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Uridine Insertion into Preedited mRNA by a Mitochondrial Extract from *Leishmania tarentolae*: Stereochemical Evidence for the Enzyme Cascade Model

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An RNA editing-like internal uridine (U) incorporation activity (G. C. Frech, N. Bakalara, L. Simpson, and A. M. Simpson, EMBO J. 14:178–187, 1995) and a 3′-terminal U addition activity (N. Bakalara, A. M. Simpson, and L. Simpson, J. Biol. Chem. 264:18679–18686, 1989) have been previously described by using a mitochondrial extract from *Leishmania tarentolae*. Chiral phosphorothioates were used to investigate the stereoconfiguration requirements and the stereochemical course of these nucleotidyl transfer reactions. The extract utilizes (S_P)-α-S-UTP for both 3′ and internal U incorporation into substrate RNA. The internal as well as the 3′ incorporation of (S_P)-α-S-UTP proceeds via inversion of the stereoconfiguration. Furthermore, internal U incorporation does not occur at sites containing thiophosphodiesters of the R_P configuration. Our results are compatible with an enzyme cascade model for this in vitro U insertion activity involving sequential endonuclease and uridylyl transferase directly from UTP and RNA ligase steps and are incompatible with models involving the transfer of U residues from the 3′ ends of guide RNAs.

The mechanism of the uridine (U) addition-deletion type of RNA editing that occurs in the mitochondria of kinetoplastid protozoa is not yet understood. It is clear however that the sequence information for editing is provided by guide RNAs (gRNAs), which are complementary to mature edited mRNA if G · U base pairing is allowed. A single gRNA mediates the editing of a short sequence block, and multiple overlapping gRNAs are required to mediate the editing of extensively (pan-) edited domains (3, 18, 39, 43).

Three models for the mechanism of U incorporation, which differ with respect to the source of the incorporated uridines and the proposed enzymatic machinery, have been proposed. In the original enzyme cascade model (4), uridine residues derived from UTP are inserted via the sequential actions of three enzymatic activities: an RNA endonuclease, a terminal uridylyl transferase (TUTase), and an RNA ligase. Candidate activities for all three of these enzymes have been identified in mitochondrial extracts of *Leishmania tarentolae* (2, 37) and *Trypanosoma brucei* (19, 29). U deletions in this model occur by 3′ exonuclease trimming prior to religation. In a modified enzyme cascade model (20, 42), the source of the inserted U residues is the gRNA 3′ oligo(U) tail but the enzymes involved are the same. The transesterification model (6, 10) proposes that U incorporation occurs via two successive transesterification reactions such as occur in RNA splicing. The gRNA-mRNA chimeric molecules predicted by the modified enzyme cascade and transesterification models have been shown to be present in steady-state mitochondrial RNA (6). However, it is unclear if these molecules are true editing intermediates or aberrant by-products.

An in vitro system in which a mitochondrial extract directs an exogenous gRNA-dependent deletion of uridines from the first editing site of a synthetic ATPase 6 preedited mRNA has been described for *T. brucei* (12, 35, 36). No U insertions were observed in this system, although the exogenously added gRNA could have been expected to also direct U addition editing at upstream sites.

An in vitro activity which directs an imprecise incorporation of U's into the preedited regions of cytochrome *b* and NADH dehydrogenase subunit 7 synthetic mRNAs has also been reported by using a mitochondrial extract from *L. tarentolae*. This activity sediments in glycerol gradients as a 25S complex and contains a micrococcal nuclease-sensitive component(s) (14, 27). Internal U incorporation was not stimulated and in fact was even inhibited by the addition of exogenous gRNA. The involvement of endogenous gRNAs in this reaction is uncertain.

In this work we have analyzed the mechanism involved in the *L. tarentolae* internal U incorporation activity by introducing phosphorothioates into a substrate mRNA. Substitution of sulfur for one of the two nonbridging oxygens in a phosphodiester link creates a chiral center around the phosphorus with two diastereomeric configurations (S_P and R_P). The two stereoisomers commonly exhibit different reactivities in enzyme-catalyzed as well as ribozyme-catalyzed reactions (13, 15, 23, 25, 26, 41). We have investigated the stereoconfiguration requirements for U insertions and the stereochemical course of events. The results were interpreted on the basis of the following predictions: (i) involvement of an RNA ligase would be expected to give rise to a ligated bond having the R_P configuration, in analogy to the stereospecificity of the ligation process catalyzed by T4 RNA ligase (8), and (ii) transesterification would proceed via inversion of the stereoconfiguration, by analogy to pre-mRNA, group I intron, and group II intron splicing (23–

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26, 32). Our results suggest that the internal U incorporation in this in vitro system occurs by a cleavage-ligation mechanism and not by transesterification.

