In the chytridiomycete fungus, *Spizellomyces punctatus*, all eight of the mitochondrially encoded tRNAs are predicted to have one or more base pair mismatches at the first three positions of their aminoacyl acceptor stems. These tRNAs are edited post-transcriptionally by replacement of the 5′-nucleotide in each mismatched pair with a nucleotide that can form a standard Watson-Crick base pair with its counterpart in the 3′-half of the stem. The type of mitochondrial tRNA editing found in *S. punctatus* also occurs in *Acanthamoeba castellanii*, a distantly related amoeboid protist. Using an *S. punctatus* mitochondrial extract, we have developed an in vitro assay of tRNA editing in which nucleotides are incorporated into various tRNA substrates. Experiments employing synthetic transcripts revealed that the *S. punctatus* tRNA editing activity incorporates nucleotides on the 5′-side of the stem. Editing of a triphosphorylated 5′-end in the absence of ATP but requires ATP to add nucleotides to a monophosphorylated 5′-end; moreover, it functions independently of the state of tRNA 3′ processing. These data parallel results obtained in a previous in vitro study of *A. castellanii* tRNA editing, suggesting that remarkably similar activities function in the mitochondria of these two organisms. The evolutionary origins of these activities are discussed.

RNA editing encompasses particular forms of RNA processing that change the primary sequence of an RNA molecule from that predicted by the gene sequence. The term “RNA editing” was coined to describe post-transcriptional U insertions within the mitochondrial cox2 transcript of two trypanosome species: insertions that correct a gene-predicted frameshift, thereby generating a functional mRNA (1). Editing has since been found to be an essential process in many diverse, predominantly organellar, systems (2). Many cases of mRNA editing have been identified, including uridylic insertion/deletion in trypanosome mitochondria (3, 4), co-transcriptional insertion of nucleotides in myxomyocyte mitochondria (5, 6), C-to-U and U-to-C editing in plant mitochondria and chloroplasts (7), A-to-I (8, 9) and C-to-U (10) base deamination in animal nuclei, co-transcriptional nucleotide insertion in viruses (11), and the recently discovered nucleotide replacement editing in dinoflagellate mitochondria (12). In addition to mRNAs, ribosomal RNAs (13–15) and transfer RNAs (tRNAs) (16) can be substrates for editing.

Editing of tRNA has been described in the mitochondria of many organisms. In plant mitochondria, C-to-U editing repairs mismatches in tRNA stems (17–20). C and U insertion occurs within myxomyocyte mitochondrial tRNAs, creating base pairs in stems as well as restoring the GUUC sequence in the T stem-loop (21). In marsupial mitochondria, C-to-U editing alters the anticodon of mitochondrial tRNA<sup>ROP</sup>, changing the decoding potential (22–24). Similarly, C-to-U editing changes the decoding properties of trypanosome mitochondrial tRNA<sup>Trp</sup> (25). Non-templated editing within the 3′-half of tRNA acceptor stems, including the discriminator position, has been described in the mitochondria of several animals (26–28). Further, in the centipede *Lithobius forficatus*, nucleotides are added to the 3′-end of mitochondrial tRNAs, apparently using the 5′-half of the acceptor stem as a template (29). Intriguingly, editing similar to that found in *L. forficatus* repairs mismatches in mitochondrial tRNA acceptor stems in the protist *Seculamonas eucadoriensis*, a jakobid flagellate (30).

An additional type of editing, and the first example of tRNA editing to be described, occurs in the mitochondria of the amoeboid protist *Acanthamoeba castellanii* (31). In this organism, 12 mtDNA<sup>1</sup>-encoded tRNAs are predicted to contain mismatches in one or more of the first three base pairs of the acceptor stem. These mismatches are such that they appear incompatible with canonical tRNA folding and function. Direct sequencing of tRNA 5′-ends (31) as well as a tRNA circularization/RT-PCR approach (32) have revealed that these mismatches are repaired in vivo by removing the 5′-nucleotide from each mismatched pair and replacing it with a nucleotide that has the potential to form a Watson-Crick base pair with its counterpart in the 3′-half of the stem. The *A. castellanii* editing activity is hypothesized to consist of at least two components: an endonuclease and/or 5′-to-3′ exonuclease that removes mismatched nucleotides from tRNA 5′-ends, and a template-directed 3′-to-5′ nucleotidyltransferase activity that restores acceptor stem base pairing (31, 33). An in vitro assay developed to study this form of editing (33) has demonstrated nucleotide

