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METHOD

A hammerhead ribozyme substrate and reporter for in vitro kinetoplastid RNA editing

BINGBING WANG,[1,2] REZA SALAVATI,[1,2] STEFAN HEIDMANN,[3] and KENNETH STUART[1,2]

[1]Department of Pathobiology, University of Washington, Seattle, Washington 98195, USA
[2]Seattle Biomedical Research Institute, Seattle, Washington 98109, USA
[3]Department of Genetics, University of Bayreuth, 95440 Bayreuth, Germany

ABSTRACT

Current in vitro assays for RNA editing in kinetoplastids directly examine the products generated by incubation of pre-mRNA substrate with guide RNA (gRNA) and mitochondrial (mt) extract. RNA editing substrates that are modeled on hammerhead ribozymes were designed with catalytic cores that contained or lacked additional uridylates (Us). They proved to be sensitive reporters of editing activity when used for in vitro assays. A deletion editing substrate that is based on A6 pre-mRNA had no ribozyme activity, but its incubation with gRNA and mt extract resulted in its deletion editing and production of a catalytically active ribozyme. Hammerhead ribozymes are thus sensitive tools to assay in vitro RNA editing.

Keywords: gRNA; *Trypanosoma brucei*; uridylate deletion; uridylate insertion

INTRODUCTION

RNA editing is a type of posttranscriptional RNA processing that occurs in the mitochondrion of trypanosomatid protozoa (for review, see Alfonzo et al., 1997; Stuart et al., 1997). In *Trypanosoma brucei*, RNA editing is characterized by frequent uridylate (U) insertion and less frequent U deletion in precursor mitochondrial mRNAs (pre-mRNAs) to create the functional mRNAs. The pre-mRNAs are encoded in the maxicircle of the kinetoplastid DNA (kDNA). The edited sequences are specified by small (~60 nt), transacting guide RNAs (gRNAs) that are encoded in kDNA minicircles (Blum & Simpson, 1990; Pollard et al., 1990).

The gRNAs have 5′ anchor, central guiding, and 3′ oligo-U domains. The 4–20-nt anchor sequence is complementary to the region of the pre-edited mRNA that is immediately 3′ to that which will be edited. The gRNA duplexes with this region during editing and the guiding portion of gRNA directs U insertion and deletion and hence the edited sequence (Seiwert & Stuart, 1994; Kable et al., 1996; Seiwert et al., 1996). The role of the ~5–20-nt oligo (U) tail in RNA editing is uncertain, but it may stabilize the interaction with the 5′ region of the pre-mRNA and/or possibly increase the access of the editing complex to the editing sites by reducing secondary structure in the pre-mRNA in the editing domain (Leung & Koslowsky, 1999).

Editing is catalyzed by a multiprotein complex, or editosome (Read et al., 1994; Rusche et al., 1997; Panigrahi et al., 2001). This complex contains the endoribonuclease, 3′ exouridylylase, terminal uridylyl transferase (TUTase), and RNA ligase activities that are needed for RNA editing. The development of an in vitro assay system for kRNA editing (Kable et al., 1996; Seiwert et al., 1996) has provided a tool for monitoring editing activity in the purification and analysis of the *T. brucei* editosome. However, this assay has a low detection limit and thus the development of a more sensitive specific assay is desirable. The highly efficient cleavage of a substrate RNA by a hammerhead ribozyme, which may be created from a preedited ribozyme by in vitro RNA editing, makes it an attractive candidate for such a sensitive in vitro RNA editing assay. The hammerhead ribozyme is the smallest catalytic RNA motif that can cleave substrate RNA efficiently either *in cis* or *in trans* at a specific phosphodiester bond (Uhlenbeck, 1987; Haseloff & Gerlach, 1988; Symons, 1992). The 5′ cleavage product has a 2′, 3′ cyclic phosphate end whereas the 3′ cleavage product has a 5′ hydroxyl terminus. Any substrate RNA with an NHH sequence appropriately positioned relative to the

Reprint requests to: Kenneth Stuart, Seattle Biomedical Research Institute, 4 Nickerson Street, Seattle, Washington 98109, USA; e-mail: kstuart@u.washington.edu.

base-paired stem can be cleaved by the ribozyme at the phosphodiester bond 3′ to the second H, where N is any nucleotide and H can be either A, C, or U (Ruffner et al., 1990; Kore et al., 1998).

