

RNA-editing Terminal Uridyl Transferase 1

IDENTIFICATION OF FUNCTIONAL DOMAINS BY MUTATIONAL ANALYSIS*

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The catalytic, RNA-binding and oligomerization domains of the RNA-editing terminal uridylyl transferase 1 (RET1) from *Leishmania tarentolae* mitochondria were characterized by mutational analysis. Significant N- and C-terminal portions of the protein were found to be dispensable for UTP polymerization *in vitro*. Changes of conserved amino acids in the active site demonstrated a general similarity of sugar-phosphate moiety recognition of the incoming ribonucleotide triphosphate by RET1 and eukaryotic poly(A) polymerases. Overlapping RNA-binding and oligomerization regions were mapped to the C-terminal region, which is conserved only among trypanosomatid RET1 enzymes. In the absence of an RNA primer, RET1 can use UTP itself to initiate nucleotide transfer and produce poly(U) molecules of several hundred nucleotides. An N-terminal zinc finger motif is essential for enzyme activity; deletion of this motif or chelation of zinc inhibits activity.

Transfer of a nucleotide to an acceptor hydroxyl group is a fundamental chemical reaction involved in a variety of biological processes. Among many enzyme superfamilies that perform this reaction, the DNA polymerase β -type group of nucleotidyltransferases is one of most widespread. Members of this group are involved in such diverse pathways as DNA repair, RNA processing, antibiotic resistance, and signal transduction (1). They include poly(A) polymerase (PAP),¹ CCA-adding enzymes, terminal deoxynucleotidyl transferase, kanamycin nucleotidyltransferase, and others. A characteristic feature of the superfamily is the presence of conserved catalytic domain with three metal-coordinating invariant carboxylates and a helical turn motif hG(G/S)^{9–13}XDh(D/E)h (where X represents any amino acid, and h is a hydrophobic amino acid) (2). Two classifications have been proposed based on sequence similarities. The class I subfamily includes the archaeal CCA-adding enzyme, eukaryotic PAP, DNA polymerase β , terminal deoxynucleotidyltransferase, and kanamycin nucleotidyltransferase, whereas the class II subfamily contains eubacterial and eukaryotic CCA-adding enzymes and eubacterial PAP (3). Ara-

vind and Koonin (1) expanded the DNA polymerase β -type nucleotidyltransferase superfamily to contain several more groups of proteins and divided the enlarged set into nine families.

Until recently, PAPs were the only known template-independent enzymes that exhibited specificity toward a particular nucleotide in an otherwise typical polymerization reaction. Atomic structures have been solved for yeast (4) and bovine (5) PAP, and several conserved amino acids were proposed to direct the specificity of the enzyme for ATP, albeit not by direct contacts between the nucleotide base and active center residues.

Terminal uridylyltransferases (TUTases), enzymatic activities that add UMP residues to the 3'-hydroxyl group of RNA, have been described in mammalian cells (6–8), plants (9), and trypanosomes (10, 11). No such activity has yet been found in prokaryotes. A biochemical analysis of protein complexes involved in RNA editing in trypanosomatid mitochondria (reviewed in Ref. 12), identified two TUTases, RNA-editing TUTase 1 (RET1) (13) and RNA-editing TUTase 2 (RET2) (14–16). The former was implicated in the addition of the nonencoded 3' oligo(U) tail to guide RNAs (17), and the latter was shown to be responsible for U insertion into the mRNA during the editing reaction (16, 17). These proteins differ in size and quaternary structure. The RET1 from *Leishmania tarentolae* is a tetramer of 121-kDa subunits, and the RET2 is a 57-kDa integral component of the multiprotein core-editing L-complex (18, 19). Although both TUTases utilize UTP as the preferred nucleotide, RET1 adds hundreds of uridines to an RNA primer *in vitro*, whereas RET2 transfers mainly one residue.

In order to assess the roles of amino acids that are conserved among PAPs and TUTases, we have tested a number of point mutants of recombinant RET1 in nucleotide incorporation assays. In addition, a series of N- and C-terminal truncated RET1 mutants were constructed and analyzed for UTP incorporation, RNA binding, and oligomerization properties. RET1 activity requires tightly bound zinc atoms, which are probably involved in stabilization of a functionally important C2H2 zinc finger domain in the N-terminal part of the protein.

EXPERIMENTAL PROCEDURES

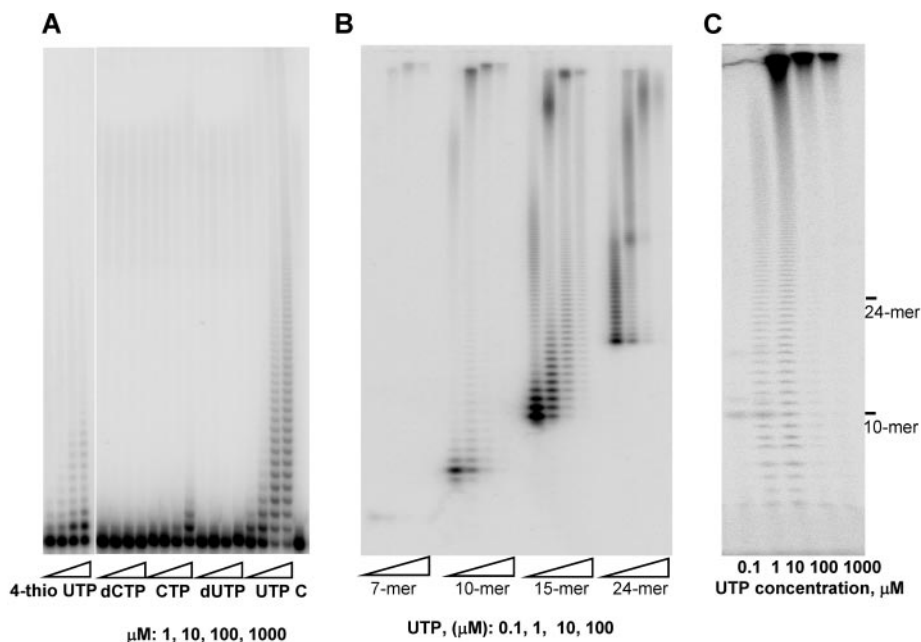
Mutagenesis, Protein Expression, and Purification of RET1—All *L. tarentolae* RET1 point and deletion mutants were prepared by site-directed mutagenesis using the QuikChange™ kit (Stratagene) and expressed in the pET-29a vector (Novagen) as fusion proteins with C-terminal His₆ affinity tags. All mutations were confirmed by DNA sequencing. Plasmids were transformed into BL 21(DE3) Codon Plus RIL (Stratagene) *Escherichia coli*. The bacterial cells were grown in 2× YT medium with 1% glucose to an A₆₀₀ of 0.3, and the temperature was then lowered to 22 °C over 1 h. Expression was induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside for 3 h. All chromatography steps were performed at 4 °C. Active or partially active mutants were followed through purification by activity. Inactive proteins were traced with penta-His monoclonal antibodies against His₆ tag (Qiagen). Cell

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¹ The abbreviations used are: PAP, poly(A) polymerase; L-complex, ligase-containing complex; TUTase, 3' terminal uridylyltransferase; nt, nucleotides; aa, amino acids; pol- β , polymerase β ; RET, RNA-editing TUTase; gRNA, guide RNA.

