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Post-transcriptional Regulation of the Steady-state Levels of Mitochondrial tRNAs in HeLa Cells*

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In human mitochondrial DNA (mtDNA), the tRNA genes are located in three different transcription units that are transcribed at three different rates. To analyze the regulation of tRNA formation by the three transcription units, we have examined the steady-state levels and metabolic properties of the tRNAs of HeLa cell mitochondria. DNA excess hybridization experiments utilizing separated strands of mtDNA and purified tRNA samples from exponential cells long term labeled with [^{32}P]orthophosphate have revealed a steady-state level of 6×10^5 tRNA molecules/cell, with three-fourths being encoded in the H-strand and one-fourth in the L-strand. Hybridization of the tRNAs with a panel of M13 clones of human mtDNA containing, in most cases, single tRNA genes and a quantitation of two-dimensional electrophoretic fractionations of the tRNAs have shown that the steady-state levels of tRNA^{Phe} and tRNA^{Val} are two to three times higher than the average level of the other H-strand-encoded tRNAs and three to four times higher than the average level of the L-strand-encoded tRNAs. Similar experiments carried out with tRNAs isolated from cells labeled with very short pulses of [5-^{3}H]uridine have indicated that the rates of formation of the individual tRNA species are proportional to their steady-state amounts. Therefore, the ~25-fold higher rate of transcription of the tRNA^{Phe} and tRNA^{Val} genes relative to the other H-strand tRNA genes and the 10–16-fold higher rate of transcription of the L-strand tRNA genes relative to the H-strand tRNA genes are not reflected in the steady-state levels or the rates of formation of the corresponding tRNAs. A comparison of the steady-state levels of the individual tRNAs with the corresponding codon usage for protein synthesis, as determined from the DNA sequence and the rates of synthesis of the various polypeptides, has not revealed any significant correlation between the two parameters.

The analysis of *in vivo* mitochondrial DNA (mtDNA) transcription in HeLa cells by S1 mapping of primary transcripts (Montoya *et al.*, 1982; Yoza and Bogenhagen, 1984; Chomyn and Attardi, 1992) and kinetic experiments (Montoya *et al.*, 1983) has revealed the presence of three transcription units, one in the light (L)[1]-strand and two in the heavy (H)-strand. The product of one of the H-strand transcription units is a polycistronic RNA molecule corresponding to nearly the entire length of the H-strand and destined to produce the majority of the tRNAs and mRNAs. The other H-strand unit, which is transcribed ~25 times more frequently per cell generation than the former (Attardi *et al.*, 1989), includes only the rDNA region and is responsible for the vast majority of the rRNA formation (Montoya *et al.*, 1983). This transcription unit also codes for two tRNAs, tRNA^{Phe} and tRNA^{Val}. The L-strand transcription unit is transcribed, in terms of transcription cycles/cell generation, at 40–65% the frequency of the H-strand rDNA transcription unit and 10–16 times more frequently than the whole H-strand transcription unit (Attardi *et al.*, 1989). Eight tRNA genes are included in this transcription unit. Thus, the human mitochondrial tRNA genes are located in three different transcription units that are transcribed at three different rates.

To determine if there is any regulation of tRNA formation from the three transcription units, we have examined the steady-state levels and metabolic properties of the tRNAs of HeLa cell mitochondria. The results show that the steady-state levels and the rates of formation of the different tRNAs encoded in the H- and L-strand of mtDNA are remarkably uniform, indicating the importance of post-transcriptional control in the maintenance of the steady-state levels of the mitochondrial tRNAs.

MATERIALS AND METHODS

Cell Culture and Labeling Conditions—The S3 clonal strain of HeLa cells was grown in suspension in Dulbecco's modified Eagle's phosphate medium supplemented with 5% calf serum (Attardi and Montoya, 1983). The doubling time of these cells was approximately 20 h. Long term labeling of cells with [^{32}P]orthophosphate was carried out by exposing them to 4.375 μCi of [^{32}P]orthophosphate/ml for 24 or 48 h in Dulbecco's modified Eagle's phosphate medium containing 10^{-3} M cold phosphate and supplemented with 5% dialyzed calf serum. Pulse labeling of long term ^{32}P-labeled cells was carried out in Dulbecco's modified Eagle's phosphate medium containing 10^{-3} M cold phosphate and supplemented with 5% dialyzed calf serum in the presence of 4.375 μCi of [^{32}P]orthophosphate/ml and 75 μCi of [5-^{3}H]uridine/ml. Cell growth was exponential for the duration of the experiments.

Mitochondrial tRNA Isolation—The preparation of twice EDTA-washed mitochondrial fraction from HeLa cells has been described previously (Attardi *et al.*, 1969). After SDS-Pronase treatment, phenol extraction, and ethanol precipitation, the nucleic acids, dissolved in 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.001 M EDTA, 0.5% SDS, were layered on 15–30% sucrose gradients (5–6 A_{260} units/gradient) in the same buffer and centrifuged in an SW 27 rotor at 95,000 × *g* for 12–20 h at room temperature. The top 10 ml of each gradient were pooled and ethanol precipitated. This material was used for two-dimensional analysis of the tRNAs. For hybridization analysis, the same material was electrophoresed through a 5% polyacrylamide, 7 M urea gel. The tRNA region, visualized with ethidium bromide, was cut out, and the nucleic acids were isolated by the crush and soak method followed by ethanol precipitation. This material, resuspended in TE (0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA), was used for DNA excess hybridizations. For RNA excess hybridizations, residual acryl-

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[1] The abbreviations used are: L, light; H, heavy.

amide and other contaminants were removed by dissolving the precipitated nucleic acids in 0.2 M NaCl in TE, passing them through a 0.5-ml Sephadex A-25 column, and eluting them with 1 M NaCl, 1% SDS in TE, at 60 °C.

mtDNA and M13 Clones of mtDNA—mtDNA and separated strands of mtDNA were isolated as described previously (Aloni and Attardi, 1971). M13 clones of mtDNA were obtained by insertion of various restriction fragments of HeLa S3 mtDNA into M13mp8 and M13mp18 (Messing and Vieira, 1982; Yanisch-Perron *et al.*, 1985). In most cases, the opposite insertion orientation to that of the first set of clones was obtained by directional subcloning into M13mp9 and M13mp19. A list of clones used in this study is given in Table I. Single-stranded phage DNA was isolated from CsCl-banded phage as described (Strauss *et al.*, 1986).

RNA-DNA Hybridizations—Separated strands of mtDNA and single-stranded phage DNA from M13 clones of mtDNA were hybridized with ^{32}P-labeled RNA in solution. For DNA excess hybridizations, DNA (generally 5–10 μg of phage DNA or 0.1–10 μg of mtDNA) and RNA samples in TE were heated in 1.5-ml Eppendorf tubes at 95 °C for 5 min, quickly cooled in an ice water bath, and brought to 0.02 M Tris-HCl, pH 7.5, 0.4 M NaCl, 0.002 M EDTA in a final volume of 20–50 μl. The tubes were incubated under water at 68 °C for 16–24 h. The samples were quickly cooled, diluted with 0.5 ml of 2–4 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na_3 citrate), and incubated with

