Campbell, I. D., Dobson, C. M., Jeminet, G., & Williams, R. J. P. (1974) *FEBS Lett. 49*, 115–119.
Douthwaite, S., Garrett, R. A., Wagner, R., & Feunteun, J. (1979) *Nucleic Acids Res. 6*, 2453–2470.
Dovgas, N. V., Markova, L. F., Mednikova, T. V., Vinokurov, L. M., Alakhov, Yu. B., & Ovchinnikov, Yu. A. (1975) *FEBS Lett. 53*, 351–354.
Johnston, P. D., & Redfield, A. G. (1978) *Nucleic Acids Res. 5*, 3913–3927.
Kime, M. J., & Moore, P. B. (1982) *Nucleic Acids Res. 10*, 4973–4983.
Kime, M. J., & Moore, P. B. (1983a) *Biochemistry 22*, 2622–2629.
Kime, M. J., & Moore, P. B. (1983b) *FEBS Lett. 153*, 199–203.
Kime, M. J., & Moore, P. B. (1983c) *Biochemistry 22*, 2615–2622.
Kime, M. J., Ratcliffe, R. G., Moore, P. B., & Williams, R. J. P. (1981) *Eur. J. Biochem. 116*, 269–276.
McDonald, C. C., & Phillips, W. D. (1969) *J. Am. Chem. Soc. 91*, 1513–1521.
O'Connor, T. P., & Coleman, J. E. (1983) *Biochemistry 22*, 3375–3381.
Stubbs, G. J., Warren, S. G., & Holmes, K. C. (1976) *Nature (London) 267*, 216–221.

Nature of Steady-State and Newly Synthesized Mitochondrial Messenger Ribonucleic Acids in Mouse Liver and Ehrlich Ascites Tumor Cells†

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ABSTRACT: The steady-state mitochondrial mRNAs in Ehrlich ascites tumor cells and mouse liver were identified by the Northern blot analysis using nick-translated mtDNA and ^{32}P-labeled cDNA probes. The steady-state mRNA species were compared with the poly(A)-containing RNA synthesized in vitro in isolated mitoplasts. The results show that the isolated mitoplast system can efficiently transcribe almost all of the poly(A)-containing RNAs detected in the steady-state RNA population. It is also seen that the mode of transcription and maturation of mitochondrial mRNAs in different mouse tissues are identical. The results of Northern blot analyses suggest that there may be at least two different modes of mRNA maturation depending upon if the reading frames are interrupted by tRNA cistrons or not. mRNAs for reading frames with adjacent tRNA cistrons downstream appear to be processed from very short-lived precursors. In contrast, mRNAs coded by adjacently located reading frames with no interrupting tRNA genes such as URF3–cyt ox III and URF5–cyt *b* are processed from relatively long-lived precursors. The in vitro pulse-labeling studies also show that almost all of the poly(A)-containing mRNAs are transcribed at nearly identical rates, suggesting that the major regulation of mt gene expression may occur at the level of translation or mRNA decay. The present experiments have also identified a 1.85-kb poly(A)-containing RNA as the putative URF5 mRNA.

Mitochondria from different mammalian cells contain a circular genome of about 16-kb[1] DNA (Borst, 1972; Dawid et al., 1976). Recent DNA sequence analyses have shown that mt genomes from human (Anderson et al., 1981), mouse (Bibb et al., 1981), and bovine (Anderson et al., 1982) cells contain information for coding 2 rRNAs, 22 tRNAs, and 13 potential mRNAs. Of the 13 mRNA reading frames, 5 have been identified as genes coding for 3 mitochondrially synthesized subunits of cytochrome *c* oxidase, designated as cyt ox I, cyt ox II, and cyt ox III, subunit 6 of ATPase, and the 42-kdalton cyt *b* protein (Anderson et al., 1981, 1982; Bibb et al., 1981). The products of the remaining eight reading frames designated as URFs have not yet been identified. By use of partial nucleotide sequencing and physical mapping methods, putative mRNAs coded by 12 different reading frames located on the H strand of the mt genome have been identified in both human (Gelfand & Attardi, 1981; Montoya et al., 1981; Ojala et al., 1981) and mouse (Battey & Clayton, 1978; Van Etten et al., 1982) systems. The precise mode of transcription and the maturation pathway for a number of mRNAs coded by the mt genome in mammalian cells, however, remain to be elucidated. Similarly, it is unknown if the mt genome is expressed uniformly irrespective of tissue types or if there are differential rates of transcription and turnover of mRNAs in different tissues.

Recent studies in our laboratory showed that digitonin-treated mitoplasts can actively synthesize proteins resembling in vivo mt translation products (Bhat et al., 1981, 1982) and also accurately transcribe and process mt-specific rRNA in vitro (Kantharaj et al., 1983). In the present paper, we have used this in vitro mitoplast system to study the mode of synthesis of poly(A)-containing RNAs, putative mt mRNAs, and compared them with the steady-state mt mRNAs from mouse liver and Ehrlich ascites tumor cells. Our results show that the in vitro mitoplast system can synthesize almost all of the

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[1] Abbreviations: kb, kilobase, kbp, kilobase pair; mt, mitochondria; mtDNA, mitochondrial DNA; rRNA, ribosomal RNA; mRNA, messenger RNA; tRNA, transfer RNA; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); EDTA, disodium ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; URF, unidentified reading frame; H strand, heavy strand; L strand, light strand; cyt ox (CO in Figure 1), cytochrome oxidase; cyt *b*, cytochrome *b*; dT, thymidine; Tris, tris(hydroxymethyl)aminomethane.

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poly(A)-containing mRNAs detected in the steady-state mtRNA. Furthermore, the mRNA patterns in Ehrlich ascites mt and mouse liver mt are identical, suggesting no detectable tissue-specific variations in mt gene expression.