FIG. 1. RNA and NTP substrate requirements of recombinant wild type RET1 TUTase. A, recognition of nucleotide triphosphate substrates by *L. tarentolae* RET1. Chemically synthesized 5'-labeled 6[U] RNA (0.1 μ M final concentration) was incubated with RET1 in the presence of increasing concentrations of 4-thio-, ribo-, or deoxy-NTPs for 15 min. Lane C, control without NTP. B, minimal length of RNA primer. RNA oligonucleotides labeled at the 5'-ends were incubated with increasing concentrations of UTP under standard conditions. Products were separated on a 15% acrylamide/urea gel. C, UTP polymerization by RET1. The enzyme was incubated with the indicated concentrations of UTP in the presence of 1 μ Ci of [α - 32 P]UTP, and the products were analyzed on a 15% acrylamide/urea gel.



paste (10 g) was resuspended in 60 ml of lysis buffer (50 mM Hepes, pH 7.6, 50 mM KCl, 1 mM dithiothreitol, 3000 units of DNase I (Sigma)), passed through a French press cell (SLM Instruments) at 1200 p.s.i., and centrifuged at 45,000 rpm for 1 h. The extract was diluted 3-fold with 50 mM Hepes, pH 7.6, and loaded onto a 10-ml Sepharose SP (Amersham Biosciences) column. RET1 was step-eluted between 100 and 300 mM of KCl and loaded onto a 1 ml Talon SuperFlow column (Clontech) pre-equilibrated with 50 mM Hepes, pH 8.1, 300 mM KCl. Proteins were eluted with 100 mM imidazole (Sigma) after washing the column repeatedly with loading buffer. Eluted fractions were concentrated by centrifugal filtration using a 10K membrane (Millipore Corp.) to 200 μ l and loaded onto a Superose 6 gel filtration column (Amersham Biosciences). The final enzyme preparations were generally more than 90% pure. A highly purified wild type RET1 preparation for the measurement of pyrophosphorolytic activity was obtained by a similar procedure that also included MonoS and MonoQ chromatographic steps. Briefly, the fraction from the Talon column was diluted 3-fold, loaded on a 5/5 MonoS (Amersham Biosciences) column, and developed with a 20-ml gradient from 100 to 500 mM of KCl in 50 mM HEPES, pH 7.6, 0.1 mM EDTA. Active fractions were diluted 5-fold and separated on a 5/5 MonoQ (Amersham Biosciences) column under the same salt conditions as for the MonoQ column in 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. Analysis of 2 μ g of protein from the final Superose 6 fraction by SDS gel electrophoresis followed by Sypro Ruby staining revealed a ~98% pure preparation. The *Trypanosoma brucei* RET1 was purified as described (13).

Protein and Enzymatic Assays—Protein concentrations were determined by quantitation of Sypro Ruby-stained bands in the SDS-gel against prediluted bovine serum albumin standards (Pierce). The molar concentration was calculated for that of a monomeric enzyme. UTP analogs were purchased from Molecular Probes and BD Biosciences. The steady-state kinetic parameters were measured in a filter binding UTP incorporation assay. *L. tarentolae* RET1 was used in all experiments unless otherwise stated. Reactions were started by the addition of enzyme, unless otherwise noted, to 2.5 nM for RET1. Inactive mutants were tested in the reaction with up to 50 nM of enzyme. Reaction mixtures containing 50 mM HEPES, pH 8.0, 10 mM MgOAc, 1 mM dithiothreitol, 100 μ M [α - 32 P]UTP (~2,000 cpm/pmol) and 1 μ M of 12[U] RNA were incubated at 27 °C for 0.2–25 min. Reactions were stopped by adding an equal volume of 0.5% SDS in 0.25 M NaH₂PO₄, spotted on DE81 filters (Whatman), washed extensively with 0.5 M NaH₂PO₄ plus 0.5% sodium pyrophosphate and counted. The apparent Michaelis constant, K_m , and the limiting rate, V_{max} , were determined by fitting the initial velocities as a function of the corresponding substrate concentrations into a standard Michaelis model. The Prism4 (GraphPad) software package was used for all calculations. Errors were quoted as standard estimations from three independent experiments. The TUTase sequencing gel assay was performed under identical conditions with 0.1 μ M 5'-labeled RNA, 100 μ M UTP, and 2–10 nM enzyme for 15 min. Products were separated on 14% acrylamide/urea sequencing gel. The

UTP polymerization reaction contained enzyme and radioactive UTP only.

RNA Substrates and Gel Retardation Analysis—The following RNAs were purchased from Dharmacon Inc.: 12[U] RNA, 5'-GCUAUGUCU-GCUAACUUGUUUUUUUUUUUU-3'; 7-mer RNA, 5'-GCUAUGU-3'; 10-mer RNA, 5'-GCUAUGUCUG-3'; 15-mer RNA, 5'-GCUAUGUCUG-CUAAC-3'; 6[U] RNA, 5'-GCUAUGUCUGCUAACUUGUUUUUU-3'; 6[A] RNA, 5'-GCUAUGUCUGCUAACUUGAAAAA-3'.

The ND7[4 \times] guide RNAs (28) with or without 15 uridine residues at the 3'-end, were synthesized *in vitro* by T7 transcription. Proteins were incubated with 1 pmol of 5'-labeled RNA for 15 min at 27 °C in 10 μ l of reaction mixture containing 5% glycerol, 2% polyvinyl alcohol, 100 mg/ml bovine serum albumin, 25 mM Tris-HCl, pH 7.9, 0.01% Nonidet P-40, 1 mM dithiothreitol, 1 mM EDTA, 5 mM KCl, and 2 mM MgCl₂. Protein concentration varied in the 0.1–2 μ M range. The reactions were loaded on a 7% acrylamide/bisacrylamide (40:0.5) gel and electrophoresed in 0.5 \times TBE buffer for 4 h at 150 V. Dried gels were exposed to a PhosphorImager screen for analysis by ImageQuant software (Amersham Biosciences).

