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A novel nucleotide incorporation activity implicated in the editing of mitochondrial transfer RNAs in *Acanthamoeba castellanii*

DAVID H. PRICE and MICHAEL W. GRAY[1]

Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

ABSTRACT

In *Acanthamoeba castellanii*, most of the mtDNA-encoded tRNAs are edited by a process that replaces one or more of the first three nucleotides at their 5′ ends. As a result, base pairing potential is restored at acceptor stem positions (1:72, 2:71, and/or 3:70, in standard tRNA nomenclature) that are mismatched according to the corresponding tRNA gene sequence. Here we describe a novel nucleotide incorporation activity, partially purified from *A. castellanii* mitochondria, that has properties implicating it in mitochondrial tRNA editing in this organism. This activity is able to replace nucleotides at the first three positions of a tRNA (positions 1, 2, and 3), matching the newly incorporated residues through canonical base pairing to the respective partner nucleotide in the 3′ half of the acceptor stem. Labeling experiments with natural (*Escherichia coli* $tRNA^{Tyr}$) and synthetic (run-off transcripts corresponding to *A. castellanii* mitochondrial $tRNA^{Leu1}$) substrates suggest that the nucleotide incorporation activity consists of at least two components, a 5′ exonuclease or endonuclease and a template-directed 3′-to-5′ nucleotidyltransferase. The nucleotidyltransferase component displays an ATP requirement and generates 5′ pppN... termini in vitro. The development of an accurate and efficient in vitro system opens the way for detailed studies of the biochemical properties of this novel activity and its relationship to mitochondrial tRNA editing in *A. castellanii*. In addition, the system will allow delineation of the structural features in a tRNA that identify it as a substrate for the labeling activity.

Keywords: 3′-to-5′ tRNA nucleotidyltransferase; nucleotide addition; pre-tRNA processing

INTRODUCTION

RNA editing may be defined as a programmed alteration of RNA primary structure such that the resulting sequence could have been directly encoded in the corresponding gene. Although transcripts of protein-coding genes are the usual substrates for the various RNA editing systems that have been described to date (Smith et al., 1997), structural RNAs such as rRNA and tRNA also undergo editing (Mahendran et al., 1994; Price & Gray, 1998). Since the first report of tRNA editing, in the mitochondria of the amoeboid protozoon, *Acanthamoeba castellanii* (Lonergan & Gray, 1993a), a number of other, mechanistically distinct tRNA editing systems have been described (reviewed in Price & Gray, 1998).

In the case of *A. castellanii*, secondary structure modeling predicted that of the 15 bona fide tRNAs encoded by the mitochondrial genome, 12 would have one or more mismatches within the first three base pairs (1:72, 2:71, and 3:70, following standard tRNA nomenclature) of the aminoacyl acceptor stem (Lonergan & Gray, 1993a,b; Burger et al., 1995). Because the acceptor stem region is important in defining the tertiary structure of tRNA molecules (Dirheimer et al., 1995; Martin, 1995), and in some cases provides major identity elements for tRNA recognition by aminoacyl tRNA synthetases (Hou & Schimmel, 1988; McClain, 1995; Martinis & Schimmel, 1995; Schimmel & Ripmaster, 1995; Ibba et al., 1996), these predicted mismatches were judged to be incompatible with tRNA function. In fact, analysis of mature *A. castellanii* mitochondrial tRNAs (mt-tRNAs) subsequently revealed that the sequences of the 5′ portions of all of the anomalous acceptor stems are altered by a form of RNA editing that restores canonical (mostly G:C or A:U) base pairing at these positions (Lonergan & Gray, 1993a; Price & Gray, 1999). The end result of this process is to make the secondary

Reprint requests to: Michael W. Gray, Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada; e-mail: mwgray@is.dal.ca.

[1]This paper is dedicated to the memory of Robert Cedergren (d. Oct. 14, 1998), a cherished colleague and friend, and an avid exponent of all things RNA.

structure of each edited tRNA conform to that of a conventional tRNA.

Because this type of RNA editing involves not only purine-to-purine and pyrimidine-to-pyrimidine changes, but also pyrimidine-to-purine changes, it has been suggested (Lonergan & Gray, 1993a) that editing proceeds by way of base or nucleotide replacement rather than base modification. One model (Lonergan & Gray, 1993a) envisages a process in which mismatched nucleotides within the 5′-terminal three positions of the acceptor stem are removed and then replaced, using the 3′ half of the acceptor stem as a guide sequence. This process would require both an endo- or exonuclease (5′-to-3′) to remove nucleotides, and (presumably) a 3′-to-5′ nucleotidyltransferase activity to replace them. The latter activity, like 5′-to-3′ RNA polymerases, would form canonical (A:U, G:C) base pairs between the newly synthesized strand and its template, in this case the 3′ half of the acceptor stem. Unlike standard RNA polymerases, the editing polymerase would act in a 3′-to-5′ direction, adding nucleotides to the 5′ rather than the 3′ end of a growing chain. In this respect the putative 3′-to-5′ polymerase would resemble the activity of histidine tRNA guanylyltransferase (Williams et al., 1990), which adds a single guanylate residue to the 5′ end of histidine tRNA, opposite a cytidylate at the discriminator position (N_{73}).

The particular form of editing first described in *A. castellanii* mitochondria (Lonergan & Gray, 1993a) has also been observed to act on mt-tRNAs in a number of other species of *Acanthamoeba* (D.R. Ledee & T.J. Byers, pers. comm.), as well as on the mt-tRNAs of certain chytridiomycete fungi (Laforest et al., 1997; B.F. Lang, pers. comm.). The latter organisms are not known to be specific evolutionary relatives of *Acanthamoeba*. This form of editing may therefore be more widespread than is currently appreciated, and may even have arisen in evolution more than once (Price & Gray, 1998).

Here we report the partial purification, from *A. castellanii* mitochondria, of a previously unreported nucleotide incorporation activity (NIA) that has many of the characteristics expected of the enzyme(s) that mediate(s) the editing of mt-tRNAs in this organism. We describe the development of a reproducible and stable in vitro system that we have used to investigate the properties, reaction requirements and substrate specificity of this novel activity.

RESULTS

Labeling of tRNA-sized RNA species *in organello*

A potential tRNA editing activity in *A. castellanii* mitochondria first emerged during experiments utilizing an *in organello* assay, based on one described for another protist, *Physarum polycephalum* (Visomirski-Robic & Gott, 1995), to test for incorporation of radiolabeled nucleotide precursors into mitochondrial transcripts. Incubation of *A. castellanii* mitochondria in the presence of [α-^{32}P]ATP and [α-^{32}P]CTP efficiently labeled both heterodisperse, high-molecular-weight transcripts and discrete, tRNA-sized species (data not shown). Incubation with [α-^{32}P]UTP and [α-^{32}P]GTP produced a similar pattern of labeling (Fig. 1). *In organello* labeling of tRNA-sized species had not been observed in the *Physarum* system (Visomirski-Robic & Gott, 1995); furthermore, although labeling of high-molecular-weight RNAs *in organello* could be ascribed to resumption of RNA synthesis from stalled transcription complexes, it seemed unlikely that the relatively intense labeling of discrete, tRNA-sized species could be accounted for by the processing of radiolabeled, tRNA-containing run-on transcripts. Finally, whereas labeling of tRNA species with [α-^{32}P]ATP or [α-^{32}P]CTP could potentially reflect the activity of ATP(CTP):tRNA nucleotidyltransferase, this enzyme is not known to efficiently incorporate GTP into tRNAs (Sprinzl & Cramer, 1979). The intense labeling of tRNAs with [α-^{32}P]GTP, and the labeling of a larger number of species than in the case of [α-^{32}P]UTP (compare A and B, Fig. 1), was particularly encouraging in view of the fact that there is a G residue at position 1 (the 5′ terminus) in nine of the 12 *A. castellanii* mt-tRNAs in which editing has been demonstrated (Lonergan & Gray, 1993a; Price & Gray, 1999), and five of these tRNAs also have a G at position 2.

Labeling of mitochondrial tRNAs in vitro

The *in organello* labeling results could be replicated using crude and partially fractionated mitochondrial extracts, which had the advantage of eliminating the background of heterodisperse, high-molecular-weight labeled RNA species resulting from run-on transcription in the *in organello* experiments. In the presence of added *A. castellanii* mitochondrial RNA (mt-RNA) and [α-^{32}P]GTP, labeling was restricted almost exclusively to tRNA-sized species, with nucleotide incorporation activity present in the S100 fraction of a mitochondrial lysate (Fig. 2, lane 1) and various subfractions obtained by ammonium sulphate fractionation (Fig. 2, lanes 2 and 3) and DEAE-Sephacel chromatography (Fig. 2, lane 6).

In the absence of added RNA, the fraction eluting from DEAE-Sephacel at 0.4 M KCl (DS Fraction) did not support the labeling of any product. The DS Fraction would be expected to be devoid of RNA species (including tRNA) that are present in the S100 and ammonium sulphate fractions but that are retained on DEAE-Sephacel in the presence of 0.4 M KCl. Thus, background labeling could essentially be eliminated by passing mitochondrial extracts through a DEAE-Sephacel column, rendering such depleted extracts dependent on added substrate RNA.

