Kinetoplastid RNA editing does not require the terminal 3' hydroxyl of guide RNA, but modifications to the guide RNA terminus can inhibit in vitro U insertion

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ABSTRACT

During RNA editing in kinetoplastid parasites, *trans*-acting guide RNAs (gRNAs) direct the insertion and deletion of U residues at precise sites in mitochondrial pre-mRNAs. We show here that some modifications to the 3' terminal ribose of gRNA inhibit its ability to direct in vitro U insertion. However, we found that gRNAs lacking this moiety in some circumstances support in vitro editing. Thus, the 3' OH is not required. Inhibition resulting from gRNA modification can be overcome by increasing the gRNA–pre-mRNA base-pairing potential upstream of the editing site, suggesting an importance for this interaction to productive processing.

Keywords: guide RNA; *Kinetoplastidae*; RNA editing; trypanosomes

INTRODUCTION

The formation of many mature mRNAs in the mitochondrion of Kinetoplastids relies on a form of RNA processing, termed RNA editing, that inserts and deletes uridylates (Us). This editing of *Trypanosoma brucei* precursor mRNA (pre-mRNA) involves the precise deletion of hundreds and insertion of thousands of Us at numerous sites. Production of the edited sequence requires a class of mitochondrially encoded small RNAs, called guide RNAs (gRNAs), which appear to specify the sequence remodeling through base-pairing interactions with pre-mRNA. The gRNAs have three sequence domains: an anchor sequence near the 5' end, a central informational region, and a 3' oligo (U) tail (see Fig. 1). The anchor can form a 4–14 bp duplex with its cognate pre-mRNA just downstream to the sequence to be edited. The informational region is complementary to the mature edited mRNA (allowing G:U pairs) and thus appears to specify the edited sequence. It is about 35 nt long on average and can specify the editing of 4–10 sites depending on the number of Us

inserted or deleted. A 3' oligo U tail encompassing 5–25 residues is added posttranscriptionally to gRNAs.

The function of the gRNA oligo-U tail is unknown. Two models for the general mechanism of RNA editing proposed that the U tail serves as a reservoir for the inserted and deleted Us (Blum et al., 1991; Sollner-Webb, 1991). They proposed that gRNA/pre-mRNA chimeric molecules in which the gRNA U tail is linked to the pre-mRNA at an editing site are editing intermediates. However, detailed studies of the editing mechanism in vitro strongly support a cleavage/ligation pathway, similar to that originally proposed by Blum et al. (1990), during which the pre-mRNA is endonucleolytically cleaved at the editing site, Us are added or removed at the 3' terminus of the 5' cleavage product, and the two pre-mRNA portions are rejoined by an RNA ligase (Kable et al., 1996; Seiwert et al., 1996; Cruz-Reyes et al., 1998). Unlike the models that invoke a chimeric intermediate, this model does not specifically predict a role for the gRNA terminus and does not require participation of the gRNA 3' hydroxyl (OH) in the reaction chemistry. Nevertheless, modification of the gRNA 3' OH by phosphorylation or periodate oxidation of the terminal ribose prevents a full round of in vitro U deletion (Seiwert et al., 1996). To determine if the terminal 3' gRNA OH is required for insertion editing and to address the pathway by which edited RNA is

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FIGURE 2. Oxidation of the 3'-terminal ribose and a cyclic phosphate group at the gRNA 3' terminus can inhibit in vitro editing. **A:** Structure of modifications to the 3' terminal nucleotide. The 3' terminal U of gA6[14] was modified by periodate oxidation (left) or ribozyme cleavage (right) resulting in 2',3' aldehydes or a 2',3' cyclic phosphate, respectively. Modifications were tested to be >98% efficient. **B:** Autoradiography of electrophoretically separated products of in vitro RNA editing. The 3' end-labeled pre-mRNA substrate was partially digested with RNase T1 (lane 1) or used in in vitro reactions that lacked gRNA (lane 2), included unmodified gA6[14] (lane 3) or included gA6[14] blocked at its 3' terminus by periodate oxidation (lane 4), or the presence of a 2',3' cyclic phosphate (lane 5). The positions of the 3' cleavage product (black dot), input substrate RNA (square), edited RNA (arrow) and chimeras (bracket) are shown alongside schematic illustrations of the respective species. The labeled 3' terminus of substrate RNA is shown in gray. The thick gray line in the cartoon representing chimeras indicates gRNA sequence, which is linked to the downstream cleavage product by a variable number of Us (U_n). Edited RNA with 2 Us inserted is drawn schematically. **C:** Analysis of the samples represented in lanes 2, 3, 4, and 5 of **B** after electrophoresis for a longer period of time (lanes 1, 2, 3, and 4, respectively) to enhance resolution of edited RNA from substrate RNA.

