Interactions of mRNAs and gRNAs involved in trypanosome mitochondrial RNA editing: Structure probing of a gRNA bound to its cognate mRNA

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ABSTRACT

Expression of mitochondrial genes in *Trypanosoma brucei* requires RNA editing of its mRNA transcripts. During editing, uridylates are precisely inserted and deleted as directed by the gRNA template to create the protein open reading frame. This process involves the bimolecular interaction of the gRNA with its cognate pre-edited mRNA and the assembly of a protein complex with the enzymatic machinery required. While a considerable amount of work has been done identifying the protein components of the editing complex, very little is known about how a functional editosome is assembled. In addition, the importance of RNA structure in establishing a functional editing complex is poorly understood. Work in our lab suggests that different mRNA/gRNA pairs can form similar secondary structures suggesting that a common core architecture may be important for editosome recognition and function. Using solution structure probing, we have investigated the structure of the initiating gRNA, gCYb-558, in the mRNA/gRNA complex with pre-edited *apocytochrome b* mRNA. Our data indicate that the stem-loop formed by the guiding region of the gRNA alone is maintained in its interaction with the pre-edited message. In addition, our data suggest that a gRNA stem-loop structure is maintained through the first few editing events by the use of alternative base-pairing with the U-tail.

Keywords: *Trypanosoma brucei*; *apocytochrome b*; post-transcriptional processing; RNA–RNA interactions; RNA structure; RNA editing

INTRODUCTION

In kinetoplastid protozoa, guide RNAs (gRNAs) serve as templates for the precise insertion and deletion of uridylates during RNA editing of the mitochondrial mRNA transcripts. This post-transcriptional process produces translatable mRNAs by creating the open reading frames as well as initiation and termination signals. In addition, there is a transcript-specific mechanism that regulates editing during the life cycle of the parasite. The gRNAs are key components of the reaction, as they provide the information for the nucleotide (nt) alterations and direct the cleavage and ligation events (Simpson et al. 2004; Stuart et al. 2005). Guide RNAs have an average length of 50–70 nt

and consist of three functional elements. Contained within the 5' end of gRNAs is a short sequence known as the gRNA anchor, a 5- to 21-nt region that base-pairs with a particular mRNA just 3' of the editing domain. The second element, the guiding region, serves as a template for the editing process and is complementary (allowing G–U base pairs) to the mature mRNA. Finally, at the 3' end of the gRNA is a poly-uridylyate tail (U-tail) that is added post-transcriptionally (Blum and Simpson 1990; Blum et al. 1990).

The latest model for RNA editing in trypanosomes involves >20 proteins that form a complex structure called the editosome around an mRNA/gRNA pair (Simpson et al. 2004; Stuart et al. 2005). The insertion and deletion of uridines occur through successive rounds of enzymatic reactions coordinated by the editosome and templated by the gRNA (Blum and Simpson 1990; Blum et al. 1990; Seiwert and Stuart 1994; Adler and Hajduk 1997). Significant progress has been made in identifying proteins involved in the editing reaction, including both proteins in the complex as well as proteins that appear to be

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accessory factors that transiently act during the editing process (Koller et al. 1997; Missel et al. 1997; Madison-Antenucci et al. 1998; Vanhamme et al. 1998; Hayman and Read 1999; Aphasizhev and Simpson 2001; McManus et al. 2001; Panigrahi et al. 2001a,b; Rusche et al. 2001; Schnauffer et al. 2001; Aphasizhev et al. 2002; Aphasizhev et al. 2003a,b; Panigrahi et al. 2003a,b; Pelletier and Read 2003). However, despite this progress, very little is known about how the editing complex is assembled onto specific RNAs. No conserved sequence domains have been found in mRNAs, and the only conserved sequence domain shared between the gRNAs is the U-tail. Previous work in this laboratory suggests that different mRNA/gRNA pairs can form similar structures (Leung and Koslowsky 1999), and we believe that structure recognition may be important for efficient editosome assembly with the mRNA/gRNA pair.