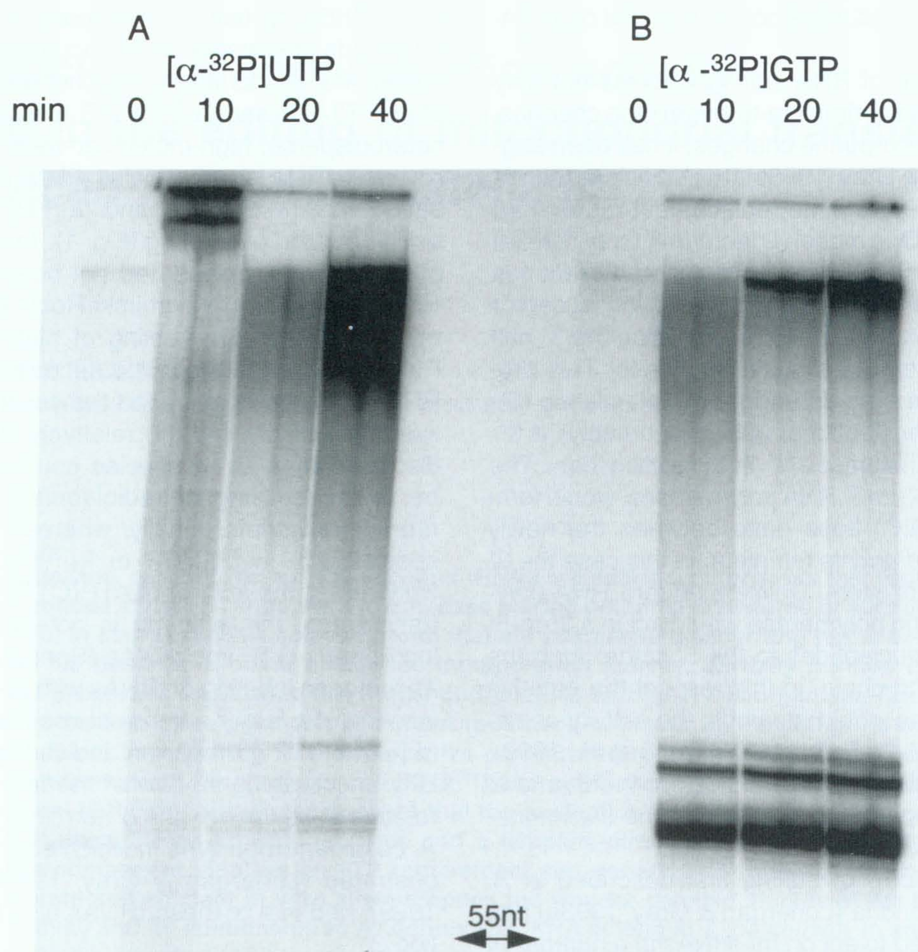

FIGURE 1. *In organello* labeling of mitochondrial transcripts with [α-^{32}P]UTP (**A**) and [α-^{32}P]GTP (**B**). Following incubation of isolated *A. castellanii* mitochondria under nonoptimized conditions (see text) in the presence of an [α-^{32}P]-labeled rNTP for varying time periods, labeled transcripts were isolated, fractionated by electrophoresis in a 10% polyacrylamide gel, and visualized by autoradiography (13-h exposure, XK-1 film with intensifying screen). The migration position of the bromophenol blue dye is indicated by the double-headed arrow.

To examine the distribution of incorporated radioactivity, the tRNA-sized products labeled in vitro were recovered and separately hydrolyzed with nuclease P1 and alkali. Depending on the nature of the 5′ terminus, treatment of [α-^{32}P]GTP-labeled material with nuclease P1 would be expected to yield pG from internal positions and pG, ppG, and/or pppG (depending on the degree of phosphorylation) from the 5′ terminus (Fig. 3A). In fact, nuclease P1 released almost all of the incorporated radioactivity as a roughly equimolar mixture of products co-migrating on PEI-cellulose with pppG and pG standards (Fig. 4, lane 1). This result indicated that (1) the labeled nucleotides were being incorporated into the main chain of the substrate (and not as nucleoside modifications), and (2) a substantial fraction of the incorporated nucleotides (those liberated as pppG) were present at the 5′ terminus of the labeled substrate.

Alkaline hydrolysis of labeled material released a major product having the chromatographic properties expected of a polyphosphorylated nucleotide (Fig. 5), and which in other experiments (see below) was identified as pppGp. This result is consistent with the incorporation of the [α-^{32}P]GTP label predominantly into the first two positions at the 5′ end of the tRNA species labeled in vitro (Fig. 3A). A small quantity of labeled Gp (from internal positions, and resolved into its 2′ and 3′ phosphate isomers in the tlc system used) was also evident among the products of alkaline hydrolysis (Fig. 5).

To examine the substrate specificity of the DS Fraction, RNAs from a variety of sources were tested. Transfer RNAs from wheat mitochondria (Fig. 6, lane 3) and cytosol (Fig. 6, lane 4) were found to be labeled by the DS Fraction, as was tRNA from *E. coli* and *Spizellomyces punctatus* (a chytridiomycete fungus) mitochondria (data not shown). In contrast, *A. castellanii* cytosolic RNA was not labeled at all (Fig. 6, lane 2) under conditions that supported efficient labeling of mt-tRNAs from this organism (Fig. 6, lane 1). Our results did, however, indicate that the *A. castellanii* cytosolic RNA

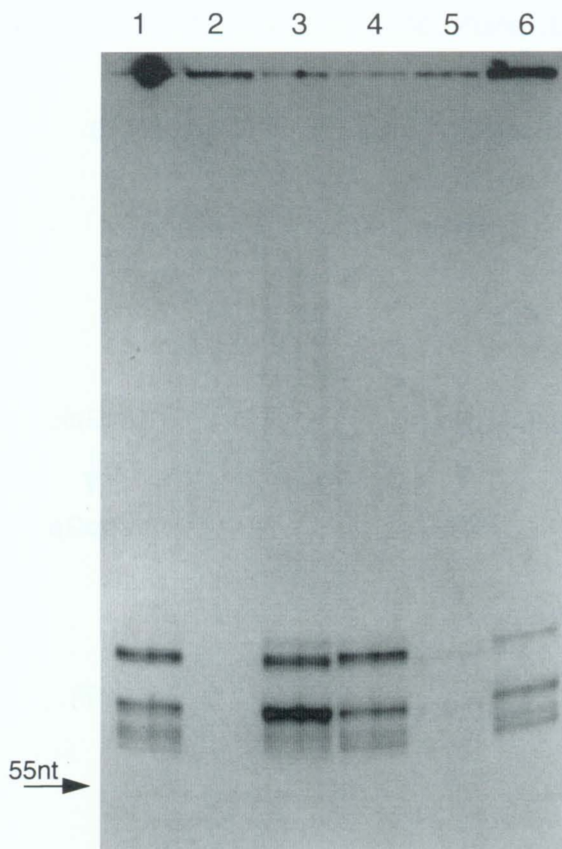

FIGURE 2. In vitro labeling of added *A. castellanii* mt-RNA by various mitochondrial protein fractions. Labeling was carried out in the presence of [α-^{32}P]GTP under nonoptimized conditions (see text) for 1 h at 30 °C, after which labeled transcripts were isolated and fractionated as described in Figure 1. The arrow indicates the migration position of the bromophenol blue dye marker. Lane 1: S100 fraction; lanes 2–5: 0–10%, 10–20%, 20–30% and 30–50% $(NH_4)_2SO_4$ fractions, respectively, of a mitochondrial S100; lane 6: fraction eluted with 0.4 M KCl during chromatography of the S100 fraction on DEAE-Sephacel ("DS Fraction"; see text). In the ammonium sulphate fractionation, labeling activity is seen to be concentrated in the 10–20% and 20–30% cuts.

fraction could act as a competitive inhibitor of the labeling of *A. castellanii* mt-tRNA (data not shown).

Labeling of *E. coli* tRNA^{Tyr}(GUA)

Optimization of labeling conditions

To be able to analyze more rigorously the pattern of incorporated radiolabeled nucleotides, and to allow optimization of reaction conditions, we investigated whether single tRNA species might serve as substrates for labeling by the DS Fraction. A positive result was obtained with *E. coli* tRNA^{Tyr}(GUA), whose aminoacyl acceptor stem is very similar to that of its counterpart in *A. castellanii* mitochondria (Fig. 7A). Upon incubation of this tRNA species with DS Fraction in the presence of [α-^{32}P]GTP and the three remaining unlabeled rNTPs, a single labeled species was produced (Fig. 7B, lane 2), which migrated in the position expected of a tRNA having a long variable loop (compare lanes 1 and 2, Fig. 7B).

With *E. coli* tRNA^{Tyr}(GUA) as substrate, the NIA displayed optimal activity between 5 and 10 mM $MgCl_2$, with incorporation at these Mg^{2+} concentrations being markedly higher (>10-fold) than at 40 mM, the concentration employed in initial experiments that used a transcription-type buffer (non-optimized conditions; see Materials and Methods). The same [Mg^{2+}] dependence was seen with *A. castellanii* mt-RNA as substrate. As divalent cation, Mn^{2+} could substitute for Mg^{2+}; in contrast, Zn^{2+}, Co^{2+}, Ba^{2+}, and Cd^{2+} did not support nucleotide incorporation. In fact, labeling was inhibited completely when Zn^{2+} was included in the reaction mix along with Mg^{2+}. Optimal [K^+] was found to be 5 mM and optimal pH (using HEPES or PIPES buffer) was 7.0. The DS Fraction showed maximal activity between 42 °C and 50 °C, with activity declining sharply by 58 °C and absent at 65 °C.

Subsequent incubations were carried out under optimized reaction conditions [40 mM HEPES (pH 7.0), 7.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT] at 37 °C. Under these conditions, incorporation of label from [α-^{32}P]GTP into *E. coli* tRNA^{Tyr} followed a linear time course over at least 4 h, with a plateau (stable up to at least 12 h) being reached between 4 and 8 h of incubation.

Pattern of nucleotide incorporation

Nuclease P1 hydrolysis of [α-^{32}P]GTP-labeled *E. coli* tRNA^{Tyr} released both [α-^{32}P]pG and [α-^{32}P]pppG, in a ratio of roughly 1:2 (Fig. 4, lane 2). Alkaline hydrolysis (in which nearest-neighbor transfer of label occurs; Fig. 3) released a highly polar, ^{32}P-labeled product (Fig. 8B) that comigrated with an authentic pppGp marker on PEI-cellulose (Fig. 8A). The results indicated that labeled G nucleotides had been incorporated next to one another, for the most part at the first two positions at the 5′ end of the molecule (see Fig. 3B,C).

When the labeling experiment with *E. coli* tRNA^{Tyr} was repeated using [α-^{32}P]UTP in place of [α-^{32}P]GTP, a single labeled product was again generated (Fig. 9, lane 1). In this case, nuclease P1 and alkaline treatment of the product released pU (Fig. 10A) and Gp (Fig. 10B), respectively. This result demonstrated that labeled U residues were being incorporated exclusively into interior position(s) of the polynucleotide chain, 3′ to G residues (i.e., as 5′...GpU...3′; see Fig. 3D).

