In *Trypanosoma brucei* RNA Editing, Band II Enables Recognition Specifically at Each Step of the U Insertion Cycle

Julie A. Law,† Catherine E. Huang,‡ Sean F. O’Hearn,† and Barbara Sollner-Webb*

Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland

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Trypanosome RNA editing is the posttranscriptional insertion and deletion of uridylicate (U) residues, often to a massive extent, through cycles of cleavage, U addition or U removal, and ligation. These editing cycles are catalyzed by a complex that we purified to seven major proteins (bands I through VII). Here we analyze the role of band II using extracts of clonal band II RNA interference (RNAi) cell lines prepared by a rapid protocol that enables retention of activities that are lost during traditional extract preparation. By individually scoring each step of editing, we show that band II is critical for all steps of U insertion but is not important for any of the steps of U deletion or for their coordination into the U deletion cycle. This specificity supports the long-standing model that U-insertional and U-deletional activities are separated within the editing complex. Furthermore, by assaying the basic activities of the enzymes that catalyze the steps of U insertion, independent of their action in editing, we show that band II is not any of those enzymes. Rather, band II enables endonuclease action at authentic U insertion sites, terminal-uridylyl-transferase (TUTase) action at cleaved U insertion sites, and U-insertion-specific ligase (band V/IREL) action in the editing complex. Thus, band II facilitates each step of U insertion by providing proper RNA and/or protein recognition. We propose that band II (TbMP81) be called IRER, indicating its essential nature in U-insertional RNA editing recognition.

Trypanosome RNA editing is an unprecedented form of transcript maturation that involves many cycles of posttranscriptional deletion and insertion of uridylicate (U) residues to generate essential mitochondrial mRNAs (reviewed in references 24, 43, and 47). These editing events can occur at over half the phosphodiester bonds of a pre-mRNA’s coding region (15), creating over three-quarters of the codons, including translation start and stop signals. Short, trans-acting guide RNAs (gRNAs), which are complementary to mature mRNA sequence, direct the editing via mismatches in base pairing (5). Furthermore, individual U deletion and U insertion cycles can be reproduced in vitro using a synthetic pre-mRNA segment, its cognate gRNA, and *Trypanosoma brucei* extract (23, 42), such as the “traditional” extract (18, 38) prepared from Percoll-enriched mitochondria.

Editing cycles involve pre-mRNA cleavage, then terminal U removal or U addition, and finally rejoining of the pre-mRNA fragments (Fig. 1A) (10, 23, 35, 41). The pre-mRNA is cleaved just upstream of an anchor duplex which forms where the pre-mRNA base pairs with the 5′-end created by the anchor duplex (12). Notably, an adenosine di- or triphosphate is required for cleavage at U deletion sites but is inhibitory for cleavage at U insertion sites (nonhydroyzable AMP-CP is convenient to use for in vitro studies) (8). Next, at the 3′ end created by the cleavage, a 3′-U-specific exonuclease (3′-U-exo) (35) removes the terminal U(s) in U deletion, whereas a terminal-U-transferase (TUTase) (4) adds a U(s) in U insertion. The two mRNA fragments are then religated, and the anchor duplex extends to the next mismatch, defining the subsequent site of editing. Thus, editing progresses sequentially 3′ to 5′ along the pre-mRNA.

Purification of the editing activities from *T. brucei* extracts indicates that they exist in a catalytic complex of ~20S (34, 35). Our purified preparations of editing complex contain seven major, approximately equimolar proteins (provisionally called bands I through VII [35]) and support full cycles of U deletion and U insertion with the greatest efficiency yet reported (9, 11, 12, 35, 45). However, the RET2 editing TUTase (2, 14) appears substoichiometric in this purified preparation, and other minor proteins could also be important. A related purification yields 13 to 15 major proteins (25, 26) that support U insertion (25). Other described purifications yield about 20 major proteins (1, 2, 29, 30, 32, 39) that actively support precleaved editing, which bypasses the cleavage step (21, 22, 31). These ~20 proteins appear to vary in relative abundances in different preparations, but they include bands I through VII (called TbMP99, -81, -63, -52, -48, -42, and -18, for the molecular sizes 20S (34, 35)). Other described purifications yield about 20 major proteins (1, 2, 29, 30, 32, 39) that actively support precleaved editing, which bypasses the cleavage step (21, 22, 31). These ~20 proteins appear to vary in relative abundances in different preparations, but they include bands I through VII (called TbMP99, -81, -63, -52, -48, -42, and -18, for the molecular sizes 20S (34, 35)). Significant effort has been focused on defining how these various proteins function in editing; multiple approaches, including biochemistry (3, 12, 14, 19, 36, 39, 40), bioinformatics (27, 29, 54), and RNA interference (RNAi) (13, 16, 20, 28, 50), have been used.

