***Trypanosoma brucei* U insertion and U deletion activities co-purify with an enzymatic editing complex but are differentially optimized**

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ABSTRACT

RNA editing, the processing that generates functional mRNAs in trypanosome mitochondria, involves cycles of protein catalyzed reactions that specifically insert or delete U residues. We recently reported purification from *Trypanosoma brucei* mitochondria of a complex showing seven major polypeptides which exhibits the enzymatic activities inferred in editing and that a pool of fractions of the complex catalyzed U deletion, the minor form of RNA editing *in vivo*. We now show that U insertion activity, the major form of RNA editing *in vivo*, chromatographically co-purifies with both U deletion activity and the protein complex. Furthermore, these editing activities co-sediment at ~20 S. U insertion does not require a larger, less characterized complex, as has been suggested and could have implied that the editing machinery would not function in a processive manner. We also show that U insertion is optimized at rather different and more exacting reaction conditions than U deletion. By markedly reducing ATP and carrier RNA and increasing UTP and carrier protein relative to standard editing conditions, U insertion activity of the purified fraction is enhanced ~100-fold.

INTRODUCTION

RNA editing is a fascinating form of RNA processing in which U residues are post-transcriptionally inserted into or deleted from primary trypanosome mitochondrial transcripts to achieve mature mRNA coding sequences (reviewed in 1–6). This editing occurs at multiple sites in substrate pre-mRNAs, can create translation start and stop codons, and inserts over half the protein coding residues of certain mRNAs. The editing is directed by guide RNAs (gRNAs), short mitochondrial transcripts whose 5'-region anchors to the pre-edited mRNA by base pairing, central region guides the editing modifications, and 3'-oligo(U) may tether the upstream, generally highly purine rich, mRNA. The mismatches in complementarity between the gRNA and pre-edited mRNA can direct U insertions and U deletions. About 90% of the ~4000 editing events in *Trypanosoma brucei*, *Leishmania tarentolae* and *Crithidia fasciculata* are U insertion, while ~10% are U deletion (2,6).

The basic mechanism of the RNA editing cycles in *T.brucei* has now been shown to involve a series of three protein enzyme catalyzed reactions (7–9; Fig. 1), much as originally suggested (10). It does not involve transesterification reactions, as has also been proposed (11,12). Initially, the mRNA is cleaved just 5' of the anchor region duplex, at the first mismatch with the gRNA. Recent evidence indicates that this initial cleavage step is different for U insertion and U deletion reactions (13). Then, for U insertion, U residues are added to the 3'-terminus of the upstream mRNA fragment by a terminal U transferase (TUTase), while for U deletion, U residues are removed from the 3'-end of the upstream mRNA fragment by a 3'-U exonuclease (Fig. 1). Finally, the upstream and downstream mRNA segments are re-joined by RNA ligase. This editing allows the mRNA-gRNA duplex to extend up to the next mismatch, which then directs cleavage and the next editing cycle (8,9). Through multiple such cycles, the pre-mRNA becomes edited progressively in a 3'→5' direction. This editing scheme has been demonstrated using ATPase 6 (A6) RNA, where the natural first editing site (ES1) is U-deletional (7,9) and the second editing site (ES2) is U-insertional (8). A similar mechanism for U insertion has also been proposed for *L.tarentolae* (14,15).

Considerable effort has focused on elucidating protein factors that catalyze the trypanosomatid editing reactions (reviewed in 1,2,6). In *T.brucei*, a gRNA-directed endonuclease, 3'-U exonuclease, TUTase, and RNA ligase activities, as well as U-deletional editing activity, all co-sediment at ~20 S and have been suggested to reside in a common complex (7,9,16–18). However, several of these individual enzymatic activities, as well as mRNAs, gRNAs and gRNA binding proteins, have been reported to additionally sediment at ~35–40 S (16,19) and thus have been suggested to also reside in a larger complex. This presumptive ~35–40 S complex could either include or not include the 20 S complex. Because U insertion was demonstrated using combined ~20 to ~35 S fractions (8), it was speculated that U insertion in *T.brucei* may require the ~35–40 S complex and not be catalyzed by the ~20 S complex that supports U deletion (6). Alternatively, however, U insertion and U deletion could be catalyzed by the same ~20 S complex. In *Leishmania*, it also remains to be determined whether the same or different complexes catalyze U insertion and U deletion (14,15).