When *E. coli* tRNA^{Tyr} was incubated with [α-^{32}P]UTP in the presence of ATP only, the product (Fig. 9, lane 2) was shorter by two nucleotides than the product generated under complete reaction conditions (Fig. 9, lane 1). This result is expected if incorporation of U is occurring at the third position from the 5′ end of the substrate tRNA (see Figs. 3D and 7A). To test whether

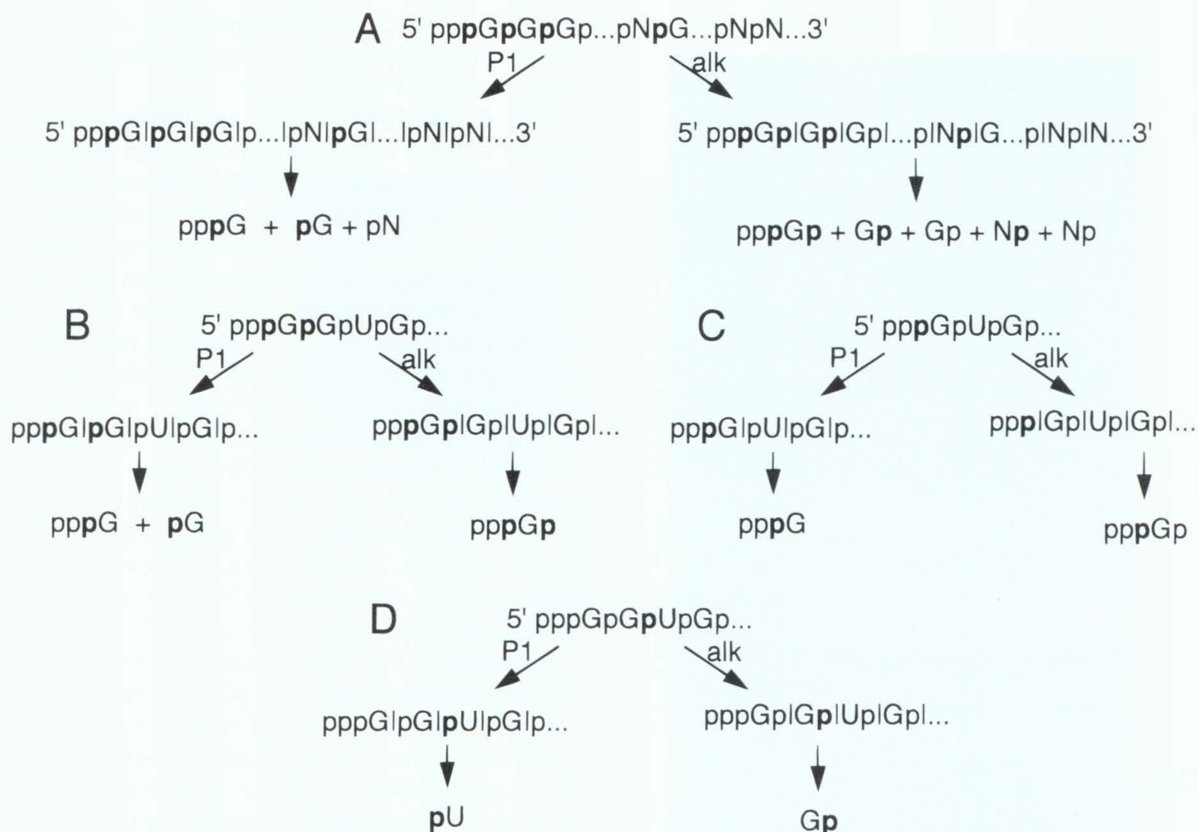

FIGURE 3. Products expected to be released by nuclease P1 and alkaline hydrolysis of various labeled RNA substrates. **A**: Transcript uniformly labeled with [α-^{32}P]GTP. **B**: Transcript labeled with [α-^{32}P]GTP at positions N_1 (5′ terminus) and N_2 only. **C**: Transcript labeled with [α-^{32}P]GTP at 5′ terminus only. **D**: Transcript labeled with [α-^{32}P]UTP at position N_3 only. The sequence in (**B**) and (**D**) corresponds to that of *E. coli* tRNA^{Tyr} (see text), whereas the sequence in (**C**) represents the same RNA starting at position N_2 (i.e., lacking the normal 5′-terminal G residue). Radiolabeled phosphate groups are shown in bold. Cleavages occurring during either nuclease P1 (P1) or alkaline hydrolysis (alk) are indicated by vertical lines.

this shorter product represents a bona fide intermediate or is a dead-end product generated under incomplete reaction conditions, further analysis was carried out. Following a 15-min incubation of the substrate in the presence of [α-^{32}P]UTP and ATP, GTP was added to a final concentration of 40 μM, and the reaction mixture was incubated for a further 15 min. The final product of this chase experiment (Fig. 9, lane 3) was found to be the same size, and to give the same nuclease P1 and alkaline hydrolysis products, as that generated when unlabeled ATP and GTP were added together rather than sequentially (Fig. 9, lane 4). This result demonstrated that the shortened species synthesized in the absence of GTP is not a dead-end product of the reaction, but rather a true intermediate.

Minimal nucleotide requirements

In initial experiments, the complete reaction mixture had included each of the four rNTPs, one [α-^{32}P]-labeled, the other three unlabeled. To investigate minimal nucleotide requirements, various combinations of unlabeled rNTPs were tested using the *E. coli* tRNA^{Tyr} substrate and [α-^{32}P]GTP as label. Strong labeling was observed in the presence of ATP + CTP + UTP (Fig. 11A, lane 1), as well as with ATP + UTP (Fig. 11A, lane 6). Weaker labeling occurred in the presence of ATP alone (Fig. 11A, lane 3) or ATP + CTP (Fig. 11A, lane 7). In the absence of ATP (Fig. 11A, lanes 2, 4, 5, and 8), no labeling was observed, demonstrating an absolute requirement for this nucleotide under these conditions. The efficiency of labeling was significantly enhanced in the presence of UTP (compare Fig. 11A, lanes 3 and 6), but this nucleotide was not absolutely required.

When the labeling experiment was repeated with [α-^{32}P]UTP in place of [α-^{32}P]GTP, strong labeling was observed in the presence of ATP alone (Fig. 11B, lane 3), ATP + GTP (Fig. 11B, lane 5), and ATP + CTP (Fig. 11B, lane 7). In the absence of ATP and presence of GTP (compare Fig. 11B, lanes 2 and 6 with lanes 5 and 7, respectively), weaker labeling was observed. In the absence of both ATP and GTP (Fig. 11B, lanes 4 and 8), no nucleotide incorporation was observed. These results demonstrated a requirement for either ATP or GTP in the labeling of *E. coli* tRNA^{Tyr} with [α-^{32}P]UTP.

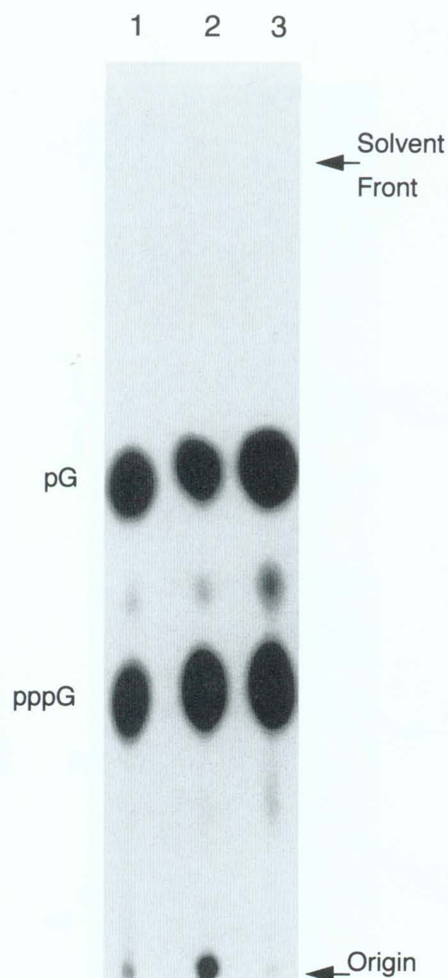

FIGURE 4. Resolution of the products of nuclease P1 hydrolysis of various [α-^{32}P]GTP-labeled RNAs. Gel-purified samples of RNAs labeled in vitro were hydrolyzed to completion with nuclease P1 and the radiolabeled nucleotide products were resolved by one-dimensional thin-layer chromatography on PEI cellulose using 0.5 M NH_4HCO_3 as solvent. The locations of marker nucleotides (pG and pppG), origin, and solvent front are indicated. Lane 1: *A. castellanii* mt-RNA (see Fig. 2); lane 2: *E. coli* tRNA^{Tyr} (see Fig. 7B, lane 2); lane 3: run-off transcript corresponding to most of the *A. castellanii* mt-tRNA^{Leu1} sequence (see Fig. 12B). In all cases, a minor product having the chromatographic mobility of guanosine 5′ diphosphate (ppG) is evident between pG and pppG.

As previously observed (see Fig. 9), the labeled product generated in the absence of GTP (Fig. 11B, lanes 3 and 7) was slightly shorter (by one or two nucleotides) than that generated in the presence of GTP. The appearance of this shorter product in the absence of GTP is consistent with a 3′-to-5′ direction of incorporation of nucleotides into positions N_1–N_3 of the substrate, because positions N_1 and N_2 are both occupied by G (see Fig. 7A). In this scenario, the labeling activity would "stall" in the absence of GTP, leaving positions N_1 and N_2 unfilled, thereby generating a slightly shorter product, as observed.

It is notable that in this particular experiment, two or three labeled products, differing in size in steps of one

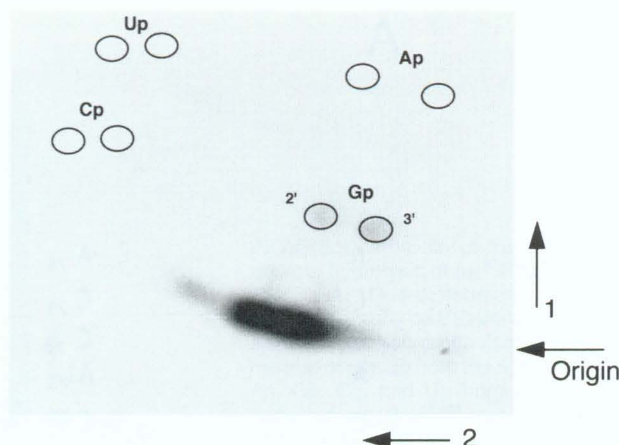

FIGURE 5. Analysis of the products of alkaline hydrolysis of mitochondrial tRNA labeled with [α-^{32}P]GTP in vitro. In vitro-labeled RNA was isolated, fractionated in a 10% polyacrylamide gel (see Fig. 2), and tRNA-sized products were eluted from the gel and hydrolyzed by incubation for 96 h at 23 °C in 1.0 M NaOH. Hydrolysis products were fractionated by two-dimensional thin-layer chromatography on cellulose plates, as described in the text. The direction of chromatography in each dimension as well as the location of the origin are indicated by arrows. The migration positions of standard nucleotides (Ap, Cp, Gp, Up) are indicated. In this system, paired spots are generated for each standard due to the separation of the 2′ and 3′ phosphate isomers released by alkaline hydrolysis of RNA.

