl_2$. When facilitator deoxyoligonucleotides were used, they were added prior to the initial 95°C annealing step. Cleavage products were detected by autoradiography and quantitated by scanning densitiometry (21).

Cleavage kinetics

The kinetics of ribozyme cleavage were measured in 10 mM $MgCl_2$ at 37°C under single turnover conditions using 10 nM βAPP-141 substrate mRNA and 10−1600 nM APPrbz-141$_s$ RNA, essentially as described in (20) and 100 nM APP-751 mRNA and 500 nM of the 917 base form of APPrbz-141 RNA, APPrbz-141$_l$ RNA, respectively.

Primer extension arrest analysis

Primer extension of full-length βAPP-751 mRNA, cleaved or uncleaved, was performed as described in (25) except that the limiting nucleotide used here was [α^{32}P]-dCTP (800 Ci/mmol) (Amersham Corp.) instead of [α^{32}P]-dATP. The primer, βAPP-181, a 21 base deoxyoligonucleotide complementary to bases 181−202 of βAPP-751 mRNA (13) was used in all of the extension reactions.

Deoxyoligonucleotide sequencing

Double-stranded DNA sequencing of plasmid pK4 (21) was performed with the Circumvent DNA polymerase sequencing kit (New England Biolabs) using βAPP-181 as a primer.

RESULTS

Description of the ribozyme

The first target sequence (5'GUC↓X3') for a trans-acting hammerhead ribozyme in βAPP mRNA occurs at position 141 (13). We chose the sequences surrounding this site to construct various RNA expression cassettes. RNAs prepared from these cassettes should fold into a cleavage-competent hammerhead ribozyme, Figure 1.

Cleavage of model βAPP mRNA substrates *in vitro*

We have previously assessed the cleavage activity of a 46 base RNA, APPrbz-141$_s$ RNA, containing the conserved core bases of the hammerhead motif interspersed between two short

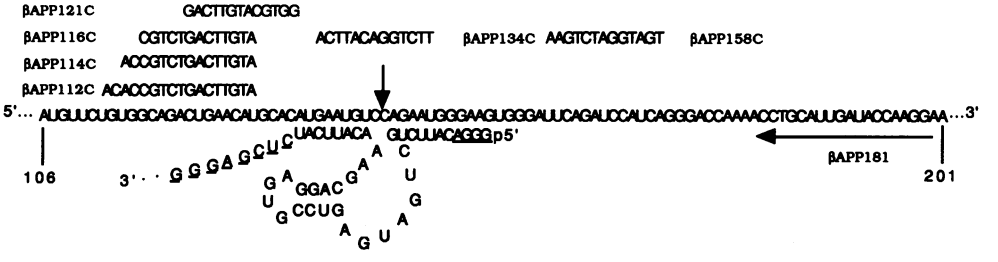

Figure 1. βAPP-141 ribozyme. The βAPP-141 hammerhead ribozyme was formed from bases 133−154 of βAPP-751 mRNA (13) and synthetic APPrbz-141$_l$ RNA containing the conserved bases of the hammerhead structure (bases 1−49 shown). Facilatator deoxyoligonucleotide sequences βAPP112c, βAPP114c, βAPP116c, βAPP121c, βAPP134c, and βAPP158c and their complementarity to βAPP-751 mRNA in the 106−201 region of the message are indicated, as is the position of the deoxyoligonucleotide used in primer extension, βAPP-181 (arrow). Vector derived sequences are underlined.

sequences that are complementary to βAPP mRNA on 26 base, model βAPP mRNA substrates. These studies demonstrated that cleavage of the model RNAs occurred in a metal-ion dependent, site-specific fashion *in vitro* (19). The kinetic parameters of this model reaction, Table 1, are similar to those published for similar systems (26, 27).

