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Interesting and unusual features in the sequence of *Neurospora crassa* mitochondrial tyrosine transfer RNA

(tRNA tertiary structure/mitochondrial evolution/*in vitro* labeling)

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ABSTRACT **The mitochondrial tyrosine tRNA from *Neurospora crassa* has been sequenced and found to have several interesting features: (*i*) It resembles prokaryotic rather than eukaryotic tyrosine tRNAs in that it possesses a large variable loop (loop III); moreover, it can be quantitatively aminoacylated by *Escherichia coli* tyrosyl-tRNA synthetase but not by yeast tyrosyl-tRNA synthetase. (*ii*) This tRNA differs from all tRNAs sequenced to date in lacking the A residue at position 14 and the constant purine residue at position 15, two nucleosides that have been found so far in loop I of all tRNAs and that have been implicated in base–base tertiary interactions, respectively, with the universal U residue at position 8 and the constant pyrimidine residue at the end of loop III. (*iii*) Unlike the *N. crassa* mitochondrial initiator tRNA, this tRNA contains the usual TΨC sequence in loop IV and the highly conserved GG sequence in loop I common to other tRNAs.**

Many features of tRNA structure have been strongly conserved through evolution. Initiator tRNAs in particular demonstrate this conservation. Although initiator tRNAs from both prokaryotes and eukaryotic cytoplasm possess specific sequence features that distinguish each class from the other and from most noninitiator tRNAs, in terms of overall nucleotide sequence the seventeen or so initiator tRNAs sequenced display a high degree of sequence homology (65–100%). The recent structural analysis of an initiator tRNA from the mitochondria of *Neurospora crassa* (1) presented an interesting exception to this pattern. Not only did this methionine tRNA differ greatly in its sequence from other initiators, both eukaryotic and prokaryotic, but it also lacked some structural features heretofore found in all tRNAs that are active in protein synthesis (2).

This unexpected finding generated several interesting questions. Are the structural features noted in the mitochondrial initiator tRNA unique to the initiator species? Will noninitiator tRNAs in the mitochondrion also show less stringent adherence to known patterns of tRNA structure and evolution compared to the known prokaryotic and eukaryotic tRNAs? If such differences do exist, do they reflect some variation in the functional demands placed upon mitochondrial tRNAs as compared to those in other systems?

In an attempt to answer some of these questions we have now purified and determined the sequence of a noninitiator tRNA, the tyrosine tRNA, of *N. crassa* mitochondria. The choice of this particular tRNA was influenced also by an evolutionary consideration. Tyrosine tRNAs from eukaryotic and prokaryotic organisms can be distinguished readily from each other. Known prokaryotic tyrosine tRNAs possess long variable loops of thirteen nucleotides, whereas the eukaryotic species (fully characterized only in baker's yeast and *Candida utilis*) has a short variable loop of five nucleotides. Also, eukaryotic and prokaryotic tyrosyl-tRNA synthetases do not aminoacylate the heterologous tyrosine tRNAs. Thus, studies on tyrosine tRNA of mitochondria could provide additional information useful for an understanding of mitochondrial evolution.

In this paper, we report the nucleotide sequence of mitochondrial $tRNA^{Tyr}$ from *N. crassa* and discuss the implications of some of the interesting and unusual features that we have found in the sequence of this tRNA.

MATERIALS AND METHODS

Cellulose acetate strips were purchased from Schleicher & Schuell, and cellogel strips were from Kalex Corp (Manhasset, NY). DEAE-cellulose thin-layer plates for homochromatography were from Analtech (Newark, DE), and Whatman DEAE-cellulose paper was from Reeve Angel and Co. (Clifton, NJ).

Triethylammonium bicarbonate was made by bubbling CO_2 into distilled triethylamine. The sources of nucleotide markers were as described (3, 4). Crude yeast RNA for homochromatography was from Sigma. Acrylamide and bisacrylamide were from Bio-Rad. [γ-^{32}P]ATP was prepared by a modification of the procedure of Glynn and Chappell (5).

Enzymes. The sources of pancreatic RNase, T1 RNase, T2 RNase, and snake venom phosphodiesterase have been described (3, 4). Nuclease P1 was from Yamasa Shoyu Co., Ltd (Chohshi, Japan). T4 polynucleotide kinase was from Boehringer Mannheim. The pyrimidine-specific RNase from *Bacillus cereus* was prepared as described elsewhere (6). Phy 1 nuclease from *Physarum polycephalum* was prepared by a modification of the method of Braun and Behrens (7).