TABLE I

Clones used in this study

Name/M13 vector[a]	Size	Location[b]	Restriction sites used		Strand[c]
			mtDNA	Vector (if different)	
	bp				
mp18.KS8.1	476	16134–41	*Kpn*I/*Sac*I		H
mp8.M9	739	1–740	*Mbo*I	*Bam*HI	H
mp9.M9	739	1–740	*Mbo*I	*Bam*HI	L
mp19.KS8.3	2,537	41–2578	*Kpn*I/*Sac*I		H
mp18.KS8.3	2,537	41–2578	*Kpn*I/*Sac*I		L
mp19.XS5.51	1,247	1194–2441	*Xba*I/*Sph*I		H
mp18.XS5.51	1,247	1194–2441	*Xba*I/*Sph*I		L
mp18.X9.2	1,760	1194–2954	*Xba*I		H
mp18.X5.5	1,760	1194–2954	*Xba*I		L
mp8.M4	1,669	1227–2896	*Mbo*I	*Bam*HI	H
mp9.M4	1,669	1227–2896	*Mbo*I	*Bam*HI	L
mp18.XS6.2	513	2441–2954	*Xba*I/*Sph*I		H
mp19.XS6.2	513	2441–2954	*Xba*I/*Sph*I		L
mp18.KR74	1,544	2578–4122	*Kpn*I/*Eco*RI		H
mp19.KR74	1,544	2578–4122	*Kpn*I/*Eco*RI		L
mp18.XB9.4	705	2954–3659	*Xba*I/*Bcl*I	*Xba*I/*Bam*HI	H
mp8.M11	596	3063–3659	*Mbo*I	*Bam*HI	H
mp9.M11	596	3063–3659	*Mbo*I	*Bam*HI	L
mp18.BR54	463	3659–4122	*Bcl*I/*Eco*RI	*Bam*HI/*Eco*RI	H
mp19.BR54	463	3659–4122	*Bcl*I/*Eco*RI	*Bam*HI/*Eco*RI	L
mp8.HE1	1,152	4122–5274	*Eco*RI		H
mp19.HE2	929	5275–6204	*Hin*dIII/*Eco*RI		H
mp8.HE2	929	5275–6204	*Hin*dIII/*Eco*RI		L
mp18.HE2	929	5275–6204	*Hin*dIII/*Eco*RI		L
mp18.XH5.2	1,237	6204–7441	*Xba*I/*Hin*dIII		H
mp8.M7	754	6904–7658	*Mbo*I	*Bam*HI	H
mp9.M7	754	6904–7658	*Mbo*I	*Bam*HI	L
mp18.XB52	217	7441–7658	*Xba*I/*Bcl*I	*Xba*I/*Bam*HI	H
mp19.XB52	217	7441–7658	*Xba*I/*Bcl*I	*Xba*I/*Bam*HI	L
mp19.X5.1	846	7441–8287	*Xba*I		H
mp18.X5.1	846	7441–8287	*Xba*I		L
mp19.XB66	629	7658–8287	*Xba*I/*Bcl*I	*Xba*I/*Bam*HI	H
mp18.XB66	629	7658–8287	*Xba*I/*Bcl*I	*Xba*I/*Bam*HI	L
mp8.M8	733	7859–8592	*Mbo*I	*Bam*HI	H
mp9.M8	733	7859–8592	*Mbo*I	*Bam*HI	L
mp18.XB53	305	8287–8592	*Xba*I/*Bcl*I	*Xba*I/*Bam*HI	H
mp19.XB53	305	8287–8592	*Xba*I/*Bcl*I	*Xba*I/*Bam*HI	L
mp18.BS51	1,056	8592–9648	*Bcl*I/*Sac*I	*Bam*HI/*Sac*I	H
mp19.BS51	1,056	8592–9648	*Bcl*I/*Sac*I	*Bam*HI/*Sac*I	L
mp8.M5	1,525	8729–10254	*Mbo*I	*Bam*HI	H
mp9.M5	1,525	8729–10254	*Mbo*I	*Bam*HI	L
mp8.M3	1,668	10254–11922	*Mbo*I	*Bam*HI	H
mp9.M3	1,668	10254–11922	*Mbo*I	*Bam*HI	L
mp19.XH5.31	1,424	10257–11681	*Xba*I/*Hin*dIII		H
mp18.XH5.31	1,424	10257–11681	*Xba*I/*Hin*dIII		L
mp18.H78	890	11680–12570	*Hin*dIII		H
mp8.HE3	890	11680–12570	*Hin*dIII		L
mp18.BR55	719	11922–12641	*Bcl*I/*Eco*RI	*Bam*HI/*Eco*RI	H
mp18.XK76	1,097	14956–16053	*Kpn*I/*Xho*I	*Kpn*I/*Sal*I	H
mp19.XK76[d]	1,097	14956–16053	*Kpn*I/*Xho*I	*Kpn*I/*Sal*I	L
mp8.M6	979	15591–16569	*Mbo*I	*Bam*HI	H

[a] The name of each mtDNA clone consists of the name of the M13 vector (mp8, mp9, mp18, or mp19) followed by the mtDNA fragment identification.

[b] Nucleotide numbering according to Anderson *et al.* (1981).

[c] Indicates that the plus strand of the phage DNA corresponds to the indicated strand of mtDNA.

[d] Deletes spontaneously at a very high frequency.

2–30 μg of RNase A and 2–30 units of RNase T_1 for 30 to 60 min at room temperature (the amount of RNase and time of digestion varied depending upon the amount of nucleic acids present in each sample). Both enzymes were pretreated at 90 °C for 15 min. The samples were brought to 10% trichloroacetic acid, 1% pyrophosphate, 1% NaH_2PO_4, and 200 μg bovine serum albumin/ml, incubated on ice 30 min, and filtered through Millipore HA filters (0.45 μm). The background for these hybridizations was determined by hybridizing equivalent amounts of labeled mitochondrial RNA with an excess of M13mp8 or M13 clones of mtDNA not containing a tRNA gene(s). RNA excess hybridizations with separated strands of mtDNA were performed as described by Cantatore and Attardi (1980).

Two-dimensional Gel Analysis of Mitochondrial RNAs—Two-dimensional gel analysis was carried out as described by deBruijn *et al.* (1980). ^{32}P-Labeled tRNAs were visualized by exposure of wet gels to x-ray film.

Estimation of the Specific Activity of the tRNAs and Possible Errors—The tRNA preparations consisted of subclasses synthesized at widely different rates. Consequently, their overall specific activity after long term labeling with [^{32}P]orthophosphate was expected to be influenced by the metabolic properties of the various components in the population and therefore not to be reliable for a quantitative interpretation of the RNA-DNA hybridization data. For the purpose of quantitating tRNA levels by DNA excess hybridization or the gene copy number in mtDNA by RNA excess hybridization, it was decided to utilize the specific activity of the phosphate in the medium, which was present in large excess, to estimate the specific activity of the hybridized tRNAs. Therefore, the specific activity of the *newly synthesized* ribonucleic acids, after pool equilibration, was expected to be 2.93×10^4 dpm of ^{32}P/μg. Since equilibration of the pools with the externally added [^{32}P]orthophosphate occurs in HeLa cells in a few hours (Jeanteur *et al.*, 1968), the deviation of the average specific activity of stable RNA species synthesized in a 24- or 48-h period from the value given above was expected to be very small. Under the conditions used here, the real specific activity of a completely stable tRNA species can be calculated to be equivalent to that of the tRNA synthesized during the 24–48-h labeling period, after correction for the 25–50% preexisting tRNA pool (depending upon the experimentally determined increase in cell mass during labeling). By contrast, the specific activity of very unstable tRNA species newly synthesized in the same period should be equivalent to the specific activity of the phosphate in the medium. Therefore, under any possible circumstance, the error in specific activity of any tRNA species would be less than 25–50%.

RESULTS

Isolation of Mitochondrial tRNAs—In the present work, to minimize the contamination of mitochondria by cytoplasmic RNAs and nuclear nucleic acids, the cells were gently homogenized, and the mitochondria, isolated by differential centrifugation, were washed extensively in isotonic solutions containing high concentrations of EDTA. The latter treatment was expected to disrupt the cytoplasmic ribosomes (Attardi *et al.*, 1969) and thus to minimize any cytoplasmic tRNA contamination of the mitochondrial fraction.