Early studies identified two *T. brucei* RNA ligases (37), bands IV and V of the editing complex (35). Band IV (TbMP52) is essential for viability (36, 40) and for sealing in U deletion (12, 19). It has been designated DREL (for U-deletion-requiring RNA editing ligase [12]) or TbREL1 (40). Band V (TbMP48) seals specifically in U insertion (12) and has been designated...
IREL (for U-insertion-specific RNA editing ligase [12]) or TbREL2 (40). This U-deletional and U-insertional assignment is reinforced by the isolation of subcomplexes that catalyze precleaved U deletion or U insertion and contain those respective ligases (39). Although DREL (TbMP52) ligates both forms of editing when IREL (TbMP48) is depleted by IREL RNAi (13, 16, 28), it remains unknown whether DREL appreciably joins U insertion in the wild-type editing complex. Analyzing other proteins of the editing complex, RNAi to band III has shown that this protein is critical for multiple steps of editing, including both forms of cleavage and retaining the DREL ligase (20). Furthermore, RNAi to each of two TUTases has shown that RET2 (TbMP57) is in the editing complex and adds the U’s in U insertion, while RET1 (TbMP108) is separate and adds U’s to gRNAs (2, 14).

Band II (TbMP81) RNAi-transfected procyclic cells have been examined by two groups (13, 28). Induction of clonal band II RNAi cell lines causes death, demonstrating that this protein is essential (28). (While induced nonclonal band II RNAi cultures can continue to grow at approximately half-maximal rate [13, 28], this appears to be due to unintended variants growing out [28].) Band II RNAi causes loss of band II (Fig. 1B) (13, 28) and corresponding loss of IREL (band V/TbMP48) protein (28), including the small fraction that is unadenylylated in the extract (13). Upon band II RNAi, the five other major proteins of our isolated editing complex remain present and associated with each other (28; see also reference 13 for earlier data). The band II-depleted complex, first thought to sediment like wild-type complex 13 (implying it is more compact), was shown to be \(15S\) when the wild-type \(20S\) complex was used as an internal marker, suggesting the depleted complex may be somewhat more extended (28). Furthermore, band II (TbMP81) RNAi was reported to decrease precleaved U insertion (both the TUTase-generated intermediate and ligated product) and about half as effectively decrease precleaved U deletion (both the \(3'\)-U-exo-generated intermediate and ligated product) (13). Additionally, a cleavage reaction was greatly reduced using band II (TbMP81) RNAi extract, raising the possibility that band II (TbMP81) may be the editing endonuclease (13; but see Discussion below).

Here we pursue functional analysis of band II. By individually assessing each step of the U-deletional and U-insertional editing cycles, we find that band II RNAi has no direct effect on U-insertional cleavage product (solid large arrowhead) generated from input mRNA (upper band) using editing complex purified to seven major proteins (purified), traditional mitochondrial extract (trad. ext), rapid extract (rapid ext), or no extract (\(-\)). nt and G indicate marker lanes in which the same mRNA was treated with hydroxide to cleave at every nucleotide or with RNase T1 to cleave after G residues, respectively (7). (Sizing markers were run, but they are not shown in subsequent figures.) In the U-deletional cleavage reactions (conducted with AMP-CP), the faint upper band (small solid arrowhead) represents a small amount of cleavage opposite the \(3'\)-end of this gRNA; this site is more efficiently cleaved in the absence of AMP-CP (see Fig. 5). In the U-deletional cleavage reactions, there are also faint bands suggesting some cleavage 1 and 2 nucleotides upstream of the major U-deletional cleavage site. In the U-insertional cleavage reactions, the band migrating \(-2\) nucleotides lower than the U-insertional cleavage band has been seen before (20) and likely represents cleavage at the next upstream U-insertional editing site.

**FIG. 1.** RNA editing pathways, band II depletion by RNAi, and assessment of editing cleavages. (A) U-deletional and U-insertional editing begins with cleavage (shown as a hollow or solid arrowhead) by a U-deletional or U-insertional endonuclease activity (del endo or ins endo, respectively) which is stimulated or inhibited, respectively, by adenosine polyphosphates. Next, a \(3'\)-U-exo or TUTase acts on the \(3'\) cleavage product, and finally the two RNA fragments are ligated by DREL or IREL, respectively. The mRNA site being cleaved and edited is immediately upstream of an anchor duplex which forms between the mRNA and the \(5'\) region of the gRNA. The G and the C represent a purine and a pyrimidine, respectively; the A represents one or more purines that guide the U insertion. (B) Western analysis showing band II depletion by RNAi in extracts used for Fig. 3 to 9 as indicated. Pre-stained size markers, in kilodaltons, are indicated. Analysis of this membrane using lipoamide dehydrogenase antibody (control) provides verification of loading. (C) Autoradiograms of gels showing U-deletional cleavage product (hollow large arrowhead) and U-insertional cleavage product (solid large arrowhead) generated from input mRNA (upper band) using editing complex purified to seven major proteins (purified), traditional mitochondrial extract (trad. ext), rapid extract (rapid ext), or no extract (\(-\)). nt and G indicate marker lanes in which the same mRNA was treated with hydroxide to cleave at every nucleotide or with RNase T1 to cleave after G residues, respectively (7). (Sizing markers were run, but they are not shown in subsequent figures.) In the U-deletional cleavage reactions (conducted with AMP-CP), the faint upper band (small solid arrowhead) represents a small amount of cleavage opposite the \(3'\) end of this gRNA; this site is more efficiently cleaved in the absence of AMP-CP (see Fig. 5). In the U-deletional cleavage reactions, there are also faint bands suggesting some cleavage 1 and 2 nucleotides upstream of the major U-deletional cleavage site. In the U-insertional cleavage reactions, the band migrating \(-2\) nucleotides lower than the U-insertional cleavage band has been seen before (20) and likely represents cleavage at the next upstream U-insertional editing site.
on U deletion but markedly impairs each step of U insertion. Obtaining these data required rapidly prepared extracts, as we found that band II-depleted complex is less stable and loses editing activities during the traditional, lengthy extract preparation. Interestingly, by assessing the “basic” activities of these enzymes, i.e., those independent of their role in editing, we additionally show that band II is not any of the U-insertional enzymes. This aggregate data indicate that in each step of U insertion, band II is needed for the enzyme’s recognition of the RNA substrate and/or editing complex.