In previous studies, we used a RNA folding program to look for conserved structures between three different mRNA/gRNA pairs. The predicted secondary structures for these mRNA/gRNA pairings incorporated a distance constraint based on photoaffinity cross-links (Leung and Koslowsky 1999). Further structure analyses for the *apocytochrome b* mRNA/gRNA complex were undertaken to look at mRNA/gRNA structure as editing progresses up through the third editing site. The predicted secondary structures for these mRNA/gRNA pairings are based on photoaffinity cross-linking studies of the 5' and 3' ends of NgCYb-558 that were mapped along 5'CYbUT (first 88 nt of CYb mRNA), 5'CYbPES1T (edited through site 1), and 5' CYbPES3T (edited through site 3) (Leung and Koslowsky 2001b). In addition, a solution structure probing study of 5'CYbUT while paired with NgCYb-558 or NgCYb-558sU (no U-tail) was done (Leung and Koslowsky 2001a). With the data from these studies, we proposed a secondary structure model that includes the following features: a gRNA/mRNA anchor helix, a U-tail/mRNA helix, and a gRNA stem-loop (Fig. 1A; Leung and Koslowsky 1999, 2001a,b). The predicted structure of the gRNA interaction with the partially edited mRNA was particularly interesting

because it suggests that the U-tail could function to maintain the gRNA stem-loop during the first few editing events (Fig. 1B). The focus of this article is on the solution structure probing of the secondary structure of the gRNA, NgCYb-558, paired with unedited and partially edited mRNAs, as we would like to understand how the gRNA structure may be changing during the early steps of the editing process. In addition, we have also probed the structure of the partially edited mRNA, CYbPES3, to corroborate our gRNA structure data. Our data indicate that the stem-loop formed by the guiding region of the gRNA alone is maintained in its interaction with the pre-edited message as predicted in the computer models. In addition, our data support the predicted structure for the gRNA interaction with its partially edited mRNA in that the gRNA stem-loop structure appears to be maintained through the first few editing events by the use of alternative base-pairing with the U-tail.

RESULTS AND DISCUSSION

Cross-linked RNAs used for solution structure probing

Editing of the *apocytochrome b* mRNA is developmentally regulated with the insertion of 34 uridylates at 13 sites near its 5' end. The editing process that creates the CYb initiation codon occurs preferentially during the procyclic (insect) and stumpy bloodstream stages of the trypanosome life cycle (Feagin et al. 1987; Feagin and Stuart 1988). In these experiments, we used modified versions of 5'CYbUT (Koslowsky et al. 1992, 1996). CYbU (U, unedited) is identical in sequence to 5'CYbUT except for the deletion of 12 nt at the 5'-most end (Fig. 2A) and is composed of 88 nt from the 5' end of the *apocytochrome b* mRNA (beginning at +13) including all 13 editing sites as well as a 24-nt vector tag at the 3' end. The partially edited substrate, CYbPES3 (PES, partially edited through site 3), is identical to CYbU except for the six uridines added so that the first three editing sites are fully edited (Fig. 2B). Our gRNA

construct NgCYb-558 (Fig. 2C) is very similar to wild-type gCYb-558 and two other redundant gRNAs, gCYb560A, B. All three gRNAs can act as the editing initiating gRNA that direct the editing of the first seven editing sites (Riley et al. 1994). NgCYb-558 is 59 nt long including a 15-nt U-tail that is added via ligation with T4 DNA ligase and a bridging deoxyoligonucleotide (Moore and Sharp 1992; Leung and Koslowsky 2001a).

The secondary structure of gCYb-558 was reported by Schmid et al. (1995) and described as two thermodynamically unstable hairpin loops separated by a single-stranded region. The smaller

FIGURE 1. The predicted models for mRNA/gRNA complex secondary structure have three helices: an mRNA/gRNA anchor helix, a gRNA stem-loop within the guiding region, and a U-tail/mRNA duplex. (A) CYbU-NgCYb-558 pair with the first editing site just 5' (mRNA) of the anchor indicated by ES1. (B) CYbPES3-NgCYb-558 pair with the fourth editing site just 5' (mRNA) of the anchor indicated by ES4.

