# tRNA Editing and Characterization of Novel Small RNAs in Protist Mitochondria

by

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## ABSTRACT

In the chytridiomycete fungus *Spizellomyces punctatus*, all eight of the tRNAs encoded in the mitochondrial genome 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, I 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 substrate tRNAs, uses the 3′ sequence as a template for incorporation, and adds nucleotides in a 3′-to-5′ direction. This activity can add nucleotides to 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 here and 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.

In the course of this work I discovered two small RNAs, one in mitochondrial RNA preparations from *A. castellanii* and the other in mitochondrial RNA preparations from the related amoeba *Hartmannella vermiformis*. Each of these RNAs has properties expected of a mitochondrial 5S rRNA, and each is mitochondrially encoded. These findings were unexpected, as analysis of the complete mitochondrial genome sequences from these two organisms had previously failed to reveal a 5S rRNA gene. These results emphasize the limitations of gene prediction based solely on DNA sequence, and at the same time suggest that the requirement for a 5S rRNA component in mitochondrial ribosomes is more stringent than previously believed.

## LIST OF ABBREVIATIONS AND SYMBOLS USED

A, adenosine

ADAR, adenosine deaminase acting on RNA

ADAT, adenosine deaminase acting on tRNA

AID, activation-induced cytidine deaminase

APOBEC, apoB mRNA editing catalytic subunit

ATCC, American Type Culture Collection

bp, base pair

BSA, bovine serum albumin

C, cytidine

$dH_2O$, distilled water

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DNase, deoxyribonuclease

DTT, dithiothreitol

EDTA, ethylenediaminetetraacetate

g, gram

*g*, unit of acceleration equal to that caused by gravity at the earth's surface

G, guanosine

h, hour

HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]

HTGT, histidine tRNA guanylyltransferase

I, inosine

L, litre

LB, Luria-Bertani culture medium

M, molar

mM, millimolar

min, minute

ml, millilitre

mm, millimetre

mRNA, messenger RNA

mtDNA, mitochondrial DNA

NDP, nucleoside diphosphate

ng, nanogram

Np, nucleoside 3′-monophosphate

nt, nucleotide

NTP, nucleoside triphosphate

OAc, acetate

O.D., optical density

PEI, polyethyleneimine

PMSF, phenylmethylsulfonyl fluoride

P100, pellet of a 100,000 *g* centrifugation

pN, nucleoside 5′-monophosphate

psi, pounds per square inch

R, purine nucleoside

RNA, ribonucleic acid

RNase, ribonuclease

rpm, revolutions per minute

rRNA, ribosomal RNA

RT-PCR, reverse transcription-polymerase chain reaction

s, second

S, Svedberg unit

SDS, sodium dodecyl sulfate

S100, supernatant of a 100,000 *g* centrifugation

T, thymidine

TLC, thin-layer chromatography

Tris, tris(hydroxymethyl)aminomethane

tRNA, transfer RNA

U, uridine

μCi, microcurie

μl, microlitre

μm, micrometre

UV, ultraviolet

WC, Watson-Crick

Y, pyrimidine nucleoside

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# I. INTRODUCTION

## A. RNA Editing

During and following the synthesis of RNA, precursor transcripts are processed and modified to generate mature molecules able to perform their various functions in the cell. For example, messenger RNAs (mRNAs) produced in the nucleus undergo intron splicing, the addition of a 5′ $m^7G$ cap and 3′ polyadenylation prior to their export into the cytoplasm. These steps affect mRNA quality control, stability in the cytoplasm and recognition by ribosomes. Similarly, ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) in the nucleus are processed by ribonucleases to generate mature 5′ and 3′ ends, and undergo (sometimes extensive) nucleotide modification prior to their export into the cytoplasm. These and many other types of RNA processing have long been recognized to be essential functions in all cells.

RNA editing encompasses particular forms of RNA processing, additional to more traditional forms such as those described above, that change the primary sequence of an RNA molecule from that predicted by the gene sequence. Editing is accomplished by the insertion, deletion or modification of nucleotides, and is an essential process in many diverse (mostly mitochondrial) systems (Gray, 2003). Editing affects mRNAs as well as non-coding RNAs such as rRNAs (Schuster *et al.*, 1991; Mahendran *et al.*, 1994; Barth *et al.*, 1999) and tRNAs (Price and Gray, 1998). Mechanistically distinct types of editing are listed in **Table 1**. Although RNA editing describes a variety of biochemically and evolutionarily unrelated processes, it remains a useful concept linking phenomena with important structural and functional consequences. All forms of RNA editing complicate the prediction of mature RNA sequences from genomic sequences and deepen our appreciation of the complexity of RNA expression.

This thesis focuses on the mechanism and evolution of a particular RNA editing process that post-transcriptionally modifies mitochondrial tRNAs in the distantly related rhizopod amoebae and chytridiomycete fungi. Editing in these systems substitutes mismatched nucleotides in regions expected to exhibit base pairing potential, thereby producing canonical tRNAs from non-canonical tRNA precursors. In this opening section, I briefly outline the principal RNA processing events generally included under the heading of RNA editing.

**Table 1:** Mechanistically distinct types of RNA editing (from Gray, 2001, with modifications)

| Editing type | Edited RNAs | Where found | Subcellular compartment[a] | References |
|---|---|---|---|---|
| U insertion/deletion | various mRNAs | kinetoplastid protozoa | M | Simpson *et al.*, 2003; Stuart et *al.*, 2001 |
| | *cob* mRNA | *T. lirellus* (nematode worm) | M | Vanfleteren and Vierstraete, 1999 |
| N insertion | mRNAs, tRNAs, SSU rRNA | *P. polycephalum* and other slime molds[b] | M | Gott, 2001; Horton and Landweber, 2002 |
| | lactonohydrolase mRNA | *Fusarium oxysporum* AKU 3702[c] | N(?) | Kobayashi *et al.*, 1998 |
| | phosphoprotein mRNA | paramyxoviruses | Cy | Hausmann *et al.*, 2001 |
| N replacement | tRNAs (5'-terminal 3 nts) | *Acanthamoeba* spp. | M | Price and Gray, 1998 |
| | | chytridiomycete fungi (e.g., *Spizellomyces*) | M | Price and Gray, 1998; Laforest *et al.*, 2004; Bullerwell and Gray, 2005 |
| | tRNAs (3'-terminal nts) | pulmonate gastropods, squid, platypus, chicken, centipede, *S. ecuadoriensis* (jakobid flgallate) | M | Price and Gray, 1998; Lavrov *et al.*, 2000; Leigh and Lang, 2004 |
| | *cox1* and *cob* mRNAs | dinoflagellates | M | Lin *et al*, 2002 |
| C-to-U conversion | apolipoprotein B mRNA | various mammals | N | Driscoll and Innerarity, 2001 |
| | neurofibromatosis mRNA[d] | humans (neural tumor, hepatoma cells) | N(?) | Skuse *et al.*, 1996 |
| | various mRNAs, tRNAs, intronic sequences | land plants | M, C | Bock, 2001 |
| | $tRNA^{Asp}$ | marsupials | M | Price and Gray, 1998 |
| | *cox1* mRNA | *Physarum polycephalum* | M | Gott *et al.*, 1993 |
| | 7SL RNA | *L. collosomo* (trypanosomatid) | N | Ben-Shlomo *et al.*, 1999 |
| | $tRNA^{Trp}$ | *L. tarentolae* (trypanosomatid) | M | Alfonzo *et al.*, 1999 |
| U-to-C conversion | various mRNAs | land plants | M, C | Bock, 2001 |
| | WT1 mRNA[e] | rat, human | N(?) | Sharma *et al.*, 1994 |
| | α-1,2-mannosidase mRNA[f] | mouse | N(?) | Schneikert and Herscovics, 1995 |
| U-to-A conversion | α-galactosidase mRNA | human | N(?) | Novo *et al.*, 1995 |
| A-to-I conversion | glutamate receptor mRNA[g] | mammalian brain | N | Emeson and Singh, 2001; Sommer *et al.*, 1991 |
| | serotonin 2C receptor mRNA | mammalian brain | N | Emeson and Singh, 2001; Burns *et al.*, 1997 |
| | α2,6-sialyltransferase mRNA[f] | rat | N(?) | Emeson and Singh, 2001; Ma *et al.*, 1997 |
| | $K^+$ channel mRNA[h] | optic lobe of squid, *Loligo peali* | N(?) | Patton *et al.*, 1997 |
| | *4f-rnp* mRNA[i] | *Drosophila* | N(?) | Petschek *et al.*, 1996 |
| | *adar2* mRNA | rat brain | N(?) | Rueter *et al.*, 1999 |
| | antigenomic RNA[j] | hepatitis delta virus (HDV) | N(?)[k] | Luo *et al.*, 1990; Casey *et al.*,1992; Polson *et al.*, 1996 |
| | transactivation response (TAR) mRNA | human immunodeficiency virus (HIV) | N(?)[l] | Sharmeen *et al.*, 1991 |

[a]M, mitochondrion; C, chloroplast; N, nucleus; Cy, cytoplasm; (? denotes probable location)
[b]Mostly single C insertions, but also U, AA, AU, CU and GU
[c]Insertion of C at one position, G at another
[d]Encodes the neurofibromatosis type I (NFI) tumor suppressor
[e]Encodes Wilms' tumor susceptibility factor
[f]Data suggestive but not definitive
[g]Encodes non-NMDA glutamate receptor subunit
[h]Encodes voltage-dependent potassium channel (sqKv2) protein
[i]Encodes putative RNA-binding protein; multiple editing (hypermutation) sites
[j]Encodes delta antigen (HDAg)
[k]Of HDV-infected cells
[l]Of HIV-infected cells

## 1. RNA Editing in trypanosomes

The term "RNA editing" was coined to describe post-transcriptional U insertions within the mitochondrial *cox2* (cytochrome oxidase subunit 2) transcript of two trypanosome species, *Trypanosoma brucei* and *Crithidia fasciculata.* These insertions correct a gene-predicted frame-shift, thereby generating a functional mRNA (Benne *et al.*, 1986). Editing in trypansome mitochondria is now among the best understood cases of RNA editing (see Simpson *et al.*, 2003 and Stuart *et al.*, 2001 for reviews). In these organisms, mRNAs undergo the deletion as well as insertion of U residues. The extent of editing varies considerably when comparing transcripts either within one species or between species. Non-coding RNAs have not been found to be edited by this mechanism.

Editing involves a macromolecular complex (the "editosome") that contains endo- and exoribonuclease, 3′-terminal uridylyl transferase and RNA ligase enzymatic activities. The complicated editing pathway is templated by small (~60 nt) guide RNAs that are encoded predominantly by minicircle DNAs. Guide RNAs consist of three portions: an anchor region that interacts with the sequence 3′ to the sequence to be edited, an informational region that guides nucleotide insertion/deletion, and a U-rich tail that can interact with purine-rich sequences upstream of the editing site. Because the anchor sequence only interacts with the mature sequence of the mRNA, editing proceeds in a 3′-to-5′ direction along the transcript. Much current research is focussed on the purification of the editosome and the identification of component proteins.

In addition to U insertion/deletion, deamination of cytidine in a tRNA (described in the section on tRNA Editing) and in the 7SL RNA (the RNA component of the signal recognition particle) of certain trypanosomes has been documented. In the latter case, two versions of the 7SL RNA were found in *Leptomonas collosoma*, despite the absence of a second gene copy in the nuclear genome (Ben-Shlomo *et al.*, 1997). These versions differed by a single base (either a C, as in the genomic version, or a U) at position 133, and it was thus presumed that editing *via* base deamination of cytidine to uridine was occurring *in vivo* (Ben-Shlomo *et al.*, 1999).

**2. In myxomycetes**

In the mitochondria of the acellular slime mold *Physarum polycephalum* (and related myxomycetes, such as *Didymium*), differences between the mitochondrial DNA and its mRNA, rRNA and tRNA products are due to hundreds of editing events, the first of which were described in 1991 by Mahendran *et al.* The vast majority of these edits are single C insertions (see Gott, 2001 and Horton and Landweber, 2002 for reviews), although insertion of U, A and G has also been observed, either in the form of single nucleotides or dinucleotides. Editing of mRNAs in this system is critical for the cell, as it creates open reading frames from genes that would otherwise be non-functional due to multiple predicted frame shifts.

Experiments using isolated mitochondria have supported the view that nucleotide insertion editing is co-transcriptional, with information directing nucleotide incorporation located in close proximity to the editing site. However, unlike the U insertion/deletion editing seen in trypanosomes, guide RNAs are not likely used, as indicated, for example, by the fact that column-purified mitochondrial transcription complexes are competent for both RNA synthesis and insertion editing (Cheng and Gott, 2000). Recent evidence (Byrne and Gott, 2002) suggests that factors (possibly proteins) associated with the DNA template are involved in directing this form of editing to the proper nucleotide insertion site.

In addition to nucleotide insertion, C-to-U editing has also been observed in *P. polycephalum*, at four sites within the *cox1* transcript (Gott *et al.*, 1993). This form of editing is predicted to occur *via* base deamination, although this remains to be confirmed experimentally. Finally, non-encoded U tails have been observed in mitochondrial transcripts of the myxomycetes *P. polycephalum* and *Stemonitis flavogenita* (Horton and Landweber, 2000). It is not known how or whether these tails are related to other editing processes. Interestingly, non-gene-encoded U tails are also found at the 3′ ends of guide RNAs as well as in the 9S and 12S rRNAs in trypanosome mitochondria (Blum and Simpson, 1990; Adler *et al.*, 1991).

**3. In plants**

RNA editing in plants was first discovered in mitochondria (Gualberto *et al.*,1989; Covello and Gray, 1989; Heisel *et al.*, 1989), and described two years later in

chloroplasts (Hoch *et al.*, 1991). Many transcripts in plant mitochondria, and to a lesser extent in plant chloroplasts, are altered by C-to-U and U-to-C editing (see Bock, 2001 for a review). The vast majority of these editing events are C-to-U transitions that likely proceed by base deamination (Rajasekhar and Mulligan, 1993; Yu and Schuster, 1995; Blanc *et al.*, 1995). Editing occurs almost exclusively in mRNAs, but also affects tRNAs (see the section on tRNA Editing) and, potentially, rRNAs (Schuster *et al.*, 1991). Editing is essential for gene expression, acting predominantly at the first and second codon positions, thereby changing amino acid identity and resulting in the interspecific conservation of organelle polypeptide sequences. As editing of this type does not occur in all bryophytes or in any members of the algae, it appears that editing emerged early in land plant evolution.

Since the discovery of RNA editing in plant organelles, research has focused on characterizing the extent of editing in various systems, identifying sequence elements that direct the editing activity to specific residues, and identifying components of the editing activity. *In vivo* studies in transgenic chloroplasts have demonstrated that mRNA sequences flanking the editing site are directly involved in editing site selection in plastids, with such sequences residing predominantly in the region upstream of the editing site (Chaudhuri *et al.*, 1995; Bock *et al.*, 1996; Reed *et al.*, 2001). These studies have also provided evidence for the participation of *trans*-acting factors in editing site selection, some of which appear to be involved in the editing of several sites having similar upstream sequence motifs (Chateigner-Boutin and Hanson, 2002). An *in vitro* system of chloroplast RNA editing has recently been developed (Hirose and Sugiura, 2001), and several potential *trans*-acting factors have been identified (Miyamoto *et al.*, 2002 and 2004). Less progress has been made in the identification of *cis*- and *trans*-acting factors in plant mitochondria, due to low activity in *in vitro* editing systems (Takenaka and Brennicke, 2003; Takenaka *et al.*, 2004) and the absence of a reliable mitochondrial transformation system. Nevertheless, some progress in the identification of *cis*-factors has recently been made using mitochondrial electroporation (Farré *et al.*, 2001; Choury *et al.*, 2004). Identification of the enzyme(s) involved in either mitochondrial or chloroplast RNA editing is a major goal of current research.

**4. In animals**

Several types of editing have been documented in animals, both within and outside of mitochondria. Transfer RNAs in the mitochondria of various animals undergo both modification and insertion/deletion types of RNA editing (described in the section on tRNA Editing). The best-characterized of editing events in animals, however, are C-to-U (see Driscoll and Innerarity, 2001 for a review) and A-to-I (see Hough and Bass, 2001 and Emeson and Singh, 2001 for reviews) editing in the nucleus. Several other types of RNA editing have been proposed to occur in animals (see **Table 1**), but they remain unconfirmed.

The editing of the apolipoprotein B (apoB) mRNA in humans was the first example of RNA editing in mammals (Powell *et al.*, 1987; Chen *et al.*, 1987). Whereas the full-length version of the apoB protein appears in the liver, a C-to-U conversion within transcripts in the intestine changes a CAA (glutamine) codon to a UAA (stop) codon, leading to a truncated form of apoB in intestinal tissues. This conversion is catalyzed by a site-specific deaminase, the apoB mRNA editing enzyme catalytic polypeptide 1 (APOBEC-1; Teng *et al.*, 1993). The C-to-U event in apoB mRNA is directed by primary and secondary structure elements in the sequences surrounding the editing site. No other substrates for APOBEC-1 have as yet been identified. Intriguingly, relatives of APOBEC-1 have been found to play roles both in antibody diversity (the activation-induced cytidine deaminase, AID; Peterson-Mahrt *et al.*, 2002) and reducing viral infectivity (APOBEC-3G in humans; Bishop *et al.*, 2004).

The first example of adenosine conversion to inosine (accomplished by the hydrolytic deamination at the C6 position of the purine ring) to be observed in mammalian mRNAs was in transcripts of glutamate-gated ion channels in the central nervous system (Sommer *et al.*, 1991), and has since been identified in other mRNAs in several mammals (see **Table 1**). This type of editing is mediated by ADARs (adenosine deaminases acting on RNA). This family of enzymes includes the ADATs (adenosine deaminases acting on tRNAs), which are responsible for the creation of inosine in tRNA (Grosjean *et al.*, 1996). The adenosine deaminase family acts on partially or fully double-stranded regions in RNAs. Interestingly, A-to-I editing serves not only to direct amino acid changes in coding regions, but also plays roles in non-coding regions. One example of this phenomenon is the partial disruption of certain long double-stranded

regions in *Caenorhabditis elegans* RNAs: in this case, the introduction of inosine residues creates UxI mismatches, thereby aborting the destruction of these RNAs by the RNA interference pathway (Tonkin and Bass, 2003). Finally, recent evidence has revealed A-to-I editing in the human genome to be extensive, with many editing sites localized within repeat regions (*e.g.*, Levanon *et al.*, 2004).

**5. In paramyxoviruses**

In the P (phosphoprotein) mRNA of paramyxoviruses, co-transcriptional insertion of a variable number of G residues leads to the creation of alternative open reading frames (see Hausmann *et al.*, 2001 for a review). In these viruses, a polymerase "stuttering" mechanism has been proposed to explain the insertion of Gs in response to a polyuridine tract followed by a C in the template sequence. Sequences upstream of this tract have an effect on the number of untemplated Gs that are inserted. This is one of only two known cases (the other being insertion editing in myxomycetes) of co-transcriptional editing: that is, cases of editing in which it is not possible to separate the editing of an RNA from its synthesis. In addition to this G insertion by stuttering, viral sequences can be modified by editing enzymes within their host cells. Both A-to-I (Bass, 2002) and C-to-U editing (Yu *et al.*, 2003; Bishop *et al.*, 2004) occur, and these changes (in some cases hypermutations) likely contribute to the diversification of viral genome sequences.

**6. In dinoflagellates**

A novel type of editing has recently been described in the mitochondria of three dinoflagellates, *Crypthecodinium cohnii*, *Pfiesteria piscicida* and *Prorocentrum minimum* (Lin *et al.*, 2002). In these organisms, cDNA sequences for *cox1* (cytochrome oxidase subunit 1) and *cob* (apocytochrome *b*) transcripts differ from the gene sequence at multiple positions, resulting in changes at approximately 2% of the total nucleotides. Most of these changes occur at the first and second codon position and alter the specified amino acid. Most changes are transitions from A-to-G (47%), U-to-C (23%) or C-to-U (17%). Although A-to-G and C-to-U changes could be the result of enzyme activities (adenosine and cytidine deaminases) known to carry out editing in other organisms, the occurrence of other changes (such as transversions) cannot. Based on this observation as well as appreciable clustering of editing sites, the authors suggest the possibility that

editing involves guide RNAs, similar to the situation in trypanosome mitochondrial editing. However, multiple mechanims may function to edit these mitochondrial mRNAs. Progress is likely to be slow in elucidating the biochemical mechanism of editing in this system, due to technical difficulties involved in working with dinoflagellate mitochondria.

**7. In mitochondria**

As evidenced by the foregoing sections, known RNA editing mechanisms are found predominantly in mitochondrial systems. Mitochondria are double membrane-bounded, ATP-producing organelles found in most eukaryotes, and are one of the few organelles in eukaryotic cells that contain DNA. Sequences of mitochondrial DNAs (mtDNAs) from diverse eukaryotes have recently become available (see Lang *et al.*, 1999 and Gray *et al.*, 1999 for reviews) and despite enormous variations in genome size ranging from the tiny apicomplexan mtDNAs (~6 kbp) to the expansive plant mtDNAs (>150 kbp), the coding function of the mitochondrial genome is relatively uniform. In general, mtDNAs code only for genes involved in the mitochondrial translation apparatus, electron transport and oxidative phosphorylation. Mitochondrial genomes often encode a number of tRNAs. The structure, function, expression and processing of these small RNAs in mitochondrial and other systems is introduced in the following sections, with an emphasis on examples of tRNA editing.

**B Transfer RNA**

**1. Structure and function**

Transfer RNAs are small RNAs that have an essential function in translation. The ribosome uses the information in the anticodons of tRNAs to decode the information found in the base triplets of mRNAs, linking each codon to a specific amino acid. By this process, repeated many times in succession, a polypeptide is created with the sequence specified by the sequence of a mRNA (note, however, that RNA editing can complicate this standard paradigm, such as in *Physarum* and trypanosome mitochondria). In addition to their role in translation, some tRNAs may act as cofactors in enzymatic reactions. To cite a few examples, the first precursor to porphyrins in many bacteria and organelles is glutamate-1-semialdehyde, which is formed after the ligation of glutamic

acid to $tRNA^{Glu}$ (Verkamp *et al.*, 1995). A $tRNA^{Gly}$ species that does not participate in protein synthesis is involved in the synthesis of peptidoglycan (Stewart *et al.*, 1971). Various retroviruses use tRNAs as primers for reverse transcriptase (Isel *et al.*, 1993). Finally, the synthesis of glutamine, asparagine, selenocysteine and N-formylmethionine are all the result of alterations of an amino acid covalently bound to a tRNA ($tRNA^{Glu}$, $tRNA^{Asp}$, $tRNA^{Ser}$ and $tRNA^{Met}$, respectively) (see Björk and Rasmuson, 1998 for an overview of the various roles of tRNAs in the cell). The role of tRNAs in translation will be highlighted in this section.

The primary, secondary and tertiary structure of tRNAs is essential for proper tRNA function. The nucleotides in every tRNA are numbered in a standard system that begins (in most cases) with the most 5′ nucleotide in a mature molecule (position 1) and ends with the discriminator nucleotide (position 73) (**Fig. 1**). The anticodon nucleotides occupy positions 34-36. Although not all tRNAs are 73 nucleotides in length (some of these 73 nucleotide positions may be entirely absent from a given tRNA), this standard numbering allows among-tRNA comparison of highly conserved structures and features. Nucleotides whose presence is more variable when comparing tRNAs in general (mostly located in the variable loop) are indicated with a letter following a number. Although tRNAs are quite variable at the primary sequence level, they retain several highly conserved sequence features, such as the GUUC sequence in the T stem-loop (positions 53-56), and a 3′ terminal 5′-$CCA_{OH}$-3′ sequence. The CCA sequence is added (in most cases) post-transcriptionally by ATP(CTP):tRNA nucleotidyltransferase (also known as the CCA-adding enzyme), but is encoded at the DNA level in some eubacteria. A CCA-adding enzyme is nevertheless present in all nuclear, organellar and bacterial systems examined to date. This enzyme participates not only in the synthesis of the CCA tail, but also in the resynthesis of degraded CCA ends (Melton *et al.*, 1980; Wolfe *et al.*, 1996).

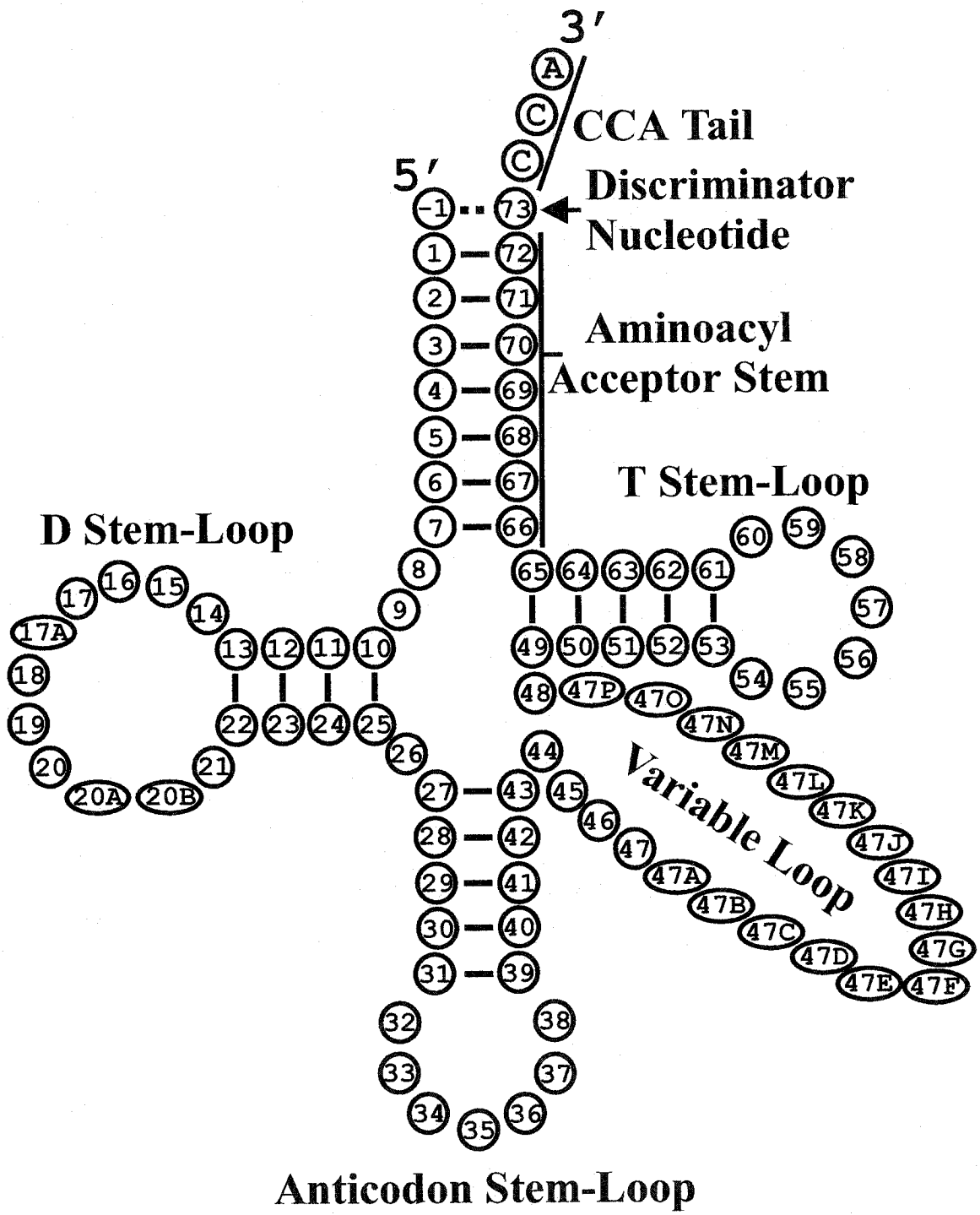
Transfer RNAs are the most heavily modified of known RNAs, and contain more than 80 different modifications either to the base or sugar moiety (see Björk, 1995 for a review). For example, in the highly conserved GUUC sequence mentioned above, U54 and U55 are modified to ribothymidine (5-methyluridine) and pseudouridine (5-ribosyluracil), respectively, in the majority of examined tRNAs. Modifications such as

these have important structural and functional consequences for transfer and other RNAs, but these will not be discussed here.

The bulk of tRNAs conform to a standard "cloverleaf" secondary structure consisting of three short stem-loops (termed the D, anticodon and T stem-loops) and a terminal aminoacyl acceptor stem (Holley *et al.*, 1965) (**Fig. 1**). The universally conserved tertiary structure of tRNAs is generally described as "L-shaped", with the anticodon at one end of the L, and the acceptor stem at the other end. The anticodon stem and the D stem interact by coaxial stacking, as do the T stem and the acceptor stem. The variable stem is located at the intersection of these two stacked helices, at the bend in the L-shaped tertiary structure. Stacking of RNA stems plays a major role in helix stabilization, as do tertiary interactions, such as those between the purines at positions 18 and 19 (in the D loop) and the pyrimidines at positions 55 and 56 (in the T loop) (see Dirheimer *et al.*, 1995 and Marck and Grosjean, 2002 for an overview of tRNA structure).

Amino acids are attached covalently to the hydroxyl group at either the 2′ or 3′ end of tRNAs by enzymes called aminoacyl-tRNA synthetases. These enzymes recognize specific features within tRNA molecules (primarily, but not restricted to, the discriminator base at position 73 and the anticodon nucleotides). These interactions aid in the selection of the correct tRNA substrate and its differentiation from other tRNAs (see Giegé *et al.*, 1998 for a review of tRNA identity). This addition reaction is highly specific for each tRNA. Indeed, this is one of the two steps of protein synthesis where the fidelity of the genetic code is maintained, the other being anticodon-codon interactions within the ribosome during translation. Once a tRNA has been aminoacylated, it is then available to fulfill its role in protein synthesis. During elongation of the polypeptide chain, each aminoacyl-tRNA is bound by an elongation factor, and the complex of these molecules enters the aminoacyl site of the ribosome where the anticodon nucleotides of the tRNA base pair with the nucleotides in their cognate codon. The polypeptide chain is transferred onto the attached amino acid (thus growing by one amino acid) as the tRNA bound to the protein chain is transferred to the peptidyl site to allow entry of the next tRNA into the aminoacyl site. This process continues until a complete protein is produced.

