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Kinetoplastid RNA editing ligases: complex association, characterization, and substrate requirements

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Abstract

RNA editing processes kinetoplastid mitochondrial transcripts post-transcriptionally by inserting and deleting uridylates (Us) to produce functional mRNAs. The activities of the RNA ligases in the multienzyme complex (the editosome) that catalyzes editing and of the recombinant proteins were characterized and found to be similar. Ligation of two RNA fragments was enhanced when bridged by a complementary RNA or DNA, which left no gaps or overhangs. An acceptor nucleotide preference of G > U > C > A was observed in the absence of exogenous ATP but U was preferred upon addition of ATP and ligase activity was increased. The substrate specificity and catalytic characteristics indicate that RNA ligase activity contributes to the accuracy of RNA editing.

Keywords: RNA editing; Editosome; RNA ligase; *Trypanosoma brucei*; Kinetoplastid

1. Introduction

The trypanosome mitochondrion contains a concatenated network of DNA, known as kinetoplastid DNA (kDNA) that consists of tens of maxicircles and thousands of minicircles. The maxicircles encode proteins that are homologous to those encoded in the mtDNA of other organisms. However, most maxicircle genes are encrypted and their transcripts undergo a post-transcriptional processing known as RNA editing that inserts and deletes uridylates (Us) to produce mature mRNAs [1,2]. The edited sequence is specified in trans by small guide RNAs (gRNAs) that are transcribed from minicircles. Editing occurs by a series of steps that are catalyzed by a multienzyme complex, the editosome [3,4]. The initial steps in editing are the formation of gRNA–mRNA duplexes, followed by endonucleolytic cleavage of the pre-mRNA. Cleavage usually occurs at the first mismatch 5′ to the duplex and selects the site to be edited. Cleavage is followed by U addition or removal by 3′ terminal uridylyl-transferase (TUTase) or a 3′ exouridylylase (ExoUase), respectively, depending on whether the gRNA specifies U insertion or deletion. The processed mRNA fragments are joined by RNA ligase to complete one round of editing. Each gRNA directs several rounds of editing and thus specifies the sequence of several editing sites and several gRNAs are used to edit most pre-mRNAs.

Ligase activity was observed prior to the discovery of RNA editing [5], and later shown to be localized in the kinetoplast-mitochondrion [6]. Two mitochondrial RNA ligases co-purify with editosomes and with in vitro editing activity [7–10]. These RNA ligase activities have characteristics that indicate they contribute to the specificity of the RNA editing process by preferential ligation of pre-mRNA fragments that are processed as specified by the gRNAs [11,12]. Two related RNA ligase genes (*TbMP52* named *REL1* or band IV ligase, and *TbMP48* named *REL2* or band V ligase) were identified in biochemically purified editosomes and the RNA ligase activity of the recombinant REL1 protein demonstrated its catalytic capability [9,10,13,14]. The presence of REL1 protein in editosomes was demonstrated by immunoprecipitation of active editosomes with monoclonal antibodies specific for REL1 protein [9,15]. In

Abbreviations: gRNAs, guide RNAs; REL1 & 2, RNA editing ligase 1 & 2; MAb, monoclonal antibody; Us, uridylates

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addition, REL1 has been shown by co-immunoprecipitation experiments to interact with the TbMP63 zinc finger protein, another protein component of the editosome [15]. Blocking expression of the larger of these genes, *REL1*, resulted in the loss of editing [10,14] but blocking expression of the smaller RNA ligase gene did not [16]. Thus, *REL1* is essential for editing while *REL2* is either not essential for editing or there is compensation for its loss, perhaps by *REL1*.

This paper reports the characteristics of highly purified *Trypanosoma brucei* native and recombinant editing RNA ligase activities using the precleaved in vitro assay and demonstrates catalytic activity of recombinant REL2 protein. These characteristics include terminal nucleotides preferences that become preferential for U and more active with added ATP. Efficient ligation requires a bridging polynucleotide (e.g. gRNA), and bridges that bring nucleotides adjacent contribute to the accuracy of editing, and vary from observed characteristics of other RNA ligases. Except for the poor ligation of molecules with overhangs or gaps by the recombinant ligases, we could not detect any differences in the activities between the recombinant ligases and the native ligases within the editosome.