The use of eucaryotic expression vectors to deliver trans-acting ribozymes *in vivo* results in modification of the basic ribozyme RNA. Various studies have demonstrated that additional sequences both 5′ and 3′ to the ribozyme RNA motif can confer added stability toward nucleases *in vivo* (28, 29). This is sometimes accomplished by embedding the ribozyme within a known stable highly expressed RNA (30, 31, 32). Often, ribozyme RNAs derived from minigene constructs include variable length viral mRNA sequences at their 3′end. However, added sequences may affect the ability of the ribozyme to fold into a cleavage-competent structure (33, 34, 35). Since we are interested in delivering ribozymes to cells in this fashion, we examined the effect sequences added to the 3′ end of APPrbz-141$_s$ RNA had on the cleavage of model βAPP mRNA substrates in more detail. For these experiments we used a capped *in vitro* transcript derived from *BamHI* cut pMAMneo-Ribozyme (19). This RNA is referred to as APPrbz-141$_l$ RNA. The first 46 bases of this 917 base RNA contains APPrbz-141$_s$ RNA while the remaining 871 bases code for the early splicing and polyadenylation signal sequence of SV40 RNA. Thus, with the exception of a polyA tail, APPrbz-141$_l$ RNA closely mimics that which would be transcribed from the pMAMneo-Ribozyme minigene *in vivo*. Figure 2 shows that APPrbz-141$_l$ RNA (lanes 1 and 2) cleaved the 26 base model βAPP mRNA substrate *in vitro*, but APP-Dead$_l$ RNA in which two of the conserved core bases of the hammerhead motif were mutated was unable to cleave the same substrate (lanes 3 and 4). Furthermore, the ratio k_{cat}/K_m, which is a measure of the relative efficiency of enzymatic activity (36) for this reaction, was virtually identical to APPrbz-141$_s$ RNA, Table 1. In this case then, neither the added trimethyl cap nor the additional 3′ sequences had an effect upon the ability of the ribozyme to form or cleave its target message.

Table 1. βAPP-141 ribozyme kinetic parameters

Substrate[1]	Core[1]	$k_{cat}/K_m(\mu M\text{-}hr)^{-1}$
26	46	72 (10)
26	917	60 (10)
2500	917	4 (0.03)

[1]The βAPP substrates, βAPP-141 (26 bases) and βAPP-751 (2500 bases) RNA and the ribozyme cleaving strands, APPrbz-141$_s$ (46 bases) and APPrbz-141$_l$ (917 bases), containing the core bases of the hammerhead motif were obtained by transcription from cDNA cassettes, plasmid pK4 or plasmid pMAMneo-Ribozyme (19), respectively. The relative efficiency, k_{cat}/K_m, are mean values of three separate determinations. Values in parentheses represent standard deviation. k_{cat} and K_m values determined from plots of the apparent first order rate constant, k_{obs} versus ribozyme concentration (56) were 0.13 min^{-1} and 108 nM respectively for the 26 base substrate and 46 base core RNA.

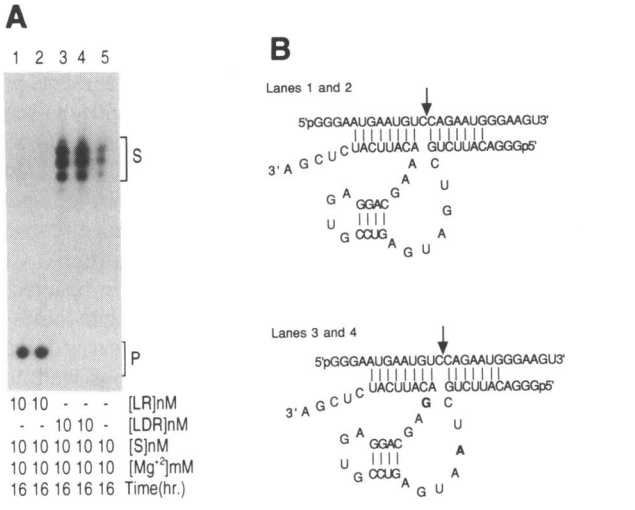

Figure 2. Cleavage of [α^{32}P]-labeled model βAPP mRNA substrates *in vitro*. **A.** Cleavage of the βAPP-141 model substrate (S) comprising bases 133–154 of βAPP-751 mRNA (13) by unlabeled APPrbz-141$_l$ RNA, lanes 1 and 2 or APPrbz-Dead$_l$ RNA, lanes 3 and 4. *In vitro* transcription of the βAPP model substrate cDNA cassette produces three products, a 26 base RNA which coincides with the end of the cassette and two other bands which are one base longer and one base shorter, respectively. The position of the 5′, 13 base cleavage product (P) is marked. Incubations were carried out at 37°C in 10 mM $MgCl_2$ for 16 hr. **B.** Secondary structure models of the hammerhead motif formed by each set of RNAs. Mutations at the conserved positions G14 and A33 are shown in bold-faced type.