We report here that the sensitivity of the assay for in vitro RNA editing has been increased using the hammerhead ribozyme activity. A pre-ribozyme was faithfully edited in the presence of *T. brucei* mitochondrial extract by removal of three uridylates as directed by an appropriate gRNA. The edited ribozyme was functional and capable of cleaving its targeted substrate.

RESULTS

Deletion editing of a hammerhead ribozyme

The deletion editing pre-ribozyme substrate (pre-A6RZ) was accurately edited and the edited product (A6RZ) efficiently cleaved its substrate (SubA6RZ). Pre-A6RZ was based on ATPase 6 pre-mRNA whereas gRNA, based on gA6[14], was modified to form an anchor duplex with pre-A6RZ and three C residues in the guiding portion to facilitate deletion editing (Seiwert et al., 1996; Cruz-Reyes et al., 2001). Incubation of 3′-end-labeled pre-A6RZ with gA6RZ (Fig. 1A) and mitochondrial extract of *T. brucei* resulted in edited RNA that is 3 nt shorter than the input RNA as specified by the gRNA and a 3′ cleavage product with the size predicted for cleavage at the editing site (Fig. 1B). The production of the edited RNA and cleavage product required both the gA6RZ gRNA and mitochondrial extract, as they were absent in reactions that lacked either the gRNA or mitochondrial extract (Fig. 1B, lanes 4 and 5). The identity of the edited RNA was confirmed by enzymatic sequencing (Fig. 1C). The input and edited RNAs were recovered from a polyacrylamide gel from a preparative reaction and were partially digested with RNase T1 (cleaves 3′ to Gs) and RNase Phy M (cleaves 3′ to As and Us). Comparison of input RNA with edited product showed that three Us had been