MATERIALS AND METHODS

Cell lines and extract preparation. The T. brucei procyclic cell lines used here carry an ectopically integrated plasmid that directs band II RNAi, and they were cloned from single cells by extreme dilution, as described previously (28). This transfected plasmid carries a 700-nucleotide internal fragment of the band II coding region flanked by T7 promoters and tetracycline (TET) operators (51, 53) from the pZJM vector (51). Because the recipient 29-13 cells synthesizes T7 RNA polymerase and T7 replicator (52), TET (1 μg/ml) induces expression of double-stranded band II RNA (28, 51). Before growth phenotypes become evident (5 days postinduction for the band II RNAi cells [28]), extracts were prepared from several independent, clonal band II RNAi cell lines (RNAi+). Extract preparation followed either the traditional protocol for trypanosome mitochondrial extract (18, 38) or a rapid extract protocol used previously for small-scale preparation followed either the traditional protocol for trypanosome mitochondrial extract at 106 cell equivalents/l. The rapid, small-scale extract streamlines this protocol to ~1.5 h. This protocol starts with ~2 × 106 cells, which allows shorter centrifugations in a microcentrifuge tube following the initial pelleting, lyse the cells quickly by vortexing rather than by Dounce homogenization plus passage through a syringe needle, and uses half the time of DNase treatment. Additionally, it omits the Percoll gradient and all but two of the STE (250 mM sucrose, 10 mM Tris [pH 8], 2 mM EDTA) washes. Finally, for the 0.5% Triton X-100 treatment (0°C, 5 min), the sample is suspended in 10 mM KCl-MRB (25 mM Tris-HCl [pH 8.0], 10 mM Mg acetate, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, and 10 mM KCl), 20-fold more dilute than in the traditional mitochondrial extract, and then the supernatant is aliquoted without recrystallation, generating extract at 106 cell equivalents/ml. In parallel, extract was also prepared from control cultures (RNAi−), using 29-13 cells for traditional extracts and uninduced band II RNAi cells for rapid extracts. Such rapid extracts of control cell support all the steps of editing (see Fig. 3 to 9 and reference 20), including U-deletional ligation by DREL, which joins gapped as well as unadenapped substrates (12), while U-insertional ligation is specific for unadennlated substrates (12, 21). To specifically assay the ligation step of U insertion, it is coupled to the TUTase step (21), but that is not possible for band II RNAi extracts, where the TUTase step is inactive. (vi) Full-round U deletion (see Fig. 9A) was assayed using the same RNAs and conditions as those used for U-deletional cleavage, except that they were supplemented with 0.15 mM ATP and 5 mM CaCl2, omitting Pi (8, 19). (vii) Coupling between the cleavage step and the 3′-U-exo step (Fig. 9B) was assayed using the same RNAs and conditions as those for the U-deletional cleavage reaction, except the mRNA was 5′-labeled to visualize U removal from the upstream fragment. For all these reactions, the mRNA (25 to 30 fmol) and gRNA (1.25 pmol) or the preadenylylated RNAs (0.1 to 0.2 pmol of upstream mRNA, 1 pmol of downstream mRNA, and 0.5 pmol of gRNA) were preadenylylated as described previously (19, 21).

(ii) Basic enzymatic assays. The enzymes that catalyze the steps of the editing cycles were assayed independently of their ability to act at an editing site, used minimal substrates. (i) Basic U-insertion-like cleavage activity (see Fig. 2C and 5) was assayed using reaction conditions for U-insertional cleavage and 3′-end-labeled mRNA (44) annealed to D33′ (45). In this reaction, cleavage is directed by the 3′ end of the gRNA, which forms an anchor-like duplex with a region of the upstream mRNA that is entirely devoid of U’s (see Fig. 5). (ii) Basic 3′-U-exo activity (Fig. 6B) (35) was assayed using ~0.05 pmol of a gel-isolated 5′-end-labeled RNA band from ance+U6 RNA (11) that has a 14-nucleotide 3′ oligo(U) tail. (iii) Basic TUTase activity (see Fig. 7B) (4) was assayed using 0.1 pmol of unlabeled m[0,4] and 1.5 pmol of [α-32P]UTP (3,000 Ci/mmol). (iv) Basic U-deletional ligase activity (see Fig. 8B) was assayed using ~0.15 pmol of 5′-end labeled polynucleotide from ECoRI digestion of pH170 (R. Voelcker and M. White, unpubl.) and 0.15 mM ATP (20, 35). The last three reaction mixtures were incubated at 22°C.