Primer extension arrest analysis

To date, much of the published literature concerning the use of hammerhead ribozymes as site-specific mRNA cleavage reagents has involved rather short model substrates (18, 26). In the few instances where comparisons have been made, the cleavage activity of longer or full-length substrates was much less than shorter model substrates (37, 38, 39). We have assessed the

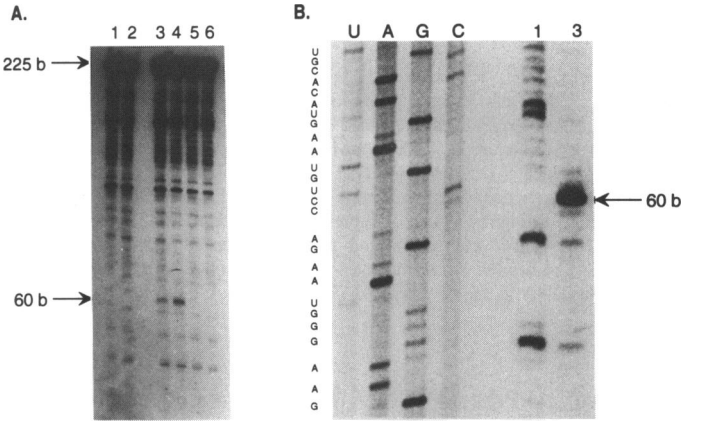

Figure 3. Primer extension arrest analysis of ribozyme cleaved βAPP-751 mRNA. **A.** Cleavage of *in vitro* transcribed, full length βAPP-751 mRNA (100 nM) by APPrbz-141$_l$ RNA (35 nM) (lanes 3 and 4) or mutant APPrbz-Dead$_l$ RNA (35 nM) (lanes 5 and 6) was performed at 37°C in 10 mM $MgCl_2$ for 60 min. with prior heat denaturation (lanes 1, 3, 5) or in the presence of 2 M urea without heat denaturation (lanes 2, 4, 6). Following cleavage the RNAs were isolated by ethanol precipitation. Primer extension of the isolated RNAs (25) was performed with AMV reverse transcriptase (Promega) and a 21 base primer, βAPP-181, targeted 40 bases from the consensus hammerhead ribozyme cleavage site. Lane 1 represents primer extension in the absence of APPrbz-141$_l$ RNA. Full length (225 base) and ribozyme cleaved (60 base) cDNA products are indicated. **B.** Identification of the site of ribozyme cleavage of βAPP-751 mRNA. Primer extension reactions, lanes 1 and 3, were run along with DNA sequencing reactions of plasmid pK4, the vector used to generate βAPP-751 mRNA. βAPP-181 was used to prime both the extension and sequencing reactions. The site of cleavage is indicated.

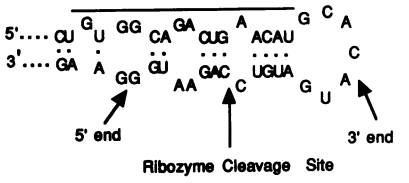

Figure 4. Secondary structure model of βAPP-751 mRNA in the vicinity of the APPrbz-141 RNA binding site. The first 1300 nucleotides of βAPP-751 mRNA was folded into its optimal secondary structure by the program RNAFOLD (Genetics Computer Group, Madison WI). A portion of that model, bases 99–153 is shown here. The 5′ and 3′ ends of the ribozyme binding site as well as the consensus cleavage site are marked by arrows. The deoxyoligonucleotide binding site of βAPP-112c is overlined.

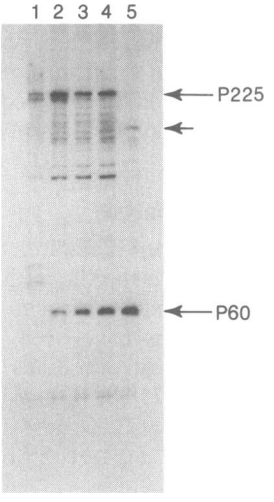

Figure 5. Effect of facilitator deoxyoligonucleotides on ribozyme cleavage. Cleavage of full-length βAPP-751 mRNA (100 nM) by APPrbz-141$_1$ RNA (500 nM) was performed at 37°C in 10 mM $MgCl_2$ for 16 hr. in the absence (lanes 1, 2) or presence of 0.1 μM (lane 3), 1.0 μM (lane 4) or 10 μM (lane 5) of the 13 base facilitator deoxyoligonucleotide βAPP116c. Primer extension arrest was performed as in Figure 3. Lane 1 represents primer extension in the absence of APPrbz-141$_1$ RNA. Full-length and ribozyme cleaved cDNA products are indicated. The shorter arrow indicates the product obtained from extension of βAPP116c DNA to the 5′ end of βAPP-751 mRNA (lane 5).