Preparation of RPC-5 Resin. RPC-5 resin was prepared by a simplified protocol. To 500 ml of 0.4 M NaCl/0.01 M sodium acetate, pH 4.4, in a Waring Blendor, 14 ml of Adogen 464 was added, and the mixture was homogenized for 5 min at room temperature. Then 300 g of Plaskon powder was added, and the mixture was homogenized for 10 min. The resin was then thoroughly rinsed with the same buffer–salt mixture on a Buchner funnel. It was stored as a slurry at 4°C after addition of 0.01% sodium azide.

Purification of Mitochondrial $tRNA^{Tyr}$. The growth of *N. crassa*, preparation of mitochondria, and isolation of mitochondrial tRNAs have been described (1).

Approximately 22 mg of total mitochondrial tRNA was fractionated on an RPC-5 column, 0.6 × 100 cm, 300 pounds/in^2 (2.07 MPa), at room temperature by using a 500-ml concave gradient from 0.4 M to 0.75 M NaCl in 0.01 M Tris·HCl, pH 7.5/0.01 M $MgCl_2$. Tyrosine acceptance activity was assayed by using total *Escherichia coli* aminoacyl-tRNA synthetases (8). Fractions showing tyrosine acceptance were pooled, dialyzed extensively against distilled water, concentrated by lyophilization, and used for further purification.

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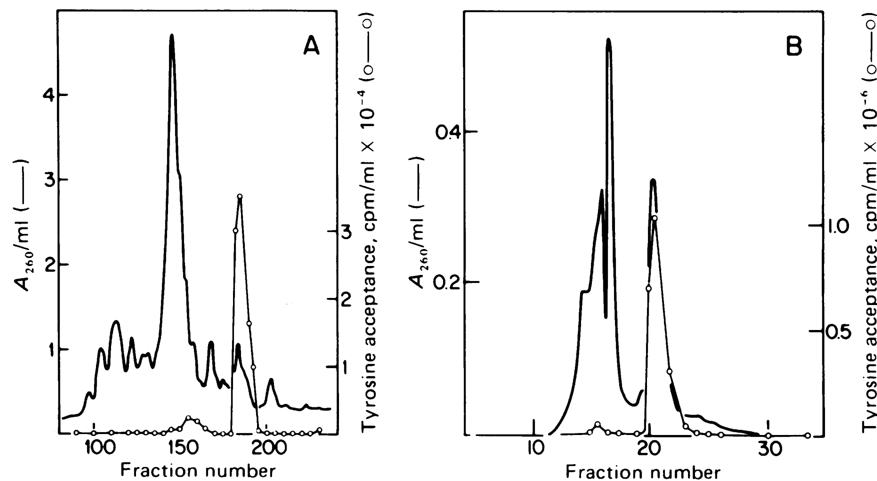

FIG. 1. Purification of *N. crassa* mitochondrial tyrosine tRNA. (*A*) Chromatography of crude mitochondrial tRNA on RPC-5, pH 7.5, at room temperature with a concave gradient of NaCl. (*B*) Chromatography of partially purified $tRNA^{Tyr}$ on RPC-5, pH 5.5, at room temperature. Tyrosine acceptance was assayed by using *E. coli* aminoacyl-tRNA synthetases.

A portion of the enriched $tRNA^{Tyr}$ fraction (250 μg) was applied to a second RPC-5 column (0.2 × 50 cm) equilibrated with 0.4 M NaCl/0.01 M sodium acetate, pH 5.5/0.01 M $MgCl_2$. The column was then eluted at room temperature with a linear gradient (100-ml total volume) of 0.4 to 0.8 M NaCl in 0.01 M sodium acetate, pH 5.5/0.01 M $MgCl_2$. The recovery of $tRNA^{Tyr}$ was approximately 35 μg; the tRNA was at least 90% pure with respect to tyrosine acceptance activity.

