Characterization of Primary Transcripts and Identification of Transcription Initiation Sites on the Heavy and Light Strands of Mouse Mitochondrial DNA†

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ABSTRACT: Total RNA from Ehrlich ascites mitochondria pretreated with RNase-free DNase was capped in vitro with [α-^{32}P]GTP and guanylyl transferase. The cappable RNAs representing the primary transcripts show a heterogeneous size distribution with four major species of 46, 63, 94, and 152 nucleotides and four minor species of 19, 24, 104, and 790 nucleotides in size. Hybridization with the D-loop DNA probes shows that the 19-nucleotide-long capped RNA is coded by the H-strand of mitochondrial DNA while the rest are coded by the L-strand. S1 nuclease mapping and primer extension analyses suggest the occurrence of a transcription initiation of H-strand at about 19 nucleotides upstream from the start of the tRNA^{Phe} gene. All of the L-strand cappable RNAs have a common 5′ end mapping to nucleotide 16 183 ± 5 of the genome. The 3′ ends of four major cappable RNA species line up to the conserved sequence boxes, putative start sites of DH-DNA; and in fact about 2% of these cappable species are found to exist as DNA-linked RNA under steady-state conditions. The 3′ end of the 790-nucleotide cappable RNA lies close to the start of the tRNA^{Pro} gene, suggesting that it may be the true precursor of L-strand transcript endonucleolytically processed at the 3′ end. The level of L-strand-coded cappable RNAs varies markedly under different growth conditions. Treatment with cycloheximide results in a reduction while chloramphenicol caused over 3-fold induction, suggesting that these "primer" RNAs may have an additional regulatory function.

The displacement loop (D-loop)[1] structures, commonly found in the mitochondrial genomes of vertebrates, represent the most rapidly evolving portion of the organelle DNA (Dawid, 1972; Upholt & Dawid, 1977; Brown et al., 1979, 1986; Lanave et al., 1984). This region consists of a triple-stranded structure where the parental H-strand is displaced by 500–700-base-long single-stranded DNA molecules called DH-DNA that are complementary to the L-strand (Clayton, 1982). The D-loop region houses the origin of H-strand synthesis (oriH), which is the starting point of DH-DNA (Clayton, 1982, 1984). In vitro transcription using partially purified RNA polymerase (Walberg & Clayton, 1983; Chang & Clayton, 1984, 1986; Bogenhagen et al., 1984) and mapping of the primary transcripts by S1 nuclease protection and primer extension (Clayton, 1984; Montoya et al., 1982; Yoza & Bogenhagen, 1984; Chang et al., 1985; Bogenhagen et al., 1986; King & Low, 1987) have firmly established that transcription initiation on both the H- and L-strands of animal mitochondrial DNA occurs at promoter regions located in the D-loop. In human (Clayton, 1984; Chang & Clayton, 1984; Bogenhagen et al., 1984), mouse (Chang et al., 1986), bovine (King & Low, 1987), and *Xenopus* (Bogenhagen et al., 1986) systems, the H- and L-strand promoters are separated by 80 and 120 bases. Despite this overall structural conservation, there are subtle differences between the three systems with respect to nucleotide sequences (Brown et al., 1986) and the organization of regulatory elements. Using a RNA polymerase directed in vitro transcription system, it was recently shown that both of the transcription promoters on the human mitochondrial genome function bidirectionally, although the rate of transcription of the opposite strand was considerably lower than the transcription of the resident strand (Chang et al., 1986). Similarly, the major H-strand promoter in the *Xenopus* mitochondrial system functions bidirectionally (Bogenhagen et al., 1986). In the bovine mitochondrial system, the 5′ termini of DH-DNA and also H-strand primary transcripts were different under different growth conditions (King & Low, 1987). Further, using S1 nuclease protection and primer extension analyses, it was shown that the synthesis of DH-DNA in mouse mitochondria may be initiated at the same site where the L-strand transcription is initiated (Chang et al., 1985). In the present study, using in vitro capped RNA, we provide confirmative evidence that 46–150-nucleotide-long cappable RNAs with identical 5′ map positions act as primers for the synthesis of DH-DNA. A 790-nucleotide-long cappable RNA of relatively low abundance appears to be the 3′ end processed primary transcript. Finally, the relative abundance of L-strand-coded cappable RNA varies markedly with respect to cellular growth conditions.

Materials and Methods

Isolation of Mitochondrial Nucleic Acids. Mitochondria were isolated from Ehrlich ascites tumor cells and other tissues by using the sucrose–mannitol buffer system (Bhat et al., 1982) and banded in a discontinuous sucrose gradient as described by Bogenhagen and Clayton (1974). The resultant mitochondrial preparations were treated with digitonin (75 μg/mg of protein) and washed 3 times with sucrose–mannitol–EDTA [see Bhat et al. (1982)], and total nucleic acids were isolated by repeated extraction of the SDS-lysed mitochondria with hot phenol–chloroform followed by ethanol precipitation (Avadhani, 1979). The resultant nucleic acids were treated with RNase-free DNase (Worthington Biochemicals, Malvern, PA) in the presence of calf intestinal RNase inhibitor (Amersham Corp., Arlington Heights, IL) as described by Yoza

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[1] Abbreviations: D-loop, displacement loop; H-strand, heavy strand; L-strand, light strand; EDTA, ethylenediaminetetraacetate disodium salt; DH-DNA, D-loop H-strand DNA; bp, base pair(s).