Experimental Procedures

Materials. Ultrapure grade guanidinium thiocyanate, formamide, and formaldehyde were purchased from Fluka Chemical Corp. Electrophoresis grade agarose, restriction endonucleases, and the nick-translation kit were purchased from Bethesda Research Laboratories. AMV reverse transcriptase was obtained from Life Sciences Inc. $(dT)_{8-12}$ was purchased from Collaborative Research Laboratories. Sequenal grade $NaDodSO_4$, optical grade CsCl, and ultrapure sucrose were from Pierce Chemical Co. Poly(U)–Sepharose 4B was purchased from Pharmacia. Methylmercury hydroxide (1 M solution) was from Alfa Ventron Corp. Other biochemicals were purchased from Sigma Chemical Co. [^{32}P]-UTP (>600 Ci/mmol), [^{32}P]CTP (>600 Ci/mmol), and [^{32}P]dCTP (>3000 Ci/mmol) were purchased from New England Nuclear Corp. Nitrocellulose membrane sheets for RNA blot transfer and Na56 DEAE paper were purchased from Schleicher & Schuell.

Isolation of Mitochondria. Mitochondria were isolated from Ehrlich ascites tumor cells and also from mouse liver by the differential centrifugation method using sucrose–mannitol buffer (4 mM Hepes, pH 7.4, 70 mM sucrose, 220 mM mannitol, and 2 mM EDTA) essentially as described before (Niranjan & Avadhani, 1980; Bhat et al., 1982; Kantharaj et al., 1983). Crude mitochondria were washed twice with mitochondrial isolation buffer containing 10 mM EDTA and used for preparing mitoplasts by the digitonin fractionation method (50 μg of digitonin/mg of mitochondrial protein) as described before (Bhat et al., 1982).

In Vitro Labeling of Mitochondrial RNA. Mitochondrial RNA was labeled with [^{32}P]UTP and [^{32}P]CTP by using the in vitro mitoplast system described before (Kantharaj et al., 1983). Freshly isolated mitoplasts were incubated in a buffer system containing 5 mM Hepes (pH 7.4), 60 mM KCl, 6 mM $Mg(CH_3COO)_2$, 5 mM 2-mercaptoethanol, 3 mM KH_2PO_4 (pH 7.4), and 0.14 M sucrose at a final concentration of 10 mg of protein/mL. The incubation mixture was supplemented with 2 mM ATP, 1 mM GTP, 5 mM creatine phosphate, 4 mM pyruvate, 0.2 mg/mL creatine phosphokinase, and 100 μM each of 20 L-amino acids. The suspension was shaken at 35 °C with added [^{32}P]UTP (>600 Ci/mmol) and [^{32}P]CTP (>600 Ci/mmol), 150 μCi/mL each, for the required length of time. The mitoplasts were pelleted, washed twice with sucrose–mannitol buffer, and used for isolating the RNA.

Isolation of RNA. Mitochondria (10–50 mg) were dissociated in 5 mL of guanidinium thiocyanate buffer (25 mM sodium citrate, pH 7.0, 5.0 M guanidinium thiocyanate, 0.1 M 2-mercaptoethanol, and 0.5% sodium laurylsarcosinate), and RNA was isolated by repeated extraction with phenol–$CHCl_3$ essentially as described before (Kantharaj et al., 1983). Finally, RNA was pelleted through CsCl as described by Chirgwin et al. (1979). Poly(A)-containing RNA was isolated by the poly(U)–Sepharose adsorption method (Ricca et al., 1981).

Preparation of Mitochondrial DNA Probes. Escherichia coli C600 transformed with pAM1 DNA was obtained from Dr. David Clayton. pAM1 DNA consists of the entire mouse mitochondrial genome cloned in pACYC 177 plasmid at the unique *Hae*II site (Martens & Clayton, 1979). Cells were grown in L broth (Martens & Clayton, 1979), and plasmid DNA was isolated by the clear lysis method of Clewell & Helinski (1972). The plasmid DNA was digested with restriction enzymes *Eco*RI, *Bgl*II, *Hpa*I, *Hin*dIII, and *Apa*I as required and resolved on 0.8% agarose slab gels. The DNA from gel slices were electroblotted to DEAE paper, eluted by extraction with 1 M NaCl, and precipitated with 2.5 volumes of ethanol. DNA restriction fragments were nick translated with [^{32}P]dCTP (>3000 Ci/mmol) by using a kit supplied by Bethesda Research Laboratories.

Preparation of cDNA. cDNA to mitochondrial poly(A)-containing RNA was prepared as described before (Avadhani, 1979). The incubation mixture (50 μL) contained 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 5 μg of actinomycin D, 5 μL of $(dT)_{12-18}$, 40 μg of total mitochondrial RNA, 200 μM each of dATP, dGTP, and dTTP, 50 μCi of [^{32}P]dCTP (>3000 Ci/mmol), and 40 units of AMV reverse transcriptase. The incubation was carried out at 42 °C for 1 h. The unincorporated ^{32}P-labeled nucleotide was removed by passing the reaction mixture through Sephadex G-100 following alkaline hydrolysis (Venetiener & Leader, 1974).

Electrophoresis of RNA. Mitochondrial RNA was electrophoresed on denaturing methylmercury–agarose gels (Bailey & Davidson, 1976). In some experiments, mtRNA was also electrophoresed on formaldehyde–agarose gels (Lehrach et al., 1977). The gels were stained with ethidium bromide, and RNA bands were visualized under UV light. In Northern blot experiments (Alwine et al., 1977), RNA from the gel was blotted onto nitrocellulose sheets (Thomas, 1980) and probed with ^{32}P-labeled nick-translated DNA or ^{32}P-labeled cDNA as described before (Kantharaj et al., 1983).