(iii) Adenylylation assay. Ligase proteins were detected by adenylylation (see Fig. 8C), the covalent transfer of AMP from [α-32P]ATP. To detect preadenylylated as well as unadenylylated molecules (35), the ligases were first deadenylylated as described previously (35) and 0.15 mM ATP (20, 35). The last three reaction mixtures were incubated at 22°C.

RESULTS

Utility of nontraditional extract preparation for RNAi cells. Using the traditional extract protocol (18, 38) (see Materials and Methods) that has been routinely used to study editing (6, 11, 23, 33, 35, 41), we prepared extracts from four clonal lines of band II RNAi cells induced for 5 days (before they exhibited appreciable growth effects) (28) and parallel control cultures. In contrast to the active control extracts, these band II RNAi extracts were markedly diminished in numerous editing activities, including U-deletional cleavage (Fig. 2A), U-insertional cleavage (Fig. 2B), basic U-insertion-like cleavage (Fig. 2C),

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not withstand the traditional extract protocol, which entails ever, we also considered that band II-depleted complex might gest that band II is important for all the above activities. How-
and basic 3'/H11032-U-exo activities (data not shown; see Materials and Methods for descriptions of these assays). This could sug-
gest that band II is important for all the above activities. How-
ever, we also considered that band II-depleted complex might not withstand the traditional extract protocol, which entails ~6 to 8 h of handling following cell lysis (18, 38). We therefore also examined extract prepared more rapidly, using a protocol that reduces the handling time postlysis by ~80% (20, 28, 36) yet is compatible with all the steps of editing (Fig. 1C; see also Fig. 3 to 9). As will be shown below, such rapid extracts, prepared in parallel from uninduced and 5-day-induced clonal band II RNAi cell lines, reproducibly retained U-deletional cleavage, basic U-insertion-like cleavage, and basic 3'-U-exo activities (see Fig. 3, 5, and 6) that were depleted in the traditional extracts (Fig. 2). This indicates that these activities were present in the RNAi cells and were lost during the longer traditional extract preparation, evidently due to instability of the band II-depleted complex. Thus, all subsequent experiments used these rapid extracts.

The cleavage steps. Using 3'-end-labeled A6 pre-mRNA and a gRNA to target the U-deletional (first) editing site, plus pyrophosphate (PPi) to inhibit ligation, we specifically assessed the U-deletional cleavage step of editing (8, 10, 19). The rapid extracts of band II RNAi and control cells are similarly active for this editing step (Fig. 3A). Titration shows that some RNAi extracts retain full U-deletional cleavage activity while others exhibit slightly less (within twofold), likely reflecting a small extent of the lability that this activity shows in traditional extracts (Fig. 2). Verifying its U-deletional nature (8), this cleavage by the rapid RNAi extracts is quite inactive without adenosine nucleotides, and it is strongly stimulated by AMP-CP (Fig. 3B). Furthermore, these RNAi extracts cleave at the same site as control extracts (Fig. 3A). Thus, by all established criteria, U-deletional cleavage appears normal upon band II RNAi.

When instead using a gRNA to target the U-insertional (second) editing site of 3'-end-labeled A6 pre-mRNA, and again including PPi to inhibit ligation, we specifically assessed the U-insertional cleavage step of editing (8, 10, 19). In contrast to U-deletional cleavage, U-insertional cleavage is markedly diminished in rapid extracts of band II RNAi cell lines (Fig. 4A). Extract titration shows that the amount of U-insertional cleavage activity remaining upon band II RNAi is obtained with only ~1/10th as much control extract. Nonetheless, this residual cleavage activity appears normal, since it is inhibited by AMP-CP (Fig. 4B) (8) and shows the same site specificity as the control extract. Because induction of the RNAi leaves some residual band II and therefore some wild-type editing complex (28), the actual band II-depleted complexes must be even more deficient in U-insertional cleavage than it appears from Fig. 4. Thus, band II is critical for endonucleolytic cleavage in U insertion but not in U deletion.

Since U-insertional cleavage is absent from rapid extracts of band II RNAi cells, we assayed for the basic enzymatic activity of the U-insertional endonuclease independent of its ability to

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**FIG. 2.** All cleavage activities are diminished in traditional extracts of band II RNAi cells. Analysis of editing cleavage reactions, using traditional extracts of control (RNAi −) and induced (RNAi +) band II RNAi cells. (A) The U-deletional cleavage step was assayed using 0.4 and 0.2 μg of extract. (B) The U-insertional cleavage step was assayed using 4 and 0.8 μg of extract. (C) Basic U-insertion-like cleavage was assayed using 0.4 and 0.2 μg of extract. In all experiments, the comparable RNAi − and + lanes used equal amounts of extract. [ext], extract titration. The sizes of the cleavage products are indicated in nucleotides (nt).

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**FIG. 3.** U-deletional cleavage is not diminished upon band II RNAi. Analysis used rapid extracts of induced band II RNAi cells (RNAi +) and control cells (RNAi −), PPi, and AMP-CP unless otherwise indicated. −, reaction without extract. (A) U-deletional cleavage using 2.4 and 0.8 μg of the extracts shown in Fig. 1B, lanes 3 and 4. (B) AMP-CP effect on U-deletional cleavage using 2.4 μg of these same extracts. (C) A larger segment of the first lane of the gel shown in panel B showing specificity of cleavage. In this and subsequent figures, the reaction is diagrammed underneath the gel. [ext], extract titration; *, 32P label; nt, nucleotide.