**Figure 1:** Standard representation of tRNA secondary structure and numbering of nucleotides. Primary sequence and secondary structure features of tRNA discussed in the text are indicated. (adapted from Dirheimer *et al.*, 1995)


3′
A
C
C
CCA Tail
5′
-1
73
Discriminator
Nucleotide
1
72
2
71
3
70
Aminoacyl
Acceptor Stem
4
69
5
68
6
67
T Stem-Loop
7
66
D Stem-Loop
60
59
58
8
65
64
63
62
61
57
16
15
17
9
17A
14
49
50
51
52
53
56
13
12
11
10
18
54
55
48
47P
47O
47N
22
23
24
25
19
47M
47L
26
44
47K
20
21
47J
Variable Loop
27
43
45
47I
20A
20B
46
28
42
47
47H
47A
29
41
47B
47G
47C
30
40
47D
47E
47F
31
39
32
38
33
37
34
36
35
Anticodon Stem-Loop

**(Figure 1)**

## 2. Expression and processing

In general, tRNAs in eukaryotes are synthesized individually by RNA polymerase III using promoter sequences located both in close proximity to and within the gene sequence (see Sprague, 1995 for a review). These monomeric transcripts are subsequently processed by nucleases at the 5′ end (by RNase P; see Altman *et al.*, 1995 and Shön, 1999 for reviews) and 3′ end (by various endo- and exonucleases), and the 3′-terminal CCA sequence is added by ATP(CTP):tRNA nucleotidyltransferase (see Wolin and Matera, 1999, Schürer *et al.*, 2001 and Mörl and Marchfelder, 2001 for reviews of 3′ tRNA processing and CCA addition). CCA addition, various nucleoside modifications, intron splicing (some tRNAs contain non-spliceosomal introns; see Belfort and Weiner, 1997 for a review) and aminoacylation appear to be essential steps in tRNA maturation prior to tRNA export from the nucleus into the cytoplasm (*e.g.*, Lund and Dahlberg, 1998).

In contrast to the situation in the nucleus, tRNAs in prokaryotes and organelles are generally synthesized as part of multi-gene precursors, from which they are subsequently processed on the 5′ side by RNase P (as in the nucleus) and on the 3′ side by various other nucleases (see Deutscher, 1995 for an introduction to tRNA 3′ processing nucleases in the best understood system, *E. coli*; see Martin, 1995 and Mörl and Marchfelder, 2001 for reviews of the 3′ processing of organellar tRNAs; see Kunzmann *et al.*, 1998 and Schiffer *et al.*, 2002 for an introduction to RNase Z, an endoribonuclease that is involved in tRNA 3′ processing in nuclear, organellar and archaeal systems). The nuclease cleavages that excise tRNAs from precursor transcripts also contribute to the processing of other RNAs in the transcript in some mitochondrial systems (*e.g.*, Ojala *et al.*, 1980; Bibb *et al.*, 1981; Lang *et al.*, 1983; de Vries *et al.*, 1985; Burger *et al.*, 1985; Dyson *et al.*, 1989). As mentioned above, the universal CCA sequence is either encoded by the gene or added to tRNA 3′ ends following 3′-end processing in bacteria and organelles. Non-spliceosomal introns (which must be spliced out of the transcript) of the same type as those found in eukaryotes have been identified in Archaea (see Belfort and Weiner, 1997 for a review), and introns of either group I and group II have been found in bacterial, mtDNA-encoded, and chloroplast DNA-encoded

tRNAs (*e.g.*, Oda *et al.*, 1992; Lang *et al.*, 1997). Additional tRNA processing events will be presented in the section on tRNA Editing.

Although many organellar genomes encode a set of tRNAs apparently sufficient to decode all of the codons found in organellar mRNAs, many others encode an insufficient number of tRNAs for this task (Lang *et al.*, 1999). The tRNAs "missing" from organelles are, or have been inferred to be, imported from the cytoplasmic pool of tRNAs. For example, the mtDNA of the apicomplexan parasite *Plasmodium falciparum* encodes no tRNAs, and thus all tRNAs must be obtained from outside the mitochondrion. In contrast, the mtDNA of the jakobid flagellate *Reclinomonas americana* encodes an apparently complete set of tRNAs. Nevertheless, the presence of a complete tRNA set encoded in an organellar genome does not preclude tRNA import: *e.g.*, in the budding yeast *Saccharomyces cerevisiae*, $tRNA^{Lys}$(cuu) is imported into mitochondria from the cytoplasm (Martin *et al.*, 1979) although it is not predicted to be necessary for mitochondrial translation (see Hopper and Phizicky, 2003 for a review).

## C. tRNA Editing

As the preceding section summarizes, a number of processes are involved in the creation of mature tRNAs ready to carry out their role in translation. In the case of organelles, the system is even more complex as it often involves tRNAs encoded in the organelle genome as well as tRNAs imported from the cytoplasmic pool. In this section, I will introduce all the known types of editing that affect tRNAs (see Price and Gray, 1998 for a review). To date, all of these RNA editing events have been found in mitochondrial systems.

### 1. In plants

Three cases of tRNA editing have been identified in plant mitochondria (reviewed in Fey *et al.*, 2002). The first of these to be discovered was that in the $tRNA^{Phe}$(gaa) of two dicots: bean and potato (Marechal-Drouard *et al.*, 1993). In these cases, a C at position 4 is converted to a U, thereby "correcting" a C4-A69 mismatch (non-Watson-Crick pair) by replacement with a standard U4-A69 pair. The same editing event occurs in $tRNA^{Phe}$(gaa) of *Oenothera berteriana* (evening primrose, another dicot) (Binder *et al.*, 1994). This editing event has obvious structural implications (*i.e.*, it

corrects a mismatch), and was subsequently shown to be prerequisite for the 5′ and 3′ processing of these tRNAs (Marchfelder *et al.*, 1996; Marechal-Drouard *et al.*, 1996a; Kunzmann *et al.*, 1998).

A second case of editing was identified in larch $tRNA^{His}$(gug), where three mismatches (C6-A67, C12-A23 and A29-C41) are converted to their standard Watson-Crick U-A or A-U counterparts by C-to-U editing (Maréchal-Drouard *et al.*, 1996b). As in the case of $tRNA^{Phe}$, these editing events are required for proper 5′ and 3′ processing (Maréchal-Drouard *et al.*, 1996b).

A third case of plant mitochondrial tRNA editing is found in $tRNA^{Cys}$(gca) of *Oenothera berteriana* (Binder *et al.*, 1994). In this case, C at position 28 is altered to a U. In contrast to the situation in $tRNA^{Phe}$, this change does not result in a Watson-Crick base pair, but instead exchanges a C28-U42 mismatch for a U28-U42 mismatch. Since this editing event was not found to affect tRNA processing, CCA addition or aminoacylation, the function of this alteration was not immediately apparent (Fey *et al.*, 2000). It had previously been noted that U was often encoded by the $tRNA^{Cys}$ gene in many other plants, (*e.g.*, in the bryophyte *Marchantia polymorpha*), and mitochondrial gene-encoded Cs at this position in two cycads (prespermaphytes) were subsequently found to be edited to Us (Fey *et al.*, 2002). Thus, it became apparent that U28 might have functional significance. Fey *et al.* (2002) went on to demonstrate that U28 is, in fact, a modified U (pseudouridine) in potato mitochondria. This led to the speculation that the role of C-to-U editing in $tRNA^{Cys}$ is to generate the precursor for the subsequent isomerization of uridine to pseudouridine. However, the significance of this modified nucleotide for the structure of this tRNA is unknown.

**2. In myxomycetes**

Single C and U insertions have been observed within several mitochondrial tRNAs from the myxomycetes *Physarum polycephalum* ($tRNA^{Met1}$, $tRNA^{Lys}$, $tRNA^{Pro}$ and $tRNA^{Glu}$) and *Didymium nigripes* ($tRNA^{Met1}$, $tRNA^{Met2}$, $tRNA^{Lys}$ and $tRNA^{Pro}$) (Antes *et al.*, 1998). These editing events are believed to be mediated by the same editing mechanism that affects other RNA transcripts in myxomycete mitochondria (see section on RNA editing in myxomycetes). Editing in myxomycete mitochondrial tRNAs increases the similarity of the gene-encoded tRNAs to canonical tRNAs. Acceptor,

anticodon, T and D stems of standard length are formed by editing, a standard 7 nucleotide anticodon loop is generated in one case, and the standard GUUC sequence in the T stem is created in two other cases. Thus this type of editing appears to be essential for the synthesis of functional tRNAs in myxomycete mitochondria.

**3. In trypanosomes**

In the mitochondrial $tRNA^{Trp}$ of the trypanosome *Leishmania tarentolae*, a C-to-U editing event has been documented that changes the decoding properties of the tRNA (Alfonzo *et al.*, 1999). The codon UGA (which codes for "stop" in the standard genetic code) specifies tryptophan in the mitochondria of trypanosomes and many other mitochondrial and *Mycoplasma* systems. As a result, the mitochondrial $tRNA^{Trp}$(s) in these systems must be able to decode both standard UGG tryptophan codons as well as UGA codons. In most systems where UGA codes for tryptophan, this problem is addressed by the presence of a $tRNA^{Trp}$(uca) in place of the usual $tRNA^{Trp}$(cca), as a 5′-CCA-3′ anticodon can only base pair with UGG codons, whereas a 5′-UCA-3′ anticodon can base pair with both UGG and UGA codons. However, no tRNAs are encoded in the mitochondrial DNA of *L. tarentolae*, and thus all tRNAs must be imported from the cytoplasm. Because the cytoplasmic $tRNA^{Trp}$ has a standard 5′-CCA-3′ anticodon, read-through of UGA stop codons in mitochondrial mRNAs should not be possible. Alfonzo *et al.* (1999) discovered that this trypanosome has evolved an elegant solution to this problem: the C in the wobble position (position 34) of the antiocodon is modified to a U after import into mitochondria, allowing read-through of UGA codons in mitochondrial mRNAs. This solution also avoids the inappropriate read-through of UGA stop codons in cytoplasmic mRNAs by segregating the two anticodon variants of this $tRNA^{Trp}$.

**4. In animals**

Several types of tRNA editing occur in the mitochondria of animals. One of the first types of tRNA editing to be discovered was modification editing in the mitochondria of the marsupial *Didelphis virginiana*. In this organism, C-to-U editing at position 35 converts a portion of the mitochondrial $tRNA^{Gly}$(gcc) to $tRNA^{Asp}$(guc) (Janke and Pääbo, 1993; Mörl *et al.*, 1995; Börner *et al.*, 1996), altering a major identity element for glycyl- and aspartyl-tRNA synthetases. Indeed, when this tRNA has the anticodon sequence 5′-GCC-3′ it is specifically charged with glycine, whereas when this

tRNA has the anticodon sequence 5′-GUC-3′ it is specifically charged with aspartate (Börner and Pääbo, 1996). In addition to the role of this modification in altering tRNA identity, C35-to-U35 editing also appears to be a prerequisite for the conversion of G34 into queosine (a modified guanosine) (Mörl *et al.*, 1995).

Other types of editing have been shown to affect the 3′ half of tRNA acceptor stems, including the discriminator position, in the mitochondria of several animals. In the platypus *Ornithorhynchus anatinus*, the acceptor stem of $tRNA^{Ser}$(gcu) contains predicted mismatches based on the mitochondrial gene sequence that would result in a highly unstable acceptor stem. The acceptor stem of this tRNA is acted upon by an apparently untemplated (as it results in a single A2-C71 mismatch) editing process that adds three Cs ( at positions 70-72) to the recessed 3′-side of the tRNA acceptor stems, as well as an A at the discriminator nucleotide (position 73) (Yokobori and Pääbo, 1995a). Similar untemplated editing of the 3′ side of mitochondrial tRNAs has been observed in land snails, squid and chicken (Yokobori and Pääbo, 1995b and 1997; Tomita *et al.*, 1996). In contrast to the situation in platypus mitochondria, however, these editing processes appear to involve only the addition of As to truncated tRNA 3′ ends.

An intriguing activity occurs in the centipede *Lithobius forficatus* wherein nucleotides are added to the 3′ end of mitochondrial tRNAs in an apparently templated manner (as editing results exclusively in Watson-Crick base pairs) (Lavrov *et al.*, 2000). In this case, tRNAs (encoded by overlapping genes in the mtDNA in some cases) with predicted mismatches at the first 5 positions of the acceptor stem are processed to generate recessed 3′ ends. A nucleotide incorporation activity then adds nucleotides to these recessed ends, apparently using the 5′ half of the acceptor stem as a template. In the eight cases examined by Lavrov *et al.* (2000), an A was further added at the discriminator position, followed by the CCA sequence.

**5. In jakobids**

Editing similar to that found in *L. forficatus* mitochondria repairs several mismatches in mitochondrial tRNA acceptor stems in the protist *Seculamonas ecuadoriensis*, a jakobid flagellate (Leigh and Lang, 2004). In this protist, predicted mismatches in the last three positions of the acceptor stem of the mitochondrial $tRNA^{Ser}$(gga) and $tRNA^{Glu}$(uuc) are repaired by replacing the 3′ nucleotide in each

mismatch pair with a nucleotide capable of forming a Watson-Crick base pair. In addition, an A is added to each of these tRNAs at the discriminator position (position 73). Interestingly, three other tRNAs containing UxU and G-U mismatches are not altered by editing of this type. It is therefore not clear how substrates for this type of editing are selected by the editing activity.

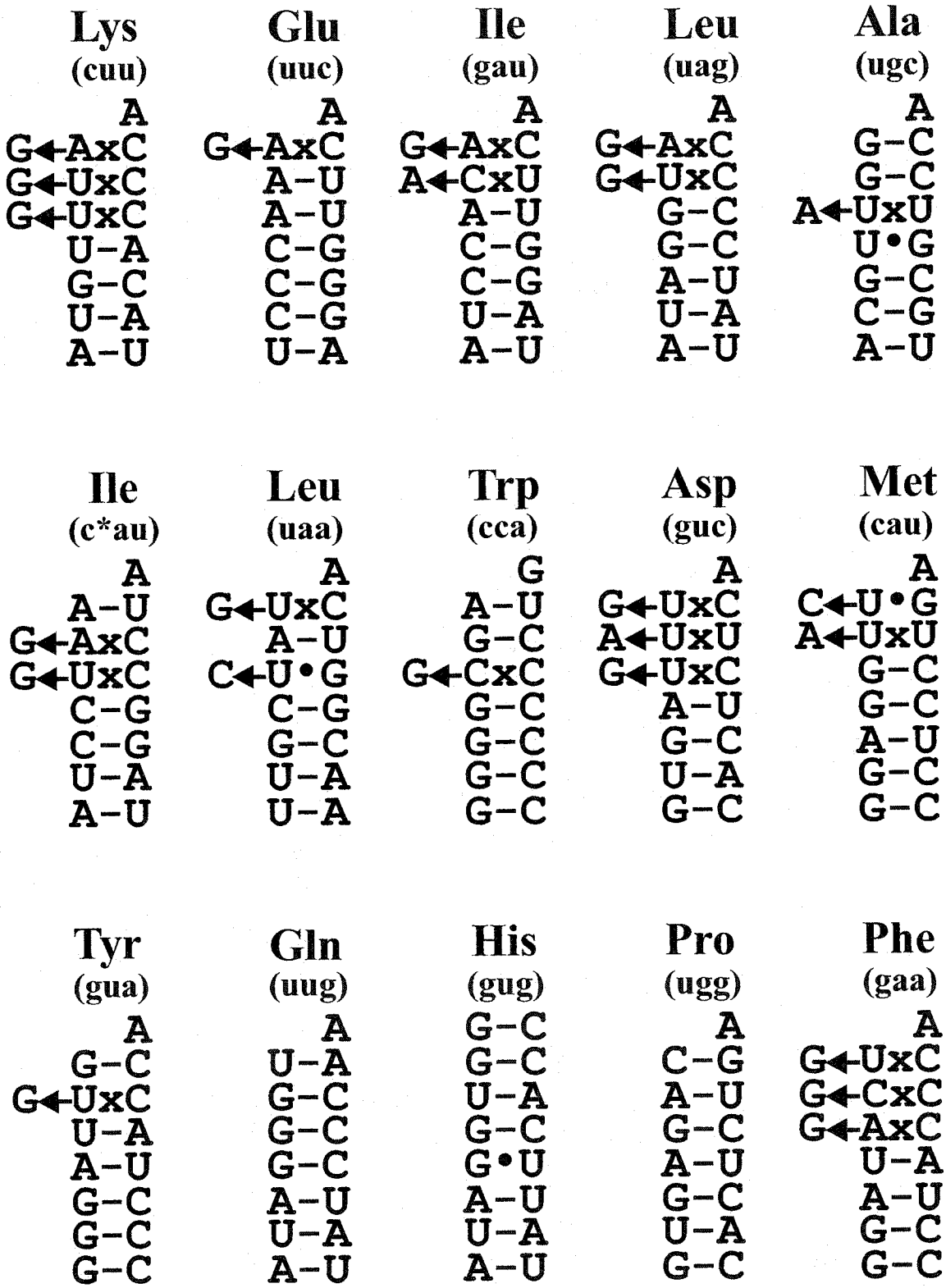
The similar form of editing observed in *L. forficatus* and *S. ecuadoriensis* mitochondria is of particular relevance to the results presented in this thesis, as both forms involve the repair of acceptor stem mismatches by a templated nucleotide replacement process. In the next two sections I will introduce the 5′ tRNA editing that occurs in *Acanthamoeba castellanii* and *Spizellomyces punctatus*.

**6. In *Acanthamoeba castellanii***

The first example of tRNA editing to be described occurs in the mitochondria of the unicellular amoeboid protist *Acanthamoeba castellanii* (Lonergan and Gray, 1993). This protozoan is abundant and widespread in nature, inhabiting both soil and water environments (including chlorinated pool and tap water). It is typically found in a trophozoite (or feeding) form, a motile cell stage characterized by pointed projections termed acanthapodia (*acanth* is from the Greek for thorn). When conditions become unfavourable, this organism is able to form a resistant cyst. *A. castellanii* is an excellent organism for mitochondrial studies because it is easy to culture and maintain, is able to grow to a high cell density in a relativley short period of time, and produces an abundance of mitochondria, mtDNA and mitochondrial RNA. Although it is a facultative pathogen (causing two diseases in humans: *Acanthamoeba* keratitis and granulomatous amoebic encephalitis), it generally only infects patients with reduced immune systems.

In *A. castellanii*, 12 of the 15 mtDNA-encoded tRNAs are predicted to contain non-Watson-Crick pairs (non-G-C/C-G/A-U/U-A) in one or more of the first three base pairs of the acceptor stem (**Fig. 2**). It should be noted that the majority of, but not all, acceptor stem base pairs found in tRNAs are of the Watson-Crick type. Non-Watson-Crick base pairs can be divided into "wobble" pairs (G-U/U-G pairs) and "mismatch" pairs (non-Watson-Crick/non-wobble). Wobble base pairs are well known to be able to substitute for Watson-Crick pairs in certain contexts (Masquida and Westhof, 2000) and,

**Figure 2:** Confirmed sites of editing in the acceptor stems (positions 1-7 and 66-73) of *A. castellanii* mitochondrial tRNAs. tRNA species are identified by their cognate amino acid (in three-letter code), with the sequence of the anticodon indicated in parentheses. Mismatched nucleotides are indicated with an X, nucleotides replaced by editing are indicated with an arrow.


Lys
(cuu)
Glu
(uuc)
Ile
(gau)
Leu
(uag)
Ala
(ugc)
Ile
(c*au)
Leu
(uaa)
Trp
(cca)
Asp
(guc)
Met
(cau)
Tyr
(gua)
Gln
(uug)
His
(gug)
Pro
(ugg)
Phe
(gaa)

**(Figure 2)**

as shown in **Table 2**, wobble base pairs are tolerated to a relatively high percentage in the tRNA acceptor stems of all the organisms compared. For example, 8.6% of acceptor stem base pairs in the mitochondrial tRNAs of *Schizosaccharomyces pombe* are wobble base pairs. However, mismatches are tolerated only to a very low percentage (**Table 2**). For example, in the archaeal tRNA acceptor stems analyzed, no mismatch pairs were identified. Thus the extent of mismatches in the mitochondrial tRNAs of *A. castellanii* (20% of total acceptor stem base pairs and 46.7% of base pairs at the three 5′-terminal position; **Table 2**) was remarkable, and appeared incompatible with canonical tRNA folding and function.

Editing was subsequently revealed to resolve the surfeit of acceptor stem mismatches with the requirement for functional tRNAs. Direct sequencing of tRNA 5′ ends (Lonergan and Gray, 1993) as well as a tRNA circularization/RT-PCR approach (Price and Gray, 1999a) revealed that these mismatches (as well as two U-G wobble pairs) 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 (**Fig. 2**). An *in vitro* assay developed to study this form of editing (Price and Gray, 1999b) has demonstrated radiolabelled nucleotide 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. Based on these results, 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 (Lonergan and Gray, 1993; Price and Gray, 1999b).

High percentages of mismatches are also observed in the three 5′-terminal acceptor stem base pairs of certain mtDNA-encoded tRNAs in the cellular slime mold *Dictyostelium discoideum* (24.1% of base pairs at these positions; **Table 2**) and the heterolobosean amoeba *Naegleria gruberi* (19.0% of base pairs at these positions; **Table 2**), two organisms in which 5′ tRNA editing is likely to occur (Ogawa *et al.*, 2000; M.W.

**Table 2**: Watson-Crick (WC) and non-WC base pairs in tRNA acceptor stems[a] (from Laforest *et al.*, 2004, with modifications)

| | | ACCEPTOR STEM BASE PAIRS (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1-72 to 7-66 | | | | 1-72 to 3-70 | | | |
| Organism (m, mitochondrial) | # of tRNAs | WC | non-WC | G-U U-G | mm | WC | non-WC | G-U U-G | mm |
| Eukarya[b] | 2025 | 89.5 | 10.5 | 9.3 | 1.1 | 92.8 | 7.2 | 6.9 | 0.2 |
| Archaea[b] | 581 | 97.6 | 2.4 | 2.4 | 0.0 | 97.4 | 2.6 | 2.6 | 0.0 |
| Eubacteria[b] | 1598 | 94.7 | 5.3 | 4.6 | 0.7 | 94.0 | 6.0 | 4.8 | 1.2 |
| *S. pombe* (m) | 25 | 89.1 | 10.9 | 8.6 | 2.3 | 88.0 | 12.0 | 10.7 | 1.3 |
| *A. macrogynus* (m) | 25 | 91.4 | 8.6 | 7.4 | 1.1 | 88.0 | 12.0 | 10.7 | 1.3 |
| *R. brooksianum* (m) | 7 | 93.9 | 6.1 | 6.1 | 0.0 | 90.5 | 9.5 | 9.5 | 0.0 |
| ***N. gruberi* (m)** | 21 | **88.4** | 11.6 | 3.4 | 8.2 | **76.2** | 23.8 | 4.8 | 19.0 |
| ***D. discoideum* (m)** | 18 | **78.6** | 21.4 | 11.1 | 10.3 | **63.0** | 37.0 | 13.0 | 24.1 |
| ***A. castellanii* (m)** | 15 | **76.2** | 23.8 | 3.8 | 20.0 | **48.9** | 51.1 | 4.4 | 46.7 |
| ***S. punctatus* (m)** | 8 | **67.9** | 32.1 | 3.6 | 28.6 | **29.2** | 70.8 | 4.2 | 66.7 |
| ***Monoblepharella*15 (m)** | 9 | **74.6** | 25.4 | 9.5 | 15.9 | **40.7** | 59.3 | 22.2 | 37.0 |
| ***Harpochytrium*94 (m)** | 8 | **76.8** | 23.2 | 1.8 | 21.4 | **45.8** | 54.2 | 4.2 | 50.0 |
| ***Harpochytrium*105 (m)** | 8 | **71.4** | 28.6 | 1.8 | 26.8 | **33.3** | 66.7 | 4.2 | 62.5 |
| ***H. curvatum* (m)** | 7 | **83.7** | 16.3 | 8.2 | 8.2 | **76.2** | 23.8 | 9.5 | 14.3 |

[a] Bold type indicates organisms with confirmed/predicted editing and highlights the percentage of WC base pairing in their mitochondrial tRNA acceptor stems. WC, Watson-Crick; mm, mismatch.

[b] Data from the analysis of Marck and Grosjean, 2002.

Genbank accession numbers for mitochondrial data: *S. pombe*, NC001326; *A. macrogynus*, NC001715; *R. brooksianum*, NC003053; *N. gruberi*, NC002573; *D. discoideum*, AB000109; *A. castellanii*, U12386; *S. punctatus*, NC003052; *Monoblepharella*15, AY1820007; *Harpochytrium*94, AY182005; *Harpochytrium*105, AY1820006; *H. curvatum*, NC003048.

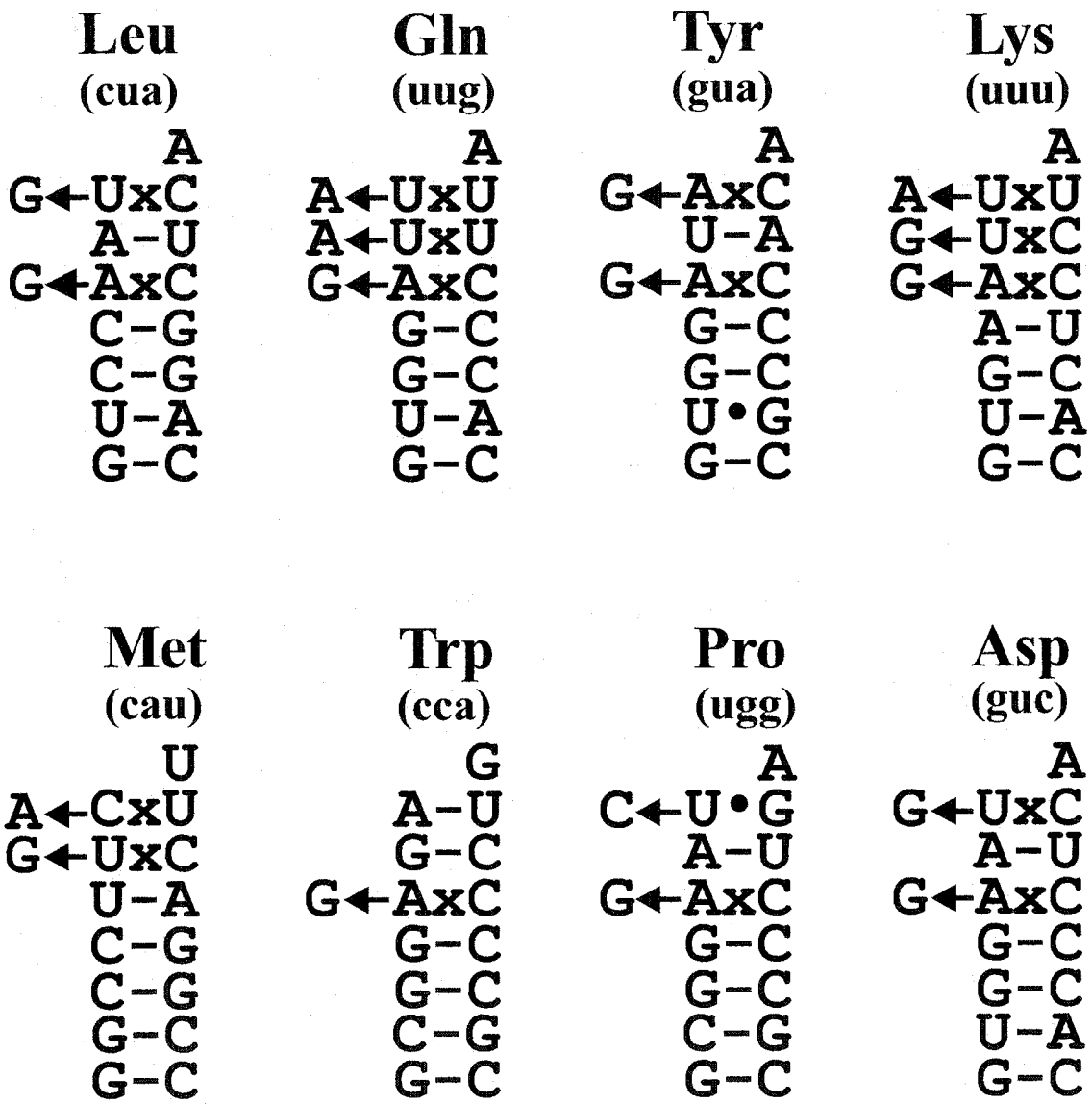
Gray, unpublished observation). However, the occurrence of this type of 5′ tRNA editing has to date only been confirmed experimentally in *A. castellanii*, certain fungi (see below) and a close relative of *D. discoideum*, *Polysphondylium pallidum* (E. Schindel, A.J. Lohan and M. W. Gray, unpublished results).

**7. In *Spizellomyces punctatus***

Chytridiomycetes are a group of organisms, once classified as protists, that are now known to diverge deeply within the Kingdom Fungi (see Bullerwell *et al.*, 2003a; Bullerwell and Gray, 2004). Although most chytrids are primary decomposers feeding off detritus in soil and water environments, some members are parasites of plants and animals (for example, they are believed to be responsible for amphibian mortality associated with major population declines; Berger *et al.*, 1998), and certain anaerobic chytridiomycetes play important roles in digestion in the guts of ruminants (see Li and Heath, 1993 for a review). This group is also of particular evolutionary interest, as its members conserve ancestral features not found in other fungi. For example, chytridiomycetes are the only fungi that display a flagellated growth stage, called the zoospore. Until recently, very little molecular data was available from members of Chytridiomycota.

*Spizellomyces punctatus* is a unicellular, saprophytic chytridiomycete. Based on the complete mitochondrial genome sequence, all eight of the encoded tRNAs in *S. punctatus* are predicted to contain mismatches at one or more of the first three acceptor stem positions (Laforest *et al.*, 1997) (**Fig. 3**). The extent of these mismatches (66.7% of base pairs at these positions; **Table 2**) appears to be incompatible with normal tRNA folding and function. These mismatches (as well as one U-G wobble pair) are repaired *in vivo*, as in *A. castellanii*, by replacement of the 5′ nucleotide in the pair to form Watson-Crick base pairs, as shown by direct sequencing of tRNA 5′ ends (Laforest *et al.*, 1997) and tRNA circularization/RT-PCR (Laforest *et al.*, 2004) (**Fig. 3**). The discovery of this type of tRNA editing in *S. punctatus* was unexpected, as the mtDNA-encoded tRNAs of all other fungi examined at the time (including the chytridiomycete *A. macrogynus*; Paquin and Lang, 1996) did not appear to require editing of this nature. Subsequent research, however, determined that other chytridiomycetes (but not all) have

**Figure 3:** Predicted and confirmed sites of editing in the acceptor stems of *S. punctatus* mitochondrial tRNAs. tRNA species are identified by their cognate amino acid (in three-letter code), with the sequence of the anticodon indicated in parentheses. Mismatched nucleotides are indicated with an X, nucleotides replaced by editing are indicated with an arrow.




**(Figure 3)**

acceptor stem mismatches that are repaired by this type of tRNA editing (Laforest *et al.*, 2004).