Results

Characteristics of Mitochondrial DNA Probes. The entire mouse mt genome cloned in pACYC 177 plasmid (Martens & Clayton, 1979) was used to prepare the DNA probes specific for various mRNA reading frames. The restriction maps for *Hin*dIII, *Hpa*I, and *Apa*I are shown in Figure 1A. The positioning of 12S, 16S, rRNAs, tRNAs, and presumptive mRNA genes on the mt genome as indicated in Figure 1A was based on the DNA sequence data reported by Bibb et al. (1981). The 3.7-kb pACYC plasmid is inserted through a unique *Hae*II site on the mt genome located about 70 nucleotides upstream from the 3′ end of the 16S rRNA coding sequence as shown in Figure 1A. A complete digestion of pAM1 DNA with *Hin*dIII, *Hpa*I, and *Apa*I yields 10 fragments of 4.14, 3.77, 2.59, 2.41, 1.94, 1.46, 1.25, 0.98, 0.88, and 0.53 kb as shown in Figure 1B. These restriction fragments will be referred to as fragments 1–10 as indicated in Figure 1B. It should be noted that under the electrophoretic conditions used, fragments 7 and 8 migrate as a doublet. DNA from the top eight bands (including the doublet containing fragments 7 and 8) were eluted and used for preparing the nick-translated probes. Fragment 10 was not used to prepare the probe because of insufficient recovery.

Identification of Transcripts. The transcripts corresponding to different reading frames were identified by the Northern blot analysis. As shown in Figure 2, total RNA from Ehrlich ascites mt was electrophoresed on methylmercury gels, blotted onto a nitrocellulose membrane (Thomas, 1980), and probed with ^{32}P-labeled nick-translated restriction fragments 1–9. As shown in Figure 2 and Table I, the results of Northern blot analysis indicate the presence of some long-lived precursors in addition to at least 10 transcripts which map precisely at the regions of 12 different mRNA reading frames. For example, fragment 1, which contains the 3′-end region (740 nucleotides) of the 16S rRNA gene and over 90% of the

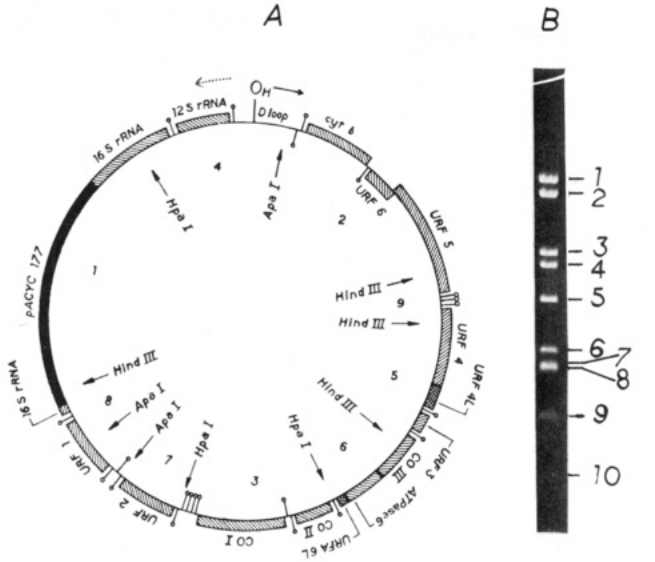

FIGURE 1: Genetic and restriction endonuclease maps of pAM1 DNA. (A) The positioning of tRNA (♀), 12S and 16S rRNAs, and genes for various known and unknown (URF) reading frames is according to Bibb et al. (1981). The tRNAs projecting outside the circles are coded by the H strand, and those projecting inside are coded by the L strand. The dashed areas denote the position of various reading frames. The dashed area positioned inside the circle represents the only L strand open reading frame, designated URF6 (Bibb et al., 1981). OH and the arrow (with a solid line) indicate the origin and direction of H strand synthesis, respectively. The arrow with a dotted line indicates the direction of H strand transcription. The restriction sites for *Hin*dIII, *Hpa*I, and *Apa*I are indicated by the arrows inside the circular map. (B) The pAM1 DNA was digested to completion with *Hin*dIII, *Hpa*I, and *Apa*I and electrophoresed on 0.8% agarose gels, and the DNA bands were visualized under UV light after staining with ethidium bromide as described under Experimental Procedures. The *Eco*RI fragments of λ and φX174 DNA were run as standard markers.

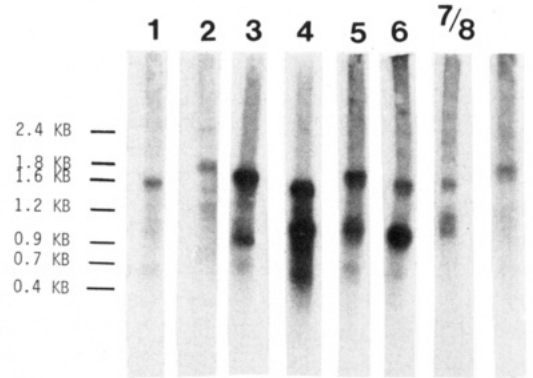

FIGURE 2: Northern blot analysis of Ehrlich ascites mitochondrial RNA using ^{32}P-labeled nick-translated DNA probes. mtRNA (8–10 μg) was electrophoresed on 1.7% agarose gels containing 10 mM methylmercury hydroxide, transblotted on nitrocellulose paper, and probed with nick-translated DNA as described under Experimental Procedures. The number on top of each lane indicates the DNA fragment number as shown in Figure 1 and Table I. 16S, 12S, 18S, and 5S RNAs were used as molecular weight markers.

plasmid vector, hybridizes to the 1.6-kb 16S rRNA. In addition, to two smaller transcripts of 0.7 and 0.4 kb of unknown function detected by this probe, two minor transcripts of about 2.1 and 1.3 kb are also detected in the total mtRNA from Ehrlich ascites cells. Preliminary results indicate that these two minor transcripts are the L strand transcription products. Furthermore, fragment 4, which contains the remaining portion of 16S rRNA gene, the 12S rRNA gene, and the D loop area, hybridizes to 16S (1.6 kb) and 12S (0.96 kb) rRNAs and also to 0.7- and an 0.4-kb transcripts of unknown function.

