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The Mitochondrial RNA Ligase from *Leishmania tarentolae* Can Join RNA Molecules Bridged by a Complementary RNA*

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A biochemical characterization was performed with a partially purified RNA ligase from isolated mitochondria of *Leishmania tarentolae*. This ligase has a K_m of 25 ± 0.75 nM and a V_{max} of $1.0 \times 10^{-4} \pm 2.4 \times 10^{-4}$ nmol/min when ligating a nicked double-stranded RNA substrate. Ligation was negatively affected by a gap between the donor and acceptor nucleotides. The catalytic efficiency of the circularization of a single-stranded substrate was 5-fold less than that of the ligation of a nicked substrate. These properties of the mitochondrial RNA ligase are consistent with an expected *in vivo* role in the process of uridine insertion/deletion RNA editing, in which the mRNA cleavage fragments are bridged by a cognate guide RNA.

RNA ligases are present in a large variety of organisms, but only a few have been implicated in specific metabolic pathways. For example, T4 RNA ligase repairs nicks in the anticodon domains of tRNAs in T4-infected *Escherichia coli* cells (1). In eukaryotes and Archea, a tRNA ligase is involved in tRNA splicing; this RNA ligase contributes to the maturation of tRNAs by joining tRNA half molecules generated by the removal of introns from tRNA precursors (2). Recently, a 2′-5′ RNA ligase has been characterized in uninfected bacteria; the function of this RNA ligase remains unclear, but it may reveal an unexpected step in *E. coli* RNA metabolism (3). An RNA ligase has also been invoked for the final step of uridine insertion/deletion RNA editing in mitochondria of kinetoplastid protozoa (4).

RNA editing in kinetoplastid mitochondria is a posttranscriptional maturation of pre-edited mRNA (5). This modification process consists of insertions and, to a lesser extent, deletions of U residues in the pre-edited mRNA, usually within coding regions. The information for the specific insertions or deletions is present as complementary sequences (allowing G-U base pairs) in short guide RNA molecules (gRNAs).[1] The gRNAs are encoded by both the maxicircle and minicircle components of the mitochondrial DNA (kinetoplast DNA) of trypanosomatids (4). Evidence to date suggests that the modified enzyme cascade model (4–9) is essentially correct, but many details remain to be established. A specific gRNA first forms a duplex region just downstream of the editing site. This is followed by a precise endonucleolytic cleavage at the first mismatched base (10, 11), addition of Us to the 3′ end of the 5′ cleavage fragment, trimming of non-base-paired Us by a 3′ to 5′ exonuclease, and finally a religation of the two cleavage fragments. An RNA ligase activity has been detected in isolated mitochondria of *Leishmania tarentolae* (12) and *Trypanosoma brucei* (13). Ligase activity sedimented as a major 20 S peak and a minor 10 S peak in glycerol gradients of mitochondrial extract from *L. tarentolae*. In *T. brucei*, mitochondrial ligase activity sedimented as two peaks of equal size (14). Two proteins of 50 and 45 kDa in both species were detected comigrating with the ligase activities. These two proteins could be adenylated by [α-^{32}P]ATP and deadenylated by incubation with ligatable RNA substrates (13) and therefore may represent putative components of the mitochondrial RNA ligase (14). Comigration of *in vitro* gRNA-dependent U insertion and U deletion activities in *T. brucei* (15, 16), gRNA-independent U insertion activity in *L. tarentolae* (17, 18) with the 20 S ligase activity, and the inhibition of the *in vitro* editing activities by ATP analogs nonhydrolyzable at the α-β bond were consistent with a role for the RNA ligase in the editing reaction. A band on a native gel has been identified as the 20 S complex that contains the two adenylatable proteins in a mitochondrial extract from *L. tarentolae* (14).

In this paper, we show that a partially purified mitochondrial RNA ligase from *L. tarentolae* can join two RNA molecules that are bridged by another RNA molecule in a model system similar to that occurring in the editing reaction.

EXPERIMENTAL PROCEDURES

RNA Substrates—The mRNA substrates were synthesized by T7 RNA polymerase transcription. A 210-nt edited cytochrome b (Cyb) mRNA (19) was transcribed from a recombinant plasmid linearized with *Ear*I. T7 transcription was performed in 50-μl reactions containing 2 μg of template DNA, 5 mM NTPs, 40 mM Tris-HCl (pH 8.0), 2 mM spermidine, 10 mM dithiothreitol, 20 mM $MgCl_2$, 5 mM NaCl, 0.05% Nonidet P-40, 12.5 units of inorganic pyrophosphatase and 100 units of T7 RNA polymerase at 37 °C for 15 h (20). The transcripts were purified from acrylamide gels by elution at 4 °C in 0.5 M sodium acetate (pH 5.2), 1 mM EDTA. After ethanol precipitation, the RNAs were resuspended in water. RNAs longer than 30 nt were commercially synthesized using a DNA nucleotide attached to the column because this provided a more robust synthesis (Oligo Therapeutics Inc.). This type of synthesis produced RNAs with a 3′ terminal DNA nucleotide. Short RNAs were synthesized using only ribonucleotides (Oligo Therapeutics, Inc.). The RNAs were purified by polyacrylamide gel electrophoresis, resuspended in water at a concentration of 100 μM, and stored at −80 °C.

Labeling of RNAs—T7-synthesized RNAs (10 μg) were dephosphorylated by incubation with 0.01 unit of calf intestinal alkaline phosphatase (Life Technologies, Inc.) at 37 °C for 1 h in 100 μl of 50 mM Tris-HCl (pH 8.5), 1 mM EDTA. The reaction was stopped by phenol extraction, followed by ethanol precipitation. The 5′ OH-RNA was labeled in 30 μl of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM

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[1] The abbreviations used are: gRNA, guide RNA; nt, nucleotide(s); AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; AMP-CPP, adenosine 5′-(α,β-methylene)triphosphate; bp, base pair(s).

