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Mode of Transcription and Maturation of Ribosomal Ribonucleic Acid in Vitro in Mitochondria from Ehrlich Ascites Cells†

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ABSTRACT: An in vitro system using mitoplasts from Ehrlich ascites mouse tumor cells was shown to be highly active in in vitro protein synthesis [Bhat, N. K., Niranjan, B. G., & Avadhani, N. G. (1982) *Biochemistry 21*, 2452–2460]. In the present studies, this system was used to investigate the mode of transcription and maturation of mitochondrial 12S and 16S rRNA. The in vitro labeled RNA hybridizes to mitochondrial DNA restriction fragments corresponding to both ribosomal and nonribosomal coding sequences. The hybridization pattern suggests that the entire mitochondrial genome is transcribed under these in vitro conditions. The extent of hybridization to various restriction fragments suggests that the rDNA region is transcribed at 20–40 times higher rates than the rest of the genome. Over 60% of the in vitro labeled RNA is adsorbed to cellulose-linked DNA restriction fragments containing rRNA coding sequences and resolves as characteristic 12S and 16S species on denaturing agarose gels. Electrophoretic analysis of in vitro pulse-labeled RNA and Northern blot analysis of steady-state mitochondrial RNA have failed to detect significant levels of common rRNA precursors, suggesting that the major pathway for mitochondrial rRNA maturation may involve endonucleolytic cleavage of nascent transcripts. Our results also indicate that the "D" loop area does not contribute to stable transcripts in the mouse mitochondrial system.

Mitchondria from a variety of animal cells contain a 16 kilobase pair (kbp)[1] ciruclar DNA genome (Borst, 1972; Dawid et al., 1976) which contains information for 2 mt rRNAs, 22 different mt tRNAs, and potentially 13 different polypeptides (Barrell et al., 1980; Anderson et al., 1981; Bibb et al., 1981; Montoya et al., 1981; Ojala et al., 1981). Recent DNA sequence analyses have revealed a remarkable constancy between bovine, mouse, and human mitochondrial systems with respect to size of various rRNA, tRNA, and mRNA genes and also the order in which they are organized on the genome (Anderson et al., 1981, 1982; Bibb et al., 1981). Available information in rat (Parker & Watson, 1977; Saccone et al., 1980) and *Xenopus* (Rastl & Dawid, 1979) suggests that a similar orgainzational scheme might exist in other animal mt systems as well.

Detailed studies in HeLa cell system (Aloni & Attardi, 1971; Murphy et al., 1975) have provided evidence on the symmetrical and complete transcription of both H and L strands of mtDNA. It is also suggested that the transcription of both strands may be initiated at single promoter sites located near the origin of replication (Montoya et al., 1981; Ojala et al., 1981). These results, along with the DNA and RNA sequence data (Anderson et al., 1981; Bibb et al., 1981; Montoya et al., 1981; Ojala et al., 1981) showing the absence of 3′- and 5′-untranslated regions on the mt mRNAs, and close proximal arrangement of genes with no in between spacers suggest that various RNA species such as rRNA, tRNA, and mRNAs in animal cell mt are derived through an intricate transcription and maturation pathway different from known pro- and eukaryotic systems.

Since mtRNA represents a very small fraction of total cell RNA (Avadhani et al., 1975; Lewis et al., 1976; Batty & Clayton, 1978), studies on the transcription and maturation of mtRNAs have been difficult particularly because most animal cells under tissue culture conditions present considerable barriers in the efficient labeling of mt transcription products. Also, specific inhibitors like camptothecin, which have been successfully used in some cells for preferential inhibition of nuclear transcription, are not effective in other cell types (B. G. Niranjan and N. G. Avadhani, unpublished results) either due to their inefficient transport across the cell membranes or due to their nonspecific effects. We have therefore attempted to develop a subcellular system for efficient labeling of mt transcription products. In a previous study from

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[1] Abbreviations: kb, kilobase; kbp, kilobase pair; mt, mitochondria; mtDNA, mitochondrial DNA; rRNA, ribosomal RNA; tRNA, transfer RNA; mRNA, messenger 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.

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this laboratory, we showed that Ehrlich ascites tumor cell mitoplasts actively incorporate [^{35}S]methionine into proteins resembling in vivo mt translation products (Bhat et al., 1982). In the present study we have determined the usefulness of this mitoplast system to study mt transcription process, and as a first step toward this goal, we show that the in vitro mitoplast system is active in transcription and is capable of accurate processing of mt rRNA.