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1 The abbreviations used are: mtDNA, mitochondrial DNA; nt, nucleotide; HTGT, histidine tRNA guanylyltransferase; RT-PCR, reverse transcription-polymerase chain reaction; TLC, thin-layer chromatography.
incorporation into various tRNA substrates. Nucleotide addition occurs at recessed tRNA 5'-ends, requires ATP when the 5'-terminus is monophosphorylated, and proceeds in a 3' to 5' direction using the 3'-half of the acceptor stem as a template.

In *Spizellomyces punctatus*, a chytridiomycete fungus, all eight of the mtDNA-encoded tRNAs are predicted to contain mismatches at one or more of the first three acceptor stem positions (34). These mismatches are repaired in vivo, as in *A. castellanii*, by replacement of the 5'-nucleotides in the pair to form Watson-Crick base pairs, as shown by direct sequencing of tRNA 5'-ends (34) and tRNA circularization/RT-PCR (35).

In the present study, we first demonstrate and then examine an enzymatic activity in *S. punctatus* mitochondrial extracts that incorporates nucleotides into tRNA molecules, an activity that has the properties expected of the in vivo mitochondrial tRNA editing activity. We compare the biochemistry of the *S. punctatus* and *A. castellanii* editing activities and discuss how they may have evolved.

**MATERIALS AND METHODS**

**Culture Conditions and Preparation of Mitochondrial S100 Extracts**—*S. punctatus* strain BR117 was kindly provided by B. F. Lang (Université de Montréal), maintained on agar plates, and grown in a liquid medium containing 0.5% yeast extract and 3% glycerol (adjusted to pH 5.5 with KH2PO4). Cells were grown with hard shaking (100 rpm) in 100-ml cultures for ~48 h until the majority of the culture consisted of zoospores, which were found to be much less resistant to mitotic lysis than mature cells. Swimming zoospores were streaked on a 15-min incubation on ice, pelleted by centrifugation at 2000 × g for 10 min, and the supernatant was collected with hard shaking (100–200 rpm) in 100-ml cultures for ~30 s, followed by 1 min rests between bursts. The supernatant was then subjected to ultracentrifugation at 100,000 × g for 1 h in a fixed angle rotor, and the supernatant (S100) was snap frozen in liquid nitrogen and stored at −70 °C in 50-μl aliquots. Alternatively, RNA was prepared from purified, intact mitochondria (see below). S100 fractions were active for nucleotide incorporation for more than a year under these conditions. For most experiments, the S100 was incubated on ice for 20 min with 1/5 volume of QAE-Sephadex A-50 strong anion exchange resin (ion exchange group: diethyl-2-hydroxypropylaminomethyl; stored in sonication buffer) prior to *in vitro* assays.

**Preparation of Mitochondrial and Cytoplasmic RNA**—Purified mitochondria were gently lysed in a solution containing 2% Triton X-100, 10 mM Tris-HCl, pH 8.5, 50 mM KCl, and 10 mM MgCl2, centrifuged at 9000 × g for 10 min, and nucleic acids were prepared from both the resultant pellet (mito-P) and supernatant (mito-S) fractions. Cytoplasmic RNAs were prepared from the supernatant of the first 9000 × g spin in the protocol for preparation of a mitochondrial S100 fraction (see above). RNA was isolated as described (36). To assess mitochondrial enrichment, 5 μg of RNA from each fraction was treated with 2.5 units of DNase I for 30 min and resolved by agarose gel electrophoresis.