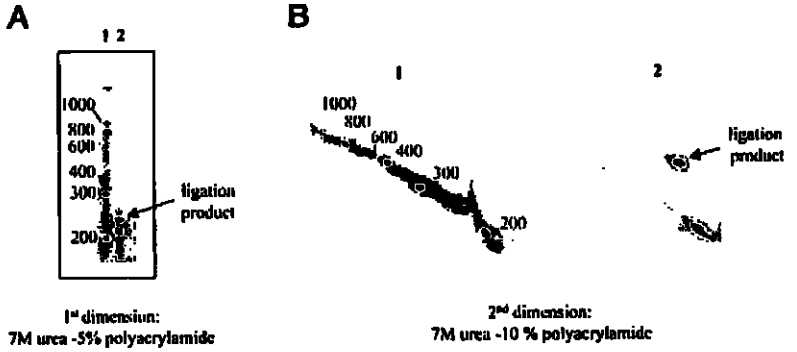

FIG. 1. **The mitochondrial RNA ligase can circularize a substrate RNA.** *A, lane 1,* 5′-labeled RNA standards (Life Technologies, Inc.) on a 7 M urea-5% acrylamide gel; *lane 2,* self-ligated 5′-labeled Cyb RNA. *B,* the lanes from *A* were excised and electrophoresed on a 7 M urea-10% acrylamide gel. *1,* migration of the RNA standards along a diagonal; *2,* abnormal migration of the Cyb ligation product off the diagonal. The *arrows* indicate the position of the major ligation product. *Numbers* indicate the size in nt of unrelated RNAs used as markers during electrophoresis.

spermidine, 0.1 mM EDTA, 2 μM ATP, 5 pmol [α-^{32}P]ATP (6000 Ci/mmol) and 5 units of T4 polynucleotide kinase (Life Technologies, Inc.). The reaction was incubated at 37 °C for 1 h and the 5′ ^{32}P-labeled RNA was purified on a 7 M urea-10% polyacrylamide gel.

3′ End Labeling of RNA with [α-^{32}P]pCp—The 3′ mRNA fragments (2 μg) were resuspended in 20 μl of ligation mixture containing 50 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 3 mM dithiothreitol, 0.1 mM ATP, 20% Me_2SO, 16 pmol [α-^{32}P]pCp and 10 units of T4 RNA ligase (Life Technologies, Inc.). The ligation reaction proceeded overnight at 4 °C. The 3′-labeled RNAs were purified on 7 M urea-10% polyacrylamide gels.

RNA Ligase Assay—Either 5′- or 3′-labeled RNAs were incubated at 27 °C for the appropriate times in 50 mM HEPES (pH 7.5), 1 mM dithiothreitol, 20 mM KCl, 1 mM ATP, 0.2 mM EDTA, 1 unit of RNasin, in the presence or absence of enzyme. For the bridged-ligation reactions, samples (5′ fragment, 3′ fragment, and bridge RNA) were annealed prior to enzyme addition by heating at 65 °C for 10 min followed by cooling at room temperature for 15 min. A similar annealing procedure was used to generate the $tRNA^{Trp}$ substrate shown in Fig. 9*A*. For the circularization reactions, substrates were used in the reaction without prior treatment. Reactions were terminated by phenol extraction and ethanol precipitation, followed by centrifugation in an Eppendorf microcentrifuge at 14,000 × *g* at 4 °C for 30 min. Reaction products were resuspended in gel loading buffer (formamide with 0.01% of xylene cyanole, 0.01% bromphenol blue and 30 mM EDTA), heated for 2 min at 80 °C, and electrophoretically separated on a 7 M urea-5% acrylamide at 500 V for 2 h. Following electrophoresis, gels were dried and exposed to a PhosphorImager screen (Molecular Dynamics). For analysis of the initial velocity kinetic constants, increasing concentration of either 5′-labeled substrates (circularization assays) or 3′-labeled substrates (bridged-ligation reactions) were incubated with constant concentration of enzyme as described above. The optimal reaction time to ensure steady-state conditions was determined by a reaction-progress curve (data not shown). Reactions were terminated by phenol extraction and ethanol precipitation. The velocity of conversion of substrates into ligated products was calculated for each substrate and plotted as a function of substrate concentration. These data were plotted as double-reciprocal plots, allowing graphical calculation of the first-order rate constants (K_m and V_{max}) for the various substrates.

Native Gel Electrophoresis—RNA substrates were annealed as for the bridging ligation followed by the addition of Tris-HEPES buffer (pH 7.6) to 50 mM, $MgCl_2$ to 10 mM, and glycerol to 10%. After incubation for 30 min at 30 °C, samples were loaded on pre-electrophoresed 12% acrylamide gel (acrylamide:bisacrylamide ratio, 29:1) with 50 mM Tris-HEPES, 10 mM $MgCl_2$. Electrophoresis was performed at a constant voltage of 150 V using 0.01% bromphenol blue as a mobility marker. After the dye migrated 10–12 cm from the top of the gel, the gel was fixed, dried, and exposed to a PhosphorImager screen (Molecular Dynamics). The dissociation constant for the 3′-fragment-bridge RNA complex was determined using increasing concentrations of bridge RNA in several independent experiments.

RESULTS

Circularization of a 210-nt RNA by the Mitochondrial RNA Ligase—The mitochondrial RNA ligase could circularize substrate RNAs that had 3′ OH and 5′ PO_4 termini. The major ligation product migrated off the diagonal formed by linear marker RNAs in two-dimensional acrylamide gel electrophoresis (Fig. 1*B, 2*). This anomalous electrophoretic migration is characteristic of circular molecules (21).