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and Bogenhagen (1984). The RNA was extracted with phenol–chloroform and recovered by ethanol precipitation. Poly(A) RNA was isolated by binding to poly(U)–Sepharose (Ricca et al., 1981).

In Vitro Capping of Nucleic Acid. Mitochondrial nucleic acids (30–35 μg of DNase-treated or untreated) were capped in a 50-μL reaction mixture containing 50 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 6 mM KCl, 1 mM dithiothreitol, 200 μM *S*-adenosylmethionine, 160 μM ATP, 3.5 nmol of [α-^{32}P]GTP (3000 Ci/mmol, Amersham Corp.), 20 units of calf intestinal RNase inhibitor, and 4–5 units of vaccinia guanylyl transferase (Bethesda Research Laboratories, Rockville, MD) at 37 °C for 60 min. The nucleic acids were ethanol precipitated following extraction with phenol–chloroform and used for further analysis.

S1 Nuclease Mapping and Primer Extension Analysis. The conditions for S1 protection analysis were essentially as described before (Bhat et al., 1985). Typically, 2–5 μg of capped RNA was hybridized to about 30 ng of heat-denatured DNA probes or 5 μg of H- or L-strand-specific single-stranded DNAs shown in Figure 2 under the conditions described before (Bhat et al., 1985), and the S1-resistant fragments were analyzed on 4–6% sequencing gels (Maxam & Gilbert, 1977). In primer extension studies, about 15 ng of 5′ end-labeled DNA primer was annealed to 3.5 μg of DNase-treated RNA in 12 μL of 10 mM Tris-HCl (pH 8.0) and 120 mM KCl. After 20 min at 68 °C, the mixture was cooled to 42 °C over a 2–2.5-h period. The reaction mixture was diluted with 12 μL of solution containing 100 mM Tris-HCl (pH 8.0), 120 mM KCl, 20 mM $MgCl_2$, 10 mM DTT, 1.6 mM each of the four dNTPs, and 10 units of AMV reverse transcriptase (Life Sciences Inc., St. Petersburg, FL). The reaction mixture was incubated at 42 °C for 2 h, and the extended primer was denatured in a formamide-containing buffer and resolved on a 5% sequencing gel.

Other Procedures. Dot blot analysis of mitochondrial RNA was carried out essentially as described by Kafatos et al. (1979). Single-stranded probes labeled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase were used for hybridization. For preparing the L-strand of probe A (see Figure 2), P407 DNA was cut with *Ssp*I, labeled at the 5′ end with [γ-^{32}P]ATP, and digested again with *Sca*I. The *Ssp*I–*Sca*I single-stranded probe was isolated by electrophoresis under denaturing conditions (Maxam & Gilbert, 1977). The cytochrome *b* specific single-stranded probe was prepared by labeling at the *Bgl*II site of pAM1 DNA [see Bhat et al. (1985)] and isolating the 5′-labeled *Bgl*II–*Xho*I strand (nucleotides 13 552–15 330) from the denaturing gel as described above. The specific activities of all of the probes were adjusted to 1 pmol of ^{32}P/100 pmol of DNA 5′ end. RNA was resolved on 4–6% sequencing gels (Maxam & Gilbert, 1987) or on formaldehyde-containing agarose gels (Lehrach et al., 1977). The conditions for agarose gel electrophoresis and Northern blot hybridization were essentially as described before (Bhat et al., 1985). The relative purities of various mitochondrial RNA preparations were estimated by determining the ratios of cytoplasmic 18S + 28S rRNA to mitochondrial 12S + 16S rRNA from the UV scans of polyacrylamide gels. The levels of cytoplasmic contamination estimated by this method ranged from 6 to 10% for mitochondrial RNA from Ehrlich ascites cells and from 9 to 16% for mouse liver and mouse embryonic mitochondrial RNAs.

Results

Steady-State RNA Hybridizing to D-Loop DNA Probes. The DNA probes used in this study were derived from two

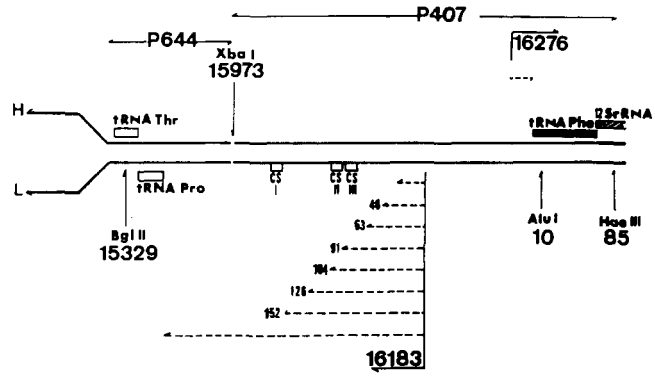

FIGURE 1: Physical map of the mouse mitochondrial D-loop and the flanking region. The relative locations of 12S rRNA, $tRNA^{Phe}$, $tRNA^{Pro}$, $tRNA^{Thr}$, the conserved sequence boxes designated as CSI, CSII, and CSIII, and the restriction sites are according to Bibb et al. (1982). The dashed lines starting from nucleotides 16 276 and 16 183 indicate the H- and L-strand transcription initiation sites as determined by the 5′ map positions of in vitro capped RNAs.