The mitochondrial RNAs were size fractionated by sedimentation through a sucrose gradient. The low molecular weight species were then fractionated by polyacrylamide gel electrophoresis, as shown in Fig. 1. The *left lane* shows an autoradiogram of the low molecular weight RNAs long term ^{32}P-labeled *in vivo*, after fractionation on a sucrose gradient and electrophoresis through a 5% polyacrylamide, 7 M urea gel. One recognizes a group of closely migrating species in the *lower portion* of the gel corresponding to the mitochondrial tRNAs, a pronounced 5 S RNA band, and a few minor species migrating more slowly than the 5 S RNA. These minor species presumably represent contaminating small nuclear RNAs. There is no evidence of any significant degradation of higher molecular weight RNAs. Indicated in this *lane* is the region of the gel from which the material was eluted for hybridization analysis. A sample of a similar preparation of low molecular weight RNAs was electrophoresed through a 20% polyacrylamide, 7 M urea gel, and the *right lane* of Fig. 1 shows an

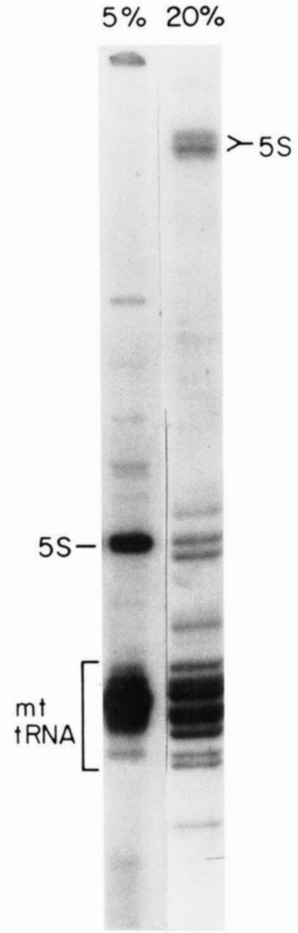

FIG. 1. **Autoradiograms of long term labeled mitochondrial tRNAs electrophoresed through 5 or 20% polyacrylamide, 7 M urea gels.** Low molecular weight RNAs, long term labeled with [^{32}P]orthophosphate, were size selected by sucrose gradient centrifugation and electrophoresed through either 5 or 20% polyacrylamide, 7 M urea gels. The indicated region of the 5% polyacrylamide gel was excised, and the RNA was eluted and subsequently used for hybridization analysis. *5S*, cytoplasmic 5 S rRNA.

autoradiogram of a portion of this gel. In the *lower part* of this *lane*, one can see a group of closely migrating bands representing the mitochondrial tRNA species, expected to have sizes ranging from 62 to 78 nucleotides including the noncoded CCA (Anderson *et al.*, 1981). Near the *top* of this *lane* is a small amount of cytoplasmic 5 S rRNA migrating as a doublet band (Ikemura and Dahlberg, 1973). Cytoplasmic tRNAs are generally larger than the mitochondrial tRNAs (Sprinzl *et al.*, 1989) and would migrate in an area of the gel extending from a position well above the mitochondrial tRNAs to the area including the top several mitochondrial tRNA species (data not shown; Roe *et al.*, 1981). Bands were not detected in the portion of the gel immediately above the mitochondrial tRNAs, pointing to the purity of the mitochondrial tRNA preparation. From the migration of the tRNAs relative to the 5 S RNA (120 nucleotides) and assuming that the two faster migrating bands in the tRNA region represent the mitochondrial $tRNA^{Ser(AGY)}$ (62 nucleotides), one could estimate that the mitochondrial tRNAs, excluding the two strong upper bands, had sizes of 62–72 nucleotides, with the latter mentioned species being 77 and 78 nucleotides in length.

The purity of the mitochondrial tRNAs is further indicated by the results of DNA excess or RNA excess hybridization of *in vivo* labeled tRNAs with mtDNA. As shown in Fig. 2*a*, the

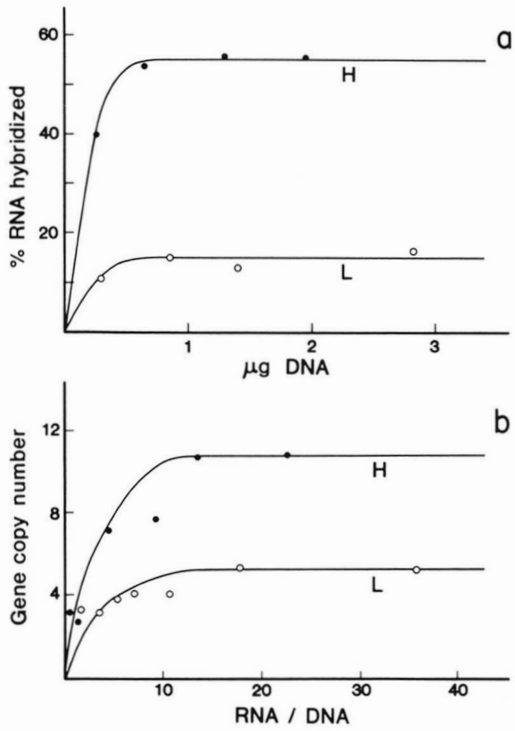

FIG. 2. **Hybridization of mitochondrial tRNAs with mtDNA.** *Panel a*, an excess of H-strand (*H*) or L-strand (*L*) mtDNA was annealed with a constant amount of purified tRNAs, labeled *in vivo* with [^{32}P]orthophosphate for 48 h. The percentage of the tRNA samples hybridized with each strand at varying DNA concentrations is plotted. *Panel b*, an excess of long term (48 h) ^{32}P-labeled tRNAs was annealed with a constant amount of H-strand (*H*) or L-strand (*L*) mtDNA. The relative gene copy number per DNA molecule, at various ratios of tRNAs to mtDNA (w/w), is plotted. This number was calculated from the radioactivity of the tRNAs hybridized with the DNA, knowing the specific activity of the RNA (considered to be equal to that of the medium) and the amount of DNA in the hybridization reaction.

results of a typical experiment in which an excess of H-strand or L-strand mtDNA was annealed with the ^{32}P-labeled mitochondrial tRNA preparation indicate that more than 70% of the labeled material hybridized with the mtDNA. The remaining 30% may represent mitochondrial tRNAs for which the hybridization conditions were not optimal. The tRNAs that are transcribed from the H-strand have on the average only a 35% (G + C) content, with four having less than 30% (G + C), whereas L-strand-encoded tRNAs average a 43% (G + C) content. However, the hybridization conditions utilized in the present work (68 °C in 0.4 M salt) were close to optimal for RNAs of higher (G + C) content (~44%). These conditions were chosen to favor the melting of the secondary structure of the tRNAs, but they may have been too stringent for the hybridization of the tRNAs with the lowest (G + C) content with the mtDNA. It is doubtful that the nonhybridizing material represents cytoplasmic species because, in another study of tRNAs from HeLa cell mitochondria, using a similar procedure to isolate mitochondria but a different methodology to estimate the purity of the tRNAs, it was estimated that less than 10% of the tRNAs isolated represented cytoplasmic tRNA species (Lynch and Attardi, 1976).