Protein Sequence Analysis—Multiple protein alignments were performed using the AlignX program of Vector NTI (Informax). Functional motifs were analyzed with SMART (available on the World Wide Web at smart.embl-heidelberg.de) and PFAM (available on the World Wide Web at www.sanger.ac.uk/Software/Pfam/search.shtml). Secondary structure predictions were performed with NNPREDPREDICT (available on the World Wide Web at www.cmpfarm.ucsf.edu/~nomi/nnpredict.html).

RESULTS

RET1 Does Not Incorporate dNTPs or Modified Uridine Nucleotides—It has been shown previously that RET1 TUTase requires no additional factors for enzymatic activity *in vitro* and utilizes UTP as a preferred substrate. CTP is incorporated with much lower efficiency, and there is no preference for the 3'-terminal nucleotide of the substrate RNA (13). Also, unlike poly(A) polymerase (20), RET1 displayed no reactivity with up to 1 mM of dNTPs (Fig. 1A), a concentration ~20-fold above the apparent K_m for UTP (see below). This is strikingly different from the properties of PAP, for which 2'-dATP is routinely used as a substrate for 3'-end labeling of RNAs (20). Attempts to determine the K_i for 2'-dUTP were unsuccessful due to this NTP being an extremely poor competitive inhibitor. The recombinant enzyme was inactive with UTP derivatives such as digoxigenin-11-UTP, Cascade Blue-7-UTP, and Biotin-21-UTP at a 1 mM concentration in the reaction mixture (not shown). Incorporation of 4-thio-UTP is markedly reduced under identical conditions, which indicates a possible role of hydrogen bond formation and involvement of position 4 in uridine recognition.

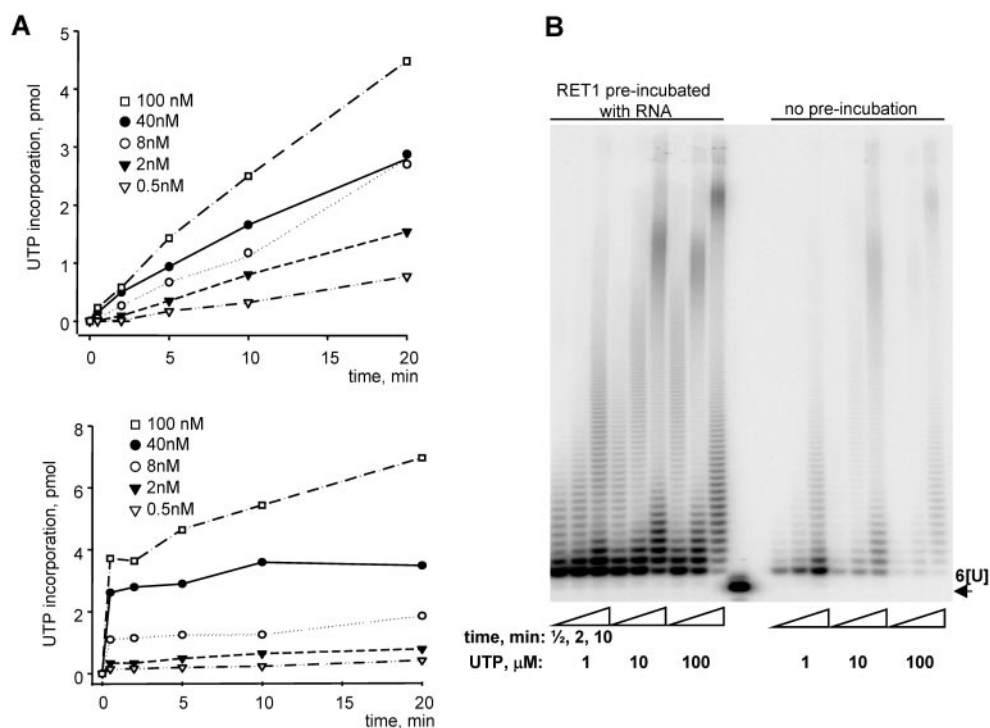


FIG. 2. **Enzyme kinetics of RET1 reaction.** *A*, total UTP incorporation assay. *Upper graph*, the RNA primer was mixed with UTP, and the reaction was started by the addition of the enzyme. *Lower graph*, RET1 was incubated with the 6[U] RNA primer for 15 min prior to the addition of radioactive UTP to 100 μM . RNA concentrations used in the reaction are indicated, and UTP and enzyme concentrations were as in the standard filter assay. *B*, analysis of reaction products for the two reactions shown in *A*. The reactions were performed in the presence of 100 nM 6[U] RNA, 0.5 μCi of [α - ^{32}P]UTP, and UTP as indicated. Products were analyzed on 15% acrylamide/urea gels. 6[U] indicates the position of the 5-labeled RNA primer.

Minimal RNA Substrate Length and RNA-independent UTP Polymerization—Several 5'-labeled synthetic oligoribonucleotides ranging in size from 7 to 24 nt were tested for primer activity with recombinant RET1. For the operational purpose of selecting an appropriate RNA primer for further enzyme characterization, the minimal substrate length should be greater than 10 nt (Fig. 1*B*). Shortening the primer from 15 to 10 nt resulted in \sim 10-fold less UTP incorporation. Further reduction to 7 nt essentially abolished its priming capacity. However, once UTP polymerization is initiated, the products of the processive reaction reach the same length as those started by primers >15 nt.

Surprisingly, polymerization also occurred in the absence of an RNA substrate (Fig. 1*C*), provided at least 1 μM UTP is present in the reaction. To eliminate the possibility that some cellular RNA co-purified with the recombinant RET1, the Sepharose SP fraction was incubated with RNase A (0.1 mg/ml) and RNase T1 (0.1 mg/ml) prior to loading on the Talon affinity resin (see "Experimental Procedures"). The observed UTP concentration threshold of between 1 and 10 μM was confirmed in a filter binding TUTase assay (not shown). The length of polymerization products grew to several hundred nucleotides as the concentration of UTP increased. No other NTP was active in this reaction. However, in the presence of 0.1 μM unlabeled RNA primer 12[U], a concentration that is 4-fold above the apparent K_m for RNA (see below), only extended primer RNA products were detected at all UTP concentrations (not shown). This finding implies that RNA and UTP may compete at the primer binding site, resulting in formation of different products, but that the affinity for RNA is higher than for UTP.

Enzyme Kinetics of RET1 Reaction—The addition of enzyme to a mixture of RNA and UTP resulted in a linear accumulation of product over time (Fig. 2*A*, *upper graph*), and these data could be fit with high confidence into a standard Michaelis

model. The K_m and V_{max} values for both UTP and RNA are shown in Table I.