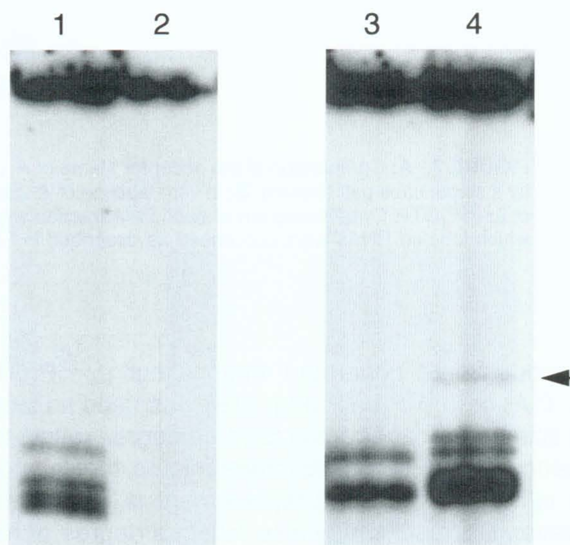

FIGURE 6. In vitro labeling of *A. castellanii* and wheat RNA fractions with [α-^{32}P]GTP. Lane 1: 1.0 μg *A. castellanii* mt-RNA; lane 2: 10 μg *A. castellanii* cytosolic RNA; lane 3: 2.0 μg wheat mitochondrial tRNA; lane 4: 2.0 μg wheat cytosolic tRNA. Labeling was conducted under optimized conditions (see text) in the presence of DS fraction for 1 h at 37 °C, following which labeled RNAs were isolated, electrophoresed in a 10% polyacrylamide gel, and visualized by autoradiography on XK-1 film (12-h exposure with intensifying screen). With these reaction conditions, labeling of the different RNA fractions was considerably more uniform than under nonoptimized conditions; however, *A. castellanii* cytosolic tRNAs were not labeled under either condition. The arrow points to the position of the wheat cytosolic 5S rRNA, which is present in the cytosolic tRNA fraction and which appears to be labeled, presumably by virtue of the fact that its 5′- and 3′-terminal sequences base pair in a way that resembles a tRNA acceptor stem.

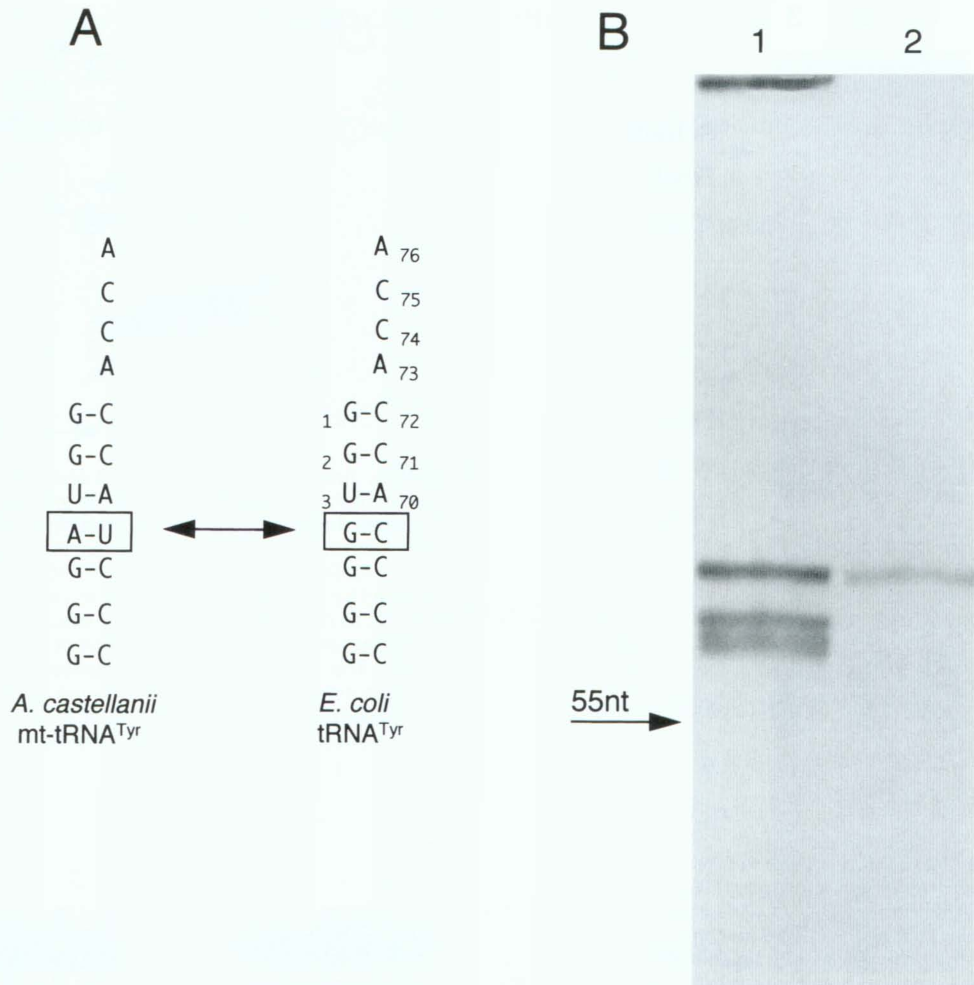

FIGURE 7. A: Comparison of the acceptor stems of *A. castellanii* mt-tRNA^{Tyr} and *E. coli* tRNA^{Tyr}. The two sequences differ by a single base pair (boxed). **B:** In vitro labeling of *A. castellanii* mt-RNA (lane 1) and *E. coli* tRNA^{Tyr} (lane 2) in the presence of [α-^{32}P]GTP. One microgram of each RNA fraction was labeled for 1 h at 30 °C under nonoptimized conditions, following which labeled RNAs were processed as described in Figure 6.

nucleotide, were generated when either [α-^{32}P]GTP (Fig. 11A) or [α-^{32}P]UTP (Fig. 11B) was used as label. This size heterogeneity may to some degree reflect the presence of partially edited intermediates, that is, products in which nucleotide replacement is incomplete. However, the results may also reflect a degree of heterogeneity in the 3′-terminal -CCA$_{OH}$ extension of the tRNA^{Tyr} substrate, a possibility that was not investigated further.

Labeling of a run-off transcript corresponding to *A. castellanii* mt-tRNA^{Leu1}

With *E. coli* tRNA^{Tyr} as substrate, the nucleotide incorporation data indicated that the NIA was generating the 5′-terminal sequence pppGpGpU…, which (excepting the degree of 5′ phosphorylation) is identical to the 5′-terminal sequence of *E. coli* tRNA^{Tyr} (Fig. 7A). The labeling results were consistent with the idea that the incorporated nucleotides were replacing the nucleotides originally present at positions N_1–N_3 of the substrate.

An alternative explanation that needed to be excluded was that the 5′ end of the tRNA substrate was being extended by an additional three nucleotides (at positions N_{-1}–N_{-3}, opposite N_{73}–N_{75}; Fig. 7A). This possibility could apply in the case of *E. coli* tRNA^{Tyr}, whose 3′-terminal sequence (starting at position N_{71}) is …CCACCA<u>**CC**</u>**A**$_{OH}$. In this instance, nucleotides could potentially have been incorporated opposite the 5′…ApCpC…3′ sequence (underlined) formed by the discriminator nucleotide (A_{73}) and the two C residues (positions N_{74} and N_{75}) of the 3′ -CCA$_{OH}$ extension (shown in bold), rather than opposite positions N_{70}–N_{72} (see Fig. 7A). The same concern applied to most of the 15 tRNAs encoded by *A. castellanii* mtDNA, 13 of which have an A residue in the discriminator position, followed by the 3′-terminal -CCA$_{OH}$ extension.

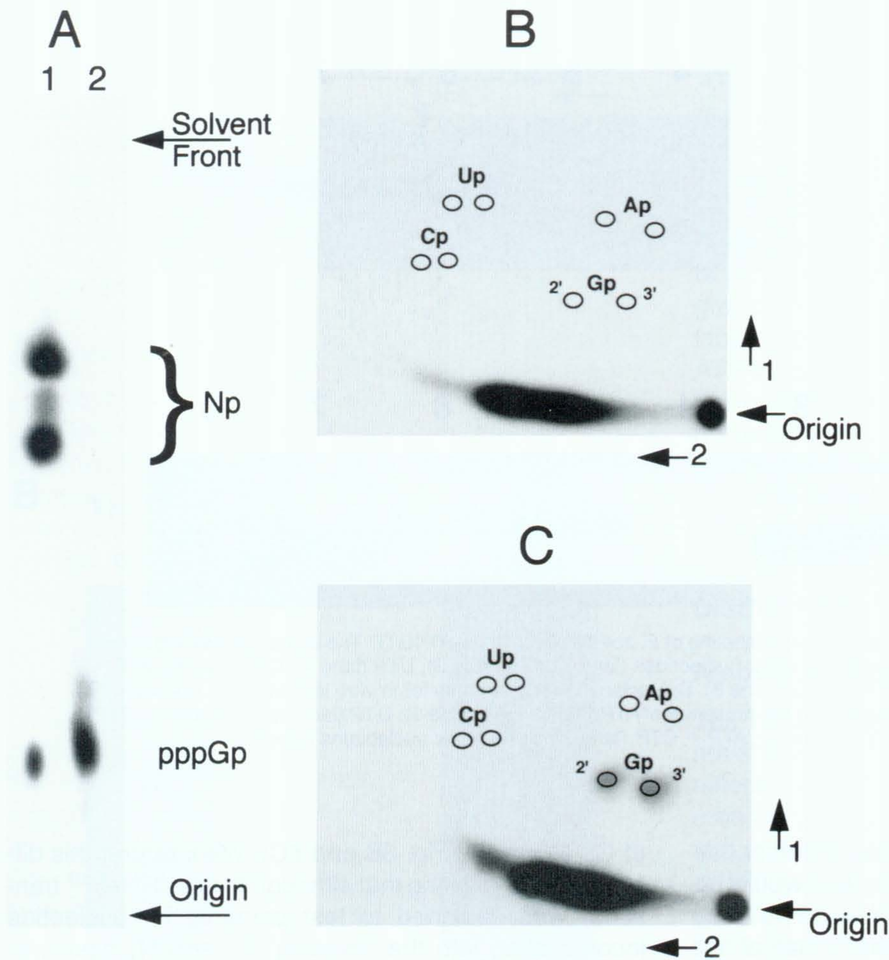

FIGURE 8. Analysis of the products of alkaline hydrolysis of [α-^{32}P]GTP-labeled RNAs. **A**: One-dimensional thin-layer chromatography on PEI-cellulose. Lane 1: alkaline hydrolysis products (a radiolabeled mixture of pppGp from the 5′ terminus and Ap, Gp, Cp, and Up from internal positions) of mt-tRNA^{Leu1} run-off transcript uniformly labeled with [α-^{32}P]GTP during synthesis; lane 2: alkaline hydrolysis products of *E. coli* tRNA^{Tyr} labeled by DS Fraction. **B**: Two-dimensional thin-layer chromatography on cellulose of alkaline hydrolysis products of *E. coli* tRNA^{Tyr} labeled by DS Fraction. **C**: Two-dimensional thin-layer chromatography on cellulose of alkaline hydrolysis products of *A. castellanii* mt-tRNA^{Leu1} labeled by DS Fraction. Note the partial separation of the pppGp label into the expected 2′ and 3′ isomers in (**B**) and (**C**).