The results of a representative experiment of mitochondrial tRNA excess hybridizations with H- and L-strands of mtDNA are shown in Fig. 2*b*. These data indicate that there is very little, if any, contamination of the tRNAs by degradation products of other mtDNA-coded RNAs. The levels of hybridization shown in Fig. 2*b*, calculated using the specific activity of the phosphate in the medium to estimate the specific activity of the tRNAs (2.93×10^4 dpm/μg of RNA), indicate an approximate gene copy number of 11 for the tRNA genes transcribed from the H-strand and 5 for those transcribed from the L-strand. These levels may be below the expected values (14 and 8 genes, respectively, for the H- and L-strand (Anderson *et al.*, 1981)) for the same reason mentioned for the DNA-excess hybridizations, *i.e.* nonoptimal hybridization conditions for all of the tRNA species. Furthermore, in these calculations it was assumed that the tRNAs were uniformly labeled. However, the human mitochondrial tRNAs have been found to have a high metabolic stability (Knight, 1969; Zylber and Penman, 1969; Attardi and Attardi, 1971). Therefore, assuming a complete metabolic stability, after a 48-h labeling with [^{32}P]orthophosphate (as used in this experiment), the specific activity of the tRNAs would have been ~20% lower than if there was a complete turnover of the tRNA population (based on a 5-fold increase in cell mass during this 48-h labeling; see "Materials and Methods"). When this correction was applied to the tRNA saturation values, the numbers of 13.4 tRNA genes transcribed from the H-strand and 6.6 tRNA genes transcribed from the L-strand were calculated. The absence of any significant contamination of the tRNAs by degradation products of other mtDNA-coded RNAs, which is indicated by the results discussed above, is further supported by the lack of hybridization of the tRNA preparations with M13 clones of mtDNA not containing tRNA genes (data not shown).

Fig. 3 shows an autoradiogram of a two-dimensional separation of the mitochondrial tRNAs long term labeled with [^{32}P]orthophosphate. The tRNAs were separated in the first

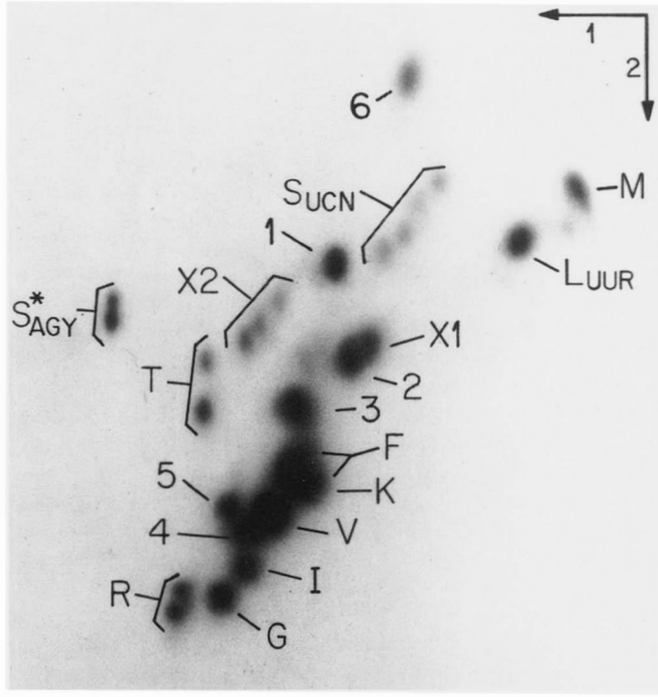

FIG. 3. **Two-dimensional polyacrylamide gel fractionation of purified mitochondrial tRNAs.** Autoradiogram of long term (24 h) ^{32}P-labeled tRNAs separated by two-dimensional electrophoresis, carried out as described by deBruijn *et al.* (1980). The mitochondrial tRNAs, indicated by their single letter amino acid code, were identified by hybridizing individual eluted tRNA species with M13 clones of mtDNA. Species *X1* and *X2* are either $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, or $tRNA^{Tyr}$. $tRNA^{Ser(AGY)}$, marked with an *asterisk*, was tentatively identified on the basis of its unique migrational properties (see "Results"). Species *1–6* are presumably mitochondrial tRNAs but have not been identified.

dimension by electrophoresis through a 20% polyacrylamide, 7 M urea gel and in the second dimension by electrophoresis through a 20% polyacrylamide, 3 M urea gel. More than 20 distinct species are resolved in this pattern.

To identify the tRNA species present in this two-dimensional separation, individual species were eluted from the gel and hybridized with M13 clones of mtDNA containing tRNA genes. Ten tRNA species that were identified by this method are designated in Fig. 3. Two of these species, $tRNA^{Ile}$ and $tRNA^{Met}$, were identified as hybridizing with a single clone containing both tRNA genes. Assignment of the identity of these two species was based on the characteristics of their migration in the two-dimensional separation. One, $tRNA^{Met}$, possesses a very high content of G:C base pairs in stem structures, 67%, which apparently contributed to its unexpectedly slow migration in both the first and second dimensions. The other species, $tRNA^{Ile}$, had migration properties comparable to tRNA species with similar lengths and content of G:C base pairs in stems. Two additional species have been identified as belonging to the group of tRNAs encoded in the L-strand four-tRNA gene cluster consisting of the $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$ genes. Because it was not possible to assign their identity unambiguously they are labeled *X1* and *X2*. One other tRNA (marked by an *asterisk*) has been tentatively identified based solely upon its unique migration properties. This species, $tRNA^{Ser(AGY)}$, the smallest mitochondrial tRNA with 62 nucleotides, was present as two RNA species and was recognized from its rapid mobility during electrophoresis in the first dimension, a separation based primarily on molecular weight. The remaining RNA species, labeled *1–6*, have not been identified but presumably are mitochondrial tRNAs.

Several tRNAs are represented as multiple species in the two-dimensional separation. These include $tRNA^{Ser(UCN)}$, $tRNA^{Phe}$, $tRNA^{X2}$, $tRNA^{Thr}$, $tRNA^{Ser(AGY)}$, and $tRNA^{Arg}$. Where multiple RNA species are shown to represent a single tRNA, the identity of each individual species was confirmed by separate hybridization analyses. The presence of multiple species was observed previously for mitochondrial tRNAs isolated from bovine liver and was attributed to varying degrees of completion of the -CCA addition at their 3′-terminus (Roe *et al.*, 1981). Alternatively, the multiple species may represent varying degrees of other types of post-transcriptional modification of the tRNAs. The mammalian mitochondrial tRNAs are known to contain numerous base modifications (Dubin and Friend, 1974; Chia *et al.*, 1976;, Roe *et al.*, 1981). In addition, 5′-end modification, in the form of a guanylate residue, has also been observed in vertebrate mitochondrial tRNAs (L'Abbé *et al.*, 1990).

Steady-state Levels of the Mitochondrial tRNAs—The steady-state level of the mitochondrial tRNAs was determined in experiments in which the mitochondrial tRNAs, labeled for 24 or 48 h *in vivo* with [^{32}P]orthophosphate, were hybridized with an excess of H- or L-strands of total mtDNA. By determining the total number of cpm which are hybridizable with the H- and L-strands of mtDNA and knowing the specific activity of the RNA synthesized in the labeling medium, the number of tRNAs present in each preparation was calculated. The values determined from four independent labeling experiments ranged from 3.7×10^5 to 6.0×10^5 molecules/cell (mean ± S.D., $5.0 \times 10^5 \pm 1.1 \times 10^5$ molecules/cell). Because no corrections for losses during recovery were made (except the percentage of cells broken during homogenization), these values are very likely to underestimate the steady-state levels of the mitochondrial tRNAs. In addition, the assumption has been made that the tRNAs are uniformly labeled. Since, as mentioned above, there is strong evidence of a high metabolic stability of mitochondrial tRNAs, it has to be expected that a significant percentage of the tRNAs would not be labeled (up to 25–50% in the present experiments, depending upon the length of labeling). When this correction for incomplete labeling of the tRNAs was applied, the calculated average number of tRNA molecules/cell in the four labeling experiments was found to be $7.6 \times 10^5 \pm 1.8 \times 10^5$ molecules/cell. In the present work, the maximum uncorrected recovery, 6×10^5 tRNA molecules/cell, was chosen as a reliable estimate of the actual number of tRNA molecules. However, it must be emphasized that this is a conservative, minimum estimate. The average ratio of tRNAs transcribed from the H-strand to those transcribed from the L-strand was 3.0 ± 0.7. Using the above estimate, this would correspond to 4.5×10^5 molecules of H-strand-encoded tRNAs and 1.5×10^5 molecules of L-strand-encoded tRNAs per cell.