Interestingly, the order of substrate addition had a profound effect on the TUTase reaction. Preincubation of RET1 with different amounts of the RNA primer followed by the addition of UTP resulted in a rapid polymerization which reached a plateau within several seconds; the value of the plateau level depended on the RNA concentration (Fig. 2*A*, *lower graph*). In an identical experiment with the RNA concentration fixed at 100 nM and UTP varying between 1 and 500 μM , similar reaction curves were observed (not shown). Attempts to challenge the preincubated reactions after reaching the plateau with a 10-fold excess of RNA or UTP substrates did not stimulate UTP incorporation (not shown). Gel analysis of products from preincubated and nonpreincubated reactions revealed more efficient UTP incorporation in the former reaction but little difference in the average product length (Fig. 2*B*). These data suggest that in the absence or low concentrations of competing UTP, RET1 may form a stable high affinity complex with an RNA primer, and the enzyme remains bound to the reaction product.

Pyrophosphorolytic Activity of RET1—For most polymerase enzymes, inorganic pyrophosphate (PP_i) stimulates pyrophosphorolysis of nucleic acids, an activity that is essentially a reversal nucleotide transfer (21). This reaction occurs by a nucleophilic attack of PP_i on the phosphodiester backbone of the RNA or DNA terminus, and the result is the release of one NTP from the 3'-end of the polynucleotide chain. To assay for pyrophosphorolytic activity of recombinant RET1, 5'-labeled 12[U] RNA primer was incubated with a highly purified recombinant RET1 preparation (see "Experimental Procedures") at a 10:1 molar ratio for 10 min. PP_i or UTP was then added to assess whether degradation of RNA or UTP polymerization could be detected (Fig. 3*A*). The control lane demonstrates the absence of hydrolytic activity as-

TABLE I
Steady-state kinetic parameters of *L. tarentolae*
RET1 deletion mutants

The range of values obtained from at least two independent measurements is provided.

Mutants	UTP		RNA	
	K_m	V_{max}	K_m	V_{max}
	μM	$\mu\text{M}/\text{min}$	nM	nM/min
RET1	45–65	2.5–4	18–30	2–3.5
N71C120	9–17	0.4–0.8	61–80	1–2
N155C120	20–38	1.9–3	77–130	1.8–3
N220C120	40–58	2.8–3.5	33–60	3.5–5
N282C120	— ^a	—	—	—
N282	—	—	—	—
N302C120	—	—	—	—
C120	34–40	2.6–3.5	43–60	0.7–1.3
C173	11–22	0.7–1	750–1120	2.8–3
C205	4–7	0.2–0.7	1084–1908	1–1.4
C236 ^b	3–9	0.1–0.2	980–1202	0.3–0.5
C370	—	—	—	—
400–543 ^c	—	—	—	—

^a —, activity undetectable in the filter assay.

^b Activity was measured for the “tetramer” fraction in Fig. 5A.

^c Internal deletion.

sociated with RET1. A pyrophosphate-dependent degradation of RNA is evident in the presence of PP_i. UTP polymerization is shown as a control. Mg²⁺ was essential for both activities (not shown). Interestingly, RET1 catalyzed pyrophosphorolysis of 6[U] or 12[U] synthetic RNAs but not the 6[A] RNA, whereas in the UTP polymerization reaction no such preference for substrate RNA was found (13). The polymerization reaction is obviously more energetically favorable, since no pyrophosphorolysis could be detected at a 1:100 molar ratio of UTP *versus* PP_i in the reaction (Fig. 3B).

Oligomerization Domain of RET1—Oligomerization was suggested by the observation that a significant portion of the total RET1 in mitochondrial extract from both *L. tarentolae* (13) and *T. brucei* (16) existed as a particle of ~500 kDa, which probably represents a homotetramer (13). In addition, a separate stable complex of ~700 kDa that contains RET1 and several yet unidentified proteins has also been detected in *L. tarentolae* mitochondrial extract, but its organization has not been analyzed (13). Since most nucleotidyltransferases do not form oligomeric structures, sequence comparisons of RET1 and enzymes belonging to the DNA polymerase β -type nucleotidyltransferase superfamily suggested that the oligomerization domain may reside in the RET1-specific N-terminal or C-terminal extensions (Fig. 4). There is also a ~150-amino acid unique insertion between the second and third aspartates of the catalytic domain in RET1. In addition, a C2H2 zinc finger motif in the N-terminal extensions of RET1 from both *L. tarentolae* (225–249 aa) and *T. brucei* (193–216 aa) is a potential homodimerization fold (22).

To address this question experimentally, a series of N- and/or C-terminally truncated RET1 proteins were constructed, expressed in *E. coli*, purified, and assayed for TUTase activity. Since the final purification step was gel filtration on a Superose 6 column, an estimation of the quaternary structure of the mutant proteins could also be obtained from the fractionation behavior (Fig. 6A). As shown in Table I and Fig. 4B, removal of up to 220 N-terminal amino acids and 120 C-terminal amino acids had no effect on TUTase activity or the apparent quaternary structure (not shown). This is consistent with the fact that the N-terminal 220-amino acid peptide has no significant homology beyond *Leishmania* and is not even conserved between the *Leishmania* and *Trypanosoma* RET1 sequences. The N282 and N302 deletion mutant proteins lacking C2H2 zinc finger were completely inactive enzymatically. Apparent molecular