The terminal 3' OH of gRNA is not required for editing

The above results with modified gRNAs suggest that the 3' OH of the gRNA may be required in the editing reaction series, and thus that the cleavage/ligation model for the production of edited RNA could be incomplete. The UTP dependence of insertion editing (Byrne et al., 1996; Kable et al., 1996) as well as its blockage by modifications of the gRNA terminal 3'OH, when taken together, raise the possibility that Us derived from UTP may first be shuttled onto the terminus of the gRNA before insertion into the editing site. We assessed this possibility by using gRNA that was minimally modified at its terminal ribose, as it lacks only

the 3' OH (Fig. 4A). When gA6[14] with a terminal 3' deoxyadenosine (cordycepin) was substituted for unmodified gRNA in an editing reaction, edited RNA was produced (Fig. 4B, lane 3). Previous studies have shown that gRNAs terminating in nucleotides other than U still support U-deletion editing (Seiwert et al., 1996). These results indicate that this is also true for U-insertion editing. Chimeras were not produced, as expected, because the gRNA 3'OH is required for gRNA ligation (Piller et al., 1995; Sabatini & Hajduk, 1995).

As an independent means to assess the possible requirement for the gRNA 3'OH, a "cis-substrate" in which gA6[14] and the pre-mRNA substrate are combined in the same RNA molecule (see Fig. 4C) was tested for its ability to support in vitro editing. The gRNA

FIGURE 3. U addition to the 5' cleavage product. **A:** Electrophoretic analysis of [α - 32 P] GTP 5' end-labeled A6-eES1 substrate RNA digested with RNase T1 (lane 1) or used in in vitro reactions that lacked gRNA (lane 2), or included unmodified gA6[14] (lanes 3 and 4) or gA6[14] modified at its terminus by periodate oxidation (lane 5), or the presence of a 2'3' cyclic phosphate (lane 6). UTP was left out of the reaction in lane 4. A synthetically transcribed RNA corresponding in sequence to the expected upstream cleavage product (A6CP) was 5' end-labeled and used under standard in vitro conditions in reactions that lacked gRNA (lane 8), or lacked both gRNA and UTP (lane 7). The positions of the upstream cleavage product (dot), input substrate RNA (square), and edited RNA (arrow) are indicated. **B:** Aliquots of the same samples as in lanes 2, 3, 5, and 6 of **A**, subjected to gel electrophoresis for a longer period of time to enhance resolution of edited RNA from input (lanes 1, 2, 3, and 4, respectively). The positions of edited RNA (arrow) and substrate RNA (square) are indicated.

sequence is 5' to the pre-mRNA substrate sequence and thus there is no free gRNA 3' end and consequently no gRNA 3' OH. When incubated in a standard in vitro reaction, a product with the mobility expected of edited RNA is generated (Fig. 4D, lane 3). This product was not generated if either mitochondrial extract (Fig. 4D, lane 1) or UTP (Fig. 4D, lane 2) was not included. Comparison of the fragmentation pattern of this species after partial digestion with RNase T1

(Fig. 4E, lane 2) with that of the input RNA (Fig. 4E, lane 1) showed the shift in size of 2 nt of all digestion products corresponding to fragments 5' to the editing site, confirming its identity as edited RNA. The size of the edited product also indicates that it was not generated as a result of cleavage within the oligo (U) portion of the gRNA sequence. These results confirm that the 3' OH of the gRNA is not required for the reaction chemistry of RNA editing and also show that a gRNA terminus per se is not required for editing.