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**FIG. 4.** U-insertional cleavage is diminished upon band II RNAi. Analysis used rapid extracts, PPi, and no AMP-CP, unless indicated. (A) U-insertional cleavage using 1.15, 0.4, and 0.1 μg of the extracts used for Fig. 3A. (B) AMP-CP effect on U-insertional cleavage using 1.8 μg of another pair of extracts. (C) A larger segment of the second lane of the gel depicted in panel B showing specificity of cleavage. The faint bands seen in the reaction without extract do not represent editing cleavages. nt, nucleotide; [ext], extract titration; *, 32P label.
U insertion site but not for this enzyme to be present or active. U-insertional endonuclease to productively recognize an actual lead to the interesting conclusion that band II is needed for the control extract (data not shown). The data of Fig. 4 and 5 also cleaves a basic U-deletion-like substrate as efficiently as AMP-CP inhibition (Fig. 5B). As expected because U-deletional cycle is removal of U’s by the 3′-U-exo step of U deletion.

FIG. 5. Basic U-insertion-like cleavage is not diminished upon band II RNAi. Analysis used rapid extracts, PPI, and no AMP-CP, unless otherwise indicated. (A) U-insertion-like cleavage using 2.4 and 0.8 μg of the extracts used for Fig. 3A and 4A, (B) AMP-CP effect on U-insertion-like cleavage using 2.4 μg of the extracts shown in Fig. 1B, lanes 1 and 2. (C) A larger segment of the second lane of the gel depicted in panel B showing specificity of cleavage. This basic U-insertion-like cleavage assay uses the same U-deletional substrate as that in Fig. 3 but without AMP-CP, so cleavage is opposite the gRNA 3′ end, which is base paired to the pre-mRNA within a polypurine region. Because the gRNA is synthesized using T7 RNA polymerase, an appreciable fraction of the molecules acquire an extra 3′ U. When annealed with the pre-mRNA, that U fortuitously aligns opposite an A and extends the base pairing by 1 residue. Thus, this assay generates doublet bands of 44 and 45 nucleotides (nt) in length. [ext], extract titration; *, 32P label.

FIG. 6. 3′-U-exo is not diminished upon band II RNAi. Analysis using rapid extracts and PPI. (A) The 3′-U-exo step of U deletion, scored using a precleaved substrate and 2.4 and 0.8 μg of the extracts used for Fig. 3A to 5A. The input RNA and products lacking 1, 2, or 3 terminal U’s are scored on an RNA with a 14-nucleotide (nt) 3′-terminal oligo(U) track, using 0.5 and 0.25 μg of the extracts used for Fig. 4B. The input RNA and the extent of the oligo(U) track that gets removed by the 3′-U-exo are indicated. [ext], extract titration; *, 32P label.

act at an editing site. The editing endonuclease activities have been found to cleave at a point abutting an anchor duplex, not only at natural editing site but also when only one strand extends (5′) from a duplex (Fig. 5) (45). Reactions using such minimal substrates mimic U-deletional or U-insertional cleavage depending on whether there are U’s or a non-U, respectively, just 5′ of the duplex (see the diagrams in Fig. 1), and this specificity is verified by its AMP-CP stimulation or inhibition, respectively (45; A. Zhelonkina, J. Cruz-Reyes, and B. Sollner-Webb, unpublished data). Remarkably, cleavage of such a basic U-insertion-like substrate remains as active with rapid extracts of the RNAi cells as with control extracts (Fig. 5A).

The 3′-U-exo step of U deletion. The second step in the U-deletional cycle is removal of U’s by the 3′-U-exo at the 3′ end generated in the cleavage step. This TUTase step was assessed using a precleaved U-insertional substrate and PPI (21), analogous to the above-described assay of the 3′-U-exo step. However, reactions with rapid band II RNAi extracts support only minimal U addition (Fig. 7A), indicating that the TUTase step of editing is inactive without band II. Thus, band II appears critical for both the first and second steps of U deletion.

The basic TUTase enzymatic activity was scored independently of editing using a minimal substrate by following transfer of UMP (from [α-32P]UTP) to the 3′ end of a single-stranded RNA (4, 35). It has been shown that the TUTase that adds the U’s in U insertion (RET2) (2), can be distinguished from the TUTase that adds the U’s onto the gRNA (RET1) (2) when using such a minimal substrate, because RET2

studied above (Fig. 3), with the upstream fragment 5′-end labeled to reveal the U removal. Using this substrate, we found that the 3′-U-exo step is as efficient with band II RNAi extracts as with control extracts (Fig. 6A).

As above, the basic enzymatic activity of the 3′-U-exo can be assayed using a minimal substrate, in this case a single-stranded RNA with a 3′ oligo(U) tail that the enzyme preferentially removes (35). Rapid extracts of band II RNAi cells are active in this assay (Fig. 6B). In fact, they reproducibly remove U’s from a larger fraction of the substrate molecules and, on average, remove a larger number of U’s than do their control extracts (Fig. 6B; see also Discussion). The combined data in Fig. 3 and 6 demonstrate that band II is not important for either of the first two steps of U deletion.