Table I: Assignment of Reading Frames to Transcripts Detected by the Northern Blot Analysis[a]

probe used (fragment no.)	estimated transcript size (kb)	gene assignment	predicted values
1	1.60	16S rRNA	(1.60)
	0.70	unknown function	
	0.40	unknown function	
2	2.40	precursor of URF5	
	1.80	URF5	(1.85)
	1.20	cytochrome *b*	(1.20)
3	1.56	cyt ox I	(1.59)
	0.72	cyt ox II	(0.93)
4	1.60	16S rRNA	(1.60)
	0.92	12S rRNA	(0.93)
	0.70	unknown function	
	0.40	unknown function	
5	1.72	URF4L–URF4	(1.71)
	1.67	precursor of cyt ox III–URFA6L–ATPase 6	
	0.82	cyt ox III	
	0.40	URF3	
6	1.67	precursor of cyt ox III–URFA6L–ATPase 6	
	0.85 (doublet)	ATPase 6–URFA6L/cyt ox III	(0.89)
7/8	1.60	16S rRNA	(1.60)
	1.05	URF2	(1.08)
	0.97	URF1	(1.00)
9	2.40	precursor of URF5	
	1.80	URF5	(1.85)
	1.72	URF4L–URF4	(1.73)

[a] The molecular weights of RNA as shown in Figure 2 were calculated on the basis of migration in comparison with standard RNA markers (16S, 12S, 18S, and 5S). The predicted values presented in the parentheses were calculated on the basis of DNA sequence analysis of Bibb et al. (1981) and further corrected to account for an average 50–60 poly(A) residue.

Similar low molecular weight rRNA-related transcripts were also detected in mtRNA from mouse LA9 cells by Van Etten et al. (1982).

Fragment 2 contains the most part of URF5 gene, genes coding for URF6 and cyt *b* and a small portion of D loop area (see Figure 1). Use of nick-translated fragment 2 as a probe identifies three transcripts of 2.4, 1.8, and 1.2 kb and also some minor low molecular weight RNA. We have recently obtained evidence that the low molecular weight species are the L strand transcripts of unknown function (K. S. Bhat and N. G. Avadhani, unpublished results). The 2.4- and 1.8-kb transcripts appear to be related to the URF5 reading frame since both of them are also identified by fragment 9 which contains the 5′ end of this reading frame (Bibb et al., 1981). In addition, fragment 9 also identifies a smaller transcript of about 1.72 kb which appears to correspond to URF4L-URF4 reading frames which contain overlapping sequences [see Anderson et al. (1981, 1982) and Bibb et al. (1981)]. Fragment 5 contains the remaining 5′ end of URF4L–URF4 reading frames, the entire length of URF3 reading frame, and the 3′ end of cyt ox III gene. This probe identifies three relatively more abundant transcripts of 1.72, 0.85, and 0.4 kb and a less abundant transcript of 1.67 kb. On the basis of the previous observations in HeLa and mouse mt systems (Montoya et al., 1981; Van Etten et al., 1982) and also the hybridization pattern obtained with fragment 9 above, the 1.72-kb transcript appears to be mRNA coding for URF4L–URF4, and the 0.85- and 0.4-kb transcripts are putative mRNAs coding for cyt ox III and URF3 gene products. The low abundant 1.67-kb RNA not detected in previous experiments (Montoya et al., 1981;

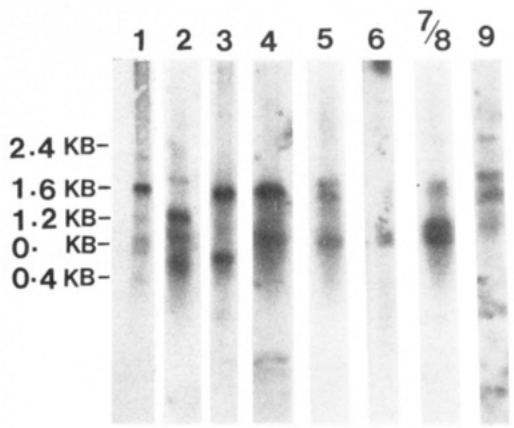

FIGURE 3: Northern blot analysis of mouse liver mtRNA using ^{32}P-labeled nick-translated DNA probes. The details are as described in Figure 2 except that mouse liver mtRNA was used. Various rRNAs were used as molecular weight markers as shown in Figure 2.

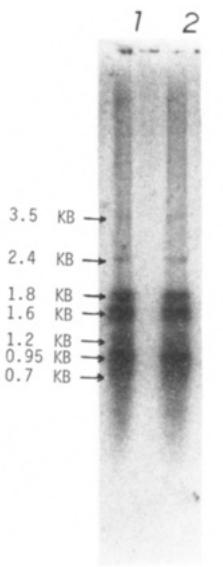

FIGURE 4: Detection of steady-state poly(A)-containing RNA species in total mtRNA using a cDNA probe. mtRNA from mouse liver and Ehrlich ascites cells (10 μg each) were electrophoresed on a methylmercury–agarose gel, transblotted on nitrocellulose membrane paper as described under Experimental Procedures, and probed with ^{32}P-labeled cDNA (about 10^8 cpm) to Ehrlich ascites mt poly(A)-containing RNA. The details of hybridization and autoradiography were described under Experimental Procedures. Lane 1, mtRNA from Ehrlich ascites tumor cells; lane 2, mtRNA from mouse liver. 16S, 12S, 18S, and 5S RNAs were used as molecular weight markers.

Attardi et al., 1982; Van Etten et al., 1982) appears to be the common precursor of cyt ox III–URFA6L–ATPase 6 reading frames. This assumption is supported by the fact that fragment 6 which contains the 5′ end of the cyt ox III gene and URFA6L–ATPase 6 reading frames also identifies a relatively less abundant 1.67-kb transcript in addition to a major transcript of about 0.85 kb. The latter is a doublet (Figure 2, lane 6) and contains 0.82- and 0.89-kb transcripts representing matured cyt ox III and URFA6L–ATPase 6 mRNAs. Fragments 7 and 8 together hybridize to 1.6 kb and 16S rRNA and two transcripts of about 0.97 and 1.05 kb. In separate experiments (not reported here) using an *Eco*RI fragment containing a portion of 16S rRNA gene and the entire URF1 coding region (Kantharaj et al., 1983), we have observed that the 0.97-kb RNA is the product of the URF1 gene. The 1.05-kb RNA, therefore, appears to be transcribed from URF2 reading frame.