FIGURE 3. Structure probing of the gRNA, NgCYb-558. Each solution structure probing gel has three sections showing the digestion pattern of NgCYb-558 alone, NgCYb-558 cross-linked to CYbU (+CYbU), and NgCYb-558 cross-linked to CYbPES3 (+CYbPES3). Digestion times in minutes are shown at the top. RNase T1 and U2 ladders with nucleotide numbers are indicated. A line diagram indicating the anchor (bold line), extended anchor (double line), guiding region (thin line), and U-tail of the gRNA is shown next to each digestion pattern. (A) Mung bean nuclease gel, single-strand specific. (B) RNase T2, single-strand specific. (C) RNase T1, single-strand guanosine specific. (D) RNase V1, double-strand specific.

the gRNA was cross-linked to its cognate mRNA (+CYbU and +CYbPES3), the anchor duplex formed and was no longer sensitive to the single-strand-specific RNases. The RNase V1 (V1) sensitivity pattern generally complemented

these data. We did observe two V1 cleavages in the unpaired gRNA at positions A12 and C13 (Fig. 3D), located near the end of the anchor region, that were also sensitive to the single-strand-specific nucleases.

FIGURE 4. Structure probing of the partially edited CYb mRNA, CYbPES3. Each solution structure probing gel has three sections showing the digestion pattern of CYbPES3 alone, CYbPES3 cross-linked to NgCYb-558sU (no U-tail), and CYbPES3 cross-linked to NgCYb-558. Digestion times in minutes are shown at the *top*. RNase T1 and U2 ladders with nucleotide numbers are indicated. A diagram of the mRNA is next to each digestion pattern showing the orientation 3' to 5' with the extended anchor (bold line). (A) Mung bean nuclease gel, single-strand specific. (B) RNase T2, single-strand specific. (C) RNase T1, single-strand guanosine specific. (D) RNase V1, double-strand specific.

While V1-sensitive cleavages were clearly visible within the anchor duplex of the paired gRNA (+CYbU; Fig. 3) and within the extended anchor duplex (+CYbPES3; Fig. 3), it was interesting that the cleavages were concentrated in the

5' half (mRNA orientation) of the anchor duplex. Lack of cleavage in the G1–A7 region may be due to the presence of the cross-link. For NgCYb-558 paired with CYbU, V1 cleavages within the anchor were surprisingly weak and

FIGURE 5. Chemical structure probing of the partially edited CYb mRNA, CYbPES3. The diethyl pyrocarbonate (DEPC) chemical structure probing gel has three sections showing the difference in digestion pattern of CYbPES3 alone, CYbPES3 cross-linked to NgCYb-558sU (no U-tail), and CYbPES3 cross-linked to NgCYb-558. Digestion times in minutes are shown at the top. RNase T1 and U2 ladders with nucleotide numbers are indicated; an alkaline hydrolysis ladder (-OH) was included as well. A diagram of the mRNA is next to each digestion pattern showing the orientation 3' to 5' with the extended anchor (bold line).

limited to A8–C13 (Fig. 3D). This region was also very poorly cleaved in the NgCYb-558/CYbPES3 pairing, where the anchor is extended to U26. In this extended anchor, strong V1 cleavages were observed in the 3' most end of the duplex (A21–U26; Fig. 3D). The limitation of strong RNase V1 cleavage to the A21–U26 region of the extended anchor duplex was unexpected and suggests that most of the anchor duplex may be protected by tertiary interactions.

Structure of NgCYb-558 guiding region

Secondary structure analyses of several unpaired gRNAs indicate that the guiding regions (nucleotides between the 5' anchor and 3' U-tail) form a gRNA stem-loop element (Schmid et al. 1995). In our digests of unpaired NgCYb-558, we also observe the presence of a gRNA stem-loop (Fig. 6). Weak but consistent single-strand-specific cleavages at nt A14–A20 indicate that the area adjacent to the anchor (containing the template for the first few editing sites) is single-stranded until nt A21 (Fig. 3). Distinct cleavages using the single-strand-specific RNases were also observed for nt A27–A36. The lack of single-stranded specific cleavages in the U22–U26 and G37–G39 flanking