MATERIALS AND METHODS

Cell culture, mitochondrial isolation, and preparation of mitochondrial extracts. *L. tarentolae* UC cells were grown as described previously (38) and used for isolation of the kinetoplast-mitochondrion fraction by flotation in Renografin density gradients (7, 40). The isolated mitochondria were resuspended at a protein concentration of approximately 5 mg/ml in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–KOH (pH 7.5)–0.1 M KCl–0.2 mM EDTA–10% glycerol. Mitochondrial detergent extracts were prepared as described previously (37) and kept frozen at −80°C.

Preparation of substrate RNA. The 280-nucleotide pNB2 RNA (37) was transcribed in vitro as described previously (2). To obtain RNA containing approximately 50% phosphorothioates, transcriptions were performed with α-thio-nucleoside triphosphate (α-S-NTP)/NTP ratios of 1:1 (11). To generate RNA containing 15 and 5% phosphorothioates, the corresponding transcription reactions were supplied with 100% CTP, 100% UTP, and α-S-ATP/ATP and α-S-GTP/GTP ratios of 3:7 or 1:9, respectively. Uniformly labeled transcripts were obtained by adding [α-^{32}P]UTP to the transcription reaction mixtures.

Creation of circular RNA. The pNB2 RNA was transcribed in the presence of 2 mM GMP-S (DuPont NEN) and 0.2 mM GTP. Gel-isolated RNA (3 μg) was 3′ labeled in a 30-μl reaction mixture containing 1 μM [α-^{32}P]ATP (800 Ci/mmol; DuPont NEN), 50 mM Tris-HCl (pH 7.9), 0.25 M NaCl, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, bovine serum albumin (0.35 mg/ml), and 2 U of poly(A) polymerase (Bethesda Research Laboratories) and incubated at 37°C for 1 h. Approximately 0.5 μg of gel-isolated, labeled RNA was then incubated in a 50-μl reaction mixture in the presence of either 10 μl of mitochondrial extract, 20 U of T4 RNA ligase (Bethesda Research Laboratories) (positive control), or no extract or ligase (negative control). The ligation reaction mixture contained 50 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 7 mM dithiothreitol, 0.2 mM ATP, 20% dimethyl sulfoxide, and 0.6 U of human placenta RNase inhibitor (Pharmacia) per μl and was allowed to proceed for 2 h at 27°C. The circular RNA was separated from the linear RNA on a 7.5 M urea–8% polyacrylamide gel and recovered by passive elution in 0.5 M ammonium acetate (pH 7.5)–10 mM magnesium acetate–0.1 mM EDTA–0.1% sodium dodecyl sulfate followed by phenol-chloroform extraction and ethanol precipitation.

Labeling of RNA with α-^{35}S-UTP in the presence of mitochondrial extract. One microgram of pNB2 RNA was incubated in a 100-μl reaction volume containing 1.6 μM α-^{35}S-UTP (1,000 to 1,500 Ci/mmol; DuPont NEN), 5 mM HEPES (pH 7.5), 60 mM KCl, 3 mM potassium phosphate (pH 7.5), 6 mM magnesium acetate, 20 mM dithiothreitol, 2 mM spermidine, 1 mM ATP, 1 mM GTP, and 4 μl of mitochondrial extract (protein concentration, approximately 5 mg/ml) at 27°C for 100 min. The gel-isolated RNA was annealed to the DNA oligomer S-165 and cleaved by incubation with RNase H as described previously (14). The 5′ and 3′ cleavage fragments were separated on a 7.5 M urea–8% polyacrylamide gel and recovered by elution.