The steady-state levels of the individual tRNAs were also determined. Because $tRNA^{Val}$ was the most abundant tRNA (see below) and was well resolved in the two-dimensional separations and because the levels of $tRNA^{Val}$ were determined within every quantitation experiment and were relatively uniform, it was decided to base the absolute quantitation of the other tRNA species on their relative level compared with that of $tRNA^{Val}$. The steady-state level of $tRNA^{Val}$, 8.4×10^4 molecules/cell, was calculated from the proportion of radioactivity found in the corresponding species in three two-dimensional gel fractionations as compared with the total radioactivity recovered in all tRNA species (0.14 ± 0.01), multiplied by the total number of tRNA molecules/cell (6×10^5 molecules) and corrected for its length. To quantitate the levels of each of the other individual tRNA species, their levels were determined in different experiments by DNA excess hybridization with M13 clones of mtDNA containing tRNA genes or by quantitation of two-dimensional fractionations of the tRNAs. Then, within each labeling experiment, the radioactivity corresponding to each species relative to that in $tRNA^{Val}$ was determined, and from the product of the average of these proportions and of the number of molecules of $tRNA^{Val}$, the number of tRNA molecules/cell of each species was calculated (Table II). Again, corrections for size variations of the tRNAs were made.

Direct comparison of the steady-state levels of the same tRNA species determined by the two methods described above usually yielded very similar results (*e.g.* $tRNA^{Leu(UUR)}$, $tRNA^{Ile}$, $tRNA^{Met}$, $tRNA^{Ser(UCN)}$, $tRNA^{Gly}$, and $tRNA^{Thr}$). The largest discrepancies occurred with $tRNA^{Arg}$ and with the combined $tRNA^{Phe}$ and $tRNA^{Lys}$, where two-dimensional analysis gave values of ~50 and 25%, respectively, higher than the values determined by hybridization analysis. The most likely explanation of this result is the low (G + C) content of the $tRNA^{Arg}$, $tRNA^{Phe}$, and $tRNA^{Lys}$ genes (23, 41, and 33%, respectively) and the suboptimal conditions used for DNA excess hybridizations with these genes. On the other hand, the higher levels of $tRNA^{Phe}$ and $tRNA^{Lys}$ determined by the two-dimensional analysis may indicate the presence of another tRNA species comigrating with them in the corresponding region of the gel.

Analysis of the steady-state levels of the tRNA species indicates that the levels of $tRNA^{Phe}$ (4.9×10^4 molecules/cell) and $tRNA^{Val}$ (8.4×10^4 molecules/cell), transcribed from the rRNA transcription unit, are 1.9, and respectively, 3.2 times higher than the average value for the other 12 species transcribed from the H-strand (~2.6×10^4 molecules/cell). Among these 12 tRNA species there is only a small variation in their steady-state levels, $tRNA^{Met}$ being the most abundant (3.1×10^4 molecules/cell) and $tRNA^{Thr}$, the least abundant ($1.7 \times$

TABLE II

Steady-state levels of the mitochondrial tRNAs

H-strand			L-strand			Unidentified		
tRNAs	Two-dimensional[a]	Hybridization[b]	tRNAs	Two-dimensional	Hybridization	tRNAs	Two-dimensional	Hybridization
	molecules/cell × 10^{-4}			*molecules/cell* × 10^{-4}			*molecules/cell* × 10^{-4}	
F	5.0 ± 0.4[c]	4.9 ± 1.0 (9)	A,N,C,Y	ND[d]	1.6 ± 0.3[e] (7)	1	2.5 ± 0.4	ND
V	8.4[f]	8.4[g]	X1	2.2 ± 0.6	ND	2	2.9 ± 0.3	ND
L(UUR)	2.8 ± 0.2	2.8 ± 0.7 (8)	X2	1.7 ± 0.2	ND	3	4.7 ± 0.7	ND
I	2.6 ± 0.5	2.8 ± 0.3[h] (7)	S(UCN)	2.1 ± 0.4	1.5 ± 0.3 (9)	4	3.5 ± 1.1	ND
M	3.1 ± 0.4	2.8 ± 0.3[h] (7)	P	ND	1.7 ± 0.1 (3)	5	2.8 ± 0.3	ND
W	ND	2.9 ± 0.2 (2)				6	1.0 ± 0.2	ND
D	ND	2.6 ± 0.9 (7)						
K	5.0 ± 0.4[c]	2.8 ± 1.0 (8)						
G	2.6 ± 0.1	2.9 ± 0.9 (9)						
R	3.0 ± 0.3	1.9 ± 0.4 (9)						
L(CUN)	ND	2.0 ± 0.1[i] (3)						
S(AGY)	2.0 ± 1.0	2.0 ± 0.1[i] (3)						
H	ND	2.0 ± 0.1[i] (3)						
T	1.7 ± 0.1	2.2 ± 1.2 (9)						

[a] Values shown are the means of the levels of tRNA molecules/cell × 10^{-4} ± 1 S.D. determined from three independent labeling and two-dimensional fractionations of the mitochondrial tRNAs. The maximum standard error for net counting rate in the three experiments for any of the tRNAs varied between 0.6 and 13% of the signal. The background was determined from a portion of the gel lacking visible spots. See "Materials and Methods" for details.

[b] Values shown are the mean levels of tRNA molecules/cell × 10^{-4} ± 1 S.D. determined by hybridization of purified tRNAs with M13 clones of mtDNA; values in parentheses are the number of independent labelings used in these determinations. The maximum standard error for net counting rate in the different experiments for any individual tRNA species varied between 1.8 and 8.3% of the signal. The background was determined using M13mp8 or M13 clones of mtDNA not containing a tRNA gene(s). See "Materials and Methods" for details.

[c] Value shown is the mean of the comigrating species $tRNA^{Phe}$ and $tRNA^{Lys}$.

[d] Not determined.

[e] Value shown is the mean for the four tRNAs hybridizing with a single M13 clone.

[f] The signal for $tRNA^{Val}$ in two-dimensional separations averaged 1,157 cpm (range 605–1,602 cpm) with a standard error of the net counting rate in each determination of less than 5% of the signal.

[g] The signal for $tRNA^{Val}$ in the nine hybridization experiments averaged 219 cpm (range 34–557 cpm). The standard error of the net counting rate in each determination was less than 2% of the signal.

[h] Value shown is the mean for the two tRNAs hybridizing with a single M13 clone.

[i] Value shown is the mean for the three tRNAs hybridizing with a single M13 clone.

10^4 molecules/cell). The tRNAs transcribed from the L-strand occur at an average level (1.9×10^4 molecules/cell), which is approximately 70% of the average level of the tRNAs transcribed from the H-strand (excluding $tRNA^F$ and $tRNA^V$). Among the L-strand encoded-tRNAs analyzed, species X1 ($tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, or $tRNA^{Tyr}$) is the most abundant, at 2.2×10^4 molecules/cell.