regions and weak cleavages of A41 to U42 suggested that the U22–U42 region base-pairs to the G37–U42 region and forms a stem with a large 10-nt terminal loop (A27–A36) (Fig. 3). The presence of this stem-loop is confirmed by the pattern of V1 sensitive cleavages of nt U22–26 and nt G37–U42 (Fig. 3D). When the gRNA was paired with the unedited mRNA (+CYbU) or the partially edited CYbPES3, the patterns of nuclease cleavages for the guiding region were almost identical to those observed in the gRNA alone (Fig. 3). This was particularly surprising for the NgCYb-558/CYbPES3 pair, as the anchor duplex has doubled in length (26 bp vs. 13 bp) and has incorporated the U22–U26 5'-stem region from our gRNA stem-loop. All three gRNA structures (gRNA alone, paired with CYbU, and paired with CYbPES3) showed a pattern of single-strand RNase sensitivity in the A27–A36 region flanked by RNase V1 sensitivity in the A21–U26 and G38–A42 regions (Figs. 3, 6). While the RNase cleavage patterns of the guiding region were very similar, we did see some consistent differences. For the NgCYb-558/CYbU pair, weak but consistent MBN and RNase T2 cleavage sensitivity could be seen in A15 to U18. In addition, both G17 and G19 are accessible to RNase T1, suggesting the presence of a 5- to 6-nt bulge between the anchor duplex and the gRNA guiding region stem (Fig. 3A–C). The corresponding region is completely protected when the gRNA is paired with CYbPES3, as would be expected with the extension of the anchor duplex to U26. The NgCYb-558 paired to CYbPES3 also showed a subtle difference in the pattern of RNase V1 cleavage in the A21–U26 region (Fig. 3). Both the gRNA alone and gRNA paired with unedited CYb had stronger RNase V1 cleavages at U22 and C25 when these nucleotides are forming the initial gRNA stem-loop. When the gRNA is paired with CYbPES3 and nt U22 and C25 have become part of the anchor duplex, the U22 V1 cleavage appears to be intense, while the other sites in this region having relatively equal cleavage intensities (Fig. 3D). We also observed a slight increase in the RNase V1 sensitivity in the U33–G37 region for the gRNA when paired to CYbPES3 (Fig. 3D, panel 3). In addition, the single-strand-specific cleavages in the A27–A36 region were consistently less intense for the gRNA when paired with CYbPES3 (Fig. 3). The cleavage patterns of the gRNA were incorporated into summary figures based on computer modeling of the predicted secondary structure of the gRNA alone (Fig. 6A) as well as the mRNA/gRNA complex with both unedited and partially edited mRNA (Fig. 6B,C). Our cleavage patterns support the computer predicted models and illustrate the change in gRNA structure produced by editing of the mRNA. The unpaired gRNA forms a gRNA stem-loop that is maintained when the gRNA pairs with the pre-edited mRNA (Fig. 6A,B). Editing of the first three sites extends the anchor duplex by 13 bp, incorporating one side of the gRNA stem into the anchor helix. The nucleotides that were located within the terminal loop remain single-stranded, but are now located

FIGURE 6. Summary figures from solution structure probing gels that show predicted secondary structures with cleavages from all four nucleases and DEPC chemical. Three sizes of symbols indicate the intensity of cleavage. Circles indicate mung bean nuclease (single-strand specific) cleavages, squares indicate RNase T2 (single-strand specific) cleavages, crosses indicate RNase T1 (single-strand guanosine specific) cleavages, cylinders indicate DEPC (single-strand adenosine specific), and triangles indicate RNase V1 (double-strand specific) cleavages. (A) NgCYb-558 alone. (B) CYbU+NgCYb-558 complex. (C) CYbPES3+NgCYb-558 complex.

within a bulge region between the anchor duplex and the new gRNA stem-loop. However, a new gRNA stem-loop is formed by alternative base-pairing of A36–A41 with part of the U-tail (Fig. 6C).