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Figure 1. Mechanism of RNA editing. U insertion and U deletion (~90 and ~10% of the *T.brucei* RNA editing events *in vivo*, respectively) have been shown to involve the three indicated enzymatic reactions, as described in the text. Y, pyrimidine; R, purine (very rarely there is a C residue at this position in the mRNA). The enzymatic activities of the first and second steps of editing are different for U insertion and U deletion (9,13,18).

From *T.brucei* mitochondria, we have chromatographically purified a complex that contains seven different detectable major polypeptides, two of which have the features of RNA ligase catalytic subunits (18). Notably, RNA ligase, gRNA-directed endonuclease, 3'-U exonuclease and TUTase activities all co-purify with the complex, and pooled fractions across this isolated complex exhibit U-deletional editing (18). In this purification, the specific activity of RNA ligase is increased ~500-fold and U-deletional editing activity is also highly enriched (18). The observation that these polypeptides and individual editing activities also remain together upon subsequent non-denaturing gel electrophoresis and velocity centrifugation, where they sediment at ~20 S, provides additional support that they are associated in a complex. However, when those studies were performed we were unable to reproduce U-insertional editing *in vitro*, so its relationship to the purified enzymatic complex was not examined.

Here we demonstrate that U insertion as well as U deletion activities chromatographically co-purify with the protein complex and also co-sediment at ~20 S. This implies that a single complex can catalyze both kinds of editing, even though at least the first and second steps of U-deletional and U-insertional editing utilize different activities (9,15,18). Furthermore, although *T.brucei* U insertion was initially reported using similar reaction conditions to U deletion (7,8), we find that the two activities are optimal under very different conditions.

MATERIALS AND METHODS

Preparation of editing complexes

Trypanosoma brucei line TREU 667 was grown and mitochondrial extract prepared as described (18). The editing complex was

purified by chromatography on Q-Sepharose and DNA-cellulose as described (18) or fractionated by 10–30% glycerol gradient centrifugation (9,16) as modified (17).

Editing reactions

U deletion and U insertion reactions were modified from the previously described conditions (8,9,20), using 3'-end-labeled pre-mRNA with the products directly visualized (12,18). The mRNA and gRNA were synthesized as described (15,20). We used 1 meter long 9% polyacrylamide, 8.5 M urea gels, run at 1600 V for 14 h, to maximally resolve 1 nt size differences. The [³²P]pCp 3'-end-labeled mRNA (~6000 c.p.m., ~0.1–0.5 pmol) and gRNA (~1.3 pmol) for each reaction were pre-annealed in 10 mM Tris-HCl (pH 8), 0.1 mM EDTA at 37°C for 10 min and then at 22°C for 5 min prior to addition of the rest of the reaction mix, enhancing editing ~2-fold. After optimizing *T.brucei* U insertion (Fig. 2), we used 0.1–30 μM ATP, 0–0.3 ng/μl torula RNA, 150 μM UTP and 20–100 ng/μl added carrier protein, such as BSA or hexokinase. These conditions support reaction by fractions of the purified polypeptide complex ~100-fold more efficiently than the conditions that were previously used for *T.brucei* U insertion (3 mM ATP, 33 ng/μl torula RNA and 50 μM UTP; 8). For U deletion by the purified fraction, editing efficiency is improved 10–20-fold using 0.1–300 μM ATP, 3 mM ADP, 33 ng/μl torula RNA and 25–100 ng/μl carrier protein (Fig. 2; 13) over the previous conditions of 3 mM ATP, 33 ng/μl torula RNA (7,19). All reactions also contained 10 mM MgCl₂, 10 mM KCl, 5 mM CaCl₂, 1 mM EDTA and 0.5 mM DTT (7,8). MgCl₂ concentrations from 6 to 13 mM support maximal editing activity (see below). Generally, the 20 μl reactions utilized 2 μl purified editing complex (~20 ng) or 10 μl glycerol gradient enriched fraction and incubations were for 1 h. It remains possible that optimization, especially of non-specific RNA and protein, may vary at different stages of editing complex purification. Nucleotides, torula RNA and hexokinase were from Sigma, acetylated BSA from Biolabs. One experiment of our recent article (13) shows U insertion and refers to the current study for its verification and reaction conditions.