Metabolic Properties of the Mitochondrial tRNAs—Considering the very different rates of transcription of the mitochondrial tRNA genes and the relatively uniform steady-state levels of the mature tRNAs, instability of the transcripts undoubtedly plays an important role in the regulation of the steady-state levels of the tRNAs. To investigate at which step this regulation occurs, HeLa cells, long term labeled with [^{32}P] orthophosphate, were exposed to [5-^{3}H]uridine in the presence of [^{32}P]orthophosphate for short periods of time. The mitochondrial tRNAs were isolated, and their ^{3}H to ^{32}P ratios were determined after hybridization with H- or L-strands of mtDNA or with M13 clones of mtDNA. The results of several pulse labeling experiments are shown in Fig. 4 for the total tRNAs hybridized with the H- and L-strands (*panel a*), as well as with M13 clones of mtDNA containing tRNA genes representative of the three transcription units (*panel c*). These results indicate that the ^{3}H to ^{32}P ratios for the tRNAs transcribed from the H- and L-strands, after different [5-^{3}H] uridine pulses, are quite similar. This similarity extends to the individual tRNA species, irrespective of the transcription unit from which they are transcribed. The ^{3}H to ^{32}P ratios, which reflect the turnover rate of the tRNAs, thus demonstrate a very similar half-life for the various species of tRNA.

Fig. 4 also shows the kinetics of accumulation of [5-^{3}H] uridine in the total tRNAs hybridized with the separated strands of mtDNA (*panel b*) and in the same individual tRNAs analyzed in *panel c* (*panel d*). The shapes of the curves of [5-^{3}H]uridine labeling after different pulse times (Fig. 4*d*), as well as the curves representing the changes in ^{3}H to ^{32}P ratios with pulse length (Fig. 4*c*), despite certain fluctuations (*e.g.* the 8 min points are abnormally low and the 10 min points are abnormally high, probably because of uncontrollable variations in the [5-^{3}H]uridine labeling experiments), show a progressive increase with time in the level of [5-^{3}H] uridine incorporation into tRNAs. For some of the individual tRNA species, including $tRNA^{Phe}$ and $tRNA^{Val}$, this incorporation was also measured after 20- and 30-min [5-^{3}H]uridine pulses and showed further increases (data not shown). These results are in agreement with previous observations of a linear increase in [^{3}H]uridine incorporation into the total tRNA population for up to 4 h (Attardi and Attardi, 1971). It is clear from Fig. 4*d* that the accumulation of label in $tRNA^{Phe}$ and $tRNA^{Val}$ is more rapid than in the other H-strand-encoded tRNAs. This difference is particularly evident after longer pulse times. These differences in ^{3}H labeling are indicative of the higher rate of synthesis of these two tRNAs and correlate well with their higher steady-state levels. However, it is clear that the rate of labeling of $tRNA^{Phe}$ and $tRNA^{Val}$ is not 25-fold higher than that of the other H-strand-encoded tRNAs, as expected from the relative rates of transcription of the two transcription units from which the two sets of tRNAs derive. Furthermore, there is no evidence of saturation of labeling of $tRNA^{Phe}$ and $tRNA^{Val}$ which would be indicative of a rapid rate of turnover of the two species.

The points discussed above are also applicable with respect

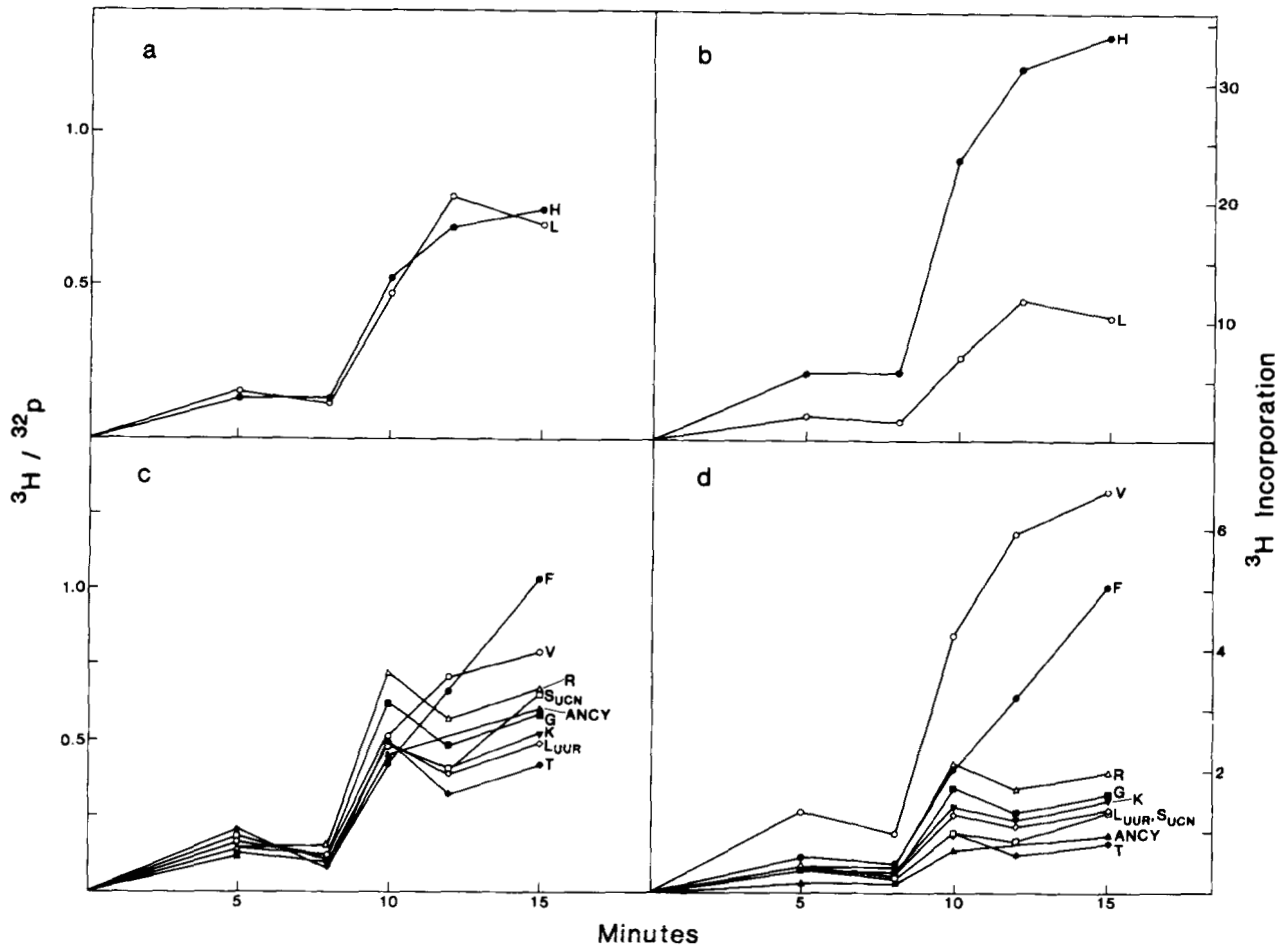

FIG. 4. **^3H to ^{32}P ratios and ^3H accumulation in mitochondrial tRNAs isolated from long term (24 h) ^{32}P-labeled HeLa cells exposed for 5, 8, 10, 12, or 15 min to [5-^3H]uridine and hybridized with H-strand and L-strand mtDNA or with individual M13 clones of mtDNA.** The ^3H to ^{32}P ratios for each time point were determined by hybridizing samples of H- and L-strands of mtDNA (*panel a*) or M13 clones of mtDNA (*panel c*) with samples of total purified tRNA preparations. These individual values were corrected for the different uridine contents of the individual tRNAs. The ^3H accumulation data for each time point were derived by multiplying the ^3H to ^{32}P ratios determined for the total tRNAs encoded in the H- or L-strand (*panel b*) or for the individual tRNAs encoded in the M13 cloned mtDNA fragments (*panel d*) by the steady-state levels of the corresponding tRNAs (Table II). Results are expressed in arbitrary units. The tRNAs are indicated by their single letter amino acid code.

to the tRNAs transcribed from the L-strand. The accumulation of [5-^3H]uridine in these tRNAs is significantly slower than in $tRNA^{Phe}$ and $tRNA^{Val}$ and, in general, also slightly slower than in the other tRNAs transcribed from the H-strand. Thus, there is no evidence of a rapid rate of synthesis or of a rapid turnover of the tRNAs transcribed from the L-strand.