Enzymatic determination of thiophosphodiester stereoconfiguration. Nuclease P1 digestion of labeled RNA was performed in 5-μl reaction volumes containing 50 mM sodium acetate (pH 5.2), 0.1 mM $ZnCl_2$, 0.5 μg of *Escherichia coli* tRNA per μl and 0.2 μg of nuclease P1 (Boehringer Mannheim) per μl at 37°C for 90 s. Snake venom phosphodiesterase (SV-PDE) digestion of labeled RNA was carried out at 37°C for 1 h in 2.5-μl reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 0.4 μg of *E. coli* tRNA per μl, and 0.13 μg of SV-PDE (Boehringer Mannheim) per μl. The reactions were stopped by the addition of urea to a final concentration of 3 M. The samples were heated to 95°C for 3 min and run through a 0.4-mm-thick 7 M urea–25% polyacrylamide gel, and this was followed by PhosphorImager (Molecular Dynamics) analysis.

RESULTS

The phosphodiester bond formed by the *L. tarentolae* mitochondrial RNA ligase has the R_P configuration. Two of the three models for trypanosomatid RNA editing invoke the involvement of an RNA ligase. Although various ligases described for different organisms utilize structurally distinct substrates, their mechanisms are, in general, fairly similar (16, 21, 44). The stereochemical details of the ligation process catalyzed by T4 RNA ligase have been elucidated, and the ligated thiophosphodiester linkage has been shown to have the R_P configuration (8).

A mitochondrial RNA ligase activity has previously been reported for *L. tarentolae* and *T. brucei* and shown to utilize 3′ hydroxyls and 5′ phosphates (2, 45). To investigate whether the stereospecificity of this activity emulates the specificity of T4 RNA ligase, the procedure diagrammed in Fig. 1A was followed. Preedited RNA (pNB2 RNA) was synthesized by T7 transcription in the presence of excess GMP-S in order to obtain RNA with a prochiral 5′ phosphorothioate rather than a 5′ triphosphate. This RNA was then 3′ end labeled with poly(A) polymerase and [α-^{32}P]ATP. Incubation with *L. tarentolae* mitochondrial extract led to circularization of the labeled RNA by the endogenous RNA ligase activity. The circular RNA was gel isolated and digested with two stereoselective nucleases: nuclease P1, which cleaves S_P but not R_P thiophosphodiesters (31), and SV-PDE, which cleaves R_P thiophos-