2. Materials and methods

2.1. Mitochondrial extract

Mitochondria were isolated from log phase procyclic *T. brucei* strain IsTar 1.7a by differential centrifugation and Percoll floatation as described [17]. Editosomes were fractionated from lysed mitochondria by sequential SP Sepharose cation-exchange, Q Sepharose anion-exchanage, and Superose 6 gel filtration column chromatography as described [9]. Fractions from peak II of Superose 6 column were concentrated approximately three-fold by volume using Centricon-YM50 membrane (Amicon) at 3000 × *g* when used to assess no., 0A, 1A, and 2A gRNAs.

2.2. Preparation of RNAs and DNA oligonucleotides

All RNA substrates were transcribed in vitro from PCR generated DNA templates using T7 RNA polymerase (Promega), except for the 3′ fragment (3′CL13pp) that was purchased as a synthetic RNA oligonucleotide from Oligos, etc. Template DNA for 5′CL18 RNA transcription was derived from PCR amplification of an oligonucleotide pair 5′-GGC GGA ATT CTG TAA TAC GAC TCA CTA TAG GAA GTA TGA GAC GTA GG-3′ and its complementary sequence; using primers *Eco*RI T7 (5′-CGG CGG AAT TCT GTA ATA CGA CTC ACT ATA G-3′) and 5′CL18 (5′-CCT ACG TCT CAT ACT TCC TAT AG-3′). Substrate for the overhang experiment was derived from the same template using *Eco*RI T7 and 5′CL3U-3′ (5′-AAA CCT ACG TCT CAT ACT TCC TAT AG-3′) primers. Substrates for terminal nucleotide variation experiments also used the same PCR template and *Eco*RI T7 oligo, but the following oligonucleotides: terminal A (5′-TCT ACG TCT CAT ACT TCC TAT AG-3′), terminal C (5′-GCT ACG TCT CAT ACT TCC TAT AG-3′), and terminal U (5′-ACT ACG TCT CAT ACT TCC TAT AG-3′). gRNAs were transcribed from PCR products derived from gpCA6-3A-Tmpl: (5′-GGC GGA ATT CTG TAA TAC GAC TCA CTA TAG GAT ATA CTA TAA CTC CGA TAA ACC TAC GTC TCA TAC TTC C-3′, and complementary sequence) with primers *Eco*RI T7 and gpCA6—0A, 1A, 2A, 3A (5′-GGA AGT ATG AGA CGT AGG $[T]_n$ ATC GGA GT-3′, where *n* is equivalent to the number of A nucleotides in the gRNA). Substrates in DNA ligation experiments were purchased as oligonucleotides from GIBCO-BRL, 5′CL18 DNA (5′-GGA AGT ATG AGA CGT AGG-3′), 3′CL13pp DNA (5′-ATT GGA GTT ATA G-3′), and gpCA6 DNA splint 3′ (5′-CCT TCA TAC TCT GCA TCC TAG CCT CAA TAT CAT ATA GG-3′). Transcription products were purified by electrophoresis through 9% (w/v) polyacrylamide denaturing gels. 5′ cleavage RNA fragments were radiolabeled by either 5′ end capping using guanylyltransferase and [α^{32}-P]GTP or by phosphorylation of alkaline phosphatase treated RNAs with [γ^{32}-P]ATP and T4 polynucleotide kinase, followed by gel purification. Synthetic DNA substrates were radiolabeled with T4 polynucleotide kinase and [γ^{32}-P]ATP.

2.3. RNA ligase assays

Ligation reactions were performed as previously described [11], but without UTP. Reactions were performed in 25 mM HEPES (pH 7.9), 10 mM $Mg(OAc)_2$, 5 mM $CaCl_2$, 50 mM KCl, 0.5 mM DTT, 1 mM EDTA and 0.3 mM ATP (except in ATP reactions) in 15 μl volume and contained 50 fmol labeled 5′ fragment, 0.5 pmol gRNA, 1 pmol 3′ fragment, and appropriate extract with incubation at 27 °C. T4 RNA ligase was tested at various dilutions in RNA editing conditions to attain a linear range of ligation activity.