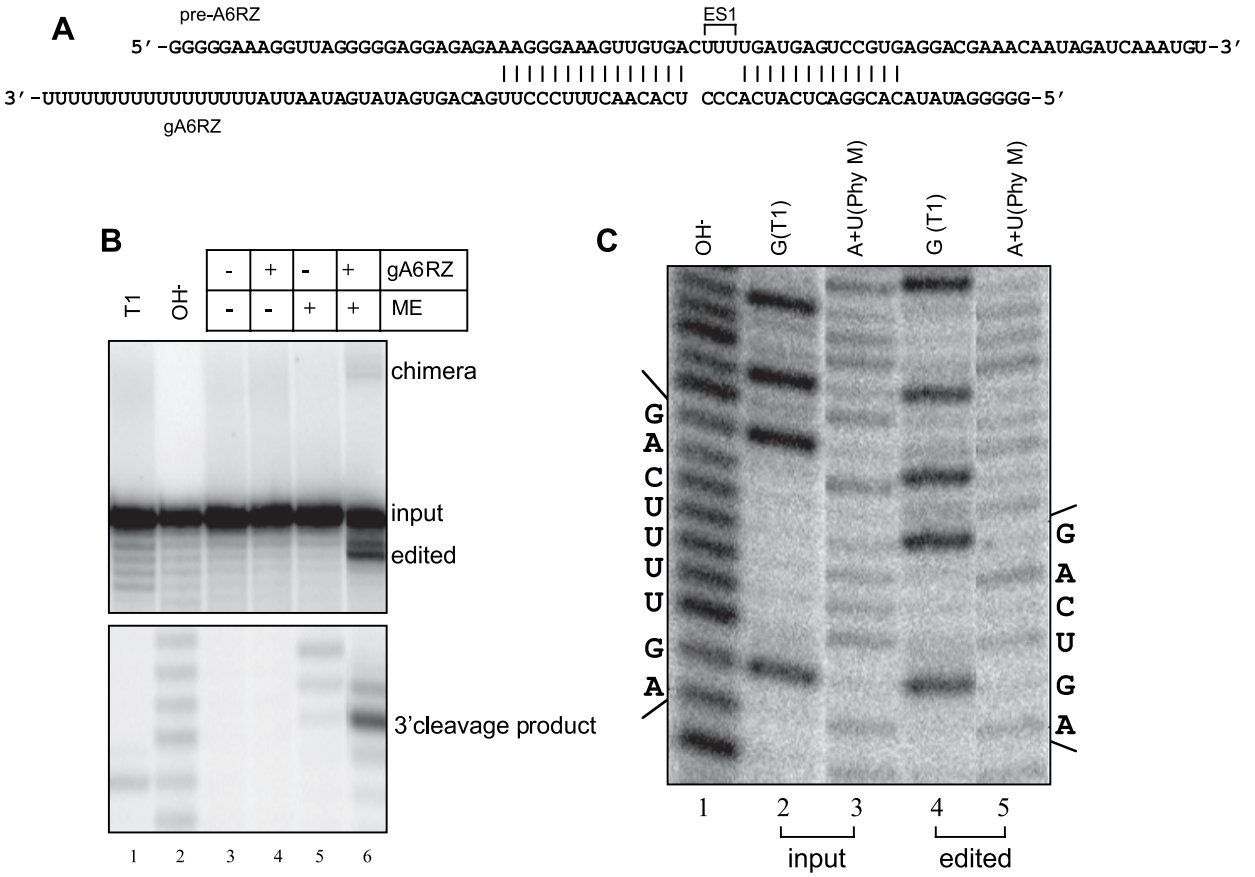

FIGURE 1. Editing of the pre-ribozyme. **A:** The pre-edited ribozyme pre-A6RZ is shown in association with the gA6RZ gRNA that specifies the deletion of three Us (bracket) from editing site 1 (ES1). **B:** The edited product and 3′ cleavage product were generated only when both gA6RZ and mitochondrial extract (ME) were present (lane 6). Pre-A6RZ RNA that was 3′ end labeled and subjected to partial digestion by RNase T1 (lane 1) or partial alkaline hydrolysis (lane 2) is used as markers. **C:** Enzymatic sequencing of editing reaction products from preparative reactions by partial digestion with RNase T1 (lanes 2 and 4) and RNase Phy M (lanes 3 and 5). The sequences at the editing site of pre-A6RZ and edited A6RZ are indicated on left and right, respectively. Pre-A6RZ RNA that was 3′ end labeled and partially hydrolyzed with alkali is used as a size marker (lane 1).

deleted at the editing site (Fig. 1C, compare lanes 2 and 3 with 4 and 5).

The edited ribozyme is catalytically active

Incubation of unlabeled in vitro-transcribed pre-A6RZ and its gRNA with mitochondrial extract resulted in cleavage of 3′ end labeled substrate for the ribozyme, SubA6RZ, at the site predicted for ribozyme cleavage (Fig. 2). This simultaneous editing of the pre-A6RZ preribozyme and the cleavage by the edited product of the ribozyme substrate, SubA6RZ, used incubation conditions that were identical to those used for pre-A6RZ deletion editing except that pre-A6RZ was not labeled and 3′-end-labeled SubA6RZ was included. RNAs were phenol extracted and ethanol precipitated prior to electrophoresis in acrylamide/urea gels as described in Materials and Methods. Production of the cleavage product that is predicted from SubA6RZ was dependent on the presence of pre-A6RZ, gA6RZ, and mitochondrial extract. The cleavage site was determined by comparison to the products of partial digestion by RNase T1 and alkaline hydrolysis of labeled SubA6RZ (Fig. 2B, lane 7) and by incubation of the substrate with a synthetic equivalent of the edited ribozyme. The cleavage product that is 1 nt smaller is a by-product of the ribozyme, as it was also produced when the synthetic equivalent to the edited pre-A6RZ was used (Fig. 2B, lane 3). Other cleavage products are nonspecific (single star) or due to a cleavage activity in mitochondrial extract (double stars). Thus the pre-A6RZ was edited and this RNA subsequently cleaved SubA6RZ.

We also modeled pre-ribozymes based on ES2 of *T. brucei* Cytochrome b (Cyb) mRNA. A synthetic Cyb-based ribozyme with a normal catalytic core (one uridylate) efficiently cleaved its substrate, and one lacking the essential uridylate in the catalytic core was inactive, but one with two uridylates in the core had reduced cleavage activity (data not shown). Kinetic analyses determined that the K_ms were 2.7×10^{-7} and 3.6×10^{-6} mol/L and k_{cats} were 4.4 and 1.1 min^{-1} for the synthetic ribozyme based on the normal catalytic core and that with two additional uridylates, respectively. Thus, the change in the catalytic core reduced the catalytic rate, but not the substrate affinity of ribozyme.