ability of APPrbz-141$_1$ RNA to cleave a full length form of βAPP mRNA *in vitro*. For these experiments, a 2500 base RNA transcript containing the entire coding region of βAPP-751 as well as 389 bases of the 3′ untranslated region was used (21). Primer extension arrest analysis was used to determine both the location and kinetics of cleavage of the the full-length βAPP-751 mRNA by APPrbz-141$_1$ RNA. This assay has previously been used to identify tRNA/rRNA crosslinking sites (25, 40, 41), the presence and location of modified bases in 16S rRNA (42) as well as chemically modified bases in 16S rRNA (43). In this particular adaptation, a 21 base primer, βAPP-181, placed 3′ to the consensus cleavage site of the hammerhead ribozyme was annealed to βAPP-751 mRNA following ribozyme cleavage, Figure 1. Extension of the annealed primer with AMV reverse transcriptase in the presence of [α^{32}P]-dCTP produces a labeled cDNA copy of the βAPP mRNA population in the reaction mixture. Arrests of the extension reaction occur for three reasons.

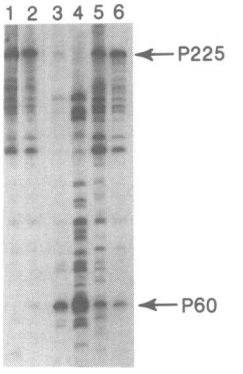

Figure 6. Comparison of the efficacy of facilitator deoxyoligonucleotides of differing lengths and target sequences upon ribozyme cleavage. Full-length βAPP-751 mRNA was cleaved for 60 min. under single-turnover conditions as in Figure 5 in the absence (lanes 1 and 2) or presence of 10 μM of βAPP116c (lane 3), 10 μM βAPP114c (lane 4), 10 μM βAPP134c (lane 5) or 10 μM βAPP158c (lane 6). Primer extension arrest was performed as in Figure 3. Lane 1 represents primer extension in the absence of APPrbz-141$_1$ RNA. Full length and ribozyme cleaved cDNA products are indicated.

First, arrest(s) occur at breaks in the RNA template. These may be caused by either specific cleavage or RNA degradation. Therefore, extension of a population of βAPP mRNAs, some of which are site-specifically cleaved at the hammerhead consensus cleavage site, will result in the production a 60 base cDNA that is absent from uncleaved control samples, Figure 1. By contrast, extension to the 5′ end of the uncleaved message will produce a 225 base cDNA. Second, the use of a limiting amount of labeled deoxynucleotide triphosphate causes AMV reverse transcriptase to pause at the point of its incorporation (42, 44). The paused transcriptase/template complex can have two possible fates. It can find the labeled nucleotide and continue the extension or it can fall off the RNA template, thereby generating a fragment of known length. These fragments produce a defined background of bands that correlate with the sequence of the RNA. Finally, transcriptase pausing can also occur in highly structured regions of RNA (43). Again, the outcome of the paused extension is the formation of background bands. The results obtained when a 60 min. ribozyme cleavage reaction was subjected to primer extension are shown in Figure 3A. Clearly, addition of APPrbz-141$_1$ RNA prior to extension resulted in the formation of a unique band ~60 bases in length (lanes 3 and 4). This band was absent from mock reactions carried out in the absence of APPrbz-141$_1$ RNA (lanes 1 and 2) as well as those in which mutant, APPrbz-Dead$_1$ RNA was added (lanes 5 and 6). Confirmation that this additional band was due to cleavage at the hammerhead consensus cleavage site is shown in Figure 3B. It is clear from the nucleotide sequence of a βAPP-751 expression plasmid primed with βAPP-181, that the position of the unique, primer arrested band coincides with the hammerhead consensus cleavage site. Sequencing analysis also confirmed the identity of the full length, 225 base, product (not shown). These data demonstrate conclusively that full-length βAPP-751 mRNA can be cleaved by a ribozyme RNA that closely mimics one transcribed *in vivo*.