**In Vitro Assay of Nucleotide Incorporation**—*In vitro* assay contained 2.5 μl of the mitochondrial S100 extract, 40 mM HEPES (pH 7.0 with NaOH), 15 mM MgCl2, 1 mM dithiothreitol, 100 μM NTPs (when included), 10 μCi of [α-32P]NTP (when included), and exogenous tRNA substrate (when included; RNA was either 1 μg of yeast soluble RNA, 1/20 of the product of a T7 transcription reaction, or a small portion of a 32P-end-labeled T7 transcription product). Reactions (in a final volume of 50 μl) were performed at room temperature (~22 °C) for 1 h (or less, as indicated in the text). The linearized products of in vitro assays were extracted with phenol–chloroform and precipitated with ethanol using linear polyacrylamide as carrier (37). Samples were electrophoresed through 10% polyacrylamide gels containing 7 M urea (36).

**Preparation of Synthetic tRNA Substrates**—Oligonucleotides used in this study are shown in Table I. The *A. castellanii* mitochondrial tRNA Glu(aaag) gene cloned into the pT7Blue vector (see Ref. 33; referred to here as plasmid 1) was used to generate the PCR templates for T7 RNA polymerase transcribed tRNA constructs 1 and 4–9 (see Figs. 3 and 4 for sequences of constructs). The PCR templates for T7 RNA constructs 2 and 3 were generated by first introducing 5′ or 3′ extensions, respectively, into the cloned tRNA Glu(aaag) plasmid 1. This was accomplished by PCR using divergently oriented primers, to generate plasmid 2 (using oligonucleotides 1 and 2) and plasmid 3 (using oligonucleotides 3 and 4). PCR amplification was performed with Vent DNA polymerase in all cases. The linearized plasmids 2 and 3 thus produced were treated with T4 polynucleotide kinase and circularized with T4 DNA ligase. Plasmids 1, 2, and 3 were cloned in competent *Escherichia coli* cells (strain DH5α), and plasmid DNA was prepared using an alkaline lysis procedure. The sequence of the tRNA Glu(aaag) region of the plasmids was confirmed by DNA sequencing.

**Short DNA templates for in vitro** transcriptions—Reactions with T7 RNA polymerase were produced using PCR with primers designed to incorporate specific sequences at the 5′- and 3′-termini of these templates. Plasmids were treated with the endonuclease HindIII (which cuts outside the tRNA Glu(aaag) region in the multiple cloning site) prior to amplification in order to eliminate longer, unwanted products observed in PCR reactions using uncleaved plasmids. T7 templates for synthesis of tRNA constructs 1–9 were generated using the oligonucleotide combinations shown in Table II. Each T7 template contained a 6 nt 5′-leader, a T7 promoter, and the desired tRNA Glu(aaag) sequence. The products were purified as described (36). Materials and methods: Preparation of Mitochondrial and Cytoplasmic RNA—Purified mitochondria were gently lysed in a solution containing 2% Triton X-100, 10 mM Tris-HCl, pH 8.5, 50 mM KCl, and 10 mM MgCl2, centrifuged at 9000 × g for 10 min, and nucleic acids were prepared from both the resultant pellet (mito-P) and supernatant (mito-S) fractions. Cytoplasmic RNAs were prepared from the supernatant of the first 9000 × g spin in the protocol for preparation of a mitochondrial S100 fraction (see above). RNA was isolated as described (36). To assess mitochondrial enrichment, 5 μg of RNA from each fraction was treated with 2.5 units of DNase I for 30 min and resolved by agarose gel electrophoresis.

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of PCR were extracted with phenol-cresol, precipitated with ethanol, and 1/10 of each product was used as template for each 50 μl of T7 in vitro transcription reaction. Products of in vitro transcription were treated with 2.5 units of DNase I, separated in 10% denaturing polyacrylamide gels, excised, and eluted. An approximately equal amount of T7 product (several micrograms) was generated for each construct, as estimated by ethidium bromide staining of polyacrylamide gels.

End Analysis of RNA—Transfer RNAs labeled by the S100 extract in the presence of [α-32P]GTP were eluted from a homogenized gel slice by shaking overnight at 4 °C in a 1:1 mixture of phenol-cresol:buffer (0.5 M NH4OAc, 10 mM Mg(OAc)2, 1.0 M EDTA) (36). Extracted RNAs were precipitated with ethanol and treated with either nuclease P1, which generates nucleoside 5'-phosphates (pN), or RNase T2, which generates nucleoside 3'-phosphates (Np). The products of nuclease P1 digestion were separated by thin-layer chromatography (TLC) using polyethyleneimine cellulose plates (predevolved with distilled H2O and allowed to dry) and 0.5 M (NH4)2 CO3 as the solvent. The products of RNase T2 digestion were separated by TLC on cellulose plates (predipped in a 10-fold dilution of a saturated solution of (NH4)2SO4 and allowed to dry) using a 4:1 mixture of 95% ethanol-water as the solvent (38).