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The ligation steps. The ligation step of U deletion is catalyzed by the DREL ligase (12, 19, 39), which remains present upon band II RNAi (see Fig. 8C and reference 28). To assay the ligation step of U deletion, we again used the precleaved approach (21) with RNAs (see the diagram in Fig. 8A) that mimic a U deletion site following endonucleolytic cleavage and 3’ U removal (19). Such U-deletional ligation requires addition of ATP (21), which is needed to activate the DREL ligase (12, 20, 35) that seals this form of editing (12, 19). Using this assay, rapid band II RNAi extracts were found to retain full activity relative to control extracts (Fig. 8A). Thus, the third step of U deletion also remains active upon band II RNAi.

To assay the basic enzymatic activity of this U-deletional ligase, we scored dimer formation of an unstructured RNA (Fig. 8B). Previous studies have shown that this ligation is catalyzed by DREL (36), not IREL (12, 20). This assay shows that the basic DREL enzymatic activity is as robust in band II RNAi extracts as in control extracts.

The second ligase of the editing complex, IREL (TbREL2), is specific for U insertion (12, 39). Adenylylation assays, which include an initial deadenylylation step to detect all ligation molecules (35), show that the IREL protein is lost from band II RNAi cells (Fig. 8C; see also reference 28). When absent, IREL surely cannot serve its function in ligating U insertion. Western and Northern blotting plus sedimentation analysis (28) affirmed that IREL loss occurs at the protein level, not the mRNA level, evidently due to degradation of ligase that is not in the editing complex (28, 36). Thus, band II appears critical for the IREL protein to be recognized and retained in the editing complex and hence to ligate U insertion. Taken together, these analyses reveal that band II is important at each step of U insertion, yet it provides none of these basic enzymatic activities.

Coordination of the individual U deletion steps within the editing cycle. Although we have shown that each step of U deletion remains active upon band II RNAi, this does not ensure that these individual steps will function in a coordinated manner to catalyze a full cycle of editing. This is especially a concern because the depleted complex may be more extended (see Discussion) (28) than the wild-type editing complex. If coordination between the cleavage and 3’-U-exo or between the 3’-U-exo and ligase was impaired, the full-round U deletion reaction (Fig. 9A) should generate more molecules that are cleaved but not ligated (designated 3’ frag) and/or that exhibit partial U removal (−2 edited product). However, there is no accumulation of these molecules with band II RNAi extracts (Fig. 9A), indicating that the cleaved fragments are converted to fully edited product (−3) as efficiently as with control extracts. To verify coordination between the cleavage and 3’-U-exo steps (Fig. 9B), we used 5’-end-labeled pre-mRNA in a U deletion reaction to directly visualize 3’-U-exo action on the terminal U5 of the upstream cleavage fragment (10, 19), with PPI added to prevent ligation (Fig. 9B). The lack of discernible partial U removal products (the position indicated by cleavage; −1U and −2U) affirms that the 3’-U-exo acts efficiently on the cleavage product in both the RNAi and control extracts (Fig. 9B). A repeat of this assay using a gRNA that is less efficient for the 3’-U-exo step (D32 [11]) reinforced this conclusion (data not shown). The results shown in Fig. 9
thus demonstrate that band II is also unnecessary for coordination of the steps of U deletion into a complete editing cycle.

**DISCUSSION**

In the present study, we define the role of band II in editing by individually assessing each enzymatic step of the U deletion and U insertion cycles and additionally assessing the basic activities of those enzymes independent of their action in editing. Furthermore, these assays include extract titrations to ensure that band intensities actually represent activity. The data demonstrate that every step of U deletion remains active (Fig. 2 and data not shown). This experiment used the same RNAi extract as that used for panel A, and although it generates a slightly reduced amount of cleaved RNA, the 3'-U-exo reaction appears as complete as that with control extract. [ext], extract titration; *, 32P label; nt, nucleotide.

![Image](https://example.com/image.jpg)

**FIG. 9.** Full-round U deletion occurs despite band II RNAi. (A) Full-round U deletion assay using 1.2, 0.4, and 0.12 μg of the rapid extracts used for Fig. 5B, 7B, and 8B, as well as AMP-CP and no PPI. The upper panel shows the gel region containing the input and fully edited (−3) RNA; the lower panel shows the region of the same gel that contains the cleaved 3′ fragment. The bands representing the product ligated with incomplete U removal (−2) and the cleaved but unligated fragment (3′ frag) are no more intense, relative to the complete −3 U deletion product, than with control extract, even though this particular experiment used an RNAi extract where U-deletional cleavage is slightly reduced. (B) The 3'-U-exo step of U deletion assayed in combination with gRNA-directed cleavage (as in Fig. 3A, except with 5' end-labeled mRNA) using 1.2 and 0.4 μg of the rapid extracts used for panel A. The 3'-U-exo efficiency is the fraction of the upstream cleavage fragments (the sum of the cleavage, −1U, −2U, and −3U products) from which all three U's (−3U) have been removed. The various fragments are distinguished relative to sequence-specific markers (data not shown). This experiment used the same RNAi extract as that used for panel A, and although it generates a slightly reduced amount of cleaved RNA, the 3'-U-exo reaction appears as complete as that with control extract. [ext], extract titration; *, 32P label; nt, nucleotide.