Since the intensity of primer arrested bands can be reliably estimated for distances of 200 nucleotides from the site of priming (43), we used this assay to measure the kinetics of ribozyme cleavage under single turnover conditions. The results of these

Table 2. Effect of facilitator deoxyoligonucleotides on cleavage of βAPP-751 mRNA and βAPP-141 model substrate RNA by a hammerhead ribozyme

Oligo	Length[b]	T_m (°C)[a]	mRNA Region[b]	k (hr^{-1})[c] for βAPP-751	k′ (hr^{-1})[d] for βAPP-141
–	–	–	–	–	5.1
–	–	–	–	0.19	–
112C	17	58	112–128	2.77	ND
114C	15	51	114–128	0.90	ND
116C	13	37	116–128	0.42	5.1
121C	13	43	121–133	0.86	ND
134C	13	39	134–146	0.23	5.1
158C	13	38	158–162	0.20	ND

[a]T_m calculation based on Freier *et al* (57).
[b]Sequence to which βAPP mRNA facilitator deoxyoligonucleotide hybridizes. Sequence numbering according to Kang *et al* (13).
[c]The apparent first order rate constant for cleavage of βAPP-751 mRNA by APPrbz-141_1 RNA was determined under single turnover conditions from semilogarithmic plots of the fraction of substrate remaining, Frac(S_t)*, vs. time as: $k=0.693/t_{1/2}$ in the absence or presence of 10 μM of each facilitator deoxyoligonucleotide.
[d]The apparent first order rate constant for cleavage of βAPP-141 RNA by APPrbz-141_1 RNA was determined under single turnover conditions from semilogarithmic plots of Frac(S_t)* vs. time as: $k'=0.693/t_{1/2}$ in the absence or presence of 10 μM of each facilitator deoxyoligonucleotide.

experiments, shown in Table 1., reveal that the cleavage reaction is approximately twenty-times slower than that observed with βAPP-141 model RNA substrates under the same conditions. One explanation for the decrease in activity is due to the presence of added secondary or tertiary structure in the full-length substrate. Two lines of evidence suggest that this indeed is the case. First, computer-generated secondary structure models of the first 1300 bases of βAPP-751 mRNA show that the putative ribozyme binding site is in a structured region of the RNA, Figure 4. Secondly, secondary structure effects are also hinted at in the results in Figure 3A which show that the extent of ribozyme cleavage in reactions carried out in the presence of 2 M urea (lane 4) are increased two-fold over those performed in the absence of urea (lane 3). Previous studies (45) have shown that added denaturants can effect an increase in the extent of ribozyme cleavage, presumably by decreasing RNA secondary structure interactions (33, 46).

Effect(s) of facilitator deoxyoligonucleotides on βAPP mRNA cleavage

Combinations of short oligonucleotides with contiguous binding sites have been shown to act synergistically in hybrid-arrest translation assays and in binding of triple helix DNAs to their targets (47, 48). Recently, enhanced catalytic activity of a hammerhead riboyzme targeted to an HIV-1 model mRNA was observed in the presence of deoxyoligonucleotides that were contiguous with the 5′ end of ribozyme RNA (49). We have investigated whether short deoxyoligonucleotides targeted upstream of the APPrbz-141 RNA binding site could enhance the cleavage of full-length βAPP-751 mRNA. For our initial studies, a set of three 13 base deoxyoligonucleotides, βAPP116c, βAPP134c and βAPP158c that bind two nucleotides 5′ to APPrbz-141_1 RNA, coincident with APPrbz-141_1 RNA or ten nucleotides 3′ to APPrbz-141_1 RNA, respectively, were synthesized. Of these, only βAPP116c was found to have any effect upon APPrbz-141_1 RNA cleavage of βAPP-751 mRNA. βAPP134c which might have been expected to inhibit APPrbz-141_1 RNA cleavage of both βAPP-751 mRNA and βAPP-141 RNA by competition for the substrate had no effect, Table 2. Presumably this is due to the difference in the ability of a 13 base deoxyoligonucleotide versus a 15 base RNA to bind