3' and 5' End-labeling and RNA Sequencing—RNAs were 3' end-labeled with [γ-32P]ATP and RNA ligase (39) or dephosphorylated with bacterial alkaline phosphatase and 5' end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (40). Labeled products were purified by electrophoresis in polyacrylamide gels, excised and eluted.

Partial sequences of the 3' and 5' portions of 3' and 5' end-labeled RNAs were determined by separation of the products of partial alkaline hydrolysis and partial digestion with RNase T1 (which cleaves 3' to G residues) in adjacent wells of a 20% polyacrylamide gel. Further 3' end-analysis was performed by TLC separation of an RNase T2 digest of 3' end-labeled material (see above for TLC conditions).

Periodate Treatment of RNA—One-twentieth of the RNA product of a T7 transcription reaction or a small portion of a 32P-end-labeled T7 transcription product was dissolved in a 40-μl solution containing 150 mM NaOAc (pH 5.3) and 1 mM Na2OAc, incubated on ice in the dark for 1 h, and precipitated with ethanol.

RESULTS

Development of an in Vitro Assay of Nucleotide Incorporation—Intact mitochondria were recovered from S. punctatus zoospores by lysis under relatively mild conditions in a French pressure cell followed by differential centrifugation. This procedure resulted in significant mitochondrial enrichment, as estimated by the ratio of mitochondrial to cytoplasmic rRNAs in mitochondrial RNA profiles (Fig. 1). Purified mitochondria were disrupted by sonication, and an S100 fraction was obtained by ultracentrifugation of the sonicate.

In order to test for a tRNA editing activity in the S. punctatus mitochondrial S100 fraction, an in vitro assay developed to study A. castellanii 5'-tRNA editing (33) was employed. This assay monitors the incorporation of [α-32P]GTP into tRNA molecules following incubation with a mitochondrial extract. As in A. castellanii, incubation of the S. punctatus mitochondrial S100 fraction in the presence of [α-32P]GTP resulted in the incorporation of label into tRNAs present in the S100 fraction (Fig. 2A, lane 1). Yeast tRNAs were also found to be substrates for the labeling activity (Fig. 2A, lane 2).

Nucleotide incorporation by the S. punctatus mitochondrial S100 fraction was initially observed using the assay conditions optimized for the A. castellanii editing activity (33). Optimal temperature and salt concentration for the S. punctatus activity were subsequently determined (not shown); the activity was found to function equally well from room temperature (−22 °C) to 37 °C, with reduced activity at 42 °C and loss of activity at 48 °C. Added monovalent cations (in the form of KCl) were not required and did not affect labeling efficiency. Added divalent cation was required, with peak activity at 15 mM MgCl2 and loss of activity at ≥30 mM MgCl2. The results in Fig. 2A were obtained using conditions optimized for the S. punctatus mito-

**Table II**

| Oligonucleotide combination for PCR generation of T7 templates |
|----------------------|----------------------|
| T7 template for construct |
| 1 | 5 (sense), 6 (antisense) |
| 2 | 7 (sense), 6 (antisense) |
| 3 | 5 (sense), 8 (antisense) |
| 4 | 9 (sense), 6 (antisense) |
| 5 | 10 (sense), 6 (antisense) |
| 6 | 5 (sense), 11 (antisense) |
| 7 | 5 (sense), 12 (antisense) |
| 8 | 9 (sense), 11 (antisense) |
| 9 | 9 (sense), 12 (antisense) |

**Fig. 1.** Profiles of RNAs isolated from S. punctatus cytoplasm (cyto); depleted of mitochondria (mito) and mitochondria (mito). Purified mitochondria were lysed with Triton X-100 and fractionated by centrifugation at 9000 × g into pellet (mito-P) and supernatant (mito-S) fractions. Samples were incubated with DNase I, electrophoresed through a 1% agarose gel, and visualized by ethidium bromide staining. The positions of the large subunit (LSU) and small subunit (SSU) ribosomal RNAs and tRNAs are indicated.