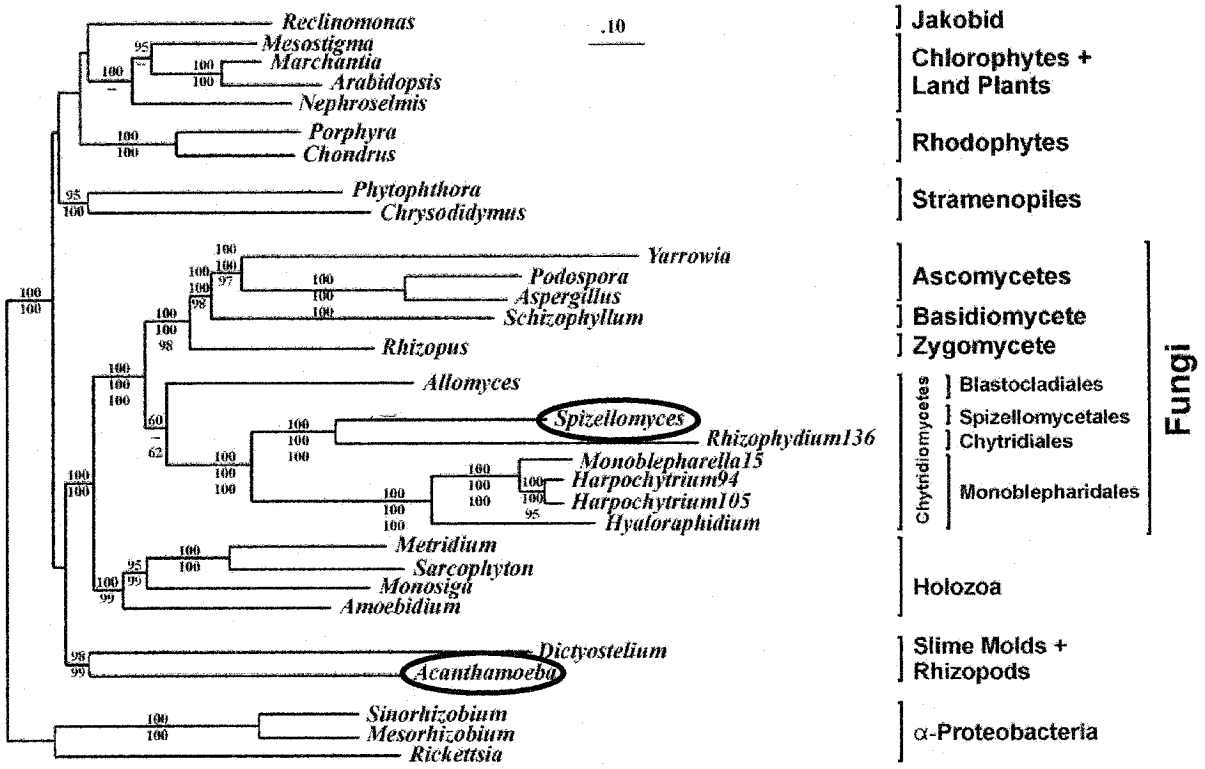
This discovery was even more unexpected as the chytridiomycete lineage branches specifically with other members of the fungal clade, whereas *A. castellanii* branches within Amoebozoa (an assemblage of protists including slime molds; Cavalier-Smith, 1998), to the exclusion of the monophyletic clade formed by fungi and metazoans (organisms in this clade are called "opisthokonts") (**Fig. 4**). Based on the lack of a specific evolutionary relationship between chytridiomycete fungi and this amoeboid protist, it was unclear how such similar types of editing could have evolved.

As a preliminary exploration to compare/contrast biochemical mechanisms of tRNA editing, the main objective of my PhD research was to develop an *in vitro* assay of editing for *S. punctatus*, and then compare the editing activities in the *A. castellanii* and *S. punctatus* mitochondrial systems. In this thesis, I first demonstrate and then examine an enzymatic activity in *S. punctatus* mitochondrial extracts that incorporates nucleotides into tRNA molecules. This activity has the properties expected of the *in vivo* mitochondrial tRNA editing activity. I further compare the biochemistry of the *S. punctatus* and *A. castellanii* editing activities and discuss how they may have evolved.

**D. Mitochondrial 5S Ribosomal RNA**

In the course of my work on tRNA editing, I identified and characterized two novel RNAs: the *A. castellanii* mitochondrial 5S rRNA, and a potential mitochondrial 5S rRNA in another amoeboid protist, *Hartmannella vermiformis*. 5S rRNA is a highly conserved and universal component of eubacterial, archaeal, plastid and eukaryotic cytoplasmic ribosomes. This small (~120 nucleotide) structured RNA interacts with ribosomal proteins (L5 in eukaryotes; L5, L18 and L25 in bacteria and organelles), and the resulting RNA-protein complex is found in the large ribosomal subunit (Moore, 1996). Despite the fact that 5S rRNA was discovered some 40 years ago (Rosset and Monier, 1963), its function is still not precisely defined; what is known is that the 5S ribonucleoprotein complex contributes importantly albeit indirectly to many of the functions of large ribosomal subunits that contain it (Moore, 1996).

**Figure 4:** Phylogeny of eukaryotes based on concatenated mitochondrial protein sequences. The positions of the genera *Acanthamoeba* and *Spizellomyces* are circled. (from Bullerwell *et al.*, 2003a, with modifications)


.10
Reclinomonas
Mesostigma
Marchantia
Arabidopsis
Nephroselmis
Porphyra
Chondrus
Phytophthora
Chrysodidymus
Yarrowia
Podospora
Aspergillus
Schizophyllum
Rhizopus
Allomyces
Spizellomyces
Rhizophydium136
Monoblepharella15
Harpochytrium94
Harpochytrium105
Hyaloraphidium
Metridium
Sarcophyton
Monosiga
Amoebidium
Dictyostelium
Acanthamoeba
Sinorhizobium
Mesorhizobium
Rickettsia
Jakobid
Chlorophytes + Land Plants
Rhodophytes
Stramenopiles
Ascomycetes
Basidiomycete
Zygomycete
Chytridiomycetes
Blastocladiales
Spizellomycetales
Chytridiales
Monoblepharidales
Fungi
Holozoa
Slime Molds + Rhizopods
α-Proteobacteria

**(Figure 4)**

Surprisingly, in view of its otherwise ubiquitous distribution, 5S rRNA appears not to be universally present in mitochondrial systems. Plant mitochondrial ribosomes do contain a distinctive 5S rRNA species (Leaver and Harmey, 1976; Cunningham *et al.*, 1976; Spencer *et al.*, 1981), encoded by the mitochondrial genome (Bonen and Gray, 1980; Oda *et al.*, 1992; Unseld *et al.*, 1997; Kubo *et al.*, 2000). A recognizable 5S rRNA gene is also present in some protist mitochondrial genomes, notably those of certain green, red and brown algae (Wolff *et al.*, 1994; Turmel *et al.*, 1999, 2002a,b; Ohta *et al.*, 1998; Burger *et al.*, 1999; Oudot-Le Secq *et al.*, 2001, 2002) and jakobid flagellates (Lang *et al.*, 1996, 1999). However, an obvious 5S rRNA gene has not been identified in other protist mtDNAs (Gray *et al.*, 1998), or in any of the more than one hundred animal mitochondrial genomes completely sequenced to date. Nor has a 5S rRNA species been detected in isolated animal mitochondrial ribosomes (O'Brien and Denslow, 1996). By the same token, fungal mitochondrial systems evidently lack a 5S rRNA component (Lizardi and Luck, 1971), although the possibility of preparative loss of 5S rRNA during isolation of fungal mitochondrial ribosomes has been debated (Datema *et al.*, 1974; Michel *et al.*, 1977).

A number of explanations could account for the absence of 5S rRNA in any given mitochondrial translation system. For example, the functional role of 5S rRNA might simply be dispensable in some cases. Other possibilities are that the functional role of 5S rRNA has been assumed by other ribosomal components (ribosomal proteins?) or that a 5S-equivalent sequence is covalently imbedded in the sequence of the large subunit (LSU) rRNA (Nierlich, 1982; Thurlow *et al.*, 1984). There is no evidence to support the former suggestion, whereas the latter can be discounted by comparative analysis of rRNA secondary structure (Lang *et al.*, 1987; Gutell *et al.*, 1993). A fourth possibility is that the function of 5S rRNA has been assumed in mitochondria by an extra-mitochondrial 5S rRNA species imported into mitochondria. In this regard, intriguing recent evidence suggests that nucleus-encoded (cytoplasmic) 5S rRNA is a *bona fide* intra-mitochondrial component in animals (Yoshionari *et al.*, 1994; Magalhães *et al.*, 1998), and that human mitochondria are able to import 5S rRNA (Entelis *et al.*, 2001). On the other hand, a mitochondrial 5S rRNA species may be present but be too divergent in sequence and higher-order structure to be readily recognized as such. I here

describe examples of the latter scenario, and discuss the significance of these discoveries.

## II. Materials and Methods

### A. Cell Culture

#### 1. *S. punctatus*

*Spizellomyces punctatus* strain BR117 was kindly provided by Dr. B. F. Lang (Université de Montréal), maintained on agar plates and grown in a liquid medium containing 0.5% yeast extract and 3% glycerol. The pH of the medium was adjusted to 5.8 with $KH_2PO_4$ before autoclaving. Cells were grown with shaking (~100 rpm) at room temperature (~22°C) in 100-ml cultures (in 250 ml conical flasks) for approximately 48 h. By this point the majority of the culture consisted of zoospores (the flagellated growth stage), which were found to be much less resistant to lysis than mature cells, likely due to the absence of a cell wall.

Swimming zoospores were slowed by a 15-min incubation on ice, pelleted by centrifugation at 2000 *g* in a swinging bucket rotor and washed twice with 500 ml phosphate-buffered saline (4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl). Washes were sometimes alternatively performed in a fixed angle rotor with smaller volumes (<50 ml), resulting in a tighter pellet and less loss of material. Cells from 1-liter of culture (1-3 g of cells were routinely obtained) were mixed with 10 ml *Spizellomyces* homogenization buffer (0.35 M sucrose, 50 mM Tris-HCl (pH 8.0), 3 mM EDTA, 1 mM DTT, 0.1% BSA) and re-suspended with a teflon homogenizer.

#### 2. *A. castellanii*

*Acanthamoeba castellanii* strain Neff (ATCC 30010; kindly provided by Dr. A.J. Lohan, Dalhousie University) was maintained in 5 ml liquid cultures at room temperature without shaking. Growth was in liquid medium at 30°C with moderate shaking (~50 rpm) to an $O.D._{550}$ of ≤1.0 (this optical density was usually attained within 7 days). Liquid medium consisted of 0.75% yeast extract, 0.75% proteose peptone, 2 mM $KH_2PO_4$, 1 mM $MgSO_4$, 1.5% glucose, 0.1 mM ferric citrate, 0.05 mM $CaCl_2$, 1mg/L thiamine, 0.2 mg/L biotin and 1 μg/L vitamin $B_{12}$. After the addition of the first four components, the medium was adjusted to pH 7 with NaOH, autoclaved and allowed to cool. The remaining components were then added by injection through a 0.22 μm filter in the vicinity of a flame (*i.e.*, sterile conditions). Cells were pelleted by centrifugation at 2000 *g* in a swinging bucket rotor, washed twice with 500 ml

phosphate-buffered saline and resuspended in 25 ml *Acanthamoeba* homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl (pH 7.6), 1 mM $ZnCl_2$, 1 mM DTT, 0.1% BSA).

**3. *H. vermiformis***

*Hartmannella vermiformis* (ATCC 50236; kindly provided by Dr. A.J. Lohan, Dalhousie University) was maintained in 5 ml liquid cultures at room temperature without shaking. Growth was in liquid medium at room temperature without shaking to an $O.D._{550}$ of $\leq 0.4$ (this optical density was usually attained within 7 days). Liquid medium consisted of 0.1% yeast extract, 2% proteose peptone, 4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 3.4 mM NaCl, 50 μM $Fe(NH_4)_2(SO_4)_2$, 2.5 mM $Na_2HPO_4$, 2.5 mM $KH_2PO_4$ and 0.1 M glucose. After the addition of all components except glucose, the medium was adjusted to pH 6.05 with HCl, autoclaved and allowed to cool. Glucose was then added by injection through a 0.22 μm filter in the vicinity of a flame (*i.e.*, sterile conditions). Cells were pelleted by centrifugation at 2000 *g* in a swinging bucket rotor and washed twice with 500 ml phosphate-buffered saline and resuspended in 10 ml *Spizellomyces* homogenization buffer.

## B. Preparation of Mitochondria and Mitochondrial S100 Extracts

**1. *S. punctatus***

Cells were disrupted by passage through a French pressure cell at 1000 psi. If >80% cell breakage was not observed by microscopy, cells were passed through the cell a second time. Cellular debris and intact cells were removed by centrifugation at 440 *g* for 10 min, and the supernatant was further clarified by centrifugation at 1780 *g* for 10 min. The supernatant of this spin was then centrifuged at 9000 *g* for 20 min, yielding a yellow-brown pellet containing intact mitochondria. The supernatant from this centrifugation step was retained for the preparation of cytoplasmic RNA (see below). The pellet was re-suspended in 10 ml homogenization buffer and residual cellular debris was removed by centrifugation at 1780 *g* for 10 min. The supernatant was then centrifuged at 9000 *g* for 20 min and the resulting mitochondrial pellet was washed twice by re-suspension in 10 ml homogenization buffer followed by centrifugation at 9000 *g* for 20 min.

Mitochondria were re-suspended in 1 ml sonication buffer (7.5% glycerol, 10 mM Tris (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF) and disrupted by sonication with a Fisher Scientific Sonic Dismembrator Model 100 at 1/3 maximum power for 3 x 30 s, 1 x 20 s, with 1-min rests between bursts. The sonicate was subjected to ultracentrifugation at 100,000 *g* for 1 h in a Ti50 fixed angle rotor (Beckman), 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-hydroxy-propyl)aminoethyl; stored in sonication buffer) prior to *in vitro* assays.

**2. *A. castellanii***

The mitochondrial purification strategy described here is based on that of Price and Gray (1999b). Cells were disrupted in a Dounce homogenizer by 35 strokes with pestle A (tight) and 5 with pestle B (loose). Centrifugations and washes to obtain a mitochondrial pellet were performed as for *S. punctatus*. However, the pellet was further purified by fractionation on a discontinuous sucrose gradient as follows. The pellet was resuspended in 10 ml pre-gradient buffer (50 mM Tris-HCl (pH 8), 3 mM EDTA, 0.25 M sucrose, 0.1% BSA, 1 mM DTT) and layered onto three gradients (each consisting of a 7.5 ml bottom layer of 1.55 M sucrose and a 15 ml layer of 1.3 M sucrose; sucrose solutions contained 50 mM Tris-HCl (pH 8) and 3 mM EDTA). The mitochondrial suspension was subjected to ultracentrifugation at 22,500 rpm for 1 h in a SW25.1 swinging bucket rotor (Beckman), and mitochondria on the 1.55 M sucrose shelf (at the interface of the 1.3 M and 1.55 M solutions) were recovered with a bent needle. Gradient-purified mitochondria were diluted gradually (over 15 min) with 2 volumes of post-gradient buffer (20 mM Tris-HCl (pH 8), 0.5 mM EDTA, 0.25 M sucrose, 15% glycerol, 0.1% BSA, 1 mM DTT) and pelleted by centrifugation at 9,000 *g* for 30 min.

Mitochondria were re-suspended in 1 ml sonication buffer (as above, but also containing 100 mM KCl) and disrupted by sonication as for *S. punctatus*. The sonicate was subjected to ultracentrifugation at 100,000 *g* for 1 h in a Ti50 fixed angle rotor

(Beckman), 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 1/2 volume of DEAE-Sephacel weak anion exchange resin (ion exchange group: diethyl-aminoethane; stored in sonication buffer) prior to *in vitro* assays.

**3. *Hartmannella vermiformis***

Cells were disrupted by passage through a French pressure cell at 1500 psi. Centrifugations and washes to obtain a mitochondrial pellet were performed as for *S. punctatus*. RNA was prepared from purified, intact mitochondria (see below).

## C. Preparation of Cytoplasmic and Mitochondrial RNA

**1. *S. punctatus***

Purified mitochondria were resuspended in 9 ml mitochondrial lysis buffer (10 mM Tris-HCl (pH 8.5), 50 mM KCl and 10 mM $MgCl_2$) with a teflon homogenizer. Mitochondria were lysed by the addition of 1 ml of 20% Triton X-100, followed by five cycles of 30 s mixing using a vortex followed by a 30 s incubation on ice. The resulting mixture was centrifuged at 9000 *g* for 10 min, and RNAs were prepared from both the resulting 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 an *S. punctatus* mitochondrial S100 fraction (see above).

RNAs from the mito-S and cytoplasmic fractions were prepared by the addition of a 1/10 volume of 10X detergent mix (10% sarkosyl, 0.5 M NaCl) to these fractions. RNAs from the mito-P fraction were prepared by resuspending the fraction in 10 ml of mitochondrial lysis buffer and adding a 1/10 volume of 10X detergent mix. One volume of phenol-cresol was mixed with each fraction and the phases were separated by centrifugation. Phenol-cresol was prepared as follows: 0.5 g or 0.1% final hydroxyquinolone was added to a mixture of 500 ml liquid phenol, 55 ml $dH_2O$, and 70 ml m-cresol, and equilibrated to pH 8 with 10 mM Tris-HCl. One-tenth volume of 5 M NaCl was added to the upper (liquid) phase, and this phase was re-extracted with phenol-

cresol. To precipitate the RNA in these samples, 2.5 volumes of 95% ethanol were added to the liquid phase, and the mixture was incubated for 30 min at –20°C. RNAs were pelleted by centrifugation, washed with 80% ethanol and dried. The pelleted mRNA was redissolved in a small volume of $dH_2O$, extracted twice with phenol-cresol and precipitated twice with ethanol. RNAs were stored at -20°C in 50% ethanol. To assess mitochondrial enrichment, 5 µg of RNA from each fraction was treated with 2.5 U of DNase I (following the manufacturer's instructions) for 30 min and resolved by agarose gel electrophoresis. Methods for cell lysis, organelle purification and the preparation and visualization of nucleic acids are based on Spencer *et al.* (1992).

Loading dye (5X) for agarose gels consisted of 100 mM EDTA (pH 7.6), 25% glycerol and 5% Ficoll. This mixture was heated to 65°C and allowed to cool. Xylene cyanol was added to 0.05% final, and the loading dye was stored at room temperature. Loading dye (1X) for polyacrylamide gels consisted of 600 µl deionized *N*-methylformamide, 80 µl $dH_2O$, 10 µl 1 M Tris-HCl (pH 7.6), 25 µl 250 mM EDTA and 300 mg enzyme-grade urea. This mixture was heated to 65°C, allowed to cool and 50 µl of a solution containing 2% bromophenol blue and 2% xylene cyanol was added. The loading dye was stored in the dark at -20°C.

**2. *A. castellanii***

Cytoplasmic RNAs were prepared as described for *S. punctatus*. Purified mitochondria were gently lysed in a 2% Triton X-100 solution followed by centrifugation at 9000 *g* for 10 min, and RNA was prepared from both the resulting pellet (mito-P) and supernatant (mito-S) fractions, as described for *S. punctatus*. To assess mitochondrial enrichment, 5 µg of RNA from each fraction was treated with 2.5 U of DNase I (following the manufacturer's instructions) for 30 min and resolved by agarose gel electrophoresis. Alternatively, the mito-S fraction was further resolved by ultracentrifugation at 100,000 *g* for 1.5 h in a Ti50 fixed-angle rotor into supernatant (S100) and pellet (P100) fractions.

RNAs prepared by extraction with phenol-cresol and precipitation with ethanol were separated on 1.5 mm-thick 10% polyacrylamide gels (all polyacrylamide gels contained 19:1 acrylamide:*bis*-acrylamide and 7 M urea) and visualized by either ethidium bromide staining or UV shadowing (Hassur and Whitlock, 1974). Certain

RNAs (*e.g.*, the *A. castellanii* 5S rRNA) were eluted from homogenized polyacrylamide gel slices by shaking overnight at 4°C in a 1:1 mixture of phenol-cresol:buffer (0.5 M $NH_4OAc$, 10 mM $Mg(OAc)_2$, 1.0 mM EDTA), precipitated with ethanol and analyzed by RNA sequencing (see below).

**3. *H. vermiformis***

Cytoplasmic and mitochondrial (mito-S) RNAs were prepared and visualized as described for *S. punctatus*. Alternatively, mito-S was further fractionated by ultracentrifugation in a 10-30% continuous glycerol gradient (prepared with mitochondrial lysis buffer; see above) at 21,000 rpm for 18.5 h in an SW27 swinging bucket rotor (Beckman). Fractions were collected from the top (fraction 1) to the bottom (fraction 20 and the pellet) of the gradient, precipitated with ethanol, redissolved in dH2O, extracted with phenol-cresol and precipitated with ethanol. RNAs were separated on a 1.5 mm-thick 10% polyacrylamide gel and visualized by ethidium bromide staining. Certain RNAs (*e.g.*, hvRNA) were eluted from homogenized polyacrylamide gel slices by shaking overnight at 4°C in a 1:1 mixture of phenol-cresol:buffer [0.5 M $NH_4OAc$, 10 mM $Mg(OAc)_2$, 1 mM EDTA], precipitated with ethanol and analyzed by RNA sequencing (see below).

**D. *In vitro* Editing Assay**

**1. *S. punctatus***

Each assay contained 2.5 µl of the mitochondrial S100 extract, 40 mM HEPES (pH 7.0 with NaOH), 15 mM $MgCl_2$, 1 mM DTT, 100 µM NTPs (when included), 10 µCi [$\alpha$-$^{32}$P]NTP (when included), and exogenous tRNA substrate (when included; RNA was either 1 µg yeast soluble RNA, 1/20 of the product of a T7 transcription reaction, or a small portion of a $^{32}$P-end-labelled T7 transcription product). Reactions (in a final volume of 50 µl) were performed at room temperature for 1 h or less, as indicated. Products of *in vitro* assays were extracted with phenol-cresol and precipitated with ethanol using linear polyacrylamide as carrier (Galliard and Strauss, 1990). Samples were separated on 0.4 mm-thick 10% polyacrylamide gels and visualized by autoradiography.

**2. *A. castellanii***

Each assay (reaction conditions are as specified in Price and Gray, 1999b) contained 2.5 µl of the mitochondrial S100 extract, 40 mM HEPES (pH 7.0 with NaOH), 7.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 100 µM NTPs (when included), 10 µCi [α-$^{32}$P]NTP (when included), and exogenous tRNA substrate (when included; RNA was either 1 µg yeast soluble RNA, 1/20 of the product of a T7 transcription reaction, or a small portion of a $^{32}$P-end-labelled T7 transcription product). Reactions (in a final volume of 50 µl) were performed at 30°C for 1 h. Products of *in vitro* assays were prepared and visualized as for *S. punctatus*.

**E. Preparation of Competent Cells, Transformation and Preparation of Plasmid DNA**

*Escherichia coli* cells (strain DH5-α) were grown overnight on LB (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl, adjusted to pH 7 with NaOH) agar plates containing 15 µg/ml nalidixic acid. A single colony was used to innoculate a 1 ml LB liquid culture that was incubated overnight at 37°C with mild agitation. This culture was used to innoculate a 50 ml LB culture, which was incubated at 37°C for 1-2 h with shaking (250 rpm) until it had reached an $O.D._{600}$ of 0.3-0.5. The cells in this culture were pelleted by centrifugation, gently resuspended in 40 ml ice-cold, sterile 100 mM $CaCl_2$, pelleted a second time, gently resuspended in 20 ml ice-cold, sterile 100 mM $CaCl_2$ and incubated on ice for 30 min. The cells were pelleted a third time, gently resuspended in 2 ml ice-cold, sterile 100 mM $CaCl_2$ and incubated overnight at 4°C. Aliquots (80 µl) of competent cells were added to 35 µl aliquots of 50% glycerol (sterilized by passage through a 0.22 µm filter), snap frozen in liquid nitrogen and stored at –70°C.

To transform competent *E. coli* cells, 100 µl aliquots of cells were added to ~10 ng of plasmid DNA (containing a gene conferring resistance to the antibiotic ampicillin) in pre-chilled microcentrifuge tubes, and DNA and cells were mixed by gentle inversion for 10-30 min on ice. The mix was then incubated ("heat shocked") at 42°C for 90-120 s, and then quickly transferred to ice for a further 2 min. Transformed cells were then mixed with 800 µl of SOC (20 g/L tryptone, 1 g/L yeast extract, 0.1 g/L NaCl, 0.5 mM

KCl, adjusted to pH 7 with NaOH), incubated for 45 min at 37°C and spread on LB agar plates. After allowing time for them to dry, the plates were incubated at 37°C overnight.

Individual colonies were used to innoculate 2 ml cultures of LB containing 100 μg/ml ampicillin, which were grown overnight at 37°C with agitation. Cells were pelleted by centrifugation, washed once with STE (10 mM Tris-HCl (pH 8), 1 mM EDTA, 100 mM NaCl), resuspended and incubated 5 min at room temperature in 100 μl GTE (25 mM Tris-HCl (pH 8), 10 mM EDTA, 500 mM glucose). A 200 μl volume of cell lysis solution (1% SDS, 0.2 M NaOH) was then added to the resuspended cells, gently mixed and incubated on ice for 5 min. A 150 μl volume of neutralizing solution (29.5% glacial acetic acid, adjusted to pH 4.8 with KOH) was then added, mixed vigorously on a vortex mixer and incubated on ice for 5-10 min. Cell debris was removed by centrifugation, and the supernatant was incubated for 10 min at 37°C with 1 U of RNase A, then extracted twice with phenol-cresol and precipitated twice with ethanol. DNA was redissolved in 50 μl 10 mM Tris-HCl (pH 8), 0.1 mM EDTA and stored at 4°C.

### F. Preparation of Synthetic tRNA Substrates

Oligonucleotides used in this study are shown in **Table 3**. The *A. castellanii* mitochondrial gene encoding $tRNA^{Leu}$(uag) (*trnL*) cloned into the *Eco*RV site in the multi-cloning site of the pT7Blue vector (see Price and Gray, 1999b; referred to here as plasmid 1) was used to generate the PCR templates for T7 RNA polymerase synthesis of tRNA constructs 1 and 4-10 (see **Figs. 7A** and **15A** for sequences of constructs). The PCR templates for tRNA constructs 2 and 3 were generated by first introducing 5′ or 3′ extensions, respectively, into the *trnL* gene in 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, following the manufacturer's instructions. PCR conditions were as follows: 5 min at 95°C (during which time the polymerase was added), 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s, and a final 5 min incubation at 72°C. The linearized plasmids 2 and 3 thus produced were phosphorylated with T4 polynucleotide kinase and circularized with T4 DNA ligase,

**Table 3:** Oligonucleotides used for PCR amplification.

| # | Sequence |
|---|---|
| 1 | 5′-GTTCACATAAATGGATATGCTGAAATGGTAGACAGG-3′ |
| 2 | 5′-TTTAGTGAGGGTTAATAATCGG-3′ |
| 3 | 5′-TGCATAATCCATATGACTAGTAGATCC-3′ |
| 4 | 5′-TTAAATACGATTTGGGGATAATGGGACTCGAACC-3′ |
| 5 | 5′-AAGCACTAATACGACTCACTATAGGATATGCTGAAATGGTAG-3′ |
| 6 | 5′-TGGGGATAATGGGACTCG-3′ |
| 7 | 5′-AAGCACTAATACGACTCACTATAGGTTCACATAAATGGATATGC-3′ |
| 8 | 5′-AAGTTAAATACGATTTGGGGATAATGGGAC-3′ |
| 9 | 5′-AAGCACTAATACGACTCACTATAGGGATATGCTGAAATGGTAG-3′ |
| 10 | 5′-AAGCACTAATACGACTCACTATAGGGGATATGCTGAAATGGTAG-3′ |
| 11 | 5′-TGTGGATAATGGGACTCGAACC-3′ |
| 12 | 5′-TTGGGATAATGGGACTCGAACC-3′ |
| 13 | 5′-TGGTGATAATGGGACTCGAACC-3′ |

following the manufacturer's instructions. Plasmids 1, 2 and 3 were cloned in competent *E. coli* cells and plasmid DNA was prepared using an alkaline lysis procedure (see above for preparation of competent cells and plasmid DNA). The sequence of the *trnL* region of the plasmids was confirmed by DNA sequencing.

Plasmids were restricted with the DNA endonuclease *Hin*dIII (which cuts once outside the *trnL* region in the multiple cloning site) prior to amplification in order to eliminate longer, unwanted products observed in PCR reactions using uncleaved plasmids. Short DNA templates for *in vitro* transcription reactions with T7 RNA polymerase were produced using PCR (under the conditions above, except: 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s) with primers designed to incorporate specific sequences at the 5′ and 3′ termini of these templates. T7 templates for synthesis of tRNA constructs 1-10 were generated using the oligonucleotide combinations shown in **Table 4**. Each T7 template contained a 6-nt 5′ leader (5′-AAGCAC-3′), a T7 promoter (5′-TAATACGACTCACTATA-3′), and the desired *trnL* sequence. The products of PCR were extracted with phenol-cresol, precipitated with ethanol, and 1/10 of each product was used as template for each 50 µl T7 *in vitro* transcription reaction (following the manufacturer's instructions, except that 10 mM $MgCl_2$ was used instead of the suggested 6 mM, as this change resulted in a significant increase in RNA production). Products of *in vitro* transcription were treated with 2.5 U DNase I, separated in 10% denaturing polyacrylamide gels, excised and eluted. An approximately equal amount of T7 product (several µg) was generated for each construct, as estimated by ethidium bromide staining of polyacrylamide gels. End labelling and sequencing (see below) of constructs 1 and 10 confirmed the purity and identity of these RNAs.

## G. 5′- and 3′-End Labelling of RNA

### 1. 5′-End labelling

RNAs were dissolved in 50 µl 50 mM Tris-HCl (pH 8), incubated at 65°C for 5 min, quickly cooled on ice and dephosphorylated by incubation with 0.2 U bacterial alkaline phosphatase. RNAs were extracted three times with phenol-cresol and precipitated twice with ethanol using linear polyacrylamide as carrier. Dephosphorylated RNAs were redissolved in 15 µl TE (10 mM Tris-HCl (pH 7.6), 0.1

**Table 4:** Oligonucleotide combinations for PCR generation of T7 templates.

| T7 template for construct: | Oligonucleotide combination |
|---|---|
| 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) |
| 10 | 5 (sense), 13 (antisense) |

mM EDTA), incubated at 65°C for 5 min and quickly cooled on ice. RNAs were 5′-end-labelled (following the protocol of Schnare *et al.*, 1985) with T4 polynucleotide kinase and [γ-$^{32}$P]ATP in a reaction mix that consisted of the following components added in the given order: 2.5 µl DMSO, 2.5 µl 10X kinase buffer (500 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$), 2.5 µl 50 mM DTT, 0.5 µl 50 mM spermidine-HCl, 10 U polynucleotide kinase and 200-800 µCi [γ-$^{32}$P]ATP. Incubation was at 37°C for 45 min. Labelled products were precipitated twice with ethanol, separated in polyacrylamide gels, excised and eluted from gel slices.

**2. 3′-End labelling**

[5′-$^{32}$P]pCp was prepared by incubation of 200-800 µCi [γ-$^{32}$P]ATP with 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 5 mM DTT, 1.5 mM spermidine-HCl, 0.15 mM Cp and 20 U polynucleotide kinase at 37°C for 16 h. The quality of radiolabeled pCp and the degree of conversion of radioactivity from [γ-$^{32}$P]ATP to [5′-$^{32}$P]pCp (to ensure that the reaction was complete) were checked periodically by thin-layer chromatography (TLC) on polyethyleneimine (PEI) cellulose plates (pre-developed with $dH_2O$ and allowed to dry) using 0.5 M $(NH_4)_2CO_3$ as the solvent. RNAs were 3′-end-labelled with [5′-$^{32}$P]pCp and RNA ligase (Peattie, 1979), according to the manufacturer's instruction. Labelled products were precipitated twice with ethanol, separated in polyacrylamide gels, excised and eluted from gel slices.