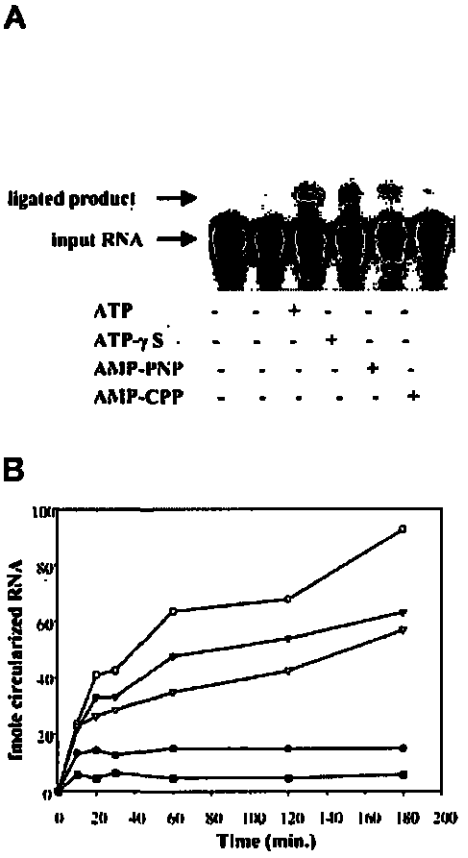

FIG. 2. **ATP requirement for the mitochondrial RNA ligase activity.** *A,* the mitochondrial RNA ligase was incubated with 0.3 pmol of the 195-nt 5′-labeled Cyb mRNA as described under "Experimental Procedures." The presence (+) or absence (−) of specific cofactors (1 mM) is indicated below each lane, and a quantitation of the results is shown as fmol of circularized RNA *versus* time at 27 °C (*B*). ATPγS, adenosine 5′-[γ-thio]triphosphate. *Open circles,* ATP; *filled triangles,* ATPγS; *open triangles,* AMP-PNP; *filled circles,* no ATP; *filled squares,* AMP-CPP.

Circularization of the 210-nt substrate RNA was used as an assay for the partial purification of the mitochondrial RNA ligase. The enzyme was partially purified from an S100 extract of a highly purified *L. tarentolae* mitochondrial fraction by chromatography through Q Sepharose (Amersham Pharmacia Biotech), Poros HS and Poros HQ columns (PerSeptive Biosystems), and isoelectric focusing in solution (Rotophor Apparatus, Bio-Rad) (data not shown). Although the extent of purification varied with different isolations, bridging experiments with less pure ligase samples gave results identical to those of the more

TABLE I
L. tarentolae mt RNA ligase requirements

Assay conditions are as described under "Experimental Procedures." Activity is expressed in fmol of ligated product generated per 30 min.

Assay conditions	Activity
	fmol/30 min
Complete reaction	6
− Mg^{2+}	0
− ATP	0.6
− ATP, + UTP	0
− ATP, + CTP	0
− ATP, + GTP	1.3
− ATP, + ADP	1.5
− ATP, + dATP (2 mM)	0.36
− ATP, + NAD^+ (5 mM)	0
Complete, + PPi (50 mM)	5.1
Complete, + PPi (1 mM)	0.36

highly purified preparations. In the ligase fraction used for the experiments reported in this paper, the enzyme was purified 14,000-fold (based on specific activity) with a yield of 6%. This fraction contained the adenylatable 50- and 45-kDa proteins (data not shown), which previous evidence indicated represented ligase intermediates (14). The partially purified RNA ligase was free of detectable DNA ligase, 3′ exonuclease and 3′ terminal uridylyl transferase activities (data not shown). The details of the enzyme purification will be presented elsewhere.

The L. tarentolae Mitochondrial RNA Ligase Requires α-β Bond Hydrolysis of ATP—The *L. tarentolae* mitochondrial RNA ligase activity was assayed in the presence of several nonhydrolyzable ATP analogs. The data in Fig. 2 show that ligase activity was inhibited when AMP-CPP, an ATP analog not hydrolyzable at the α-β bond, was present during the reaction. When ATP analogs not hydrolyzable at the β-γ bond (*e.g.* AMP-PNP) were used, ligation was either not inhibited or inhibited to a lesser extent. These results are consistent with previous results with crude mitochondrial extracts, suggesting that the *L. tarentolae* ligation reaction involved formation of an activated RNA intermediate. This is similar to the enzymatic mechanism of T4 RNA ligase, which involves an initial hydrolysis of ATP at the α-β bond, followed by the formation of an AMP-RNA intermediate (22, 23).

Cofactor Requirements—Nucleotide cofactor and divalent cation requirements of the ligase reaction are shown in Table I. The *L. tarentolae* RNA ligase has an absolute requirement for ATP and Mg^{2+}. Neither UTP nor CTP could substitute for ATP; GTP could substitute for ATP, but to a lesser extent. The optimum Mg^{2+} concentration was 5 mM; concentrations greater than 10 mM were inhibitory (data not shown). No ligase activity was detected in the presence of 0.5–10 mM $CaCl_2$, $ZnCl_2$, or $MnCl_2$. The ligase did not require monovalent cations for activity, and no stimulation was observed in the presence of KCl, NaCl, NH_4Cl, or NH_4OAc (data not shown). The ligase activity was inhibited 22-fold by 200 mM KCl (data not shown) and showed a narrow optimal pH range between pH 7.5 and 9.0 (data not shown).

The Mitochondrial RNA Ligase Requires 5′ Phosphate and 3′ OH Termini—RNA ligases can be divided into several classes, depending on the preference for given termini on the substrate RNA. To investigate the requirements of the *L. tarentolae* mitochondrial RNA ligase, two RNAs containing either 5′ OH and 3′ phosphate or 5′ phosphate and 3′ OH termini were used as substrates in the circularization assay. As shown in Fig. 3, ligated products appeared only with the latter substrate. These results indicated that *L. tarentolae* mitochondrial RNA ligase is similar to T4 RNA ligase in that it requires a 5′ phosphate and a 3′ OH.