**Fig. 2.** Incorporation of label from [α-32P]GTP into natural tRNA substrates by an S. punctatus mitochondrial S100 extract. A. Autoradiogram of labeled tRNAs following incubation of the S. punctatus mitochondrial S100 extract, unincubated (+) or preincubated (+) with QAE-Sephadex A-50 resin (QAE), with (+) or without (−) addition of yeast transfer RNA (tRNA) and in the presence of [α-32P]GTP and unlabeled CTP, ATP, and UTP. The labeled tRNAs in lane 4 were eluted from the gel and digested with either nuclease P1 (B) or RNase T2 (C) and separated by one-dimensional TLC. The migration positions of nucleotide markers are indicated. The labeled material that migrates between ppGp and pG in B is presumed to be ppGp. D. Expected products of nuclease P1 and RNase T2 digestion of yeast tRNAs with [α-32P]GTP incorporated at the first and second 5'-positions. Asterisks indicate the positions of 32P.
Following optimization, endogenous tRNAs were removed from the S100 by incubation of the extract with QAE-Sephadex A-50 anion exchange resin, rendering this fraction dependent on addition of exogenous tRNA substrates (compare lanes 3 and 4, Fig. 2A). QAE-Sephadex-treated S100 fractions (hereinafter referred to as “the S100 extract”) were used for all subsequent experiments.

When a nuclease P1 digest (which generates nucleoside 5’-phosphates, pN) of yeast tRNAs labeled with [γ-32P]GTP, with a minority incorporated as a nucleoside monophosphate (pG) (Fig. 2B). This experiment indicated that nucleotide incorporation had occurred both internally (possibly at position 2) and at tRNA 5’-ends. The result also indicated that 5’-ends are left as triphosphates by the activity of mitochondrial tRNALeu(uag). RNase T2 digests (which generate nucleoside 3’-monophosphates, pN) of the same material revealed only a product that did not migrate from the origin in the TLC conditions used. Only acceptor stem sequences are known to migrate little, if at all, from the origin (Fig. 2C). Because multiphosphorylated nucleotides are known to migrate little, if at all, from the origin (Fig. 2D). Based on these results, the activity in the S. punctatus mitochondrial S100 extract was further characterized as a potential tRNA editing activity analogous to that described in A. castellanii.
Mitochondrial tRNA Editing in S. punctatus

require no additional nucleotides, having a completely base-paired acceptor stem. As expected, construct 4 was found to be a very good substrate for the nucleotide incorporation activity (Fig. 4B, lane d). Construct 5, on the other hand, was a poor substrate for the activity (Fig. 4B, lane e). These results, together with those for construct 1, indicate that the activity adds nucleotides up to and including tRNA position 1, whether 1 or 2 nucleotides are missing from the 5′-end. When the 5′-terminal sequence of the added synthetic tRNA is complete, no nucleotides are incorporated.

cis-Templated 3′-to-5′ Nucleotide Incorporation—Constructs 6–9 (Fig. 4A) were designed to directly test whether the sequence on the 3′-side of the acceptor stem templates nucleotide addition to the 5′-side of the acceptor stem, and whether addition proceeds in a 3′-to-5′ direction, as it does in the case of tRNA editing in A. castellanii mitochondria (33).

Construct 6 was designed to incorporate U at position 2 and G at position 1. Labeled products were observed after incubation with the extract in the presence of [α-32P]GTP and UTP (Fig. 4B, lane f). When UTP was not present during incubation, no labeled products were observed (Fig. 4B, lane g). These results support addition at position 2 followed by addition at position 1: i.e. sequential incorporation in a 3′-to-5′ direction. When [α-32P]UTP was used, labeling occurred in both the presence and absence of GTP; however, the product formed in the absence of GTP (lane o) was shorter than in its presence (lane n), as expected if U addition at position 2 is required for G addition at position 1.