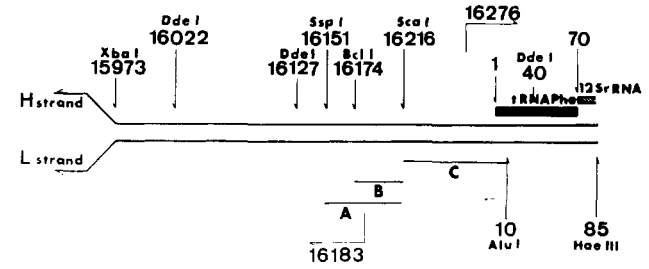

FIGURE 2: Detailed restriction map of the P407 D-loop DNA region. A, B, and C indicate the map positions of the three DNA probes (A is 65 nucleotides, B is 42 nucleotides, and C is 89 nucleotides in length) used in this study. The half-arrow below probe C indicates the position of (H-strand homologous) synthetic primer TTGTTAATGT used for primer extension analysis shown in Figure 7.

mitochondrial D-loop DNA clones designated as P407 and P644 as shown in Figure 1. Clone P407 represents the *Xba*I to *Hae*III (nucleotides 15 973–85 of the mouse mitochondrial genome) fragment, cloned in the *Xba*I and *Sal*I sites of plasmid pUR250 (see Figure 1). The H- and L-strand-specific clones were generated by forced cloning a 450 bp *Eco*RI–*Hind*III fragment from P407 DNA in M13mp8 and M13mp9 vectors. These two restriction sites are in the polylinker region flanking both sides of the insert DNA. The strand specificities of the two M13 clones were established by DNA sequencing, and as indicated in Figure 2, M13mp9-407 contains the H-strand, and M13mp8-407 contains the L-strand. A detailed restriction map of P407 and the map positions of various DNA probes used in this study have been presented in Figure 2. The second D-loop probe was derived by cloning the 644-base-long *Bgl*II to *Xba*I (nucleotides 15 329–15 973) fragment in *Bam*HI and *Xba*I sites of the pUR250 plasmid.

As shown in the Northern blot experiment in Figure 3, the L-strand clone M13mp8-407 hybridizes to two poly(A)-containing RNAs of 920 and 790 nucleotides. It is also seen that this probe hybridizes with relatively abundant nonpolyadenylated RNAs of heterogeneous size. The H-strand-specific M13mp9-407 DNA, on the other hand, hybridizes with a 960-nucleotide poly(A) RNA and a relatively abundant nonpolyadenylated RNA of about 60–70 nucleotides. Since this probe contains about 15 nucleotides from the 5′ end of the small rRNA gene and all of the $tRNA^{Phe}$ gene, it is likely that the 960-nucleotide poly(A) RNA may be the 12S rRNA and the 60–70-nucleotide RNA in the total RNA fraction may be $tRNA^{Phe}$. Even an overexposure of the blot (up to 3 days)

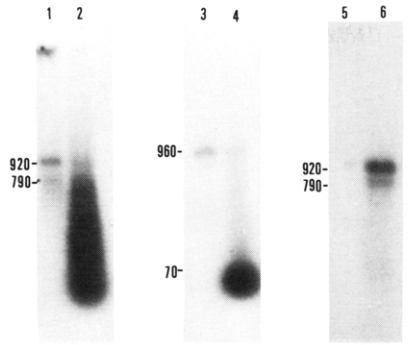

FIGURE 3: Northern blot analysis of mitochondrial DNA using the D-loop DNA probes. Three micrograms of poly(A) + RNA and 12 μg of total RNA from Ehrlich ascites mitochondria were electrophoresed on a 1.6% agarose gel in the presence of formaldehyde and transblotted to Gene Screen (New England Nuclear Corp.) as described under Materials and Methods and hybridized with ^{32}P-labeled DNA probes (10^7 cpm) as described before (Bhat et al., 1985). Lanes 1, 3, and 6 represent poly(A) RNA and lanes 2, 4, and 5 total RNA. Lanes 1 and 2 were probed with L-strand DNA clone M13mp8-407, lanes 3 and 4 were probed with H-strand DNA clone M13mp9-407, and lanes 5 and 6 were probed with nick-translated P644 DNA. Both of the M13 DNA probes were labele with [^{32}P]dCTP by using the hybridization probe primer as described by Hu and Messing (1982). *Hin*fI-digested pUR250 DNA was run as a standard.

failed to detect a putative precursor containing the tRNA^{Phe} and 12S rRNA sequences. In order to determine the 3′ boundaries of the L-strand-specific RNA identified by the single-strand DNA probe M13 mp8-407, the total as well as the poly(A)-containing RNA species were hybridized with nick-translated P644 DNA. As seen from lanes 5 and 6 (Figure 3), this probe hybridizes to the two poly(A) RNAs of 790 and 920 nucleotides, suggesting that the 3′ ends of these two RNAs extend into the P644 region. It is also seen that the heterogeneous nonpolyadenylated RNAs fail to hybridize with this probe, suggesting that their coding region resides within the boundaries of P407 DNA.

