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Ribozyme and Antisense RNAs Inhibit Coupled Transcription Translation by Binding to Rabbit Polyribosomes

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The behavior of ribozyme and antisense RNAs was analyzed in a coupled rabbit reticulocyte transcription translation system. Both ribozyme and antisense RNAs were efficiently produced and bound tightly to polyribosomes at 30°C, but did not produce a protein product. Antisense and ribozyme RNA binding depended upon the presence of intact ribosomes, was specific since, plasmid DNA did not associate with either ribosomes or polyribosomes, and was temperature dependent. Ribozyme-specific mRNA cleavage in the coupled system was inferred from translation inhibition studies and was confirmed by primer extension analysis. Thus, ribozyme RNA can inhibit target protein production in the coupled transcription translation system by competing out cellular mRNAs and *via* targeted message degradation. © 1997 Academic Press

The internal milieu of eukaryotic cells is a complex mixture of organelles attached to a cytoskeleton, and bathed in a cytosol filled with carbohydrates, proteins and RNAs. It is within this heterogeneous amalgam that therapeutic agents are targeted. One set of therapeutic compounds, antisense and ribozyme RNAs, potentially affect their targets in several ways. By hybridizing to specific regions of a targeted message, antisense and ribozyme RNAs form substrates for double-stranded RNAses (1, 2). In addition, double-stranded antisense/target or ribozyme/target complexes may inhibit the nuclear export or translation of the targeted message (3). Finally, ribozyme RNAs can potentially cleave targeted mRNAs and concomitantly reduce the level of the target protein in cells (4). To date, it has been difficult to demonstrate these actions directly in cells; however, for antisense and ribozyme RNAs to be useful, it is essential that the mechanisms by which their effects are elicited *in vivo* be understood. We have chosen to address some of these questions using a coupled rabbit reticulocyte transcription translation system. This system closely mimics *in vivo* conditions, but is more amenable to biochemical manipulation. In this paper we directly show (i) antisense and ribozyme RNAs inhibit protein synthesis by competing with sense mRNAs for polyribosome binding and (ii) ribozyme-mediated mRNA cleavage enhances that inhibition.

MATERIALS AND METHODS

Plasmid DNAs. pNR-11 containing βAPP$_{751}$ cDNA, and pMAMneo-Rz and pMAMneo-Dead containing active and inactive hammerhead ribozymes targeted to βAPP RNA, respectively, have been described previously (5, 6, 7); the cDNAs of each of these constucts is under the control of a bacteriophage T7 RNA polymerase promoter. pFMR1-22 and pFMR1-36 identical original clones containing the human FMR1 coding sequence under the control of a bacteriophage T7 RNA polymerase promoter were kind gifts of W. T. Brown. pMAMneo DNA was obtained from Clontech; Sp6 and T7-control DNAs encoding firefly luciferase cDNA were obtained from Promega.

Transcription translation of plasmid DNAs. Twenty five microliters of TNT^{Tm} rabbit reticulocyte lysate were combined with 2 μl of TNT^{Tm} T7, or Sp6 RNA polymerase, 1 μl of 1 mM amino acid mix minus methionine, 4 μl (40 μCi) of TRAN ^{35}S-LABEL^{Tm}, 1 μl of RNasin and 1 μg of plasmid DNA in a total volume of 50 μl. DEPC-treated sterile water was added to bring the volume up to 50 μl. The contents of the tube was thoroughly mixed by pipetting. Samples were incubated at 30°C for 90 min.; 5 μl aliquots were assayed for the incorporation of ^{35}S-methionine into protein by electrophoresis through 10% Laemmeli gels (8). Following electrophoresis, the gels were fixed for 20 min. and then vacuum dried. Dried gels were subjected to autoradiography for 1-8 hr.