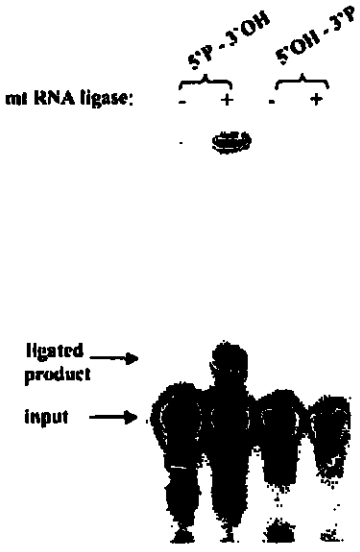

FIG. 3. **3′ OH and 5′ PO_4 termini are required for circularization of a linear RNA molecule by the mitochondrial RNA ligase.** The 210-nt Cyb mRNA (0.3 pmol) with either a 5′-labeled and a 3′ OH terminus, or a 5′ OH and a 3′ [α-^{32}P]pCp-labeled terminus was incubated in the presence (+) or absence (−) of mitochondrial RNA ligase in a standard circularization reaction as described under "Experimental Procedures." Reaction products were separated on a 7 M urea-5% acrylamide gel.

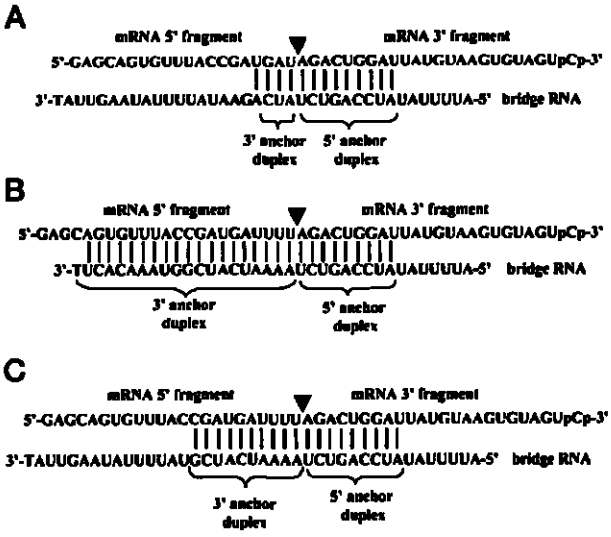

FIG. 4. **Representative sequences of several nicked double-stranded RNA substrates for the bridged ligation reaction.** Synthetic RNAs representing the 5′ and 3′ cleavage fragments of a pre-edited ND7 mRNA cleaved at editing site 1 are shown annealed to bridge RNAs mimicking cognate gRNAs. The 9 bp 5′ anchor duplex in all constructs is identical to the wild type situation. The 3′ nucleotide of all bridge RNAs is dT. *A*, the 3′ cleavage fragment was 3′ end-labeled with [α-^{32}P]pCp to follow the ligation. The location of the nick at editing site 1 is indicated by an *arrowhead*. This construct has a 4-bp 3′ anchor duplex. *B*, the bridge RNA was mutated to create a 15-bp 3′ anchor duplex. *C*, the bridge RNA was mutated to create a 10-bp 3′ anchor duplex.

Substrate Specificity of the Ligation Reaction—The enzyme cascade model for RNA editing postulates a mitochondrial RNA ligase that can join two mRNA cleavage fragments bridged by a cognate gRNA. The substrates constructed to assay the activity of the mitochondrial RNA ligase were based on the sequence of the internal editing domain of pre-edited ND7 mRNA and the cognate gRNA, which *in vivo* mediates the insertion of one U at editing site 1, three Us at site 2, and one U at site 3. A nick was present at editing site 1 in these constructs to mimic