DISCUSSION

Regulation of the Steady-state Levels of the Mitochondrial tRNAs—This report has demonstrated that the levels of the different mitochondrial tRNA species in exponentially growing HeLa cells are relatively uniform. This is a striking finding, considering the widely varying rates at which the corresponding tRNA genes are transcribed. Thus, although transcriptional control plays an important role in the regulation of the steady-state levels of the rRNAs and mRNAs (Gelfand and Attardi, 1981), it is clear that post-transcriptional mechanisms must be the predominant means by which the steady-state levels of the tRNAs are regulated.

In mammalian mtDNAs, the mitochondrial tRNAs are transcribed as part of larger polycistronic RNA molecules in which tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences. In these polycistronic molecules, the tRNA structures are believed to act as recognition signals for the processing enzymes, which make precise endonucleolytic cleavages in the primary transcripts yielding the mature rRNAs, mRNAs, and tRNAs. Several enzymatic activities are required for the excision and maturation of the tRNAs. In particular, two endoribonucleases are needed, one to make a cleavage precisely on the 5′-side of each tRNA sequence, and another to make a cleavage on the 3′-side. In addition, since the -CCA of the tRNAs is not encoded in the DNA, a nucleotidyl transferase is needed to add these nucleotides to the 3′-end of the tRNAs. Finally, some nucleotides of the tRNAs undergo post-transcriptional base modifications.

The ribosomal DNA transcription unit, one of three polycistronic transcription units of human mtDNA, starts upstream of the $tRNA^{Phe}$ gene (Montoya *et al.*, 1983; Chang and Clayton, 1984; Bogenhagen *et al.*, 1984), continues through the 12 S rRNA, $tRNA^{Val}$, and 16 S rRNA genes and terminates at the 3′-end of the 16 S rRNA gene. This transcript is processed to yield the mature rRNAs and, because of its very high rate of synthesis, is responsible for the bulk of the rRNA formation (Montoya *et al.*, 1983). Mapping and sequencing of

the ends of the rRNAs (Crews and Attardi, 1980; Dubin *et al.*, 1982) have indicated that the endonucleolytic cleavages that precisely excise $tRNA^{Val}$ also form the 3′-end of the 12 S rRNA, to which an oligo(A) stretch is attached and the mature 5′-end of 16 S rRNA. -CCA addition and other post-transcriptional modifications would complete the maturation of this tRNA. However, the analysis of the steady-state amount of $tRNA^{Val}$ determined in the present work has shown that it is present at a level only about 3-fold higher than the levels of the tRNAs derived from the large polycistronic transcript of the whole H-strand, which is synthesized at a ~25-fold lower rate than the rRNAs. Thus, it appears that the differential stability of the newly synthesized $tRNA^{Val}$ as compared with the other H-strand-encoded tRNAs, plays a major role in determining the relative similarity of their steady-state amounts.

Endonucleolytic cleavage at the 3′-end of $tRNA^{Phe}$ results in the formation of the 5′-end of the mature 12 S rRNA. The primary transcript must also undergo cleavage at the 5′-end of $tRNA^{Phe}$ for the removal of the short leader. The removal of the leader is a late step in the formation of $tRNA^{Phe}$ and 12 S rRNA (Gaines and Attardi, 1984a, 1984b; Yoza and Bogenhagen, 1984), and this cleavage may not be an obligatory step in the formation of the rRNAs and $tRNA^{Val}$. This may explain the slightly lower steady-state level of $tRNA^{Phe}$ as compared with $tRNA^{Val}$. Thus, in the case of $tRNA^{Phe}$, differential stability of the processed tRNA may combine with incomplete processing of the primary transcript to make the steady-state level of this tRNA relatively similar to those of the other H-strand-encoded tRNAs.

The steady-state levels of tRNAs transcribed from the L-strand are also surprisingly low if correlated with the high rate of transcription of the corresponding genes. The L-strand is transcribed 10–16 times more frequently per cell generation than the whole H-strand transcription unit (Attardi *et al.*, 1989), yet the tRNAs encoded in this strand are present in mitochondria at an average level that is only 70% of the average level of the tRNAs transcribed from the H-strand (excluding $tRNA^{Phe}$ and $tRNA^{Val}$). However, it is known that the L-strand transcripts have a much shorter half-life than the H-strand transcripts and do not accumulate to any significant extent (Aloni and Attardi, 1971; Cantatore and Attardi, 1980). It seems a plausible hypothesis that the vast majority of these transcripts may decay before any processing occurs. So, in contrast to the situation with $tRNA^{Val}$ and $tRNA^{Phe}$, the tRNA sequences would be degraded before they are excised from the polycistronic RNAs. Thus, it appears possible that very different mechanisms, differential stability of the mature or near mature tRNAs, and the differential utilization of the primary transcripts by the mitochondrial processing enzymes may be used in mammalian mitochondria to maintain the relatively uniform levels of the mitochondrial tRNA species.

Experiments examining the metabolic properties of the mitochondrial tRNAs by double labeling with [5-^{3}H]uridine and [^{32}P]orthophosphate strongly suggest that the majority of newly transcribed $tRNA^{Phe}$ and $tRNA^{Val}$ and L-strand-encoded tRNA sequences do not become mature tRNAs and are rapidly degraded. In fact, those experiments indicated that all of the mature mitochondrial tRNAs, irrespective of the transcription unit from which they were derived, have rates of formation proportional to their steady-state levels and have similar rates of turnover. These observations suggest that the mechanism for maintaining the uniform steady-state levels of the tRNAs must operate by eliminating the excess of tRNA sequences transcribed from the rDNA and L-strand transcription units before they reach the pool of mature tRNAs. At least for the H-strand-encoded tRNAs, a plausible mechanism would involve the presence of factors capable of stabilizing the newly formed tRNAs. These factors should be specific for the various species of tRNA and should be present in limiting, approximately equivalent amounts for the different tRNAs. Ideal candidates for these hypothetical stabilizing factors would be the tRNA synthetases. In *Escherichia coli*, the different aminoacyl-tRNA synthetases are present in similar amounts and are rate-limiting for growth (Neidhardt *et al.*, 1977). Thus, it is possible that limiting amounts of the aminoacyl-tRNA synthetases for $tRNA^{Phe}$ and $tRNA^{Val}$ may prevent the acylation of the excess of these newly synthesized tRNA species and thus lead to their rapid degradation.

Mitochondrial tRNAs and Translation—In the nucleo-cytoplasmic compartment, the rRNAs are under independent transcriptional control from the mRNAs and tRNAs, and it is mainly at this level that the relative amounts of the three main RNA classes are regulated. In mitochondria, although transcriptional control does play a role, post-transcriptional mechanisms appear to be of primary importance. Similarly in *E. coli*, post-transcriptional mechanisms predominate. Yet, as seen in Table III, the relative amounts of ribosomes, mRNA molecules, and tRNA molecules found in the cytoplasmic and mitochondrial compartments of HeLa cells and in *E. coli* are nearly identical. Based upon the data of Mueller and Getz (1986), the three major classes of RNA species are present in similar relative amounts also in the mitochondria of *Saccharomyces cerevisiae*. However, if one examines the absolute concentrations of the RNA components within each compartment, striking differences are apparent. The concentrations of the three RNA classes are 8–11-fold lower in the nucleo-cytoplasmic of HeLa cells as compared with their concentrations in *E. coli*. More striking are the differences

TABLE III

Average number and molar concentration of ribosomes, mRNA molecules, and tRNA molecules in HeLa cell cytoplasm and mitochondria and in E. coli