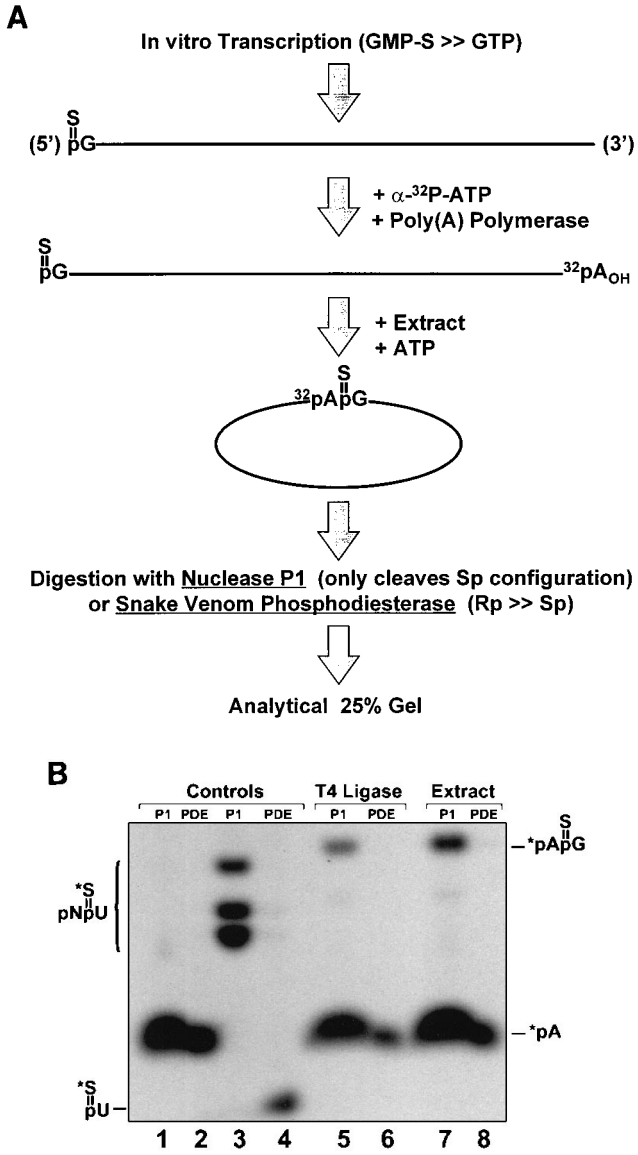

FIG. 1. Investigation of the stereoconfiguration of thiophosphodiester bonds formed by mitochondrial RNA ligase. (A) Diagram of strategy; (B) PhosphorImager analysis of an analytical 25% polyacrylamide gel of nuclease P1 and SV-PDE digestions (odd- and even-numbered lanes, respectively). Lanes: 1 and 2, RNA 3′ end labeled by poly(A) polymerase; 3 and 4, RNA uniformly labeled with α-^{35}S-UTP; 5 and 6, 3′-end labeled RNA circularized by T4 RNA ligase; 7 and 8, 3′-end-labeled RNA circularized by incubation with mitochondrial extract. The positions of the expected mono- and dinucleotide cleavage products are indicated. ^{32}P and ^{35}S radioisotopes are shown as *p and *S, respectively. The three bands labeled pNp*SU (pGp*SU, pAp*SU, and comigrating pUp*SU and pCp*SU) represent the dinucleotides resistant to cleavage by P1 nuclease.

phodiesters approximately 3 orders of magnitude more readily than S_P thiophosphodiesters (9). The digestion products of both enzymes contain 5′ phosphates. RNA uniformly labeled with α-^{35}S-UTP served as a control for the stereospecificity of nuclease P1 and SV-PDE (Fig. 1B, lanes 3 and 4), since T7 RNA polymerase produces RNA containing thiophosphodiester linkages of only the R_P configuration (17). PhosphorImager analysis of the digestion products separated in a polyacrylamide gel revealed that the thiophosphodiester bond produced by the mitochondrial RNA ligase, like T4 RNA ligase, had the R_P configuration: only nuclease P1- and not SV-PDE-digested material was partially resistant to digestion down to mononucleotides (Fig. 1B, lanes 5 to 8). The observation that the majority of the P1-digested material is monomeric rather than dimeric is explained by the fact that poly(A) polymerase extended the 3′ ends with several adenosine nucleotides, even though the reactions were carried out at low ATP concentrations to minimize the processivity of the enzyme.

Mitochondrial extract can utilize (S_P)-α-S-UTP for both 3′ and internal U incorporation into substrate RNA. The RNA editing-like activity in the *L. tarentolae* mitochondrial extract directs the internal incorporation of U's into the preedited domains of certain synthetic mRNAs (14). The extract also contains a TUTase activity which adds U residues to the 3′ ends of RNAs (2). Preedited substrate RNA labeled in vitro by incubation with mitochondrial extract was digested with RNase H after hybridization with a specific oligodeoxynucleotide to release two fragments that can be separated by gel electrophoresis (14). Both 3′ and internal U incorporation still occurred when UTP was replaced with (S_P)-α-S-UTP. A small amount of incorporation was also detected by using an (R_P)-α-S-UTP preparation, but this was at a level expected from the quoted stereochemical purity of the material (data not shown).

Internal as well as 3′ incorporation of (S_P)-α-S-UTP proceeds via inversion of the stereoconfiguration. To determine the stereochemistry involved in the incorporation of uridine thiophosphate, pNB2 RNA was incubated with mitochondrial extract in the presence of (S_P)-α-^{35}S-UTP. The intact labeled RNA was gel isolated and then digested with RNase H after hybridization to a DNA oligomer complementary to a sequence downstream of the preedited region (Fig. 2A). The 5′ fragment containing internally inserted U's was separated by gel electrophoresis from the 3′ fragment containing U residues added by TUTase. Each fragment was then digested with nuclease P1 and SV-PDE to determine the stereoconfiguration of the thiophosphodiester linkages.

A PhosphorImager analysis of the digestion products in a polyacrylamide gel is shown in Fig. 2B. For both the 5′ and 3′ fragments, digestion with SV-PDE released mononucleotides (Fig. 2B, lanes 7 and 10, respectively). In contrast, both RNA fragments were resistant to complete digestion by nuclease P1, as indicated by the presence of di- and oligonucleotides and by the absence of mononucleotides (Fig. 2B, lanes 6 and 9). This evidence suggests that the thiophosphodiester linkages formed by the TUTase activity as well as by the internal U incorporation activity are in the R_P stereoconfiguration.