Experimental Procedures

Materials. Electrophoresis grade agarose and restriction endonucleases were purchased from Bethesda Research Laboratory. Ultrapure sucrose was from Schwarz/Mann. Sequanal grade $NaDodSO_4$ and optical grade CsCl were purchased from Pierce Chemical Co. Poly(U)–Sepharose 4B was purchased from Pharmacia. Methyl mercury hydroxide (1 M solution) was from Alfa Ventron Corp. Most of the inhibitors like chloramphenicol, ethidium bromide, and cordecepin triphosphate were purchased from Sigma Chemical Co. [^{3}H]CTP (18 Ci/mmol), [^{3}H]UTP (59 Ci/mmol), [^{32}P]UTP (>600 Ci/mmol), and [^{32}P]dCTP (>3000 Ci/mmol) were obtained from Amersham Corp. Nitrocellulose membrane sheets for RNA blot transfer were purchased from Schleicher & Schuell.

Isolation of Mitochondria. Ehrlich ascites mouse tumor cells were grown in the peritoneal cavity of Swiss mice (Chun et al., 1969) and used as the source of mitochondria. Disruption of cells by homogenization in sucrose–mannitol buffer (4 mM Hepes, pH 7.4, 220 mM mannitol, 70 mM sucrose, and 2 mM EDTA) and isolation of crude mitochondria by differential centrifugation were as described before (Lewis et al., 1976; Niranjan & Avadhani, 1980; Bhat et al., 1981). Mitochondria were washed once with sucrose–mannitol buffer containing 20 mM EDTA, and mitoplasts were isolated by the digitonin fractionation method using 0.1 mg of digitonin/mg of mt protein, also as described before (Bhat et al., 1982).

In Vitro Labeling of Mitoplasts and Isolation of mtRNA. mtRNA was labeled with ^{3}H-labeled or ^{32}P-labeled nucleotides by using an in vitro system previously described for labeling mt translation products (Bhat et al., 1981, 1982). Freshly prepared mitoplasts were suspended in the RNA synthesis buffer [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 concentration of 6–10 mg of mitoplast protein/mL and 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 mixture was gently shaken at 35 °C, and the labeling was initiated by adding 100 μCi/mL each of [^{3}H]CTP (18 Ci/mmol) and [^{3}H]UTP (50 Ci/mmol) or 100 μCi/mL [^{32}P]UTP (>600 Ci/mmol). Unless otherwise stated, the labeling was continued for 60 min. Aliquots (2.5 μL) were withdrawn at intervals, adsorbed onto filter disks, and assayed for radioactive RNA synthesis by cold CCl_3COOH method (Mans & Novelli, 1961).

The labeled mitoplasts were pelleted at 10000*g* for 10 min at 4 °C, washed once with sucrose–mannitol buffer, and used for isolation of mtRNA by the phenol–chloroform method (Avadhani, 1979) with the following modifications. The mt pellet (5–10 mg) was suspended in 2.5 mL of guanidinium thiocyanate buffer (25 mM sodium citrate, pH 7.0, 5 M guanidinium thiocyanate, 0.1 M 2-mercaptoethanol, and 0.5% sodium laurylsarcosinate) by homogenization with a Dounce homogenizer (Chirgwin et al., 1979). The clear lysate was extracted with equal volumes of phenol saturated with water and $CHCl_3$. The aqueous phase was separated and saved, and the interphase was resuspended in guanidinium thiocyanate buffer as above and extracted again with phenol–chloroform. The combined aqueous phases were reextracted once with phenol–chloroform and once with $CHCl_3$–isoamyl alcohol (95:5) and adjusted to pH 5.0 by adding 0.025 volume of 1 M CH_3COOH. RNA was precipitated with an equal volume of ethanol at −20 °C overnight. The RNA precipitate was dissolved in 0.5–1.0 mL of H_2O and reprecipitated with 2 volumes of ethanol in the presence of 0.3 M CH_3COOK (pH 5.0). RNA was further pelleted through CsCl (Chirgwin et al., 1979) to eliminate contaminating DNA.