Qualitative and Quantitative Comparison of Transcription Pattern in Different Tissues. In order to determine variations in the relative levels of expression of mt genes or any tissue-specific differential expression of the mt genome, we have compared the transcription patterns of mouse liver and Ehrlich ascites cell mt using two different procedures. First, the steady-state mRNA in total mtRNA from mouse liver was studied by the Northern blot experiments using the specific DNA restriction fragments (see Figures 1 and 2 and Table I) as hybridization probes. These results, presented in Figure 3, show that the steady-state transcripts detected in mouse liver mtRNA are nearly identical with those detected in total mtRNA from LES cells with respect to size. The only exceptions are the minor species of 2.1- and 1.3-kb RNA which are identified by probe 1 in the Ehrlich ascites mtRNA but not in the mouse liver mtRNA (Figure 3). Although not shown here, mtRNA from other tissues such as kidney and brain yield essentially similar patterns. Second, qualitative and quantitative comparisons were made with a cDNA probe. In this experiment, total mtRNA from mouse liver and Ehrlich ascites cells was probed with ^{32}P-labeled cDNA prepared to poly(A)-containing RNA from Ehrlich ascites mt. This probe detects transcripts of about seven different size classes in total mtRNA from Ehrlich ascites cells (Figure 4, lane 1). RNA populations of identical sizes and relative intensities are also detected in the total RNA from mouse liver mt (Figure 4, lane 2), suggesting that the overall transcription patterns in different cell types are identical. In both cases, the poly(A)-containing RNA species resolve as major complex bands in the size range of about 1.8, 1.5–1.6, 1.1–1.2, 0.9–1.0, and 0.7–0.8 kb. There are also two less abundant species in the size range of 2.3–2.4 and 3.5–3.6 kb. A 2.4-kb RNA having the properties of a URF5 precursor was also detected with specific DNA probes (Figure 2 and Table I). Although not shown here, the 3.5–3.6-kb species detected by the cDNA probe hybridizes to fragment 2, suggesting that it may be a common precursor of URF5–cyt *b* mRNAs. Use of ^{32}P-labeled cDNA prepared to mouse liver mt poly(A)-containing RNA yields essentially an identical pattern of transcripts in the Northern blot experiments (results not shown), confirming the results obtained with nick-translated DNA probes (Figures 2 and 3). These results collectively show that the overall mode of mtDNA transcription and the relative levels of expression in different cell types are nearly identical.

Analysis of Newly Synthesized Mitochondrial mRNA. In an attempt to determine the rates and extent of transcription of various mRNA reading frames, we have studied the nature of poly(A)-containing RNA pulse labeled in vitro in isolated mitoplasts. As shown in Figure 5, the in vitro system transcribes the entire mt genome as shown by in vivo pulse labeling of HeLa cell mtRNA (Murphy et al., 1975) and S1 nuclease mapping of DNA–RNA heteroduplexes in mouse LA9 cells (Battey & Clayton, 1978). The in vitro pulse-labeled RNA hybridizes to all three large fragments of 11.8-, 5.8-, and 2.2-kb DNA fragments obtained by *Bgl*II and *Eco*RI digestion. A small fragment of 0.2-kb DNA electrophoreses out of the gel under these conditions. Further digestion of the 11.8-kb DNA containing 12 different reading frames [from URF2 to cyt *b*; see Kantharaj et al. (1983)] to completion with *Hpa*I and *Hin*dIII yields 6 fragments all of which hybridize to in vitro pulse-labeled poly(A)-containing RNA (see Figure 5, lane 2), suggesting that the entire genome is transcribed under these conditions. Although not shown here, the in vitro labeled RNA contains both the H strand and L strand complements.

The electrophoretic patterns of in vitro ^{32}P-labeled RNA [total RNA, poly(A)-lacking RNA, and also poly(A)-con-

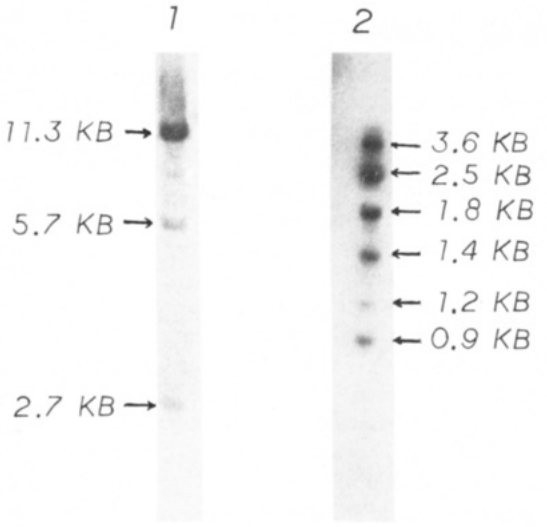

FIGURE 5: Extent of transcription of mt genome under in vitro conditions. Ehrlich ascites mitoplasts were pulse labeled for 20 min with [^{32}P]UTP and [^{32}P]CTP as described under Experimental Procedures, and the labeled RNA was hybridized to mtDNA restriction fragments by the Southern procedure. Lane 1, pAM1 DNA digested to completion with *Eco*RI and *Bgl*II; lane 2, the 11.8-kb DNA from lane 1 further digested to completion with *Hpa*I and *Hin*dIII. In both cases, DNA fragments were electrophoresed on 0.8% agarose gels. About 15 μg of total RNA (3.2×10^4 cpm/μg) was used as the probe. The *Eco*RI fragments of λ and ϕX174 DNA were run as standard markers.