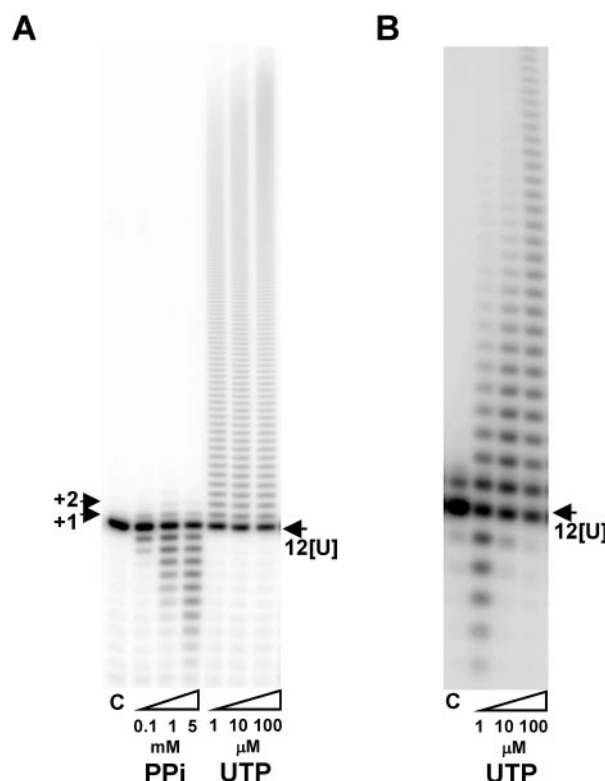


FIG. 3. Pyrophosphorolytic activity of RET1. A, inorganic pyrophosphate stimulates pyrophosphorolysis of RNA. The 5'-labeled 12[U] RNA (100 nM) was incubated with RET1 (10 nM) and the indicated concentrations of tetrasodium pyrophosphate for 15 min (*PP_i lanes*) under standard conditions. An identical reaction was performed with increasing amounts of UTP in the absence of pyrophosphate (*UTP lanes*). Lane C, control for hydrolytic activity. RNA was incubated in the presence of enzyme. The positions of the input 12[U] RNA and the +1 and +2 additions are shown by the arrows. B, transferase activity of RET1 in the presence of inorganic pyrophosphate. The reaction was performed in the presence of 1 mM PP_i and the indicated concentrations of UTP.

masses of N220 and N302 mutants calculated based on their elution profiles were 251 ± 15 and 220 ± 15 kDa. This corresponds to a decrease in molecular mass due to a loss of ~30 kDa, close to what would be predicted from an 82-amino acid deletion times 4 to account for a tetramer. A change to a monomeric state would have produced a protein with an estimated elution volume of ~17 ml, which is a much more dramatic effect than had been observed.

Truncation of the C terminus by 205 amino acids led to a moderate decrease in the K_m for UTP, probably due to more complete protein folding during expression in *E. coli*. An effect on quaternary structure was observed only with a C-terminal deletion of 236 amino acids. This mutant protein migrated in gel filtration as two peaks corresponding to a dimer and tetramer (Fig. 5A). The tetramer peak was isolated and shown to exhibit the same K_m for UTP as the C205 deletion. The dimer peak was active immediately after isolation but was unstable, preventing collection of reproducible kinetic data. The appearance of two forms due to the C236 deletion suggests that the oligomerization domain was partially affected. However, the properly assembled tetramer fraction of C236 mutant was as active as the C205 form. Removal of 370 amino acids from the C terminus led to a complete loss of enzymatic activity. The C370 protein was soluble but eluted from the Superose 6 gel filtration column in the void volume, suggesting aggregation. This deletion protrudes into the region that is highly conserved among TUTases (Fig. 4) and, although it shares no homology with the C-terminal domain of PAPs (4, 5), is clearly important

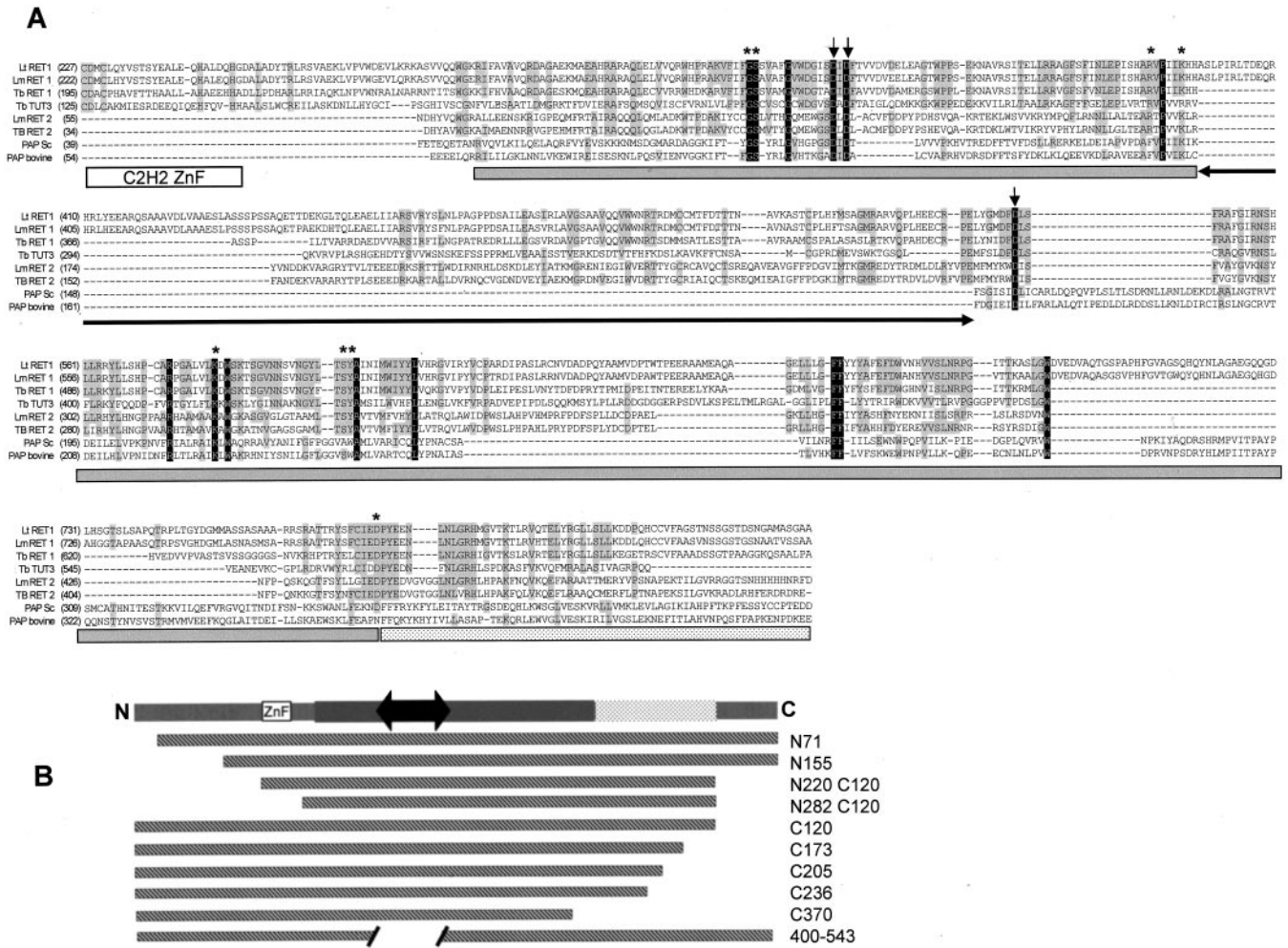


FIG. 4. Multiple sequence alignment of trypanosomatid RET1, RET2, yeast, and bovine poly(A) polymerases and diagram of *L. tarentolae* RET1 deletion mutants. A, bovine PAP domains are shown as predicted by PFAM. Central domain is shown in gray; poly(A) polymerase RNA binding is shown in a dotted pattern. Note the virtual absence of homology between PAP and TUTases in the RNA binding (C-terminal) region. The C2H2 zinc finger is boxed. Metal-coordinating aspartate residues are indicated by the arrows. The TUTase-specific insertion in the central domain is shown by a black arrow. Identical residues are white on black; conservative residues are in black on gray background. Point mutations are shown by asterisks. B, sequence diagram of deletion mutations. Central domain and insertion are as depicted in A. Truncated forms are shown in a downward diagonal pattern.