Preparation of Plasmid DNA. Escherichia coli C600 r^-m^- transformed with pACYC 177 plasmids carrying the entire mouse mt genome (designated as pAM1) was a gift from Dr. David A. Clayton (Martens & Clayton, 1979). The growth of cells in low PO_4 L broth and lysis of cells with lysozyme–EDTA and Triton X-100 were according to Battey & Clayton (1978). The lysate was clarified at 75000*g* for 30 min at 4 °C and was made to 10% with poly(ethylene glycol). The nucleic acid precipitate was collected by centrifugation at 25000*g* for 30 min at 4 °C, digested with pancreatic RNase (DNase free), and used for the isolation of closed circular DNA by CsCl banding (Clewell & Helinski, 1970).

Isolation and Nick Translation of Restriction Fragments. The plasmid DNA carrying mouse mt genome (10–50 μg) was digested to completion with *Eco*RI and *Bgl*II under standard conditions recommended by the vendor. The DNA fragments were resolved by electrophoresis on 0.6% agarose slabs and localized by staining with 0.5 μg/mL EtBr. The DNA from gel slices were electroblotted on DEAE paper (NA 45, Schleicher & Schuell), eluted by extraction with 1 M NaCl, and precipitated with 2.5 volumes of ethanol. The DNA restriction fragments were nick translated with [^{32}P]dCTP (>3000 Ci/mmol) by using a kit supplied by Bethesda Research Laboratory.

Covalent Binding of DNA to Cellulose. About 200–250 μg of mtDNA (restriction fragments) in 1 mL of H_2O was sonicated for 10 s and denatured by heating at 100 °C, followed by quick chilling in ice. The single-stranded DNA fragments were covalently linked to "epoxycellulose" by using the method of Moss et al. (1981).

Electrophoretic Analysis of RNA. RNA was separated on denaturing agarose–methyl mercury hydroxide gels (Bailey & Davidson, 1976). After the electrophoretic run, the gels were stained with 1 μg/mL ethidium bromide in 0.5 M NH_4CH_3COO, and the RNA bands were visualized under UV light. ^{3}H-Labeled RNA bands were localized by fluorography by using EN^{3}HANCE (New England Nuclear). In Northern blot experiments (Alwine et al., 1977), RNA from the gels was blotted onto nitrocellulose sheets as described by Thomas (1980) and probed with ^{32}P-labeled DNA fragments (Alwine et al., 1977).

DNA–RNA Hybridization. DNA (2–10 μg) in 100 μL of 0.3 M NaCl and 0.3 M NH_4OH was denatured by heating at 100 °C for 15 min following quick chilling in ice. DNA was spotted on nitrocellulose disks and immunobilized by heating at 80 °C under vacuum for 2 h. Hybridization was carried out in 0.5–1.0 mL of reaction mixture containing 5× SSPE (1× SSPE = 0.18 M NaCl, 10 mM sodium phosphate, pH 7.1, and 1 mM EDTA), 50% formamide, 2× Denhardt's solution [0.04% each Ficoll, poly(vinylpyrrolidone), and bovine serum albumin], 50 μg/mL *yeast* tRNA, 0.1% $NaDodSO_4$, and 0.4–1 μg of ^{3}H-labeled mtRNA at 42 °C for 48 h. The filters were washed with 25 mL of 2× SSPE and 25 mL of 0.1× SSPE. The filters were digested with 50 μg of pancreatic

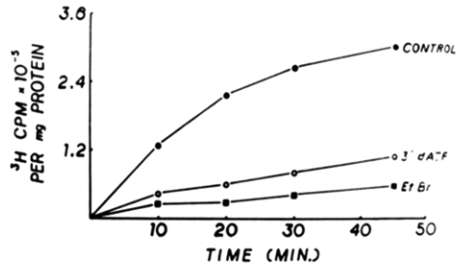

FIGURE 1: Mitoplasts from Ehrlich ascites tumor cells isolated by digitonin fractionation were labeled with 100 μCi/mL [^{3}H]UTP and [^{3}H]CTP as described under Experimental Procedure. Aliquots (2.5 μL) were assayed for cold CCl_3COOH insoluble cpm. Ethidium bromide when added (■) was at 2 μg/mL, and 3′-dATP when added was at 25 μg/mL level (○).