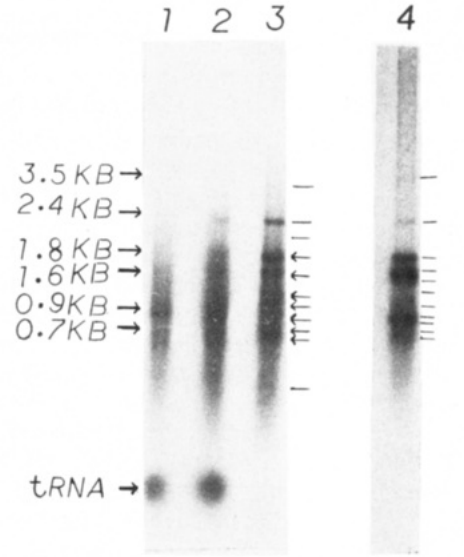

FIGURE 6: Comparison of newly synthesized and steady-state poly(A)-containing RNA. In vitro pulse-labeled RNA as described in Figure 5 was fractionated into poly(A)-containing and poly(A)-lacking species by poly(U)–Sepharose chromatography. About 2×10^5 cpm each of ^{32}P-labeled total RNA (lane 1), poly(A)-lacking RNA (lane 2), and poly(A)-containing RNA (lane 3) as well as 10 μg of RNA from unlabeled mitoplasts was electrophoresed on 1.7% agarose–methylmercury gels as described under Experimental Procedures. Lanes 1–3 were processed directly for autoradiography. Lane 4 was transblotted on nitrocellulose paper and probed with ^{32}P-labeled cDNA to Ehrlich ascites mt poly(A)-containing RNA as in Figure 4. Arrows in lane 3 indicate major poly(A)-containing RNAs detected in steady-state mt RNA. 16S, 12S, 18S, and 5S RNAs were used as molecular weight markers.

taining RNA] are shown in Figure 6 (lanes 1–3). Both the total RNA and poly(A)-lacking RNA contain 12S and 16S rRNA and 4S RNA presumed to be tRNAs as major components, in addition to RNAs of heterogeneous size distribution. The poly(A)-containing RNA contains at least 14 different species, 9 of which appear to be relatively more abundant. A comparison of the in vitro labeled RNA with steady-state poly(A)-containing RNA detected by the cDNA probe (Figure 6, lane 4) shows a striking similarity in that all of the transcripts detected in the steady-state RNA are present in the in vitro pulse-labeled species. These results suggest that the in vitro system accurately transcribes and processes all of the mRNAs expressed under the in vivo conditions. The minor species of in vitro labeled RNA not detected in the steady-state RNA may represent short-lived precursors. Furthermore, the major populations of poly(A)-containing RNA indicated by the arrows in Figure 6 (lane 3) corresponding in size with various mRNA reading frames shown in Figure 2 and Table I are detected in nearly equimolar ratios (cpm in the band/molecular weight), suggesting that almost all of the mRNAs are transcribed at nearly the same rates. Finally, although results are not shown, the newly synthesized poly(A)-containing RNAs in mouse liver mitoplasts are exactly similar to those of Ehrlich ascites mitoplasts shown in Figure 6 (lane 3).

Discussion

A recent study from our laboratory showed that digitonin-treated mitoplasts from Ehrlich ascites cells can accurately transcribe and process mt 16S and 12S rRNAs (Kantharaj et al., 1983). Also, two groups have independently shown that *yeast* mt particles can synthesize precursors of 21S and 15S rRNAs under in vitro conditions (Boerner et al., 1981; Groot et al., 1981). This in vitro system was also shown to convert the "pre-21S" rRNA to mature 21S rRNA by splicing a 1.1-kb intervening sequence (Boerner et al., 1981; Groot et al., 1981). Furthermore, several investigators have shown that isolated mt particles from Ehrlich ascites cells as well as from rat liver can synthesize poly(A)-containing RNA (Aujame & Freeman, 1976; Rose et al., 1975; Cantatore et al., 1976). The accurate synthesis and processing of poly(A)-containing RNAs having the properties of matured mt mRNAs have not yet been reported in any of the mt systems. The results presented in this paper show that the entire mt genome is transcribed under these in vitro conditions. Furthermore, all of the putative mRNAs detected in the steady-state mt RNA are transcribed and processed accurately in this in vitro system. Our inability in the past to demonstrate the synthesis of poly(A)-containing RNA by isolated mt particles, therefore, appears to be largely due to suboptimal labeling conditions employed (Avadhani et al., 1974, 1975).

With a view to understand the mode of transcription and maturation of mouse mt mRNA, we have studied the nature of steady-state mRNA coded by the mt genome and compared them with newly synthesized mRNA species. The results of Northern blot analysis using specific nick-translated mt DNA probes show the presence of 12 discrete transcripts of 0.4–2.4 kb in addition to 12S and 16S rRNA in the total mt RNA (steady-state RNA). The results also show that the patterns of steady-state transcripts are identical in both Ehrlich ascites tumor cell and mouse liver mt RNA. The general pattern of steady-state RNA observed in this study is similar to those previously reported from HeLa (Gelfand & Attardi, 1981; Montoya et al., 1981; Ojala et al., 1981) and mouse liver mt (Van Etten et al., 1982) systems. A relatively abundant 7S poly(A)-containing RNA species believed to be the L strand product of the D loop area in the HeLa cell mt system (Attardi et al., 1982) was not detectable either in the steady-state RNA or in the newly synthesized species of mouse mt system. In agreement with previous studies in HeLa (Attardi et al., 1982) and mouse (Van Etten et al., 1982) mt systems, the nick-translated DNA probe used in the present study also failed

to detect significant levels of steady-state transcripts corresponding to the only L strand open reading frame, URF6. It is likely that these L strand products in the mouse mt system are rapidly degraded without significant accumulation.