act by two distinct means. First, through protein recognition, band II retains IREL (Fig. 8C) (28, 39), the U-insertion-specific ligase. Band II holds IREL in the editing complex (28), evidently by a direct interaction (39) without which this ligase becomes degraded (28), in analogy with band III holding DREL in the editing complex (20), and thereby protecting it from degradation (19, 36). Second, Schnaufer et al. (39) have presented intriguing evidence that band II may provide part of the RNA recognition function for the IREL (TbREL2) ligase. An oligonucleotide-oligosaccharide binding (OB) fold domain 27a that is present in most RNA ligases and needed to recognize the RNA substrate (48, 49) is lacking in IREL (TbREL2), but it is likely provided in trans by such a domain in band II (TbMP81) (39). Thus, band II may facilitate U-insertional ligation by binding both the enzyme and its RNA substrate (39). In considering the U addition step of U insertion, band II could function by one or both of those means to enable TUTase action on the editing substrate. By directly binding RET2 (39), band II could correctly hold this TUTase in the editing complex, which in turn could facilitate the enzyme's recognition of the editing substrate. Additionally, or alternatively, band II could present the U-insertional substrate to RET2 by directly binding the RNA, possibly using its OB fold domain (14). For the cleavage step of U insertion, band II could enable specific recognition of the editing substrate, similar to its action at the ligation step, by helping tether the endonuclease within the editing complex and/or by directly binding the U-insertional RNA. It is intriguing to hypothesize that the OB fold domain (and/or zinc finger) of band II could hold the U-insertional substrate throughout all three editing steps. Such retention of the substrate RNA could favor the concerted action of the three U-insertional editing activities. Observing the basic activities of the U-insertional endonuclease and the RET2 TUTase enzymes when band II is depleted (Fig. 5 and 6B) indicates that these enzymes remain present without band II and therefore that they must require aspects of the band II-containing complex to recognize their U-insertional substrate. It follows that if these enzymes are not retained in the band II-depleted editing complex, they must remain stable when separated, which would be different from what happens with the ligases. In any case, band II provides critical recognition for all three steps of U insertion, and we therefore propose that band II be called IRER, for U-insertional RNA editing recognition.

**Extract preparation.** Although traditional mitochondrial extract (see Materials and Methods and references 18 and 38) is frequently used for the study of editing complex from wild-type cells (12, 23, 34, 35, 41), we considered that band II-depleted complex might be less stable and thus might not withstand this lengthy preparation. We therefore compared extract from band II RNAi cells prepared by this protocol and that prepared by a protocol that takes <1/5 the time (20, 28, 36) yet is compatible with all steps of editing (for an example, see Fig. 1C). Interestingly, the U-deletional cleavage, basic U-insertion-like cleavage, and basic 3'-U-exo activities that are retained in rapid extracts of band II RNAi cells (Fig. 3, 5, and 6), and thus surely were in the original cells, are lost in traditional extracts of those cells (Fig. 2 and data not shown). This indicates that band II-depleted editing complex is less stable than wild-type complex, and the apparent lack of these activities in...
traditional extracts is not a direct effect of band II loss but rather an indirect effect due to lability of the band II-depleted complex. This lability presumably explains why occasional rapid extracts of band II RNAi cells exhibit slightly reduced U-deletional cleavage activity (Fig. 9). In contrast, all extracts of the RNAi cells show greatly reduced U-insertional editing activities, evidently reflecting a direct effect of band II depletion.

Cleavage steps. While band II loss affects the U-insertional cleavage step, it does not affect the U-deletional cleavage step (Fig. 3 and 4) or the basic U-insertion-like cleavage activity (Fig. 5), indicating that the band II protein is not the endonuclease. This challenges an earlier proposal that band II may be the editing endonuclease, based on a TbMP81 (band II) RNAi extract exhibiting reduced cleavage of a CYb substrate (13). However, that cleavage activity appeared to sediment somewhat faster than the editing proteins, minimally overlapping the DREL (TbMP52) ligase (13). Indeed, there is another endonuclease activity that sediments slightly faster than the editing proteins and is known to efficiently cleave this CYb substrate adjacent to its editing site (33), suggesting that study (13) may not have scored an editing endonuclease. In contrast, our data demonstrate that band II enables the U-insertional endonuclease to productively recognize its editing substrate, a novel activity for an editing protein.

3′-U-exo and TUTase steps. Our assays for the individual 3′-U-exo and TUTase steps suggest that band II plays a recognition role at the second step of editing, specifically in U insertion, much like that at the first step. The initial study of band II (TbMP81) RNAi (13) suggested that both the 3′-U-exo activity in precleaved U deletion and the TUTase activity in precleaved U insertion were diminished, the latter approximately two- to threefold more than the former, as quantitated from the residual unligated reaction intermediate. Their reported decrease in the TUTase intermediate is consistent with our data (Fig. 7A), but we suspect their reported decrease in the 3′-U-exo intermediate (13) is not a direct effect of band II RNAi but rather an indirect effect due to lability of the complex. Supporting this contention, the 3′-U-exo remains fully active in rapid extracts of band II RNAi cells (Fig. 6) yet is diminished during a lengthy extract preparation. Our additional assessment of the basic TUTase enzymatic activity (Fig. 7B) by RET2 (2, 14) demonstrates that its inactivity in U insertion upon band II RNAi is not due to loss or inactivation of the TUTase enzyme (Fig. 7B). Instead, all data support band II facilitating specific recognition by the TUTase.