To address this issue, and at the same time to determine whether a synthetic tRNA would serve as substrate for the NIA, a run-off transcript corresponding to most of the *A. castellanii* mt-tRNA^{Leu1} was designed

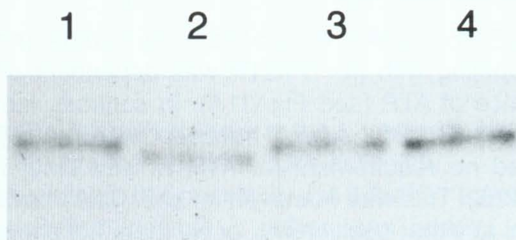

FIGURE 9. Effect of different combinations of unlabeled rNTPs on in vitro labeling of *E. coli* tRNA^{Tyr} with [α-^{32}P]UTP in the presence of DS Fraction. The labeling reaction was carried out at 37 °C under non-optimized conditions (see text). Lane 1: ATP, GTP, and CTP all present throughout a 30-min incubation; lane 2: only ATP present throughout a 15-min incubation; lane 3: ATP present during the first 15 min of incubation, GTP then added and the reaction allowed to proceed for a further 15 min; lane 4: ATP + GTP both present throughout the 30-min incubation period. Labeled RNAs were isolated, fractionated by polyacrylamide gel electrophoresis, and visualized on XK-1 film (12-h exposure with intensifying screen).

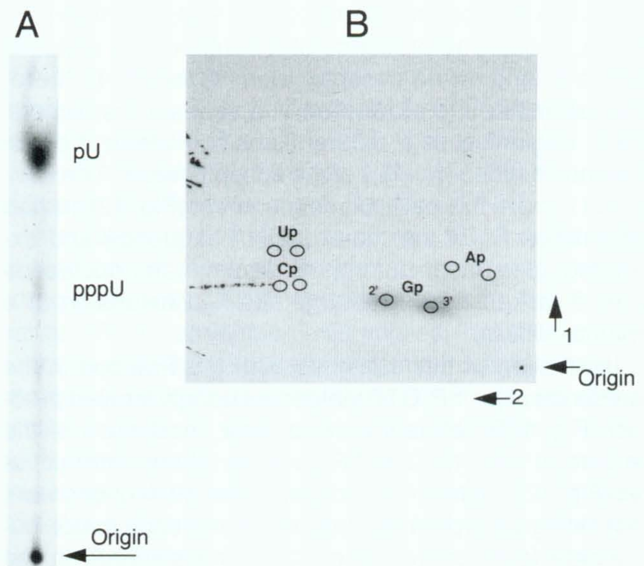

FIGURE 10. Analysis of the products of nuclease P1 hydrolysis (**A**) and alkaline hydrolysis (**B**) of *E. coli* tRNA^{Tyr} labeled with [α-^{32}P]UTP by DS Fraction. The RNA sample used for this analysis was that shown in Figure 9, lane 1. Hydrolysis products were resolved by one-dimensional thin-layer chromatography on PEI cellulose (**A**) and two-dimensional thin-layer chromatography on cellulose (**B**), as described in the text.

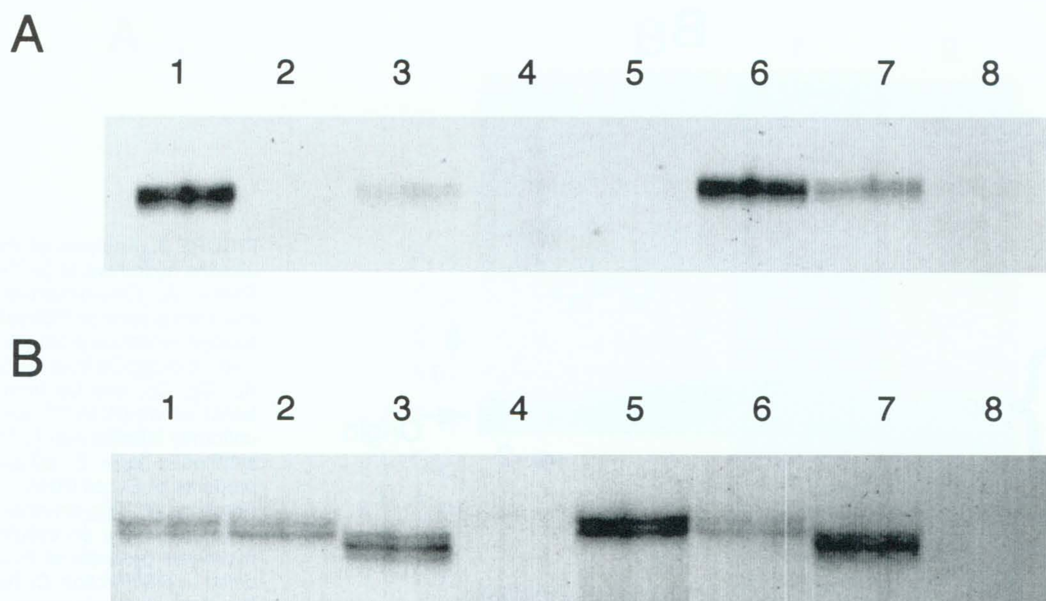

FIGURE 11. **A**: Nucleotide requirements for in vitro labeling of *E. coli* tRNA^{Tyr} with [α-^{32}P]GTP. The substrate was incubated in the presence of ATP, CTP + UTP (lane 1); no other nucleotides (lane 2); ATP (lane 3); UTP (lane 4); CTP (lane 5); ATP + UTP (lane 6); ATP + CTP (lane 7); or CTP + UTP (lane 8). **B**: Nucleotide requirements for in vitro labeling of *E. coli* tRNA^{Tyr} with [α-^{32}P]UTP. The substrate was incubated in the presence of ATP, CTP + GTP (lane 1); GTP (lane 2); ATP (lane 3); CTP (lane 4); ATP + GTP (lane 5); CTP + GTP (lane 6); ATP + CTP (lane 7); or no other nucleotides (lane 8).

(Fig. 12A). This particular tRNA was selected because its 3′-terminal sequence (...CpCpCpCpA$_{73}$) would be expected to support the incorporation of [α-^{32}P]GTP by the NIA into terminal positions within the 5′ half of the acceptor stem. As well, T3 RNA polymerase, used to synthesize the run-off transcripts, has a strong (if not exclusive) preference for a G residue at the first position of the transcript (Maitra et al., 1980; Chamberlin & Ryan, 1982). The presence of a number of G residues at the 5′ end of the acceptor stem of this tRNA therefore permitted the production of a series of transcripts, each beginning at a different position within the first four nucleotides (N$_1$–N$_4$) of the acceptor stem. The transcript used in this particular experiment (Fig. 12) started at position N$_3$ of the mature tRNA sequence and extended up to and including the discriminator nucleotide (i.e., it lacked the 3′-terminal -CCA$_{OH}$ extension of a mature tRNA).

Incubation of this substrate with DS Fraction in the presence of [α-^{32}P]GTP yielded a strongly labeled product (Fig. 12B, left lane); in contrast, incubation of the substrate with [α-^{32}P]UTP gave no labeled product at all (Fig. 12B, right lane), indicating that nucleotides were not being incorporated opposite the discriminator nucleotide (position N$_{73}$, Fig. 12A) or elsewhere in the molecule. As before, nuclease P1 hydrolysis of the labeled product released pppG and pG (Fig. 4, lane 3), whereas alkaline hydrolysis yielded primarily pppGp (Fig. 8C). However, in addition, and in contrast to the case of *E. coli* tRNA^{Tyr}, alkaline hydrolysis of the labeled mt-tRNA^{Leu1} transcript yielded detectable amounts of Gp (compare Fig. 8B and 8C). We interpret this difference as indicating that although the mt-tRNA^{Leu1} transcript was designed to test primarily for nucleotide incorporation into the "missing" N$_2$ and N$_1$ positions, the extract was also capable of removing the 5′ terminus (G at position N$_3$) in a proportion of the substrate molecules before incorporating G residues back into this position, as well as into N$_2$ and N$_1$, to generate a 5′-terminal pp$\underline{p}$G$\underline{p}$G$\underline{p}$G... sequence (radiolabeled phosphorus atoms underlined).

Requirement for an activated 5′-terminal residue

Using *E. coli* tRNA^{Tyr} as substrate, we had determined that labeling with [α-^{32}P]GTP was dependent on the presence of ATP (see Fig. 11A). In contrast, labeling of the mt-tRNA^{Leu1} run-off transcript with [α-^{32}P]GTP showed no requirement for ATP, or any other rNTP (Fig. 12C). This was true even for substrates having 5′ termini at either positions N$_1$ or N$_4$ (data not shown). A major difference between the two substrates (beyond the obvious sequence differences) is that *E. coli* tRNA^{Tyr}, like other *E. coli* tRNAs, carries a 5′-terminal monophosphate group whereas the mt-tRNA^{Leu1} substrate possesses a 5′-terminal triphosphate, as a consequence of its production by run-off transcription. We reasoned that if ATP is required to activate 5′ monophosphate-terminated substrates generated by nucleotide removal prior to replacement, the presence of

FIGURE 12. Strategy for construction of an *A. castellanii* mt-tRNA^{Leu1} run-off transcript and labeling of the transcript in vitro. **A**: Clones corresponding to the mt-tRNA^{Leu1} gene were generated by PCR using primers whose 5′ ends are coterminal with the 5′ and 3′ ends of the tRNA sequence. The 5′ primer contained a T3 RNA polymerase transcription promoter from which a run-off transcript was generated. **B**: Labeling of the mt-tRNA^{Leu1} transcript in the presence of [α-^{32}P]GTP or [α-^{32}P]UTP. **C**: Effects of unlabeled rNTPs included in the reaction mixture on labeling of the mt-tRNA^{Leu1} transcript with [α-^{32}P]GTP.

a high-energy triphosphate at the 5′ end of a substrate might obviate the need for such activation.