Nucleotide sequence analysis studies have shown that in both HeLa and mouse mt systems, reading frames for URF5 and cyt *b* are localized on the H strand and separated by a 600-nucleotide stretch which does not contribute any specific information (Anderson et al., 1981; Bibb et al., 1981). The opposite strand (L strand) of this 600-nucleotide stretch, however, contains an open reading frame designated URF6. On the basis of the 5′-end sequencing of RNA it was reported that a 2.4-kb poly(A)-containing RNA mapping between the start of the URF5 reading frame and the beginning of the cyt *b* gene may be putative mRNA for the URF5 gene (Anderson et al., 1981; Montoya et al., 1981; Ojala et al., 1981). On the basis of hybridization with DNA restriction fragments, a transcript of similar size was thought to be putative URF5 mRNA in the mouse mt system (Van Etten et al., 1982). In the present study, in addition to this 2.4-kb RNA, we have identified a transcript of 1.85 kb coded by the URF5 reading frame (Figures 2 and 3 and Table I). In steady-state RNA the 2.4-kb RNA appears to be the less abundant of the two species as determined by both nick-translated DNA and cDNA probes. In the newly synthesized RNA, on the other hand, the 2.4-kb species is a relatively more abundant species, suggesting that it may be an immediate precursor of the mature 1.85-kb mRNA. In addition to the 1.2-kb cyt *b* mRNA, 1.85-kb URF5 mRNA, and a 2.4-kb putative precursor, a low abundant transcript of 3.5 kb coded by the cyt *b*–URF5 area is detectable inthe steady-state RNA (using the cDNA probe). This 3.5-kb RNA appears to be a common precursor from the cyt *b*–URF5 region. The Northern blot analysis using specific nick-translated probes in the present study has also detected a relatively less abundant 1.67-kb species having the properties of a common precursor of the cyt ox III–URFA6L–ATPase 6 region.

The comparative analysis of steady-state and newly synthesized poly(A)-containing RNA (Figures 2–4 and 6) suggests the presence of two different types of precursor RNAs. A number of very low abundant species not detected in the steady-state RNA and three species of relatively more abundant precursors of 1.67, 2.4, and 3.5 kb correspond to the cyt ox III–URFA6L–ATPase 6 coding region and the URF5–cyt *b* region. It should be noted that both the 12S and 16S rRNA genes and most of the mRNA reading frames, other than the cyt ox III–URFA6L–ATPase 6 and URF5–cyt *b* frames, are separated by tRNA cistrons [see Figure 1 and Bibb et al. (1981)]. Thus, as indicated in our recent study on mt rRNA processing (Kantharaj et al., 1983) and also as predicted by others (Van Etten et al., 1980; Attardi et al., 1982), mRNAs for reading frames which contain juxtaposed tRNA cistrons may be processed rapidly from the nascent transcripts. In addition, the results of the present study also suggest that mRNAs for reading frames which are continually arranged without interrupting tRNAs are derived from relatively longer lived precursors. It is therefore likely that there are at least two modes of mRNA maturation: one involving the endonucleolytic cleavage of nascent transcripts using the secondary structure of tRNA as signals (Van Etten et al., 1980) and the secondary mechanism for processing relatively long-lived precursors. The molecular signal(s) for the second type of processing remains (remain) unknown.

Results showing nearly identical specific activities for all of the putative mRNAs in the newly synthesized poly(A)-containing RNA population (Figure 6) suggest that they may all be transcribed at similar rates. These results also support the view that the mRNAs or their immediate precursors are derived from a common transcript (or growing chain) as proposed by Attardi et al. (1982). The differential expression of mt genome as indicated in vastly different levels of individual mt translation products (Bhat et al., 1981, 1982; Ching & Attardi, 1982) may be determined either by differential turnover of mt mRNAs as previously indicated (Avadhani, 1979) or by the translation efficiencies of individual mRNAs. In conclusion, the mode of transcription and maturation of mt mRNA in different mouse cells appears to be identical. In this respect, a recent observation in our laboratory on the qualitative and quantitative differences between the translation products of Ehrlich ascites and mouse liver mt (Bhat et al., 1981, 1982) may be related to posttranscriptional control mechanisms. The in vitro mitoplast system capable of transcribing almost all of the poly(A)-containing RNAs resembling authentic mt mRNAs may prove useful for further elucidation of the mt mRNA maturation process and in the identification of processing signals.

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References

Alwine, J., Kemp, D., & Stark, R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5350–5354.

Anderson, S., Bankier, A., Barrell, B., de Bruijn, M., Coulson, A., Drouin, J., Eperon, I., Nierlich, D., Roe, B., Sanger, F., Schreier, P., Smith, A., Staden, R., & Young, I. (1981) *Nature (London) 290*, 457–465.

Anderson, S., de Bruijn, M., Coulson, A., Eperon, I., Sanger, F., & Young, I. (1982) *J. Mol. Biol. 156*, 683–717.

Attardi, G., Cantatore, P., Chomyn, A., Crews, S., Gelfand, R., Merkel, C., Montoya, J., & Ojala, D. (1982) in *Mitochondrial Genes* (Slonimski, P., Borst, P., & Attardi, G., Eds.) p 51, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Aujame, L., & Freeman, K. (1976) *Biochem. J. 156*, 499–506.

Avadhani, N. (1979) *Biochemistry 18*, 2673–2678.

Avadhani, N., Lewis, F., & Rutman, R. (1974) *Biochemistry 13*, 4638–4644.

Avadhani, N., Lewis, F., & Rutman, R. (1975) *Subcell. Biochem. 4*, 93–127.

Bailey, I., & Davidson, N. (1976) *Anal. Biochem. 70*, 75–85.

Battey, J., & Clayton, D. (1978) *Cell (Cambridge, Mass.) 14*, 143–156.

Bhat, N. K., Niranjan, B. G., & Avadhani, N. G. (1981) *Biochem. Biophys. Res. Commun. 103*, 621–628.

Bhat, N. K., Niranjan, B. G., & Avadhani, N. G. (1982) *Biochemistry 21*, 2452–2460.

Bibb, M., Van Etten, R., Wright, C., Walberg, M., & Clayton, D. (1981) *Cell (Cambridge, Mass.) 26*, 167–180.