Table I: Specific Activity of mtRNA Synthesized in Vitro in Isolated Mitochondria[a]

sample	time of labeling (min)	specific activity (^{3}H cpm/ μg of RNA)
control	15	2656
	30	4128
	45	6219
+ethidium bromide	45	1240
+3′-dATP	45	1362

[a] Mitoplasts from Ehrlich ascites tumor cells were labeled with 100 μCi/mL each of [^{3}H]UTP and [^{3}H]CTP as described under Experimental Procedures. At the end of labeling, mitoplasts (7.5 mg) were pelleted at 10000*g* for 10 min and washed once with sucrose–mannitol buffer. The pellets were dispersed in 2.5 mL each of guanidinium thiocyanate buffer and RNA was extracted as described under Experimental Procedures. RNA was estimated by using an extinction coefficient of 1 OD at 260 nm = 40 μg of RNA. Ethidium bromide and 3′-dATP were added at zero time at the concentrations of 2 μg/mL and 25 μg/mL, respectively.

RNase in 1 mL of 1× SSPE, washed with 10 mL of 0.1× SSPE, air-dried, and counted with 10 mL of ACS II scintillation mixture (Amersham).

Results

RNA Synthesis in Isolated Mitoplasts. In a recent study from this laboratory, it was shown that digitonin-treated mt particles from LES cells are highly active in [^{35}S]methionine incorporation (Bhat et al., 1981, 1982). Essentially, the same incubation system was used to label total mt RNA and study the synthesis and maturation of mt rRNA. The rate of incorporation of [^{3}H]CTP and [^{3}H]UTP by LES mitoplasts is presented in Figure 1. Ethidium bromide at 2 μg/mL level inhibits the incorporation by about 75%, and 3′-dATP at 25 μg/mL inhibits the incorporation by about 60%. Although not shown here, the rates of inhibition by both ethidium bromide and 3′-dATP are dose dependent. The specific activity of mtRNA labeled in vitro with [^{3}H]CTP and [^{3}H]UTP for various time intervals has been presented in Table I. It is seen that the specific activity increases from about 2500 cpm/μg of RNA at 15 min of labeling to about 6200 cpm/μg of RNA at 45 min of incubation, suggesting a steady accumulation of newly synthesized RNA. As shown for the total acid precipitable cpm above, addition of 2 μg/mL ethidium bromide or 25 μg/mL 3′-dATP results in 60–70% reduction in the specific activity of mtRNA. A nearly 60% higher specific activity is obtained with 200 μCi/mL each of [^{3}H]-UTP and [^{3}H]CTP. Also, use of 250 μCi/mL each of [^{32}P]UTP and [^{32}P]CTP yields about 3.5 × 10^4 cpm/μg of mtRNA (results not shown).

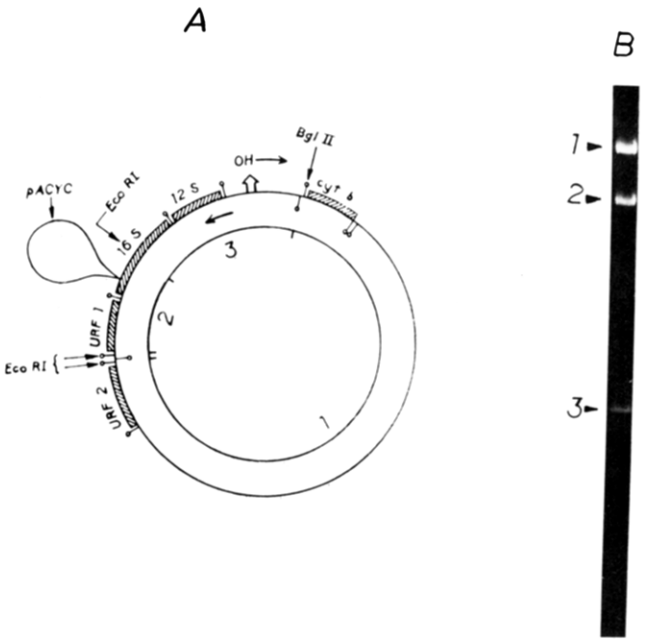

FIGURE 2: Restriction map of pAM1 DNA. (A) The relative positioning of tRNA (○); 12S and 16S rRNA; URF_1, URF_2, and cytochrome *b* genes are according to Bibb et al. (1981). The tRNAs projecting outside the circles are coded by the H strand and those projecting inside are coded by the L strand. The position of insertion of plasmid DNA (pACY 177) and the position of *Eco*RI and *Bgl*II restriction sites are indicated by the arrows. OH and the arrow outside the circle indicate the origin and direction of H strnd DNA synthesis. The arrow inside the circle indicates the direction of H strand transcription. (B) The pAM1 DNA was digested to completion with *Bgl*II and *Eco*RI and electrophoresis on 0.6% agarose gels, and the DNA bands were visualized under UV light after staining with ethidium bromide as described under Experimental Procedures.