Structure of CYbPES3

In order to corroborate the structure of the gRNA interaction with the partially edited CYbPES3, we also probed the secondary structure of CYbPES3. For comparative purposes, we investigated the structure of CYbPES3 alone as well as its structure when paired with NgCYb-558 with and without the U-tail. In previous work, the structure of the unedited CYb mRNA alone was found to be a strongly base-paired stem-loop with the first three editing sites contained in a terminal 5-nt loop (A35–U41) (Leung and Koslowsky 2001a). Editing of the first three sites appears to

add an additional six uridines to the terminal loop but otherwise does not significantly change the structure found within the stem region (Figs. 4, 5, 7). This is shown (Figs. 4, 5) by MBN (U42–A36), T2 (U42–U38), and T1 (G41, G43) cleavages within the terminal loop (Figs. 4, 7). RNase V1 data complement the data from the single-stranded nucleases (Fig. 4D, 7).

The structure of CYbPES3 cross-linked to its cognate gRNA (NgCYb-558) was much more difficult to interpret. Pairing of the partially edited mRNA with its gRNA clearly changed the structure of the mRNA, making it much more susceptible to the single-strand-specific nucleases (Fig. 4). Interestingly, while the anchor duplex region was mostly protected, we did observe distinct cleavages in the U39–G43 regions for all three single-strand-specific nucleases (Fig. 4). This corresponds to a run of four G:U base pairs within the anchor duplex created by the second and third RNA editing events. Within this region, the RNase V1 analyses did complement the single-strand-specific nucleases with distinct V1 cleavages localized in two regions flanking the U39–G43 region: A33–A37 and U47–A54. No corresponding single-strand nuclease sensitivity was observed when probing the gRNA; however, this region was also protected in our RNase V1 analyses, suggesting that tertiary interactions may be restricting nuclease access. It has been proposed that G–U

base pairs may locally destabilize helical regions, widening the major groove (Chow et al. 1992).

Upstream of the anchor duplex, nt A14–G27 were also much more sensitive to the single-stranded nucleases, indicating that interaction with the gRNA opens up the stable stem-loop structure of the mRNA (Figs. 4, 6C). Surprisingly, the run of six adenosine residues (A28–A32), located just 5' of the anchor duplex, was clearly protected from cleavage by both MBN and RNase T2 (Fig. 4). RNase V1 cleavages were observed within this region. However, RNase V1 cleavages were also observed for nt A14–G27, which also show distinct sensitivity to the single-stranded nucleases (Fig. 4D). The ability of RNase V1 to cleave stacked, highly structured regions and junctions found between two helices may explain some of the overlapping nuclease sensitivity (Lowman and Draper 1986). In an effort to resolve our conflicting nuclease results, additional

FIGURE 7. Summary figure of CYbPES3 alone from solution structure probing gels that show predicted secondary structure with cleavages from all four nucleases and DEPC using three sizes of symbols to indicate intensity of cleavage. Circles indicate mung bean nuclease (single-strand specific) cleavages, squares indicate RNase T2 (single-strand specific) cleavages, crosses indicate RNase T1 (single-strand guanosine specific) cleavages, cylinders indicate DEPC (single-strand adenosine specific), and triangles indicate RNase V1 (double-strand specific) cleavages.

structure probing experiments, using diethyl pyrocarbonate (DEPC), were performed (Fig. 5). This chemical was chosen because it is reactive with purines and because their reactivity can be monitored via chain scission, a necessity with our cross-linked substrates. DEPC is very sensitive to the stacking of base rings; therefore, adenines within a helix are not reactive. In these experiments, while the anchor is clearly protected, nt A26–A32 are reactive with DEPC, suggesting that this region is in fact single-stranded and is not highly structured (Figs. 5, 6C). Adenosine residues located further upstream (A14–A21) are not reactive, suggesting that this region is helical in nature (Figs. 5, 6C).

In comparing the cleavage patterns when CYbPES3 was paired to its gRNA with and without (NgCYb-558sU) a U-tail, we saw very little difference. Previous cross-linking experiments indicate that the U-tail does interact with the purine-rich region around G25; however, in these experiments the cross-linking efficiency was low, indicating that this interaction is not stable.