Adenylation of the editing RNA ligases [7] was carried out by incubating 10 μl of mitochondrial fraction for 15 min at 28 °C with 2.5 μCi of [α-^{32}P]ATP in 25 mM HEPES (pH 7.9), 10 mM $Mg(OAc)_2$, 50 mM KCl, 0.5 mM DTT and 10% DMSO. The radiolabeled proteins were resolved on 10% SDS-PAGE gels and visualized on a Storm PhosphorImager (Molecular Dynamics). Quantification was performed using ImageQuaNT software.

2.4. Recombinant proteins

The *REL2* gene was amplified from *T. brucei* genomic DNA by PCR (primers 3664 5′-CCG AAT TCA TGC ACC ATC ACC ATC ATC ATG TCG GTG GGG ACG GCA GCA-3′ and 3665 5′-CCG GAT CCT CAT TCG CTA AAG TCA G-3′) and cloned into pSG1 vector. The predicted protein from the construct contained a 6× His tag at the N-terminus that replaced the mitochondrial import signal (amino acids 2–17). The REL1 protein was expressed as

described [10]. Recombinant proteins were synthesized in vitro using a cell-free coupled transcription and translation system according to the manufacturer's instructions (TNT, Promega). Recombinant REL1 and REL2 proteins were immunoprecipitated with MAb P3C1 (anti-REL1) and 6× His MAb (Clontech), respectively. A total of 4×10^7 immunomagnetic beads (Dynabeads M-450; Dynal) coated with goat anti-mouse IgG plus 1% BSA were incubated with 1 ml P3C1 tissue culture supernatant or 6× His MAb diluted 1:100 in IP buffer (10 mM Tris pH 7.2, 200 mM KCl, 0.1% Triton X-100) for 1 h at 4 °C with bi-directional mixing then washed three times with IP buffer. The antibody-bound beads were incubated with 50 μl in vitro coupled transcription–translation products in 400 μl of IP buffer containing 1% BSA with bi-directional mixing for 1 h at 4 °C. The beads were washed three times with IP buffer and once with buffer containing 1× HHE (25 mM HEPES pH 7.9, 10 mM $Mg(OAc)_2$, 50 mM KCl, 1 mM EDTA). The beads were resuspended in 1× HHE and assayed for ligase activity. Serial dilutions were made of REL1 and REL2 IPs to ensure that the activity measured was within the linear range.

3. Results

3.1. Complex association and ligase activity of native protein

The RNA ligase and adenylylation activities copurified with in vitro RNA editing activity (Fig. 1A–C). The ligase activity eluted from SP Sepharose cation exchange columns as a broad peak that encompassed the peaks of both in vitro deletion and precleaved insertion editing. The breadth of the peak probably reflects complex interactions between the column and the multiple proteins of the editosome that contains the RNA ligases. A major peak of adenylylation co-eluted with the ligase activity and in vitro deletion editing. Additional peaks of adenylylation eluted in later fractions. These may represent fragments that dissociated from the editosome or cellular ligases that contaminate the isolated mitochondria. The RNA ligase activity and most of the adenylylation activity in the pooled fractions from the SP Sepharose column co-eluted from the Q Sepharose anion exchange column with each other, with in vitro editing, and with an elution profile narrower than the SP Sepharose column. A smaller