gRNA/pre-mRNA interactions upstream of the editing site

The inhibition of insertion editing observed by some modifications to the gRNA terminus is not due to the involvement of the nucleophilic terminal 3' OH in the reaction mechanism, and therefore must be due to other factors. One factor that has been proposed to be important for productive processing is an interaction between gRNA and substrate RNA upstream of the editing site (Blum & Simpson, 1990). Similar to the observed effect of oxidation or phosphorylation of the terminal gRNA's ribose, sequence modifications that destroy this upstream interaction appear to inhibit editing, but not pre-mRNA cleavage (Kable et al., 1996; Seiwert et al., 1996). As shown in Figures 5A and 5B, elimination of the U tail from the gRNA substantially reduced insertion editing but not pre-mRNA cleavage or chimera formation. The smaller size of the chimeras reflects the lack of the gRNA U tail and their size diversity probably reflects U addition to the gRNA 3' end prior to ligation with the pre-mRNA 3' cleavage fragment. It is possible that some modifications to the gRNA terminus affect the interaction of the gRNA with the upstream portion of the substrate RNA after it is cleaved from the anchor duplex. If true, one would predict that increasing the strength of the RNA–RNA duplex upstream of the editing site might overcome the inhibition observed when the terminus of the gRNA is modified. To test this possibility, a gRNA, termed gA6[14]COMP (see Fig. 5C), which has no U tail but contains a 3' sequence that is predicted to form a more stable duplex with the 5' region of the substrate pre-mRNA than possible with gA6[14] [−30.5 kcal/mol vs. −6.9 kcal/mol (Zuker & Stiegler, 1981)] was used in in vitro processing reactions. This gA6[14]COMP supports in vitro editing even when its 3' end is modified by periodate oxidation (Fig. 5D). Chimeras are not detected with unmodified gA6[14]COMP, presumably reflecting the strong interaction of the terminus of the gRNA with the pre-mRNA. The ability of increased base-pairing stability between the 3' region of gRNA and the 5' region of pre-mRNA to overcome the inhibition of editing by periodate oxidation of gRNA suggests that the inhibition is due to destabilization of this interaction.

FIGURE 4. In vitro editing does not require a free 3' gRNA hydroxyl group. **A:** Structure of terminus of gRNA after addition of 3' deoxyadenosine. **B:** In vitro U insertion directed by a guide RNA terminating in 3' deoxyadenosine (cordycepin). Electrophoretic analysis of 3' end-labeled substrate RNA and processing products from in vitro reactions that lacked gRNA (lane 1) or contained either unmodified gA6[14] (lane 2) or gA6[14] modified by the addition of a 3' deoxyadenosine nucleotide (lane 3). The positions of chimeras (bracket), edited RNA (arrow), and substrate RNA (square) are shown. **C:** The sequence of the *cis*-substrate RNA is shown paired through its anchor duplex and the possible interaction between the U-stretch and the stretch of purine nucleotides in the pre-mRNA portion. **D:** Polyacrylamide gel electrophoresis of products from reactions with 3' end-labeled *cis*-substrate RNA that were complete (lane 3), lacked mitochondrial lysate (lane 1), or lacked UTP (lane 2). The positions of substrate RNA and the predicted position of edited RNA are indicated as in Figure 2. **E:** Comparison of the RNase T1 digestion pattern of the gel-isolated product of the size expected for edited RNA (from a complete reaction like that shown in **B**, lane 3) with the digestion pattern of input substrate RNA.

DISCUSSION

This article reports that, although gRNAs with some particular modifications of their 3' OH groups inhibit editing, the gRNA 3' OH and, indeed, a free gRNA 3' end is not required for editing. The gRNA terminal 3' OH, which has been shown to be critical for in vitro chimera formation (Harris & Hajduk, 1992; Piller et al., 1995; Sabatini & Hajduk, 1995) and has been proposed to be required for the production of edited RNA either by participation in the reaction chemistry of editing (Blum et al., 1991; Cech, 1991; Harris & Hajduk, 1992) or as an essential part of a recognition domain for binding of a component of the editing machinery (Seiwert et al., 1996), is clearly dispensable to editing.