To investigate this possibility, mt-tRNA^{Leu1} transcripts having 5′-terminal pG... rather than pppG... were synthesized in vitro (Sampson & Uhlenbeck, 1988; Fig. 13A). In the experiment whose results are presented in Figure 13B, the proportion of 5′ pG-terminated transcripts within the total run-off transcript population was increased stepwise by inclusion of increasing amounts of free pG (Fig. 13B, lanes 1–4 and 5–8) in the transcription reaction. As the proportion of 5′ pG-terminated transcripts increased, the extent of labeling of the substrate decreased in the absence of ATP (Fig. 13B, lanes 1–4). In the presence of ATP, this trend was not seen (Fig. 13B, lanes 5–8). In the absence of ATP, neither UTP nor CTP supported labeling of run-off transcripts of 5′ monophosphorylated mt-tRNA^{Leu1} with [α-^{32}P]GTP above background levels (data not shown).

These results demonstrated a requirement for ATP in the labeling of substrates having 5′ monophosphate ends (compare Fig. 13B, lanes 4 and 8) and the relief of this requirement by the presence of a triphosphate group at the 5′ end of the substrate (compare Fig. 13B, lanes 1 and 5; see also Fig. 12C). This suggests that ATP is used to activate the 5′ end of a tRNA substrate via formation of a high-energy phosphate bond prior to nucleotide incorporation. This conclusion is supported by the observation that although ATP and dATP both supported labeling of a 5′ monophosphate-terminated tRNA substrate, ADP and AMP did not (data not shown).

DISCUSSION

A novel nucleotide incorporation activity

The results summarized here provide evidence of a novel nucleotide incorporation activity in *A. castellanii* mitochondria. This activity displays many of the properties expected of the mitochondrial tRNA editing system in this organism. First, the activity acts selectively on tRNAs. Second, it is limited in its site of action to the first three positions (N_1, N_2, and N_3) on the 5′ side of the acceptor stem of the tRNA substrate. Third, the activity generates a 5′-terminal trinucleotide sequence in a template-directed fashion, evidently using the 3′ half of the acceptor stem (positions N_{70}, N_{71}, and N_{72}) to guide nucleotide incorporation. As a result, the 5′-terminal sequence is able to form canonical base pairs with the complementary sequence on the 3′ side of the acceptor stem. This is precisely the consequence of mitochondrial tRNA editing in *A. castellanii*, which effectively corrects mismatches within the first three positions of the acceptor stem of edited tRNAs.

In addition to acting on synthetic tRNA substrates lacking one or more nucleotides at the 5′ end, the incorporation activity is able to act on mature, fully base-paired tRNA substrates. This implies that the activity contains a component (endo- or exonuclease) that is capable of removing the first three 5′ nucleotides of the acceptor stem, as well as a template-directed 3′-to-5′ nucleotidyltransferase that acts sequentially to restore nucleotides at these positions. Observations that support the existence of the nucleotide excision component include the following:

1. With [α-^{32}P]UTP as the labeled precursor, many fewer tRNAs are labeled *in organello* or in vitro than are labeled with [α-^{32}P]GTP (see Fig. 2). This would not be expected if the incorporation activity was simply extending the mature 5′ end of its tRNA sub-

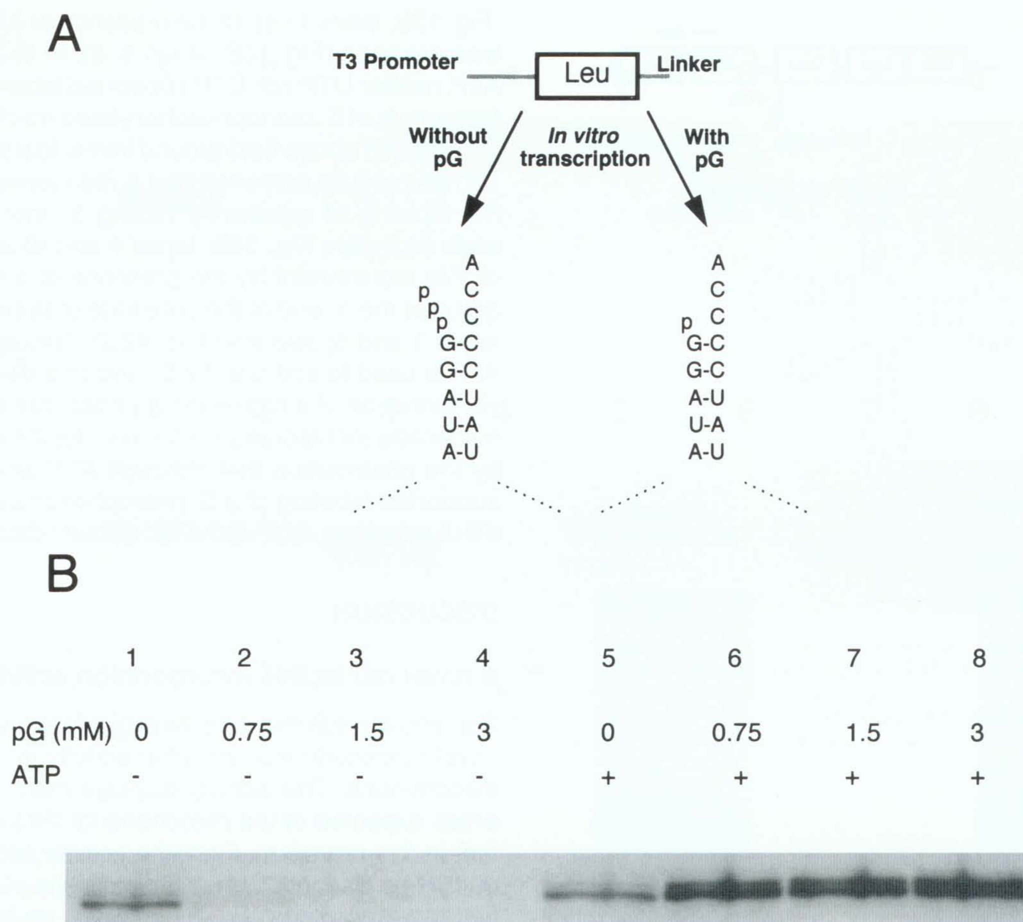

FIGURE 13. **A**: Strategy for generation of mt-tRNA^{Leu1} run-off transcripts having 5′ monophosphate or 5′ triphosphate termini. **B**: Effect of ATP on labeling of mt-tRNA^{Leu1} run-off transcripts having different proportions of 5′ monophosphate and 5′ triphosphate termini. Run-off transcripts having an increasing proportion of 5′ monophosphate termini were synthesized in the presence of increasing concentrations of pG (0–3 mM) in the transcription mix during generation of the substrate. Labeling reactions were carried out with [α-^{32}P]GTP in the presence or absence of ATP and in the absence of other rNTPs. The increased labeling in lanes 6–8 compared to lane 5 is attributed to stimulation of RNA synthesis by inclusion of pG during run-off transcription; as a result, the labeling reactions corresponding to lanes 6–8 contained a larger amount of substrate than that corresponding to lane 5.

strates in a 3′-to-5′ direction, using as template the discriminator nucleotide at N_{73} (an adenylate in most cases) and residues C_{74} and C_{76} of the 3′ -CCA_{OH} extension.

2. With *E. coli* tRNA^{Tyr}(GUA) as substrate, incorporation of label from [α-^{32}P]GTP occurs (albeit with reduced efficiency) in the absence of unlabeled UTP (Fig. 11A), a result that is also not expected if sequential extension of the mature 5′ terminus is occurring, starting opposite the discriminator nucleotide.
3. With a run-off mt-tRNA^{Leu1} transcript beginning at the N_3 position and lacking a 3′ -CCA_{OH} extension, efficient labeling occurs with [α-^{32}P]GTP but no labeling is detectable with [α-^{32}P]UTP (Fig. 12B). In this case, the labeling pattern is consistent with incorporation of G into the missing N_2 and N_1 positions (opposite C_{71} and C_{72}, respectively), but no incorporation opposite the discriminator nucleotide (A_{73}).
4. In the case in (3), a detectable amount of Gp was liberated during alkaline hydrolysis of the labeled product (Fig. 8C), a result consistent with the removal and reincorporation of a proportion of the 5′-terminal nucleotide (corresponding to the guanylate at position 3).
5. Finally, mt-tRNA^{Leu1} transcripts were efficiently labeled with [α-^{32}P]GTP even when they retained nucleotides at the N_1 and N_2 positions (data not shown).

That the incorporation activity acts in a 3′-to-5′ direction is most clearly evidenced in experiments in which *E. coli* tRNA^{Tyr}(GUA) (whose 5′-terminal sequence is pGpGpU...) was labeled with [α-^{32}P]UTP in the absence of GTP. In this case, the product was two nucleotides shorter than when unlabeled GTP was also present in the reaction mixture (Figs. 9 and 11B), which is expected if the activity is able to incorporate the first nucleotide (a uridylate) but is not able to incorporate

the next two (both guanylates). This result argues against the alternative model in which the incorporation activity first synthesizes a trinucleotide (pppGpGpU) in the usual 5′-to-3′ direction, using positions N_{70}–N_{72} in the 3′ half of the acceptor stem as template, with the trinucleotide then being ligated to the 5′ end of a tRNA substrate lacking the first three nucleotides.