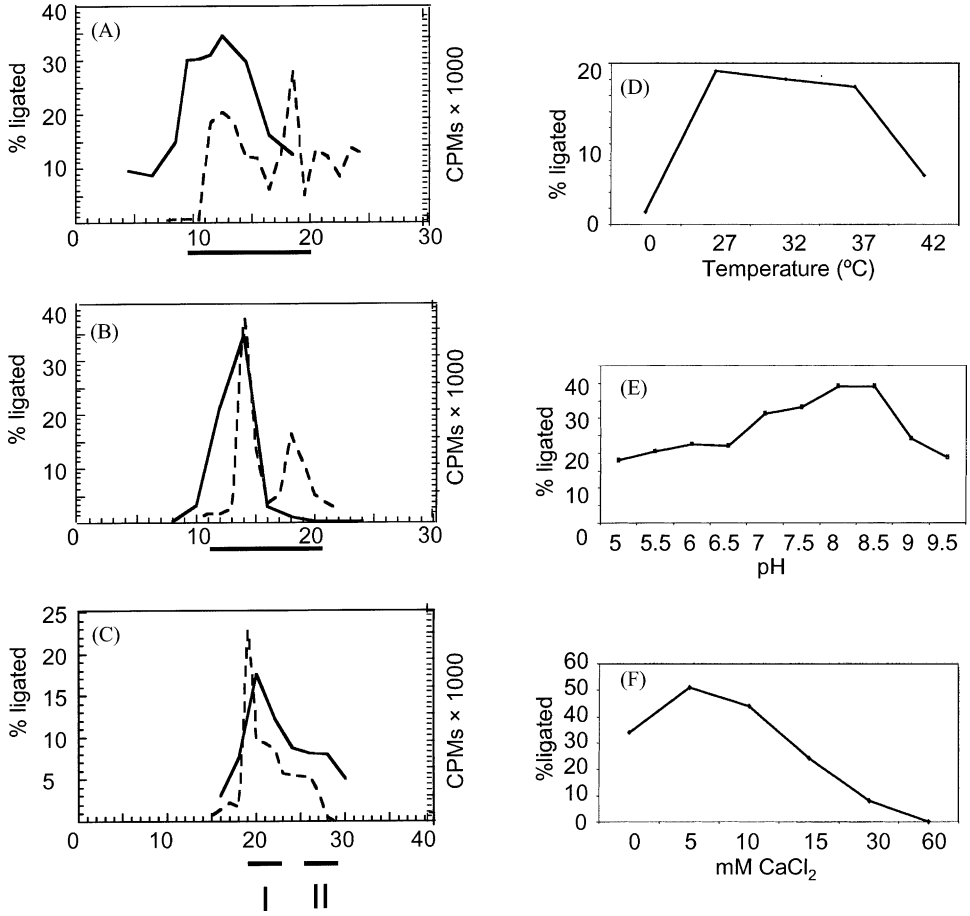

Fig. 1. Ligase (solid) and adenylylation (dashed) activities fractionated from *T. brucei* mitochondria. Underlined fractions indicate fractions with active in vitro precleaved insertion editing which were pooled and fractionated with subsequent columns. (A) SP Sepharose fractionation of cleared mitochondrial lysates. (B) Q Sepharose separation of fractions from (A). (C) separation of fractions from (B) using Superose 6. (D–F) Ligase activities at different temperatures, pH, and $CaCl_2$ concentrations of Superose 6 peak I.

second peak of adenylylation was observed, again perhaps reflecting fragmentation. The ligase, adenylylation and editing activities [9] from the pooled fractions co-eluted from Superose 6 size exclusion columns with a major peak (I) at ~1600 kDa and a shoulder (II) at ~500 kDa. Peak I coincides with complexes that are capable of a full round of editing, while II lacks some editosome proteins, and editing associated activities [15,18]. Thus, the larger peak appears to contain more intact editosomes than does the smaller peak. Overall, the bulk of the mitochondrial RNA ligase and adenylylation activities are associated with the editosomes. The ligase activity in Superose 6 peak I had broad temperature optima between 27 and 37 °C, a pH optimum between 7 and 8.5, and maximal activity at 5 mM $CaCl_2$ (Fig. 1D–F).