Comparison of edited ribozyme editing with conventional in vitro RNA deletion editing

The fractionation of the ribozyme-mediated editing activity was compared to that of the conventional assay of editing activity with the A6 or pre-A6RZ substrates to assess the association of the assayed activity with the editing complex. Mitochondrial lysate was fractionated by Mono Q ion exchange chromatography and the active editing fractions were subsequently fractioned by SP Sepharose ion exchange chromatography according to the editing complex purification protocol (Panigrahi et al., 2001). All three activities had a similar elution profile from the SP Sepharose column with activities in fractions 12 through 21 and an activity peak in fraction 14 (Fig. 3). Notably, a greater proportion of SubA6RZ substrate was processed by the ribozyme.

Ribozyme assay sensitivity

A greater percentage of products were generated from the combined editing/ribozyme cleavage than those generated from the direct editing of both the A6short/TAG and pre-A6RZ substrates using fraction 13 (Fig. 3) as the source of mitochondrial extract. There was little difference between the three assays for the first 30 min,

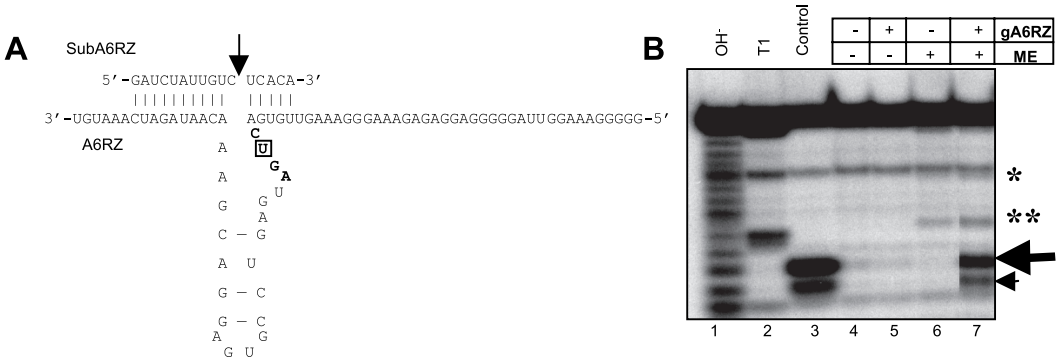

FIGURE 2. Activity of the ribozyme. **A:** Ribozyme A6RZ is shown in association with its substrate SubA6RZ with the cleavage site of SubA6RZ indicated by an arrow. The conserved 5′-CUGA-3′ of A6RZ (in bold) is essential for ribozyme activity and the box indicates ES1, where three Us are removed from pre-A6RZ by editing. **B:** The same major and minor cleavage products of 3′-end-labeled SubA6RZ (large and small arrows, respectively) were generated upon incubation with synthetic ribozyme (lane 3) or with unlabeled pre-A6RZ, gA6RZ RNA, and mitochondrial extract (ME; lane 7). These products were not obtained upon omission of either gA6RZ or ME (lanes 5 and 6). A minor band (single star) was present in the input RNA and a nonspecific cleavage product was generated by endonuclease activity in ME (double stars). The partial alkaline hydrolysis (lane 1) and RNase T1 digestion (lane 2) of 3′-end-labeled SubA6RZ serve as markers.