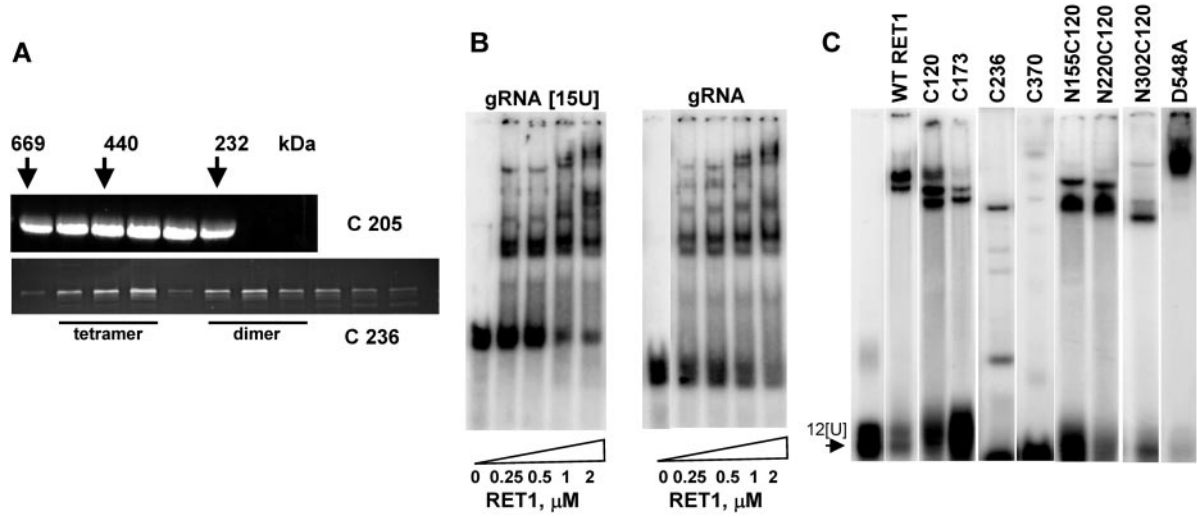


FIG. 5. RNA binding and oligomerization regions of RET1 TUTase overlap in the C-terminal region. A, oligomerization of RET1 involves the C-terminal region of the protein. C-terminal deletion mutants were separated on a Superose 6 size fractionation column, and each fraction was analyzed by SDS gel electrophoresis followed by staining with Sypro Ruby. B, the presence of oligo[U] tail did not affect gRNA binding to RET1. Uniformly labeled *in vitro* transcribed ND7[4×]gRNA was used in a gel retardation assay with RET1. See “Experimental Procedures” for details. C, RNA binding region was localized in the C-terminal region of RET1. The 5'-labeled 12[U] RNA was used in a gel retardation assay with 1 μM truncated versions of RET1. Deletions in N and/or C terminus and the length of deleted fragments are indicated.

for proper protein folding. Deletion of the TUTase-specific insertion (aa 400–543) within the catalytic core abolished UTP incorporation activity (Table I) without a major effect on the Superose 6 elution profile.

RNA Binding Domain of RET1—An indication that the RNA binding domain is also localized in the C-terminal region was provided by showing that the K_m for RNA increased nearly 2 orders of magnitude by deletion of 205 amino acids from the C terminus, whereas N-terminal deletions up to 282 amino acids had no effect (Table I). A partial separation of RNA binding and oligomerization domains is indicated by the lack of any effect on oligomerization by the C205 deletion (see above).

RNA binding was also assayed directly by a gel retardation procedure using a synthetic guide RNA, which is a natural substrate for RET1 (17). The synthetic guide RNA with and without 15 uridine residues at the 3'-end were incubated with RET1 and separated on a native gel. The appearance of one or more retarded labeled bands with increasing amounts of RET1 can be seen in Fig. 5B. The presence of the oligo[U]-tail did not affect the gRNA-RET1 interaction, which is consistent with the previous observation that the presence of the oligo[U] tail in synthetic gRNA did not affect the cross-linking efficiency with RET1 in the mitochondrial extract (13). A K_d of 0.55–0.7 μM was calculated from these data.

A synthetic RNA with 12 U nucleotides at the 3'-end (12[U] RNA) that was used in the kinetic analysis produced simpler gel shift patterns than gRNA and therefore was adopted for gel retardation assays with truncated proteins (Fig. 5C). The C120 mutant protein was fully active in RNA binding. Further C-terminal deletions gradually impaired and, in the case of the C370 mutant, totally abolished binding of RNA. It is possible that deletion of this region disturbed proper folding of RET1 (above) and that the observed inhibition of RNA binding was a secondary effect.

Deletion of 220 amino acids from the N terminus had no effect on either enzymatic activity or RNA binding. Removal of 302 amino acids, which inactivated UTP incorporation, also did not significantly inhibit RNA binding. The point mutation in the carboxylate triad, D548A, although deleterious for UTP incorporation (see below), served as a control that the catalytic residues did not contribute to RNA binding. Thus, it appears that the RNA binding and oligomerization domains of RET1 are ~200 amino acids long and occupy an overlapping region between positions 800 and 1000.

Identification of the Active Site—The two metal ion catalytic reaction mechanism postulated for pol- β and other polynucleotide polymerases (21) assumes the coordination of Mg^{2+} ions in the active center by a triad of carboxylate residues. These three or possibly two amino acids in the case of class II CCA-adding enzymes (23) have been identified in a variety of enzymes. We found that mutation either of two aspartic acid residues (Asp³⁴² and Asp³⁴⁴) in RET1 that are part of the pol- β superfamily signature motif led to complete inactivation of UTP polymerization activity (Table II).