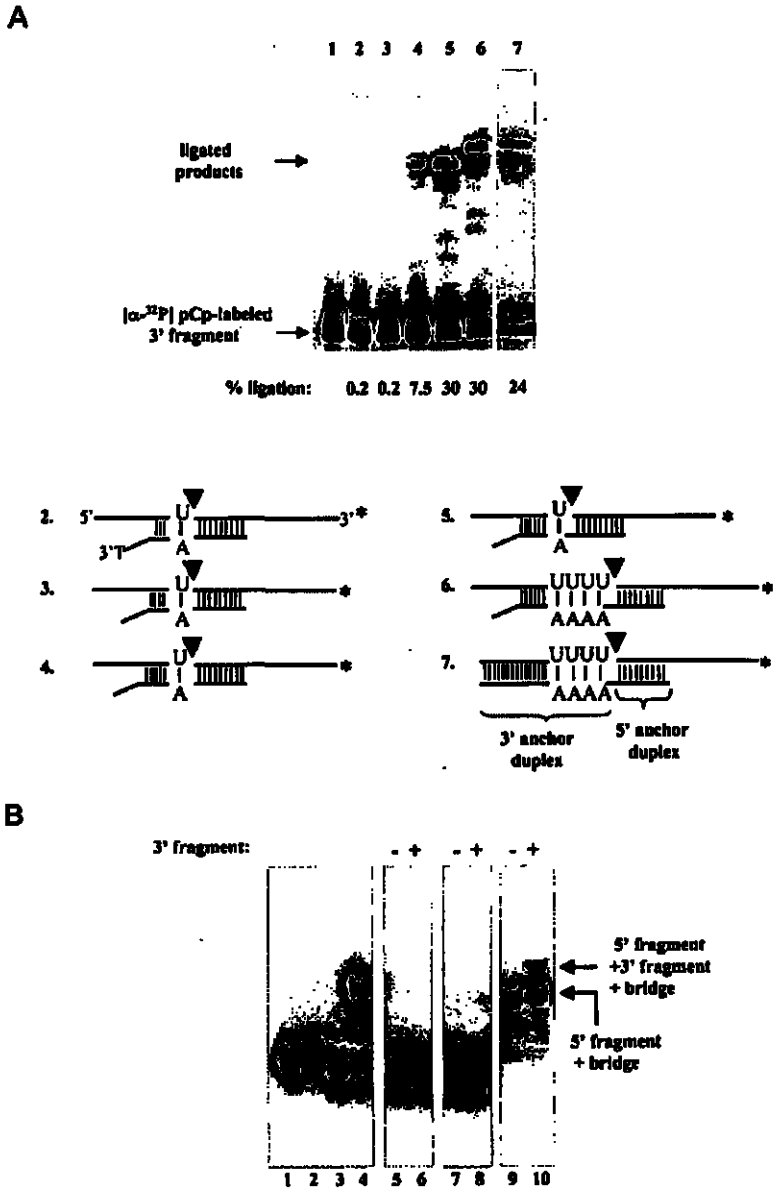

FIG. 5. **Effect of varying the length of the 3′ duplex on the efficiency of bridged ligation.** *A*, the constructs for lanes 2–7 are shown diagrammatically below the gel. A control ligation reaction in the absence of bridge RNA is in lane 1. The nick in each construct is indicated by an *arrowhead*. The length of the 3′ duplex varies from 4–19 bp. The base pairs added in constructs 3–7 as compared with *construct 2* are indicated by *bold lines*. The synthetic 23-nt mRNA 3′ fragment was 3′ end-labeled with [α-^{32}P]pCp (*). The 3′ terminal dT nucleotide in the bridge RNA is only shown in *construct 2*. The *% ligation* is the percentage of input substrate converted to product. *B*, native gel analysis of the interaction between the mRNA 5′ and 3′ fragments and the bridge RNA in the absence of protein. The 5′ fragment in *lane 1* is from the constructs 2–5 in *A*. The 5′ fragment in *lane 2* is from constructs 6 and 7 in A. The 3′ fragment in *lane 3* is identical in all constructs. *Lane 4* shows the interaction of the labeled 3′ fragment and unlabeled bridge RNA. *Lanes 5* and *6* show the labeled 5′ fragment from *construct 2* in the presence of the bridge RNA with and without the unlabeled 3′ fragment. *Lanes 7* and *8* show the labeled 5′ fragment from *construct 6* in the presence of the bridge RNA from *construct 6* with and without the unlabeled 3′ fragment. *Lanes 9* and *10* show the labeled 5′ fragment from *construct 7* in the presence of the bridge RNA from *construct 7* with and without the unlabeled 3′ fragment.

the final ligation step in the editing reaction. The sequences of three constructs used in these experiments are shown in Fig. 4, together with an indication of the 5′ and 3′ mRNA cleavage fragments and the 5′ and 3′ anchor duplexes.

The mitochondrial RNA ligase was found to efficiently ligate two synthetic RNAs in the presence of a bridge RNA that could base pair with both fragments (Fig. 5). In these constructs, the 5′ anchor duplex of 9 bp is identical to the wild type anchor duplex formed between the gRNA and the mRNA. The effect of varying the length of the 3′ anchor duplex between the 3′ end of the gRNA and the 5′ mRNA cleavage fragment on the efficiency of bridged ligation is shown in Fig. 5*A*. A 3′ anchor of 5 bp yielded 0.2% ligation, whereas an anchor of 6 bp yielded 7.5% ligation. The maximum value of 30% ligation was achieved with a 3′ anchor of 7 bp, and increasing the length of the 3′ anchor to 19 bp did not increase the extent of ligation.

A native gel analysis of the interactions in the absence of proteins between these 5′ and 3′ cleavage fragments and the bridge RNAs is shown in Fig. 5*B*. The interaction of labeled 3′ fragment from construct 2 in Fig. 5*A* and unlabeled bridge RNA is shown in Fig. 5*B*, *lane 4*. Note the almost complete upward shift of the 3′ fragment from the position in *lane 3*. In Fig. 5*B*, *lanes 5–10*, the interactions of labeled 5′ fragments from constructs 2, 6, and 7 in Fig. 5*A* with the unlabeled bridge RNAs and the respective unlabeled 3′ fragments are shown. In Fig. 5*B*, *lanes 5* and *6*, the bridge RNA, which could potentially form a 3′ anchor duplex of 4 bp, showed no detectable interaction with the 5′ fragment, whereas in *lanes 7* and *8*, a detectable interaction of the bridge RNA, which could form a 3′ anchor duplex of 7 bp, with the 5′ fragment was seen. A more efficient interaction of the bridge RNA with a potential 19-bp 3′ anchor duplex with the 5′ fragment is shown in Fig. 5*B*, *lanes 9* and *10*. These results allowed the determination of dissociation constants (24). Annealing of the 3′ fragment and the cognate bridging RNA in *lane 4* resulted in the formation of a stable complex with K_d of 0.8–1 nM, which is in the same range (0.5–2 nM) as the dissociation constant observed for the interaction of a fragment of the pre-edited mRNA for ribosomal protein S12 and its cognate gRNA (data not shown).

A comparison of the extent of ligation of bridged RNAs with different lengths of gaps separating the two fragments under excess enzyme conditions is shown in Fig. 6. The nicked sub-

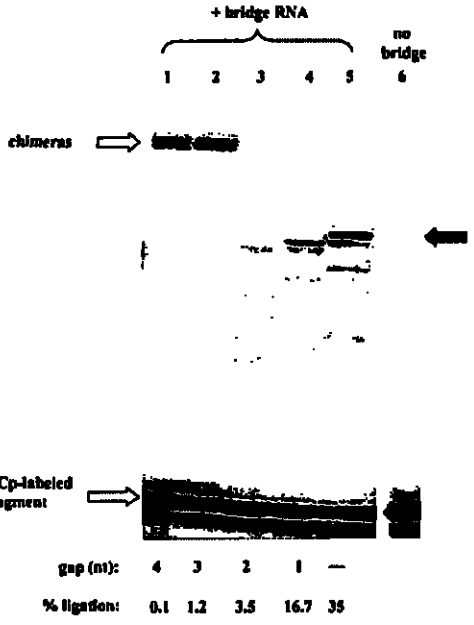

FIG. 6. **Effect of the length of the gap on the efficiency of bridged ligation of two RNAs.** *Construct 6* in Fig. 5*A* was modified by deletion of 1–4 3′ nucleotides from the 5′ mRNA cleavage fragment. The 5′ and 3′ mRNA fragments (0.5 pmol) were incubated with the mitochondrial RNA ligase in the presence of 7 pmol of the same bridge RNA. The extent of ligation of the two mRNA fragments and the length of the gap are indicated below each lane. The nicked substrate is indicated by a *dash*. *Lane 6* is the ligation reaction in the absence of bridge RNA. * denotes the position of the radioactive label. The *black arrow* indicates the position of the ligation product. The minor, low molecular weight bands in *lanes 3–5* represent ligation of nonspecific degradation products of the substrates in this experiment.