In Vitro ^{32}**P Labeling and Sequence Analysis.** Techniques for *in vitro* labeling of tRNA and oligonucleotides by using T4 polynucleotide kinase and [γ-^{32}P]ATP for separation of labeled oligonucleotides and for sequence analysis of these oligonucleotides were as described (1, 3, 9, 10) and have been reviewed elsewhere (11). Methods for sequence analysis of end-labeled tRNA by partial digestion with specific enzymes and analysis of partial digests by polyacrylamide gel electrophoresis (12, 13)

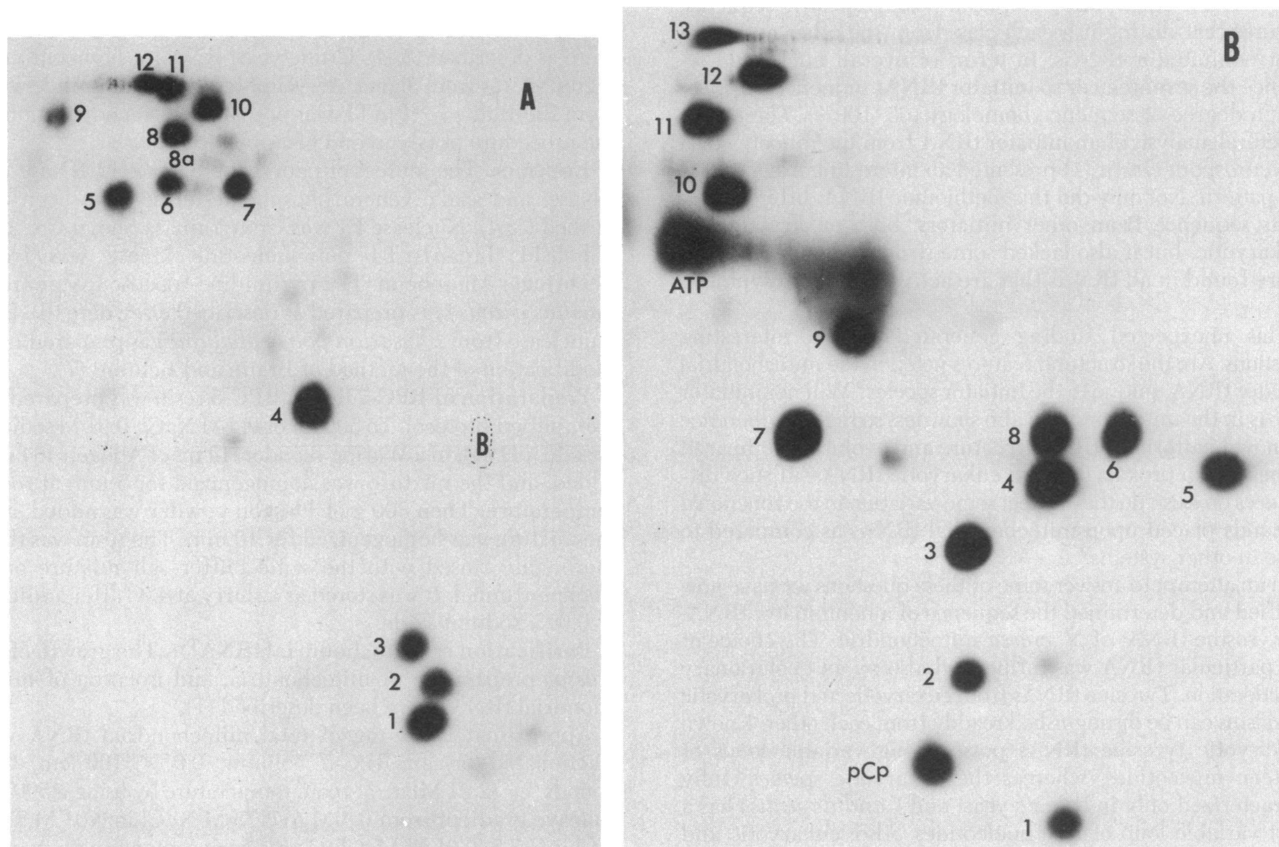

FIG. 2. Autoradiograms of 5′-^{32}P-labeled oligonucleotides from *N. crassa* mitochondrial tyrosine tRNA (9). Oligonucleotides were obtained from T_1 RNase (*A*) and pancreatic RNase (*B*) digests of mitochondrial $tRNA^{Tyr}$. Electrophoresis in the first dimension was on cellulose acetate and, in the second dimension, on DEAE-cellulose paper. Spot B surrounded by dots is blue dye marker. In *B*, blue dye comigrates with spot 6. The location of oligonucleotides in *B* is quite different from that expected on the basis of their sequences (14). This is due to the use of a new batch of Whatman DEAE-cellulose paper for the second dimensional electrophoresis compared to those used previously or in *A*.