	HeLa cells		*E. coli*[c]
	Cytoplasmic compartment[a]	Mitochondrial compartment[b]	
Ribosomes/cell	5.6×10^6	3.4×10^4	2.6×10^4
Ribosome concentration (M)	2.6×10^{-6}	2.4×10^{-7}	2.5×10^{-5}
mRNA molecules/cell	6.4×10^5	7.0×10^3	3.3×10^3
mRNA concentration (M)	3.0×10^{-7}	4.9×10^{-8}	3.2×10^{-6}
tRNA molecules/cell	7.7×10^7	6.0×10^5	3.0×10^5
tRNA concentration (M)	3.6×10^{-5}	4.2×10^{-6}	2.9×10^{-4}
Ribosomes/mRNA molecule	9	5	8
tRNA molecules/ribosome	14	18	12
tRNA molecules/mRNA molecule	120	86	91

[a] The amounts/cell of the three main classes of RNA were derived from data by Darnell (1968) and Murphy and Attardi (1977) for the cytoplasmic compartment, assuming a total RNA content of 30 pg/cell. Ribosome number was determined from the rRNA content. The volume of the cytoplasmic compartment was based on the cytoplasmic compartment occupying 72% of a cell volume of 4.9 pliters (Posakony *et al.*, 1977; King and Attardi, 1989).

[b] The RNA contents were derived from Attardi *et al.* (1982) and this report. The volume of the mitochondrial compartment was calculated from the volume of the cytoplasmic compartment and the percentage of the cytoplasmic compartment occupied by mitochondria (10.5%; Posakony *et al.*, 1977) and the percentage of mitochondria occupied by the matrix (64%; Schwerzmann *et al.*, 1986).

[c] The rRNA and tRNA contents and cell volume data were derived from Gouy and Grantham (1980), and the mRNA content was derived from data by Dennis and Bremer (1974) and Gillespie *et al.* (1973). RNA contents and cell volume were based on two population doublings/h.

between *E. coli* and the mitochondrial compartment. Each of the RNA classes is 60–100-fold less concentrated in mitochondria as compared with *E. coli*. If one calculates the number of ribosomes, mRNA molecules, and tRNA molecules present in a single mitochondrion (based upon a value of 500 mitochondria/cell (Posakony *et al.*, 1977)), only 68 ribosomes, 14 mRNA molecules, and 1,200 tRNA molecules are present in each organelle. It is not known to what extent these differences in the absolute concentrations or numbers of components of the translational apparatus would contribute to differences in the absolute rate or efficiency of translation.

The levels of individual tRNAs also appear to be regulated in prokaryotic and eukaryotic organisms. When the relative steady-state levels of the tRNAs have been examined in various species, they have rarely been found to vary more than 10-fold (Ikemura, 1981, 1982; Ikemura and Ozeki, 1982; Yamao *et al.*, 1991). For the majority of the tRNAs the variation was less than 3-fold, quite similar to the variations reported here for HeLa cell mitochondria. It has been observed in a number of systems that an extreme imbalance of tRNAs can lead to alterations of translation. Ribosomal frameshifting, amino acid misincorporation, and suppression of termination codons can occur when individual tRNAs are present in excess over their normal physiological concentrations (Atkins *et al.*, 1979; Pure *et al.*, 1985; Weiss and Friedberg, 1986; Lin *et al.*, 1986; Spanjaard *et al.*, 1990; Ulrich *et al.*, 1991). Thus, maintaining individual tRNAs to similar levels is physiologically advantageous for the cell. Therefore, it seems reasonable to postulate that the relative steady-state levels of the mitochondrial tRNAs are adjusted to optimize the rate and accuracy of mitochondrial translation.

A variety of organisms have been found to have strongly nonrandom usage of synonymous codons. In many of these organisms, whenever a codon is frequently used, the level of the corresponding tRNA is also found to be high. This strong correlation between the most frequently used synonymous codon and the most prevalent isoaccepting tRNA species has been shown to be true for *E. coli* (Ikemura, 1981), *Salmonella typhimurium* (Ikemura and Ozeki, 1982), *Mycoplasma capricolum* (Yamao *et al.*, 1991), and *S. cerevisiae* (Ikemura, 1982). When this situation is examined in HeLa cell mitochondria, no such correlation is seen. There are two cases in which there are two isoaccepting tRNAs (for serine and leucine), and in both cases there is a 5–7-fold bias for one set of codons over the other (Anderson *et al.*, 1981). However, the levels of the serine-isoaccepting tRNAs are nearly identical. In the case of the leucine isoacceptors, the tRNA for the less frequent group of codons (UUR) is one of the most abundant tRNAs. Furthermore, the evidence strongly suggests that the other leucine tRNA, $tRNA^{Leu(CUN)}$, occurs at a lower level than $tRNA^{Leu(UUR)}$.

A second correlation that has been found in some organisms is between the tRNA levels and the frequency of use of the corresponding amino acids in cellular proteins (Yamao *et al.*, 1991). Mitochondria offer an excellent opportunity to examine the possible presence of this correlation because not only have the levels of most individual tRNAs been determined, but the codon usage of all translated proteins and their relative rates of translation are also known. Fig. 5 shows the steady-state level of the individual tRNA species *versus* the corresponding codon usage in the mitochondrially translated proteins. The codon usage has been corrected for the relative rates of individual polypeptide synthesis. The data were analyzed by linear regression, and the regression line is shown in the figure. For the data set shown, or when $tRNA^{Phe}$ and $tRNA^{Val}$ are excluded from the analysis, or for the data points

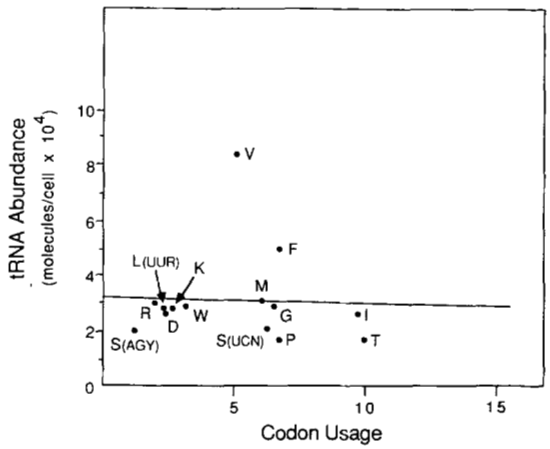

FIG. 5. **Correlation of tRNA abundance and frequency of its usage in translation.** For each identified tRNA, its steady-state level (molecules/cell) is plotted against its relative codon usage in translation (arbitrary units). The codon usage was determined by multiplying the codon usage in each polypeptide (Anderson *et al.*, 1981) by the relative rate of polypeptide synthesis estimated from the labeling of proteins following a 15-min [^{35}S]methionine pulse in the presence of emetine (Chomyn and Attardi, 1987). Linear regression analysis of all the data points is shown by the *solid line* ($r = -0.03$). The tRNAs are indicated by their single letter amino acid code.

when codon usage is not corrected for rates of protein synthesis (data not shown), there is no evidence of a significant positive slope of the regression line, indicative of a dependence of tRNA abundance on its use.

Mitochondrial tRNAs and Human Disease—The mitochondrial encephalomyopathies, a clinically, morphologically, and biochemically diverse group of disorders, have recently begun to be described genetically (Moraes *et al.*, 1991; Wallace, 1992). In a number of these diseases, point mutations within the genes for the mitochondrial tRNAs have been found to be the underlying genetic lesion. For two of these diseases, myoclonic epilepsy and ragged-red fiber and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes, alterations of mitochondrial translation have been observed and correlated with the mutation (Kobayashi *et al.*, 1991; Chomyn *et al.*, 1991, 1992; King *et al.*, 1992). Quantitative information on the mammalian mitochondrial tRNAs, such as that presented in this report, will help us better understand the pathogenesis of these diseases.

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