Identification of Primary Transcripts. The primary transcripts coded by the mitochondrial DNA were identified by in vitro capping of the total RNA fraction (DNase-treated nucleic acids) from digitonin-treated and gradient-purified Ehrlich ascites mitochondria. As shown in Figure 4, the RNA capped with [α-^{32}P]GTP resolves into a number of species in the range of 19–152 nucleotides in length. Further, the patterns of cappable RNAs from two different isolates are nearly identical, suggesting that the multiple species of cappable RNAs detected are not due to artifacts of the isolation procedure. When the capped RNA species are electrophoresed on a denaturing formaldehyde-containing gel, an additional large RNA of about 790 nucleotides is also seen (see Figure 4, lane 4). The strand specificities of the capped RNAs were determined by hybridization to single-stranded DNA clones M13mp9-407 (H-strand) and M13mp8-407 (L-strand). Analysis of RNA eluted from the DNA–RNA hybrids shows that the 19-nucleotide-long cappable RNA is coded by the H-strand (Figure 5, lane 1) and the rest of the RNAs are coded by the L-strand (Figure 5, lane 4). Further, S1 protection of RNA–DNA hybrids presented in lanes 2 and 5 (Figure 5) suggests that all of the cappable RNAs of 19–152 nucleotides map within the boundaries of P407 DNA (nucleotides 15 973–85).

Characterization of Primary Transcripts. The 5′ ends of cappable RNAs coded by the L-strand were further characterized by S1 nuclease protection as shown in Figure 6. In this experiment, in vitro capped RNAs hybrid selected on the L-strand DNA M13mp8-407 were hybridized to DNA probes

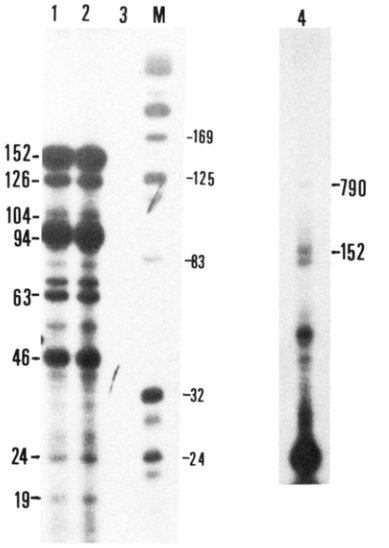

FIGURE 4: Electrophoretic patterns of in vitro capped RNA. Mitochondrial RNA prepared by DNase treatment of total nucleic acid was capped using [α-^{32}P]GTP and vaccinia guanylyl transferase as described under Materials and Methods. Electrophoresis was carried out either on a 6% sequencing gel (lanes 1–3 and M) or on a 1.5% agarose gel containing 2 M formaldehyde (lane 4). Lanes 1 and 2, two separate preparations of RNA capped under identical conditions [(5–6) × 10^4 cpm]. Lane 3, capped RNA as in lane 2 was digested with 1 μg of RNase for 10 min at 37 °C. Lane M, molecular weight markers as indicted. Lane 4, capped RNA as in lane 2 was electrophoresed on a formaldehyde-containing agarose gel. Alongside lane 4, *Hin*fI-digested pUC19 DNA was run as a molecular weight marker and visualized by ethidium bromide staining (not shown).

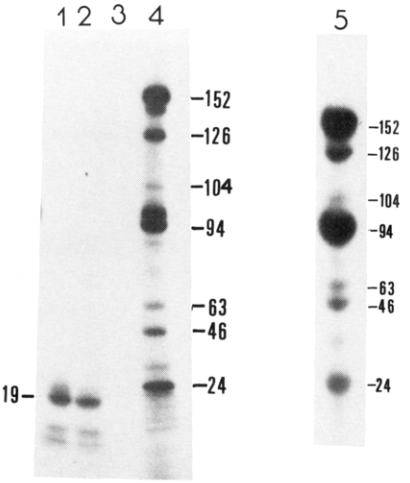

FIGURE 5: Specificities of cappable RNase for hybridization with the H- and L-strands of D-loop DNA. Capped RNA (10^5 cpm) was hybridized to 5 μg each of M13mp8-407 (L-strand) and M13mp9-407 (H-strand) immobilized on APT paper and eluted with 80% formamide under conditions described before (Bhat et al., 1985). In parallel experiments, capped RNA was also hybridized with similar amounts of single-stranded DNAs in solution and digested with S1 nuclease as described under Materials and Methods. Resultant RNA samples were analyzed on a 6% sequencing gel using molecular weight standards shown in Figure 4. Lanes 1 and 4, RNAs eluted from H-strand-specific M13mp9-407 DNA and L-strand-specific M13mp8-407 DNA, respectively. Lane 2, S1 protection of RNA hybridized to H-strand DNA in solution. Lane 3, same as in lane 2 except that no DNA was added. Lane 5, S1 protection of RNA hybridized to L-strand DNA in solution.

A and B shown in Figure 2. As seen from Figure 6A (lanes 1–3), hybridization with increasing amounts of probe A and subsequent digestion with S1 nuclease yielded a prominent 32-nucleotide fragment, whereas probe B protected only an 8–12-nucleotide-long capped RNA. In another series of ex-