Construct 7 was designed to incorporate G at position 2 and U at position 1. Labeled products were observed with [α-32P]GTP in both the presence and absence of UTP, with the product in the absence of UTP (Fig. 4B, lane i) shorter than when UTP was present (lane h). When [α-32P]UTP was used, labeling occurred in the presence of GTP (lane p) but not in its absence (lane q), again supporting the inference of incorporation at position 2 followed by incorporation at position 1.

Construct 8 was designed to require the removal of a G at position 2 (which is involved in a G2A mismatch) prior to addition of U at position 2 and G at position 1. However, no labeled products were observed for this construct following incubation with the extract and either [α-32P]GTP (Fig. 4B, lanes j and k) or [α-32P]UTP (lanes r and s). These results, together with those obtained for construct 2, suggest that nucleotides are not efficiently removed from the 5′-ends of these synthetic tRNA substrates in this assay (see “Discussion”), and that the in vitro activity is only able to extend a 5′-terminus that forms a standard base pair with its partner nucleotide on the 3′-side of the acceptor stem.

Finally, construct 9 was designed to incorporate U at position 1. This construct was not labeled with [α-32P]GTP (Fig. 4B, lanes l and m), but gave the same-sized labeled products with [α-32P]UTP in the presence (lane t) and absence (lane u) of GTP, as expected. These combined results strongly support a mechanism of nucleotide addition to tRNA 5′-ends, templated by the 3′-side of the acceptor stem and proceeding in a 3′-to-5′ direction.

Nucleotide Requirements for Incorporation—To determine the nucleotide requirements of the incorporation activity, construct 1 (Fig. 4A) was incubated with [α-32P]GTP, the S100 extract and various combinations of CTP, ATP and UTP. The latter three NTPs were not required for incorporation of label from [α-32P]GTP into this construct (Fig. 7A, lanes b–i). The size and intensity of the labeled products, however, varied depending on which of these NTPs were included in the reaction mix. Through subsequent analysis, the observed size variation was attributed to an activity incorporating C residues at

![Figure 6](http://www.jbc.org/)

**Fig. 6.** Effect of periodate treatment on incorporation of label from [α-32P]GTP into tRNA construct 3. A, acceptor stem sequence of construct 5. 32P-labeled G residues are predicted to be incorporated at positions 1 and 2 after incubation with the S100 extract. Asterisks indicate the positions of 32P. The 15-nt 3′ extension (relative to construct 1) is boxed. B, autoradiogram of construct 3, untreated (−) or pretreated (+) with sodium periodate (NaIO4) prior to incubation with the S100 extract in the presence of [α-32P]GTP and unlabeled CTP, ATP, and UTP. The predominant labeled band obtained with periodate-treated tRNA construct 3 (96 nt) is indicated with an arrow.
Mitochondrial tRNA Editing in S. punctatus

An in Vitro Assay of Mitochondrial tRNA Editing in S. punctatus—We have obtained a mitochondrial extract from S. punctatus, a chytridiomycete fungus, that supports specific and efficient incorporation of nucleotides into natural and in vitro-transcribed tRNAs. Incorporation is at the 5'-end of tRNA substrates at both internal and 5'-terminal positions (positions 2 and 1), templated by the 3'-half of the acceptor stem (positions 71 and 72) with nucleotide addition proceeding in a 3'-to-5' direction, leaving triphosphorylated 5'-ends in vitro. These features are consistent with this activity being the one that carries out in vivo editing of mitochondrial tRNAs. The development of an in vitro assay is an important step in the study of this putative editing activity, particularly as a tool to monitor the progress of any future enzyme purification.

The results obtained here are remarkably similar to those obtained with a previously reported in vitro assay of mitochondrial tRNA editing in the amoeboid protozoon, A. castellanii (33). The determination of mature tRNA acceptor stem sequences from S. punctatus and A. castellanii had demonstrated that these activities corrected very similar patterns of pre-
dicted base pair mismatches in tRNA acceptor stems, by 5′-nucleotide replacement to produce standard Watson-Crick base pairs (31, 32, 34, 35). The results in the present study indicate that these activities are not only similar in effect, but also in the biochemistry of the reactions they catalyze. The replacements mediated by the two activities are directed by the sequence of the 3′-half of the acceptor stem, both activities incorporate nucleotides in a 3′-to-5′ direction, and both require a 5′-triphosphate to begin nucleotide incorporation (or a 5′-monophosphate plus ATP).