**H. Periodate Treatment**

RNAs were dissolved in a 40-µl solution containing 150 mM NaOAc (pH 5.3) and 1 mM $NaIO_4$, incubated on ice in the dark for 1 h, and precipitated by the addition of 120 µl of 100% ethanol.

**I. RNA Sequencing**

**1. Enzymatic sequencing**

To determine partial RNA sequences, 3′- and 5′-end-labelled RNAs were subjected to partial alkaline hydrolysis and partial digestion with RNase T1 (which cleaves 3′ to G residues), and sometimes also with RNase U2 (which cleaves 3′ to A>G

residues) and RNase PhyM (which cleaves 3′ to U>A residues). The products of these digestions were separated in adjacent wells of 6% and 20% polyacrylamide gels.

Alkaline hydrolysis was performed as follows: RNAs were dissolved in 20 µl 0.15 M ammonium hydroxide (1/100 dilution of the concentrate) on ice, incubated at 90°C for 15-45 s, quickly cooled on ice, frozen at –75°C and lyophilized for ~1 h. The pellet was redissolved in 50 µl $dH_2O$, frozen at -75°C and lyophilized for ~2 h. Dried RNAs were redissolved in 5 µl RNase U2 digestion buffer [700 µl 10 M urea (made fresh), 20 µl 1 M citric acid (adjusted to pH 5 with NaOH), 10 µl 0.1 M EDTA, 25 µl 2% xylene cyanol, 30 µl yeast carrier tRNA (5 mg/ml, extracted with phenol-cresol and precipitated with ethanol) and 215 µl $dH_2O$] and stored at -20°C.

Enzymatic digests were prepared as follows: RNAs were dissolved in 2 µl $dH_2O$, 4 µl of 95% ethanol was added and the mix was allowed to evaporate in a dessicator. Dried RNAs were dissolved in digestion buffer (same as for RNase U2, except that for T1 and PhyM, citric acid was adjusted to pH 3.5 with NaOH), ~1 µl of the appropriate RNase was added and partial digests were incubated at 50°C for 30 min. Following this incubation, digests were stored at -20°C. Samples were heated to 65°C for 5 min and allowed to cool to room temperature before being separated on 0.4 mm-thick 6% or 20% polyacrylamide gels, and visualized by autoradiography.

5′-End analysis was performed by digestion of 5′-end-labelled RNAs (produced by either the kinase reaction described above, or by labelling with [α-$^{32}$P]GTP and a mitochondrial S100 extract) with P1 nuclease, an endoribonuclease that generates nucleoside 5′-monophosphates (pN). RNase P1 digests (10 µl), consisting of 100 ng P1 nuclease, 45 mM $NH_4OAc$ (pH 5.3) and 5 µg yeast tRNA, were incubated at 37°C overnight. The products of P1 digestion were separated by one-dimensional TLC using PEI cellulose plates (pre-developed with $dH_2O$ and allowed to dry) with 0.5 M $NH_4CO_3$ as the solvent. 3′-End analysis was performed by digestion of 3′-end-labelled RNAs with RNase T2, a random endoribonuclease that generates nucleoside 3′-phosphates (Np). RNase T2 digests (10 µl), consisting of 0.5 U RNase T2, 10 mM $NH_4OAc$ (pH 4.5) and 5 µg yeast tRNA, were incubated at 37°C overnight. Ammonium formate (2 µl of a 500 mM solution) was added to each digest following incubation. The products of T2 digestion were separated by one-dimensional TLC on cellulose plates (pre-dipped in

a 10% dilution of a saturated solution of $(NH_4)_2SO_4$ and allowed to dry) using a 4:1 mixture of 95% ethanol:water as the solvent (Lane, 1963). Radioactive products of P1 and T2 digests were visualized by autoradiography. The digestion products (mononucleotides) of the unlabelled yeast tRNAs added to each nuclease P1 and RNase T2 digestion were visualized by UV shadowing, and served as migration markers for radiolabelled products.

**2. Chemical sequencing**

To determine partial RNA sequences, 3′-end-labelled RNAs were subjected to nucleotide-specific chemical reactions that result in RNA cleavages (following the protocols of Peattie, 1979). The products of these reactions were separated in adjacent wells of 6% and 20% polyacrylamide gels. Chemical sequencing reactions were as follows: RNA was dissolved in 20 µl $dH_2O$, heated to 65°C for 5 min, allowed to cool to room temperature and 5 µl was aliquoted to each of 4 tubes, labelled G, A, U and C.

G residues: 200 µl 50 mM cacodylic acid (pH 4.5) was added to the dissolved RNA, on ice. Dimethyl sulfate (1 µl) was then added with thorough mixing, and after incubation for 45 sec at 90°C, the mixture was cooled quickly on ice. A solution of 1 M β-mercaptoethanol, 1.5 M NaOAc (50 µl) and 750 µl 95% ethanol were added, and the RNA products were precipitated at -75°C for 30 min and then reprecipitated with ethanol. Precipitated RNAs were dissolved in 5 µl $dH_2O$. Tris-HCl (pH 8) (10 µl) and 0.4 M $NaBH_4$ (5 µl) were added and the mixture was incubated in the dark for 30 min on ice. RNAs were precipitated by the addition of 100 µl 3 M acetic acid (pH 4.7 with NaOH) and 300 µl 95% ethanol.

A residues: 200 µl 50 mM NaOAc (pH 4.5) was added to the dissolved RNA, on ice. Diethyl pyrocarbonate (1 µl) was then added with mixing, and the mixture was incubated for 4 min at 90°C, then chilled on ice. A solution containing 1 M β-mercaptoethanol and 1.5 M NaOAc (50 µl), and 750 µl of a 95% ethanol solution were added. RNAs were precipitated at -75°C for 30 min, and then reprecipitated with ethanol.

U residues: Dissolved RNAs were frozen at -75°C and lyophilized. Dried RNAs were redissolved in 10 µl 50% hydrazine and incubated on ice for 8 min. RNAs were

precipitated by the addition of 200 µl 0.3 M NaOAc and 750 µl 95% ethanol, and then reprecipitated with ethanol.

C residues: Dissolved RNAs were frozen at -75°C and lyophilized. Dried RNAs were redissolved in 10 µl of a solution of 17.5 mg NaCl in 100 µl ice-cold hydrazine (to give a final concentration of 3 M NaCl) and incubated 25 min on ice. RNAs were precipitated by the addition of 500 µl 80% ethanol, and then reprecipitated with ethanol.

All precipitated RNAs were dissolved in 6.4 µl $dH_2O$, 13.6 µl aniline: $dH_2O$:acetic acid (in a 20:110:20 ratio) was added and the mixture was incubated at 65°C for 20 min in the dark. Samples were frozen at 75°C, lyophilized for ~1 h, redissolved in 50 µl $dH_2O$ and lyophilized for ~2 h. Samples were heated to 65°C for 5 min and allowed to cool to room temperature before being separated on 0.4 mm-thick 6% or 20% polyacrylamide gels, and visualized by autoradiography.

## III. RESULTS

### A. *In vitro* Characterization of a tRNA Editing Activity in the Mitochondria of *Spizellomyces punctatus* (Bullerwell and Gray, 2005)

#### 1. Development of an *in vitro* assay of nucleotide incorporation

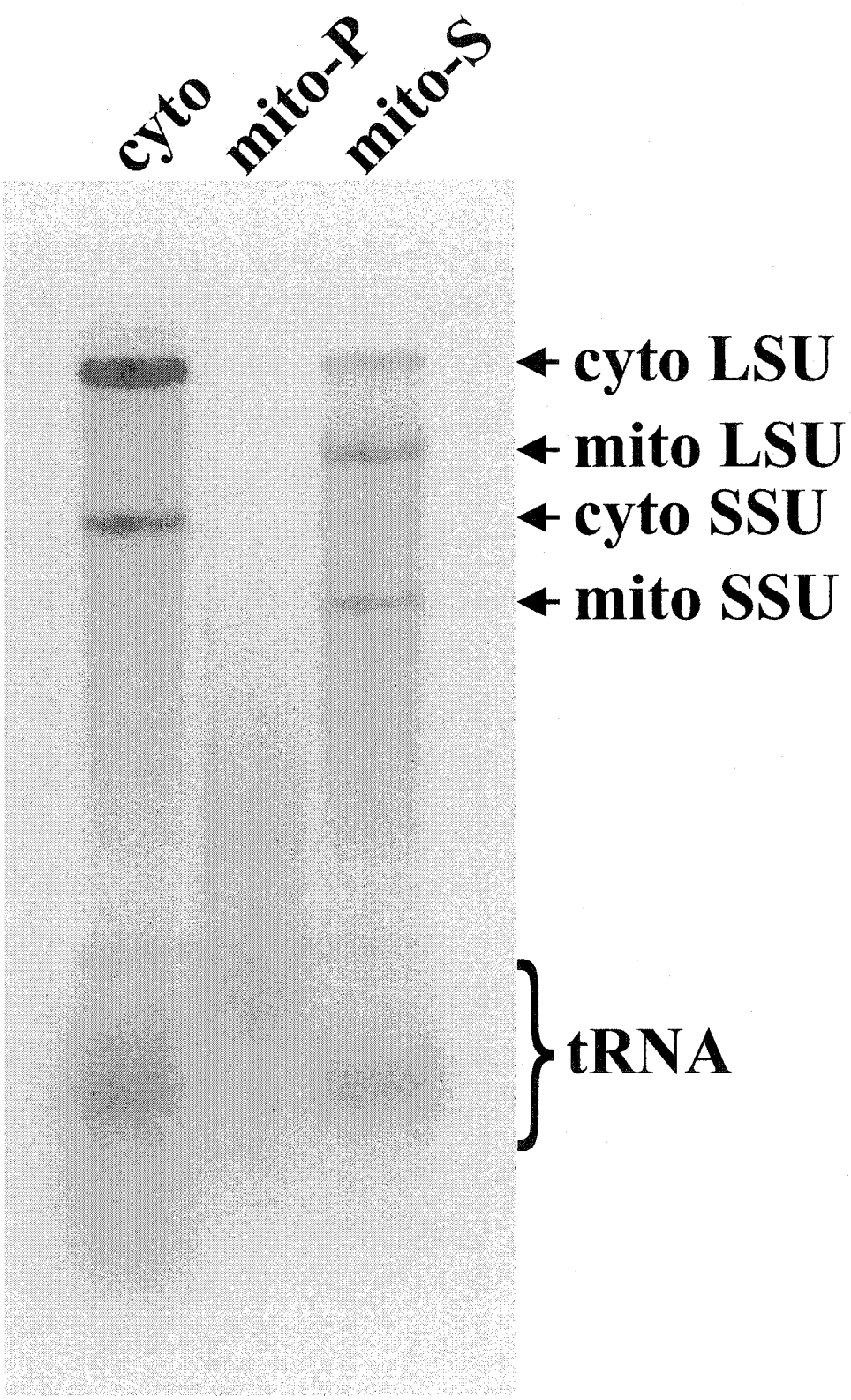
Intact mitochondria were isolated from *S. punctatus* zoospores by lysis under relatively mild conditions in a French pressure cell followed by differential centrifugation. This procedure resulted in significant mitochondrial enrichment, as estimated by the ratio of mitochondrial to cytoplasmic rRNAs in mitochondrial RNA profiles (**Fig. 5**). Purified mitochondria were disrupted by sonication, and an S100 fraction was obtained by ultracentrifugation of the mitochondrial lysate.

In order to test for a tRNA editing activity in the *S. punctatus* S100 fraction, an *in vitro* assay developed to study *A. castellanii* 5′ tRNA editing (Price and Gray, 1999b) was employed. This assay monitors the incorporation of [α-$^{32}$P]NTPs 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-$^{32}$P]GTP resulted in the incorporation of radioactive label into tRNAs present in the S100 fraction (**Fig. 6A**, *lane 1*). Yeast tRNAs were also found to be substrates for the labelling activity (**Fig. 6A**, *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 (Price and Gray, 1999b). Optimal temperature and salt concentrations for the *S. punctatus* activity were subsequently detérmined (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 labelling efficiency. Added divalent cation was required, with peak activity at 15 mM $MgCl_2$ and loss of activity at ≥30 mM $MgCl_2$. The results in **Fig. 6A** were obtained using conditions optimized for the *S. punctatus* mitochondrial nucleotide incorporation activity.

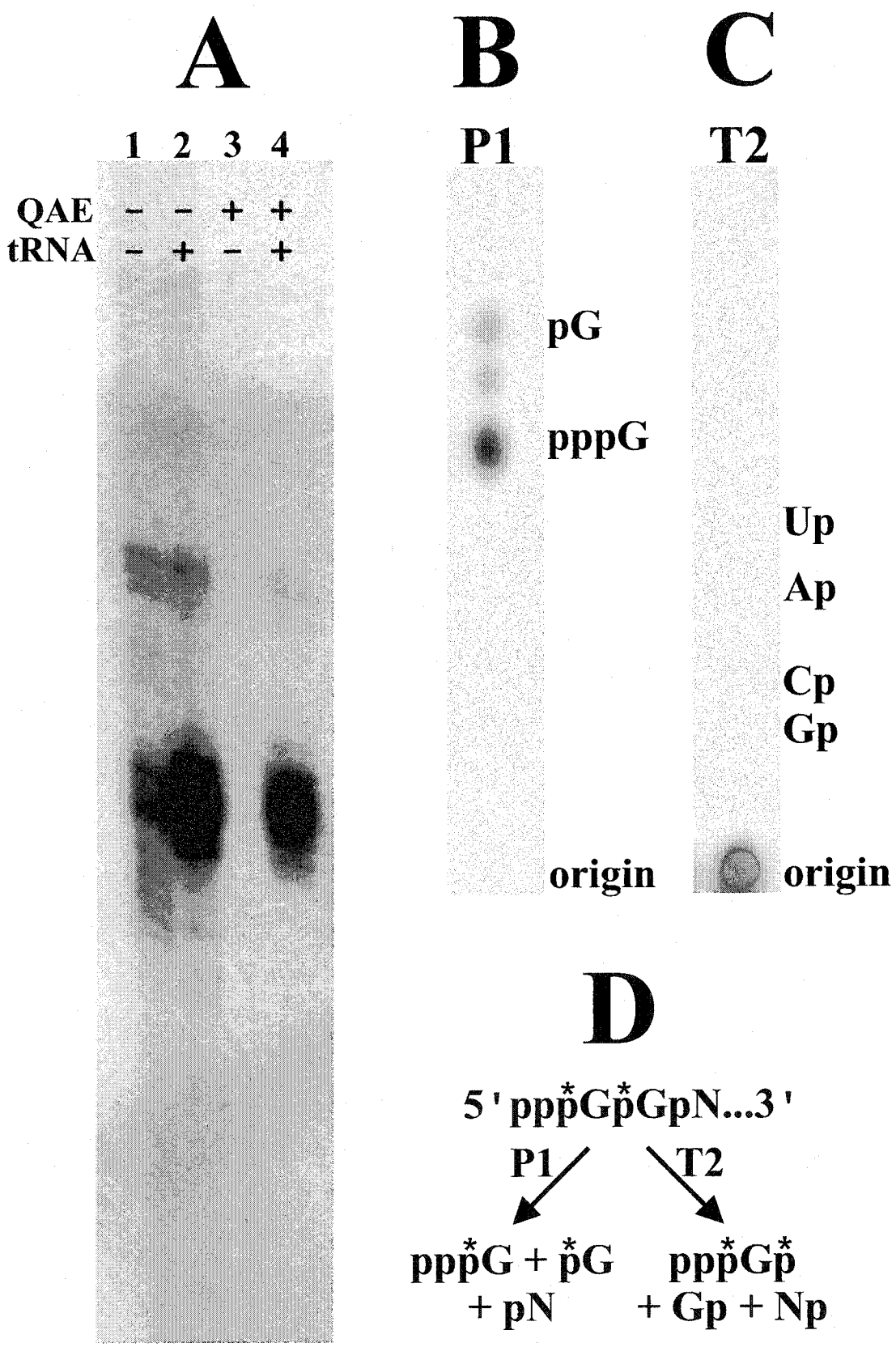
Following optimization, endogenous tRNAs were removed from the S100 by incubation of the extract with QAE-Sephadex A-50 anion exchange resin, rendering this fraction dependent on addition of exogenous tRNA substrates (compare *lanes 3* and *4*,

**Figure 5:** Profiles of RNAs isolated from *S. punctatus* cytoplasm (cyto; depleted of mitochondria) 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) rRNAs and tRNA are indicated.




**(Figure 5)**

**Figure 6:** Incorporation of label from [α-$^{32}$P]GTP into natural tRNA substrates by an *S. punctatus* mitochondrial S100 extract. **A**, autoradiogram of labelled 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 tRNAs and in the presence of [α-$^{32}$P]GTP and unlabelled CTP, ATP and UTP. The labelled tRNAs in *lane 4* were eluted from the gel and digested with either (**B**) nuclease P1 or (**C**) RNase T2 and separated by one-dimensional TLC. The migration positions of nucleotide markers are indicated. Markers in **B** were [α-$^{32}$P]GTP and non-radioactive GMP, which were separated adjacent to the P1-digested sample on the same TLC plate and visualized by autoradiography and UV-shadowing, respectively. Markers in **C** were the products of digestion of non-radioactive yeast tRNAs added to the RNase T2 digestion. The visualization (by UV-shadowing) in **C** of only monophosphate products indicates that RNase T2 was active, and that the radiolabel was not contained in a monophosphorylated species. The labelled material that migrates between pppG and pG in **B** is presumed to be ppG. **D**, expected products of nuclease P1 and RNase T2 digestion of yeast tRNAs with [α-$^{32}$P]GTP incorporated at the first and second 5′ positions. Asterisks indicate the positions of $^{32}$P.


A
1 2 3 4
QAE – – + +
tRNA – + – +
B
P1
pG
pppG
origin
C
T2
Up
Ap
Cp
Gp
origin
D
5 ' ppp*Gp*GpN...3 '
P1
T2
ppp*G + p*G
+ pN
ppp*Gp*
+ Gp + Np

**(Figure 6)**

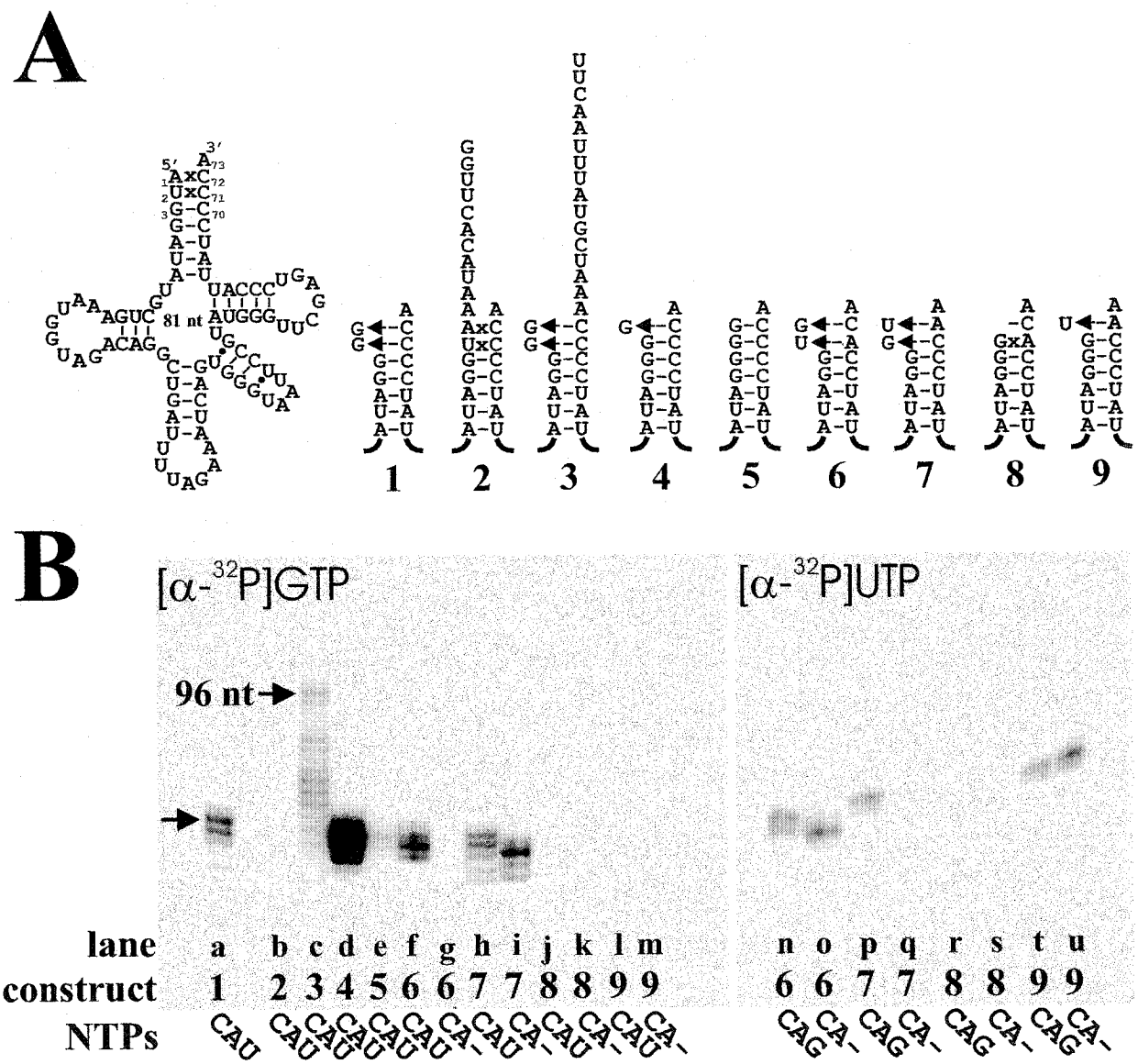
**Fig. 6A**). QAE-Sephadex-treated S100 fractions (hereinafter referred to as "the S100 extract") were used for all subsequent experiments.

When a nuclease P1 digest (which generates nucleoside 5′-phosphates, pN) of yeast tRNAs labelled with [α-$^{32}$P]GTP was analyzed by thin-layer chromatography (TLC), the majority of the label was found to be incorporated as a nucleoside triphosphate (pppG), with a minority incorporated as a nucleoside monophosphate (pG) (**Fig. 6B**). 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 *in vitro*, as is also the case for the *A. castellanii* activity (Price and Gray, 1999b). Consistent with incorporation at tRNA positions 1 and 2, TLC analysis of an RNase T2 digest (which generates nucleoside 3′-phosphates, Np) of the same material revealed only a product that did not migrate from the origin (**Fig. 6C**). Because multi-phosphorylated nucleotides are known to migrate little, if at all, from the origin in the TLC conditions used (Price and Gray, 1999b), it was possible that this product represented pppGp, the only product expected if all label was incorporated at positions 1 and 2 (**Fig. 6D**). 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*.

**2. Characterization of nucleotide incorporation using synthetic tRNA substrates**

To examine in more detail the mechanism of nucleotide incorporation into tRNA molecules, *in vitro*-transcribed tRNA constructs, based on an *A. castellanii* mitochondrial tRNA$^{Leu}$(uag) scaffold (**Fig. 7A**), were produced using T7 RNA polymerase. Specific sequence changes were engineered into the 5′ and 3′ side of the acceptor stem of this tRNA to create RNA constructs (**Fig. 7A**) that were then tested as substrates for the *S. punctatus* nucleotide incorporation activity. Transcripts were incubated with the *S. punctatus* mitochondrial S100 extract and either [α-$^{32}$P]GTP or [α-$^{32}$P]UTP, and the products were separated by denaturing polyacrylamide gel electrophoresis (**Fig. 7B**). The labelling results shown in **Fig. 7B** were consistent with the expected positions and identities of incorporated nucleotides (indicated with arrows in **Fig. 7A**).

**Figure 7:** Incorporation of label from [α-$^{32}$P]GTP and [α-$^{32}$P]UTP into *in vitro*-transcribed tRNA substrates by an *S. punctatus* mitochondrial S100 extract. **A**, tRNA constructs (based on *A. castellanii* mitochondrial tRNA$^{Leu}$(uag)) were generated by *in vitro* transcription with T7 RNA polymerase. Only acceptor stem sequences are shown; curved lines represent the remainder of the tRNA sequence, shown at left, which is identical for each construct. Arrows indicate nucleotides predicted to be incorporated after incubation with the *S. punctatus* mitochondrial S100 extract. **B**, autoradiograms of tRNA constructs 1-9 after incubation with the S100 extract, [α-$^{32}$P]GTP (*lanes a-m*) or [α-$^{32}$P]UTP (*lanes n-u*) and the combination of unlabelled nucleotides (NTPs) indicated below each lane. The labelled product indicated with an arrow in *lane a* was further analyzed (see **Fig. 8**). The size of the expected full-length (96 nt) labelled product of construct 3 is indicated in *lane c*.


A
81 nt
1
2
3
4
5
6
7
8
9
B
[α-32P]GTP
[α-32P]UTP
96 nt
lane a b c d e f g h i j k l m n o p q r s t u
construct 1 2 3 4 5 6 6 7 7 8 8 9 9 6 6 7 7 8 8 9 9
NTPs CAU CAU CAU CAU CAU CA_ CAU CA_ CAU CA_ CAU CA_ CAG CA_ CAG CA_ CAG CA_ CAG CA_

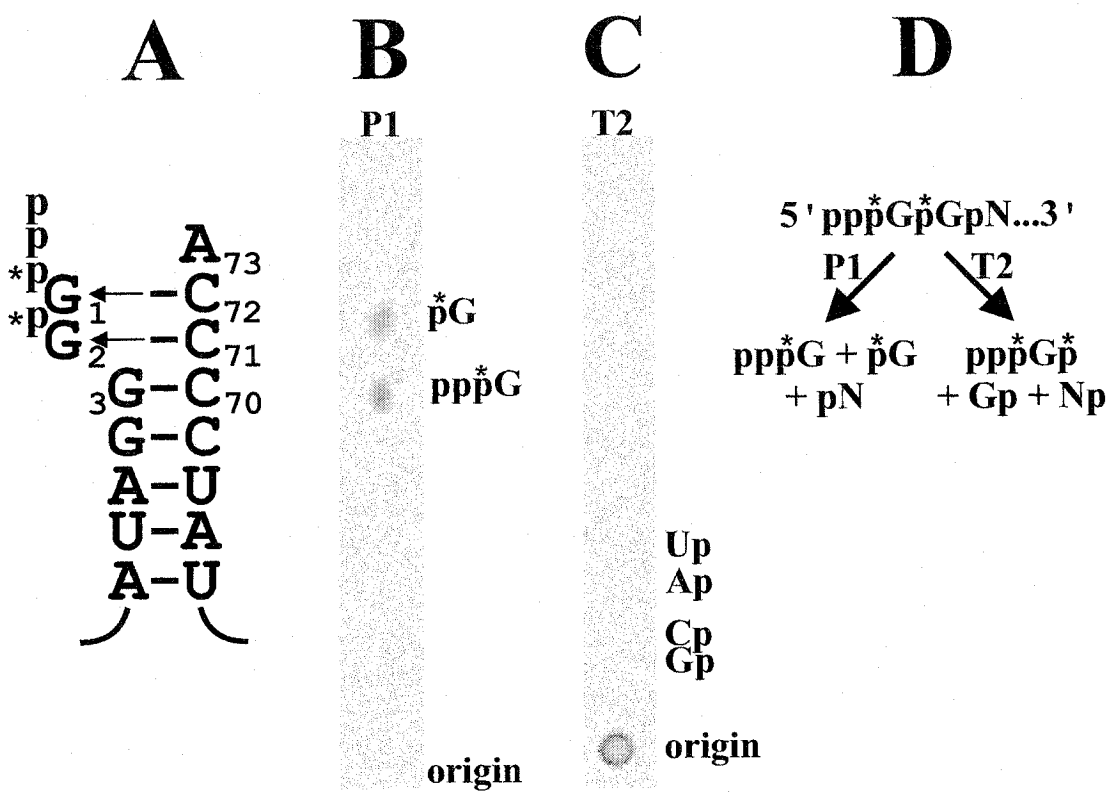
**(Figure 7)**

Construct 1 (**Fig. 8A**) was designed to direct G incorporation at positions 2 and 1, templated by C71 and C72, respectively. Construct 1 was labelled by incubation with the S100 extract in the presence of [α-$^{32}$P]GTP (**Fig. 7B**, *lane a*). TLC analysis of a nuclease P1 digest of the largest labelled band (indicated with an arrow in **Fig. 7B**, *lane a*) revealed that incorporation was 1 pG:1 pppG (**Fig. 8B**). TLC analysis of an RNase T2 digest of the same material gave a product that did not migrate from the origin (**Fig. 8C**), assumed to be pppGp (see above). These results indicated that nucleotide incorporation was occurring as expected at the 5′ end of this construct, specifically at positions 2 and 1 (**Fig. 8D**).

Construct 2 (**Fig. 7A**) had a 13-nt 5′ extension relative to construct 1. No radioactive products were observed after incubation of this RNA with [α-$^{32}$P]GTP and the S100 extract (**Fig. 7B**, *lane b*). The absence of labelled products suggests that processing of tRNA 5′ extensions occurs inefficiently or not at all in the *S. punctatus* mitochondrial extract with this particular substrate. In contrast, label from [α-$^{32}$P]GTP was incorporated into construct 3 (**Fig. 7B**, *lane c*), which had a 15-nt 3′ extension relative to construct 1 (**Fig. 9A**). This result suggests that the 5′ nucleotide incorporation activity is relatively insensitive to the state of 3′ processing.