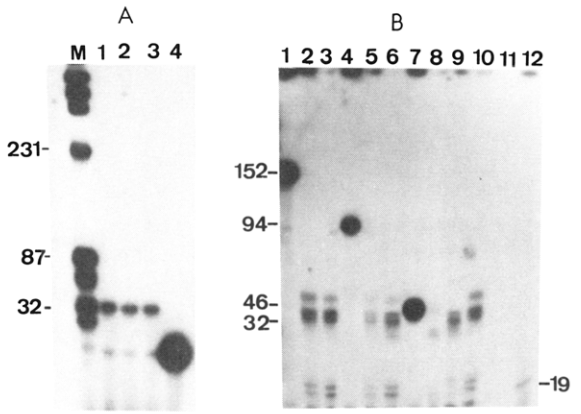

FIGURE 6: Mapping of 5′ ends of in vitro capped RNAs by S1 nuclease protection. Panel A represents S1 protection analysis using the total population of capped RNA hybrid selected on L-strand-specific DNA M13mp8-407 (see Figure 5) using D-loop DNA probes A and B shown in Figure 2. Lanes 1, 2, and 3, capped RNA (6×10^4 cpm) was probed with 30, 40, and 50 ng, respectively, of heat-denatured probe A in S1 protection analysis. Lane 4, S1 analysis was carried out using 12×10^4 cpm of capped RNA and 50 ng of probe B. Panel B represents S1 analysis using individual RNA species hybridizing with the L- and H-strand D-loop DNA recovered from acrylamide gels. Lanes 1, 4, and 7, 152-nucleotide, 94-nucleotide, and 46-nucleotide-long capped RNAs, respectively, recovered from the gel. Lanes 2 and 3, S1 protection of RNA as in lane 1 using 20 and 30 ng, respectively, of probe A. Lanes 5 and 6, S1 protection of RNA as in lane 4 using 20 and 30 ng, respectively, of probe A. Lanes 8 and 9, S1 protection of RNA as in lane 7 using 10 and 30 ng, respectively, of probe A. Lane 10, S1 protection of 790-nucleotide capped RNA using 40 ng of probe A. Lanes 11 and 12, S1 protection of H-strand hybridizing 19-nucleotide RNA using probe B and probe C, respectively. Digestion with S1 nuclease (80–90 Vogt units) was carried out for 35 min for samples in (A) and for 25 min in (B). Electrophoresis was carried out on a 6% sequencing gel.

periments, the ^{32}P-capped RNAs of 790, 152, 94, and 46 nucleotides hybridizing to the L-strand-specific single-stranded DNA clone and the 19-nucleotide RNA hybridizing to the H-strand were eluted from the acrylamide or agarose gels (see Figure 6B) and used for S1 nuclease mapping. It is seen that all of the L-strand-coded RNAs capped in vitro yield nearly identical S1 fragments of about 32 nucleotides against probe A. An additional band in the region of about 45 nucleotides may represent incomplete digestion at the 3′ ends since a longer digestion with S1 nuclease as in Figure 6A eliminated this band (results not shown). These results suggest that all of these L-strand-hybridizing RNAs have the same or similar 5′ ends. Also, since probe A with an L-strand complement spanning nucleotides 16 151–16 216 protects about 32-nucleotide segments of each of the four RNAs and probe B with an L-strand complement spanning nucleotides 16 174–16 216 protects only a 9–10-nucleotide segment (see Figure 6A), it is apparent that the 5′ ends of L-strand primary transcripts map to nucleotide 16 183 ± 5. These results are in agreement with a previous report showing a major L-strand transcription initiation at this site using partially purified RNA polymerase from mouse LA9 cell mitochondria (Chang & Clayton, 1986). It is also seen that the 19-nucleotide-long RNA hybridizing to the H-strand is completely protected by the DNA probe C (see Figure 6B, lane 12). However, probe B fails to protect any portion of this RNA (lane 11). The putative 5′ end of the H-strand-coded RNA was further mapped by primer extension of a synthetic decanucleotide homologous to nucleotides 16 286–16 295 of the H-strand. It is seen that the size of extended primer (about 19 nucleotides) closely resembles the size of the cappable RNA hybridizing to the H-strand (see Figure 7). Further, omission of RNA or addition of RNA pretreated with RNase for various lengths of time yields none to limited extension, suggesting that the extension of decanucleotide primer is indeed dependent on template RNA. These results suggest a transcription initiation on the H-strand around nucleotide 16 276 ± 3.

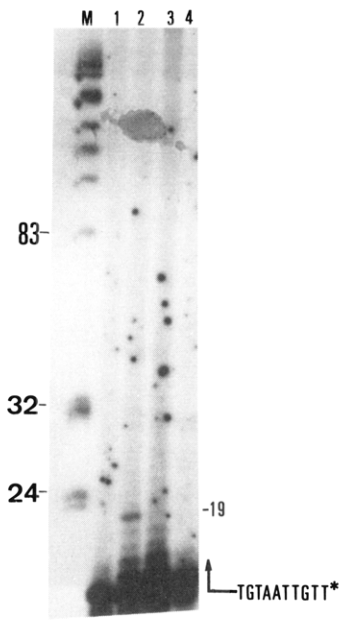

FIGURE 7: Primer extension analysis using an H-strand-specific synthetic probe. A decadeoxynucleotide probe corresponding to H-strand sequences (nucleotides 16 286–16 295) as shown in the figure was synthesized using an Applied Biosystems Model 380-A DNA synthesizer. The probe was labeled at the 5′ end using [γ-^{32}P]ATP and polynucleotide kinase. About 15 ng of labeled primer (4×10^4 cpm) was annealed to 3.5 μg of total RNA, and primer extension was carried out as described under Materials and Methods. Lane M, molecular weight markers; lane 1, primer alone; lane 2, primer annealed to control RNA; lanes 3 and 4, primer extension carried out with RNA treated with RNase for 1 and 5 min, respectively.