Coupled transcription/translation inhibition studies were performed using a Master Mix of 100 μl TNT^{Tm} rabbit reticulocyte lysate, 8 μl TNT^{Tm} reaction buffer, 4 μl TNT^{Tm} T7 RNA polymerase, 4 μl of 1 mM amino acid mix minus methionine, 16 μl of TRAN ^{35}S-LABEL^{Tm}, 2 μl RNasin, 4 μg pNR-11 DNA and 4 μg of TNT^{Tm} T7 control plasmid DNA. The mixture was adjusted to 200 μl with DEPC-treated sterile water and mixed well by pipetting. Ten microliters of the Master Mix was then added to 1 μl aliquots of ribozyme or control DNAs. The samples were incubated at 30°C for 90 min.; 5 μl aliquots were assayed for the incorporation of ^{35}S-methionine into protein as

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described above. The relative inhibition of βAPP_{751} synthesis was determined by scanning densitiometry (6) and calculated as: 100 × [βAPP_{751}/luciferase in the presence of inhibitor]/[βAPP_{751}/lucifease in the absence of inhibitor].

RNA transcription in rabbit reticulocyte lysate. [α-^{32}P]CTP (3000Ci/mmol) incorporation into TCA precipitable ribo-oligonucleotides was performed in a standard 50 μl transcription/translation reaction containing 25 μl of TNT^{Tm} rabbit reticulocyte lysate, 2 μl of TNT^{Tm} T7, or Sp6 RNA polymerase, 1 μl of 1 mM complete amino acids, 1 μl of Rnasin and 1 μg of plasmid DNA. Two microliter aliquots were removed at 30 minute intervals for up to three hours and precipitated with 5 ml of 5% TCA; the precipitated material was separated from unincorporated [α-^{32}P]CTP by filtration through 0.45 mm HAWP filters (Millipore Corp). The TCA precipitable radioactivity bound to the membrane was measured by liquid scintillation counting in the presence of 10 ml of Filtron-X (National Diagnostics Labs).

RNA incorporation into polyribosomes. Radiolabeled RNA from 3 hr., 50 μl transcription translation reactions (above) was assessed for its ability to incorporate into polyribosomes by pelleting the polyribosomes (50 K, 16 hr, 4°C) through a cushion of 50% [w/w] sucrose in 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 25 mM KCl (TMK) buffer (6). Polyribosome pellets were dissolved in 50 μl of 5 M urea to dissociate any bound proteins. The mixture was then precipitated with 5 ml of 5% TCA; the precipitated material was filtered through 0.45 μm HAWP filters (Millipore Corp), dissolved in 10 ml of Filtron-X, and quantified by liquid scintillation counting.

Analysis of polyribosome mRNAs by RT-PCR. Transcription translation reactions were prepared and treated with exogenous mRNAs, 370 mM NH_4Cl (9), 5 mM EDTA (10) (as described in the text). Polyribosomes were separated from unincorporated RNA and mRNPs by pelleting through sucrose cushions (above). RNA was isolated from polyribosome pellets and sucrose cushions using TRI-Reagent (Molecular Research Center) according to the manufacturer's directions. βAPP and ribozyme RNAs were amplified by RT-PCR as previously described (11). Under these conditions the amount of PCR product is linearly related to input RNA. RNA levels were determined by scanning densitometry and expressed as the percentage of the total RNA in the gradient. pMAMneo-Rz DNA was amplified under the same conditions as Rz cDNA except a plasmid-specific 5′-amplimer $pMAMneo_{1521\text{-}4}$, 5′ACGGACTCACCATAGGGACCA3′, was used in the PCR reaction.

Primer extension arrest. Primer extension arrest of 1 μg RNA isolated from transcription translation mixtures was performed essentially as previously described (12, 13). Primer extension products were resolved on 8% PAGE/7M Urea gels and subject to autoradiography for 1-2 weeks.