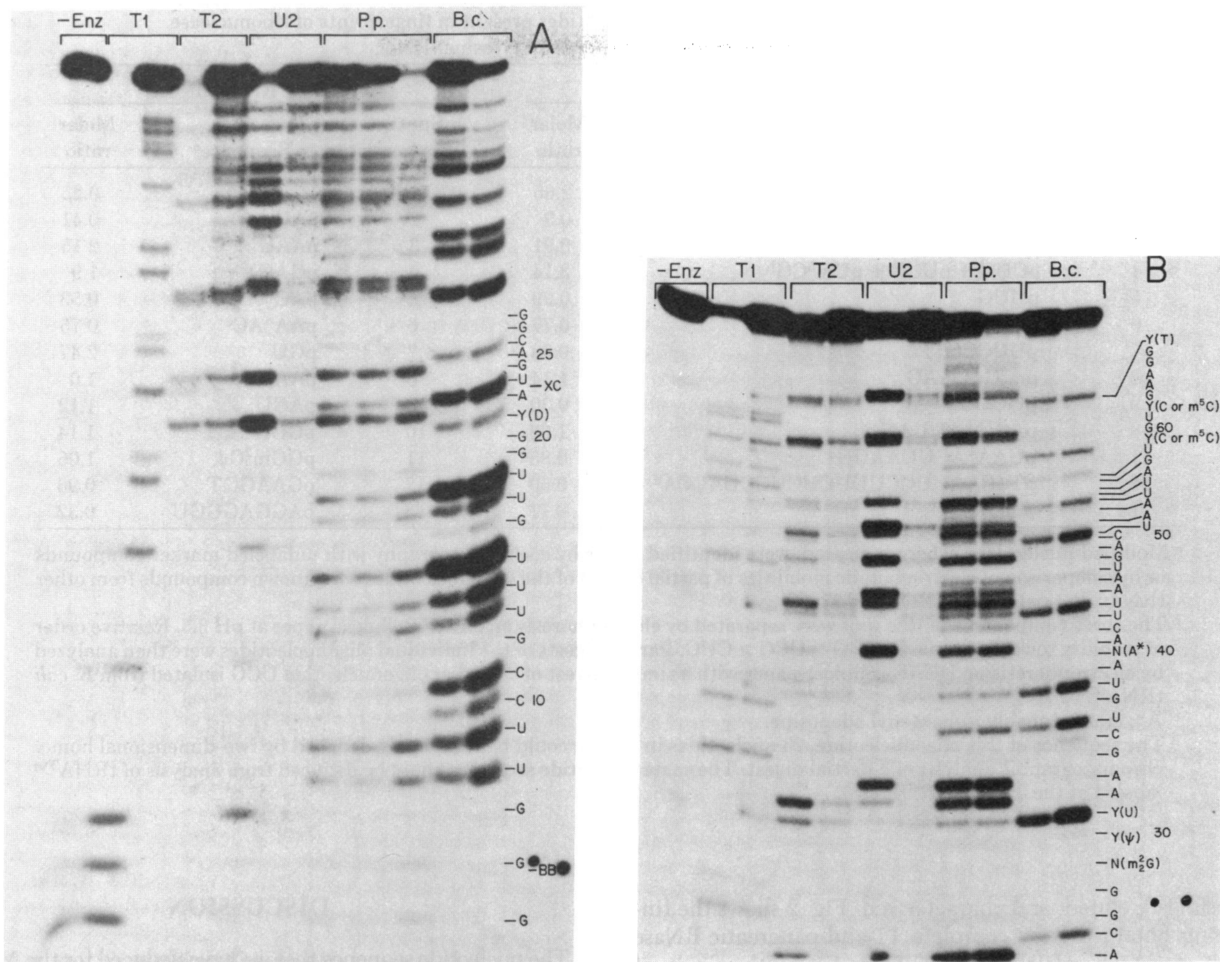

FIG. 3. Autoradiograms of partial enzymatic digests of 5′-^{32}P-labeled *N. crassa* mitochondrial tRNA^{Tyr}. From left to right: (−Enz) incubated in the absence of enzyme; (T1, T2, U2) incubation of end-labeled tRNA at two different concentrations of these ribonucleases; (P.p.) incubation of the tRNA with two (or three) different concentrations of the cytidine-negative ribonuclease from *P. polycephalum;* (B.c.) incubation with two concentrations of a pyrimidine-specific ribonuclease from *B. cereus.* Details are in ref. 8.

were as described (6) except for one modification. A nuclease from *P. polycephalum* was used to help distinguish between uridine and cytidine residues in the gel pattern (12, 13).