CONCLUSIONS

In this study, we investigated the structure of NgCYb-558 alone and its structure when paired with its cognate

unedited mRNA or partially edited mRNA. From our previous structure studies (Leung and Koslowsky 1999), the computer-predicted models suggested that the gRNA stem-loop (A29–U42) is maintained when the initial mRNA/gRNA complex is formed (Fig. 6B). In addition, 3' cross-linking studies with partially edited substrates suggested that a gRNA stem-loop structure is preserved as editing proceeds through the third editing site via interaction with the U-tail (A36–U53) (Fig. 6C). The results of this study support the predicted models and suggest that the gRNA stem-loop may be an important structural component of the initial editing complex. We hypothesize that the formation of multiple helices surrounding the first few editing sites may allow for tertiary interactions that help stabilize the initial gRNA/mRNA interaction. Increasing the amount of editing increases the number of base pairs within the anchor duplex, and this may negate the need for tertiary interactions during the later stages of the gRNA interaction. Alternatively, the multiple helices may limit the number of potential gRNA/mRNA interaction sites, and this may help increase the accuracy of the editing process. This suggests that the U-tail may enhance the editing process in a number of ways. The U-tail is added post-transcriptionally and has been shown to be unnecessary for *in vitro* cleavage of editing substrates (Kable et al. 1996; Seiwert et al. 1996; Burgess et al. 1999). However, sequence modifications that disrupt upstream base-pairing interactions severely diminish formation of edited product. Likewise, sequence modifications that increase the stability of the upstream duplex are known to increase the efficiency of *in vitro* editing, suggesting that the upstream gRNA/mRNA interaction is important (Burgess et al. 1999; Kapushoc and Simpson 1999; Igo et al. 2000; Cruz-Reyes et al. 2001). Previous work in our laboratory using gel shift analyses indicates that the U-tail is very important for stabilization of the interaction of some mRNA/gRNA pairs (Koslowsky et al. 2004). RNAi studies have also shown that when the RNA editing terminal uridylyl transferase (RET1), responsible for adding the U-tail to gRNAs, is down-regulated, there is a decrease in edited mRNAs and inhibited growth of the trypanosome (Aphasizhev et al. 2002). This suggests that the U-tail is necessary for *in vivo* editing (Gott 2003; Nebohacova et al. 2004). We know that the RNA editing process must involve the formation and disruption of intramolecular helices to form a number of intermediate complexes (Nordgren et al. 2001). Guide RNAs appear to be able to take advantage of U-tail flexibility through the ability of uridines to base-pair with both purine bases. By employing an uridylylate tail, the gRNA may increase mRNA/gRNA complex stability, without hampering the U-tail migration needed within the complex during the editing process. In addition, the editing process may take advantage of the special properties of G–U base pairs. The G–U base pair has a distinctly different geometry and may locally destabilize helical regions creating

protein recognition elements (Chow et al. 1992; Batey and Williamson 1996). Editing also creates G–U base pairs, raising interesting possibilities for protein recognition of the edited mRNA/gRNA complex and the recruitment of RNA helicases necessary for multiple gRNA utilization.

We have reported here the structure of the naked RNA for the dynamic CYb mRNA/gRNA complex. It will be interesting to discover in future studies how much these RNA structures and dynamics change in the presence not only of the editosome proteins but also with the accessory factors assembling and disassembling from the mRNA/gRNA complex.

MATERIALS AND METHODS

Oligodeoxyribonucleotides

All oligodeoxynucleotides (Table 1) were ordered from Integrated DNA Technologies, Inc.

Oligoribonucleotide

Oligoribonucleotide was ordered from Dharmacon Research, Inc. U₁₅-tail: 5'-UUUUUUUUUUUUUUU-3', 15 nt.