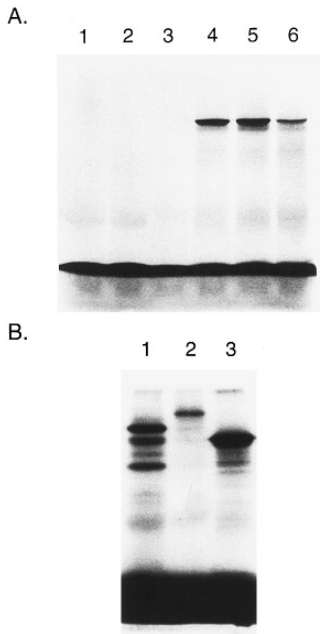

FIG. 1. Coupled transcription translation of pFMR1-22. A. pFMR1-22 (0.1, 0.2, 0.3 μg; lanes 1-3) and Sp6-Control DNAs (lanes 4-6) were subjected to coupled transcription translation with Sp6 RNA polymerase. B. pFMR1 (lane 1), pNR-11 (lane 2) and T7-Control DNAs were subject to coupled transcription translation with T7 RNA polymerase. Sp6-Control and T7-Control DNAs encode the 61 kDa firefly luciferase; pNR-11 encodes the 96 kDa βAPP, and pFMR1-22 encodes the 66 kDa FMR1 protein. Note the sensitivity of FMR1 to proteolytic degradation.

RESULTS

Antisense and Ribozyme mRNAs Are Efficiently Produced in a Coupled Rabbit Reticulocyte Transcription Translation System

We have previously shown that ribozyme plasmid vectors that transcribe relatively small RNAs do not yield detectable protein products in coupled transcription translation (8). Here, we use a human FMR1 cDNA, cloned between apposing T7 and Sp6 promoters, to demonstrate that this is also true of antisense RNA. Figure 1A shows that plasmid pFMR1-22 does not produce protein in Sp6 coupled transcription translation reactions in which FMR1 cDNA is in an antisense orientation with respect to the promoter, but does produce protein in T7 coupled transcription translation reactions where it is sense-oriented with respect to the promoter, Figure 1B. Since antisense and ribozyme plasmid vectors do not yield detectable protein products in the coupled transcription translation system, [α-^{32}P]-CTP incorporation into ribo-oligonucleotides was measured to insure that their RNAs were being made during the reaction. Table 1 shows that βAPP ribozyme RNA (Rz RNA) and antisense FMR1 RNA (aFMR1 RNA) were transcribed from pMAMneo-Rz, pFMR1-22 and pFMR1-36 DNAs during coupled transcription translation. Comparison with protein coding control vectors pNR-11 and Sp6-Control revealed that each plasmid produced unique amounts of RNA; however, the T7-based vectors were generally more active than Sp6-based vectors.

Antisense and Ribozyme RNAs Associate with Rabbit Polyribosomes

The fate of antisense and ribozyme RNAs in the coupled transcription translation system was further examined by measuring their ability to associate with rabbit polyribosomes under a variety of conditions.

TABLE 1

RNA Production in Coupled Transcription Translation

Plasmid	Polymerase	RNA transcription rate[a] (μCi/hr/μg DNA)	Standard deviation
pNR-11	T7	0.57	0.14
pMAMneo-Rz	T7	0.28	0.10
pMAMneo-Dead	T7	0.23	0.06
pSP6-Control	Sp6	0.23	0.08
pFMR1-22	Sp6	0.06	0.02
pFMR1-36	Sp6	0.10	0.05

[a] Incorporation of [α-^{32}P]CTP (3000 Ci/mmol) into TCA precipitable cpm was measured at 30 minute intervals for up to three hours in a standard 50 μl transcription/translation reaction. The slopes from Incorporation vs. Time plots for each plasmid, obtained by regression analysis, were normalized for the amount of plasmid DNA input into the reaction. The data are the mean values of four independent experiments for each plasmid.

First, the amount of radiolabeled aFMR1 RNA that co-sedimented with polyribosomes was compared to the amount transcribed during coupled transcription translation. As a control, the amount of luciferase mRNA that co-sedimented with polyribosomes was also examined. In these experiments polyribosomes were isolated from cytosolic proteins, RNAs and mRNPs by centrifugation through 50% [w/w] sucrose cushions (6). The results shown in Table 2, reveal that 90% of luciferase mRNA and 63% of aFMR1 RNA associated with polyribosome pellets. Thus, a significant amount of antisense RNA appears to bind to polyribosomes under these conditions. A control experiment, in which radiolabeled RNA extracted from identical coupled transcription translation reaction mixtures was layered on to sucrose cushions, was performed to demonstrate that sucrose gradient centrifugation specifically isolated messages bound to polyribosomes. Table 2 shows that the extracted luciferase (SP6-Control) and aFMR1 RNAs were not pelleted, indicating that both RNAs must associate with a larger complex *i.e.* polyribosomes for sedimentation to occur.