Identification of the third functionally important residue, which is typically found ~50 amino acids toward the C terminus, required an extensive carboxylate scan. Positions 406, 424, 473, 507, 546 (not shown), and 775 (Table II) were mutated without any effect on UTP incorporation activity, within experimental variation. However, the D548A substitution completely abolished TUTase activity without compromising the quaternary structure or RNA binding (Fig. 5C). The ~150-amino acid insertion in the catalytic domain of RET1 can also be seen in the alignment analysis in Fig. 4A. The insertion displays only a moderate conservation in length, sequence, or secondary structure among the known TUTases (Fig. 4) yet

TABLE II
Kinetic constants for selected point mutants

Mutants	UTP		RNA	
	K_m	V_{\max}	K_m	V_{\max}
	μM	$\mu\text{M}/\text{min}$	nM	nM/min
LtRET1	45–65	2.5–4	18–30	2–3.5
D342A	— ^a	—	—	—
D344A	—	—	—	—
D548A	—	—	—	—
R390F	—	—	—	—
K395D	—	—	—	—
F328G	77.0	3.1–4.2	92–102	0.8–1.2
S330A	4–9	0.5–0.7	1220–1640	2.3–3
K580A	352–443	4.7–4.9	20–28	0.9–1.3
S598A	52–80	5.0–7.0	16.0	3.4
Y599F	67.6	17.5	72.7	18.3
D775A	22	9.3	776	39.2

^a —, activity undetectable in the filter assay.

may be a characteristic feature of this class of enzymes. The deletion of this region (positions 443–500) inactivated the enzyme (Table I).

The hydrophobic residue preceding the TUTase signature motif (position 328 in RET1) has been placed within the van der Waals radius from the sugar and base moieties of the incoming nucleotide in bovine PAP (5) and apparently affects the V_{\max} for ATP incorporation (24). In RET1, this mutation had little effect on UTP binding but increased the K_m for RNA 3-fold. More surprisingly, the serine residue of the signature sequence (position 330), which in the PAP structure contacts the γ - and β -phosphates of the incoming ATP, appears to be involved more in recognition of RNA rather than UTP (Table II). Mutation of another amino acid, K580A, that can be expected to contribute to pyrophosphate binding based on the yeast and bovine PAP structures indeed increased the apparent K_m for UTP by 10-fold. Finally, replacement with aspartate of a conserved positively charged residue Lys³⁹⁵, which could be involved in hydrogen bond formation with the metal-coordinating Asp⁵⁴⁸, had a devastating effect on activity.

Most amino acids in the active center in PAP are conserved in TUTases, suggesting that these are undergoing contacts with the ribose and phosphate moieties. The exceptions are few but noteworthy. The Lys³⁹⁰ of RET1 corresponds to a conserved phenylalanine in PAP, and K390F mutation inactivated RET1. It is interesting that the nucleotide specificity of all of the active RET1 mutant proteins was unchanged (not shown). One possibility is that base specificity may be governed by a dynamic protein template that forms hydrogen bonds between protein and the nucleotide base, as demonstrated for the CCA-adding enzyme (25). The fact that such divergent enzymes as RET1 and RET2 are both U-specific suggests that a very few structural features are sufficient to confer UTP specificity to a general pol- β catalytic scaffold, but this remains to be investigated.

RET1 Is a Zinc-dependent Enzyme—The crucial role of divalent metals for polymerization reactions is well established, and the amino acids that were predicted to coordinate magnesium ions in the active center are indeed required for TUTase activity (see above). However, deletion of another potential metal-coordinating domain, the C2H2 type zinc finger shown in Fig. 4A, completely inactivated the enzyme (the N282C120 mutant in Table I). In order to assess the possible role of zinc ions for RET1 activity, a highly purified recombinant RET1 preparation was dialyzed against the zinc-chelating agent, o-phenanthroline. A 90% reduction of activity was observed after 12 h of dialysis, and complete inactivation followed repetition of the procedure (Fig. 6A). It should be noted that no protein loss or precipitation occurred. Identical results were observed using a

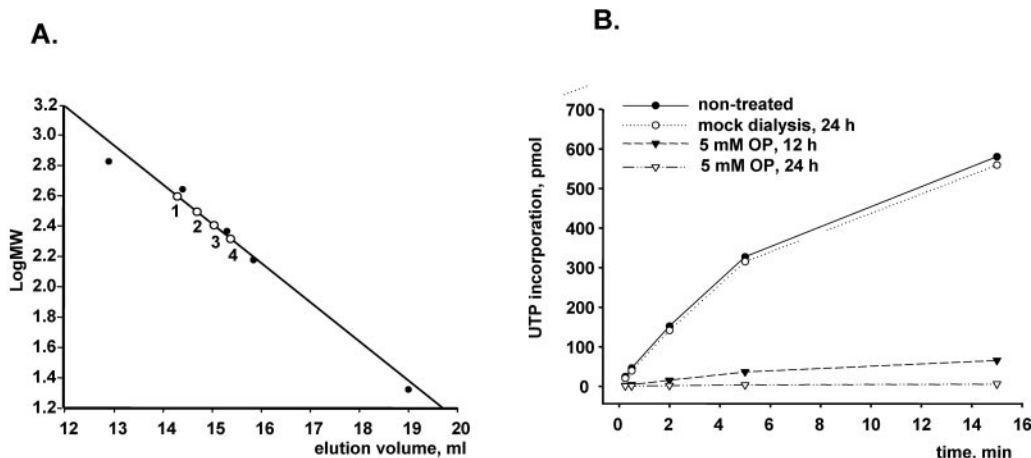


FIG. 6. Zinc is required for RET1 activity. *A*, deletion of the C2H2 zinc finger did not affect the oligomerization state of RET1. Purified N-terminal deletion mutants (*open circles*) were fractionated on a Superose 6 gel filtration column. 1, full-length RET1; 2, N150; 3, N220C120; 4, N302C120. Calibration standards (*filled circles*) were thyroglobulin (669 kDa), ferritin (438 kDa), catalase (232 kDa), aldolase (150 kDa), and trypsin inhibitor (20.1 kDa). *B*, inhibition of RET1 activity by dialysis against zinc-chelating agent. Recombinant RET1 was dialyzed against 50 mM HEPES, pH 7.5, 10% glycerol, 1 mM dithiothreitol, 50 mM KCl in the presence or absence of 5 mM 1,10-orthophenanthroline (OP) for 12–24 h. In order to remove 1,10-orthophenanthroline from the sample, additional dialysis was performed for 2 h in the same buffer. The protein concentration after dialysis was verified by SDS gel electrophoresis. The UTP incorporation activity was measured in a standard filter assay with 50 nM enzyme.

sequencing gel assay for enzymatic activity (not shown). In an attempt to restore TUTase activity, the sample was further dialyzed against a buffer with no *o*-phenanthroline for 2 h and then against a buffer with 50 μ M ZnSO₄ for 6 h. This led to only ~5% restitution of activity (not shown). These data indicate that RET1 requires zinc binding for activity. The involvement of zinc atoms in catalysis or RNA binding is unlikely. Rather, it may be a manifestation of C2H2 zinc finger involvement in protein folding. A lack of involvement of the zinc finger in oligomerization was evidenced by the fact that the N220 and N302 truncation mutants had similar apparent molecular weights, although the latter lacks the entire zinc finger (Fig. 6A).