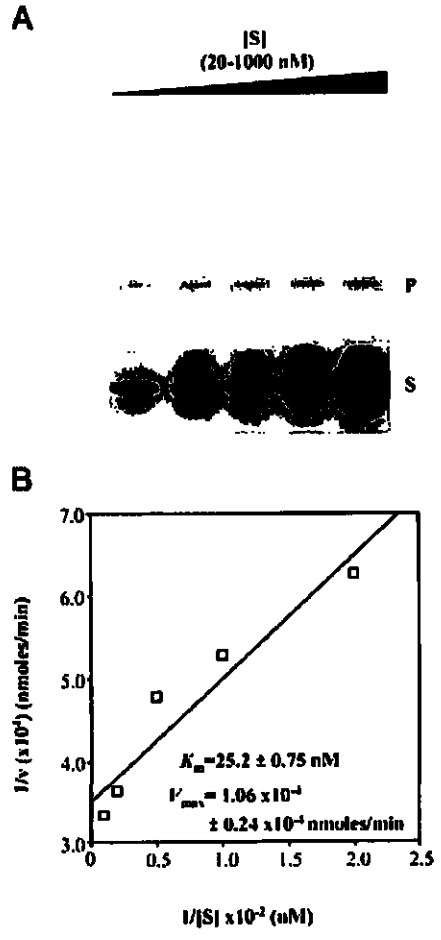

FIG. 7. **Kinetic analysis of bridged ligation by the mitochondrial RNA ligase.** *A*, gel analysis of a representative ligation reaction of increasing concentrations (20–1000 nM) of the nicked substrate with constant and undersaturating concentration of enzyme. *S* refers to the input substrate, and *P* refers to the ligated product. The reaction was performed as described under "Experimental Procedures." *B*, a double-reciprocal plot of velocity *versus* substrate concentration was used to calculate the K_m and V_{max} for the mitochondrial RNA ligase for the reaction in *A*. The fraction of the various concentrations of the input RNA converted to ligated product was determined by PhosphorImager analysis of the dried gels. Fraction ligated ($P/(P + S)$) divided by the reaction time was used to calculate velocity, the amount of ligated product made per min. Reactions similar to those in *A* and calculations as in *B* were performed for all of the substrates tested. The data are summarized in Table II.

strate in *lane 5* is shown as construct 6 in Fig. 5*A*, and the gapped substrates represent successive deletions of the 3′ nucleotides from the 5′ fragments. The decreased ligation efficiencies of the bridged substrates with gaps of 1–4 nucleotides are apparent. It is of interest that the efficiency of formation of gRNA-mRNA chimeras increases as the bridged ligation efficiency decreases. Because the 3′ terminal nucleotide of the bridge RNA is dT, the formation of chimeras must be due to nibbling of the end of the RNA.

A Nicked Double-stranded RNA Is the Preferred Substrate for Mitochondrial RNA Ligase: A Kinetic Analysis—If the ligase described in this report has an involvement in the editing process *in vivo*, then it should show a substrate preference for double-stranded nicked RNA substrates formed by the bridging of mRNA 5′ and 3′ cleavage fragments by a cognate gRNA. Several RNA constructs were made to test the partially purified *L. tarentolae* mitochondrial RNA ligase for this activity. The different synthetic substrates were tested under steady-state conditions (Fig. 7), and the first-order rate constants for each substrate were calculated, as shown in Table II. These data indicate that the preferred substrate for the ligase is a double-stranded RNA with a nick. As the distance separating the 3′ end of the 5′ fragment from the 5′ end of the 3′ fragment was increased to create a gap of 1, 2, or 3 nt, a decreased efficiency of ligation was observed. With a 3-nt gap separating the two fragments, a 416-fold decrease in ligation efficiency was observed, as shown by comparing the relative V_{max}/K_m of the nicked and the 3-nt gapped substrates in Table II. The mitochondrial RNA ligase also favored the ligation of the nicked substrate over the circularization of longer RNAs lacking a bridge by a factor of approximately 4, as shown by comparing the relative V_{max}/K_m of the nicked and the Cyb-210 substrates in Table II.

Comparison of the ligation efficiency of the mitochondrial RNA ligase and T4 RNA ligase indicated that the mitochondrial ligase was almost 100-fold more efficient in catalyzing the ligation reactions of the substrates used under the ligation conditions described. Interestingly, we observed that the T4 RNA ligase could ligate the nicked substrate with an efficiency comparable to that of the circularization reaction (Table II). This observation is in contrast with previous reports that showed a lack of ligation by this enzyme when the ends to be ligated were fully base paired (25).

The efficiency of ligation by the mitochondrial ligase was also affected by the length of the substrate used in the circularization reaction. Whereas the 210-nt Cyb-210 substrate was efficiently ligated, the 30-nt EP1–30 substrate (Table II) was an extremely poor substrate for this enzyme, with a 4166-fold lower V_{max}/K_m relative to that of the nicked substrate.