The formation of gRNA/pre-mRNA chimeras was uncoupled from the generation of edited RNA by increasing the potential base pairing between the gRNA and pre-mRNA, using gRNA with 3' terminal cordycepin, or by using a gRNA/pre-mRNA *cis*-substrate. These results strongly support the conclusion that chimeras are not intermediates in the editing reaction series and that Us are added from UTP directly to the terminus of the upstream cleavage product. Hence, the 3' end of the gRNA does not participate in the reaction chemistry of editing. These results confirm that the general mechanism of editing occurs by an enzyme cascade similar to that initially proposed by Blum et al. (1990), and involves endoribonuclease cleavage, uridylylate addition or removal, and RNA ligation (Kable et al., 1996; Seiwert

FIGURE 5. Interactions upstream of the editing site. **A:** Processing directed by a gRNA lacking a U tail. Electrophoretic analysis of products from in vitro processing reactions performed without gRNA (lane 1), with gA6[14]Δtail (lane 2), and with unmodified gRNA (lane 3). The positions where the 3' cleavage product (dot), input substrate RNA (square), edited RNA (arrow), and chimeras (bracket) migrate are shown. Input substrate RNA partially digested with RNase T1 (lane 4) was used as a size marker. **B:** The same samples as in **A**, lanes 1, 2, and 3, subjected to electrophoresis for a longer time to visualize edited RNA. Only the region of the gel in which input substrate pre-mRNA (square), edited RNA (arrow), and chimeras (bracket) migrate is shown. **C:** gA6[14]COMP, a mutated version of gA6[14], in which the U tail has been replaced with a sequence predicted to form a stable upstream duplex, is shown paired to A6-eES1. The interaction based on calculation of the Gibbs free energy for this duplex (−30.5 kcal/mol) is much more favorable than for the duplex formed with the U tail (−6.9 kcal/mol). A modified 3' end of gA6[14]COMP is indicated by a gray square. **D:** Electrophoretic analysis of products resulting from in vitro reactions performed without guide RNA (lane 1), with gA6[14] (lane 2), with gA6[14]COMP (lane 3), or with gA6[14]COMP that had been oxidized by treatment with periodate (lane 4). Substrate RNA, chimeras, and edited RNA are indicated as in Figure 2.

et al., 1996). The data reported here support a role for the gRNA in directing the catalytic steps of editing, but not itself participating in the reaction chemistry.

Additionally, these results also provide insight into possible RNA:RNA and RNA:protein interactions required for in vitro editing. It has been proposed that an interaction between gRNA and substrate RNA upstream of the editing site may be important to the production of edited RNA (Blum & Simpson, 1990; Kable et al., 1996; Seiwert et al., 1996). This is supported by our finding that a mutated version of gA6[14] that lacks a U tail is greatly compromised in its ability to promote the formation of RNA edited by U insertion. gRNA-directed cleavage at the editing site and chimera formation are not inhibited, suggesting that the ligation of the properly processed 5' cleavage product is prevented. The U tail, through its interaction with the stretch of purines upstream of the editing site, may help stabilize the association of the upstream portion of this pre-mRNA (Kable et al., 1996; Seiwert et al., 1996). Consistent with this hypothesis, direct mapping of the contacts between gA6[14] and A6 pre-mRNA indicates that the terminus of the U tail of gA6[14] is preferentially constrained to a region of pre-mRNA about 10–12 nt 5' of

the anchor duplex (Leung & Koslowsky, 1999). In in vitro experiments of U deletion, a reduction in base-pairing potential upstream of the editing site favors chimera formation, whereas an increase in base-pairing potential favors the production of edited RNA (Seiwert et al., 1996). This is consistent with the results obtained with in vitro processing reactions containing (unmodified) gA6[14]COMP, which did not promote the formation of chimeras to a detectable level. Presumably the strong interaction with the pre-mRNA substrate makes the gRNA terminus unavailable for ligation at the editing site.

The underlying basis for the inhibition by the modified gRNAs has not been definitively determined. However, the finding that this inhibition is overcome by increasing the predicted stability of the interactions between the 3' region of gRNA and the 5' region of the pre-mRNA suggest that it is due to destabilization of molecular interactions involving the upstream portion of the pre-mRNA. It is somewhat surprising that the relatively modest modifications at the 3' terminus of the gRNA, such as oxidation of the terminal 2' and 3' hydroxyl groups, addition of a 3' phosphate, or addition of a 2'3' cyclic phosphate, could have such a dramatic