It will be of considerable interest to determine which enzymes are responsible for this form of 5′-tRNA editing, particularly as editing appears to involve a novel 3′-to-5′ nucleotidyltransferase. The sequences of these enzymes may reveal how the activity recognizes its substrates and carries out catalysis, by comparison with other enzymatic activities that carry out similar reactions. For example, histidine tRNA guanylyltransferase (HTGT) is the enzyme responsible for the non-templated, post-transcriptional addition of a G residue at the −1 position of tRNAHis in all eukaryotes (42–44). HTGT is the only activity other than the mitochondrial 5′-tRNA editing activities known to extend a polynucleotide chain in a 3′-to-5′ direction via formation of a normal phosphodiester bond. To accomplish this extension, HTGT first activates the 5′-end of 5′-monophosphorylated tRNAHis by adding an adenylate (AMP) moiety to the tRNA, forming a 5′-5′ phosphoanhydride bond. The covalently bound AMP residue is subsequently displaced by the attack of the 3′-hydroxyl of the GTP that provides the incorporated GMP moiety. Activation of monophosphorylated 5′-ends by adenylate is also employed by DNA and RNA ligases (45, 46). Because the S. punctatus and A. castellanii mitochondrial activities catalyze a reaction similar to the one mediated by HTGT, both activities requiring ATP for nucleotide addition to monophosphorylated tRNA 5′-ends (likely via an adenylated tRNA intermediate), it is possible that HTGT and the 5′-tRNA nucleotide incorporation activities have common evolutionary origins as well as biochemical mechanisms.

Absence of 5′-tRNA Processing Prior to Nucleotide Incorporation—Mitochondrial genomes are generally transcribed to produce several multigene transcripts from which tRNAs are processed. In all systems examined to date, the 5′-ends of mature tRNAs are generated by RNase P, an essential endonuclease that cleaves the phosphodiester bond between the nucleotides at positions 1 and −1 (in most cases), thereby removing 5′ extensions and producing monophosphorylated 5′-ends. Evidence for S. punctatus mitochondrial tRNAs being processed from large RNA transcripts has been presented (34). However, as all mtDNA-encoded tRNAs in S. punctatus undergo 5′ editing, processing by RNase P between positions 1 and −1 would have to be followed by the further removal of nucleotides at the first three 5′-positions before the nucleotide incorporation activity described in the present study could restore proper acceptor stem base pairing. Alternatively, S. punctatus mitochondrial RNase P may have an altered cleavage site (i.e., between positions 4 and 3) thus removing the requirement for additional 5′ nuclease events prior to nucleotide incorporation. In support of this idea, RNase P has been shown to cleave sites other than between positions 1 and −1 in some systems. For example, RNase P cleaves 5′ to position −1 in certain prokaryotes to generate the mature tRNAHis (47).

In a recent analysis of cDNA sequences of acceptor stems produced by RT-PCR from circularized tRNAs in three chytrid fungi (Monoblepharella15, Harpochytrium94 and Harpochytrium105), partially and completely unedited tRNAs were found that support the existence of a 5′-to-3′ exonuclease acting to remove 5′-nucleotides at tRNA positions 1–3, prior to 5′-nucleotide incorporation (35). In contrast, analysis of cDNAs of tRNA acceptor stems from S. punctatus mitochondria did not reveal any partially or completely unedited tRNAs (35). Similarly, no partially or completely unedited tRNAs were reported from cDNA studies of A. castellanii mitochondrial tRNAs (32). The possibility of an altered RNase P cleavage site in the latter two systems therefore remains an intriguing possibility.