DNA templates and RNA synthesis

DNA templates of the partial mRNAs: CYbU and CYbPES3 templates were created by ligating two oligodeoxyribonucleotides, the 5' (5'ShortCYb and 5'CYbPES3Short) (Table 1) and 3' (3'ShortCYb and 3'CYbPES3Short) (Table 1) halves of the molecules using T4 DNA ligase (Roche) and a complementary DNA bridge (ShortCYb bridge and CYbPES3ShortDNAbridge) (Table 1; Moore and Sharp 1992). The ligated templates were then PCR amplified with T7 and Big SK oligonucleotides (Table 1) and Taq polymerase (Promega) as per the manufacturer's directions. The gRNA constructs (NgCYb558 and NgCYb558(sU)) (Table 1) have been previously described (Leung and Koslowsky 2001b). The mRNA transcripts were synthesized either by T7 RNA polymerase (Ribomax, Promega) according to the manufacturer's directions or in the presence of 5 mM guanosine as described

previously (Leung and Koslowsky 2001a). NgCYb-558 and NgCYb-558sU were synthesized using a T7 RNA polymerase using a Ribomax kit (Promega) in the presence (Burgin and Pace 1990; Harris and Christian 1999) or absence of Guanosine 5'-monophosphorothioate (GMPS) (Biolog Life Science Institutes or Harris Laboratory). If GMPS was incorporated at the 5' end of the NgCYb-558 transcripts (with and without U-tail), then 20 μ Ci of [³²P]rATP (Perkin-Elmer) was added for visualization, the rGTP was reduced to 80 nmol, and 8 μ L of 100 mM MgCl₂ was added per 80 μ L reaction. The mRNAs and gRNAs were gel-purified on 6% and 15% 7 M urea acrylamide gels, respectively (Leung and Koslowsky 2001a). The oligoribonucleotide U₁₅-tail was phosphorylated using T4 kinase (Invitrogen) and ligated to NgCYb-558sU using 25 U T4 DNA ligase (Roche) and the NgCYb-558(sU) bridge (Table 1; Moore and Sharp 1992; Leung and Koslowsky 2001a).

RNA cross-linking and end labeling

Attachment of p-azidophenacyl bromide (Sigma) to GMPS NgCYb-558 and GMPS NgCYb-558sU transcripts as well as cross-linking of gRNAs and mRNAs was done as described previously (Leung and Koslowsky 1999, 2001a, b). The mRNA (50 pmol of free and 5 pmol of cross-linked mRNA) was 5'-end-labeled using 50–100 μ Ci of [γ -³²P]rATP (Perkin-Elmer) and T4 Kinase (Invitrogen) as per the manufacturers' directions and gel-purified as above. The gRNA was 3'-end-labeled by ligating 0.5 nmol of U₁₅-tail to 130 μ Ci of [5'-³²P]cytidine 3',5'-bisphosphate (pCp) (Perkin-Elmer) using T4 RNA ligase (New England Biolabs) as per the manufacturer's directions. The labeled U₁₅-tail was ethanol-precipitated, 5'-phosphorylated with T4 Kinase (Invitrogen) according to the manufacturer's directions, and ligated to NgCYb-558sU (no U-tail) using 25 U high concentration T4 DNA Ligase (Roche) and the NgCYb558(sU) bridge (Table 1) as previously described.

Structure-specific enzymatic probing

In these experiments, 150–200 kcpm of labeled RNA sample and 10 μ g of tRNA were heated to 50°C, cooled slowly, and incubated at 27°C for 20 min. Enzyme was then added to the sample (0.1 U RNase T1 [Industrial Research, LTD], 0.18 U RNase V1 Cobra