DISCUSSION

The principal goal of this study was to define functional regions and substrate requirements of the RNA-editing TUTase 1 (13), which has been implicated in the 3' addition of uridines to guide RNAs to form the 3' oligo(U) tails. RET1 is still the only essential enzymatic activity involved in U insertion/deletion editing in trypanosomes for which an active and properly folded recombinant protein can be obtained in preparative amounts. Combined with the availability of specific and sensitive enzymatic assays, this makes RET1 a good candidate for biochemical and structural studies as well as a possible chemotherapeutic drug target.

The results of a "carboxylate scan" point to aspartate residues at positions 342, 344, and 548 as constituents of a metal-coordinating catalytic triad found in most polymerase enzymes. A remarkable feature of all TUTase enzymes known to date is an insertion of variable length within the catalytic domain that moves the third aspartate further away from the two carboxylates that are part of the pol- β superfamily signature sequence. In the three-dimensional structure, these residues probably remain in close proximity, and the insertion is likely to fold into a structure independent of the catalytic domain. The mutant that underwent a deletion of this insertion sequence, however, was not active in the TUTase reaction and appeared to have a general folding problem. This mutant protein was partially degraded in the bacterial cells and had chromatographic properties uncharacteristic of the other RET1 mutant proteins examined. Otherwise, the amino acids implicated in interactions with ribose and phosphate moieties appear to be conserved among poly(A) polymerases and RET1 TUTase. A notable ex-

ception is a major contribution of the 2'-OH, which is probably involved in UTP binding. Also, it can be speculated that the inability of RET1 to incorporate modified nucleotides reflects an involvement of the uridine base, probably through positions 1 and 4, in hydrogen bond contacts with the enzyme. This would be consistent with our finding of 4-thio-UTP being a poor substrate for RET1.

One major difference with other nucleotidyltransferases is that RET1 has an oligomeric (probably tetrameric) configuration in the active state, whereas all other transferases are active as single polypeptides. The oligomerization domain was localized by gel filtration analysis of a series of N- and C-truncated mutant proteins to a C-terminal region (aa 800–900). Deletion C236 yielded two forms of the protein, which could be separated by gel filtration and that most closely match tetramer and dimer forms of RET1. This mutant had a markedly reduced RNA binding affinity that may indicate dual functions of the aa ~880–915 region of the protein as a part of oligomerization and RNA binding domains. Further C-terminal deletion (C370) completely abolished RNA binding, but these data should be interpreted cautiously, since the protein, although soluble during expression and purification, seemed to have formed an aggregate. It is possible that the effect on folding occurred due to removal of the aa 760–800 region in RET1. This fragment is highly conserved among RET1 and RET2 sequences, and, given the major differences in oligomeric structure and RNA substrates of these enzymes *in vivo*, it is unlikely to be involved in either oligomerization or RNA binding. The extended RNA-binding region of RET1 may be relevant to the finding that in the mitochondrial extract, RET1 interacts via an RNA linker with the ~16-polypeptide L-complex (14) and the MRP1/2 RNA chaperone complex (26).

Another interesting feature of RET1 is that it exhibits a primer-independent UTP polymerization activity at high concentrations of UTP. This suggests that the 3'-OH of a single UTP can be properly positioned in the active center to serve as a nucleophile. In the presence of RNA, however, this reaction was not observed. Presumably, multiple contacts render a higher affinity to an RNA primer, but even a single ribonucleotide can be properly positioned in the active center to act as a nucleophile in the transferase reaction. It is unclear whether the primer-independent reaction takes place *in vivo*, given the

3 orders of magnitude lower apparent K_m for RNA. Yeast PAP is also capable of primer-independent poly(A) synthesis, and this activity can be stimulated by dinucleoside polyphosphates (27).

A model for nucleotide recognition by another template-independent nucleotidyltransferase, the CCA-adding enzyme, has been proposed (25). Several conserved amino acids are believed to form a dynamic template for incoming nucleotides by hydrogen bonding. In RET1, the mechanism of U specificity remains a mystery. Mutations in some positions that are apparently conserved in known TUTases (aa 598 and 599 in *L. tarentolae* RET1) did not affect activity or nucleotide specificity. The nature of the 3'-end nucleotide of the RNA primer does not influence UTP incorporation by RET1 (13, 16). Thus, it seems likely that no base-specific contacts are involved in binding of the 3'-end of the RNA substrate, as may not be the case with RET2 (16).

Another activity of RET1 described here is pyrophosphorolysis. We have not observed a 3'-5' hydrolytic activity of RET1 toward any RNA tested, but the addition of inorganic pyrophosphate greatly stimulated a distributive 3'-5' degradation of synthetic RNA ending with uridine residues. Pyrophosphorolytic activity of the mutant enzymes correlated with their polymerase activity profiles and was undetectable in carboxylate triad mutants involved in coordination of Mg^{2+} . This reaction is essentially a reversal of the transferase reaction and, predictably, was inhibited by the first non-U base in the RNA sequence. This finding may be of technical value for future research on the structure of the active center, since it may allow the use of RNAs with a cross-linking agent at the 3'-end to map both primer and, in the presence of PP_i , incoming nucleotide binding sites. The same phenomenon has been observed with poly(A) polymerase; pyrophosphorolysis was specific for RNAs ending in oligo(A). This specificity is probably a consequence of leaving group (nucleotide triphosphate) stabilization at the incoming nucleotide binding site. In the absence of reliable data on intramitochondrial UTP and PP_i concentrations, the biological relevance of the RET1 pyrophosphorolytic activity is unclear. However, given the low apparent K_m values of the recombinant enzyme for UTP (Table I) and the fact that RET1 adds hundreds of uridines *in vitro*, it is reasonable to assume that, in the mitochondrion, RET1 activity is somehow down-modulated. Indeed, guide RNAs are the natural RET1

substrates *in vivo* (17), and the average length of the post-transcriptionally added oligo(U) tail is only ~15 nucleotides.

One of the most unexpected results presented here is the finding that RET1 requires zinc for enzymatic activity. Removal of zinc ions led to a complete inactivation of TUTase, but the exact role of zinc atoms remains to be investigated.

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