RNA sequence analysis

3'-End-labeled input and edited mRNAs were gel isolated, subjected to partial cleavage at A and U residues by digestion with PhyM RNase (Pharmacia; 21) and resolved by electrophoresis.

RESULTS

Optimization of U-insertional editing by purified extract fractions

Because ~90% of *T.brucei* RNA editing events are U insertion, while only ~10% are U deletion (6), it was of interest to determine whether U insertion could be catalyzed by the same purified polypeptide complex that has been implicated to catalyze U deletion (18) or if it requires a different machinery, specifically a ~35–40 S complex, as has been suggested (6). Our initial attempts to efficiently reproduce U insertion of A6 mRNA using mitochondrial extract or various partially or highly purified fractions were unsuccessful (data not shown). Additionally, our mitochondrial extracts yielded no detectable ~35–40 S complex of activities (9,17), which could explain our failure to observe U insertion, if that complex was needed. Alternatively, we considered

Figure 2. Optimization of U insertion using extract fractions. RNA editing was assessed under various reaction conditions using the pooled peak fractions of RNA ligase activity obtained by passage over Q-Sepharose and DNA-cellulose columns (18). U insertion reactions (**A**, **C** and **E**) used A6 pre-edited mRNA [0,4] and gRNA [2,4], while U deletion reactions (**B**, **D** and **F**) used mRNA [0,4] and gRNA [2,1], which specify insertion of two U residues or deletion of three U residues, respectively (Fig. 3A). In this [x,y] designation, x indicates the number of U residues in pre-mRNAs or guiding purines in gRNAs present at ES2 and y indicates similarly at ES1. The reactions contained varying amounts of: (**A** and **B**) ATP and non-specific RNA; (**C** and **D**) UTP; (**E** and **F**) non-specific protein. Otherwise optimized conditions were utilized for that kind of editing (Materials and Methods). The last lane (*) of (**F**) had no column fraction added. Consistent with the inferences from Cruz-Reyes *et al.* (13), U deletion occurs ~2-fold more efficiently with 0.3 mM ATP plus 3 mM ADP than with 3 mM ATP.

that the standard reaction conditions previously used for both U deletion and U insertion reactions (7–9,20) could actually be inhibitory for U insertion in our preparations. We pursued this latter possibility because those conditions include 3 mM ATP, and we recently found that U-insertional cleavage is markedly inhibited at 3 mM adenosine nucleotide (ATP or ADP) and considerably more active at <0.03 mM (13); conversely, U-deletional cleavage is far more active at 3 mM than at <0.03 mM. Furthermore, RNA ligase is fully active above 0.1 μ M ATP (13), permitting assessment for U insertion at low ATP concentration. Indeed, on studying U-insertional cleavage, we recently observed detectable levels of a mRNA product whose length (+2 relative to input mRNA) was consistent with accurate U insertion by that gRNA when the reaction contained 0.3 μ M ATP (13), but this product was not further characterized. We now examined various components of

Figure 3. Verification of the U insertion reaction. (**A**) Natural pre-edited A6 mRNA [0,4] or partly edited mRNA [0,2], the upper sequence of each set, is paired with gRNA [2,1], [2,2] or [2,4]. The indicated RNA pairs specify deletion of three U residues at ES1 (top set) or insertion of two U residues at ES2 (middle and lower set). The editing sites are indicated by numbers above the mRNAs, with a triangle indicating the site to be edited, adjoining the base paired region (shaded). The relevant U and purine residues of the editing sites are in bold. Potential base pairings that will be utilized following the current editing event are indicated by diagonal dotted lines and the 3'-end-label is shown by an asterisk. (**B**) RNA editing reactions using the noted RNAs were as in Figure 2. The regions of the gel containing the editing and cleavage products are shown. These RNA products plus chimeras are the only major bands observed in the gel. (**C**) The gel isolated -3 and +2 editing products and the input mRNA [0,4] were subjected to partial PhyM digestion (at A and U residues). Horizontal and diagonal lines indicate corresponding residues. The -3 mRNA differs from the input by lacking three U residues at ES1, while the +2 mRNA differs from the input by having an extra two U residues at ES2.