Nuclease P1 limit digestion of the 5′ fragment revealed that the internal U incorporation activity adds up to 15 to 20 U's per individual site, with the predominant addition being one or two U's (Fig. 2B, lane 6). The majority of the nuclease P1-resistant material derived from the 3′ fragment migrated as dinucleotides (Fig. 2B, lane 9). This indicates that the TUTase activity preferentially adds only one uridine to the 3′ end of pNB2 RNA under the experimental conditions used. The predominant dinucleotide spot corresponds to comigrating pCp (S)U and pUp(S)U, which is consistent with the fact that the

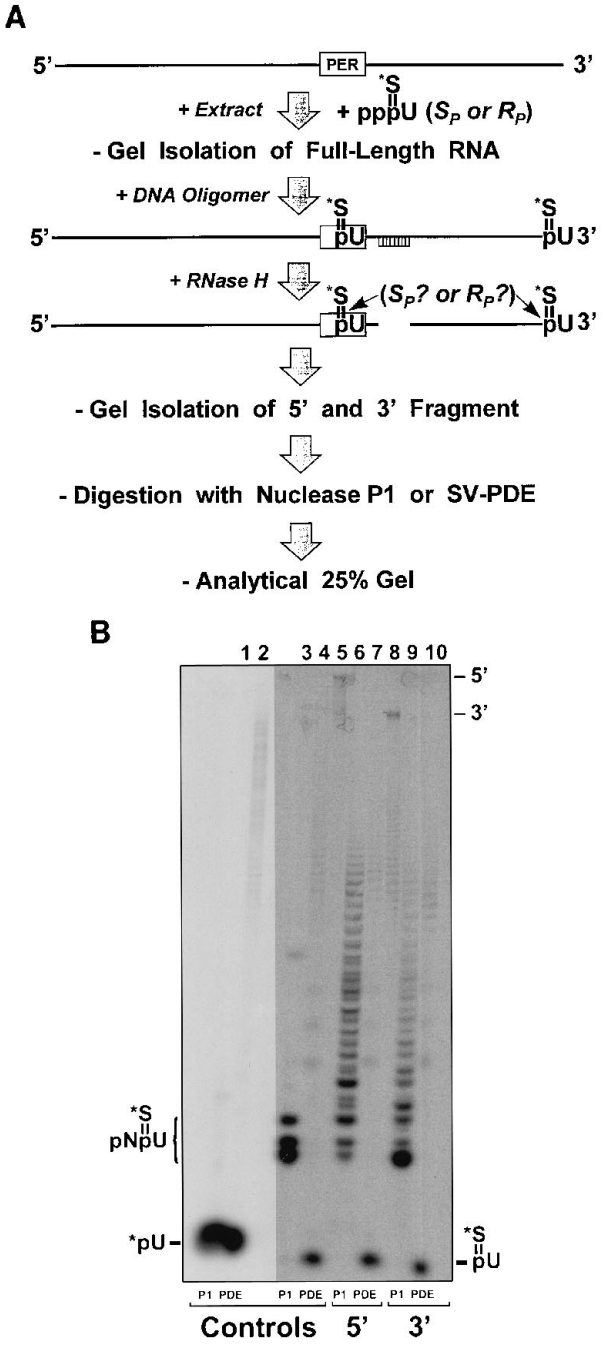

FIG. 2. Investigation of the stereoconfiguration of 5′-incorporated uridines. (A) Diagram of strategy (PER, preedited region); (B) PhosphorImager analysis of an analytical 25% polyacrylamide gel of nuclease P1 (lanes 1, 3, 6, and 9) and SV-PDE (lanes 2, 4, 7, and 10) digestions. Lanes: 1 and 2, digested RNA uniformly labeled with [α-^{32}P]UTP; 3 and 4, digested RNA uniformly labeled with α-^{35}S-UTP; 5 and 8, undigested 5′ or 3′ fragment, respectively, labeled by incubation with mitochondrial extract and α-^{35}S-UTP; lanes 6 and 7, digested 5′ fragment, labeled by incubation with mitochondrial extract and α-^{35}S-UTP; 9 and 10, digested 3′ fragment, labeled by incubation with mitochondrial extract and α-^{35}S-UTP. The positions of the undigested 5′ and 3′ fragments as well as the mono- and dinucleotide cleavage products are indicated. ^{32}P and ^{35}S radioisotopes are shown as *p and *S, respectively. The relative numbers of added U's per site can be estimated from the nuclease P1 digestion lanes after the appropriate normalization for the number of labeled U residues per band.