Peak I, but not peak II, had a consistent, low level of ligation in the absence of added ATP (Fig. 2A) while such activity, although lower than in peak I, was occasionally detected in peak II but was detectable when peak II was concentrated approximately three-fold. Most of the ligated material was specific, i.e. no ligation in the absence of a gRNA and ligation is most efficient with no gap in the guiding sequence (Table 1). Addition of ATP greatly enhanced ligation by peak I, and in substantial ligation by peak II, but resulted in a substantial increase in the proportion of non-specific ligation (i.e. with one or two nucleotide gaps). Addition of CTP and UTP did not increase ligation compared to no added nucleotide while addition of GTP stimulated ligation slightly, perhaps due to ATP contamination. There was a sharp increase in ligation activity with increasing ATP concentration above 0.03 mM in peak I up to approximately 0.6 mM above which activity was lower (Fig. 2B).

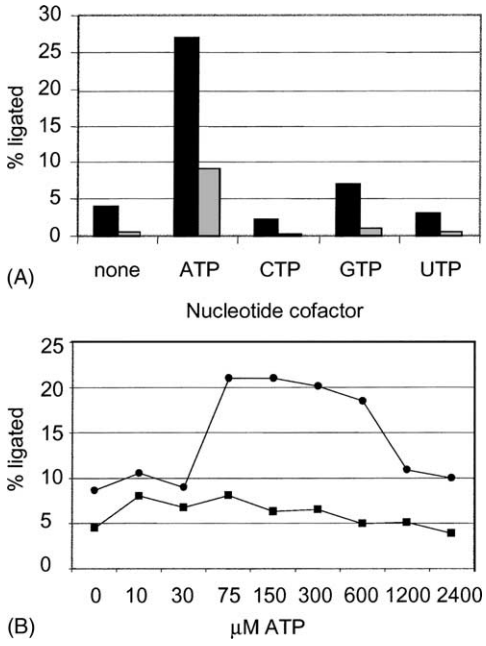

Fig. 2. Nucleotide effects on ligase activity. (A) Ligase activities of Superose 6 peaks I (black) and II (gray) in the absence of added rNTP or upon addition of 0.3 mM of each rNTP. (B) Ligase activities of Superose 6 peaks I (●) and II (■) as a function of final ATP concentration. Differences in activity in the absence of added ATP reflect experimental variation between preparations.

Table 1
Percent ligation of input RNA by recombinant REL1 or REL2, Superose 6 peak I or II in the presence or absence of 0.3 mM added ATP

	gRNA			
	None	0A	1A	2A
REL1 + ATP	0	29.6	9.9	0.21
REL2 + ATP	0	4.24	0.126	0.09
S6 I + ATP	0	53	48	28
S6 I − ATP	0	25.9	16.43	0.4
S6 II + ATP	0	36	33	26
S6 II − ATP	0	16	11	0.6
T4 RNA ligase	2	2	2.43	7.8

Ligation was assayed in the absence of complementary gRNA or with gRNAs that specify 0, 1, or 2 nucleotide gaps between 5′ and 3′ substrate RNA fragments as indicated. T4 RNA ligase was used as a control. Peak II was concentrated about three-fold.

3.2. Recombinant protein activity and bridging requirements

As was observed for REL1 protein [10], immunoprecipitated REL2 protein had RNA ligase activity confirming the function of this gene (Fig. 3). Neither anti-REL1 nor 6× His MAb immunoprecipitated any detectable ligase activity from TNT extracts alone, or controls wherein only pSG1 vector was used (data not shown). Ligation by recombinant proteins only occurred in the presence of added ATP. Ligation by recombinant ligases and purified complexes, was efficient in the presence of a bridging complementary RNA (e.g. gRNA) but undetectable in its absence under the assay conditions used. In addition, ligation by the complexes, but not recombinant ligases, was readily detected when a complementary DNA was substituted for the complementary RNA (Fig. 3 and Table 2). Superose 6 peaks I and II ligated a 5′ DNA and 3′ RNA when an RNA but not DNA bridge was present and peak I but not peak II ligated two DNAs when an RNA