Boerner, P., Mason, T., & Fox, T. (1981) *Nucleic Acids Res. 9*, 6379–6390.

Borst, P. (1972) *Annu. Rev. Biochem. 41*, 333–376.

Cantatore, C., Giorgi, C., & Saccone, C. (1976) *Biochem. Biophys. Res. Commun. 70*, 43–50.

Ching, E., & Attardi, G. (1982) *Biochemistry 21*, 3188–3195.

Chirgwin, J., Przybyla, A., MacDonald, R., & Rutter, W. (1979) *Biochemistry 18*, 5294–5299.

Clewell, D., & Helinski, D. (1970) *Biochemistry 9*, 4428–4440.

Dawid, I., Klukas, C., Ohi, S., Ramirez, J., & Upholt, W. (1976) in *Genetic Function of Mitochondrial DNA* (Sac-

cone, C., & Kroon, A., Eds.) p 3, North-Holland Publishing Co., Amsterdam.
Gelfand, R., & Attardi, G. (1981) *Mol. Cell. Biol. 1*, 497–511.
Groot, G., Van Harten-Loosbroek, N., Van Ommen, G., & Pijst, H. (1981) *Nucleic Acids Res. 9*, 6369–6377.
Kantharaj, G., Bhat, K., & Avadhani, N. (1983) *Biochemistry 22*, 3151–3156.
Lehrach, H., Diamond, J., Wozney, J., & Boedtker, H. (1977) *Biochemistry 16*, 4743–4751.
Martens, P., & Clayton, D. (1979) *J. Mol. Biol. 135*, 327–351.
Montoya, J., Ojala, D., & Attardi, G. (1981) *Nature (London) 290*, 465–470.
Murphy, W., Attardi, B., Tu, C., & Attardi, G. (1975) *J. Mol. Biol. 99*, 809–814.
Niranjan, B., & Avadhani, N. (1980) *J. Biol. Chem. 255*, 6575–6578.
Ojala, D. Montoya, J., & Attardi, G. (1981) *Nature (London) 290*, 470–474.
Ricca, G., Hamilton, R., McLean, J., Conn, A., Kalinyak, J., & Taylor, J. (1981) *J. Biol. Chem. 256*, 10362–10368.
Rose, K., Morris, H., & Jacob, S. (1975) *Biochemistry 14*, 1025–1032.
Thomas, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201–5205.
Van Etten, R., Walberg, M., & Clayton, D. (1980) *Cell (Cambridge, Mass.) 22*, 156–170.
Van Etten, R., Michael, N., Bibb, M., Brennicke, A., & Clayton, D. (1982) in *Mitochondrial Genes* (Slonimski, P., Borst, P., & Attardi, G., Eds.) p 73, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Venetierner, P., & Leder, P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3892–3897.

Integrated Function of a Kinetic Proofreading Mechanism: Steady-State Analysis Testing Internal Consistency of Data Obtained in Vivo and in Vitro and Predicting Parameter Values†

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ABSTRACT: Experimental measurements of the kinetic mechanism involving isoleucyl-tRNA synthetase proofreading valyl-tRNA^{Ile} in *Escherichia coli* have been incorporated into the conventional Michaelis–Menten model for this system. The model was subjected to a detailed mathematical analysis in the steady state. The results of this analysis provide an excellent illustration of the value of integrating fragmentary data into a model of the intact system. (1) Such integration provides a rigorous test for consistency of the individual measurements. For the above synthetase system, the published experimental data were found to be internally inconsistent. (2) Such integration predicts which experimental data are most suspect. In this case, one of the three most questionable measurements, the isoleucine pool size in vivo, was found upon reexamination to be in error by 10–15-fold. Correction of this error produced a self-consistent set of parameter values. (3) The integrated analysis provides predictions for various parameter values. In many cases, these predictions provide estimates for parameter values that are difficult to determine directly or that have yet to be measured experimentally. (4) A sensitivity analysis provides an indication of the relative importance of various parameter values and, hence, an indication of where future experimental effort might be focused most profitably.

In order to maintain biological accuracy and integrity, living systems expend a considerable fraction of their available free energy for proofreading, repair, and disposal of faulty components. These processes have a formal similarity, and we have developed a general theory for the analysis of such phenomena (Savageau & Freter, 1979a, Freter & Savageau, 1980). Although these general methods have been applied to the specific case of Michaelis–Menten mechanisms (Savageau & Lapointe, 1981) and although these methods have been used to test certain experimental measurements (Savageau & Freter, 1979a,b; Freter & Savageau, 1980), a complete characterization of a specific proofreading mechanism has never been presented. In this and the following paper (Okamoto & Savageau, 1984), we shall present such a characterization by focusing upon the single best studied proofreading mechanism—isoleucyl-tRNA synthetase proofreading valyl-tRNA^{Ile} in *Escherichia coli*. The isoleucyl-tRNA synthetase occasionally links the amino acid valine to tRNA^{Ile}. However, the synthetase is known to possess a second activity that allows it to preferentially hydrolyze valyl-tRNA^{Ile} and thereby prevent the initial error in amino acid recognition from being passed on to the finished protein product. This process, by which an enzyme "reexamines" its products and preferentially "rejects" those that are faulty before they can be utilized, has been called by various names, including verification, editing, and kinetic proofreading. We shall simply use the term *proofreading*.

We have adopted the accepted Michaelis–Menten model for this enzyme [e.g., see Yarus (1969), Eldred & Schimmel (1972), Holler & Calvin (1972), and Mulvey & Fersht (1977)], used experimental measurements made both in vitro and in vivo, and integrated these to obtain a complete characterization of the rate constants and steady-state concentrations in the model. The results of this analysis suggest three important conclusions. (a) The published experimental data are internally inconsistent. (b) The analysis predicts which experimental data are most suspect. (c) The analysis predicts values of key physiological and biochemical parameters in vivo, including amino acid step time, tRNA charging ratios, amino

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