RESULTS

Purification of *N. crassa* Mitochondrial tRNA^{Tyr}. Mitochondrial tRNA^{Tyr} was purified by two steps of high-pressure column chromatography on RPC-5 resin from total tRNA isolated from purified mitochondria (1). Fig. 1*A* shows the chromatographic profile of the total mitochondrial tRNA on RPC-5 at pH 7.5, when a concave salt gradient was used for elution. The tyrosine-accepting fractions detected by assay with *E. coli* crude synthetases were pooled and subjected to a second step of RPC-5 chromatography at pH 5.5. The results of this fractionation are shown in Fig. 1*B*. The recovered tRNA^{Tyr}, approximately 95% pure, was used both for sequence analysis by *in vitro* ^{32}P labeling and as a hybridization probe for gene mapping and cloning studies (unpublished results).

Sequence Analysis of Mitochondrial tRNA^{Tyr}. The procedures used for sequence analysis of this tRNA involving *in vitro* ^{32}P labeling were essentially the same as those used for the analysis of the mitochondrial initiator tRNA (1) and have been described in detail elsewhere (6, 10, 11). All of the oligonucleotides produced by complete T1 RNase or pancreatic RNase digestion of the tRNA were labeled at the 5′ end by using T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled oligonucleotides were separated by two-dimensional fingerprint

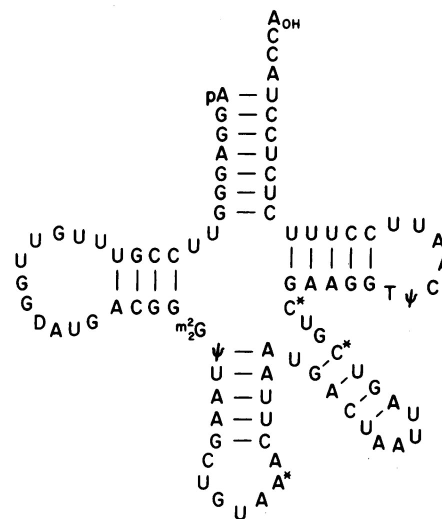

FIG. 4. Sequence of *N. crassa* mitochondrial tyrosine tRNA written in cloverleaf form. C*, one is m^{5}C and the other is C.

Table 1. Sequence and molar ratio of oligonucleotides present in fingerprints of ribonuclease digests of *N. crassa* mitochondrial tyrosine tRNA

T1 RNase digest			Pancreatic RNase digest		
Spot number	Sequence[a]	Molar ratio	Spot number	Sequence[a]	Molar ratio
1	pAG	2.65	1	X	0.25
2	pACG	0.9	2	pAU	0.41
3	pAAG	0.91	3	pAAU	2.15
4	pCUG + pUCG + pUm^{5}CG[b]	3.14	4	pGAC	1.9
5	pUUG	0.99	5	pAC	0.53
6	pDAUG	0.77	6	pAA*AC[c]	0.75
7	pUUCCG	0.93	7	pGU	2.47
8	pm$^2_2$Gψ UAAG	1.14	8	pAAGC	1.0
9	pUUUG	0.30	9	pAGU	1.12
10	pACUAAUUAG	1.08	10	pGGD	1.14
11	pUAA*ACUUAAUG[c]	0.85	11	pGGm$^2_2$Gψ	1.06
12	pTψCAAUUCCUUUCUCUCCUACCA[d]	0.60	12	pGAAGGT	0.96
8a	pψUAAG	0.11	13	pAGGAGGGU	0.32

[a] Modified nucleotides, where indicated, were identified either by cochromatography with unlabeled marker compounds or by comparison of electrophoretic mobilities of partial digests of the oligonucleotides with known compounds from other tRNAs. For details see refs. 1 and 11.