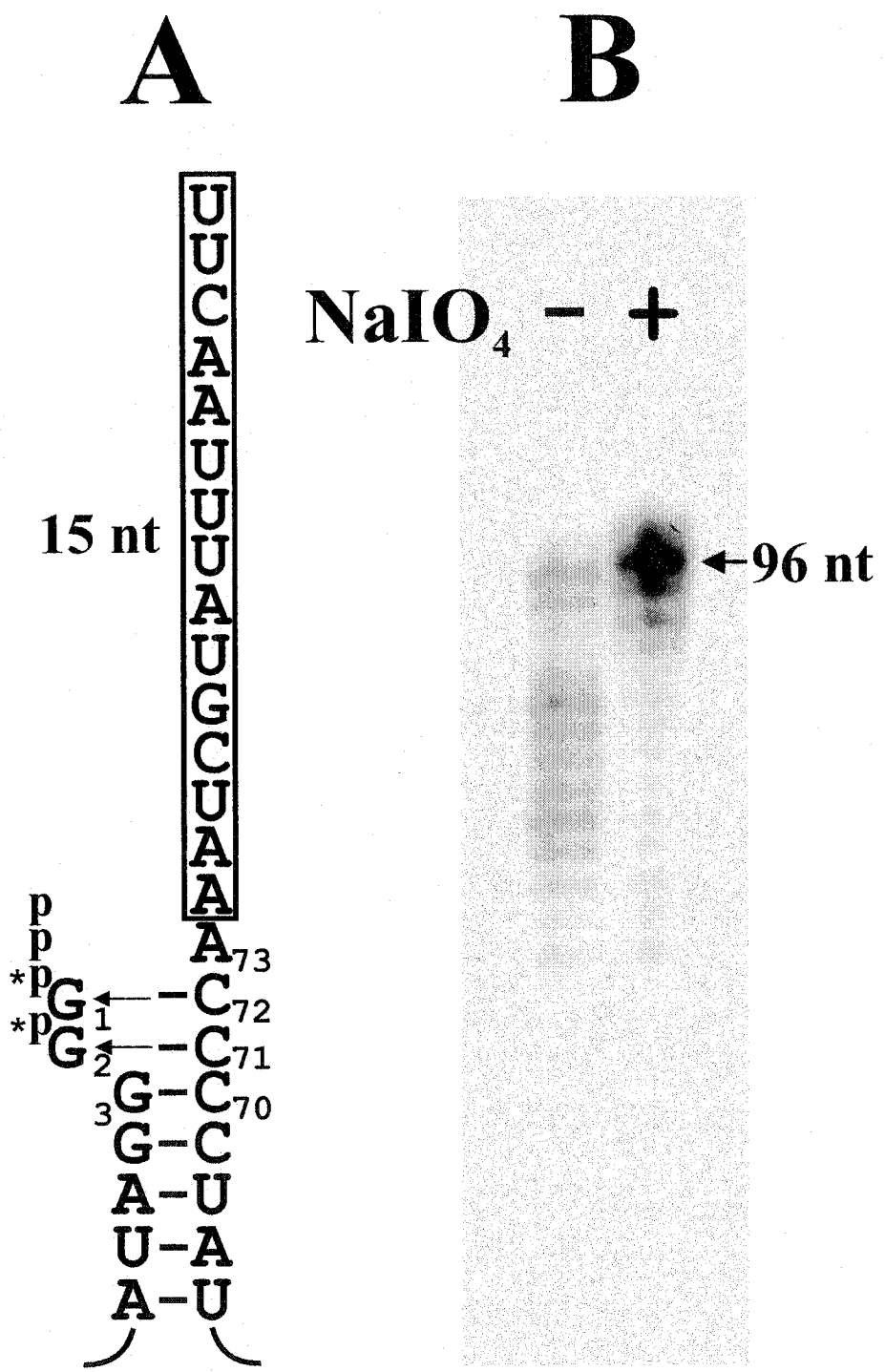
Many shorter labelled products of construct 3 in addition to the expected 96-nt (94 nt + 2 added $^{32}$P-labelled G residues = 96 nt) full-length product were observed (**Fig. 7B**, lane c), suggesting that degradation of the 3′-end of this construct was occurring during incubation with the extract. To test this possibility, the construct was subjected to periodate oxidation, a treatment that breaks the ribose ring of the 3′ nucleotide in an RNA molecule, thereby converting the 2′,3′ *cis*-diol to vicinal aldehydes. This treatment is expected to render the product a poor substrate for 3′-to-5′ exonuclease activities. As anticipated, the amount of label in the shorter products was significantly reduced by periodate oxidation (**Fig. 9B**), with the majority of label now appearing in the expected full-length product. These results indicate (i) that the activity recognizes and incorporates 2 nucleotides at the 5′ end of construct 3 despite the absence of 3′ processing; (ii) that the 3′ extension does not act as a template for further nucleotide

**Figure 8:** Incorporation of label from [α-$^{32}$P]GTP into tRNA construct 1 by an *S. punctatus* mitochondrial S100 extract. **A**, acceptor stem sequence of construct 1. $^{32}$P-labelled G residues are predicted to be incorporated at positions 1 and 2 after incubation with the S100 extract. Asterisks indicate the positions of $^{32}$P. Following incubation of construct 1 with the S100 extract in the presence of [α-$^{32}$P]GTP and unlabelled CTP, ATP and UTP, the longest labelled product (indicated with an arrow in **Fig. 7**, *lane a*) was eluted from the gel and digested with either (**B**) nuclease P1 or (**C**) RNase T2, and the products were separated by one-dimensional TLC. The migration positions of nucleotide markers are indicated. **D**, expected products of nuclease P1 and RNase T2 digestion of construct 1 with [α-$^{32}$P]GTP incorporated at the first and second 5′ positions.


A
B
C
D
P1
T2
p
p
*p
*p
G
G
1
2
3
A 73
C 72
C 71
G–C 70
G–C
A–U
U–A
A–U
*pG
pp*pG
origin
Up
Ap
Cp
Gp
origin
5 ' ppp*Gp*GpN...3 '
P1
T2
ppp*G + p*G
+ pN
ppp*Gp*
+ Gp + Np

**(Figure 8)**

**Figure 9:** Effect of periodate treatment on incorporation of label from [α-$^{32}$P]GTP into tRNA construct 3 by an *S. punctatus* mitochondrial S100 extract. **A**, acceptor stem sequence of construct 3. $^{32}$P-labelled G residues are predicted to be incorporated at positions 1 and 2 after incubation with the S100 extract. Asterisks indicate the positions of $^{32}$P. The 15-nt 3′ extension (relative to construct 1) is boxed. **B**, autoradiogram of construct 3, untreated (-) or pretreated (+) with sodium periodate ($NaIO_4$) prior to incubation with the S100 extract in the presence of [α-$^{32}$P]GTP and unlabelled CTP, ATP and UTP. The predominant 96-nt labelled product obtained with periodate-treated tRNA construct 3 is indicated with an arrow.




(Figure 9)

addition at the 5′ end past position 1; and (iii) that an unidentified 3′-to-5′ exonuclease is active in the S100 extract.

Construct 4 has an additional G at position 2 and construct 5 has additional Gs at positions 2 and 1, relative to construct 1 (**Fig. 7A**). Construct 4 was designed to incorporate one G only at position 1, whereas construct 5 was designed to 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. 7B**, *lane d*). Construct 5, on the other hand, was a poor substrate for the activity (**Fig. 7B**, *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.

### 3. *cis*-Templated 3′-to-5′ nucleotide incorporation

Constructs 6-9 (**Fig. 7A**) 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 (Price and Gray, 1999b).

Construct 6 was designed to incorporate U at position 2 and G at position 1. Labelled products were observed after incubation with the extract in the presence of [α-$^{32}$P]GTP and UTP (**Fig. 7B**, *lane f*). When UTP was not present during incubation, no labelled products were observed (**Fig. 7B**, *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-$^{32}$P]UTP was used, labelling 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. Labelled products were observed with [α-$^{32}$P]GTP in both the presence and absence of UTP, with the product in the absence of UTP (**Fig. 7B**, *lane i*) shorter than when UTP was present (*lane h*). When [α-$^{32}$P]UTP was used, labelling 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 G2xA71 mismatch) prior to addition of U at position 2 and G at position 1. However, no labelled products were observed for this construct following incubation with the extract and either [α-$^{32}$P]GTP (**Fig. 7B**, *lanes j* and *k*) or [α-$^{32}$P]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.

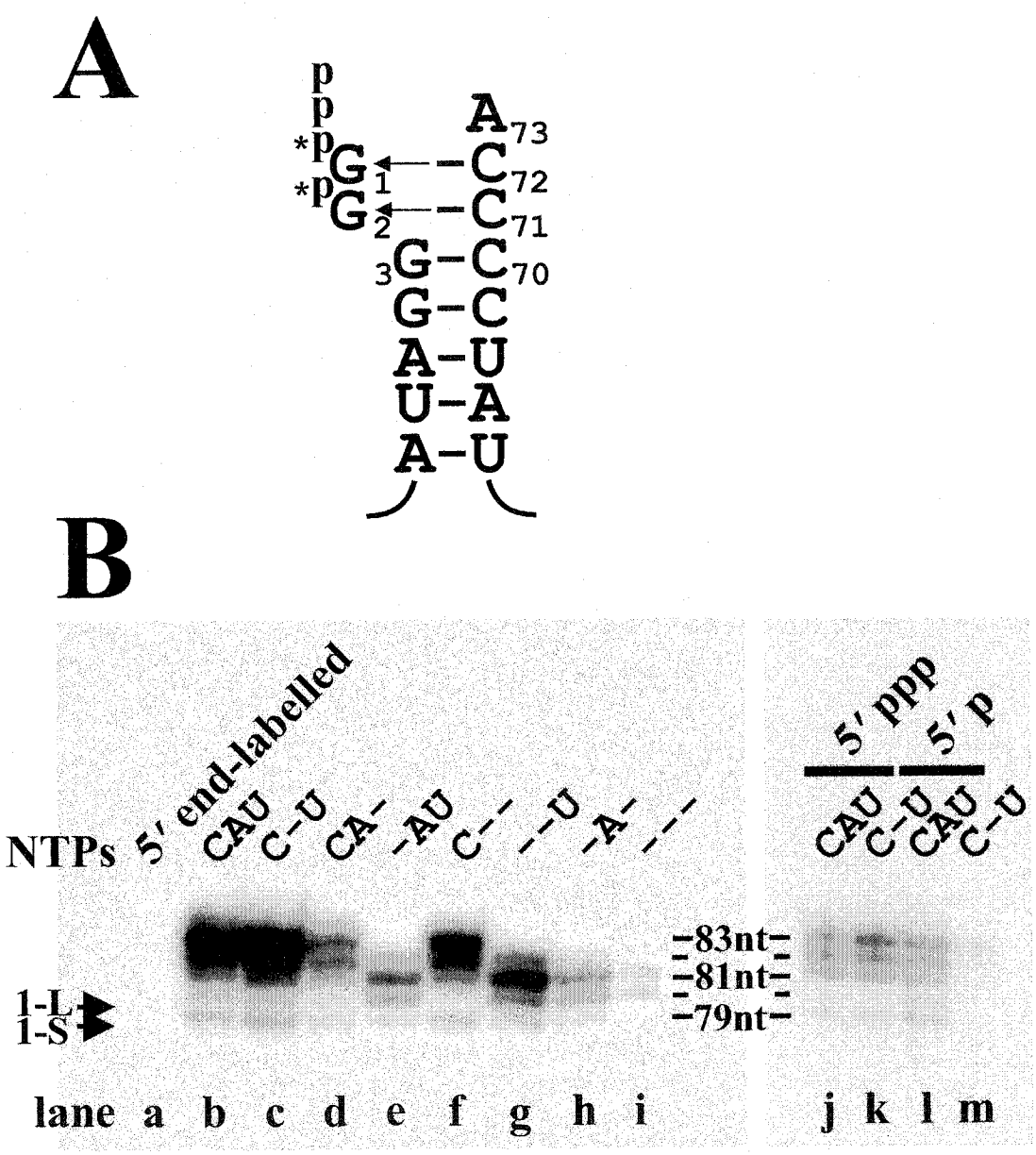
Finally, construct 9 was designed to incorporate a U at position 1. This construct was not labelled with [α-$^{32}$P]GTP (**Fig. 7B**, *lanes l* and *m*), but gave the same-sized labelled products with [α-$^{32}$P]UTP in the presence (*lane t*) and absence (*lane u*) of GTP, as expected. These combined 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.

**4. Nucleotide requirements for incorporation**

To determine the nucleotide requirements of the incorporation activity, construct 1 (**Fig. 10A**) was incubated with [α-$^{32}$P]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-$^{32}$P]GTP into this construct (**Fig. 10B**, *lanes b-i*). The size and intensity of the labelled products, however, varied depending on which of these NTPs were included in the reaction mix. Through subsequent analyses, the observed size variation was attributed to an activity incorporating C residues at tRNA 3′-ends (see below). The reason(s) for variation in labelling intensity remain(s) unknown, as further experiments did not indicate any role for CTP (see **Fig. 12**), UTP (not shown) or ATP (at least when the recessed tRNA 5′ end is triphosphorylated; see below) in this reaction.

The results shown in **Fig. 10B** were obtained with an untreated T7 transcript having a triphosphorylated 5′ end. When the 5′ end was converted from a triphosphate

**Figure 10:** Nucleotide requirements for incorporation of label from [α-$^{32}$P]GTP into tRNA construct 1 by an *S. punctatus* mitochondrial S100 extract. **A**, acceptor stem sequence of construct 1. $^{32}$P-labelled G residues are predicted to be incorporated at positions 1 and 2 after incubation with the S100 extract. Asterisks indicate the positions of $^{32}$P. **B**, autoradiograms of construct 1 after incubation with the S100 extract in the presence of [α-$^{32}$P]GTP and the combination of unlabelled NTPs indicated above each lane. 5′-End-labelled construct 1 is included in *lane a*, and the two constituent labelled RNAs of 79 nt (1-S) and 80 nt (1-L) are indicated. Prior to incubation with the S100 extract, the 5′ end of construct 1 was either triphosphorylated (*lanes a-k*) or monophosphorylated (*lanes l* and *m*).


A
p
p
*p
*p
G
G
1
2
3
A 73
C 72
C 71
G–C 70
G–C
A–U
U–A
A–U
B
NTPs
5′ end-labelled
CAU
C–U
CA–
–AU
C–
–U
–A
–
5′ ppp
5′ p
CAU
C–U
CAU
C–U
1-L
1-S
83nt
81nt
79nt
lane a b c d e f g h i
j k l m

**(Figure 10)**

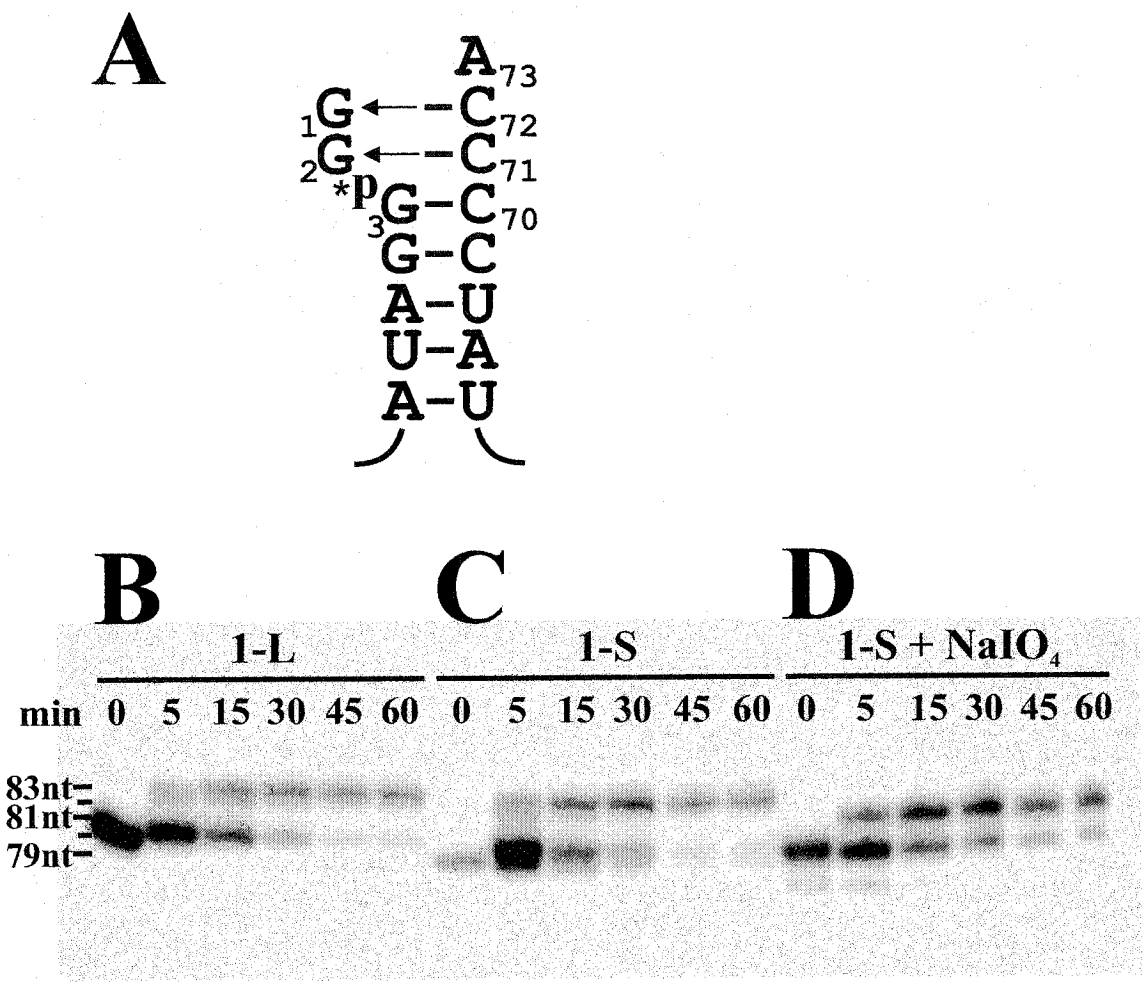
to a monophosphate by dephosphorylation with alkaline phosphatase followed by re-phosphorylation using polynucleotide kinase, ATP was then required for incorporation of label from [α-$^{32}$P]GTP (**Fig. 10B**, compare *lanes l* and *m*). No labelling was observed when the tRNA substrate had a 5′ hydroxyl terminus (not shown). These results indicate that an activated 5′ end is necessary for nucleotide incorporation, and that ATP can provide the necessary activation energy when the 5′ end of a tRNA substrate is monophosphorylated. It is likely that this activation energy comes in the form of an adenylylated tRNA intermediate (see Discussion).

In order to determine how many nucleotides were incorporated into construct 1 after incubation with the extract and [α-$^{32}$P]GTP, the T7 transcript was first 5′-end-labelled with polynucleotide kinase and [γ-$^{32}$P]ATP and then electrophoresed next to [α-$^{32}$P]GTP-labelled material. Two labelled products in a 1:1 ratio (instead of the single expected 79-nt product) were observed in the 5′-end-labelled T7 transcription reaction (**Fig. 10B**, *lane a*). Enzymatic RNA sequencing (not shown) revealed that the shorter of the two T7 products (1-S) had the desired 79-nt sequence, whereas the longer product (1-L) had 1 additional nucleotide (a mixture of G, A, U and C) at its 3′ end. Transcript 1-L is likely the result of the T7 RNA polymerase adding a random nucleotide at the 3′ end of a portion of its products, as has been documented previously (*e.g.*, Milligan *et al.*, 1987). Following incubation of construct 1 with the S100 extract and [α-$^{32}$P]GTP, and using 1-S and 1-L as size standards, the largest labelled product (in **Fig. 10B**, *lane b*) was found to be 83 nucleotides in length.

**5. Dissociation of 5′ and 3′ nucleotide incorporation activities**

5′-End-labelled 1-L and 1-S were separated from one another by polyacrylamide gel electrophoresis. The two products were shown individually to be substrates for the nucleotide incorporation activity (**Fig. 11B,C**). After a sufficient period of incubation (~30 min), the principal products with the two RNAs were single species, 83 nucleotides in length; *i.e.*, the product after incubation of 1-L was three nucleotides longer (**Fig. 11B**) and that of 1-S four nucleotides longer (**Fig. 11C**) than the corresponding unincubated material, rather than the expected two nucleotides for each RNA. This result confirmed the observation that after incubation of unlabelled construct 1 with the S100 extract in

**Figure 11:** Autoradiogram of 5′-end-labelled tRNAs after incubation (0-60 min) with an *S. punctatus* S100 extract and unlabelled CTP, ATP, UTP and GTP. The 5′-end-labelled substrates were the (**B**) longer (1-L) and (**C**) shorter (1-S) versions of tRNA construct 1 and (**D**) 1-S pretreated with sodium periodate ($NaIO_4$). **A**, acceptor stem sequence of construct 1-S (1-L has one additional nucleotide at its 3′ end). G residues are predicted to be incorporated at positions 1 and 2 after incubation with the S100 extract in all three cases. The asterisk indicates the position of $^{32}P$.


A
A73
1G
C72
2G
C71
*p
3G
C70
G-C
A-U
U-A
A-U
B
C
D
1-L
1-S
1-S + NaIO4
min 0 5 15 30 45 60 0 5 15 30 45 60 0 5 15 30 45 60
83nt
81nt
79nt

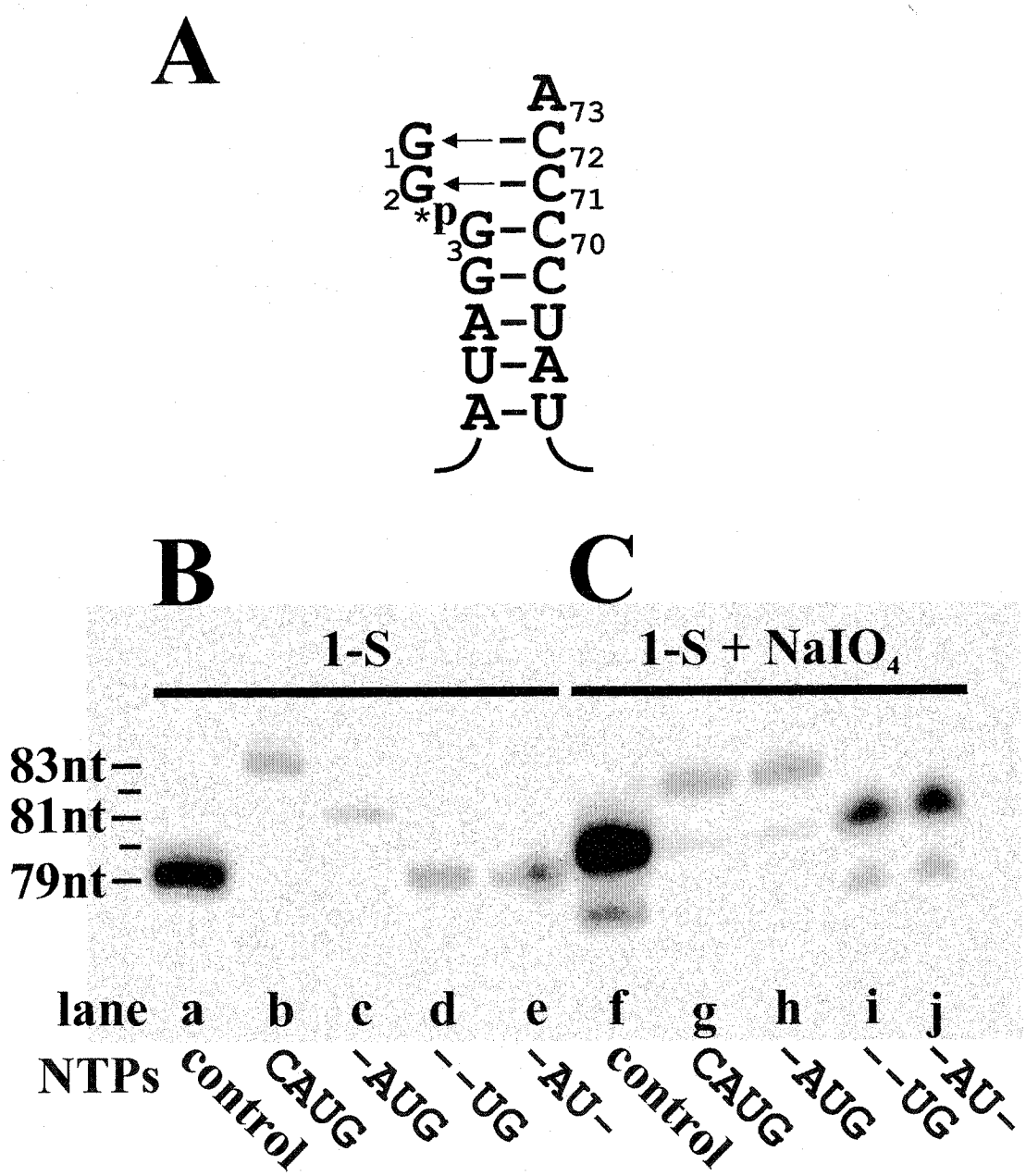
**(Figure 11)**

the presence of [α-$^{32}$P]GTP and CTP, the largest product was 83 nucleotides in length (**Fig. 10B**, *lane f*) rather than the expected 81 nucleotides. Further, in the absence of CTP, the principal labelled product was only 81 nucleotides in length (**Fig. 10B**, *lane g*).

Thus, it appeared that an activity additional to the 5′ nucleotide incorporation activity was present in the S100 extract, and that this activity was capable of incorporating C residues into tRNA substrates. A well-known activity that catalyzes this type of reaction is ATP(CTP):tRNA nucleotidyltransferase, the ubiquitous enzyme that adds the 3′-terminal CCA sequence to tRNA. To test the idea of nucleotide addition to tRNA 3′ ends, 1-S (5′-end labelled; **Fig. 11A**) was subjected to periodate oxidation and then incubated with the S100 extract and all four unlabelled NTPs. After incubation, $NaIO_4$-treated 1-S was 81 nucleotides in length; *i.e.*, only two nucleotides longer than the unincubated material (**Fig. 11D**; note that $NaIO_4$-treated RNA species migrate slightly more slowly than the corresponding untreated RNAs). This result is consistent with the interpretation that periodate oxidation blocks the addition of two nucleotides to the 3′ end of this particular tRNA substrate.

To further confirm that CTP was the nucleotide whose addition had been blocked by the periodate treatment, 5′-end-labelled 1-S (untreated or treated with $NaIO_4$; **Fig.12A**) was incubated with the S100 extract in the presence or absence of CTP. Whereas the size of the untreated product was two nucleotides shorter in the absence (81 nt) than in the presence (83 nt) of CTP (**Fig. 12B**, compare *lanes b* and *c*), the $NaIO_4$-treated product remained the same size (81 nt) in the presence and absence of CTP (**Fig. 12C**, *lanes g* and *h*). The absence of CTP and either GTP or ATP resulted in no increase in the size of the labelled material (79 nt) following incubation with the S100 extract, whether or not the substrate had been treated with $NaIO_4$ prior to incubation (**Fig. 12B**, *lanes d* and *e*; **Fig. 12C**, *lanes i* and *j*). These results, together with other data presented here, strongly support the idea that two G residues are added to the 5′ end of this tRNA in a reaction that requires ATP, and that two C residues are added to the 3′ end of the tRNA construct, likely by the mitochondrial ATP(CTP):tRNA nucleotidyltransferase. Why the full CCA sequence is not added to the 3′ end of the construct is not known; it is possible that *in vitro* conditions for the addition of the 3′-terminal A residue are sub-optimal.

**Figure 12:** Autoradiogram of 5′-end-labelled tRNAs incubated with an *S. punctatus* mitochondrial S100 extract and various NTPs. Incubations were for 30 min with the NTPs indicated below each lane. 'Control' (*lanes a* and *f*) indicates that the RNA was not incubated with the S100 extract. The 5′-end-labelled substrates were (**B**) the shorter version of tRNA construct 1 (1-S) and (**C**) 1-S pretreated with sodium periodate, $NaIO_4$). **A**, acceptor stem sequence of construct 1-S. G residues are predicted to be incorporated at positions 1 and 2 after incubation with the S100 extract in both cases. The asterisk indicates the position of $^{32}P$.


A
A73
1G
C72
2G
C71
*pG
3G
C70
G–C
A–U
U–A
A–U
B
C
1-S
1-S + NaIO4
83nt
81nt
79nt
lane a b c d e f g h i j
NTPs control CAUG -AUG --UG -AU- control CAUG -AUG --UG -AU-

**(Figure 12)**

## B. *In vitro* Characterization of a tRNA Editing Activity in the Mitochondria of *Acanthamoeba castellanii*

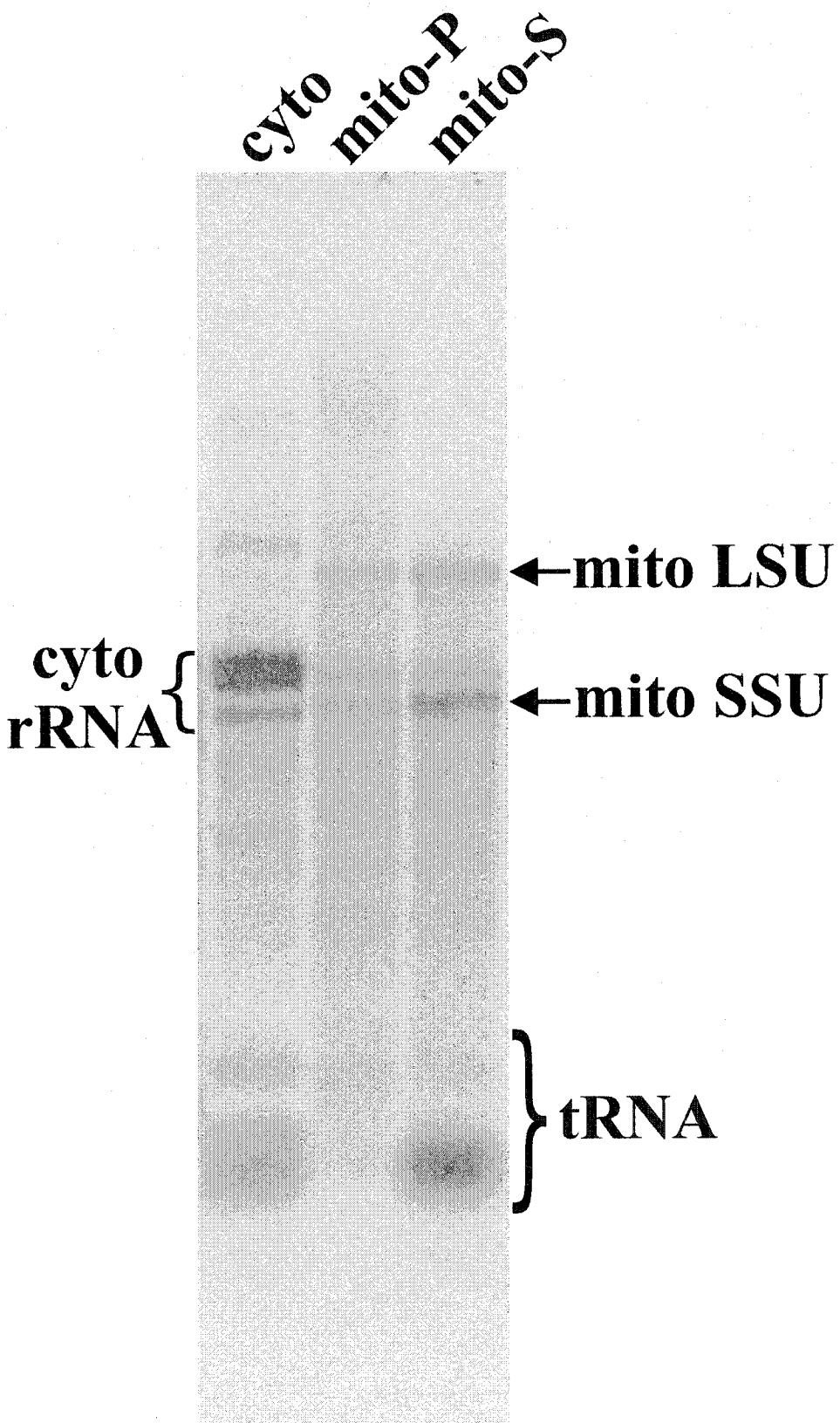
### 1. Development of an *in vitro* assay of nucleotide incorporation

The *in vitro* data presented in the preceding section demonstrate an activity in an *S. punctatus* mitochondrial S100 extract equivalent to that previously described in *A. castellanii* mitochondrial S100 extracts (Price and Gray, 1999b). To confirm and expand upon the results of Price and Gray (1999b), I prepared a mitochondrial extract following their procedures. Intact mitochondria were isolated from *A. castellanii* by cell lysis in a Dounce homogenizer followed by differential centrifugation and purification on a sucrose step gradient. This procedure resulted in significant mitochondrial enrichment, as estimated by the ratio of mitochondrial to cytoplasmic rRNAs in mitochondrial RNA profiles (**Fig. 13**). Purified mitochondria were disrupted by sonication, and an S100 fraction was obtained by ultracentrifugation of the mitochondrial lysate.