the standard (8) U insertion reaction (Fig. 2), and Figure 3 (below) shows that the +2 mRNA product obtained using purified pooled fractions and gRNA that specifies insertion of two U residues at ES2 reflects accurate insertional editing. Although almost no +2 RNA is seen using the previous conditions (Fig. 2A, lane 1), some is detected by reducing the ATP concentration (lanes 3 and 5; 13) and when the concentrations of both ATP and non-specific RNA are markedly reduced, appreciable amounts of this +2 mRNA are observed (lanes 4 and 6). Reducing both these components together increases the efficiency of U-insertional editing ~20-fold (Fig. 2A, lanes 1 and 6), while reducing only one of them has far less effect (Fig. 2A, lanes 2 and 5).

In contrast to its effect on U insertion, variations in the amount of non-specific RNA do not appreciably affect the efficiency of either accurate U deletion (the -3 product in Fig. 2B) or the cleavage reactions that initiate the U-insertional and U-deletional editing cycles (data not shown). This implies that non-specific RNA inhibits a later step specific to U insertion, possibly by acting as a competitor in the TUTase reaction, which is specific for U insertion (Fig. 1) and uses non-specific RNA as a substrate

(18). As we recently reported for the component cleavage reactions (13), adenosine nucleotides have different effects on U insertion and U deletion (Fig. 2). High concentrations of ATP and ADP inhibit U-insertional cleavage and U insertion, but are required for optimal U-deletional cleavage and full round U deletion (Fig. 2A and B and data not shown; 13). Separate experiments demonstrate that this differential effect of adenosine nucleotides on U insertion and U deletion is specific for these particular nucleotides and that it cannot be attributed to their affecting the available magnesium concentration, for maximal levels of both U insertion and U deletion are obtained with anywhere between 6 and 13 mM added $MgCl_2$ (data not shown).

UTP is a required substrate for TUTase and thus should be important for U-insertional editing (8). However, we obtain ~5-fold more +2 U insertion product with the purified polypeptide complex when UTP is increased from 0.05 mM (the concentration standardly used in *T. brucei* U insertion reactions; 8) to 0.15–0.5 mM (Fig. 2C). UTP concentrations above this relatively narrow optimum are also less effective at directing U insertion. In contrast, in U deletion reactions, all UTP concentrations from 0.5 μ M to 1.5 mM had a similar effect; accurately edited –3 product is observed, but its level is reduced 2–3-fold relative to reactions with no added UTP (Fig. 2D and data not shown). Instead, at all these UTP concentrations there is a corresponding increase in an RNA 1 nt longer than the accurate U deletion product (Fig. 2D); sequence analysis demonstrates that this –2 RNA contains one more U residues at ES1 than is directed by the gRNA (data not shown; see Discussion).

Finally, we also found that U insertion and especially U deletion, and their component cleavage reactions, are stimulated several-fold upon addition of non-specific protein, such as BSA, hexokinase, catalase, pyruvate kinase or thyroglobulin, to the purified polypeptide complex-containing fractions (Fig. 2E and F and data not shown). This may reflect stabilization of the editing complex by increasing the very low protein concentration otherwise present in reactions with purified fractions (~1 ng/ μ l); it is not attributable to the added proteins affecting ATP concentration.

The optimizations of Figure 2A, C and E together allow production of at least 100-fold more of the +2 U-insertional editing product by the purified fraction than is observed using the previous reaction conditions (8). When each kind of editing is carried out under its optimized conditions (Fig. 2), this fraction catalyzes U insertion at least as efficiently as U deletion.