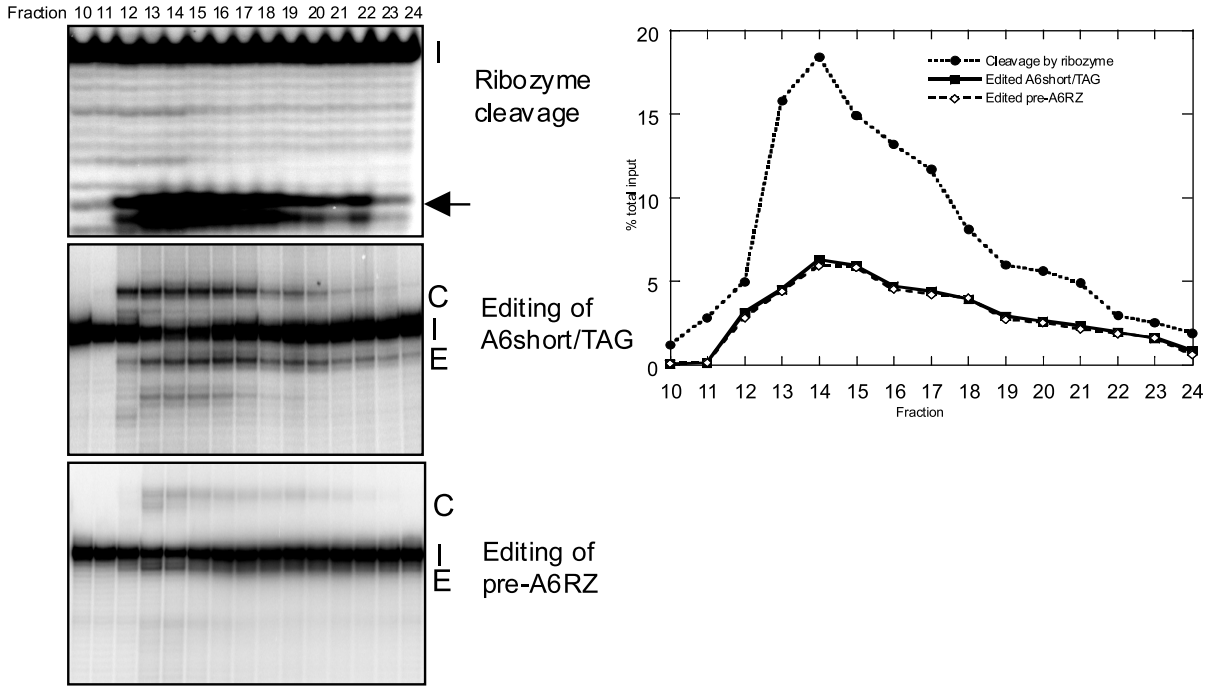

FIGURE 3. Cofractionation of ribozyme-mediated and conventional editing assays. Left panel: Fractions from Mono Q column, following fractionation of mitochondrial extract on SP Sepharose, were assayed for editing by the ribozyme-mediated assay or by the conventional editing assay as described in the Materials and Methods section. Input (I) 3′-end-labeled SubA6RZ, A6short/TAG, and pre-A6RZ, the ribozyme cleavage products (arrow), edited (E) A6short/TAG and pre-A6RZ RNAs, and chimeras (C) are indicated. Right panel: Quantitation shows the percent of total input that is edited (squares for edited A6short/TAG and diamonds for edited pre-A6RZ) or cleaved by the ribozyme (circles).

but after this time progressively more input RNA was processed by the ribozyme-mediated assay compared to the direct assays (Fig. 4A). More than twice the amount of SubA6RZ was cleaved than A6RZ and A6short/TAG were processed (16.8% vs. 5–6%). Progressive dilution of fraction 13 resulted in rates of diminishment such that the combined editing/ribozyme assay was 2–3 times more sensitive than the direct assay over most dilutions (Fig. 4B).

DISCUSSION

A convenient in vitro ribozyme-based RNA editing assay has been developed and has a greater sensitivity than the conventional assay that is based on direct visualization of the editing reaction products. This assay entails the editing of a nonfunctional pre-ribozyme into a catalytically active hammerhead ribozyme. The activity is gRNA directed and copurifies with the editing complex. Mitochondrial extract does not interfere with the assay although additions of gRNA and pre-mRNA do interfere presumably as a result of base pairing with the ribozyme and the ribozyme substrate, thus competing with a productive ribozyme–ribozyme substrate interaction (data not shown). The assay is convenient, as the production of active ribozyme and cleavage of the ribozyme substrate occur simultaneously in the same tube. It is also two to three times more sensitive than the conventional direct assay.

The coupled deletion editing/ribozyme cleavage with pre-A6RZ RNA occurred efficiently. This substrate is based on ATPase 6 pre-mRNA, whose in vitro deletion editing at the editing site (ES) 1 has been directly visualized in vitro (Seiwert et al., 1996). The pre-A6RZ is inactive as a ribozyme, presumably due to structural and base pairing changes that result from the insertion of three additional Us into the catalytic core. Deletion editing of pre-A6RZ occurred efficiently, was directly visualized, and confirmed by RNA sequencing. However, the Cyb insertion editing substrate was not edited, although it was cleaved. This may reflect rapid religation and/or inaccessibility to TUTase, perhaps as a consequence of the conformation imposed in this case by strong duplexes on either side of the ES that results from base pairing with the gRNA. The more efficient deletion editing than insertion editing may also reflect differences in these processes.