Nature of Mitochondrial DNA Probes Used. Hybridization of in vitro labeled mtRNA to mtDNA restriction fragments containing rRNA genes and various nonribosomal genes was used to determine the extent of transcription of mt genome and the nature of RNA synthesized under the in vitro conditions. In these studies, the mouse mt genome from LA9 cells cloned in pACYC-177 plasmid vector (Martens & Clayton, 1979) was used to prepare the specific probes. The restriction maps for *Bgl*II and *Eco*RI on the cloned mt genome are shown by the arrows in Figure 2. The relative positioning of 12S and 16S rRNA, the tRNAs, and presumptive mRNA cistrons on the mt genome, as shown in Figure 2, was based on the DNA sequence data reported by Bibb et al. (1981). The point of insertion of PACYC plasmid at a unique *Hae*II site on the mt genome lies about 70 nucleotides from the 3′ end of the 16S rRNA coding sequence (see Figure 2A). Double digestion of cloned DNA with *Bgl*II and *Eco*RI yields three large DNA fragments as shown in Figure 2 and one small fragment of 200 nucleotides which migrates out of the gel under the electrophoretic conditions used. Following the terminology of Bibb et al. (1981), the largest frament of 11.3-kb DNA contains 12 different reading frames potentially coding for mRNA and 15 different tRNAs. The second largest fragment of 5.7 kb contains URF_1 gene, 0.5 of the 16S rRNA gene (925 nucleotides), 3 tRNA genes, and 3.7-kb plasmid DNA. The third fragment of 2.7 kb contains the 12S rRNA gene, 0.5 of the 16S rRNA gene (725 nucleotides), 3 tRNA genes, and all of the D loop area. For the sake of presentation, these three DNA restriction fragments will be referred to as fragments 1, 2, and 3, respectively, as indicated in Figure 2B.

Extent of Transcription of mt Genome. The extent of transcription of mt genome under the in vitro conditions was

Table II: Hybridization of ^{3}H-Labeled RNA to mtDNA Restriction Fragments[a]

DNA used	^{3}H cpm hybridized using RNA labeled for		
	10 min	30 min	45 min
calf thymus DNA	19	22	16
mtDNA			
fragment 1	102	165	210
fragment 2	148	297	358
fragment 3	330	689	897

[a] Equimolar amounts of mtDNA restriction fragments 1, 2, and 3, i.e., 2, 5, and 11 μg, respectively, and 5 μg of calf thymus DNA were immobilized on nitrocellulose filters and hybridized with 0.3 μg of mtRNA for 48 h at 42 °C as described under Experimental Procedures. The input ^{3}H cpm in the case of 10-min labeled RNA was 630 and 1150 and 1465 cpm, respectively, for 30- and 45-min labeled mtRNAs.

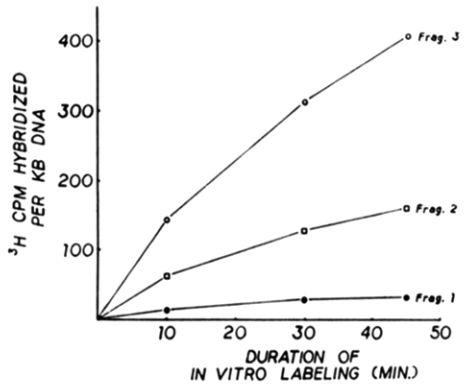

FIGURE 3: Rate of transcription of mt rRNA and nonribosomal genes. The hybridization values from Table II were normalized for 1-kb DNA in each case (i.e., fragments 1, 2, and 3) and presented as a function of time of in vitro labeling.