effect, as the 3' end of the gRNA is not essential to editing. The inhibition is not due to the presence of RNAs with oxidized, 3'-phosphorylated, or cyclic phosphorylated ends, as editing occurs in the presence of such RNAs (Kable et al., 1996; Seiwert et al., 1996; see Figs. 3 and 5). These small modifications would not be predicted to appreciably affect the RNA:RNA interactions between gRNA and substrate RNA. It is possible that the initial association of the editing machinery with the RNAs is altered, because cleavage at the editing site with the modified gRNAs seems to be somewhat diminished. However, the pre-mRNA is accurately cleaved and addition of Us to the 3' end of the 5' cleavage product is not blocked. This implies that the inhibitory effect is mainly acting to prevent a later step in the editing reaction series, perhaps RNA ligation. Byrne et al. (1996) reported that in vitro U-insertion editing was not inhibited by periodate oxidized gRNA in *Leishmania tarentolae*. However, gRNA with a 3' hydroxyl may have directed this editing. This is because 30% of the gRNA had a 3' hydroxyl after incubation and the edited product was indirectly assayed (i.e., after reverse transcription and PCR amplification), and thus the ratio of the gRNA with a 3' hydroxyl to edited pre-mRNA substrate is uncertain. Nevertheless, differences in editing between *L. tarentolae* and *T. brucei* cannot be discounted.

The generation of accurately edited RNA requires ligation of the appropriately processed 5' cleavage product with the 3' cleavage product. The observation that the predominant processed 5' cleavage product has one, and not two Us added indicates that either the editing machinery selectively ligates cleavage products with two Us added or that the steady-state level of the 5' cleavage products does not accurately reflect the population of cleavage products that are intermediates in the editing pathway. The 5' portion of the pre-mRNA, subsequent to gRNA-directed cleavage, would be expected to require association with the guiding portion of the gRNA for accuracy of ligation of the appropriately processed 5' cleavage product to the 3' cleavage product. Such an interaction may be facilitated by one or more protein components of the editing machinery. The interactions responsible for maintaining the 5' cleavage product in the appropriate manner may be fairly unstable, judging by the proportion of 3' cleavage products that end up in chimeras when the wild-type gRNA is used in in vitro editing reactions. An RNA:RNA duplex 5' to the editing site may contribute to the stability with which the 5' cleavage product is maintained. However, as many gRNA/pre-mRNA pairs are not predicted to be able to form a stable 5' duplex (Simpson et al., 1998), especially those pairs responsible for editing the 5'-most sites in an editing domain, protein factors are likely to be involved in vivo.

It is possible that some gRNA 3' end modifications are destabilizing because they disrupt the association

of this protein with the gRNA:pre-mRNA duplex (Seiwert et al., 1996). The additional negative charges of a 3' phosphate or a 2'-3' cyclic phosphate of the terminal ribose or structural alteration by periodate oxidation may destabilize molecular interactions. These treatments remove the terminal 2' and 3' OH groups, and thus hydrogen bond donors that might participate in stabilizing interactions with a protein component. This general notion is supported by editing with a gRNA containing a 3' terminal cordycepin, which has an intact ribose ring, a 2' OH potential hydrogen donor, and no terminal negatively charged phosphate group.

In other systems, the chemical structure of nucleic acid termini has been shown to be an important protein-recognition motif. For example, the presence of a cyclic phosphate at the 3' terminus of U6 snRNA disrupts binding of the nuclear antigen La in *Xenopus* oocytes (Lund & Dahlberg, 1992; Terns et al., 1992). Also, replication factor C, a protein identified in humans that activates DNA polymerase δ , binds to telomeric duplexed DNA if the telomeric sequences terminate in a 3' OH and a 5' monophosphate, but not if the 5' monophosphate is replaced by a 5' OH (Uchiumi et al., 1996). Multiple proteins have been identified in mitochondrial extracts of kinetoplastid organisms that associate with the 3' portion of gRNA. These include a 90-kDa protein that depends, for its interaction, on the U tail of gRNA (Read et al., 1994) and a 75-kDa U-binding protein that copurifies with editing activity (Leegwater et al., 1995; Vanhamme et al., 1998). Additionally, a 124-kDa protein present in mitochondrial extracts of *T. brucei* appears to require the normal terminus of gRNA for its interaction (Köller et al., 1994). More investigation is required to address the possibility that this protein could be involved in the stabilization of the 5' duplex.