Rz RNA also associated with rabbit polyribosomes. Figure 2A shows that both βAPP and Rz RNA were amplified from total RNA isolated from the polyribosome pellet of a transcription translation reaction mixture primed with equal amounts of pNR-11 and pMAMneo-Rz DNA (lanes 1-4), while only βAPP was amplified from transcription translation reactions primed with pNR-11 DNA (lanes 5-8). The specificity of Rz association was further demonstrated in the experiment presented in Figures 2B and 2C. Here, exogenously added Rz RNA was incubated at 4°C for 5 min or 12 hr with transcription translation reaction mixtures primed with pNR-11 DNA. The results clearly show that while βAPP mRNA was amplified from polyribosomal RNA isolated at each time point (lanes 3 and 4), Rz RNA was only amplified from polyribosomal RNA following prolonged incubation, Figure 2C (lanes 1 and 2). These data imply that Rz RNA does not bind to polyribosomes adventitiously. To confirm this pNR-11 or pMAMneo-Rz primed transcription translation reactions were treated with 370 mM NH_4Cl; this treatment partially disrupts polyribosomes, releasing mRNAs, but keeps ribosome subunits intact (9). The resulting mixtures were separated on 50% sucrose cushions and RNAs from both the cushion and the pellet were amplified by RT-PCR. The results in Table 3 indicate that the amount of Rz RNA in the cushion following treatment increased by 40%, while the amount of βAPP RNA in the cushions increased by 22%. In addition, in both controls and NH_4Cl-treated samples, pMAMneo-Rz DNA was only found in the sucrose cushion (data not shown). Similar results were obtained when polyribosomes were disrupted by 5 mM EDTA.

Ribozyme RNAs function in a Coupled Rabbit Reticulocyte Transcription Translation System

Two experiments were performed to address whether Rz RNA formed during coupled transcripton transla-

TABLE 2

aFMR1 RNA Is Associated with Polyribosomes in Coupled Transcription Translation

Plasmid	RNA[a]	SD	Polyribosomes[b]	SD	Polyribosome/RNA	SD
pSP6-Control	9.1	3.5	8.2	2.7	0.9	0.4
pFMR1-22	2.1	0	1.3	0.4	0.62	0.1
pFMR1-36	3.8	0.3	2.4	1.0	0.63	0.1
pSP6-ControlE[c]	6.0	1.8	0.07	0.02	0.012	0.005
pFMR1-22E[c]	1.8	0.3	0.03	0.01	0.017	0.002
pFMR1-36E[c]	2.3	0.4	0.06	0.01	0.026	0.002

[a] Percentage of [α-^{32}P]CTP incorporated into RNA following a 3 hr incubation as described in Table 1.
[b] Percentage of [α-^{32}P]CTP associated with polyribosomes.
[c] RNA was separated from polyribosomes by extraction with TRI-Reagent before sucrose gradient centrifugation.
The results are the average values of two experiments. SD are standard deviation values.

TABLE 3

Effect of NH_4Cl and EDTA on Polyribosome-bound RNA Levels

Treatment	% Rz RNA in polyribosomes	% βAPP RNA in polyribosomes
—	28 (11)	81 (7)
NH_4Cl[a]	17 (11)	63 (4)
EDTA[b]	16 (6)	ND

[a] Coupled transcription translation mixtures primed with pMAMneo-Rz DNA or pNR-11 DNA were treated at 4°C with 370 mM NH_4Cl for 10 min. prior to sucrose gradient centrifugation.

[b] Coupled transcription translation mixtures primed with pMAMneo-Rz DNA or pNR-11 DNA were treated at 4°C with 5 mM EDTA for 10 min. prior to sucrose gradient centrifugation.