determined by hybridizing in vitro labeled mtRNA to the three mtDNA restriction fragments described above (Table II). It is seen that RNA labeled for a short duration of 10 min as well as those labeled for 45 min hybridize significantly to all three mtDNA fragments, suggesting that genomic area coding for both rRNA and mRNAs are transcribed under the in vitro conditions. The rate of transcription of different genomic areas appears to vary considerably (Table II) since nearly 60% of the counts hybridize to fragment 3 which contains rRNA genes, whereas only 10–15% hybridize to fragment 1 which mainly contains the mRNA and tRNA genes. Although about 25% of the labeled RNAs hybridize to mtDNA fragment 2, it should be noted that this fragment contains a portion of the 16S rRNA coding sequence in addition to genes coding for URF_1 and two tRNAs. Further, since there is a vast difference (about 5-fold) in the size between fragment 3 and fragment 1, the hybridization results in Table II were normalized for a unit length of DNA (1 kb) and presented in Figure 3. These results clearly show that at 10, 30, and 45 min of in vitro RNA labeling, the rRNA region is transcribed 20–40 times faster than the rest of the genome. These results are in agreement with the published reports showing a considerably faster rate of metabolic labeling of mtrRNAs as compared to that of nonribosomal transcripts in HeLa cells (Gelfand & Attardi, 1981; Attardi et al., 1982) and 20–60-fold abundance of mt 12S and 16S rRNA in the steady-state mtRNA (Battey & Clayton, 1978).

Mode of Synthesis of mt rRNA. In order to study the mode of synthesis of mt rRNA and also to identify putative precursors of rRNA, mtRNAs pulse-labeled for 10, 30, and 45 min were electrophoresed on 1.6% agarose–methyl mercury

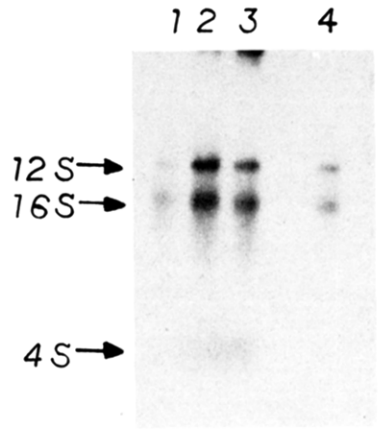

FIGURE 4: Electrophoretic pattern of in vitro labeled mtRNA. Mitochondrial RNA was labeled in vitro by using 100 μCi/mL each of [^{3}H]UTP and [^{3}H]CTP as described in Figure 1 and Table II for 10, 30, and 45 min. A 10-μg sample of RNA in each case was electrophoresed on 1.6% agarose–methyl mercury gels. Similarly, 20 μg of 10-min labeled [^{3}H]RNA was adsorbed to cellulose-bound DNA restriction fragment 3 (about 100 μg of DNA), and RNA specifically hybridized was eluted as described under Experimental Procedures and electrophoresed as above. The gels were fluorographed by using EN^{3}HANCE. (Lane 1) mtRNA labeled for 10 min; (lane 2) mtRNA labeled for 30 min; (lane 3) mtRNA labeled for 45 min; (lane 4) 10-min labeled RNA hybridized to cellulose-bound restriction fragment 3.

gels. As shown in Figure 4, all of the three [^{3}H]RNA samples contain two major components with electrophoretic mobilities characteristic of mt 12S and 16S rRNAs. These two RNA bands together account for over 60% of the input label, providing support to the hybridization results which showed that rRNA genes are transcribed at 20–40-fold higher frequency than the rest of the genome.

Studies on HeLa cell mt transcription (Gelfand & Attardi, 1981; Ojala et al., 1981; Attardi et al., 1982) suggested the occurrence of a low abundance poly(A) RNA having the properties of a common precursor to both 12S and 16S rRNA. The in vitro ^{3}H-labeled RNA, however, does not appear to contain detectable levels of putative mt rRNA precursors even during a short pulse-labeling of 10 min (Figure 4, lanes 1–3). The ribosomal nature of the two RNA bands shown in Figure 4 (lanes 1–3) was ascertained by specific hybridization to cellulose-bound mtDNA. As shown in Figure 4 (see lane 4), 10-min in vitro labeled RNA hybridized to cellulose-bound mtDNA restriction fragment 3 shows the presence of 12S and 16S rRNA with no detectable precursor species. In a separate experiment involving high specific activity labeling with [^{32}P]UTP, although the synthesis of some high molecular weight RNA in the size range of 2.5–3.6 kb is noticed, these high molecular weight mtRNAs do not hybridize to cellulose-bound DNA fragment 3 (data not presented), suggesting their nonribosomal nature.