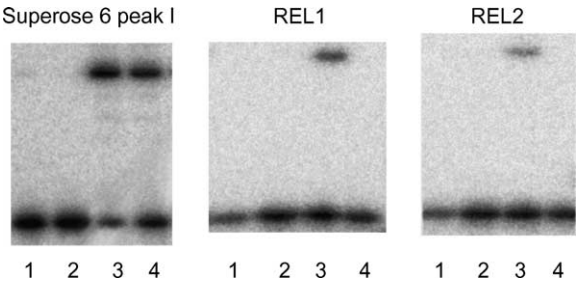

Fig. 3. Ligation by purified editosomes and recombinant editing ligases REL1 and REL2. RNA ligase activities of Superose 6 peak I, transcribed–translated REL1 (REL1) and REL2 (REL2) that were immunoprecipitated with anti-REL and 6× His MAbs, respectively. The lanes in each panel contain (1) 5′ fragment only, (2) 5′ + 3′ fragments, (3) 5′ + 3′ fragments + gRNA, and (4) 5′ + 3′ fragments + complementary DNA.

Table 2
Ligation of RNA and/or DNA fragments in the presence or absence of complementary RNA or DNA

5′	3′	Splint	S6 I	S6 II	REL1	REL2
RNA	RNA	None	−	−	−	−
RNA	RNA	RNA	+	+	+	+
RNA	RNA	DNA	+	+	−	−
RNA	DNA	None	−	−	−	−
RNA	DNA	RNA	+	+	−	−
RNA	DNA	DNA	+	+	−	−
DNA	RNA	None	−	−	−	−
DNA	RNA	RNA	+	+	−	−
DNA	RNA	DNA	−	−	−	−
DNA	DNA	None	−	−	−	−
DNA	DNA	RNA	+	−	−	−
DNA	DNA	DNA	−	−	−	−

See Section 2 for details. (+): detectable ligation; (−): ligation not detected.

but not DNA bridge was present (Table 2). These results may reflect progressively less ligase protein in the peak I, peak II, REL1, and REL2 samples, more stable RNA/RNA versus RNA/DNA interactions, and/or binding interactions with the complexes compared to the recombinant proteins. Nevertheless, the preference for a double-stranded substrate and the ability to utilize a bridging DNA is reminiscent of the sequence similarity of REL1 and REL2 to DNA ligases [10].

3.3. Preferential ligation

The RNA editing ligase activity preferentially joins RNA termini that are bridged by gRNAs so that there are no gaps or overhangs between the 5′ and 3′ fragments [11,12]. This mimics the final step of a round of editing wherein U removal or addition results in base-pairing between the mRNA and gRNA both upstream and downstream of the editing site and brings the nucleotides that will be ligated into adjacency. In contrast, T4 ligase does not require a bridging RNA and prefers non-base paired RNA termini, as previously reported [19] (Table 1). Similarly, editosomes in Superose 6 peak I preferentially ligated RNAs with no overhanging Us (Fig. 4). In experiments presented here the endogenous ExoUase activity in the editosomes removed various numbers of the overhanging Us thus presenting the ligase with a set of substrates. The ligase activity preferentially ligated the products with the numbers of Us specified by the gRNA. Hence, 5′ fragments with unpaired 3′ Us (i.e. overhangs) were ligated with lower efficiency. The 1U ligated product with the 0A gRNA may be due to competition between the exonuclease and ligases for the RNA substrate. U removal is processive and in previously reported studies [20], complete removal of Us took up to 8 h. Ligation occurs much more rapidly, and most likely joined the most favorable substrates prior to complete U removal. Studies using a purified *Leishmania tarentolae* RNA ligase used various substrates with different numbers of overhanging Us for ligation and found