The edited pre-ribozyme specifically and efficiently cleaved a ribozyme substrate RNA. The less abundant product resulting from cleavage 1 nt further 3′ with both the synthetic ribozyme and that resulting from editing may be due to an alternative secondary structure possibly as a result of the short helix I (3′ to cleavage site in Fig. 2A). However, a relatively short helix I is

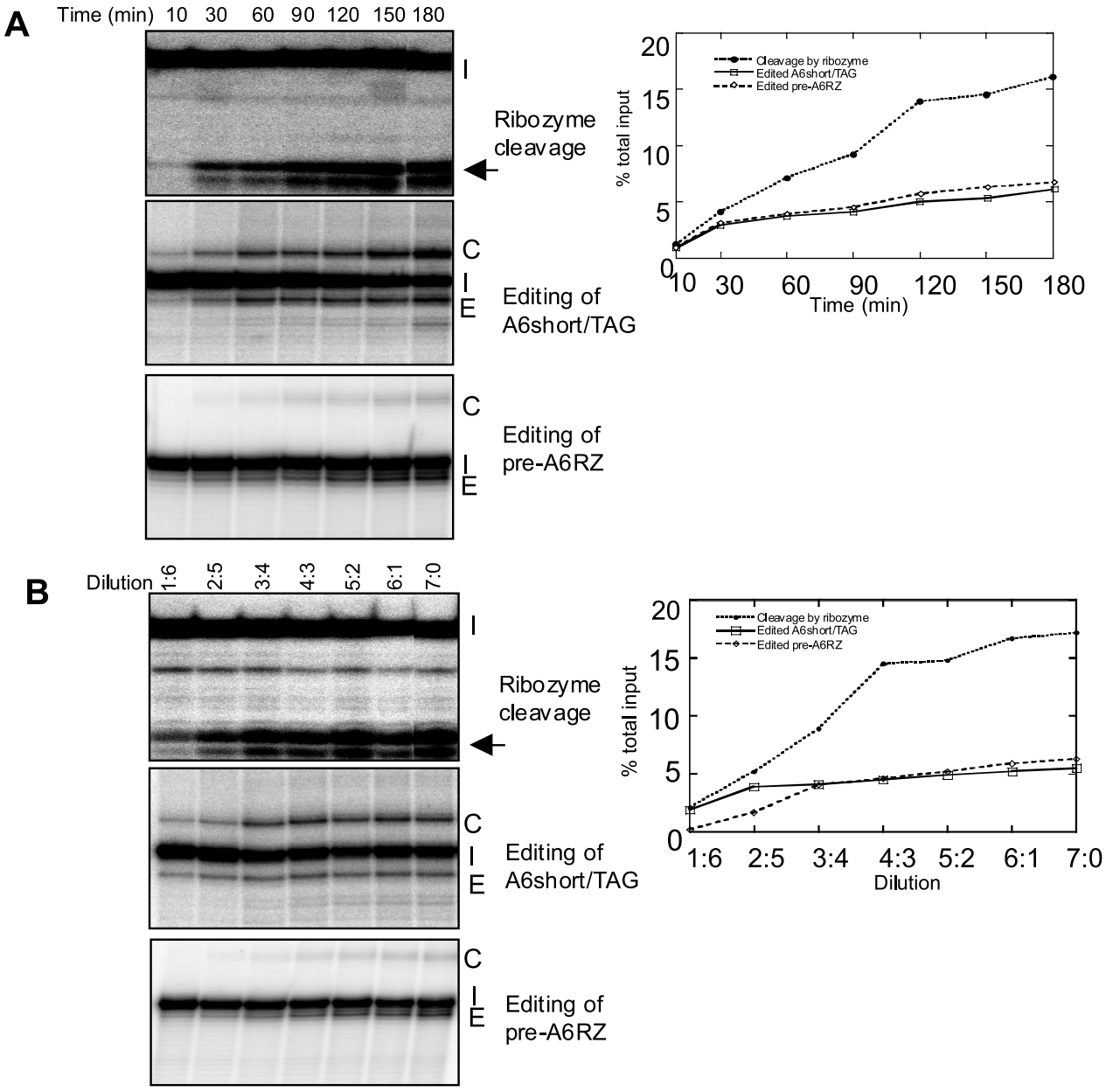

FIGURE 4. Cleavage or editing of substrate RNAs over time or with varying amounts of mitochondrial extract. 3′-end-labeled input SubA6RZ was cleaved by the coupled edited/ribozyme assay (circles). A6short/TAG and pre-A6RZ (squares and diamonds, respectively) were assayed by in vitro editing. In all assays, SP Sepharose/Mono Q fraction 13 was used. Gel autoradiograms are on the left and quantitations (as in Fig. 3) are on the right. Input (I) 3′-end-labeled SubA6RZ, A6short/TAG, and pre-A6RZ, the ribozyme cleavage products (arrow), edited (E) A6short/TAG and pre-A6RZ RNAs, and chimeras (C) are indicated. **A:** Comparison of cleavage with editing over time. **B:** Comparison of cleavage with editing using various dilutions of SP Sepharose/Mono Q fraction 13 with distilled water.

required for a higher rate of hammerhead ribozyme cleavage (Hendry & McCall, 1996; Clouet-d'Orval & Uhlenbeck, 1997). Coelution of the coupled editing/ribozyme activity with conventional in vitro editing activity as well as the requirements for gRNA and the mitochondrial extract indicates that the activity requires the functioning of the RNA editing complex. The ability to use less mitochondrial extract and the greater proportion of product resulting from the coupled editing/ribozyme activity than with the conventional assay illustrate the greater sensitivity of the former. Indeed, edited RNA can be reliably detected with yields exceeding 0.25%.

Although the efficiency of the coupled editing/ribozyme assay is higher than that of conventional RNA editing, it is much lower than the cleavage of substrate RNA by the synthetic hammerhead ribozyme alone. In addition, the substrate cleavage efficiency by the synthetic ham-