[b] The three components of this spot were separated by electrophoresis on DEAE-cellulose paper at pH 3.5. Relative order of mobility towards anode, Um^{5}CG > UCG > CUG. Partial digests of the individual oligonucleotides were then analyzed by electrophoresis on DEAE-cellulose paper with a similar digest of a marker oligonucleotide UCG isolated from *E. coli* tRNA^{fMet}.

[c] A*, most probably isopentenyl adenosine.

[d] The sequence of this oligonucleotide, 23 nucleotides in length, could be completely deduced by two-dimensional homochromatographic analysis of a partial digest. The same nucleotide sequence could be deduced from analysis of tRNA^{Tyr} labeled at the 3′ end with ^{32}P (15).

analysis (14), eluted, and characterized. Fig. 2 shows the fingerprints obtained from complete T1 and pancreatic RNase digests of mitochondrial tRNA^{Tyr}. Table 1 gives the results of analysis of each of the oligonucleotides isolated from the fingerprints.

The oligonucleotides listed in Table 1 were aligned into a unique sequence by polyacrylamide gel electrophoretic analysis of partial enzymatic digests on 5′- or 3′-^{32}P-labeled tRNA^{Tyr} (6, 11–13). Fig. 3 shows two such analyses, which together provide the sequence from residue 5 through residue 67. Overlap of the GGGU sequence in nucleotides 5–8 to the pancreatic RNase fragment P13 (Table 1) allows us to extend this sequence to nucleotides 1–67. The sequence of nucleotides 68–90 is based on the fact that there is no T1 RNase cleavage site within this region (Fig. 3*B*). This allows us to assign the T1-oligonucleotide T12 (Table 1), which is 23 nucleotides long and contains the 3′-terminal CCA at this position, extending the sequence from the beginning of loop IV to the 3′ end of the tRNA.

The complete nucleotide sequence of mitochondrial tRNA^{Tyr} deduced from these analyses is shown in cloverleaf form in Fig. 4. This sequence has several interesting features. First, the mitochondrial tRNA^{Tyr} is 90 nucleotides long and has a large variable loop similar to *E. coli* (16), *Bacillus stearothermophilus* (17), and *Bacillus subtilis* tyrosine tRNAs (G. Dirheimer, personal communication) but different from the corresponding tRNAs from baker's yeast (18) and *C. utilis* (19). Second, mitochondrial tRNA^{Tyr} lacks the A residue at position 14 and a purine at position 15, nucleosides found until now in all tRNAs that are active in protein synthesis (2). Third, unlike the mitochondrial initiator tRNA, the tyrosine tRNA contains the normal TΨC sequence in loop IV and the corresponding highly conserved GG sequence in loop I.

DISCUSSION

The nucleotide sequence that we have deduced for the *N. crassa* mitochondrial tyrosine tRNA has turned out to be interesting both because of its implications for mitochondrial evolution and because of the unique features in its sequence. Among tRNAs for the 20 different amino acids, tRNAs for 17 of them all contain a small variable loop of four or five nucleotides in both prokaryotes and in eukaryotic cytoplasm (20). Of the tRNAs for the remaining three aminoacids—leucine, serine, and tyrosine—those for leucine and serine contain a variable loop of 13 or more nucleotides in prokaryotes and in eukaryotic cytoplasm. In the case of tyrosine tRNAs, however, whereas the three prokaryotic tRNAs sequenced to date contain a large variable loop of 13 nucleotides (refs. 16, 17, and 21; G. Dirheimer, personal communication), the two eukaryotic tyrosine tRNAs sequenced (from baker's yeast and *C. utilis*) (18, 19) possess a small variable loop of 5 nucleotides. Assuming that future sequence results on other tyrosine tRNAs will continue to follow this trend, the mitochondrial tyrosine tRNA, which has a variable loop of 16 nucleotides, falls distinctly into the prokaryotic class by this criterion. In addition, we have found that although the mitochondrial tyrosine tRNA can be aminocylated quantitatively by *E. coli* tyrosyl-tRNA synthetase like the prokaryotic tyrosine tRNAs, it cannot be aminoacylated by the corresponding enzyme from yeast. The mitochondrial tyrosine tRNA is, therefore, "prokaryote-like" both in its overall structural feature and functional properties. Thus, similar to the use of formylated methionyl-tRNA for the initiation of protein synthesis in mitochondria (22), our current findings may be taken as further evidence for the endosymbiotic theory of mitochondrial evolution (23, 24).