TABLE 1. List of oligodeoxyribonucleotides

Name	Sequence	Length (nt)
Big SK	5'-GGCCGCTCTAGAACTAGTGG-3'	20
T7	5'-AATTAATACGACTCACTATAG-3'	22
NgCYb558(sU)	5'-TTATTCCTTTTATCACCTAGAAATTCACATTGTCTTTAATCCCTATAGTGAGTCGTATTAATT-3'	65
NgCYb558	5'-AAAAAAAAAAAAAAAAATTATTCCTTTTATCACCTAGAAATTCACATTGTCTTTAATCCCTATAGTGAGTCGTATTAATT-3'	80
gCYb558(sU) bridge	5'-AAAAAAAAAAAAAAAAATTATTCCTTTTATCACCT-3'	32
5'ShortCYb	5'-CTTTCTTTTTTCTCCGCTTTTATATAAAATTTATAACCTATAGTGAGTCGTATTAATT-3'	59
3'ShortCYb	5'-CCGCTCTAGAACTAGTGGATCCATATTTCTATATAAAACCTGACATTAAGACCT-3'	58
ShortCYb bridge	5'-GGAGAAAAAAGAAAGGGTCTTTTAATGTCAG-3'	31
5'CYbPES3Short	5'-CAATTTCTTTTTTCTCCGCTTTTATATAAAATTTATAACCTATAGTGAGTCGTATTAATT-3'	63
3'CYbPES3Short	5'-CCGCTCTAGAACTAGTGGATCCATATTTCTATATAAAACCTGACATTAAGACAACA-3'	61
CYbPES3ShortDNAbridge	5'-GGAGAAAAAAGAAATTTGTGTGTCTTTAATGTCAG-3'	37

Venom [Pierce_{MB}], 0.4 U RNase T2 [Invitrogen], 1 U MBN [GibcoBRL]). For RNases T1 and T2, aliquots were taken at 5, 10, and 15 min of digestion, and for RNase V1 and MBN, aliquots were taken at 2, 5, and 10 min. The aliquots were immediately phenol/chloroform-extracted, ethanol-precipitated, and then loaded on 12% or 15% denaturing acrylamide gels. Reactions were performed in 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM MgCl₂ for all enzymes except MBN. MBN had buffer conditions of 10 mM Tris (pH 7.5), 10 mM NaOAc (pH 5.0), 0.1 mM ZnOAc, 50 mM NaCl, 1 mM L-cysteine, 5% glycerol, 1 mM MgCl₂. RNA sequence ladders were made by denaturing 150 kcpm of labeled RNA and 2 µg of tRNA and digesting with 0.07 U RNase T1 and 0.2 U RNase U2 (Industrial Research, LTD) for 10 min at 55°C in the following buffers: [1 × Buffer I (T1): 19.8 mM NaCitrate (pH 5.0), 1 mM EDTA, 4.2 M Urea, 0.02% Xylene Cyanol, 0.05% Bromophenol blue], [1 × Buffer II (U2): same as above except NaCitrate at pH 3.5] (buffer recipes courtesy of Dr. Brenda Peculis, NIH, pers. comm.). The gels were fixed, dried, and exposed on a PhosphorImaging screen overnight.

Solution structure probing with chemicals

For chemical modification and cleavage, the 5'-end-labeled mRNA was annealed to the gRNA by heating to 50°C and cooling slowly to 27°C, where they were held for 30 min in 50 µL of 1 × HE buffer (25 mM Hepes at pH 7.5, 2 mM MgOAc, 50 mM KCl, 0.5 mM DTT, 0.1 mM EDTA) and then cross-linked along with controls as above. The mRNA alone samples as well as untreated samples were treated as above without cross-linking. Ten micrograms of tRNA plus 40 U of rRNasin (Promega) were added to all samples and a no chemical control aliquot (10 µL) was taken. Four microliters of 97% diethyl pyrocarbonate (DEPC; Aldrich) were added and the samples incubated at room temperature (Brunel and Romby 2000). A 20-µL aliquot was taken from each reaction at 15 and 25 min. The aliquots were immediately ethanol-precipitated twice in the presence of 0.2 M NaOAc (pH 7.0), followed by a 70% ethanol rinse. Cross-links and controls were then gel-purified on 6% acrylamide, 7 M urea gels as described above (Leung and Koslowsky 1999, 2001a, b). Strand scission of all chemical samples was accomplished by incubating samples in 20 µL of 1 M Aniline (Sigma-Aldrich) (pH 4.5) for 10 min at 55°C, ethanol-precipitating as above (Brunel and Romby 2000), and analyzed on 15% denaturing acrylamide gels. T1 RNA sequencing ladders were made as described above. An alkaline hydrolysis ladder of mRNA was generated by incubating 100 kcpm of RNA and 10 µg tRNA in 50 mM NaHCO₃/Na₂CO₃ (pH 9.0), 1 mM EDTA (pH 8.0) at 90°C for 5 min.

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