Somewhat surprisingly, the in vitro system studied here yielded no strong evidence for the removal of 5′-nucleotides from tRNA substrates prior to nucleotide incorporation. For example, construct 2 (containing a 13-nt 5′ extension; Fig. 4B, lane b), construct 5 (containing a fully base-paired acceptor stem; Fig. 4B, lane c) and construct 8 (containing a mismatch at base pair 2:71; Fig. 4B, lanes j, k, r, and s) were not labeled to a significant degree after incubation with [α-32P]GTP and the S. punctatus mitochondrial S100 extract. Further, radioactivity in 5′-end-labeled construct 1 was retained after incubation with the extract (Fig. 8). Suggestive evidence of a 5′ nuclease activity was only obtained when yeast tRNAs were used as substrate: 32P-labeled G residues were then found to be incorporated at tRNA positions 1 and 2 (Fig. 2, B and C). However, the incorporated nucleotides at these positions may constitute addition to degraded tRNA 5′-termini, and therefore this is not definitive evidence of
nuclease activity. Studies of *A. castellanii* mitochondrial tRNA editing *in vitro* have similarly yielded suggestive but not definitive evidence of 5′ nuclelease activities (33). These results may simply indicate that nuclease activities are much less efficient in comparison to the nucleotide incorporation activity under the conditions of the *in vitro* assays. Additional studies will be required to identify and characterize nuclelease activities that process tRNA 5′-ends in *S. punctatus*, as well as in *A. castellanii*, mitochondria.

5′-Nucleotide Incorporation Is Independent of 3′-tRNA Processing—Two G residues were incorporated at the 5′-end of construct 1: (i) in the presence and absence of C residues added to the 3′-end (Fig. 7A), (ii) in the presence of a 15-nt 3′ extension (Fig. 6B), and (iii) when the 3′-terminal residue (the discriminator nucleotide, position 73) had been chemically altered by periodate oxidation (Fig. 9B). The 5′-nucleotide incorporation activity therefore does not appear to be affected by the nature of the tRNA 3′-end, and 5′ editing is likely independent of 3′ processing. Interestingly, the results of Laforest et al. (35) also suggest that 5′-tRNA editing and the addition of the CCA sequence to tRNA 3′-ends are independent processes in the mitochondria of other chytridiomycetes. Further studies will be required to determine how substrates are recognized by the activity, and how the number of nucleotides to be incorporated is determined.

Evolution of 5′-tRNA Editing—The pronounced similarity of the *S. punctatus* and *A. castellanii* 5′-tRNA editing activities, in terms of both tRNA sequence changes observed *in vivo* and characteristics of activity seen *in vitro*, is surprising in light of the distant evolutionary relationship of these two organisms, as shown by phylogenies based on molecular data (e.g. Ref. 48). It is possible that this activity was present in the common ancestor of *S. punctatus* and *A. castellanii* but lost in the vast majority of descendant lineages. It is also possible that its components have been horizontally transferred between distantly related organisms. Additionally, it is possible that the activity has emerged independently in several lineages including Chytridiomycota, Amoebozoa (which includes *A. castellanii* and *Dictyostelium discoideum*, a cellular slime mold that likely has mitochondrial tRNA editing, based on an abundance of acceptor stem mismatches; Ref. 49, see also Ref. 35) and Heterolobosea (extensive mismatches have been identified in the mitochondrial tRNAs of the heterolobosean *Naegleria gruberi*; see Ref. 35).2

An intriguing possibility is that this activity is derived from phylogenetically widespread but poorly characterized enzymes that are responsible for the maintenance of tRNA 5′-ends, such as the ATP[CTP]tRNA nucleotidytransferase adds and maintains the 3′-CCA<sub>OH</sub> tail across the three domains of life. An activity of this sort would remain largely cryptic in genomic and *in vitro* studies, as it would act only on 5′-degraded tRNAs to regenerate proper acceptor stem base pairing. Such an activity would, however, assume a much more prominent role if the sequence of the first three 5′-tRNA nucleotides diverged and Watson-Crick base pairing potential with the 3′-half of the stem was lost (as is seen in *S. punctatus* mRNA-encoded tRNAs). An acceptor stem repair function would thus be rendered absolutely essential for the synthesis of functional RNAs. Identification of components of 5′-tRNA editing activities from various organisms will help to address this possibility, while at the same time clarifying the biochemistry and evolution of 5′-tRNA editing and its relationship to tRNA processing.

2 M. W. Gray, unpublished observations.
In Vitro Characterization of a tRNA Editing Activity in the Mitochondria of Spizellomyces punctatus, a Chytridiomycete Fungus
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