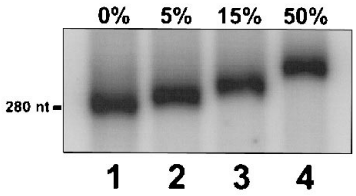

FIG. 3. Electrophoretic mobilities of RNA transcripts containing various amounts of thiophosphodiesters. Shown is the PhosphorImager analysis of an analytical 6% polyacrylamide gel containing uniformly labeled RNA in vitro transcribed in the absence of α-S-NTP (lane 1) and uniformly labeled RNA transcribed in the presence of increasing α-S-NTP/NTP ratios as indicated (lanes 2 to 4). nt, nucleotides.

predicted 3′-terminal nucleotide of full-length pNB2 RNA is a C.

Internal U incorporation does not occur at sites containing thiophosphodiesters of the R_P configuration. To investigate whether U's are inserted into substrate RNA containing R_P phosphorothioates, pNB2 RNA was transcribed by T7 RNA polymerase in the presence of α-S-NTPs. T7 RNA polymerase, like all known polymerases, utilizes only (S_P)-α-S-NTP and produces RNA containing thiophosphodiester bonds of only the R_P configuration (17). Substrate RNAs containing 5, 15, and 50% randomly distributed phosphorothioates were synthesized by taking advantage of the fact that T7 RNA polymerase does not significantly discriminate between α-S-NTP and NTP (11). The mobility of the different RNA populations in polyacrylamide gels was inversely related to the extent of sulfur substitution (Fig. 3).

The pNB2 RNA transcripts containing R_P thiophosphodiester linkages were used as substrates for in vitro internal U incorporation in the presence of mitochondrial extract and [α-^{32}P]UTP. The extent of 3′ and internal U incorporation was assayed by gel electrophoresis after digestion with RNase H in the presence of oligomers. The amount of internal U incorporation in the phosphorothioate-containing substrate RNAs was reduced, whereas 3′ U addition was not affected (data not shown).

The 5′ fragments of the labeled substrate RNAs were gel isolated after RNase H digestion and were digested with nuclease P1 and SV-PDE. All four substrate RNAs yielded only pU mononucleotide digestion products (Fig. 4, lanes 3 to 10). No dinucleotides were observed, indicating that U's were inserted only at sites containing phosphodiester but not thiophosphodiester linkages. These data suggest that internal U incorporation does not occur at sites containing R_P thiophosphodiester bonds.

DISCUSSION

Chiral phosphorothioates were used to investigate the stereospecificity of nucleotidyl transfer reactions involved in the in vitro incorporation of U residues catalyzed by enzymatic activities present in an *L. tarentolae* mitochondrial extract. The mitochondrial TUTase activity was shown to utilize α-S-UTP of the S_P but not of the R_P configuration, and the stereoconfiguration of the incorporated nucleotides was inverted to R_P. The internal U incorporation activity was likewise shown to utilize only (S_P)-α-S-UTP, which was inverted to the R_P stereoconfiguration as a result of the reaction.

Internal U incorporation did not occur at RNA substrate sites containing chiral R_P thiophosphodiester linkages. There exists a formal possibility that this is due to a general structural effect caused by a large degree of phosphorothioate substitutions in the RNA backbone rather than due to the local geometry of the R_P thiophosphodiester itself. However, this is unlikely, since the inhibitory effect was also observed with substrate RNA containing only one phosphorothioate site per 20 nucleotides, i.e., statistically only 14 phosphorothioates per RNA molecule (Fig. 4, lanes 5 and 6). Furthermore, multiple uridine nucleotides derived from α-S-UTP were incorporated into the substrate RNA, thereby creating RNA molecules containing up to approximately 20 phosphorothioates (Fig. 2B, lane 6) and potentially even more if U's were incorporated into more than one site per molecule.

The stereochemical evidence in this paper is in agreement with a cleavage-TUTase ligation model for the in vitro internal U incorporation in Fig. 5A but not with the transesterification model in Fig. 5B or the modified enzyme cascade model in Fig. 5C. The data do not speak to the question of the involvement of endogenous gRNA in this reaction, however. In the transesterification model in Fig. 5B, which involves mediation by gRNA, the two reaction steps are predicted to proceed via inversions of the stereoconfiguration, by analogy with the mechanisms for pre-mRNA- (23, 25), group I- (24, 32), and group II-splicing reactions (26). The evidence that the reactive thiophosphodiester would retain the R_P stereoconfiguration in the second reaction step (Fig. 5B) is not compatible with the transesterification model. Instead, the final product would be predicted to contain all S_P, or a mixture of S_P and R_P in the case that U's would be transferred from the gRNA U tail in blocks rather than individually. However, no evidence for the existence of any S_P phosphorothioates was obtained (Fig. 2B, lanes 6 and 7).