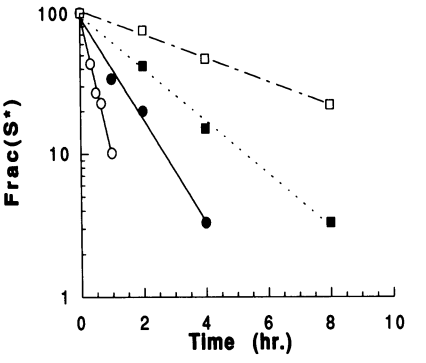

Figure 7. The effect of facilitator deoxyoligonucleotides on the rate of ribozyme cleavage. βAPP-751 mRNA cleavage reactions were carried out as in Figure 5 and 6., in the absence (open squares) or presence of 10 μM of βAPP116c (filled squares), βAPP114c (filled circles) or βAPP112c (open circles). At the times indicated, aliquots of the reaction mixture were removed and immediately ethanol precipitated (25). Primer extension arrest was performed on RNA isolated at each of these times as in Figure 3. Autoradiograms of the reaction kinetics were quantitated by scanning densitometry. The fraction of substrate remaining, Frac (S_t)*, was plotted as a function of time.

to the substrate RNA. As expected, βAPP116c had no effect upon APPrbz-141_1 RNA cleavage of βAPP-141 substrate RNA (Table 2).

Formation of the 60 base cDNA, which corresponds to the ribozyme cleavage product, was enhanced in a concentration dependent fashion by βAPP116c, Figure 5. The magnitude of this enhancement was determined by measuring the rate of βAPP-751 mRNA cleavage by APPrbz-141_1 RNA in the presence and absence of 10 μM βAPP116c. The results show that βAPP116c increased the apparent first order rate constant of the reaction by a factor of 2.2, Table 2 and Figure 7. However, despite the enhancement, the rate of cleavage of full-length βAPP-751 mRNA cleavage by APPrbz-141_1 RNA was more than ten-fold slower than cleavage of the 26 base model βAPP-141 substrate RNA.

Each of the 13 base deoxyoligonucleotides used to enhance the ribozyme cleavage reaction melting temperatures are near

that used in the βAPP-751 mRNA cleavage reactions, Table 2. In order to determine whether increasing the deoxyoligonucleotide melting temperature (T_m) would increase the enhancement of βAPP-751 mRNA cleavage, two additional deoxyoligonucleotides, βAPP114c and βAPP112c, which differ from βAPP116c by the addition of two or four bases at their 5′ ends, were synthesized, Figure 1. The added bases increase the T_m of the DNA:mRNA hybrids by 14°C and 21°C, respectively. As shown in Figure 6., both deoxyoligonucleotides enhance the rate of βAPP-751 mRNA cleavage. The rate of cleavage in the presence of 10 μM of each deoxyoligonucleotide, Figure 7, shows that the observed enhancement is dependent upon the length of the deoxyoligonucleotide used. Furthermore, the apparent first order rate constant for the reaction in the presence of βAPP112c increased ten-fold to nearly that obtained for the 26 base βAPP-141 model substrate reactions. Finally, we ascertained that base stacking interactions between the deoxyoligonucleotide and ribozyme synergistically facilitate βAPP mRNA cleavage. This was accomplished by comparing the enhancement of a 13 base deoxyoligonucleotide, βAPP121c, which abuts the 3′ end of the helix formed between the ribozyme and βAPP mRNA, and βAPP116c, which is four bases removed from this helix, Figure 1., Table 2. It is evident from these data that the additional stability due to base-stacking (49) results in a two-fold increase in the rate of enhancement in βAPP mRNA cleavage.

DISCUSSION

We have demonstrated that a *trans*-acting ribozyme designed to mimic a transcript generated *in vivo* from a eucaryotic expression vector and targeted to sequences in exon 2 of the βAPP gene was able to cleave both small model RNA substrates as well as a full-length form of the βAPP message *in vitro*. In both cases, the cleavage reaction was site-specific, occurring at the consensus cleavage site predicted for hammerhead ribozymes, Figure 3B. The relative efficiency of cleavage, k_{cat}/K_m, was unaffected by additional 3′ non-ribozyme sequences when small model RNA substrates 26 bases in length were compared, Table 1. However, the cleavage efficiency of a full length form of the βAPP message was approximately twenty-fold less than the model substrate, Tables 1 and 2.