Assessment of editing

A6 RNA has been a convenient substrate for studying editing, in part because its first two editing sites are U-deletional and U-insertional, respectively (8,20). The substrate mRNAs and gRNAs will be denoted as [x,y], where *x* indicates the number of U residues in pre-mRNAs (or guiding purines in gRNAs) present at ES2, and *y* indicates similarly at ES1 (Fig. 3A). U deletion is generally studied using either the natural [0,4] or a pseudo-natural [0,5] pre-edited mRNA and either the natural [2,2] or a pseudo-natural [2,1] gRNA, which direct removal of all but two or one U residues at ES1, respectively (7,9,20; Fig. 3A, upper). U insertion has been studied using mRNA [0,2] which is already edited at ES1 and the natural gRNA [2,2], to direct insertion of two U residues at ES2 (8; Fig. 3A, middle). However, to more readily compare U insertion and U deletion reactions, we wanted to use a common labeled input mRNA and thus pursued our

Figure 4. U insertion and U deletion catalyzed by the fractions from the final purification step of the editing complex. Fractions 22–31 of the DNA–cellulose column previously described (18) are shown, assayed by silver staining of an SDS gel (A), by adenylation to identify the RNA ligase polypeptides (B), and by editing as in Figure 2 to assess for U insertion (C) and U deletion (D). Bands IVa, IVb and V, indicated with *, were previously shown to be RNA ligase polypeptides (18). The right lane of (A) is a more concentrated preparation from a different DNA–cellulose column.

recent observation that U-insertional cleavage at ES2 is at least as efficient using pre-edited mRNA [0,4] and a mutant gRNA [2,4] (13; Fig. 3A, bottom). This mRNA:gRNA combination indeed yields a +2 editing product (Fig. 3B, lane 3), permitting the same [0,4] input mRNA (lane 1) to generate either +2 or –3 RNA, depending on the gRNA (lanes 2 and 3). Figure 3C shows a direct RNase sequencing analysis of the gel isolated +2 and –3 RNAs, demonstrating accurate insertion of two U residues at ES2 (Fig. 3C, lane 3) and confirming accurate removal of three U residues at ES1 (lane 1), respectively, relative to the input mRNA (lane 2).

A common polypeptide complex appears to catalyze both U insertion and U deletion

The experiments of Figures 2 and 3 used pooled fractions across the isolated enzymatic complex, which consist of only seven observed major polypeptides (18; Fig. 4A), suggesting that the same complex catalyzes U insertion and U deletion. To further address this possibility, we assayed individual fractions from the DNA–cellulose column that is the final chromatographic step of this purification (Fig. 4). On this column, ~95% of the loaded protein elutes in the flow-through, while the noted seven major polypeptides comprise most of the remaining <5% (~0.02% of the starting mitochondrial extract protein); the polypeptides co-elute at ~100 mM KCl, along with RNA ligase activity and the other component activities of editing (18; Fig. 4A and B). The data of Figure 4C and D now show that U-insertional editing activity and U-deletional editing activity both co-elute and exhibit the same peak of elution as the polypeptide complex (Fig. 4A and B). These results, plus the high degree of protein purification (18),

Figure 5. U insertion and U deletion catalyzed by glycerol gradient fractions. Fractions 10–21 of a glycerol gradient of mitochondrial extract are shown, assayed as in Figure 2 for U insertion (A) and U deletion (B). The ~20 S editing complex peaks in fraction 17, while a ~35–40 S complex should peak in fraction 11–12. The thyroglobulin (19 S) marker peaked at the position of fraction 17; catalase (11 S) and *Xenopus* ribosomal subunit (40 and 60 S) markers confirmed that the separation was approximately linear with sedimentation coefficient.

strongly indicated that the same complex catalyzes both U insertion and U deletion.

To further address whether U insertion and U deletion are catalyzed by the same editing complex, we examined the fractions from the Q-Sepharose column, which is the step prior to DNA–cellulose in the purification (18). As a marker for U insertion, we assayed the U-insertional cleavage reaction, since an activity that inhibits detection of the complete reaction is present in these fractions, but subsequently purifies away upon DNA–cellulose chromatography (data not shown). The U-insertional and U-deletional cleavage activities both have the same elution peak (data not shown), along with the TUTase, 3'-U exonuclease and RNA ligase activities, at ~190 mM KCl (18), supporting their presence in the same complex.