The modified enzyme cascade model (Fig. 5C), which also involves the mediation by gRNA, invokes two successive rounds of endonucleolytic cleavage and religation. The evidence that the first but not the second round would be blocked by the presence of an R_P thiophosphodiester (Fig. 5C) is incompatible with the modified enzyme cascade model. The unlikely scenario that the first step occurs via transesterification and the second step occurs via cleavage-ligation can be eliminated on grounds of parsimony.

Chimeric gRNA-mRNA molecules in various trypanosomatid species have been shown to be formed both in vivo (6) and in vitro (1, 5, 20, 22). Strong circumstantial evidence that in

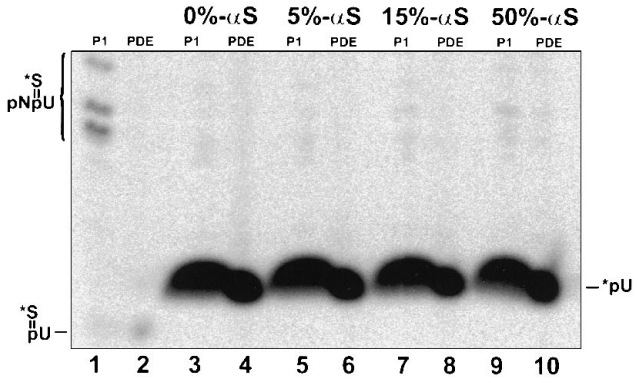

FIG. 4. Use of substrate RNA containing R_P thiophosphodiester bonds for the internal U incorporation assay. Shown is the PhosphorImager analysis of an analytical 25% polyacrylamide gel of RNAs containing various relative amounts of phosphorothioates, as indicated, incubated with mitochondrial extract and [α-^{32}P]UTP, and then digested with nuclease P1 (lanes 3, 5, 7, and 9) and SV-PDE (lanes 4, 6, 8, and 10). Lanes 1 and 2, RNA uniformly labeled with α-^{35}S-UTP and digested with nuclease P1 or SV-PDE, respectively. The positions of the mono- and dinucleotide cleavage products are indicated.