Nature and requirements of the postulated 3′-to-5′ tRNA nucleotidyltransferase in *A. castellanii* mitochondria

In its ability to add nucleotides to the 5′ end of a tRNA, the nucleotide incorporation activity from *A. castellanii* mitochondria is reminiscent of the enzyme histidine tRNA guanylyltransferase (HTGT; Williams et al., 1990; Jahn & Pande, 1991; Pande et al., 1991). HTGT adds a single guanylate residue to the 5′ end of histidine tRNA, opposite a cytidylate in the discriminator position, to form an extra G-C pair at the end of the acceptor stem (−1 position). In chicken mitochondria, addition of the extra G residue by HTGT leaves a di- or triphosphate at the 5′ end of the modified $tRNA^{His}$, as judged by the ability of the product to be "capped" (i.e., labeled with [α-^{32}P]GTP in the presence of a guanylyltransferase; L'Abbé et al., 1990). In this respect, the chicken mitochondrial enzyme resembles the *A. castellanii* NIA characterized here. On the other hand, yeast HTGT generates a 5′ monophosphate-terminated product (Pande et al., 1991), implying that the enzyme contains or is associated with a (pyro)phosphatase activity.

In preliminary capping experiments, we have not been able to demonstrate labeling of tRNA-sized molecules in the steady-state RNA population of *A. castellanii* mitochondria. If mature *A. castellanii* mt-tRNAs do possess 5′ pN... termini, and if the NIA is involved in mt-tRNA editing, then another activity must exist to convert 5′ pppN... termini to 5′ pN... Clearly, conditions for labeling *A. castellanii* mt-tRNAs, both *in organello* and in vitro, have not revealed the presence of such an activity. We have noted the presence of a small amount of product having the mobility of ppG in nuclease P1 digests of NIA-labeled material (e.g., see Fig. 4), which could be a manifestation of a slow dephosphorylation of the pppN... termini initially generated; however, we have not been able to demonstrate that ppN...-terminated tRNA molecules are intermediates in the conversion of pppN... to pN...

Another characteristic in which the NIA resembles HTGT is in its requirement for an activated 5′-terminal phosphate in the substrate, before the first nucleotide residue can be added. With the NIA acting on a 5′ monophosphate-terminated substrate, this activation step requires ATP (Fig. 13); however, this requirement is relaxed if the substrate contains a 5′ triphosphate terminus (Fig. 12). In the case of yeast HTGT, activation of the 5′ terminus occurs via an enzyme-mediated adenylation reaction, using ATP as donor and with formation of an activated A(5′)pp(5′)N... intermediate (Jahn & Pande, 1991). The nature of the first activated intermediate in the case of the NIA described here remains to be determined.

Substrate specificity

Under optimized conditions, the NIA was found to act not only on *A. castellanii* mt-tRNAs, but on tRNAs from other organisms as well (notably wheat cytosol and mitochondria and *E. coli*). On the other hand, no labeling of *A. castellanii* cytosolic tRNAs was evident in any experiments in which either total cytosolic RNA or a fraction enriched in cytosolic tRNAs was tested as substrate. Discrimination against *A. castellanii* cytosolic tRNAs (which are unlikely to require any kind of editing) and in favor of the mitochondrial tRNAs (most of which do require editing) could be taken as another indication that the activity described here is involved in tRNA editing in vivo. Failure to label the cytosolic tRNAs suggests that these species lack whatever structural features enable a tRNA to serve as a substrate for nucleotide incorporation; conversely, labeling of heterologous tRNAs may simply be fortuitous, reflecting the fact that these substrates happen to have structural features that mimic those of an homologous (i.e., *A. castellanii* mitochondrial) tRNA substrate.

It remains to be established what the structural determinants defining a labeling-competent substrate might be; it is possible such determinants are associated largely, if not exclusively, with the acceptor stem itself. For example, comparison of the acceptor stem sequences of edited mt-tRNAs from *A. castellanii* shows that none has an A at positions N_{71} or N_{72}. Further comparison between tRNAs that do and do not serve as substrates for the nucleotide incorporation activity should provide further insights into the structural determinants of this activity, as should the use of run-off transcripts specifically engineered with sequence changes in the acceptor stem and elsewhere in the tRNA structure.

Relationship between tRNA processing and tRNA editing

In the *A. castellanii* mitochondrial genome, genes are closely packed, and all are encoded on the same strand and therefore potentially expressed from a single promoter (Burger et al., 1995). This arrangement implies that long polycistronic transcripts are processed by endonucleolytic cleavages to generate mature RNA species. Previous work has provided evidence of pre-tRNA cotranscripts and suggested a tight coupling between tRNA processing and tRNA editing (Lonergan & Gray, 1993a). Normally, the enzyme RNase P mediates the endonuceolytic cleavage that creates the 5′ end of ma-

ture tRNAs; however, it has been shown that RNase P selects its cleavage site in part by "measuring" from structural features within the folded tRNA moiety of tRNA precursors (Yuan & Altman, 1995). Therefore, it is not clear how RNase P could cleave a mismatched tRNA precursor in such a way as to initiate the nucleotide excision and replacement carried out by the tRNA editing system.

Our working hypothesis is that in *A. castellanii* mitochondria, endonucleolytic cleavages occur somewhere upstream of mature 5′ tRNA ends during initial processing of pre-tRNA transcripts. Creation of a free 5′ end would then allow removal of nucleotides exonucleolytically up to and including the first three nucleotides of the acceptor stem, at which point nucleotide addition could proceed to restore proper base pairing at positions 1:72, 2:71, and 3:70. It is important to note that we have not yet tested synthetic tRNAs having 5′- and/or 3′-terminal extensions as substrates for the NIA; such RNAs should more closely approximate the structure of partially processed tRNA substrates on which the in vivo tRNA editing system presumably acts. It is conceivable that in the presence of a 3′-terminal extension, the NIA might continue to add nucleotides upstream of the position of the mature 5′ end, with the intermediates thus formed then acting as substrates for RNase P. Not only would this invest RNase P with a role in the processing of all mt-tRNAs in *A. castellanii* (whether or not they require editing), it would also provide a mechanism for creation of a monophosphorylated 5′ terminus. With the in vitro system described here, these possibilities can be tested systematically.

Does in vitro labeling correspond to in vitro editing?

The ability of the NIA to label tRNAs having a fully base-paired acceptor stem (and therefore requiring no editing) may at first sight appear paradoxical, and at odds with an activity that is seemingly designed to act at positions of mispairing. However, our view is that the NIA may be serving as a more generalized activity that is able to replace, constitutively, the first three nucleotides of a substrate tRNA, *whether or not these nucleotides are mismatched.* An analogy of sorts would be the ATP(CTP) tRNA nucleotidyltransferase that reversibly adds the -CCA_{OH} trinucleotide to the 3′ ends of tRNAs, acting as both a nucleotidyltransferase and a pyrophosphorylase. We envisage the evolutionary emergence of an activity able to remove and then restore the first three nucleotides in the 5′ half of an acceptor stem, using information in the 3′ half of the stem to guide reincorporation. We have argued (Price & Gray, 1998) that such an activity would constitute a *de facto* editing activity, effectively correcting any mismatches within the first three base pairs. In fact, the existence of such an activity could be causally related to the presence of mismatches within the first three acceptor stem positions of most *A. castellanii* mt-tRNAs, because structural constraints on these positions would be relaxed at the genome level, allowing these positions to mutate to nucleotides specifying mismatches that could be repaired at the RNA level (Price & Gray, 1998).

Besides functioning in editing, the NIA could have been adapted to serve other roles in mt-tRNA processing in *A. castellanii* mitochondria. One feature of the *A. castellanii* mitochondrial genome is the presence of a number of overlapping genes (Burger et al., 1995), among which are two overlapping tRNA genes (Lonergan & Gray, 1993b). In the latter case, the last nucleotide (equivalent to the discriminator nucleotide at N_{73}) of the upstream $tRNA^{Gln}$ coding sequence overlaps the first nucleotide (equivalent to N_1) of the downstream $tRNA^{Lys}$ sequence. Assuming that these two tRNA genes are cotranscribed and that the resulting cotranscript is processed by precise endonucleolysis, a cleavage just prior to N_{73} of the upstream $tRNA^{Gln}$ sequence would generate an intact downstream $tRNA^{Lys}$; however, the upstream $tRNA^{Gln}$ would lack a nucleotide at the discriminator position, with no obvious mechanism to recover that information. On the other hand, cleavage 3′ to N_{73} of the upstream $tRNA^{Gln}$ would generate an intact $tRNA^{Gln}$, but the downstream $tRNA^{Lys}$ would lack its 5′-terminal nucleotide. In this case, however, the editing (NIA?) activity could restore the normal 5′ terminus, using information in the 3′ half of the $tRNA^{Lys}$ acceptor stem. Thus, the tRNA editing observed in this system may be one manifestation of a more versatile activity that serves generally to maintain the integrity of the 5′ ends of *A. castellanii* mt-tRNAs, much as the ATP(CTP):tRNA nucleotidyltransferase has a role in maintaining the integrity of their 3′ ends.

MATERIALS AND METHODS

Growth of *A. castellanii* and isolation of mitochondria

A 1-L suspension culture of *A. castellanii* strain Neff (American Type Culture Collection 30010) was grown at 30 °C, with moderate shaking, to an OD_{550} of 1.0. Cells were collected by centrifugation at 900 × *g* for 3 min and washed twice in 0.5 volumes phosphate-buffered saline [4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl (pH 7.0)]. Cells were then resuspended in 25 mL of homogenization buffer [10 mM Tris-HCl (pH 7.6), 1 mM $ZnCl_2$, 0.25 M sucrose] and disrupted in a tissue grinder (Dounce; Kontes, Vineland, New Jersey). Nuclei were removed by centrifugation at 900 × *g* for 10 min. Mitochondria were recovered from the postnuclear supernatant by centrifugation at 9,000 × *g* for 20 min, then washed twice in 10 mL of mitochondria wash buffer [10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.35 M sucrose].

Preparation of mitochondrial and cytosolic RNA

Following the second wash of the mitochondrial fraction, mitochondrial nucleic acids were isolated by extraction with phenol-cresol (Parish & Kirby, 1966) and SDS and treated with DNase I (Pharmacia). Cytosolic RNA was isolated by phenol-cresol-SDS extraction of the supernatant following the first high-speed (9,000 $\times$ *g*) centrifugation, then treated with DNase I (Pharmacia).

Sucrose gradient purification of mitochondria

Following the second wash step, mitochondria were resuspended in presucrose gradient buffer [50 mM Tris-HCl (pH 8.0), 3 mM EDTA, 0.25 M sucrose, 1 mM DTT, 0.1% BSA]. The suspension was loaded onto a two-step (1.3 M and 1.55 M) discontinuous sucrose gradient and centrifuged for 1 h at 22,500 rpm in a Beckman SW25.1 swinging bucket rotor. Mitochondria recovered from the gradient were diluted slowly with two volumes postsucrose gradient buffer [20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.25 M sucrose, 15% (v/v) glycerol, 1 mM DTT] and centrifuged at 12,000 $\times$ *g* for 30 min. The resulting mitochondrial pellet was frozen in liquid nitrogen and stored at $-70\,^{\circ}$C.