Finally, to directly determine the sedimentation properties of the U-insertional editing activity in whole mitochondrial extract, we assessed fractions from a glycerol gradient centrifugation of mitochondrial extract. U deletion activity and its component cleavage, 3'-U exonuclease, and RNA ligase activities, as well as TUTase activity, were previously reported to sediment at ~20 S (9,16), as was the DNA–cellulose purified polypeptide complex (18), suggesting that U insertion activity may also exhibit this sedimentation coefficient. We now find that the peak of U insertion activity from the mitochondrial extract (Fig. 5B) indeed coincides with the ~20 S peak of U deletion activity in fraction 17 (Fig. 5A). Notably, U insertion activity is not detected in the ~35–40 S region (fraction ~11–12; Fig. 5B), where U insertion activity has been speculated to sediment (6). Thus, upon velocity centrifugation and throughout purification of the seven major polypeptide complex, the activities that catalyze U insertion and U deletion co-purify. We conclude that they are most likely components of the same ~20 S editing complex.

DISCUSSION

A fraction from *T.brucei* mitochondria that contains a seven major polypeptide complex sediments at ~20 S, catalyzes U-deletional editing and contains all the known component activities of RNA editing cycles: gRNA-directed endonuclease, 3'-U exonuclease, TUTase and RNA ligase (18; Fig. 1). Because U deletion accounts for only ~10% of trypanosome RNA editing events while U insertion accounts for the remaining ~90% (6), we wanted to determine whether this same purified fraction could also catalyze U insertion. The reported success at reproducing

U-insertional RNA editing *in vitro* using pooled fractions sedimenting from ~20 to ~35 S in glycerol gradients of mitochondrial extract (8) was consistent with U insertion being catalyzed by the same complex that catalyzes U deletion, by it plus additional components or by an entirely different complex. Indeed, others have reported a peak (19) or shoulder (16) of TUTase and RNA ligase activities at ~35–40 S, and it has been suggested that U insertion may be catalyzed by such an ~35–40 S complex rather than by the ~20 S complex (6).

The data reported in this communication indicate that the purified ~20 S polypeptide complex catalyzes both U insertion and U deletion (Figs 3 and 4) and that both kinds of editing can be similarly efficient in the purified fractions (Figs 2–5; see below). This complex behaves as a single entity upon purification on Q-Sepharose and DNA–cellulose chromatography, subsequent glycerol gradient centrifugation and non-denaturing gel electrophoresis (18). Because the U-insertional and U-deletional activities peak together with this complex during its purification (Fig. 4) and during velocity centrifugation (Fig. 5), most likely a single complex catalyzes both forms of RNA editing.

Initially, we readily observed *in vitro* U deletion but not U insertion, both using mitochondrial extract and partly or highly purified components. This apparent inefficiency of *in vitro* U insertion raised the possibility that our mitochondrial extract was deficient in some U insertion factors, possibly the ~35–40 S complex reported by others. However, our finding that U insertion and U deletion are differently affected by a number of reaction components, including ATP, UTP and non-specific RNA (Fig. 2), demonstrates that the apparent relative *in vitro* efficiency of these two kinds of editing is not a fixed value, but reflects the chosen reaction conditions. By optimizing conditions, the apparent efficiency of U insertion was enhanced ~100-fold (Fig. 2A, C and E), relative to conditions initially used for its analysis (8), and U deletion efficiency was also enhanced several-fold (Fig. 2F and legend). When assayed under their respective optimized conditions, U insertion is catalyzed by the fraction containing the purified complex at least as efficiently as U deletion.

Although the complex co-purifies with ~1/6000 of the protein of the mitochondrial extract (~1/60 000 the protein of a *T.brucei* cell; 18), quantitation of the purification of U-insertional or U-deletional editing activities has not been possible. First, there is a component (other than ATP) that inhibits U insertion in the starting extract. Second, we have not found conditions where assays for U insertion or U deletion are linear with the amount of added complex in any of the fractions examined. Furthermore, this non-linearity appears different for the two kinds of editing, with U insertion activity diminishing more rapidly than U deletion activity at decreasing amounts of complex (data not shown). This differential non-linearity may explain why U insertion and U deletion activities show the same elution peaks throughout purification but can trail to different extents (Figs 4 and 5).