Lack of Specificity for the 3′ nt of the 5′ Fragment in Bridged

TABLE II

Kinetic analysis of the L. tarentolae mitochondrial RNA ligase and the bacteriophage T4 RNA ligase

Cyb-210 and EP1–30 refer to the 210- and 30-nt RNAs used in the circularization reaction. Rel V_{max}/K_m refers to the efficiency of ligation relative to that of the nicked substrate. For the T4 RNA ligase analysis, only the Cyb-210 and the nicked substrate were tested. N/A indicates not applicable. The K_m and V_{max} values given are for "apparent" first order rate constants, as the kinetic analysis was performed with a single, constant, and saturating concentration of ATP (a cofactor in the ligase reaction). Nicked refers to a double-stranded substrate with a single nick at a position corresponding to editing site 1 (ES1) in the natural substrate. 1-, 2-, and 3-nt gaps refer to double-stranded substrates similar to the nicked substrate except that the distance between the ends to be ligated was increased accordingly. Each K_m and V_{max} value given is the average of at least three independent measurements.

Substrate	Mitochondrial RNA ligase				T4 RNA ligase			
	K_m	V_{max}	V_{max}/K_m	Rel V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	Rel V_{max}/K_m
	nM	*nmol/min*			*nM*	*nmol/min*		
Cyb-210	54.9	5.2×10^{-5}	9.5×10^{-7}	0.23	1500	1.8×10^{-4}	1.2×10^{-7}	0.029
EP1–30	445.5	6.5×10^{-7}	1.0×10^{-9}	0.00024	N/A	N/A	N/A	N/A
Nicked	25.2	1.06×10^{-4}	4.2×10^{-6}	1.0	2273	2.4×10^{-4}	6.4×10^{-8}	0.015
1-nt gap	110	1.84×10^{-4}	1.75×10^{-6}	0.42				
2-nt gap	170	1.73×10^{-4}	1.0×10^{-7}	0.024				
3-nt gap	121	1.2×10^{-6}	1.0×10^{-8}	0.0024				

Ligation—Because T4 RNA ligase shows a nucleotide preference for ligation (26), it is possible that the observed difference in the efficiency of bridged ligation of several synthetic RNAs could be due to the chemical nature of the 3′ terminal nucleotide of the 5′ fragment (as opposed to the length of the gap). As shown in Fig. 8, *A* and *B*, the mitochondrial RNA ligase joins bridged fragments having different 3′ terminal nucleotides with almost identical efficiencies.

The mitochondrial RNA ligase is less active in ligating a nicked tRNA substrate than T4 RNA ligase The native substrate for T4 RNA ligase is a tRNA that has a nick in the anticodon loop. A comparison of the time course of the ligation of the synthetic nicked $tRNA^{Trp}$ in Fig. 9*A* by the mitochondrial RNA ligase and T4 RNA ligase is shown in Fig. 9*B*. The mitochondrial ligase is much less active in ligating this substrate than the T4 ligase.

DISCUSSION

Several properties of the partially purified *L. tarentolae* mitochondrial RNA ligase are consistent with a role for this enzyme in the terminal step of RNA editing (4). The ligation of two fragments that are bridged by a complementary RNA is similar to the predicted role of the editing ligase in joining two mRNA cleavage fragments bridged by a cognate gRNA.

Blum *et al.* (4, 27) originally proposed that the 3′ oligo(U) nonencoded tail of the gRNA base pairs with the G+A rich pre-edited sequence and forms a 3′ anchor duplex that both stabilizes the initial interaction of the gRNA and the mRNA and assists in maintaining the mRNA 5′ cleavage fragment within the editing complex. The data in this paper do not directly support this hypothesis as originally proposed. In addition, we have found by native gel analysis of the ND7.4x substrate and the cognate gRNA (19) that in the absence of proteins, the gRNA 3′ oligo(U) tail does not contribute to the interaction.[3] Furthermore, Kapushoc and Simpson (28) recently showed that deletion of the 3′ oligo(U) tail from a 3′ end blocked gRNA provided *in cis* has no effect on the directed insertion of Us at editing site 1 in a synthetic ND7-1 mRNA.

It is clear, however, that stabilization of the interaction of the bridging gRNA and the mRNA 5′ cleavage fragment is critical for ligation, and a similar result was previously obtained for *in vitro* editing in the *T. brucei* system (7). *In vivo*, the number of consecutive base pairs 5′ of an editing site is usually quite limited; in the ND7-I situation, the mRNA sequence at editing site 1 could at most form a 3-bp 3′ duplex with the cognate gRNA. We show in this paper that a 5-bp 3′ duplex does not support a bridged ligation of a nicked double-stranded substrate RNA, whereas a 7-bp duplex allows 30% ligation. An interaction of the bridge RNA that could form a 3′ anchor duplex of 7-bp with the 5′ fragment was directly demonstrated by native gel analysis. We presume that the function of this 3′ anchor duplex in the *in vitro* system is to maintain the

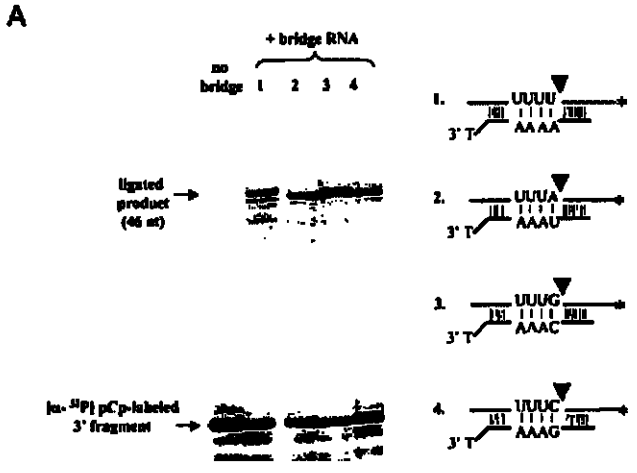

FIG. 8. **Lack of specificity for 3′ terminal nucleotide in bridging ligation.** *A*, ligations of nicked double-stranded substrates with different 3′ terminal nucleotides on the 5′ cleavage fragment. The control reaction has no bridge RNA. Diagrams of the four constructs are shown to the right of the gel. The 3′ duplex is 10 bp. The 3′ terminal nucleotide of the 5′ fragment is shown in ***boldface***. * denotes the position of the radioactive label. *B*, time course of the ligation reaction shown in *A*. ●, A; ▲, U; ■, C; ◆, G.