A surprising finding of this work is that the mitochondrial tyrosine tRNA lacks the "invariant" A residue at position 14 from the 5′ end. Every tRNA sequenced so far, with the single

exception of a *Staphyloccus aureus* glycine tRNA (25), which is inactive in protein synthesis, has been found to have A-14 (20). Because in the three dimensional structure of yeast $tRNA^{Phe}$ A-14 is involved in tertiary interaction with U-8 via a "Reversed-Hoogsteen" base pair (26, 27) and both A-14 and U-8 are "invariant" residues (2, 20), it has been assumed that a U-8•A-14 pairing is a tertiary interaction common to all tRNAs. Such an interaction is considered necessary to stabilize the sharp bend of the tRNA chain in this region of the three-dimensional structure of yeast $tRNA^{Phe}$ (28). Our finding that the mitochondrial tyrosine tRNA contains U-14 instead of A-14 suggests either that tertiary interaction between U-8 and a nucleotide in position 14 is not essential in every tRNA or that the nucleotide at position 14 need not always be A for such an interaction to occur. Some indication that the first of these possibilities may be correct is provided by the work of B. R. Reid, R. E. Hurd, and E. M. Azhderian (private communication) who have preliminary evidence (based on NMR spectroscopic analysis of specific *E. coli* tRNAs) for the absence of NMR signals assigned to the U-8•A-14 interaction in some *E. coli* tRNAs.

Another constant feature of all tRNAs is the presence of a purine residue at position 15 which also is involved in tertiary interaction with the constant pyrimidine residue [C-48 in the case of yeast $tRNA^{Phe}$ (29)] at the end of the variable loop. This interaction involves "transpairing" between the two bases (26, 27). The mitochondrial tyrosine tRNA does not have a purine residue at position 15 but has a purine, G, at position 16. Whether G-16 forms a "trans base pair" with C*-62 at the end of the variable loop (equivalent to G-15•C-48 in yeast $tRNA^{Phe}$) or whether such an interaction is also absent in this tRNA remains unknown.

As noted above, an important reason for undertaking the sequence work on a *N. crassa* mitochondrial noninitiator tRNA was based on our finding that the mitochondrial initiator tRNA (1) possessed structural features that had never been observed before in any tRNA. These included the coordinate replacement of the "invariant" GG sequence in loop I by AG and of the UC or ΨC which is part of the TΨC sequence in loop IV by GC. The mitochondrial tyrosine tRNA has the usual TΨC sequence in loop IV and the GG sequence in loop I. We have recently determined the sequence of the *N. crassa* mitochondrial alanine tRNA (S. Yin and U. L. RajBhandary, unpublished results), and, although this tRNA also contains yet another unique feature, it does contain the GG sequence in loop I and the TΨC sequence in loop IV. These findings support our previous assumption that the highly unusual sequence features of mitochondrial initiator tRNA are probably unique to the initiator species and not to mitochondrial tRNAs in general.

A feature of the tyrosine tRNA which it shares with mitochondrial tRNA in general is the high A + U content (59%) of the tRNA. This might have a bearing on its anomalously fast mobility upon electrophoresis in polyacrylamide gels in 7 M urea. For instance, although the mitochondrial tyrosine tRNA is 90 nucleotides long, we have found it to migrate considerably faster than *B. stearothermophilus* tyrosine tRNA (85 nucleotides long, a gift of J. P. Goddard). The anomalous behavior of hamster mitochondrial tRNA and of mitochondrial RNAs in general upon electrophoresis in urea/polyacrylamide gels and sedimentation has been observed also by others (30, 31). This fact must, therefore, be taken into account in any attempts to estimate and compare the size of individual mitochondrial tRNAs to the corresponding tRNAs from prokaryotes and from eukaryotic cytoplasm.

Finally, the availability of purified preparations of *N. crassa* mitochondrial $tRNA^{Tyr}$ has allowed us to use the purified tRNA as a hybridization probe in the mapping and cloning of this tRNA gene. In studies to be described elsewhere, we have shown that the gene for $tRNA^{Tyr}$ maps between the small and the large ribosomal RNAs of *N. crassa* mitochondria.

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