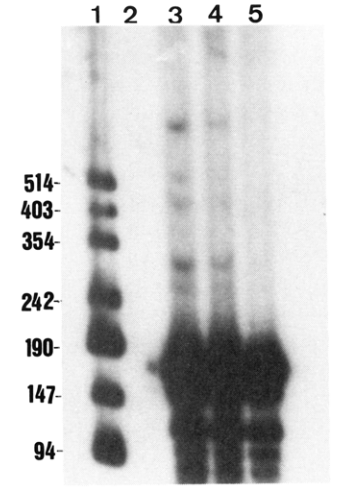

FIGURE 8: DNA-linked cappable RNA in the total nucleic acid fraction. The total nucleic acid fraction (without DNase treatment) was capped in vitro and subsequently treated either with RNase or with DNase. Lane 1, molecular weight marker; lane 2, 1 μg of capped nucleic acid treated with RNase for 10 min at 37 °C; lane 3, capped nucleic acid as in lane 1 was electrophoresed without any treatment; lanes 4 and 5, capped nucleic acid as in lane 3 was treated with DNase for 1 and 5 min respectively, at 37 °C. Electrophoresis was carried out on a 3.8% sequencing gel.

Levels of Cappable RNAs and DNA-Linked RNAs. When mitochondrial nucleic acids without treatment with DNase were capped in vitro with [α-^{32}P]GTP, there was a significant change in the gel profiles of cappable species (see Figure 8, lane 3). In comparison to the gel pattern of DNase-pretreated

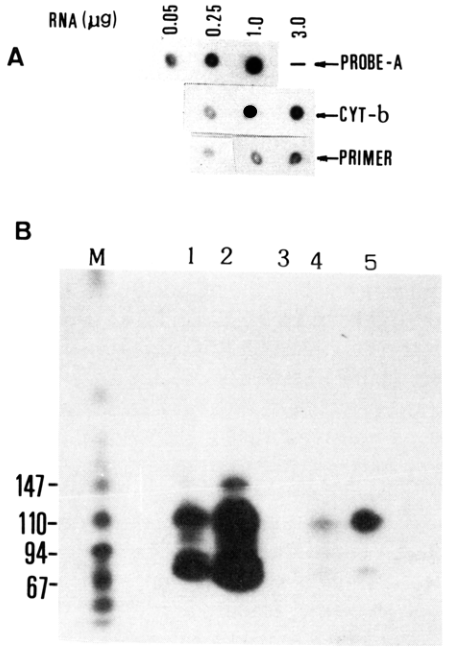

FIGURE 9: Effects of growth conditions and inhibitors on the relative levels of cappable RNAs. (Panel A) Relative levels of H- and L-strand-coded primary transcripts were compared with the level of cytochrome *b* transcripts in Ehrlich ascites mitochondria. 0.05–3.0 μg of RNA was probed with 30 pmol of 5′ end-labeled probes of nearly equal specific activties (1 pmol of ^{32}P/100 pmol of 5′ terminus) by dot blot hybridization. The three single-stranded DNA probes used were the L-strand of probe A (see Figure 2), the H-strand of the cytochrome *b* coding region [see Bhat et al. (1985)], and the synthetic decanucleotide primer shown in Figure 7. The amounts of RNA used are indicated at the top of each column. (Panel B) Relative levels of L-strand-specific primary transcripts in mitochondria under different growth and physiological conditions. 1 μg of DNase-treated nucleic acid capped in vitro was hybridized with 5 μg of L-strand DNA M13mp8-407 in solution and treated with S1 nuclease, and the S1-resistant fragments were electrophoresed on a 3.8% sequencing gel. Lane 1, capped mitochondrial RNA from control Ehrlich ascites cells incubated for 24 h under tissue culture conditions; lane 2, capped RNA from Ehrlich ascites cells treated with 500 μg/mL chloramphenicol for 24 h under tissue culture conditions; lane 3, same as in lane 1 except that the tumor cells were treated with cycoheximide (200 μg/mL) for 10 h; lane 4, capped mitochondrial RNA from adult mouse liver; lane 5, capped mitochondrial RNA from 19-day-old mouse embryos.

RNA preparations presented in Figure 4, at least four additional species of 300–700 nucleotides are seen in untreated samples. As shown in lanes 4 and 5, these additional species of nucleic acids which represent only 2–5% of the total capped population are sensitive to DNase treatment, suggesting that segments of DNA strands are covalently attached to 45–152-nucleotide-long capped RNA molecules.

The steady-state levels of L-strand- and H-strand-coded cappable RNAs were compared with the levels of relatively abundant cytochrome *b* mRNA (Bhat et al., 1985) in the total mitochondrial RNA from Ehrlich ascites cells by dot hybridization using single-strand DNA probes of nearly comparable specific activities (1 pmol of ^{32}P/100 pmol of single-stranded DNA). As seen from Figure 9A, the relative level of L-strand-coded primary transcripts is 80–100 times that of the H-strand cappable species. Further, their steady-state level in Ehrlich ascites mitochondria is about 10-fold in excess of the relatively abundant cytochrome *b* mRNA (see Figure 9A). We have also estimated the relative levels of these L-strand-coded cappable RNAs in mitochondria from adult liver and 19-day-old embryos. Results presented in Figure 9B show that the level of L-strand cappable RNA in Ehrlich ascites mitochondria is <100-fold higher than that seen in mitochondria from adult mouse liver. Although not shown, similar low levels of cappable RNA were observed in mitochondria from other adult mouse tissues such as kidney, brain, and heart, suggesting a remarkably higher pool of these RNAs in rapidly growing tumor cells. In support of this, mitochondrial RNA isolated from 19-day-old mouse embryos contains 20–50-fold higher levels of cappable RNA species than in adult liver. Further, as shown in lane 3, the level of cappable RNA species is markedly reduced by treatment with cycloheximide, an inhibitor of cytoplasmic protein synthesis. In contrast, chloramphenicol, a specific inhibitor of mitochondrial translation, induces the level of cappable RNA (see lane 2). The significance of chloramphenicol induction remains unknown at this time. These results together show that the relative levels of cappable RNAs fluctuate depending on the growth conditions and physiological states of the cells. Since the RNA preparations used in this experiment are of comparable purity (80–90%), the magnitude of difference observed (10-fold to over 100-fold) in the levels of cappable RNA is not due to varying levels of contaminants.