In organello nucleotide incorporation assay

Purified mitochondria were resuspended in postsucrose gradient buffer at a concentration of 3.0 μg/μL. NIA was assayed in a reaction containing 40 μL isolated mitochondria (about 120 μg), 15 μCi [α-^{32}P]rNTP, 50 mM Tris-HCl (pH 7.5), 36 mM $MgCl_2$, 18 mM KCl, 0.18 M sucrose, 2.5 mM DTT, 0.36 mM EDTA, 10% (v/v) glycerol, and 130 μM each of the other three rNTPs. Mitochondria were preincubated in the reaction mix, without labeled nucleotide, for 5 min at 35 °C. Following addition of the [α-^{32}P]rNTP, samples were incubated at 35 °C for various lengths of time. Reactions were terminated by the addition of SDS to a final concentration of 0.4%, and proteins were extracted with phenol-cresol/chloroform (1:1, v/v). Nucleic acids in aqueous solution were precipitated twice with ethanol and resolved by electrophoresis in a denaturing polyacrylamide gel.

Preparation of a mitochondrial S100 fraction

The postsucrose gradient mitochondrial pellet was resuspended in mitochondria lysis buffer [20 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.25 M sucrose, 15% (v/v) glycerol, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF)] and then pelleted by centrifugation at 12,000 $\times$ *g* for 30 min. Mitochondria were resuspended in 2 mL of the above lysis buffer minus sucrose. The following were then added stepwise, with homogenization for 20 strokes in a Teflon homogenizer after each addition: 414 μL distilled H_2O, 200 μL 20% (v/v) Triton X-100, and 1,060 μL 4 M KCl. The lysate was then vortexed for 10 s every 5 min during a 15-min incubation on ice, then centrifuged at 100,000 $\times$ *g* for 1 h. The supernatant was recovered and dialyzed against dialysis buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 7.5% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF].

Ammonium sulfate fractionation

The dialyzed mitochondrial extract was diluted with an equal volume of ammonium sulfate fractionation buffer [90 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 2.5% (v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF] and stirred slowly as $(NH_4)_2SO_4$ was added to a final concentration of 0.1 g/mL. The suspension was adjusted to pH 8 with 1 M NaOH, stirred for 30 min, and then centrifuged at 16,000 $\times$ *g* for 20 min. Three further steps of $(NH_4)_2SO_4$ addition (0.1 g/mL, 0.1 g/mL, and 0.2 g/mL) were carried out. The resulting four precipitates (0–10%, 10–20%, 20–30%, and 30–50%) were resuspended in buffer A [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 7.5% glycerol, 50 mM KCl, 1 mM DTT, 0.5 mM PMSF], dialyzed against buffer A, frozen in liquid nitrogen, and stored at $-70\,^{\circ}$C.

Chromatography on DEAE-Sephacel

The mitochondrial extract (either the S100 fraction or the pooled 10–30% $(NH_4)_2SO_4$ precipitate) was dialyzed into buffer A, then loaded onto a 5-mL DEAE-Sephacel column that had been preequilibrated in buffer A. The resin was washed with 4 column volumes of buffer A and then with 4 column volumes of buffer A containing 0.4 M KCl. The protein peak eluting at 0.4 M KCl was dialyzed against dialysis buffer and then distributed into small aliquots that were frozen in liquid nitrogen and stored at $-70\,^{\circ}$C.

In vitro nucleotide incorporation assays

Mitochondrial extracts were assayed for NIA, in the presence or absence of exogenous RNA substrates, in a reaction medium containing 40 mM buffer [Tris-HCl (pH 7.6) or HEPES (pH 7.0)], 5–80 mM $MgCl_2$, 5–50 mM KCl, 0–2.5 mM DTT, 5–20 μCi [α-^{32}P]rNTP, and 40–160 μM unlabeled rNTPs (when included). Initial (nonoptimized) conditions were 45 mM Tris-HCl (pH 7.6), 40 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 10 μCi [α-^{32}P]rNTP, 160 μM unlabeled rNTPs. Optimized conditions were 40 mM HEPES (pH 7.0), 7.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 10 μCi [α-^{32}P]rNTP, and 40 μM unlabeled rNTPs. Labeling reactions were incubated for 1 h at 30 °C or 37 °C, extracted once with phenol-cresol, precipitated once with ethanol, and fractionated in a fully denaturing 10% polyacrylamide (19:1 polyacrylamide:*bis*acrylamide) gel. Labeled RNAs were visualized on Kodak X-ray film (XK-1 or XAR) with or without an intensifying screen.

The *E. coli* $tRNA^{Tyr}$(GUA) (from strain MRE 600) used in some experiments was obtained from Boehringer Mannheim.

Recovery of RNA from polyacrylamide gels

Radioactively labeled RNAs were eluted from polyacrylamide gels by shaking gel slices overnight at 4 °C in a 1:1 mixture of phenol-cresol and elution buffer [0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$, and 1.0 mM EDTA] supplemented with 10 μg *E. coli* tRNA as carrier. RNAs in aqueous phase were precipitated twice with ethanol and resuspended in the appropriate volume of distilled H_2O.

Nuclease P1 hydrolysis

Gel-purified RNA samples were incubated for 1 h at 37 °C with 0.1 U nuclease P1 (Sigma) in 50 mM NH_4OAc. Reaction products were resolved by one-dimensional thin-layer chromatography on polyethyleneimine (PEI) cellulose (Sigma) using 0.5 M NH_4CO_3 as solvent.

Alkaline hydrolysis

RNA samples were incubated for 96 h at 23 °C in 1.0 M NaOH, following which the hydrolyzate was neutralized by addition of 0.1 volume glacial acetic acid. Reaction products were resolved by two-dimensional thin-layer chromatography on cellulose (Kodak, 13254 cellulose thin-layer chromatography plates with fluorescent indicator), using 95% ethanol:H_2O (4:1) as the first-dimension solvent and saturated $(NH_4)_2SO_4$:isopropanol (40:1) in the second dimension (Singh & Lane, 1964). Resolution of products was improved by developing the chromatogram twice in the first dimension before beginning chromatography in the second dimension.

Run-off transcripts of mt-tRNA^{Leu1}

Templates for mt-tRNA^{Leu1} run-off transcripts were prepared by polymerase chain reaction (PCR) amplification from a mtDNA clone spanning positions 41,167 to 801 of the mitochondrial genome (Burger et al., 1995), using primers specific for the 5′ and 3′ ends of the mt-tRNA^{Leu1} sequence. The 5′ primer (5′-ATTAACCCTCACTAAAGGATATGCTGAAATGGTA-3′) included a T3 RNA polymerase promoter sequence immediately upstream of the start of the mt-tRNA^{Leu1} sequence (underlined). The beginning of the resulting run-off transcript corresponded to position N_3 of the mature tRNA sequence. The 3′ primer (5′-**ATGCA/T**CCCGATAATGGGACTCG-3′) contained an *Nsi*I restriction site (in bold; / = cleavage site), which permitted generation of a run-off transcript (underlined sequence complementary to mt-tRNA^{Leu1} sequence) that ended at the discriminator nucleotide (i.e., the transcript lacked the normal 3′ -CCA_{OH} extension).

PCR amplifications were performed in a Perkin Elmer GeneAmp PCR System 2400. One microliter of mtDNA clone (in pBluescript KS+, Stratagene) was combined with 10 pmol each of the 5′ and 3′ primers in a 10-μL reaction mix containing 10 mM Tris-HCl (pH 9.0 at 25 °C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 μg/μL BSA or gelatin, 1.0–2.5 U Taq DNA polymerase (BRL), and 250 mM each of the four dNTPs. Cycle parameters were as follows: initial denaturation at 94 °C for 5 min; 5 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s; 25 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; final extension at 72 °C for 5 min. PCR products were visualized by ethidium bromide staining after electrophoresis in nondenaturing polyacrylamide gels. The desired products were excised from the gel and eluted by shaking for 3 h at 37 °C in a mixture of 400 μL phenol-cresol, 400 μL elution buffer [0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$, 1.0 mM EDTA] and 20 μg linear polyacrylamide. DNA was precipitated twice from aqueous solution with ethanol and redissolved in 5 μL distilled H_2O. PCR products were cloned into the pT7Blue vector (Novagen) using standard protocols (Sambrook et al., 1989). Clones were sequenced from the T7 promoter of the vector using the fmol DNA Cycle Sequencing System (Promega).

Clones containing the desired sequences were digested with 10 U of *Nsi*I (Promega) and linearized DNA was resolved from undigested recombinant plasmid in 0.8% low-melting-point agarose (BRL) gels. Insert DNA was eluted by melting the excised gel plug at 65 °C and then freezing the plug in liquid nitrogen. After thawing at room temperature, the disrupted gel plug was centrifuged for 5 min at 12,000 × *g*. The resulting supernatant was extracted once with chloroform, and DNA was then precipitated with ethanol in the presence of 20 μg linear polyacrylamide. Purified transcription templates were resuspended in 10 μL TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 7.6)].

Transcription reaction mixtures were incubated for 1 h at 37 °C and included 1–2 U/μL T3 RNA polymerase (Promega or BRL) and 1 U/μL RNAguard (Pharmacia) in a buffer containing (in addition to BRL RNA polymerase) 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine-HCl, 25 mM NaCl, 5 mM DTT, and 400 μM of each of the four rNTPs, or (with Promega RNA polymerase) 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 4 mM spermidine-HCl, 10 mM NaCl, 10 mM DTT, and 500 μM of each of the four rNTPs. To produce a transcript with a 5′ monophosphate terminus, GMP was included at concentrations up to 3 mM (Sampson & Uhlenbeck, 1988). Following incubation, DNase I was added and the reaction mixture was incubated for another 10 min at 37 °C. The reaction was stopped by heating to 65 °C for 10 min to denature the RNA polymerase and DNase I. The transcription mixture was then centrifuged through a G-25 Microspin column (Pharmacia) to remove unincorporated rNTPs, extracted once with phenol-cresol, and the RNA products in aqueous solution were precipitated twice with ethanol. Run-off transcripts generated in this way were resuspended in 20 μL distilled H_2O or TE and stored at −70 °C.

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