merhead ribozyme, A6RZ, used in this study is lower than that of previously published ribozymes. A kinetic study of this ribozyme showed that its k_{cat} is 0.8 min^{-1} with K_m of 1.8×10^{-7} mol/L (data not shown). This may be due to the relatively large size of the ribozyme (79 nt) that is less efficient than smaller hammerhead ribozymes, as it may assume multiple secondary structures that decrease both the rate of association with the substrate (increased K_m) and catalysis (decreased k_{cat}) as a long target RNA substrate does (Fedor & Uhlenbeck, 1990; Campbell et al., 1997). In addition, helix length and base composition of ribozymes are also determinants for how a particular ribozyme is functioning catalytically (Fedor & Uhlenbeck, 1990; Hertel et al., 1996).

These results show that the catalytic turnover of hammerhead ribozymes can be utilized in conditions of substrate excess (Fedor & Uhlenbeck, 1990; Williams et al., 1992) to increase the sensitivity of in vitro RNA editing assays. The coupled editing/ribozyme assay is convenient because it is performed in a single tube and because the cleavage products are readily and rapidly resolved whereas the edited RNAs require long run times on long, high resolution acrylamide gels to be resolved from their substrates from which they differ by only one or a few nucleotides.

MATERIALS AND METHODS

Production of RNA

The pre-edited ribozyme (pre-A6RZ) was transcribed from the synthetic DNA oligonucleotide 5′-ACATTTGATCTATTG TTTCGTCCTCACGGACTCATCAAAAGTCACAACTTTCCC TTTCTCTCCTCCCCCTAACCTTTCCCCCCTATAGTGAGTC GTATTA-3′ and the gRNA (gA6RZ) from 5′-AAAAAAAAAA AAAAAAATAATTATCATATCACTGTCAAGGGAAAGTTGTG AGGGTGATGAGTCCGTGTATATCCCCCCTATAGTGAGTCG TATTA-3′ after annealing of the T7 promoter oligo, 5′-TAATACGACTCACTATAGGG-3′. The T7 promoter (minus strand) is underlined. Single-strand DNA (0.2 nM) was heated to 90 °C together with 0.2 nM T7 promoter primer for 3 min and cooled to room temperature. In vitro transcription was performed overnight in 100 μL with 40 mM Tris, pH 7.6, 24 mM $MgCl_2$, 2 mM spermidine, 100 mM DTT, 0.01% Triton, 7.5 mM nucleotide mix, and 120 U T7 RNA polymerase (Promega) at 37 °C. The reaction was stopped by adding an equal volume of loading buffer (10 M urea, 0.05% bromophenol blue, and 0.05% xylene cyanole) and the RNA was electrophoresed in a 9% polyacrylamide gel containing 7 M urea. The band of the appropriate size was excised under UV shadow, eluted in RNA elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS), and ethanol precipitated. Substrate RNA (SubA6RZ, 5′-GAUCUAUUGUCUCACA-3′) was synthesized by Oligos. Etc (Wilsonville, Oregon). A6 short/TAG DNA was prepared by PCR as previously described (Seiwert et al., 1996). Synthetic DNA oligonucleotides were gel purified, resuspended in distilled water, and the concentrations were determined by UV absorbance.