This fraction was then tested for nucleotide incorporation activity. Incubation of the *A. castellanii* mitochondrial S100 fraction in the presence of [α-$^{32}$P]GTP resulted in the incorporation of label into tRNAs present in the S100 fraction (not shown). Yeast tRNAs were also found to be substrates for the labelling activity (**Fig. 14A**, *lane 5*). To remove the endogenous tRNAs present in the mitochondrial extract, I incubated the extract with DEAE-Sephacel anion exchange resin, which is known to bind the nucleotide incorporation activity (Price and Gray, 1999b). The resin-bound activity was washed with a solution containing 100 mM KCl (**Fig. 14A**, *lane 3*) and then eluted from the resin by the addition of a solution containing 300 mM KCl (**Fig. 14A**, *lane 5*). The resulting extract (hereinafter referred to as "the S100 extract"), rendered dependent on the addition of exogenous tRNA to observe labelling with radioactive nucleotides (compare *lanes 5* and *6*, **Fig. 14A**), was used for all subsequent experiments.

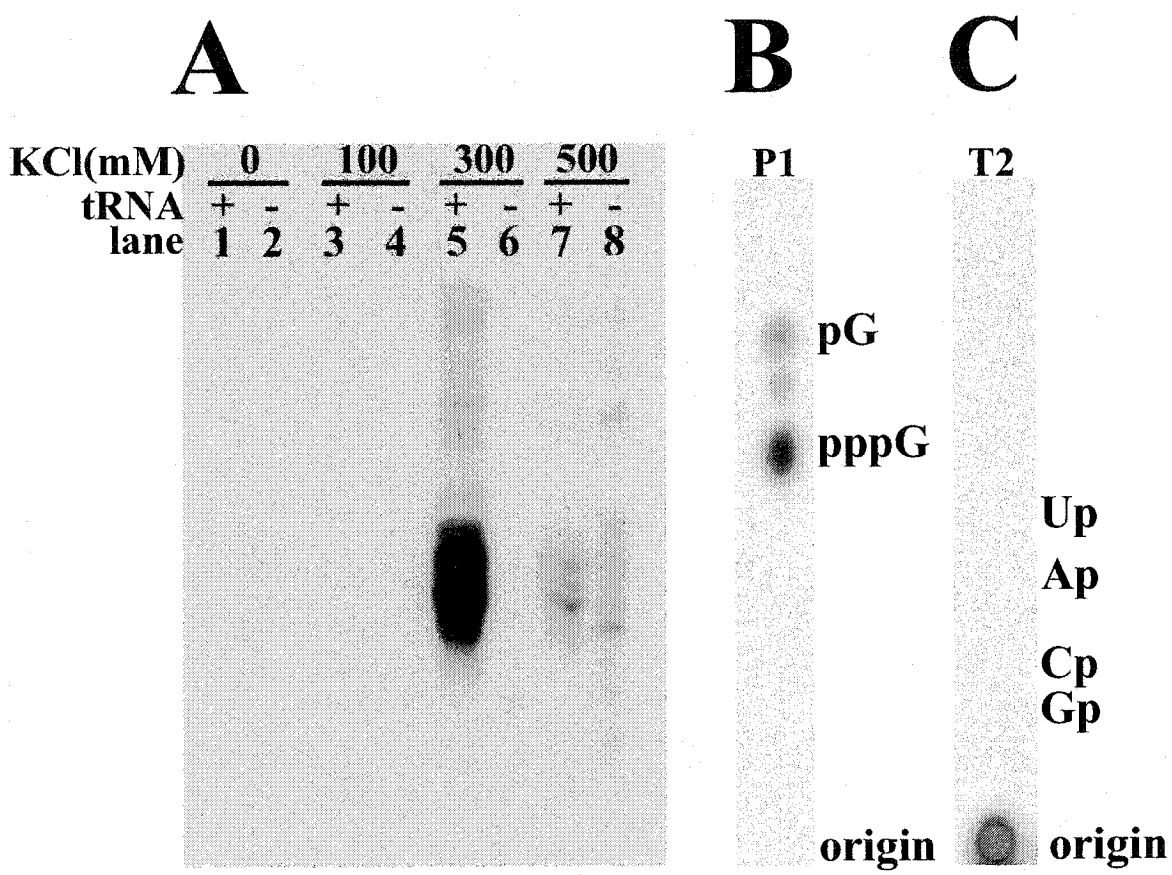
When a nuclease P1 digest (which generates nucleoside 5′-phosphates, pN) of yeast tRNAs labelled with [α-$^{32}$P]GTP by the S100 extract was analyzed by TLC, the majority of the label was found to be incorporated as a nucleoside triphosphate (pppG), with a minority incorporated as a nucleoside monophosphate (pG) (**Fig. 14B**). 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

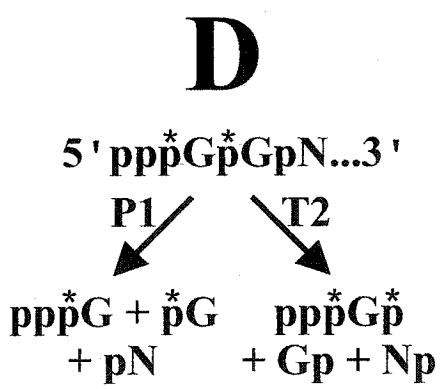
**Figure 13:** Profiles of RNAs isolated from *A. castellanii* cytoplasm (cyto; depleted of mitochondria) and mitochondria (mito). Purified mitochondria were lysed with Triton X-100 and fractionated by centrifugation at 9000 *g* into pellet (mito-P) and supernatant (mito-S) fractions. Samples were incubated with DNase I, electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining. The positions of the mitochondrial large subunit (LSU) and small subunit (SSU) rRNAs and tRNAs are indicated. The positions of the cytoplasmic LSU and SSU rRNA are uncertain, due to fragmentation of the LSU rRNA (D'Alessio *et al.*, 1981).




(Figure 13)

**Figure 14:** Incorporation of label from [α-$^{32}$P]GTP into yeast tRNAs by an *A. castellanii* mitochondrial S100 extract. **A**, autoradiogram of labelled tRNAs following incubation of the *A. castellanii* mitochondrial S100 extract, preincubated with DEAE-Sephacel resin and eluted with the concentration of KCl indicated above each lane, with (+) or without (-) addition of yeast tRNAs and in the presence of [α-$^{32}$P]GTP and unlabelled CTP, ATP and UTP. The labelled tRNAs in *lane 5* were eluted from the gel and digested with either (**B**) nuclease P1 or (**C**) RNase T2 and separated by one-dimensional TLC. The migration positions of nucleotide markers are indicated. Markers in **B** were [α-$^{32}$P]GTP and non-radioactive GMP, which were separated adjacent to the P1-digested sample on the same TLC plate and visualized by autoradiography and UV-shadowing, respectively. Markers in **C** were the products of digestion of non-radioactive yeast tRNAs added to the RNase T2 digestion. The visualization (by UV-shadowing) in **C** of only monophosphate products indicates that RNase T2 was active, and that the radiolabel was not contained in a monophosphorylated species. The labelled material that migrates between pppG and pG in **B** is presumed to be ppG. **D**, expected products of nuclease P1 and RNase T2 digestion of yeast tRNAs with [α-$^{32}$P]GTP incorporated at the first and second 5′ positions. Asterisks indicate the positions of $^{32}$P.


A
B
C
KCl(mM) 0 100 300 500
tRNA + - + - + - + -
lane 1 2 3 4 5 6 7 8
P1
T2
pG
pppG
Up
Ap
Cp
Gp
origin
origin


D
5 ' ppp*G*pGpN...3 '
P1
T2
ppp*G + p*G
+ pN
ppp*Gp*
+ Gp + Np

**(Figure 14)**

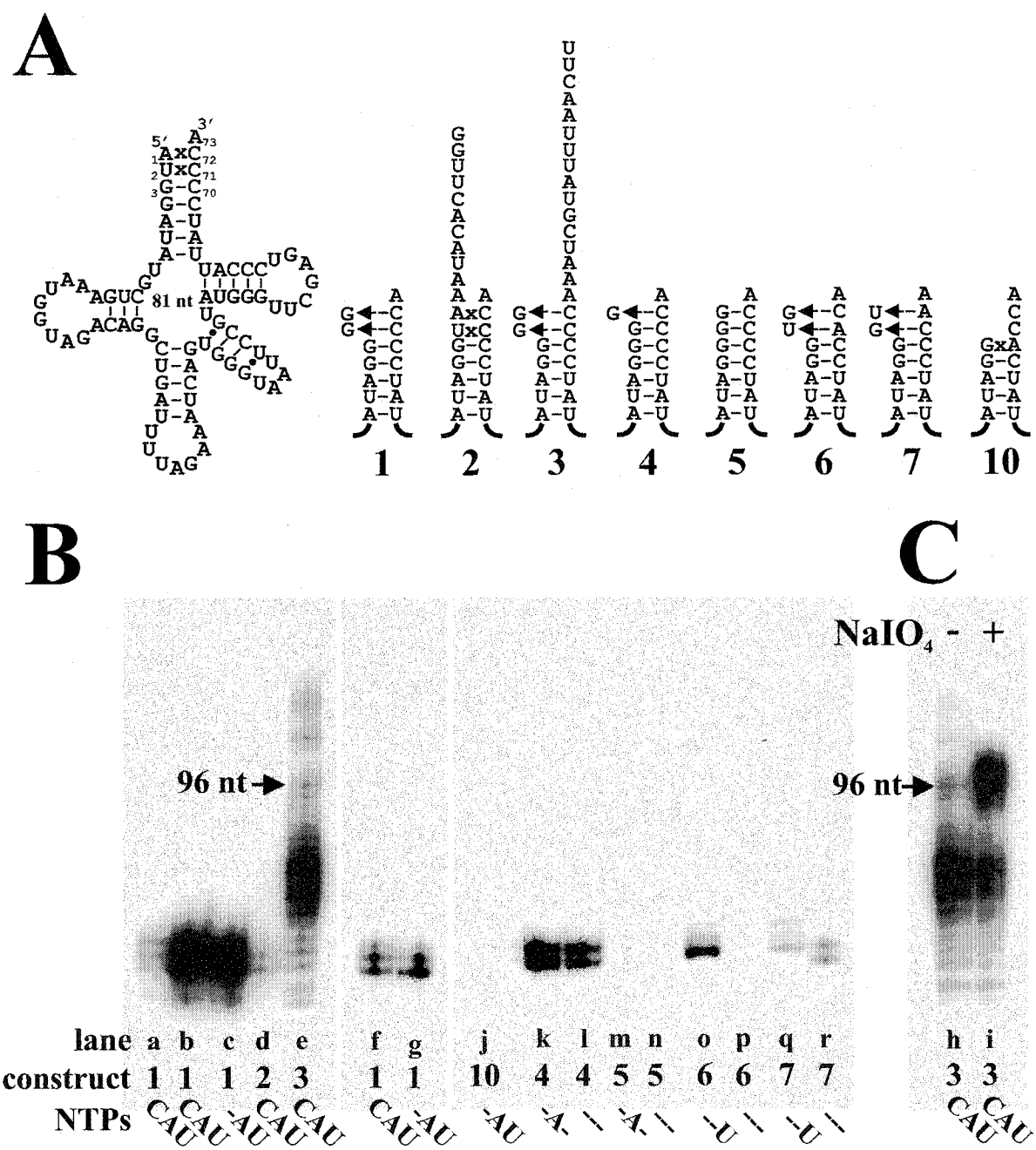
triphosphates, as was previously shown by Price and Gray (1999b). Consistent with incorporation at tRNA positions 1 and 2, TLC analysis of an RNase T2 digest (which generates nucleoside 3′-phosphates, Np) of the same material revealed only a product that did not migrate from the origin (**Fig. 14C**). Because multi-phosphorylated nucleotides are known to migrate little, if at all, from the origin in the TLC conditions used (Price and Gray, 1999b), it was possible that this product represented pppGp, the only product expected if all label was incorporated at positions 1 and 2 (**Fig. 14D**). Based on these results, it was likely that the nucleotide incorporation activity observed here was the same as that described by Price and Gray (1999b).

**2. Characterization of nucleotide incorporation using synthetic tRNA substrates**

To examine in more detail the mechanism of nucleotide incorporation into tRNA molecules, *in vitro*-transcribed tRNA constructs, based on an *A. castellanii* mitochondrial $tRNA^{Leu}$(uag) scaffold (**Fig. 15A**), were produced using T7 RNA polymerase. These constructs (**Fig. 15A**) were then tested as substrates for the *A. castellanii* nucleotide incorporation activity by incubation with the *A. castellani* mitochondrial S100 extract and [α-$^{32}$P]GTP. The products of this incubation were then separated by denaturing polyacrylamide gel electrophoresis (**Fig. 15B**). The labelling results shown in **Fig. 15B** were consistent with the expected positions and identities of incorporated nucleotides (indicated with arrows in **Fig. 15A**).

Construct 1, designed to direct G incorporation at positions 2 and 1 (**Fig. 15A**), was labelled by incubation with the S100 extract in the presence of [α-$^{32}$P]GTP (**Fig. 15B**, *lane b* and *c*; a shorter exposure is shown in *lanes f* and *g*). The main product (the smaller of the two most prominent products in *lane f*), however, was found to be 2 nucleotides smaller than the largest product obtained after labelling with the *S. punctatus* mitochondrial S100 extract and [α-$^{32}$P]GTP (**Fig. 15B**, *lane a*). As shown in the previous section, the *S. punctatus* mitochondrial S100 extract has both 5′ and 3′ nucleotide incorporation activities that add two Gs and two Cs, respectively, to this construct. As the nucleotide incorporation assay employed here traces the incorporation of radiolabelled Gs, a likely explanation for the size difference is that an activity equivalent to that responsible for the incorporation of Cs at tRNA 3′ ends in the

**Figure 15:** Incorporation of label from [α-$^{32}$P]GTP into *in vitro*-transcribed tRNA substrates by an *A. castellanii* mitochondrial S100 extract. **A**, tRNA constructs (based on *A. castellanii* mitochondrial tRNA$^{Leu}$(uag)) were generated by *in vitro* transcription with T7 RNA polymerase. Only acceptor stem sequences are shown; curved lines represent the remainder of the tRNA sequence, shown at left, which is identical for each construct. Arrows indicate nucleotides predicted to be incorporated after incubation with the *A. castellanii* mitochondrial S100 extract. **B**, autoradiograms of tRNA constructs 1-7 and 10 after incubation with the *A. castellanii* mitochondrial S100 extract, [α-$^{32}$P]GTP and the combination of unlabelled nucleotides (NTPs) indicated below each lane (*lanes b-r*). Construct 1 after incubation with the *S. punctatus* mitochondrial S100 extract, [α-$^{32}$P]GTP and the combination of unlabelled nucleotides (NTPs) indicated below each lane (as in **Fig. 7B**, *lane a*) was included as a size marker (*lane a*). Lanes *f* and *g* are shorter exposures of lanes *b* and *c*. The size of the expected full-length (96 nt) labelled product of construct 3 is indicated with an arrow in *lanes e* and *h*. **C**, autoradiogram of construct 3, untreated (-) or pretreated (+) with sodium periodate ($NaIO_4$) prior to incubation with the *A. castellanii* mitochondrial S100 extract in the presence of [α-$^{32}$P]GTP and unlabelled CTP, ATP and UTP.


A
81 nt
1
2
3
4
5
6
7
10
B
C
NaIO4 - +
96 nt
96 nt
lane a b c d e f g j k l m n o p q r h i
construct 1 1 1 2 3 1 1 10 4 4 5 5 6 6 7 7 3 3
NTPs CAU CAU -AU CAU CAU CAU -AU -AU -A_ \ -A_ \ -U \ -U \ CAU CAU

**(Figure 15)**

*S. punctatus* extract was either not active or not present in the *A. castellanii* extract. In support of this idea, the product obtained after incubation with the *A. castlellanii* mitochondrial S100 extract was the same size in the presence or absence of CTP (compare *lanes f* and *g*, **Fig. 15B**). Since labelled products are observed in both the presence and absence of CTP, it can be inferred that the CCA sequence is not required for incorporation of nucleotides at tRNA 5′ ends by the *A. castellanii* editing activity.

Construct 2 (**Fig. 15A**), with a 13-nt 5′ extension relative to construct 1, was labelled very poorly after incubation of this RNA with [α-$^{32}$P]GTP and the S100 extract (**Fig. 15B**, *lane d*). There were minor labelled products, however, that were of the same size as those obtained with construct 1 (compare *lanes d* and *f*, **Fig. 15B**). This suggests that processing of tRNA 5′ extensions occurs inefficiently in the *A. castellanii* mitochondrial S100 extract, at least with this particular substrate (see Discussion).

In contrast to construct 2, label from [α-$^{32}$P]GTP was incorporated into construct 3 (**Fig. 15B**, *lane e*), which had a 15-nt 3′ extension relative to construct 1 (**Fig. 15A**). This result suggests that the 5′ nucleotide incorporation activity is relatively insensitive to the state of 3′ processing. As was observed in the previous section with the *S. punctatus* mitochondrial S100 extract, various labelled products, mostly smaller than the expected 96-nt (94 nt + 2 added $^{32}$P-labelled G residues = 96 nt; see arrow in **Fig. 15B**, *lane e*) full-length edited product, appear after incubation with the extract. Based on this result, it seemed likely that, as in *S. punctatus*, a 3′-to-5′ exonuclease was present in *A. castellanii* mitochondrial extracts that was capable of degrading the 3′ extension on this construct. The presence of labelled products larger than 96 nucleotides further suggested that either an activity was adding nucleotides to the 3′ end of this construct, or that the 5′ nucleotide incorporation activity was continuing past position 1, possibly using the 3′ extension as a template. To test these ideas, construct 3 was subjected to periodate oxidation (**Fig. 15C**) prior to incubation with the S100 extract. The appearance of labelled products additional to the expected 96-nt product was reduced when the 3′ end of construct 3 was modified with $NaIO_4$ prior to the *in vitro* labelling assay (**Fig. 15C**, *lane i*). Further, the majority of label now appeared to be located within the expected full-length (96-nt) edited product. These results indicate (i) that the activity recognizes

and incorporates 2 nucleotides at the 5′ end of construct 3 despite the absence of 3′ processing; (ii) that the 3′ extension does not act as a template for further nucleotide addition at the 5′ end past position 1; and (iii) that an unidentified 3′-to-5′ exonuclease is active in this S100 extract, as it is in that of *S. punctatus*.

Construct 10, with a G3xA70 mismatch, was also shown to be a poor substrate for the *A. castellanii* activity (**Fig. 15B**, *lane j*). This result suggests that mismatched nucleotides are not efficiently removed from the 5′ ends of synthetic tRNA substrates in this assay. It also suggested 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.

Construct 4 has an additional G at position 2 and construct 5 has additional Gs at positions 2 and 1, relative to construct 1 (**Fig. 15A**). Construct 4 was designed to incorporate only one G at position 1, whereas construct 5 was designed to require no additional nucleotides, as it has a fully base-paired acceptor stem. As expected, construct 4 was found to be a good substrate for the nucleotide incorporation activity (**Fig. 15B**, *lanes k* and *l*), whereas construct 5 was a poor substrate for the activity (**Fig. 15B**, *lane m* and *n*). 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.

As was observed by Price and Gray (1999b), no nucleotides additional to [α-$^{32}$P]GTP were found to be required for the nucleotide incorporation when the 5′ end of the tRNA substrate was triphosphorylated (*e.g.*, **Fig. 15B**, *lane l*). All substrates in **Fig. 15B** are products of transcription with T7 polymerase, and therefore have triphosphorylated 5′ ends. However, when tRNA 5′ ends were monophosphorylated, Price and Gray (1999b) demonstrated that the activity then required ATP for nucleotide incorporation. ATP is thought to be used to activate tRNA 5′ ends *via* the formation of an adenylylated tRNA intermediate (see Discussion). In support of this idea, I have observed (not shown) labelling of 5′-monophosphorylated construct 1 when incubated with the *A. castellanii* mitochondrial S100 extract and [α-$^{32}$P]ATP in the absence of

other nucleotides. When this labelled tRNA was digested with P1 nuclease and the products of digestion separated by thin-layer chromatography, the sole radiolabelled product did not migrate from the origin. These results support the idea that ATP can be covalently linked to a tRNA by an activity in the *A. castellanii* extract, and that the bond that is created is not cleavable by P1 nuclease. Because phosphoanhydride bonds (symbolized as AppN), which an adenylated tRNA would be expected to contain, are not cleavable by P1 nuclease, these combined results support adenylylation as a likely mechanism for the activation of monophosphorylated tRNA 5′ ends. However, to firmly establish that this uncleaved radiolabelled material was indeed AppN, this experiment would have to be repeated with the inclusion of an additional digestion of the products of P1 nuclease digestion with snake venom phosphodiesterase, which is able to cleave phosphoanhydride bonds. This additional step would be predicted to yield $^{32}$pA and pN, which could then be resolved by TLC. This result would be strong evidence for the involvement of an adenylylated-tRNA intermediate in the editing reaction.

### 3. *cis*-Templated 3′-to-5′ nucleotide incorporation

Constructs 6 and 7 (**Fig. 15A**) were designed to confirm directly whether in *A. castellanii* the sequence on the 3′ side of the acceptor stem templates nucleotide addition on the 5′ side of the acceptor stem, and whether addition proceeds in a 3′-to-5′ direction. Results addressing these issues have been presented by Price and Gray (1999b).

Construct 6, designed to incorporate U at position 2 and G at position 1, was labelled after incubation with the extract in the presence of [α-$^{32}$P]GTP and UTP (**Fig. 15B**, *lane o*). When UTP was not present during the incubation, no labelled products were observed (**Fig. 15B**, *lane p*). These results support addition at position 2 followed by addition at position 1: *i.e.*, sequential incorporation in a 3′-to-5′ direction.

Construct 7, designed to incorporate G at position 2 and U at position 1, was labelled with [α-$^{32}$P]GTP in both the presence and absence of UTP (**Fig. 15B**, *lanes q* and *r*). When UTP was not present during the incubation, the labelled products were shorter (**Fig. 15B**, *lane r*) than when UTP was present (*lane q*). These combined results confirm 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, as proposed by Price and Gray (1999b).

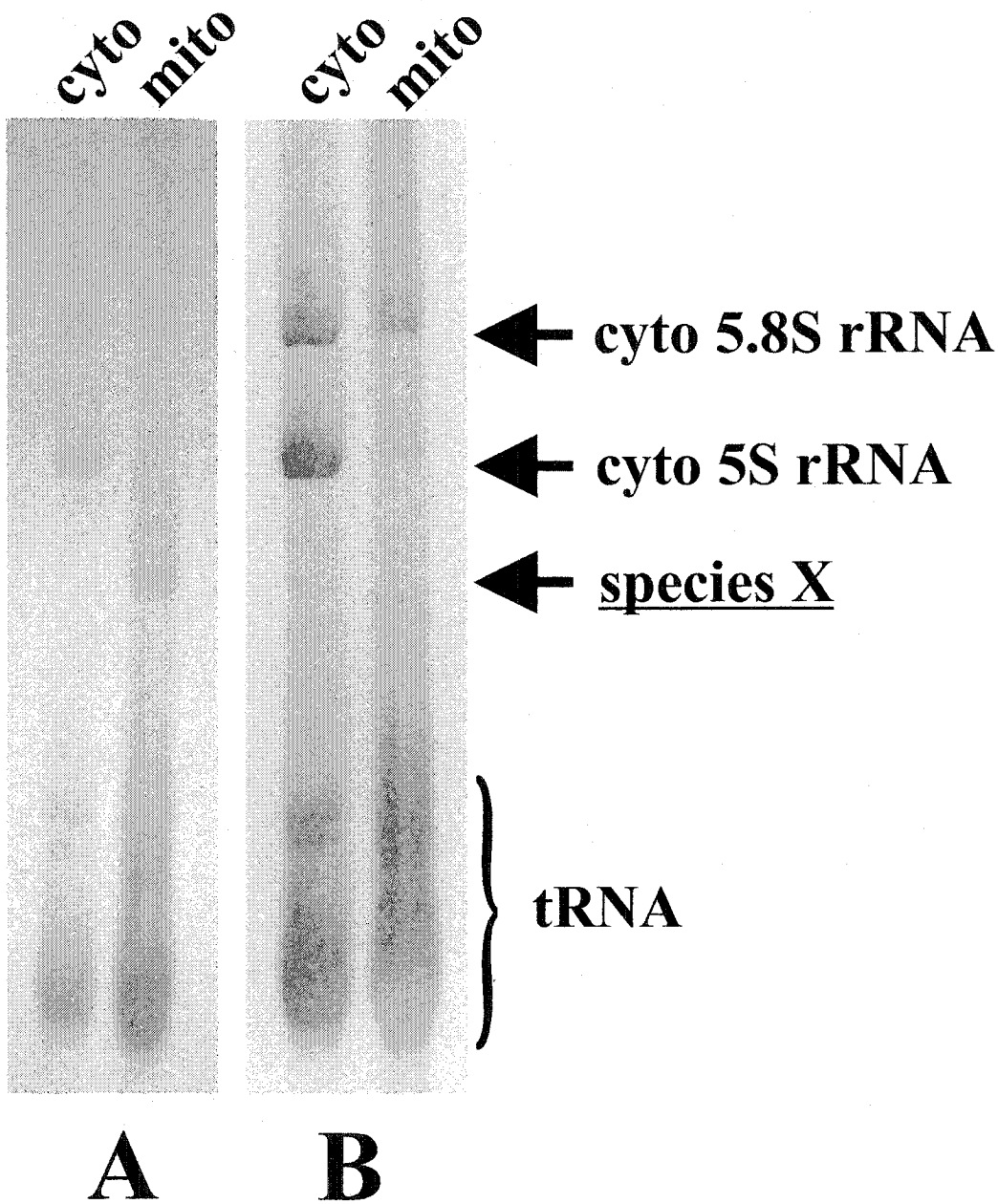
## C. Discovery and Characterization of Novel Small RNAs in Members of the Phylum Amoebozoa

### 1. Discovery and characterization of *Acanthamoeba castellanii* mitochondrial 5S rRNA (Bullerwell *et al.*, 2003b)

When *A. castellanii* RNA fractions were resolved by polyacrylamide gel electrophoresis, UV shadowing (Hassur and Whitlock, 1974) revealed the presence of a novel, highly abundant, small RNA species (X) in purified mitochondrial (but not cytoplasmic) RNA (**Fig. 16A**). Species X was, however, invisible when gels were stained with ethidium bromide (**Fig. 16B**), an intercalating agent whose interaction with nucleic acids is strongly affected by the degree and stability of base pairing. Detection and isolation of species X was simplified by the unusually slow migration (relative to other 5S rRNAs) of *A. castellanii* cytoplasmic 5S rRNA (length 119 nucleotides) under the gel electrophoresis conditions used.

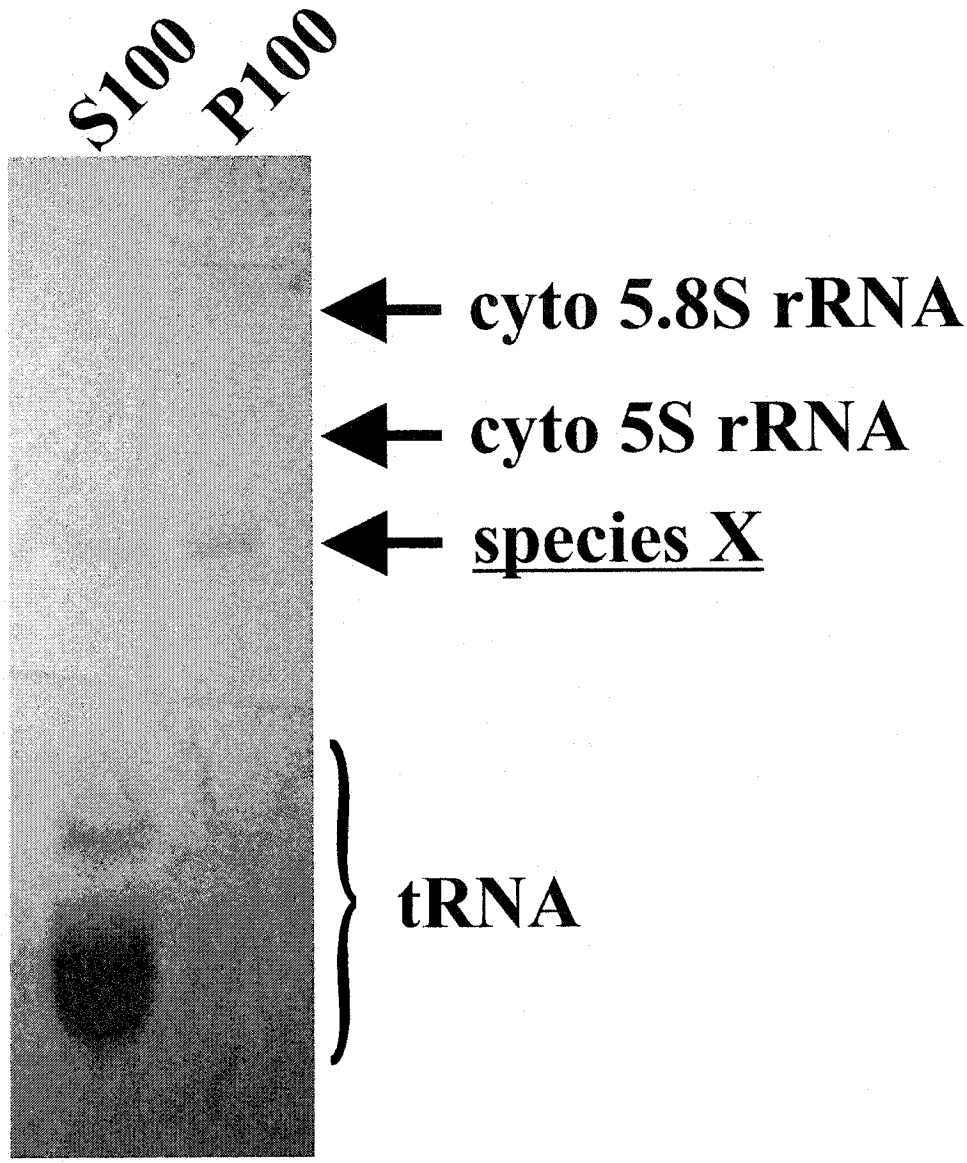
The size and abundance of species X suggested that it could be a mitochondrial 5S rRNA. To test this hypothesis, we investigated the mitochondrial localization of this RNA by subjecting a clarified Triton X-100 lysate of purified *A. castellanii* mitochondria to centrifugation at 100,000 *g* for 1.5 hr. We expected that if species X is a *bona fide* 5S rRNA, it should remain associated with ribosomes and appear in the 100,000 *g* pellet under the conditions used in this study. RNA was prepared from both the supernatant (S100) and pellet (P100) fractions and, as expected, a significant portion of species X was found in the P100 (**Fig. 17**). The species X present in the S100 might be due to incomplete sedimentation of mitochondrial ribosomes; alternatively, a low-molecular-weight ribonucleoprotein complex containing species X (a putative 5S rRNA) may have dissociated from a fraction of the ribosomes (see Moore, 1996). Localization of tRNAs exclusively in the S100 confirms that the high-speed centrifugation did not pellet small RNA species that are not associated with large complexes. Together, these observations support the hypothesis that species X is associated with mitochondrial ribosomes.