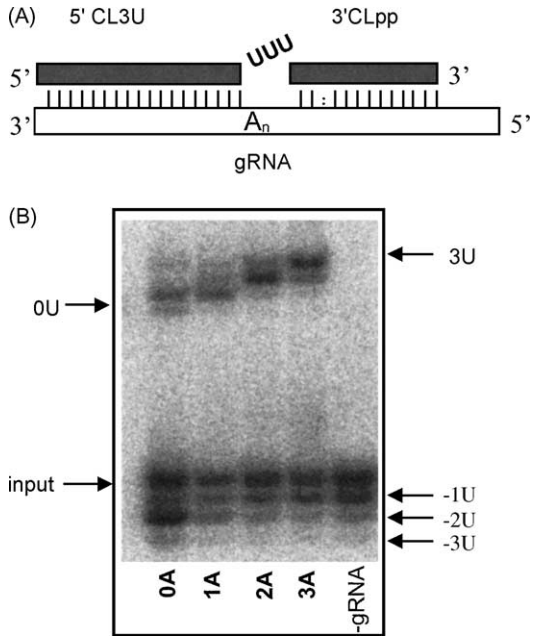

Fig. 4. Ligation of substrates with U overhangs by Superose 6 peak I. The overhang ligation substrates in which the splinting gRNA has 0, 1, 2, or 3. As opposite the ligation site to produce a corresponding number of overhanging Us are illustrated in panel A. (B) The input 5′labeled 5′CL3U is indicated in the autoradiogram as are products from which 1, 2 or 3 Us are removed by editing exonuclease and ligation products with no removed Us (0U) or with 3 removed Us (3U). Reaction in the absence of gRNA is also indicated.

similar results [12]. Immunoprecipitated recombinant REL1 and REL2 proteins also preferentially ligated splinted RNAs with no gaps compared to RNA with a two nucleotide overhang or two nucleotide gap (Fig. 5A and Table 3). However, the sensitivity to a gap or overhang was greater than with editosomes. This may be due to more stable binding of the RNA fragments by editosomes that have several RNA binding proteins compared to the recombinant proteins, or the amount of recombinant protein.

3.4. Terminal nucleotide preference

The ligase activity of Superose 6 purified editosomes (peak I) has a preference for the 3′ terminal nucleotide of the 5′ fragment (Fig. 5). In the absence of added ATP, 5′ fragments with a 3′ terminal G were preferentially ligated, reflecting the presence of adenylylated ligase. However,

Table 3
Percent ligation of the input RNA resulting from incubating the substrates with immunoprecipitated REL1 and REL2 proteins

	No gRNA	No gaps or overhangs	2U overhangs	Two gaps
REL1	0	14.5	0.2	6.4
REL2	0	4.8	0.04	0.55

The number of U overhangs or gaps in the substrate as specified by splinting gRNA is indicated.

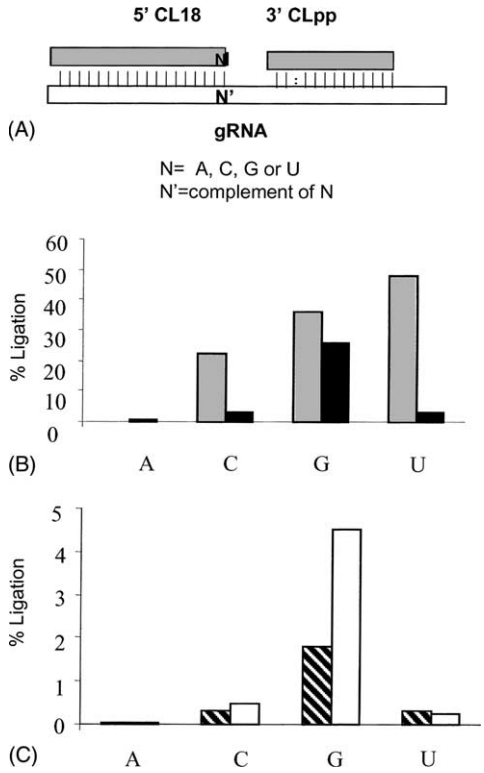

Fig. 5. Terminal nucleotide preferences of editing ligase activities. (A) Schematic representation of the substrates. Ligation activity of (B) Superose 6 peak I editosomes assayed in the absence (black) or presence (gray) of 0.3 mM ATP. Ligation activity in the presence of 0.3 mM ATP of (C) recombinant REL1 (open) or REL2 (cross-hatched). Recombinant REL1 and REL2 were immunoprecipitated with anti-REL1 and 6× His MAbs, respectively.

addition of 0.3 mM ATP resulted in ligation of 5′ fragments with 3′ U > G > C > A (Fig. 5A). Control T4 RNA ligase had A > C > G > U terminal nucleotide preference as previously reported [19]. Recombinant REL1 and REL2 ligases assayed in the presence of added ATP had a terminal nucleotide preference similar to that of Superose 6 peaks I and II complexes in the absence of ATP (Fig. 5B and C). Ligation by the recombinant proteins was not detected in the absence of added ATP.