The possible occurrence of putative rRNA precursors in the steady-state mtRNA was determined by using RNA blot transfer experiments (Alwine et al., 1977). As seen in Figure 5, the EtBr-stained pattern of mt total RNA shows the presence of two major rRNA bands and a number of minor RNA species smaller than 16S rRNA. When the RNA blots are probed with ^{32}P-labeled nick-translated mtDNA fragment 3, two major bands corresponding to 16S and 12S rRNAs are seen (Figure 5, lane 2). Even 5 times longer exposure of blots to X-ray films failed to identify RNA species larger than 16S rRNA by this probe (results not shown). Use of nick-translated total pAM1 DNA, however, shows the presence of two large RNA species (2.8 kb and 3.6 kb), presumptive mRNA

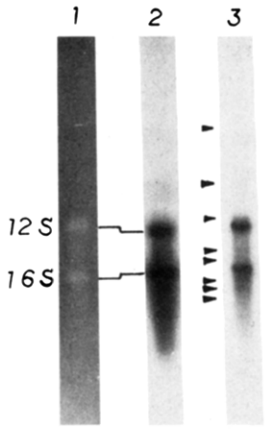

FIGURE 5: Analysis of steady-state mtRNA by Northern blot. Total mtRNA (10 μg) isolated from EDTA washed mitoplasts as described under Experimental Procedures was electrophoresed on 1.6% agarose–methyl mercury gels. The RNA was blot transferred to nitrocellulose papers and probed with nick-translated mtDNA restriction fragments (6.2×10^8 ^{32}P cpm) as described under Experimental Procedures. (Lane 1) Ethidium bromide stained pattern of mtRNA; (lane 2) mtRNA probed with nick-translated DNA fragment 3; (lane 3) mtRNA probed with nick-translated pAM1 DNA. Arrows in lane 3 indicate the presumptive mRNAs and pre-mRNAs.

precursors in addition to a number of putative mRNA species as shown for HeLa mtRNA (Gelfand & Attardi, 1981; Montoya et al., 1981). These results using specific mtDNA probes show the absence of detectable rRNA precursors in steady-state mtRNA and also in mtRNA synthesized in vitro in isolated mt particles.

Discussion

In a previous paper from this laboratory, it was shown that Ehrlich ascites tumor cell mt can synthesize heterogenous size class RNA which is found to be associated with ribonucleoprotein particles resembling polysome structures with respect to sedimentation property (Avadhani et al., 1974). Similarly, 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). To our knowledge, however, the accuracy of transcription and also the exact nature of RNA synthesized in these in vitro systems from mammalian cells remain unclear. Recently, it was shown that *yeast* mt particles are able to synthesize putative precursors of 21S and 15S rRNAs under in vitro conditions (Boerner et al., 1981; Groot et al., 1981). The labeled pre-21S rRNA in the mt particles was converted to mature 21S rRNA, a process which includes "splicing" of a 1.1-kb intervening sequence and trimming at the 3′ end. The processing of pre-15.5S rRNA to mature 15S rRNA was, however, restricted in this in vitro system (Boerner et al., 1981; Groot et al., 1981). In this paper we report on the qualitative and quantitative nature of RNA synthesized in Ehrlich ascites mt in vitro and show that both the 16S and 12S rRNAs are synthesized with accuracy in this system.