A proposed model of the effect of molecular interactions upstream of the editing site on the editing of a pre-mRNA is presented in Figure 6. Protein component(s) (referred to as X) of the editosome have been proposed to stabilize the interaction of the gRNA with the portion of the pre-mRNA 5' of the editing site (Seiwert et al., 1996). According to this model, interaction with X is not interfered with by the conservative 3' deoxy alteration, but it is disrupted by phosphorylation or oxidation of the terminus. X is not required for processing if the association of the 5' cleavage product is maintained by an RNA-RNA association, such as the duplex created with gA6[14]COMP, or the intramolecular link in the *cis*-substrate. More work is needed to establish the RNA:RNA and RNA:protein interactions required during RNA editing. Progress is being made toward purification of the molecular machinery that catalyzes RNA editing (the "editosome") (Rusché et al., 1997; Allen et al., 1998; Madison-Antenucci et al., 1998), which may lead to the identification of a protein with the expected binding characteristics.

FIGURE 6. Model of processing events during in vitro incubation of pre-mRNA substrate and gRNA. pre-mRNA is shown in black and gRNA in gray. The productive pathway for U insertion directed by gA6[14] is shown in the left panel. Pre-mRNA is cleaved at the editing site, as directed by the gRNA duplex downstream of the editing site. The portion of pre-mRNA upstream of the editing site may be held in place by base pairing, possibly augmented by unidentified proteins X (shown as a dark curved shape; Seiwert et al., 1996). After pre-mRNA cleavage, Us, donated from UTP, are added to the 5' cleavage product. The cleavage product with two Us added, which can pair with the two As highlighted in the gRNA, is ligated to the 3' cleavage product, generating RNA that is edited at one site. Processing may differ, however, if the interaction between the region 5' of the editing site and the guide RNA is destabilized. Some gRNA modifications, such as periodate oxidation and 3' phosphorylation (indicated by a diamond) may destabilize the pre-mRNA/gRNA interaction, possibly because of a disrupted association with X. After the 5' cleavage fragment is lost, Us may be added to the 5' cleavage fragment, but it is not ligated to the 3' pre-mRNA portion, and thus, a full round of editing is prevented (middle panel). A duplex between gRNA and pre-mRNA 5' of the editing site may obviate the need for stabilization by X, and promote the formation of edited RNA even when the interaction with X is disrupted by 3' gRNA modification (right panel).

MATERIALS AND METHODS

Cell culture and purification of editing activity

Cell culture of procyclic *T. brucei* cells was performed as previously described (strain IsTaR 1.7a; Stuart et al., 1984) and mitochondria were isolated by the method of Harris et al. (1990). Editing activity was enriched by glycerol gradient sedimentation as previously described (Seiwert et al., 1996).

In vitro editing

In vitro editing assays were performed as previously described (Kable et al., 1996). Briefly, 0.25 pmol pre-edited mRNA

substrate and 2.5 pmol gRNA were incubated for 2 h at 28 °C with 10 μ L (~10 μ g protein) of a 20–35S glycerol gradient fraction in a final reaction volume of 30 μ L containing 50 μ M UTP, 37 mM KCl, 10 mM MgOAc, 25 mM Tris-HCl (pH 8.0), 3 mM ATP, 0.5 mM dithiothreitol, 1 mM EDTA, 5 mM CaCl₂, and 1 μ g *Torula* yeast RNA. Reactions were stopped by the addition of 70 μ L NET-2 [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.05% Nonidet P-40] and 1 μ g glycogen. The sample was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The RNA is precipitated by the addition of 0.1 vol 3 M sodium acetate (pH ~5), and 2.5 vol ethanol. Samples were electrophoresed on 9% polyacrylamide, 7 M urea, 1 $\times$ Tris-borate-EDTA gels as described in Stuart et al. (1998) and visualized by autoradiog-

raphy. In the experiment described in Figure 5D, 62.5 pmol of unlabeled A6-eES1 were added to each of the samples, which were heated at 90 °C for 1 min, before loading. Presumably because of the strong complementarity between gA6[14]COMP and A6-eES1, this addition of competitor RNA was required to resolve the substrate RNA and edited RNA into discrete bands.