4. Discussion

Two mitochondrial editing RNA ligase activities were examined together as proteins within the editosome and as individual recombinant proteins. Both the native and recombinant ligases most efficiently ligate a 5′ RNA with a 3′ OH to 3′ RNA with a 5′ phosphate when these RNA fragments are base-paired with a bridging RNA. Editosome associated enzymes will also ligate RNA with DNA and two DNAs when duplexed with RNA but not when duplexed with DNA. Ligation is most efficient when the RNA fragments are base-paired with the bridging RNA such that there is no gap or overhang. This most closely resembles the circumstances under which ligation occurs in vivo. The preference for double-stranded substrates with no gap or overhang resembles that of DNA ligases to which the editing ligases show some sequence similarity [10] and distinguishes it from T4 RNA ligase [19]. The requirement for a bridging RNA implies that substrate recognition entails a nucleic acid duplex of which the gRNA equivalent is an RNA. The two ligases may be required to accomplish both insertion and deletion editing. Preferential ligation of molecules with the number of Us specified by the gRNA as a result of U addition by 3′ TUTase and/or U removal by 3′ ExoUase indicates that the RNA ligase activities along with specificities of TUTase and ExoUase and the interactions between pre-mRNA and gRNA contributes to the accuracy of RNA editing.

The characteristics of the ligase activities in the complex are very similar to those of individual recombinant proteins. However, ATP affects the accuracy of ligation, 3′ terminal nucleotide preference, and relative activity with bridging RNA versus DNA. These probably result from the association of the ligases with other proteins in the editosome. This association is likely to enhance the association of RNAs with the editosome perhaps by proteins that have zinc finger or other RNA binding domains [15,21]. This may affect presentation of the RNA substrates to the enzymes of the editosome and perhaps adenylylation of the ligases and also affect ligase conformation. The shift in terminal nucleotide preference from G to U upon addition of ATP in native but not recombinant ligases may reflect conformational changes in the complex. The terminal nucleotide preferences of the ligases are consistent with bias in the sequences of natural RNA editing sites [22] suggesting that the bias may reflect catalytic characteristics of these enzymes well as gRNA/mRNA interactions. The increases in both specific and non-specific ligation upon addition of ATP may be the result of increases in ligase adenylylation and indirect effects resulting from increased interaction of ATP with other editosome proteins. The activities of the RNA ligases in the purified complexes have broad temperature and pH optima. The $CaCl_2$ optimum of 5 mM is in contrast with a report on the effect of $CaCl_2$ on ligase activity in purified *L. tarentolae* extracts [12]. This may reflect species differences or technical differences between laboratories. The 1600 kDa complex from the Superose 6 column retains all editing capabilities that can be tested in vitro, while the 500 kDa complex appears to be less complete since it lacks at least one protein and endonuclease activity [15,18] and ligation in the absence of exogenous ATP and ligation of DNAs could not be detected.

The two editing ligases may have distinct but not mutually exclusive functions in editing. Gene inactivation and enzymological studies suggest that REL2 functions in insertion editing and REL1 in deletion editing [10,16,23]. However, *REL2* gene inactivation does not block editing. Also REL2 does not have a demonstrable preference for U termini as would be anticipated for an insertion ligase. Hence, ligase function may be preferential but not exclusive for insertion versus deletion editing or REL1 can compensate for the

absence REL2 but the reverse is not the case in vivo. The compensation may be functional wherein REL1 performs ligations normally performed by REL2 or it may be physical wherein REL1 replaces REL2 in the editosome. Further study is needed to resolve this issue. That in vitro editing by REL1 knockouts has an increased ligase specificity suggests that REL2 is functional and is a more stringent ligase than REL1 (Palazzo, unpublished data; [23]). This is consistent with the observed tolerance to substrate gaps and overhangs by REL1 compared to REL2.

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