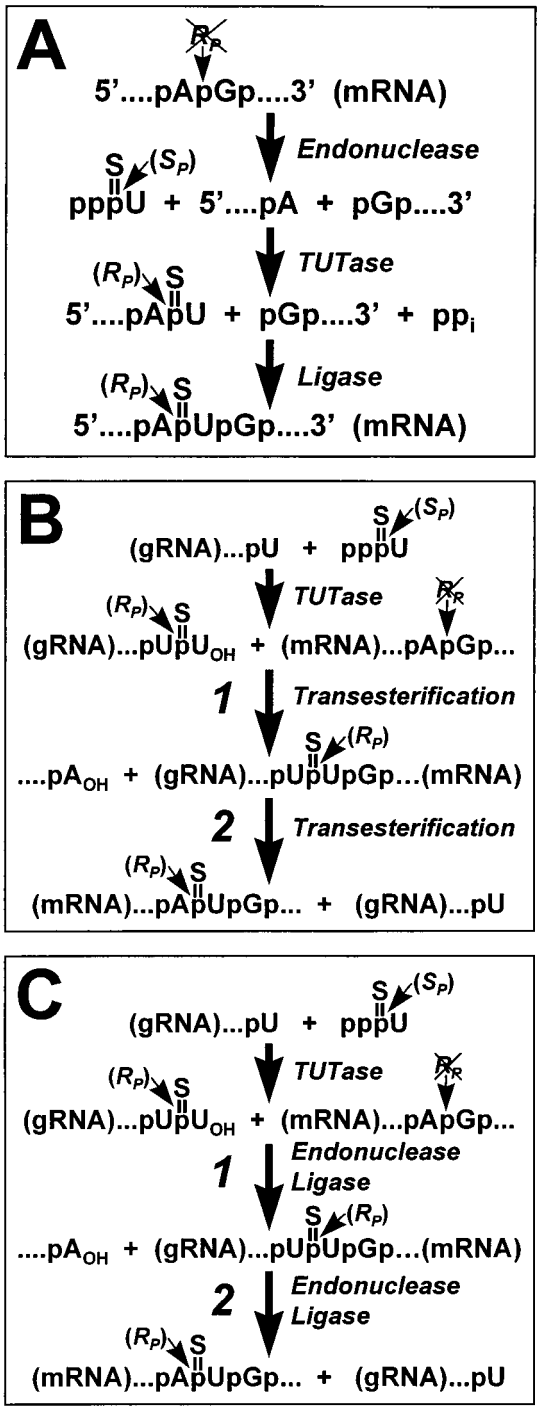

FIG. 5. Models for the mechanism of internal U incorporation. The experimentally determined stereoconfigurations (R_P and S_P) of reactive phosphorothioates are indicated. The compatibility of the experimental data with the various models is discussed in the text. (A) Original enzyme cascade model. The incorporated U residues are derived directly from UTP. Transesterification (B) and modified enzyme cascade (C) models. The uridines are incorporated via the gRNA U tail; chimeric gRNA-mRNA molecules are obligatory reaction intermediates. Incorporation occurs either via two successive transesterification reactions (B) or via two rounds of cleavage-ligation (C). Incorporation of only one uridine at one site is shown for each model. The site of U incorporation in the mRNA is shown between an A and a G residue.

vitro chimera formation in *T. brucei* mitochondrial extract occurs via a cleavage-ligation rather than a transesterification mechanism has been reported. Sedimentation of mitochondrial extract revealed that chimera-forming activity cofractionated with TUTase and RNA ligase activities (29, 30) and, under reducing conditions, also with a site-specific endonuclease activity (28). Furthermore, two cosedimenting adenylylated proteins were identified as components of an RNA ligase (34), and conditions that inhibited RNA ligase activity also inhibited chimera formation (33, 34). In vitro-synthesized chimeras were shown to be specifically cleaved by extract endonuclease within the oligo(U) tract at the editing site, and the cleavage products were shown to be joined by RNA ligase to generate partially edited mRNAs (28, 29). However, a recent study of gRNA-mediated U deletion occurring in vitro with a mitochondrial extract from *T. brucei* suggested that chimeras appeared in time after cleavage intermediates and edited products (36). In addition, a mutant gRNA with an extended 3′ oligo(U) tail promoted the formation of edited products but not of chimeras, suggesting that chimeras are nonproductive end products of editing (36).

Finally, it has been shown that the introduction of R_P phosphorothioates at sites in the mRNA substrate where endonucleolytic cleavage normally occurred completely suppressed both cleavage and chimera formation at those sites (33).

Our stereochemical results rule out a role for gRNA-mRNA chimeras derived either by cleavage-ligation or transesterification in the in vitro internal U incorporation activity we have studied. This would imply that chimeric gRNA-mRNA molecules are not true editing intermediates but rather represent nonfunctional fortuitous by-products of the editing reaction, formed within the confines of the editing complex when the gRNA is accidentally ligated to the mRNA 3′ cleavage fragment. A similar conclusion was reached in an investigation of a gRNA-mediated in vitro U deletion reaction with *T. brucei* (36).

The stereochemical data for the in vitro U incorporation reaction which we have studied are compatible with the enzyme cascade model, which predicts that U incorporation occurs by the sequential interactions of endonuclease, TUTase, and RNA ligase. The stereochemical results eliminate the possibility of the involvement of gRNA as a reservoir for U's in this in vitro activity but do not address the question of whether the observed internal U incorporation activity is dependent on or independent of endogenous gRNAs.

The stereochemical evidence would not distinguish between the original enzyme cascade model in Fig. 5A and a transesterification model in which the U's are derived from UTP (10). However, there is additional recent biochemical evidence for the involvement of cleavage and ligase activities in the in vitro internal U incorporation reaction (27a).

The precise relationship of the internal U incorporation activity to in vivo gRNA-mediated RNA editing is unclear, but there is recent evidence that the two activities might have a common mechanism (9a). This would suggest that information about the in vitro U incorporation activity could prove directly applicable to in vivo gRNA-mediated RNA editing, but further investigation is required to substantiate this hypothesis.

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