**Figure 16:** Visualization by (**A**) UV shadowing and (**B**) ethidium bromide staining of *A. castellanii* cytoplasmic (cyto; depleted of mitochondria) and mitochondrial (mito) RNAs separated on a 10% polyacrylamide gel. Note: The low-abundance RNA in the mitochondrial RNA preparation that co-migrates with cytoplasmic 5.8S rRNA has a 3′-terminal sequence identical to that of cytoplasmic 5.8S rRNA (not shown). Our data indicate that this RNA has a single 3′-terminal U residue, whereas the number of U residues is ambiguous in the published sequence (MacKay and Doolittle, 1981).


cyto
mito
cyto
mito
cyto 5.8S rRNA
cyto 5S rRNA
species X
tRNA
A
B

**(Figure 16)**

**Figure 17:** Visualization by UV shadowing of RNAs isolated from the supernatant (S100) and pellet (P100) of a 100,000 *g* ultracentrifugation of a clarified Triton X-100 lysate of purified *A. castellanii* mitochondria. RNAs were separated on a 10% polyacrylamide gel. High molecular weight RNA (presumably the mitochondrial LSU and SSU rRNAs) were present predominantly in the P100 fraction, near the top of the gel (not shown). The positions of *A. castellanii* cytoplasmic (cyto) 5.8S and 5S rRNA markers (run in a lane adjacent to the S100 and P100 samples; not shown) and tRNA are indicated.



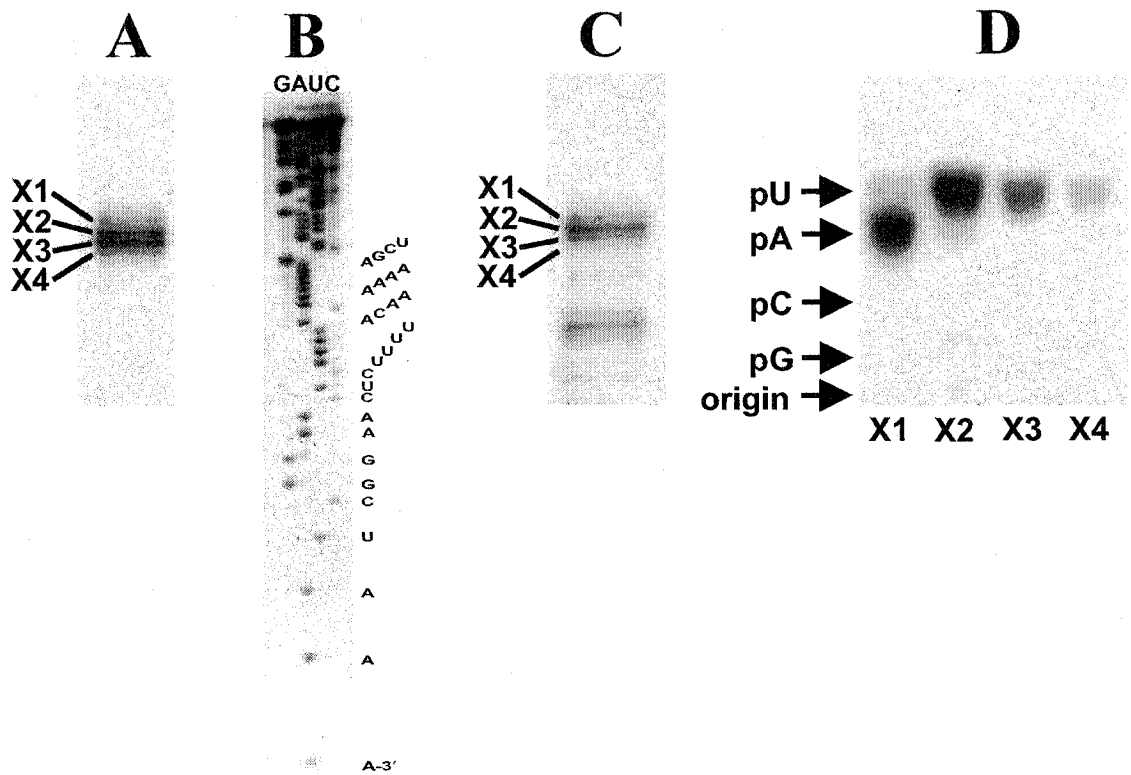

**(Figure 17)**

To further characterize this novel ribosomal component, RNA sequence data were obtained. Both 3′-end labelling (**Fig. 18A**) and 5′-end-labelling (**Fig. 18C**) of isolated species X resulted in four labelled RNA species (X1-X4), differing in length by one nucleotide. By chemical sequencing of the 3′-end-labelled RNAs (**Fig. 18B**), the four RNAs were shown to have exactly the same 3′-termini, indicating that they must be heterogeneous at their 5′-ends. Terminal analysis of 5′-end-labelled RNAs (**Fig. 18D**) revealed that the three shortest length variants (X2-X4, 116-118 nucleotides) contained a U residue at their 5′-termini, whereas the longest variant (X1, 119 nucleotides) had a 5′-terminal A. Because chemical sequencing gels of the 3′-end-labelled RNAs could be read to within a stretch of U residues near their 5′-ends, these combined analyses yielded RNA sequence data for every position in the molecule, demonstrating that species X is distinctly different in sequence from the nucleus-encoded cytoplasmic 5S rRNA of *A. castellanii* (MacKay and Doolittle, 1981).

Alignment of the experimentally determined RNA sequence of species X with mitochondrial and eubacterial 5S rRNA sequences (**Fig. 19A**) revealed nucleotide similarity within a region highly conserved in other 5S rRNAs (outside of this stretch, very little primary structure conservation is evident among mitochondrial 5S rRNAs in general). In addition to displaying a diagnostic primary sequence motif, the RNA sequence of species X can be folded into a secondary structure (**Fig. 19B**) consistent with the consensus 5S rRNA secondary structure (Moore, 1996). These data strongly suggest that species X is indeed a mitochondrial 5S rRNA. The high A+U content of this 5S rRNA (78%) with concomitant relatively weak base pairing in stem regions presumably accounts for the failure of ethidium bromide to bind efficiently to this RNA species in gels.

A search of the complete mtDNA sequence of *A. castellanii* (Burger *et al.*, 1995; GenBank acc. no. NC_001637) located the mitochondrial 5S rRNA gene (*rrn5*) within a previously unassigned 240-nucleotide spacer between the *cox1/2* and *rps4* genes. The 5′-ends of this molecule map 109-112 nucleotides downstream of the 3′-end of the *cox1/2* reading frame, whereas the 3′-end maps 22 nucleotides upstream of the 5′-end of the *rps4* coding region. The *rrn5* gene is in the same transcriptional orientation as all other genes in this mitochondrial genome. When the *A. castellanii* mitochondrial 5S

**Figure 18:** End-labelling and sequencing of species X. **A,** electrophoretic separation on a 6% acrylamide gel of 3′-end-labelled species X and (**B**) chemical sequencing of these labelled RNAs. A representative 20% polyacrylamide gel for RNA band X4 is displayed, with a portion of the deduced RNA sequence shown on the right. **C,** Electrophoretic separation on a 6% polyacrylamide gel of 5′-end-labelled species X and (**D**) one-dimensional thin-layer chromatography of the products of P1 nuclease digestion of the four largest 5′-end-labelled RNAs (X1-X4). The positions of nucleoside 5′-monophosphate markers are indicated. Autoradiograms are shown in all cases. (3′-End-labelling and chemical sequencing were performed by Dr. M. N. Schnare, Dalhousie University)



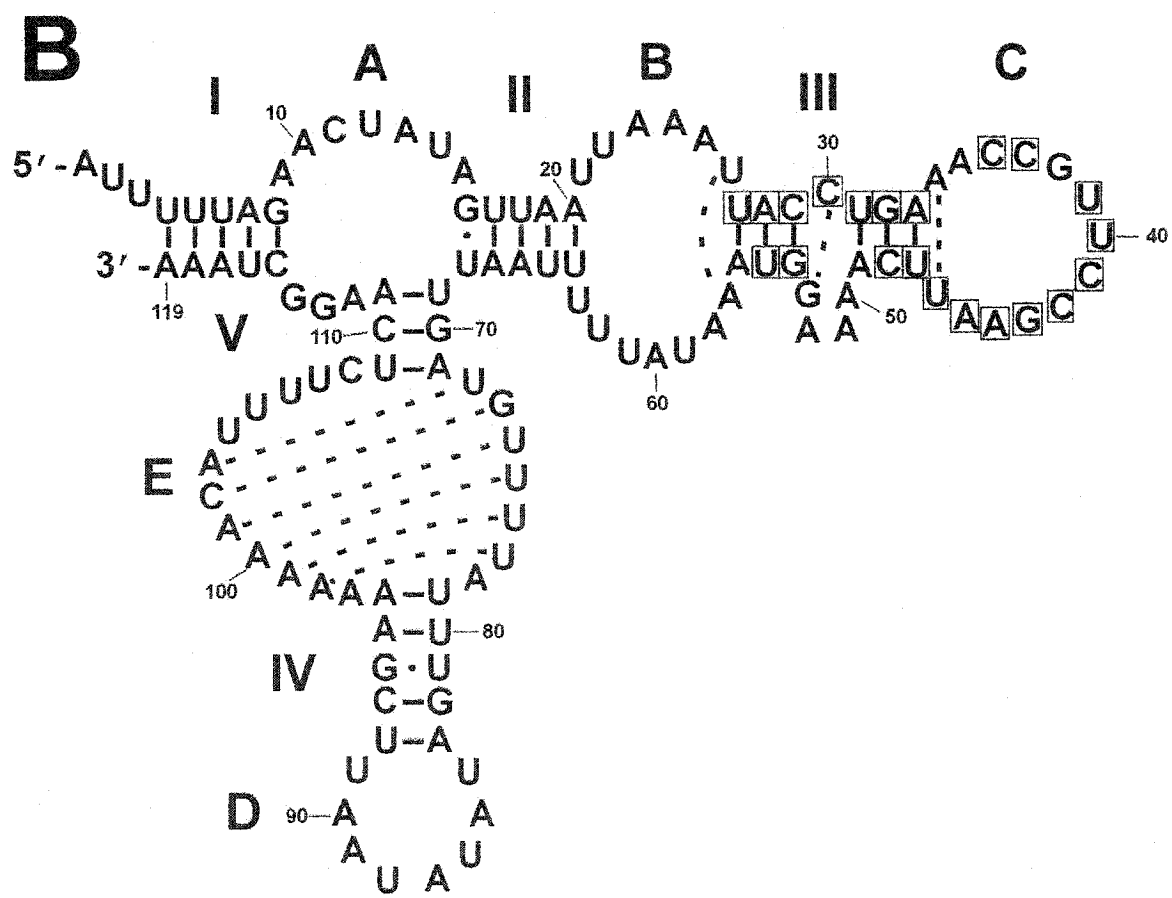

**(Figure 18)**

**Figure 19:** Potential secondary structure of species X and alignment with other 5S rRNAs. **A,** alignment of a conserved portion of mitochondrial (m) and eubacterial (e) 5S rRNA sequences. Sequences used (Genbank acc. no. in parentheses): *Acanthamoeba castellanii* (Aca; this work), *Triticum aestivum* (Tae; M10361), *Marchantia polymorpha* (Mpo; M68929), *Prototheca wickerhamii* (Pwi; U02970), *Nephroselmis olivacea* (Nol; AF110138), *Cyanidium caldarium* (Cca; Z48930), *Cyanidioschyzon merolae* (Cme; D89861), *Chondrus crispus* (Ccr; Z47547), *Pylaiella littoralis* (Pli; AJ277126), *Laminaria digitata* (Ldi; AJ344328), *Reclinomonas americana* (Ram; U59762), *Rhodobacter capsulata* (Rca; X04585) and *Escherichia coli* (Eco; X00414). The spacing in the alignment represents the alternation of single- and double-stranded regions. Dashes (-) indicate alignment gaps. Numbers in parentheses indicate nucleotides not shown. The consensus (con m) indicates positions that are either identical in at least 9 of the 11 mitochondrial sequences or of the same type (Y = pyrimidine, R = purine) in all 11 mitochondrial sequences. Asterisks indicate positions in the mitochondrial consensus that are also conserved in the eubacterial sequences. **B,** potential RNA secondary structure of the longest sequence variant of the *A. castellanii* mitochondrial 5S rRNA. Boxed nucleotides indicate positions that match the mitochondrial consensus. Additional potential base-pairing is indicated by broken lines. Helices I to V and loops A to E are denoted as in Burger *et al.* (1999).

**A**

```
                    III          C          III
Aca   m  (26)-UAC C UGA AACCGUUCCGAAU UCA AAAG---- GUA-(63)
Tae   m  (29)-CAC C CGA UCCCAUUCCGACC UCG AUAUAUAU GUG-(58)
Mpo   m  (29)-CAC C CGA UCCCAUUUCGACC UCG AUAU---- GUG-(62)
Pwi   m  (27)-CAC C CGA ACCCAUUUCGAAU UCG AUUA---- GUG-(60)
Nol   m  (27)-CAC C CGA ACCCACUCUGAAU UCG AUAAU--- GUG-(55)
Cca   m  (28)-CUC U CGA UCCCUUUUCGAAU UCG AUA----- GAG-(58)
Cme   m  (29)-CAU U CGA AACCUUUUCCGAAC UCG AAA----- AUG-(57)
Ccr   m  (29)-UAU U UGA UCCCAUUCCGAAC UCA CAU----- AUA-(54)
Pli   m  (23)-UAC C CGA ACUCUUACCGAAC UCG AGC----- GUC-(55)
Ldi   m  (23)-UAC C CGA ACUCUUACCGAAC UCG AGC----- GUC-(61)
Ram   m  (25)-CAC U UGA UCCCAUUCCGAAC UCG AAUU---- GUG-(55)
Rca   e  (26)-CAC C CGA UCCCAUCCCGAAC UCG GCC----- GUU-(61)
Eco   e  (26)-CAC C UGA CCCCAUGCCGAAC UCA GAA----- GUG-(62)
                *** * ***  *** * ****** **          **
con   m         YAY Y YGA  CCC UUYCGAAY UCG          GU
```

**B**




**(Figure 19)**

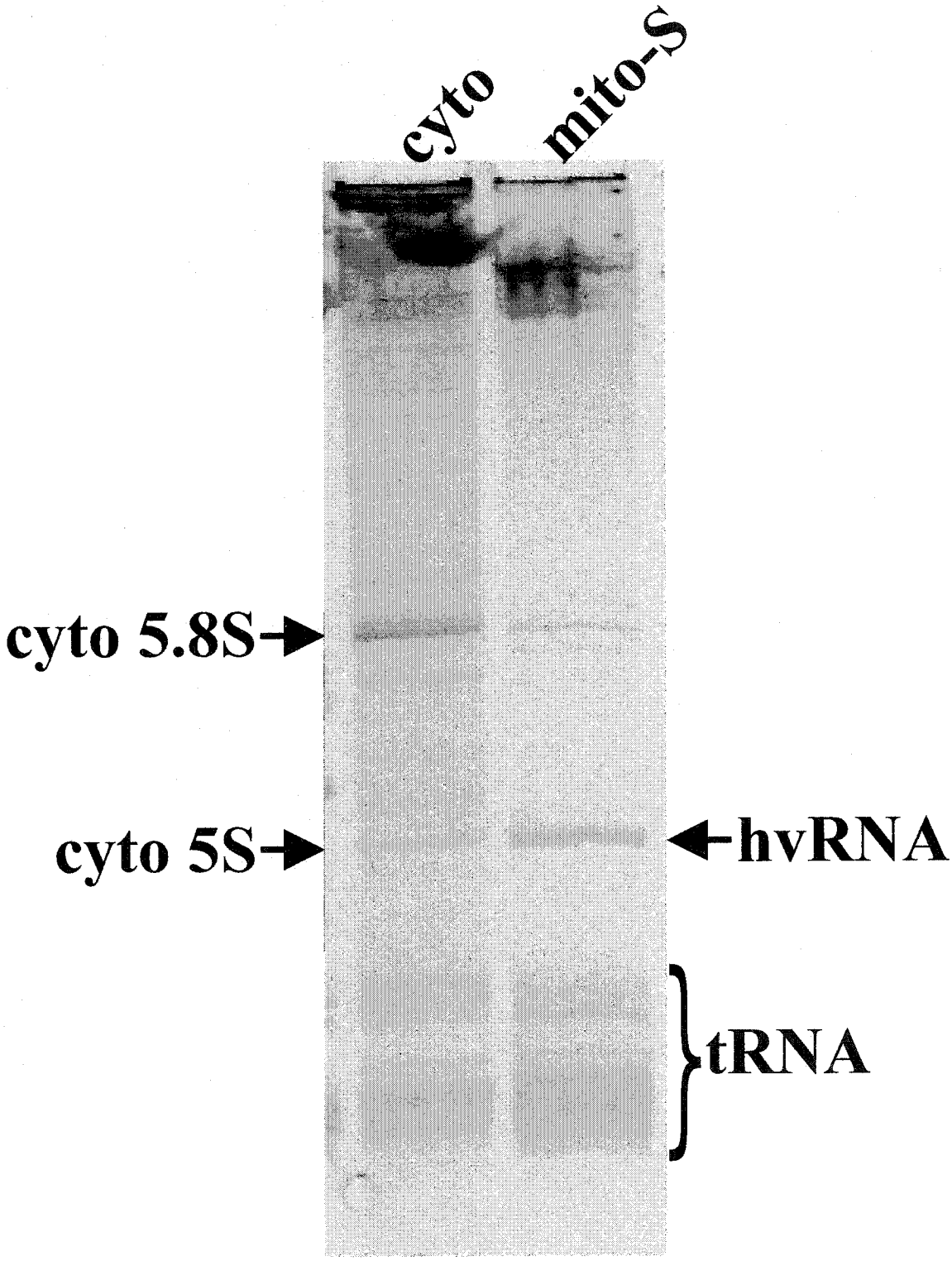
rRNA sequence was used as a query in BLAST searches of public domain databases, no other 5S rRNA sequences (including any of the known mitochondrial ones) were detected.

## 2. Discovery and characterization of a potential *Hartmannella vermiformis* mitochondrial 5S rRNA homolog

The unexpected discovery of a 5S rRNA in the mitochondria of *A. castellanii* raised the possibility that other members of Amoebozoa in which a mtDNA-encoded 5S rRNA has not been identified might nevertheless encode and express a 5S rRNA homolog. To test this possibility, mitochondria were prepared from *Hartmannella vermiformis*, an amoeboid protist that branches deeply within the Amoebozoan lineage near *A. castellanii* (according to phylogenies based on molecular data; B. F. Lang, personal communication). Searches of the recently completed mtDNA sequence (M. W. Gray *et al.*, unpublished) did not identify a potential 5S rRNA sequence. Mitochondrial RNA profiles from this organism, however, revealed a highly abundant RNA that comigrated with cytoplasmic 5S rRNA (**Fig. 20**). The size, abundance and mitochondrial localization of this novel RNA, referred to here as hvRNA, suggested that it might represent *H. vermiformis* mitochondrial 5S rRNA.

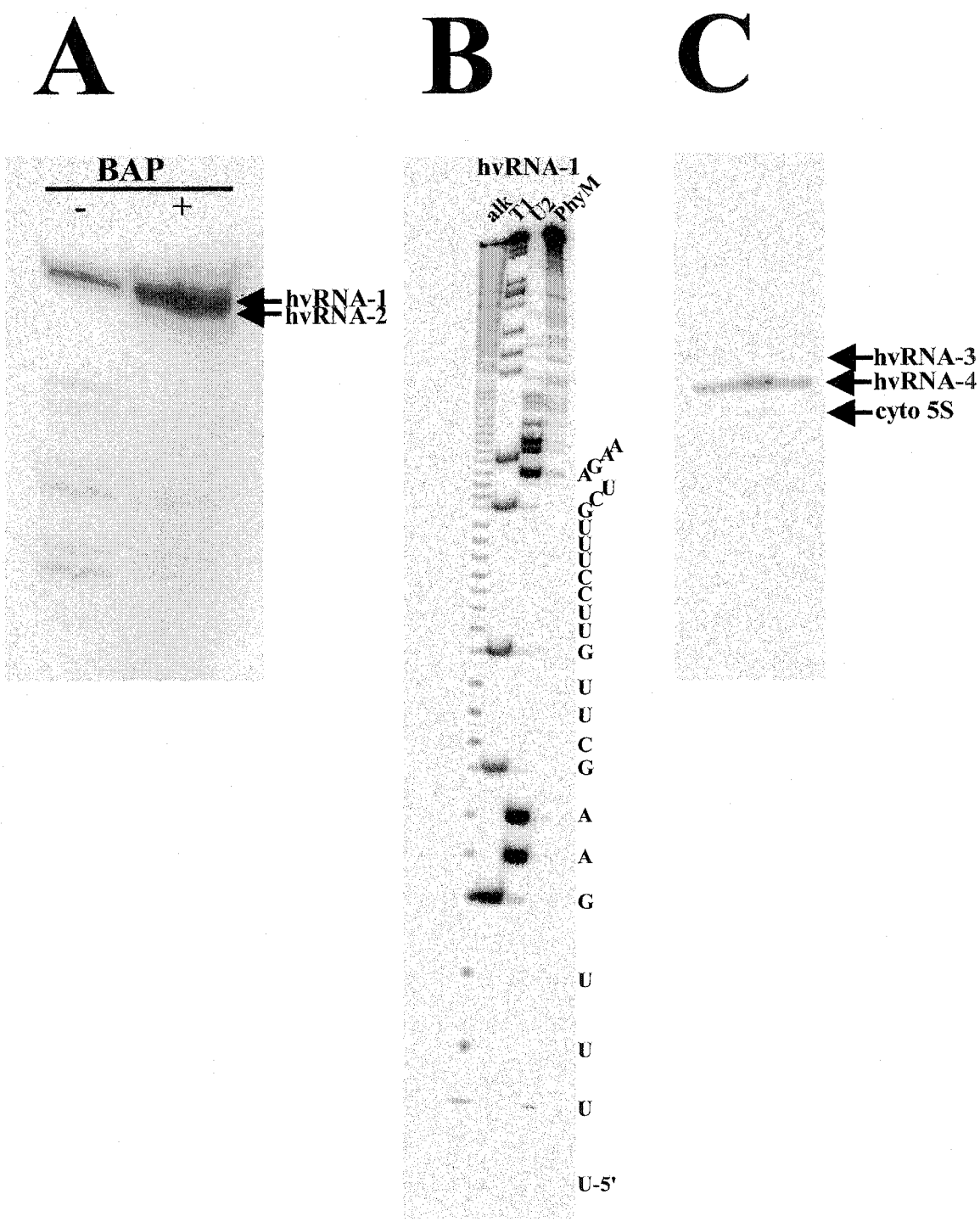
To further characterize this novel RNA, RNA sequence data were obtained. 5′-End-labelling of isolated hvRNA resulted in two labelled RNA species, differing in length by one nucleotide (**Fig. 21A**). These RNAs were determined to have identical sequences (**Fig. 21B**), except that the larger variant (hvRNA-1) contained an additional U at its 5′-terminus. The larger variant was more than 10-fold as abundant as the smaller variant. 3′-End labelling of isolated hvRNA from a separate culture of *H. vermiformis* resulted in three RNAs (**Fig. 21C**), which were shown by enzymatic sequencing (data not shown) to represent two length variants of hvRNA (hvRNA-3 and hvRNA-4) that differed by 2 nucleotides at the 3′-end, as well as what is likely contaminating cytoplasmic 5S rRNA (based on its size and high G content as determined by enzymatic sequencing; data not shown). The smaller of the two hvRNA length variants (hvRNA-4) was at least 10-fold as abundant as the larger variant. The combined sequence data reveal an RNA that shows both 5′ and 3′ length heterogeneity (although in different

**Figure 20:** Profiles of RNAs isolated from *H. vermiformis* cytoplasm (cyto; depleted of mitochondria) and mitochondria (mito). Purified mitochondria were lysed with Triton X-100 and fractionated by centrifugation at 9000 *g* into pellet and supernatant (mito-S) fractions. Samples were electrophoresed through a 10% polyacrylamide gel and visualized by ethidium bromide staining. The positions of the cytoplasmic 5.8S and 5S rRNAs, hvRNA and tRNA are indicated.




**(Figure 20)**

**Figure 21:** End-labelling and sequencing of hvRNA. **A,** electrophoretic separation on a 6% polyacrylamide gel of 5′-end-labelled hvRNA, preincubated (+) or unincubated (-) with bacterial alkaline phosphatase (BAP). **B,** enzymatic sequencing of the larger 5′-end-labelled length variant (hvRNA-1) by partial alkaline hydrolysis (alk) and partial digestion with ribonucleases T1, U2 and PhyM. Products were separated on a 20% polyacrylamide gel. A portion of the deduced sequence is indicated on the right. The same sequence, one nucleotide shorter at its 5′ end, was obtained for hvRNA-2 (not shown). **C,** 3′-end-labelled hvRNA separated on a 6% polyacrylamide gel. The identity of hvRNA-3 and hvRNA-4 was confirmed by enymatic sequencing (not shown). The identity of the smallest 3′-end-labelled RNA is uncertain, but is likely cytoplasmic 5S rRNA (see text). Autoradiograms are shown in all cases.


A
B
C
BAP
-
+
hvRNA-1
hvRNA-2
hvRNA-1
alk
T1
U2
PhyM
AGAA
C
U
GUUUCCCUUG
U
U
C
G
A
A
G
U
U
U
U-5'
hvRNA-3
hvRNA-4
cyto 5S

**(Figure 21)**

RNA preparations), with the largest possible hvRNA length variant being 124 nucleotides long. When the hvRNA sequence was used as a query in BLAST searches of public domain databases, no 5S rRNAs (or any other known RNAs) were detected.

A search of the complete mtDNA sequence of *H. vermiformis* located the gene for hvRNA within a previously unassigned repeat region, 80 nucleotides downstream of and in the same transcriptional orientation as the gene for $tRNA^{Ser}$(uga) (**Fig. 22A**). The repeat region begins 28 nucleotides upstream of the 5′-end of the largest hvRNA 5′ variant, and ends 5 nucleotides upstream of the 3′-end of the largest hvRNA 3′ variant. Each repeat unit consists of 147 nucleotides, and no sequence heterogeneity is observed between repeats. The repeat region consists of at least 9 reiterations of this sequence; however, the exact length of this region is not known, as the presence of this long repeat region complicated genome assembly (the shotgun sequencing method was used on a library consisting of small cloned mtDNA fragments). Due to this difficulty, it is not known whether the repeat region represents a telomere-like element or whether the *H. vermiformis* mitochondrial genome is circular-mapping or linear.

Upon further inspection of this repeat region, I identified a second tRNA gene missed during annotation of the *H. vermiformis* genome. This gene codes for a perfectly canonical $tRNA^{Arg}$(ucg), with its 5′ end located one nucleotide downstream of the 3′ end of the gene for $tRNA^{Ser}$(uga) (**Fig. 22A**). The *trnR*(ucg) gene overlaps the repeat region by 22 nucleotides, with the 3′ end immediately abutting a stretch of 10 Ts, 6 nucleotides upstream of the 5′ end of the sequence coding for the largest variant of hvRNA. The location of this tRNA gene suggests that the 5′ end of hvRNA could be generated by the same endonuclease cleavage that liberates the 3′-end of $tRNA^{Arg}$(ucg) prior to trimming by 3′-to-5′ exonuclease activity. The surrounding sequence gives no indication as to how the 3′ end of hvRNA is generated.

Although the sequence of hvRNA does not suggest homology to 5S rRNA or to any other known RNA, it is possible to generate a secondary structure that is consistent with that of a 5S rRNA (**Fig. 22B**). Unexpectedly, glycerol gradient ultracentrifugation of a *H. vermiformis* mitochondrial extract resulted in all detectable hvRNA being found at or near the bottom of the gradient, cosedimenting with mitochondrial ribosomes (**Fig. 23**). In contrast, tRNAs and a 5S-sized RNA were found solely at or near the top of the

**Figure 22:** Potential secondary structure of hvRNA and location of its gene sequence in the mtDNA of *H. vermiformis*. **A,** a portion of the *H. vermiformis* mtDNA coding for hvRNA. Sequences predicted to code for tRNAs and hvRNA are underlined. All genes are in the same transcriptional orientation (from left to right); only the coding strand is shown. One repeat unit (the first of at least 9 repeats) is boxed. The 22 3′ nucleotides of the *trnR*(ucg) gene included in each repeat unit are in bold. **B,** predicted secondary structure of hvRNA based on the sequence of the longest 5′ (hvRNA-1) and 3′ (hvRNA-3) length variants. Many additional/alternative base-pairings are possible.

**A**

ACTTTTCAATTTTATTAGGGGGGGCAGATGGCTGAGTGGTTTAAAGCGCTATTCTTGAAAATTAGTTCAGG

**trnY(gua)** **trnS(uga)**

CCAAAAACCTGACACGGGTTCAAATCCCGTTCTGCCCTTGCATCTATAGCTTAATTGGTAGAGCGACACTC

**trnR(ucg)**

TTCGAAAGTGTAGGTTCCA**TGTTCAAGTCGTGGTAGATGCG**TTTTTTTTTTTGAAGCTTGTTCCTTTGCTAG

**hvRNA**

**Repeat Unit**

AATATAAATAAGCCAGCATATGTTTCACATTGCTTTAGTGCTTTGTTCTTTTCTATTTTATTCTTTATGAT

TAAAGTAGTTTCTAAGGAAAACTC**TGTTCAAGTCGTGGTAGATGCG**TTTTTTTTTTTGAAGCTTGTTCCTTT

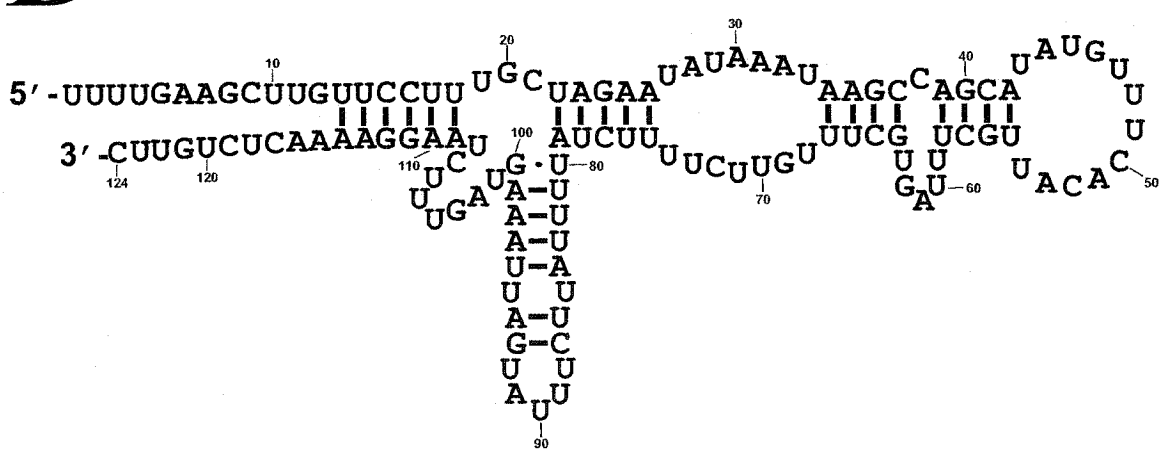
**hvRNA**

GCTAGAATATAAATAAGCCAGCATATGTTTCACATTGCTTTAGTGCTTTGTTCTTTTCTATTTTATTCTTT

ATGATTAAAGTAGTTTCTAAGGAAAACTC**TGTTCAAGTCGTGGTAGATGCG**TTTTTTTTTTTGAAGCTT...