The existence of slow cleaving RNA conformers is a well documented phenomenon. Uhlenbeck *et al*, for example, have shown that alternative RNA conformers dramatically affect both the trans-acting form of the *Tetrahymena* ribozyme (50) as well as smaller forms of the hammerhead ribozyme (26). Likewise, Silver *et al* have postulated that alternative RNA conformations in the satellite RNA of barley yellow dwarf virus may, in fact, function as a molecular switch to regulate the cleavage reaction (34). Finally, Pace *et al* have recently suggested that multiple viroid RNA conformers observed under *in vivo*-like conditions may be ideal targets for protein-RNA regulating interactions (51). Two lines of evidence suggest that the decreased cleavage efficiency of the full length form of the βAPP message may be due to the formation of a cleavage resistant RNA conformation. First, computer generated secondary structure modelling of βAPP mRNA (nucleotides 1−1300) predicted the ribozyme target sequence to be base-paired, Figure 4. Base-pairing may limit the accessibility of the ribozyme to its target site. Recently, Huillier *et al* demonstrated that ribozymes targeted to open stem loops within the α lactalbumin mRNA were more effective in reducing the level of message *in vivo* than one targeted to a base-paired region near the initiation codon (52). Similarly, Heidenreich *et al* have postulated that the observed decrease in k_{cat}/K_m of ribozymes targeted to HIV-1 RNA that accompany increases in substrate length is due to extensive base pairing of the message (37). Secondly, we demonstrated that ribozyme cleavage carried out in the presence of 2 M urea was stimulated two to three-fold over that performed in its absence, Figure 3A. Been *et al* have postulated that refolding of long-lived inactive or slow cleaving forms of hepatitis delta virus (HDV) RNAs are facilitated by either increased reaction temperatures or added denaturants (53).

To increase the rate of cleavage of the full length form of the βAPP message we added short deoxyoligonucleotides that were complementary to sites adjacent to the ribozyme binding site in ribozyme reaction mixtures. Previous studies found such a strategy synergistically stimulated triple-helix DNA binding (48), antisense mediated translational inhibition (47), as well as ribozyme mediated cleavage of short model substrates (49) *in vitro*. Our results showing a length-dependent, position-dependent increase in the cleavage of full-length βAPP mRNA, Table 2, Figures 5−7, complement these studies. In particular, we noted that addition of 10 μM βAPP112c, a 17 base oligodeoxynucleotide, increased the rate of full-length βAPP mRNA cleavage nearly fourteen-fold, bringing it close to that observed with the small model βAPP substrate RNA.

Facilitator deoxyoligonucleotides have been postulated to work by increasing the association rate of the target and targeted molecule (49). Under single-turnover conditions when the ribozyme is in excess over substrate RNA, the rate-limiting step of the ribozyme reaction is expected to be the cleavage step of the reaction and not ribozyme association (49, 54). Accordingly, Goodchild *et al* state that facilitator deoxyoligonucleotide enhancement of ribozyme cleavage did not occur under these conditions. Our results however are not in agreement with these data. We observed substantial enhancements of the rate of cleavage for all of the facilitator deoxyoligonucleotides we tried, even though the concentration of ribozyme RNA was in five-fold exess of the initial concentration of substrate RNA. Recently, Hendry *et al* have suggested that the differences observed in the k_{cat}, determined under multiple turnover conditions, and the apparent first order rate constant k_2, determined under single turnover conditions of a ribozyme (ribozyme RNA in three-fold excess of substrate RNA) indicates that the cleavage step is not rate-limiting (36). They further speculate that this phenomenon may be due to an inability of the ribozyme to bind substrate RNA. The rate-limiting step of the reaction would then be the rate of unfolding of the ribozyme into a form capable of binding substrate. This situation is analogous to the one reported here. In this case however, it is the substrate, βAPP-751 mRNA, that appears to be folded into a conformation that is inacessable to the ribozyme. The mechanism by which facilitator deoxyoligonucleotides stimulate βAPP-751 mRNA cleavage, namely by increasing the rate of association of the ribozyme with its target, may be operative; however this question is still being investigated. Regardless of the mechanism, the use of facilitator deoxyoligonucleotide/ribozyme combinations should have a distinct advantage in *in vivo* applications where steady state levels mRNA levels are determined by both the rate of ribozyme cleavage and the natural message decay rate (55).

Finally, we have demonstrated that primer extension arrest is a viable means of determining the cleavage properties of longer forms of substrate RNAs. The intensity of the cleavage product detected by primer extension arrest is dependent upon the reaction

time, the ribozyme concentration as well as exogenous modulators of the reaction. The method is quick, straightforward, adaptable to any RNA or region of RNA, and has the added advantage of not having to end label or purify substrate RNAs; each of these steps may subject the RNA to unwanted degradation.

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