The potential biological significance of nucleotide effects on editing is of particular interest. ATP, ADP and other adenosine nucleotides inversely affect both gRNA-directed cleavage and full round editing, inhibiting U insertion and stimulating U deletion (Fig. 2A; 13). These nucleotide effects on cleavage may be allosteric and are half-maximal at ~0.3 mM, within the range of measured or estimated ATP concentration in procyclic mitochondria and in whole bloodstream trypanosomes (13 and references therein). This is compatible with observation of both types of editing at these life cycle stages. However, mitochondrial

adenosine nucleotide concentration may change and could affect editing efficiency during developmental transitions, especially when ATP production switches between mitochondrial respiration in the insect form and glycolysis in the bloodstream form.

UTP also differently influences U deletion and U insertion by the purified editing fraction. While similar levels of accurate U deletion occur at all UTP concentrations between 0.5 μ M and 1.5 mM (Fig. 2D and data not shown), U insertion is efficient only between 0.15 and 0.5 mM UTP and is much less efficient at lower or higher UTP concentrations (Fig. 2C). These different effects of UTP could result because 3'-U exonuclease and TUTase are distinct activities (9,18). Although the *in vivo* mitochondrial UTP concentration is unknown, especially at the editing catalytic site (1), the relatively narrow *in vitro* optimum for UTP in U insertion suggests that physiologically relevant variations in UTP concentration could affect RNA editing efficiency *in vivo*.

Interestingly, in U deletion, all added UTP concentrations from 0.5 μ M to 1.5 mM reduce accurate U deletion ~2–3-fold relative to no added UTP, and they induce similar levels of a partial U deletion where one extra U residue is retained (Fig. 2D). This is consistent with the previous observation that UTP addition to a U deletion reaction diminishes complete 3'-U removal from the upstream cleaved mRNA fragment and instead promotes accumulation of a 5' cleavage product retaining one extra U residue (9; presumably by inhibiting the 3'-U exonuclease and/or stimulating U re-addition by TUTase). We speculate that during editing, RNA ligase efficiently re-seals all accumulated mRNA fragments, whether or not the U residues to be deleted are fully removed, resulting in the observed inaccurate editing. Indeed, examination of published partially edited *T.brucei* cDNA sequences (for example 22) provides several examples where one extra U residue is retained at a U deletion site *in vivo*, possibly reflecting the UTP-induced incomplete U removal observed *in vitro*. These observed effects of UTP support the notion that gRNAs may dictate the number of U residues to be deleted or added at a post-ligation step by proof-reading (4,23,24), rather than at the initial ligation step by directing ligase to preferentially seal correctly edited fragments, as has been suggested (6,8). Consistent with the ligation step being non-selective, *in vitro* analysis has indicated that the editing machinery exhibits an inherent proof-reading activity that could effectively repair misedited mRNA sites (9).

Future *in vivo* gene knock-out studies of candidate editing factors should provide further understanding of the editing machinery. Our laboratory has now started the cloning and expression of the seven major components of the editing complex (C.Huang *et al.*, unpublished results) and we find that addition of recombinant band VII protein to the *in vitro* reaction is strongly inhibitory for both U insertion and U deletion, while non-specific proteins added at >100-fold excess are not (data not shown). This is consistent with band VII protein specifically interacting with a common U-insertional and U-deletional editing complex.

It seems advantageous for the trypanosome that the same enzymatic complex should catalyze both U-insertional and U-deletional editing. In most edited mRNAs, these two kinds of

editing sites are interdigitated, and the same gRNA that directs U deletion also directs U insertion at adjacent sites. If different enzymatic complexes were required to catalyze U insertion and U deletion, the editing machinery would need to exchange after catalyzing each kind of editing. However, if the same enzymatic complex can catalyze both kinds of editing, it could be processive and move along the mRNA as editing proceeds. Indeed, we have now observed input mRNA carrying out two successive cycles of editing, with the second cycle appearing considerably faster than the first (J.Cruz-Reyes, unpublished results), consistent with editing being processive. A processive editing complex could also facilitate rapid proof-reading of any misediting that occurred. Especially for mRNAs that have hundreds of editing modifications, RNA editing should be much more efficient if the enzymatic machinery remains on the mRNA and catalyzes sequential editing and proof-reading cycles, rather than dissociating and re-binding between each cycle.

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