In vitro T7 transcription templates

Transcription templates for A6-eES1 and gA6[14] were synthesized as described in Kable et al. (1996). A transcription template for gA6[14] Δ tail was synthesized as described in Siewert et al. (1996). The transcription template for A6CP was constructed by PCR of A6-eES1 DNA with primers R1T7 (5'-CGGCGGAATTCTGTAATACG ACTCACTATAG-3') and 5'ES2 (5'-CACAACTTTCCCTTCTTCT-3'). A template for the *cis*-substrate RNA was constructed by mutagenic PCR of A6-eES1 DNA (Kable et al., 1996) with the TQ5'A6 (5'-AGTACTCGAGGAAAGGTTAG-3') and A6RT2 (GAT GCC AGG TAA GTA TTC TAT AAC TCC-3') primers, thereby adding a *TaqI* restriction site at the 5' end of the template. The same restriction site was added to the 3' end of the gRNA template for gA6[14] by mutagenic PCR with R1T7 and TQ3'gA6 (5'-AGTACTCGAAAAAAAAAAAAAAAAATA-3') primers. These two PCR products were digested with *TaqI* (Gibco BRL) and then ligated using T4 DNA ligase (Gibco BRL). The gel purified ligation product was used as a template for PCR amplification with oligos R1T7 and A6RT2, and the product was used as a template for transcription by T7 RNA polymerase. A transcription template for generation of gA6[14] terminating in a VS ribozyme-target sequence (ryzmA6[14]) was generated by mutagenic PCR of the template for gA6[14] with primers R1T7 and gA6TailVSzyme (5'-GGATCCTCGGGGCGACGACGCCCTTAAAAAAAAAAAAAAA AAAATAATTATC-3'). A template for gA6[14]COMP was generated by mutagenic PCR of gA6[14] DNA with primers R1T7 and UCOMP (5'-GGGGGAGGAGAGAAGAAAATATC ATATC-3').

RNA gel-purification and labeling reactions

Products from T7 RNA polymerase transcriptions (T7 Mega-Short kit by Ambion) were gel purified as previously described in Stuart et al. (1998). A6-eES1 and the *cis*-substrate RNA were radiolabeled at their 3' ends by T4 RNA ligase-mediated ligation of [5'-³²P]pCp. A6-eES1 and A6CP were 5' end labeled using [α -³²P]GTP and guanylyltransferase (Gibco BRL), the former after periodate oxidation (Seiwert et al., 1996). All labeled RNAs were purified to homogeneity on 9% polyacrylamide, 7 M urea, 1 $\times$ Tris-borate-EDTA gels (Stuart et al., 1998).

3' end modifications

The terminal 2' and 3' hydroxyls of A6-eES1 and gA6[14] were oxidized by periodate treatment as described (Seiwert et al., 1996). A cyclic 2'3' monophosphate was placed at the terminus of gA6[14] by incubation of the PCR product rzymgA6[14] in a T7 transcription reaction containing the A-3 plasmid that encodes the VS ribozyme (Guo & Collins, 1995)

and had been digested with *SspI* (Gibco BRL). A 3' deoxy A residue was added to the terminus of wild-type gA6[14] by incubating 1 nmol of gA6[14] at 30 °C for 1.5 h with 10 mM cordycepin triphosphate (Sigma) and 3,000 U yeast poly(A) polymerase in buffer supplied by the manufacturer (USB). The RNA was then precipitated with ethanol and gel purified as above. The resultant RNA was incubated with 1,000 U of poly(A) polymerase as above but with 5 mM ATP rather than cordycepin triphosphate to increase the size of RNA lacking cordycepin. The RNA was again precipitated and run on a 10% denaturing polyacrylamide gel as described above and the species with the expected size of wild-type gA6[14] with a terminal cordycepin was isolated, eluted, and ethanol precipitated as before and quantified by spectrophotometry. The efficiency of the 3' end modifications was greater than 98%, as judged by [5'-³²P]pCp labeling (Seiwert et al., 1996).

RNase T1 digestion

RNAs that were partially digested by RNase T1 (which cleaves 3' of guanylate residues, leaving a 3' phosphate) were prepared as size markers. End-labeled RNA (0.25 pmol) was incubated with 0.3 U RNase T1 (Boehringer Mannheim) for 15 min at 55 °C in a final volume of 6 μ L that contained 20 mM sodium citrate (pH 5.0), 1 mM EDTA, 4.2 M urea, 0.6 μ g/ μ L *Torula* yeast RNA, 0.02% bromophenol blue and 0.02% xylene cyanole.

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