Discussion

DNA sequence analyses have shown a considerable similarity with respect to mode of gene organization among mitochondrial genomes of diverse animal cells and conservation of sequences in various reading frames (Anderson et al., 1981, 1982: Bibb et al., 1982; Roe et al., 1985). Contrary to this, there is an apparent divergence of structure and organization of regulatory elements housed in the D-loop region. The transcription on both H- and L-strands of human and *Xenopus* mitochondrial genomes initiates from a 6–8-nucleotide consensus sequence region (Montoya et al., 1982; Bogenhagen et al., 1986) analogous to the promoter sequences of *yeast* mitochondrial DNA (Christianson & Rabinowitz, 1983; Biswas et al., 1985). There is, however, very little homology in the consensus sequences from all of these mitochondrial systems (Bogenhagen et al., 1984, 1986; Chang & Clayton, 1984). There are also subtle differences with respect to number and relative locations of the transcription initiation sites (Clayton, 1984; Bogenhagen et al., 1986; Low & King, 1987). In the present study, we have used in vitro capped steady-state RNA to study the nature of primary transcripts and transcription initiation sites on the mouse mitochondrial genome.

Studies reported in the human mitochondrial system showed the presence of two distinct species of H-strand-coded precursor RNAs spanning the $tRNA^{Phe}$ 12S and 16S rRNA genes. These RNAs had different 5′ ends: one mapping to 13 nucleotides upstream to the start of the $tRNA^{Phe}$ gene, close to a major H-strand transcription promoter called the HSP (Chang & Clayton, 1984; Yoza & Bogenhagen, 1984; Montoya et al., 1982), and the other mapping to about 2–3 nucleotides upstream from the start of the 12S rRNA gene (Montoya et al., 1982). A previous study from this laboratory failed to detect such precursors in mouse mitochondrial RNA pulse labeled in vitro in isolated mitoplasts, suggesting a rapid processing of precursor molecules in this system (Kantharaj et al., 1983). In the present study, in an attempt to determine the occurrence of such precursors, total RNA and poly(A) RNA were probed with M13 single-stranded DNA. The H-strand probe M13mp9-407 contains complete sequences of the $tRNA^{Phe}$ gene and 15 nucleotides at the start of the 12S rRNA gene, thus providing a powerful probe to detect any possible low-level precursors. Results of Northern blot presented in Figure 3 (lanes 3 and 4) show the occurrence of only mature 12S rRNA species with no detectable larger size precursors. Further, in vitro capped RNA hybridizing to the H-strand consists mainly of a short 19-nucleotide-long species

representing the 5′ end of the putative precursor.

The S1 nuclease protection of capped RNA using DNA probes B and C (Figure 6B) suggests that the 5′ terminus of the 19-nucleotide cappable RNA maps to 19–79 nucleotides upstream from the $tRNA^{Phe}$ gene. In the initial experiments (results not shown) when single-stranded DNA primers from the $tRNA^{Phe}$ and 12S rRNA regions (*Alu*I–*Hae*III) were used for primer extension analysis, we were unable to detect any extension beyond the 5′ end of $tRNA^{Phe}$. Also, a relatively more sensitive approach of in vitro capping failed to detect any $tRNA^{Phe}$ or 12S rRNA precursors with intact 5′ ends. Primer extension analysis using a decanucleotide synthetic oligonucleotide homologous to sequences immediately preceding the $tRNA^{Phe}$ gene (see Figures 1 and 7) indeed shows the occurrence of an RNA species with the 5′ terminus mapping around nucleotide 16 276 ± 3. These results lead us to conclude that transcription initiation on the H-strand of the mouse mitochondrial genome occurs at this site under in vivo conditions. In vitro transcription experiments of Chang and Clayton (1986) using purified RNA polymerase have identified a major promoter activity in this region. Minor differences (16 284 observed by these authors versus 16 276 in the present studies) with respect to the start positions of H-strand transcription may represent methodological differences although physiological differences between the in vivo versus in vitro systems cannot be excluded. Also, we have been unable to detect the putative precursors starting from nucleotide 16 290 reported by Chang and Clayton (1986) possibly because of its rapid processing as described above.