Product profiles in the basic 3′-U-exo and TUTase enzymatic assays of band II RNAi extracts are also noteworthy. The basic 3′-U-exo activity is more robust in band II RNAi extracts than in control extracts (Fig. 6B), suggesting that exogenous RNA has greater access to the 3′-U-exo in the band II-depleted complex. That could result if band II-depleted complex is more extended than the wild type, as we earlier suggested (28). In the basic TUTase assay, the profile of products is altered upon band II RNAi, with many additional molecules acquiring short U tracts (Fig. 7B). If this U addition is by the RET2 TUTase, which frequently adds short U tracts in U insertion (2, 14), it could also suggest that exogenous RNA has greater access to enzymes upon band II depletion. Alternatively, it has been proposed that U insertion arises from the TUTase generating longer U tracts which are then trimmed by 3′-U-exo (6, 21, 26). If the final U length is determined by this proposed additional step in the U insertion cycle, then the longer U tracts produced in the basic TUTase assay by the RNAi extract (Fig. 7B) would likely reflect impaired coupling of this TUTase step to the 3′-U-exo step, so all four steps of such a U insertion cycle would depend on band II. Although the RET1 TUTase appears separate from the editing complex (2, 14) and generally adds much longer U tracks when assayed for basic TUTase activity (3, 14), we cannot rule out that the short U tracts that arise upon band II RNAi could be due to this TUTase.

Ligation step. Band II is also specific for U insertion at the ligation step. DREL (band IV/TbMP52/TbREL1), which is needed to seal U deletion (12, 19, 39), remains present (Fig. 8C and reference 28) and active in ligating U deletion (Fig. 8A and 9A) upon band II RNAi. In contrast, IREL (band V/TbMP48/TbREL2), which only seals U insertion (12, 39), is lost (as revealed by adenylylation assays that include the deadenylylation step and Western analyses [Fig. 8C and reference 28]). Although U insertion can be sealed by DREL in editing complexes lacking only IREL (13, 16, 28), it is unknown whether DREL appreciably serves this function in the wild-type editing complex. Furthermore, DREL activity in the U-insertional cycle cannot be assessed in the editing complex lacking band II, because assaying for U-insertional ligation requires the TUTase step (13, 16, 28) (which is inactive upon band II RNAi) (Fig. 7A). An earlier study (13), which scored adenylylation of ligases without prior deadenylylation, showed that the IREL (TbMP48) protein was either depleted or preferentially preadenylylated in band II (TbMP81) RNAi extract. However, in their wild-type extract, most of the IREL appeared preadenylylated (13) and the preadenylylated fraction of this ligase should increase upon band II RNAi (28), suggesting that these adenylylation differences (13) may not reflect altered protein abundance. The initial band II (TbMP81) RNAi study (13) additionally showed ligation in precleaved editing assays. Those authors reported somewhat inhibited ligation in precleaved U deletion, which we did not observe and which may again reflect instability of band II-depleted complex in their extract preparation, and twofold more inhibited ligation in precleaved U insertion, which presumably reflects both TUTase inactivity and IREL loss. Thus, all data support band II being important for retention of IREL but not DREL in the editing complex.

As understanding about U deletion and U insertion has grown, models for the editing complex have evolved, oscillating between extremes. When these two forms of processing were originally found to involve three parallel reaction steps (Fig. 1A) (10, 23, 35, 41), it seemed that these parallel steps could share enzymatic activities (17, 46). However, finding that the U-deletional and U-insertional endonuclease activities have different features (8, 11), that the 3′-U-exo is not a reverse TUTase reaction (10, 35), and that the two ligases (35, 37) serve the two forms of editing differently (12, 19, 20, 39) suggested that U deletion and U insertion use separate activities, likely present in different domains of a common editing complex (8, 9, 39). While the two forms of editing could be catalyzed by separate protein complexes (46), such complexes would need to exchange many times during editing of an
mRNA, and extensive purification (1, 9, 31, 35, 39) has not separated natural complexes that catalyze only one form of editing. Interestingly, overexpressed tagged ligase proteins have enhanced the abundance and facilitated the isolation of ~10s subcomplexes, one containing DREL (TbREL1), band III (TbMP63), and band I (TbMP99) that catalyzes precleaved U insertion, and another containing IREL (TbREL2), band II (TbMP81), and TbMP57 (RET2) that catalyzes precleaved U deletion (39). Although neither subcomplex catalyzes cleavage (39), these data attest that at least the later two steps of U deletion and U insertion are catalyzed by different domains of the editing complex. Indicating that all three steps of U insertion utilize a distinct subcomplex, we find that band II is critical for the cleavage as well as the later two steps of U insertion but not for the steps of U deletion (Fig. 3 to 8). Additionally reinforcing the notion of functionally separate subcomplexes for the two forms of editing, all the steps of U deletion remain coordinated in catalyzing the full editing cycle, despite the band II-depleted complex being less stable (Fig. 2) and likely less compact (28) and having defects in all steps of U insertion (Fig. 4, 7, and 8). However, band II depletion causing the U-deletional activities to be more labile (Fig. 2A) and the U-deletional 3'-U-exo to be more accessible (Fig. 6C) implies that the subcomplexes also exhibit significant interdependence within the intact editing complex.

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