The results are the average values of four different reverse transcription reactions for each message. Numbers in parenthesis are standard deviation values.

tion cleaved βAPP mRNA. First, by titrating increasing amounts of pMAMneo, pMAMneo-Rz and pMAMneo-Dead plasmid DNAs into a coupled transcription translation assay containing equal amounts of pNR-11 and T7-control DNA, it was shown that pMAMneoRz was a significantly more potent inhibitor of βAPP translation than the other plasmid DNAs, Figure 3A. Since the transcription rates of pMAMneo-Rz and pMAMneo-Dead were nearly identical, Table 1, and pMAMneo-Dead RNA cannot cleave βAPP mRNA (12), the difference in inhibition implies that Rz cleavage occurs during the transcription translation reaction. This was directly demonstrated by primer extension analysis of total RNA isolated from transcription translation reactions primed with equal amounts of pNR-11 and pMAMneo-Rz DNA. Figure 3B shows that a 60 base arrested cDNA, derived from ribozyme-mediated cleavage was present in transcription translation reactions containing pMAMneo-Rz RNA, but not those in which it was absent. Thus, at least a portion of βAPP mRNA is not translated into full length βAPP protein because it has been cleaved by Rz RNA.

DISCUSSION

The rabbit reticulocyte lysate used in *in vitro* translation consists of the S15 supernatant of lysed red blood cells (14). As such, it contains cytosolic proteins, ribosomes and mRNAs in the form of mRNPs, but no nuclear proteins or membranes. Endogenous mRNAs are removed by micrococcal nuclease digestion, allowing exogenous messages to be efficiently, if not solely, translated. In coupled transcription translation, one or more bacteriophage promoter-based vectors are used as the source of genetic information and a bacteriophage RNA polymerase substitutes for the nuclear transcription apparatus. Canine microsomal membranes can be added to allow membrane protein insertion, translocation and post-translational modification (15, 16). Clearly, this is a complex system that closely mimics cellular metabolism in many respects.

In vitro transcription translation systems and coupled transcription translation systems have previously been used to investigate the effect of antisense oligonucleotides and antisense peptide nucleic acids on gene expression (17, 18). Antisense oligonucleotide-mediated translational inhibition occurs *via* RNase H-dependent and RNase H-independent mechanisms (19). RNase H-dependent antisense oligonucleotide inhibition results from the formation of truncated messages that cannot be fully translated. RNase H-independent inhibition occurs when antisense oligonucleotides bind to the 5′-leader region of a mRNA and compete with initiation complex formation. Both types of inhibition occur in rabbit reticulocyte lysates. The results pre-

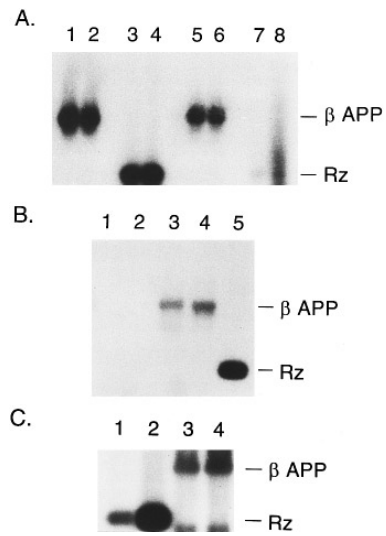

FIG. 2. Ribozyme RNA association with rabbit reticulocyte Polyribosomes. A. Southern blot of RT-PCR products from total RNA isolated from polyribosomes following coupled transcription translation of pNR-11 and pMAMneo-Rz DNAs (lanes 1-4) or of pNR-11 DNA alone (lanes 5-8). RT-PCR was performed in duplicate using βAPP amplimers (lanes 1, 2, 5 and 6) or Rz-specific amplimers (lanes 3, 4, 7 and 8); RT-PCR products were probed with βAPP or Rz-specific hybridization probes (Denman, 1994). B. 0.3 μg Rz RNA was incubated with a 15 μl pNR-11 *in vitro* transcription translation mixture at 4°C for 1 hr. Subsequently, the polyribosomes were isolated and total RNA extracted and duplicate samples subjected to RT-PCR using Rz-specific amplimers (lanes 1 and 2), or βAPP amplimers (lanes 3 and 4). Lane 5 is an RT-PCR control of the Rz RNA added to the *in vitro* transcription translation mixture. RT-PCR products were probed with βAPP or Rz-specific hybridization probes as in A. C. 0.3 μg Rz RNA was incubated with a 15 μl pNR-11 *in vitro* transcription translation mixture at 4°C for 12 hr prior to the isolation of polyribosomes. RT-PCR on duplicate sample and Southern blotting were performed as in B. Sample lanes correspond to those in B.