Hybridization of in vitro labeled RNA to mtDNA restriction fragments 1, 2, and 3 shows that both ribosomal and nonribosomal regions of mt genome are transcribed in the isolated mt particles (Table II). Further digestion of DNA fragment 1 (11.3 kb) with *Hpa*I and *Hin*dIII yields six restriction fragments of 0.9–3.8 kb, and the in vitro labeled mtRNA hybridizes to all of these six fragments (results not presented), suggesting the complete transcription of mt genome under these conditions. The rate of hybridization of in vitro labeled RNA to different fragments varies markedly (Table II). Data on the DNA excess hybridization presented in Table II and Figure 3 show that on a unit DNA basis, the rRNA region may be transcribed at >30-fold higher rates than the rest of the genome. Moreover, restriction fragment 3 (2.7 kb) containing the rRNA genes also contains a 750-nucleotide D loop area (see Figure 2A). In human mtDNA this region is actively transcribed into an abundant poly(A)-containing RNA of unknown function, designated as 7S RNA (Attardi et al., 1982). In confirmation with the results of Van Etten et al. (1982), however, we have been unable to detect the "7S RNA" or its counterpart in the steady-state mtRNA (Figure 5). Although the significance or the reasons for this apparent difference in the stability of D loop transcripts between the human and mouse mt system are unknown, it may indicate a unique inherent difference between these two systems. thus, if we account for the D loop region which corresponds to about one-third of the mtDNA restriction fragment 3, the relative rate of rDNA transcription may be over 40 times that of the rest of the genome. These results are in general agreement with higher rates of labeling of mt rRNA in vivo and the relative abundance of rRNA in steady-state mtRNA (Gelfand & Attardi, 1981; Attardi et al., 1982; Battey & Clayton, 1978; Bibb et al., 1981; Dubin et al., 1982).

The transcription of H strand is believed to be initiated at a single promoter located adjacent to the D loop area (Attardi et al., 1980; Eperon et al., 1981), and the chain elongation appears to continue until the opposite end of the molecule (Murphy et al., 1975). Therefore, the observed 20–40-fold higher rate of transcription of rDNA in the present study and also in previously reported instances (Battey & Clayton, 1978; Gelfand & Attardi, 1981; Attardi et al., 1982) raises several possiblities. First, preferential transcription of rDNA may be accomplished by chain termination at the end of 16S rRNA gene by a mechanism similar to that of bacterial transcription–attenuation (Attardi et al., 1980; Van Etten et al., 1980). In fact, sequences reminiscent of the hairpin oligo(U) structures believed to be the stop signals for *E. coli* polymerase (Rosenberg & Courts, 1979) have been reported to occur near the 3′ end of human, mouse, bovine, and hamster mt 16S rRNA (Eperon et al., 1980; Van Etten et al., 1980; Dubin et al., 1981, 1982). The second possibility is the involvement of two distinct RNA polymerases, one for the transcription of the entire genome and the second for the preferential transcription of rDNA. As yet, however, there is no indication for the occurrence of more than one type of mt-specific RNA polymerase. Thus, the precise mechanism regulating this differential expression of H strand remains to be determined.

The occurrence of a low abundance poly(A)-containing RNA in HeLa mtRNA which hybridizes to both 12S and 16S coding regions on the mtDNA (Ojala et al., 1980) suggested the possibility that the 12S and 16S mt rRNAs may be derived from a common precursor. In the present study, our attempts to detect such putative rRNA precursors either in the in vitro labeled mtRNA or in steady-state RNA have been unsuccessful. First, in vitro RNA specifically hybridized to cellulose-linked restriction fragment 3 consists of only 12S and 16S RNA species with no detectable precursor even in 10-min pulse-labeled RNA (Figure 4). Similarly, analysis of steady-state mtRNA by Northern blot with ^{32}P-labeled nick-translated mtDNA fragment 3 probe does not show the presence of rDNA transcripts larger than 16S rRNA (Figure 5). Use of mtDNA fragment 1 or total mt genome as a probe, however, detects the presence of 2.4–3.8-kb transcripts, possibly representing the mRNA precursors. Because of the limitations of the hybridization conditions, however, we cannot completely

rule out the presence of such a precursor. It is estimated that such a precursor, if present, may occur at concentrations less than 1 in 500 molecules of rRNA. Our results, therefore, demonstrate that the major pathway for rRNA maturation in mouse mitochondria does not involve the synthesis of a common precursor of 12S and 16S rRNA. This mode of maturation is consistent with the results reported for HeLa mt (Gelfand & Attardi, 1981). In conclusion, RNA synthesis in this in vitro system appears to resemble in vivo mt transcription with respect to the rate of transcription and mode of rRNA maturation.

Acknowledgments

We are thankful to Dr. David A. Clayton for a gift of cloned mtDNA used in these experiments. We are also thankful to John Cozza and Cathy Fluellen for excellent technical help and to Nina Leinwand for her help during the preparation of the manuscript.

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