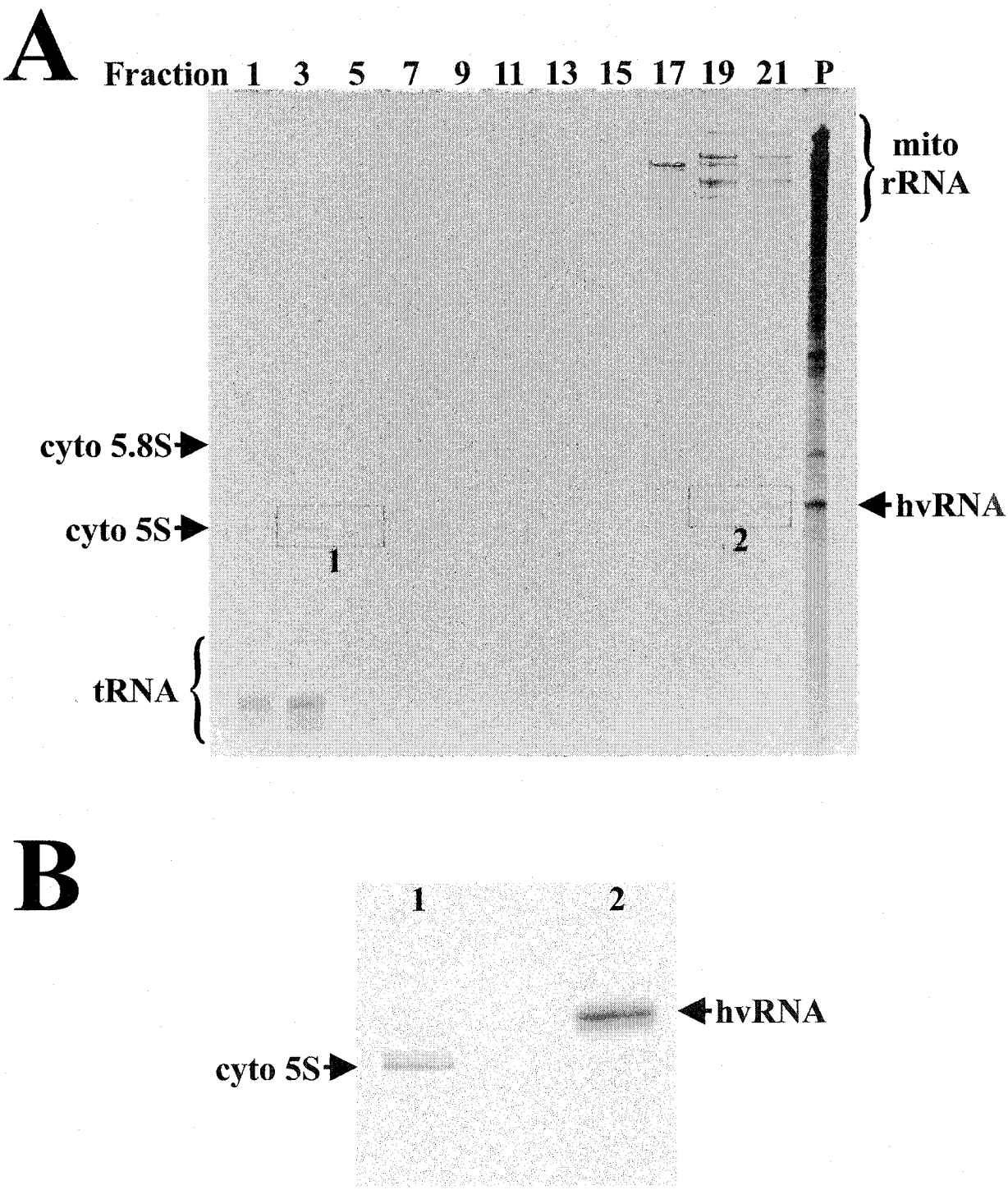
**hvRNA**

**B**




**(Figure 22)**

**Figure 23:** Fractionation of *H. vermiformis* mitochondria. **A,** visualization by ethidium bromide staining of RNAs isolated from the top (fraction 1) to the bottom (P, pellet) of a glycerol gradient fractionation (by ultracentrifugation at 21,000 rpm for 18.5 h) of a clarified *H. vermiformis* mitochondrial extract. An equal portion of every second fraction (extracted with phenol and precipitated with ethanol) was separated on a 10% polyacrylamide gel. The positions of the cytoplasmic 5.8S and 5S rRNAs, hvRNA and tRNA are indicated. The 5S-sized RNA in fractions 3 and 5 (box labelled "1") and in fractions 19 and 21 (box labelled "2") was excised from the gel, (**B**) 3′-end-labelled and separated on a 6% polyacrylamide gel. Enzymatic sequencing of the resolved RNA species (not shown) revealed that the RNA in fractions 3 and 5 has the same sequence as the smallest RNA in **Fig. 21C** (likely the cytoplasmic 5S rRNA) and that the RNA in fractions 19 and 21 is hvRNA. (3′-End-labelling and enzymatic sequencing were performed by M. N. Schnare, Dalhousie University)


A
Fraction 1 3 5 7 9 11 13 15 17 19 21 P
mito rRNA
cyto 5.8S
cyto 5S
hvRNA
1
2
tRNA
B
1
2
hvRNA
cyto 5S

**(Figure 23)**

gradient, confirming that the high-speed centrifugation did not pellet small RNA species that are not associated with large complexes. Together, these observations support the hypothesis that hvRNA is associated with mitochondrial ribosomes. The 5S-sized RNA that remained at the top of the gradient is presumed to be cytoplasmic 5S rRNA (the sequence of this RNA was found to be the same as that for the smallest pCp-labelled RNA presented in **Fig. 21C**).

Cosedimentation of hvRNA with mitochondrial ribosomes suggests that despite the absence of expected primary sequence motifs and a convincing secondary structure, hvRNA might in fact represent a highly derived 5S rRNA. To further test this idea, intact mitochondrial ribosomes could be separated into their large and small subunits and then fractionated on a sucrose gradient. If hvRNA is found to co-fractionate with the mitochondrial LSU, this would strongly support association with this ribonucleoprotein complex, and further indicate that it is indeed a 5S rRNA homolog.

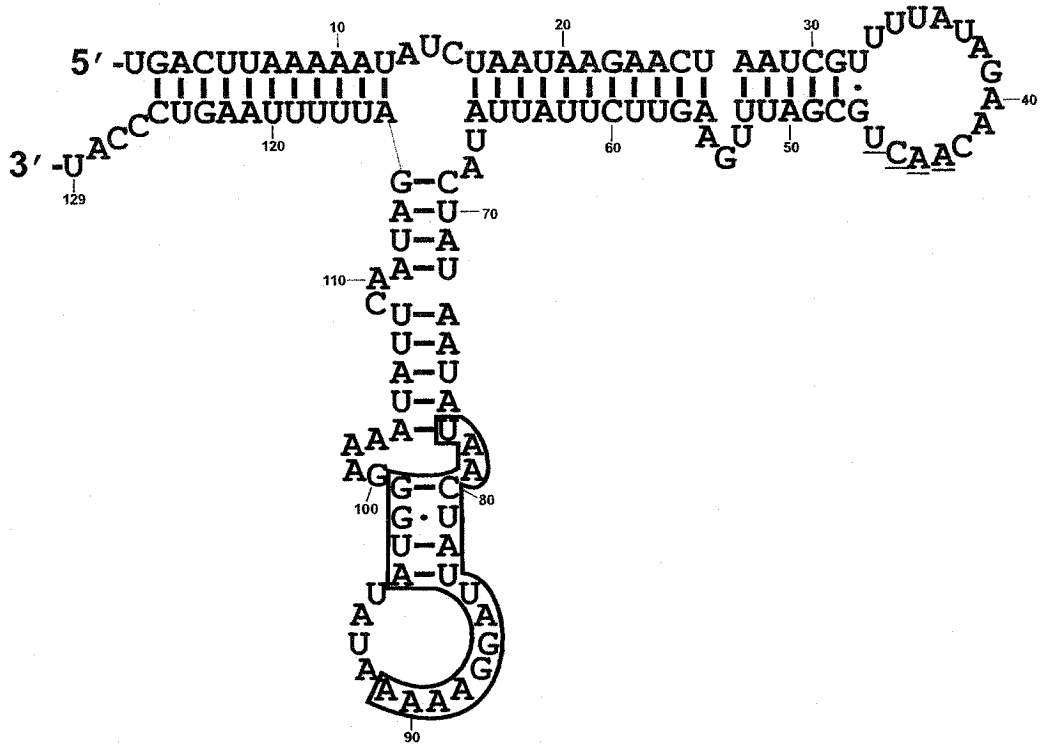
### 3. Small mitochondrial RNAs in *Dictyostelium discoideum* and *Polysphondylium pallidum*

The discovery of a highly derived 5S rRNA in *A. castellanii* mitochondria, and an even more derived 5S rRNA (if it truly represents a 5S rRNA homolog) in *H. vermiformis* mitochondria, makes it reasonable to expect that a 5S rRNA could be functional despite lacking features generally expected of such an RNA. These new data led me to reconsider a small RNA (called msRNA) that had previously been identified in the cellular slime mold *Dictyostelium discoideum* (Pi *et al.*, 1998). This RNA was suspected by the authors to be a 5S rRNA based on its size, abundance and presence specifically in mitochondrial preparations. However, although it can be folded into a 5S-like secondary structure (**Fig. 24A**), msRNA lacks primary sequence motifs characteristic of other 5S rRNAs. Further, it was found almost exclusively in the supernatant of a 250,000 *g* centrifugation of a mitochondrial lysate, rather than co-sedimenting with mitochondrial ribosomes. These data seemed to make it unlikely that msRNA represented a true 5S rRNA homolog.

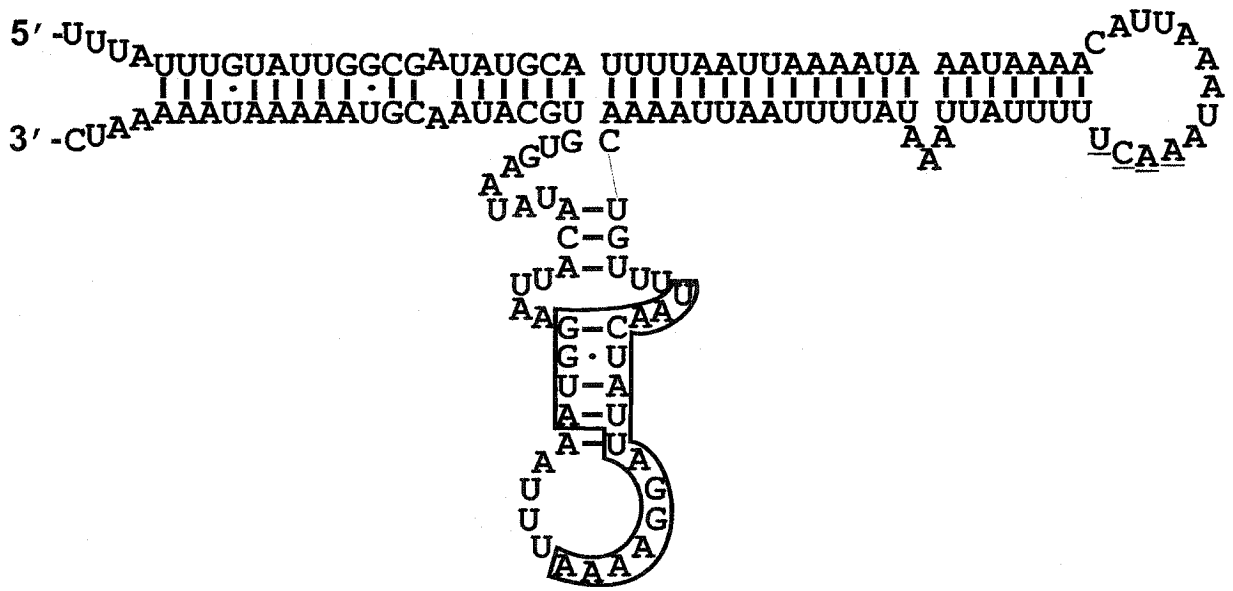
Nevertheless, a search of the mtDNA sequence of a related cellular slime mold, *Polysphondylium pallidum* (the complete sequence has been recently determined; M.W.

**Figure 24:** Potential secondary structures of (**A**) msRNA (as proposed by Pi *et al.*, 1998) and (**B**) a putative mitochondrial RNA from *P. pallidum*. A region of primary sequence and secondary structure conservation in the two structures is boxed. A second region of nucleotide sequence conservation is underlined.

**A**



**B**



**(Figure 24)**

Gray *et al.*, unpublished) using the msRNA gene sequence as the query revealed a region of identitiy in the msRNA gene and in an unassigned region downstream of the *P. pallidum rnl* gene. This genomic location was intriguing, as msRNA is encoded in the same general location, immediately downstream of the *rnl* gene in the mtDNA of *D. discoideum*. Secondary structure modeling of the region flanking this stretch of sequence identity in *P. pallidum* (**Fig. 24B**) revealed a secondary structure with similarities to that proposed for msRNA (**Fig 24A**), suggesting that these two RNAs are homologous. The presence of conserved regions of primary sequence and secondary structure, boxed and underlined in **Fig. 24A** and **B**, support this idea. The identification of an msRNA homolog in *P. pallidum* suggests a conserved function for these two small RNAs in *D. discoideum* and *P. pallidum* mitochondria. However, although the secondary structures of these RNAs are reminiscent of 5S rRNAs, no obvious 5S rRNA hallmarks (either in primary sequence or secondary structure) are evident.

# IV. DISCUSSION

## A. Comparison of tRNA Editing Activities in the Mitochondria of *S. punctatus* and *A. castellanii*

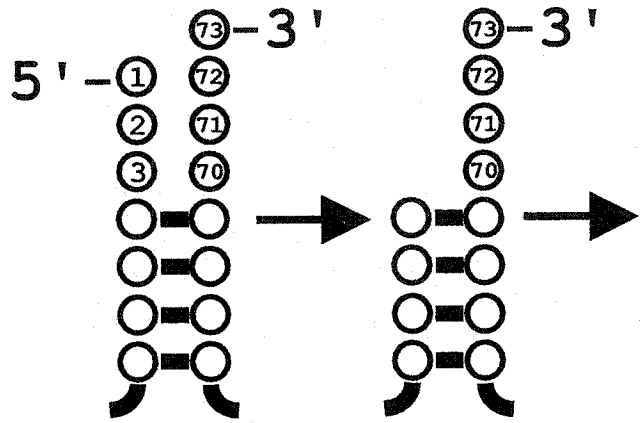
### 1. Similar biochemistry of 5′ tRNA editing in distantly related organisms

I have obtained a mitochondrial extract from *S. punctatus*, a chyridiomycete fungus, that supports specific and efficient incorporation of nucleotides into natural and *in vitro*-transcribed tRNAs. Incorporation observed *in vitro* is at the 5′ end of tRNA substrates at both internal and 5′-terminal positions (positions 2 and 1), and is 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. 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.

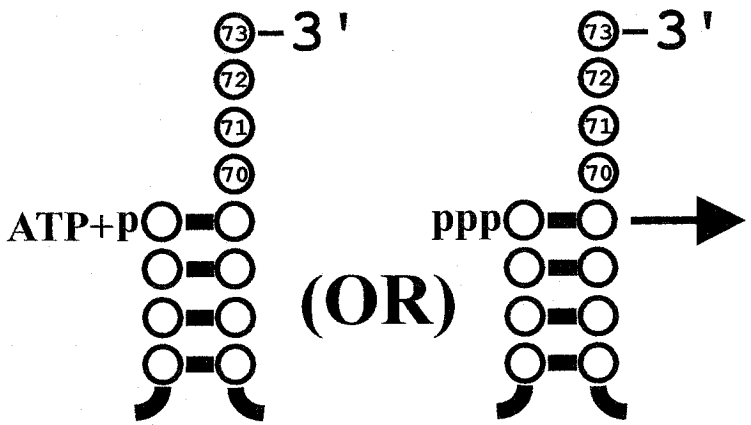
The results obtained here for *S. punctatus* are remarkably similar to those obtained here and previously (Price and Gray, 1999b) with an *in vitro* assay of mitochondrial tRNA editing in the amoeboid protozoon, *A. castellanii*. The determination of mature tRNA acceptor stem sequences from *S. punctatus* and *A. castellanii* had previously demonstrated that these activities corrected very similar patterns of predicted base pair mismatches in tRNA acceptor stems, by 5′ nucleotide replacement to produce standard Watson-Crick base pairs (Lonergan and Gray, 1993; Price and Gray, 1999a; Laforest *et al.*, 1997 and 2004). 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.

Based on the combined *in vivo* and *in vitro* data obtained to date, 5′ tRNA editing in these two organisms appears to proceed in three steps (**Fig. 25**): Step 1, nucleotide removal from tRNA 5′-ends; Step 2, 5′ activation of recessed 5′-termini; and Step 3, nucleotide incorporation to restore standard base pairing in tRNA acceptor stems. The nucleotide incorporation activity (Step 3) from both *S. punctatus* and *A. castellanii* was studied in detail in the present study (as well as in Price and Gray, 1999b). The replacements mediated by the two activities are directed by the sequence of the 3′ half of

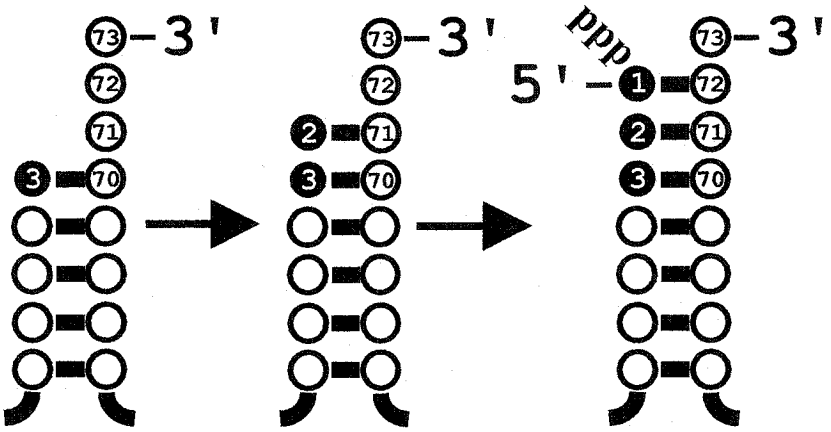
**Figure 25:** Model of 5′ tRNA editing in the mitochondria of *S. punctatus* and *A. castellanii* based on *in vivo* and *in vitro* studies. Only acceptor stem positions are shown; curved lines represent the remainder of the hypothetical tRNA. Incorporated nucleotides are indicated by filled circles.




**Step 1: Nucleotide Removal**




**Step 2: 5'-End Activation**




**Step 3: Nucleotide Incorporation**

**(Figure 25)**

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). Steps 1 and 2 are less well understood.

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 (**Fig. 1**) of $tRNA^{His}$ in all eukaryotes (Williams *et al.*, 1990; Jahn and Pande, 1991; Gu *et al.*, 2003). 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 $tRNA^{His}$ 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 adenylylation is also employed by DNA and RNA ligases (Doherty and Suh, 2000; Ho *et al.*, 2004). Because the *S. punctatus* and *A. castellanii* mitochondrial activities catalyze a reaction similar to the one mediated by HTGT, and because both activities require ATP for nucleotide addition to monophosphorylated tRNA 5′ ends (likely *via* an adenylylated tRNA intermediate), it is possible that HTGT and 5′ tRNA nucleotide incorporation activities have common evolutionary origins as well as biochemical mechanisms.

**2. Lack of evidence for 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 tRNA 5′ ends. Evidence for *S. punctatus* and *A. castellanii* mitochondrial tRNAs being processed from large RNA transcripts has been presented (Laforest, *et al.*, 1997 and Lonergan and Gray, 1993, respectively).

Processing of the majority of mtDNA-encoded tRNAs in *S. punctatus* and *A. castellanii* must be more complicated than this standard model, however, as tRNAs in these organisms undergo 5′ editing. As a result, if these tRNAs are processed by RNase P between positions 1 and –1, this cleavage would have to be followed by the further removal of nucleotides at the first three 5′ positions (Step 1, **Fig. 25**) before the nucleotide incorporation activity (Step 3, **Fig. 25**) could restore proper acceptor stem base pairing. Alternatively, the mitochondrial RNase P in these organisms 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 at 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 $tRNA^{His}$ (Orellana, Cooley and Söll, 1986). Analysis of tRNA acceptor stem cDNAs (generated by tRNA-circularization/RT-PCR) from *S. punctatus* and *A. castellanii* mitochondria did not reveal any tRNAs showing partial or complete lack of editing (Laforest *et al.*, 2004; Price and Gray, 1999a). Therefore, the possibility of an altered RNase P cleavage site in the latter two systems remains an intriguing possibility.

Somewhat surprisingly, no strong evidence for the removal of 5′ nucleotides from tRNA substrates prior to nucleotide incorporation was obtained with either *S. punctatus* or *A. castellanii* S100 extracts. For example, construct 2 (containing a 13-nt 5′ extension) and construct 5 (containing a fully base-paired acceptor stem) were poor substrates for both the *A. castellanii* and *S. punctatus* mitochondrial S100 extracts. Further, construct 8 (containing a mismatch at base pair 2:71) was not labelled to a significant degree after incubation with [α-$^{32}$P]GTP and the *S. punctatus* S100 extract, nor was construct 10 (containing a mismatch at base pair 3:70) labelled by the *A. castellanii* activity. Finally, radioactivity in 5′-end-labelled construct 1 is retained after incubation with the *S. punctatus* activity. Suggestive evidence of a 5′ nuclease activity

was only obtained when yeast tRNAs were used as substrate: $^{32}$P-labelled G residues were then found to be incorporated at tRNA positions 1 and 2 by both the *S. punctatus* and *A. castellanii* activities. However, the incorporated nucleotides at these positions may constitute addition to degraded tRNA 5′ termini, and therefore this result is not definitive evidence of nuclease activity. The results obtained in these *in vitro* assays may indicate that 5′ nuclease activities acting on tRNA 5′ ends are much less efficient than the nucleotide incorporation activity under the conditions of the assay. Additional studies will evidently be required to identify and characterize the nuclease activities that process tRNA 5′ ends in the mitochondria of *S. punctatus* and *A. castellanii*.

In a recent analysis of acceptor stems cDNA produced by RT-PCR of circularized mitochondrial tRNAs from three chytridiomycetes (*Monoblepharella*15, *Harpochytrium*94 and *Harpochytrium*105), partially and completely unedited tRNAs, which appear to represent intermediates of the editing process, were found (Laforest *et al.*, 2004). In contrast to the situation in *S. punctatus* and *A. castellanii*, these intermediates do indeed support the existence of a nuclease activity acting to remove 5′ nucleotides at tRNA positions 1-3, prior to 5′ nucleotide incorporation. No intermediates were identified with 5′ (or 3′) trailers: *i.e.*, all sequences had nucleotides up to positions 1 and 73, with no sequences from the regions flanking the tRNA genes present at the ligation junction. These data suggest not only that are there nuclease activities that act on tRNA 5′ ends in these three chytridiomycetes, but also that there are separate nuclease activities for the removal of tRNA 5′ trailer sequences and for removing mismatched nucleotides at tRNA positions 1-3. It is likely that the nuclease responsible for removing tRNA 5′ trailers by cleavage between positions 1 and –1 in these three chytridiomycetes is the mitochondrial RNase P.

### 3. 5′ Nucleotide incorporation is independent of 3′ tRNA processing

Using the *S. punctatus* mitochondrial S100 extract, two G residues were incorporated at the 5′ end of construct 1 in the presence and absence of C residues added to the 3′ end, in the presence of a 15-nt 3′ extension (construct 3), and when the 3′-terminal residue (the discriminator nucleotide, position 73) of construct 1 had been

chemically altered by periodate oxidation. Similarly, using the *A. castellanii* mitochondrial S100 extract, it has been shown that the activity can act on tRNAs either containing (*E. coli* $tRNA^{Tyr}$; Price and Gray, 1999b, and data not shown) or lacking (construct 1) a CCA tail, and in the presence of a 15-nt 3′ extension (construct 3). It therefore appears that the state of 3′ processing is independent of 5′ editing in both *S. punctatus* and *A. castellanii* mitochondria. Interestingly, the results of Laforest *et al.* (2004) also suggested that 5′ tRNA editing and the addition of the CCA sequence to tRNA 3′ ends are independent processes in the mitochondria of three distant chytridiomycete relatives of *S. punctatus*. Further studies will be required to determine how substrates are recognized by the activities and how the number of nucleotides to be incorporated is determined.

**4. 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* (summarized in **Fig. 25**) is surprising in light of the distant evolutionary relationship of these two organisms (as revealed by phylogenies based on molecular data; *e.g.*, Bullerwell *et al.*, 2003a; **Fig. 4**). There are several possibilities to explain how these similar activities could have evolved. 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 (convergently) in several lineages including Chytridiomycota, Amoebozoa [which includes *A. castellanii*, *D. discoideum* and *P. pallidum*, which are predicted (Ogawa *et al.*, 2000) or have been demonstrated experimentally (Lonergan and Gray, 1993; E. Schindel, A. J. Lohan and M.W. Gray, unpublished) to have 5′ tRNA editing of this type] and Heterolobosea (which includes *N. gruberi*, which is also predicted to have this type of 5′ tRNA editing; M. W. Gray *et al.*, unpublished).

An intriguing possibility is that these 5′ tRNA editing activities may have evolved from phylogenetically widespread but poorly characterized enzymes that are

responsible for the maintenance of tRNA 5′ ends, much as the ATP(CTP):tRNA nucleotidyltransferase adds and maintains the 3′ -$CCA_{OH}$ tail across the three domains of life. An activity of this sort would remain relatively 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* and *A. castellanii* mtDNA-encoded tRNAs). An acceptor stem 'repair' function would thus be rendered absolutely essential for the synthesis of functional RNAs.

This latter scenario is consistent with the model of Covello and Gray (1993) for the evolution of editing systems. Indeed, Gray (2001) has previously argued that this three-step model could be applied to the evolution of 5′ tRNA editing. The first step of this model is the appearance of an RNA editing activity, which might emerge by the modification of a pre-existing enzyme activity. In the case of 5′ tRNA editing, an activity would emerge with the ability to substitute nucleotides at the first three 5′ positions in mitochondrial tRNA acceptor stems with nucleotides having the potential to form Watson-Crick pairs with their 3′ counterparts. As a result of this new activity, changes in tRNA gene sequences at these positions resulting in mismatches would be rendered evolutionarily neutral, as the correct RNA sequence would either be encoded by the DNA or would be restored at the RNA level by editing. The emergence of mutations at editable positions in the DNA sequence represents the second step in the Covello and Gray model.

The third and final step of this model is the maintenance of an RNA editing activity by natural selection. If only one site was permitted to drift in the DNA sequence, the requirement for an editing activity could be lost by a single reversion at the edited site to the "correct" nucleotide. However, reversion becomes increasingly less likely as more editable sites drift from the "correct" nucleotide. Indeed, the higher the number of editable sites (and as a consequence the number of editable sites with functional importance), the lower the likelihood of reversion. In this way, an RNA editing activity could become essential for cell survival and be maintained by natural

selection. Using the Covello and Gray model, the emergence and maintenance of functionally redundant processes such as 5′ tRNA editing can be rationalized.

**B. Significance of the Discovery of Mitochondrial 5S rRNA Homologs**

Even though the *A. castellanii* mitochondrial 5S rRNA displays a degree of primary and secondary structure conservation clearly sufficient to mark it as a homolog of other 5S rRNAs, extensive analysis of the complete mtDNA sequence (Burger *et al.*, 1995) had previously failed to identify the corresponding gene. The 5S RNA species itself had also escaped detection over a number of years in experiments where ethidium bromide was routinely used to visualize gel-purified *A. castellanii* mitochondrial RNA species. Only when an alternative technique (UV shadowing) was employed was this 5S rRNA finally revealed. This result clearly demonstrates that direct characterization of mitochondrial RNAs by several methods remains the most reliable approach for the identification of mitochondrial 5S rRNAs and their genes.

The discovery of the *A. castellanii* mitochondrial 5S rRNA suggested that a 5S rRNA species might be encoded in more mitochondrial genomes than is currently appreciated. Indeed, I have presented evidence that another amoebozoan, *H. vermiformis*, also encodes an RNA in its mtDNA that likely represents a highly derived 5S rRNA homolog. Undoubtedly, many other organisms contain mitochondrial 5S rRNAs that have not been identified due to their lack of sequence conservation. The small RNA (msRNA) in *D. discoideum* and its homolog in *P. pallidum* may represent examples of such highly divergent 5S rRNAs. Although the precise role(s) of 5S rRNA in the ribsome is(are) not known with certainty, 5S rRNA is an important component in all examined cytoplasmic, bacterial and plastid ribosomes (see Moore, 1996 for a review). If mitochondrial 5S homologs are found to be similarly ubiquitous, it is a strong possibility that future research will reveal a vital function for a 5S rRNA in all mitochondrial ribosomes.

## V. CONCLUSIONS

The data presented in this thesis demonstrate that the 5′ tRNA editing activities found in the mitochondria of the distantly related organisms *A. castellanii* and *S. punctatus* have similar biochemical mechanisms. This new information sheds light on the evolution of this intriguing form of tRNA processing, raising the possibility that homologous enzymes are responsible for editing in each case. To definitively resolve questions about the evolution and mechanism of these activities, future research should focus on enzyme purification in order to identify editing components from both organisms, and characterization of these activities should continue using the established *in vitro* systems.

The discovery of a mitochondrial 5S rRNA in *A. castellanii* and putative homologs in other members of Amoebozoa suggests that mitochondrial ribosomes, like the ribosomes of eubacteria, archaea, chloroplasts and the cytosol of eukaryotes, may have a stringent requirement for a 5S rRNA component. This discovery also highlights the importance of direct characterization of RNAs using a variety of methods, as the *A. castellanii* mitochondrial 5S rRNA was not identified either by mtDNA sequence analysis or by ethidium bromide staining of mitochondrial RNAs. Further research should identify further mitochondrial 5S rRNA homologs and address the precise role(s) of this enigmatic RNA in ribosomal function.

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