[3] R. Aphasizhev and L. Simpson, unpublished results.

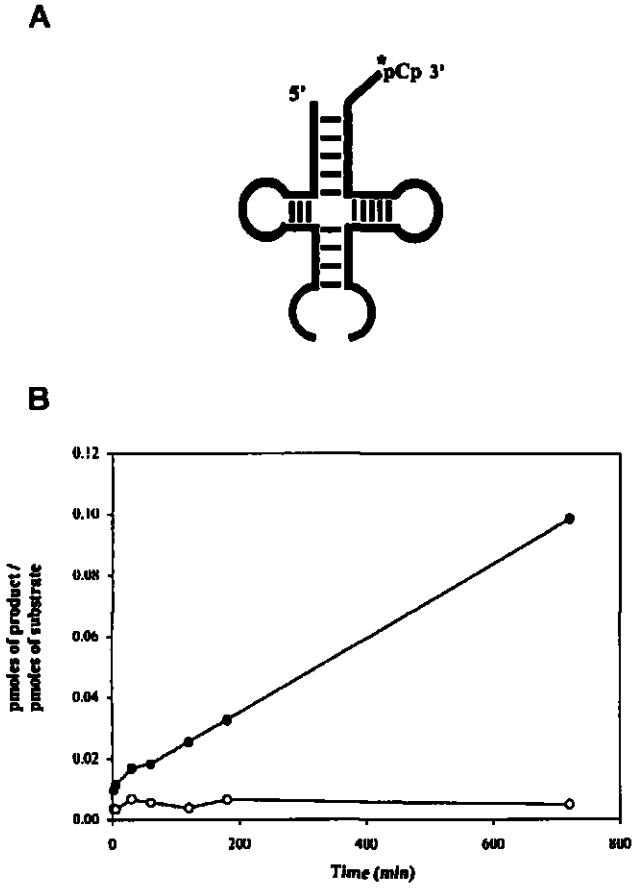

FIG. 9. **Comparison of ligation of nicked $tRNA^{Trp}$ by the mitochondrial RNA ligase and T4 RNA ligase.** *A*, diagram of the nicked substrate RNA. The substrate was constructed by annealing two synthetic RNAs to mimic a nicked $tRNA^{Trp}$ from *L. tarentolae*, as described under "Experimental Procedures." The nick is between nt 34 and 35 in the anticodon. The 3' fragment was labeled with [^{32}P]pCp as indicated. *B*, time course of the ligation reaction. *Solid circles* represent the T4 RNA ligase reaction, and *open circles* represent the mitochondrial RNA ligase reaction. Conversion of substrate to product was assayed by gel electrophoresis and PhosphorImager analysis as described under "Experimental Procedures."

5' fragment in the correct configuration and proximity for ligation to the 3' fragment. *In vivo*, proteins within the editing complex may serve the role of binding to the 5' cleavage fragment, either directly, through RNA binding, or indirectly, through protein-protein interactions. In this regard, several mitochondrial proteins have been identified that bind to gRNA and mRNA (29–34). Also, we cannot rule out the possibility that RNA ligase itself could be involved.

Another property of the mitochondrial RNA ligase consistent with a role for joining two mRNA cleavage fragments that have undergone either U addition or U deletion at the 3' end of the 5' fragment is the lack of a requirement for a specific 3' nucleotide on the 5' fragment. U deletions in particular would frequently expose a 3' terminal A, G, and sometimes C, which must be joined to the 3' cleavage fragment. Furthermore, the preference for ligation of a nicked substrate is consistent with ligation generally occurring *in vivo* after a precise trimming of the 3' single-stranded overhang (5). The less efficient ligation of bridged fragments with a gap or with a 3' overhang may be responsible *in vitro* and possibly also *in vivo* for the appearance of a variable percentage of misedited molecules that contain the incorrect number of Us at an editing site (35–37).

The dependence of ligation efficiency on the length of the gap separating the two bridged fragments is consistent with the results of Cohen and Cech (38), who quantitated by disulfide cross-linking the relative flexibility of an RNA bridged duplex consisting of three strands. They examined constructs in which a bridge RNA hybridized to two RNA molecules yielding a nick or single-stranded gaps of 1, 2, or 3 nt. The relative flexibility of the nicked duplex was 2 orders of magnitude less than that of the duplexes with gaps of 1–3 nt. The high relative rigidity of the nicked duplexes in the constructs that we have employed may be one of the major factors increasing the efficiency of ligation of the two mRNA fragments and inhibiting the formation of chimeric molecules, as we have observed. The higher flexibility of the gapped duplexes similarly may be responsible for increased formation of chimeric molecules (Fig. 6). In any case, the increased formation of chimeric molecules in the gapped substrates is entirely consistent with the kinetic data in Table II in that the decreased efficiency of bridged ligation due to the gaps would allow the ligation of the 3' end of the gRNA to the 3' cleavage fragment, which essentially represents a circularization ligation.

In a comparison of the *L. tarentolae* mitochondrial RNA ligase and T4 RNA ligase, each enzyme appears to prefer its native substrate. The mitochondrial RNA ligase is approximately 100-fold more active in ligating a nicked double-stranded RNA substrate, and the T4 RNA ligase is more active in ligating a nicked tRNA substrate.

In conclusion, we have shown that a partially purified mitochondrial RNA ligase activity from *L. tarentolae* has several properties consistent with the ligase proposed to be involved in RNA editing. Purification to homogeneity and cloning of the gene(s) coding for this enzyme should provide answers to many of the outstanding questions about this biochemical mechanism.

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