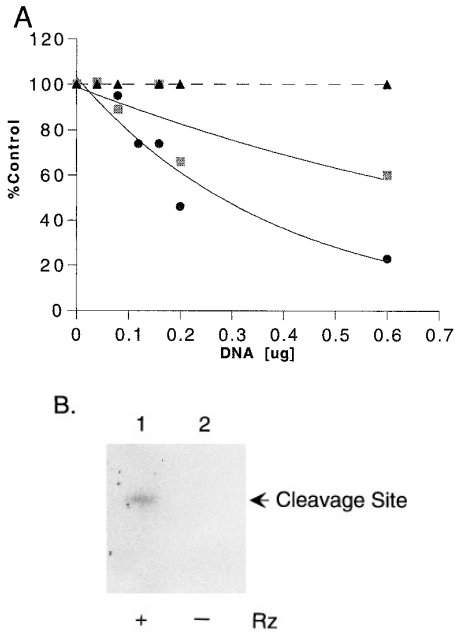

FIG. 3. Ribozyme-mediated inhibtion of βAPP$_{751}$ synthesis in a coupled transcription translation reaction. A. Relative inhibition of βAPP$_{751}$ synthesis by increasing concentrations of pMAMneo (▲), pMAMneo-Dead (■) and pMAMneo-Rz (●) DNAs. B. Primer extension analysis of 1 μg of total RNA isolated from coupled transcription translation of pNR-11 and pMAMneo-Rz (lane 1) or pNR-11 alone (lane 2). Total RNA was primed with a 21 b oligonucleotide that specifically hybridizes to βAPP mRNA and extended with AMV-RT in the presence of [α^{32}P]dCTP (Denman, 1996). The 60 b product corresponding to the ribozyme cleavage site is marked.

sented in this study show that in rabbit reticulocyte coupled transcription translation reactions both translatable (sense) and untranslatable (antisense and ribozyme) RNAs associate specifically with polyribosomes, Figure 2, Table 2 and Table 3. Therefore, antisense RNA inhibition appears to involve a competition between antisense RNA and cellular mRNAs for limiting translational factors in the mixture. Of course *in vivo,* competition with the nearly 10-15,000 messages produced by cells may minimize the effect observed in this system; nevertheless if the untranslatable RNA steady-state level is large enough and/or primary or secondary structural features allow efficient binding to polyribosomes, this mechanism may affect cellular gene expression.

Using ribozyme-containing transcription vectors a second type of translational inhibition was also observed in this study. Specifically, pMAMneo-Rz and pMAMneo-Dead DNA produced RNAs that inhibited βAPP$_{751}$ mRNA translation in a cleavage-dependent manner, Figure 3A. In fact, cleaved βAPP$_{751}$ mRNA was detected in pMAMneo-Rz-containing coupled transcription translation reactions, but not in controls, Figure 3B. It has been very difficult to demonstrate ribozyme cleavage directly *in vivo,* (20, 21) although indirect evidence relying on the inhibition differential between ribozyme and inactive ribozyme or antisense forms is abundant (22, 23, 24). However, such indirect measurements can be compromised by clonal variation resulting in (i) different expression levels between the ribozyme and inactive ribozyme cells, and (ii) transfection or integration-related differences between cell lines that are not related to ribozyme activity. In the rabbit reticulocyte lysate coupled transcription translation system, transcription rates and absolute levels of each RNA are readily measured, Table 1.; therefore differences in ribozyme and inactive ribozyme expression can be minimized. Further, since reactions are carried out *in vitro,* transfection and integration differences are not relevant. Thus, this system should be extremely useful in determining mechanisms involved in ribozyme-mediated degradation of cellular mRNAs.

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