Substrate labeling

Substrate RNAs (40 pmol) were 3′ end labeled with 30 μCi [5′-^{32}P]pCp and 45 U T4 RNA ligase in 20 μL reactions containing 50 mM HEPES, pH 7.9, 10 mM $MgCl_2$, 3 mM DTT, 100 μM ATP, and 10 μg/mL BSA at 4 °C for overnight. Radiolabeled RNAs were visualized by autoradiography after electrophoresis through a 9% polyacrylamide gel containing 7 M urea. RNAs of the appropriate size were excised, eluted overnight, and precipitated with ethanol.

Pre-ribozyme deletion editing

1 pmole radiolabeled pre-A6RZ was typically incubated with 2.5 pmol gA6RZ and 7 μL of partially purified editing complex either from glycerol gradients or SP Sepharose/Mono Q columns (Panigrahi et al., 2001) at 28 °C for 3 h as previously described (Seiwert et al., 1996). The reaction was terminated with stop buffer (130 mM EDTA and 2.5% SDS) and RNAs were extracted with phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.0) followed by ethanol precipitation. The sample was resuspended in loading buffer, separated in a 9% polyacrylamide gel containing 7 M urea, and visualized using a phosphorimager.

RNA sequencing

Pre-edited and presumptive edited RNAs that were generated in preparative reactions, using 3′ end labeled pre-edited ribozyme and gRNA, were excised after separation in a 9% polyacrylamide gel containing 7 M urea. The RNAs were subjected to partial digestion by RNase T1 in 5-μL reactions containing 20 mM sodium citrate, pH 5.0, 1 mM EDTA, 4.2 M urea, 0.6 μg/μL *Torula* yeast RNA, 0.02% bromophenol blue, 0.02% xylene cyanole, and 0.3 U enzyme at 55 °C for 15 min. Partial digestions using Phy M RNase (Pharmacia Biotech) were performed according to the manufacturer's instruction. For partial alkaline hydrolysis, 1 pmol 3′-end-labeled RNA substrate was incubated in 10 μL reactions with 50 mM sodium carbonate, pH 9.0, 1 mM EDTA, and 0.5 μg/μL yeast RNA for 7 min at 90 °C.

Cleavage of ribozyme substrate RNA

We incubated 0.5 pmol 3′-end-labeled substrate RNA SubA6RZ with 1 pmol unlabeled pre-A6RZ and 2.5 pmol gA6RZ in the presence of *T. brucei* mitochondrial extract as described in the RNA deletion editing reaction. RNAs were extracted with phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.0), precipitated with ethanol, and were electrophoresed in a 20% polyacrylamide gel containing 7 M urea. Substrate cleavage by the synthetic hammerhead ribozyme was performed in 10-μL reactions with 10 mM $MgCl_2$, 50 mM Tris, pH 7.9, and 10 mM DTT at 28 °C for 60 min. To determine the influence of pre-A6RZ, gA6RZ, and mitochondrial extract on ribozyme activity in the editing reaction, various amounts of synthetic A6RZ were added to reactions containing 1 pmol pre-A6RZ, 2.5 pmol gA6RZ, 0.5 pmol 3′-labeled SubA6RZ, and 7 μL pooled fractions of SP Sepharose/Mono Q fractions. The reaction conditions were exactly the same as those

used for RNA deletion editing except that SubA6RZ cleavage resulted from synthetic A6RZ instead of edited A6RZ.

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