The in vitro capped RNA hybridizing to the L-strand D-loop DNA clone M13mp8-407 resolves into six to nine species in the size range of 24–152 nucleotides (Figure 4). Results of S1 mapping experiments (Figure 6) using the total population of capped RNAs hybrid selected on the L-strand DNA clone M13mp8-407, as well as the gel-purified RNA species, show that they all have a common 5′ end mapping close to nucleotide 16 183 ± 5. These results are in agreement with the results of Chang et al. (1985), who reported a major L-strand transcription activity in this region using an in vitro transcription system programmed with mitochondrial RNA polymerase from mouse LA9 cells. The occurrence of multiple species of cappable RNA in mouse mitochondria represents an unusual situation different from other animal systems. In the human mitochondrial system, a relatively abundant 7S RNA species with no apparent protein-coding propensity (Ojala et al., 1981) has been implicated to serve as a primer for DH-DNA synthesis (Chang & Clayton, 1985). A recent study suggests an analogous situation in the *Xenopus* mitochondrial system (Bogenhagen et al., 1986). Similarly, on the basis of primer extension using DH-DNA isolates as templates, it was shown that the DH-DNA synthesis in mouse mitochondria is initiated at the same point as the start of L-strand transcription. Similarly, RNase sensitivity of the 5′ ends of these molecules suggested the occurrence of DH-DNA species linked to putative RNA primers (Chang et al., 1985). By virtue of size and their common 5′ termini in the region of nucleotide 16 183 ± 5, the end of many of the predominant cappable RNA species detected in this study maps to the conserved sequence boxes as indicated in Figure 1. In agreement with the results of Chang et al. (1985), these results strongly suggest that the major species of capped RNAs of 46, 63, 93, and 152 nucleotides may serve as the primers for DH-DNA synthesis. A direct proof for this possibility was provided in experiments showing that a fraction of these cappable RNA species exists as DNA-linked RNA (see Figure 8). Also, since DNase treatment effectively reduced the size of the 5′-capped nucleic acids without affecting the amount of radioactivity, it is reasonable to assume that the DNA is linked at the 3′ ends of these capped RNA species.

It is now well established that the $tRNA^{Phe}$–16S rRNA region is the most aggressively expressed region of the mitochondrial genomes from varied animal cells (Clayton, 1984; Montoya et al., 1982; Kantharaj et al., 1983). However, the relative level of the H-strand-coded 19-nucleotide cappable RNA in Ehrlich ascites mitochondria detectable by the oligonucleotide probe is only about 1–2% of the L-strand-coded cappable species, suggesting that the former is turned over rapidly after being processed at the 3′ end by endonucleolytic cleavage. The L-strand-coded cappable RNA species are also detectable in about 10–20-fold higher concentrations than the relatively abundant cytochrome *b* mRNA (see Figure 9A). Furthermore, under the steady-state condition, only 1–2% of the cappable RNA species are found associated with DH-DNA molecules (see Figure 8). These results demonstrate that the RNA primers removed from the DH-DNA by endonucleolytic processing at the 5′ ends of DH-DNA molecules are heavily conserved to maintain an abundant steady-state level. The level of these RNAs is relatively low in mitochondria from adult tissues, and they increase markedly in fast growing embryonic tissues and tumor cells, suggesting a correlation with cell division rates. These results along with the results of induction with chloramphenicol suggest an additional functional role for these L-strand-coded cappable RNAs possibly at the level of translation.

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Properties of the Telomeric DNA-Binding Protein from *Oxytricha nova*†

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ABSTRACT: Telomeres of *Oxytricha* macronuclear DNA exist as discrete DNA–protein complexes. Different regions of each complex display characteristic DNA–protein interactions. In the most terminal region, binding of a 43- and a 55-kDa protein to the telomeric DNA appears to account for all the DNA–protein interactions that can be detected by chemical and nuclease footprinting. We have used gradient sedimentation and protein–protein cross-linking to establish that the 43- and 55-kDa proteins are subunits of a heterodimer. Both subunits are very basic, which is unexpected considering the resistance of the DNA–protein interaction to high concentrations of salt. It is extremely difficult to dissociate the two subunits either from telomeric DNA or from each other. Even after extensive treatment of protein preparations with nuclease, a fragment of the 3′ tail from macronuclear DNA remains bound to the protein. A wide range of conditions was screened for dissociation of the subunits from the DNA and/or from each other. Dissociation was only obtained by using conditions that caused some inactivation of the DNA-binding capacity of the protein. The use of reagents that covalently modify sulfydryl groups during the purification procedure facilitates preparation of telomere protein with full DNA-binding activity.

Telomeres, the natural ends of chromosomes, have several properties that make them essential for proper cell growth and development [reviewed in Blackburn and Szostak (1984)]. They stabilize chromosomes by preventing end-to-end joining reactions and degradation by exonucleases (McClintock, 1941; Muller & Herskowitz, 1954), and they make possible the complete replication of linear DNA molecules so that no gap is left at the 5′ end of the daughter strand (Cavalier-Smith, 1974; Bateman, 1975; Holmquist & Dancis, 1979). They may also help to position chromosomes within cells (Dancis & Holmquist, 1979).

Telomeres from humans, plants, and unicellular eukaryotes seem to be fundamentally similar both in function and in DNA composition (Blackburn, 1984; Richards & Ausubel, 1988; Allshire et al., 1988). Nuclear telomeric DNAs from these phylogenetically diverse organisms all contain tandem repeats of simple sequences with a C-rich strand at the 5′ end and a G-rich strand at the 3′ end. Although the telomeric repeated sequences are necessary to make the end of a chromosome act as a telomere (Pluta et al., 1984), it appears that telomeric DNA-binding proteins are also required to form a functional telomere.

Telomeric DNA-binding proteins have been detected in a few organisms including yeast, *Physarum*, *Tetrahymena*, and *Oxytricha* (Berman et al., 1986; Cheung et al., 1981; Black-

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