MOLECULAR AND CELLULAR BIOLOGY, Nov. 1990, p. 5671–5678
0270-7306/90/115671-08$02.00/0

Vol. 10, No. 11

Mitochondrial DNA Structure and Expression in Specialized Subtypes of Mammalian Striated Muscle

BRIAN H. ANNEX[1] AND R. SANDERS WILLIAMS[2]†*

Departments of Medicine[1] and Microbiology,[2] Duke University Medical Center, Durham, North Carolina 27710

Received 22 February 1990/Accepted 31 July 1990

Mitochondrial DNA (mt DNA) in cells of vertebrate organisms can assume an unusual triplex DNA structure known as the displacement loop (D loop). This triplex DNA structure forms when a partially replicated heavy strand of mtDNA (7S mtDNA) remains annealed to the light strand, displacing the native heavy strand in this region. The D-loop region contains the promoters for both heavy- and light-strand transcription as well as the origin of heavy-strand replication. However, the distribution of triplex and duplex forms of mtDNA in relation to respiratory activity of mammalian tissues has not been systematically characterized, and the functional significance of the D-loop structure is unknown. In comparisons of specialized muscle subtypes within the same species and of the same muscle subtype in different species, the relative proportion of D-loop versus duplex forms of mtDNA in striated muscle tissues of several mammalian species demonstrated marked variation, ranging from 1% in glycolytic fast skeletal fibers of the rabbit to 65% in the mouse heart. There was a consistent and direct correlation between the ratio of triplex to duplex forms of mtDNA and the capacity of these tissues for oxidative metabolism. The proportion of D-loop forms likewise correlated directly with mtDNA copy number, mtRNA abundance, and the specific activity of the mtDNA (γ) polymerase. The D-loop form of mtDNA does not appear to be transcribed at greater efficiency than the duplex form, since the ratio of mtDNA copy number to mtRNA was unrelated to the proportion of triplex mtDNA genomes. However, tissues with a preponderance of D-loop forms tended to express greater levels of cytochrome *b* mRNA relative to mitochondrial rRNA transcripts, suggesting that the triplex structure may be associated with variations in partial versus full-length transcription of the heavy strand.

A naturally occurring form of vertebrate mitochondrial DNA (mtDNA) contains a triplex DNA structure known as the displacement loop (D-loop) (6, 13). Chang and coworkers have described the process by which this structure is created (10). Briefly, DNA synthesis is initiated from RNA primers transcribed from the light-strand promoter but terminates after partial replication of the heavy strand. The resulting third strand of mtDNA (7S mtDNA), which varies in size from 500 to 1,000 bases depending on the species, remains annealed to the light strand and displaces the native heavy strand in this region. The triplex DNA structure does not appear to be a replicative intermediate, because over 95% of the newly synthesized 7S mtDNA strands are turned over and not extended into full-length heavy-strand mtDNA (6).

In mammalian mitochondrial genomes, the 7S mtDNA is located between the genes encoding $tRNA^{Phe}$ and $tRNA^{Pro}$ (1, 2, 5, 11). This region is the only noncoding segment of mammalian mtDNA but contains the promoters for transcription and the origin of heavy-strand replication (11). Therefore, when present, the 7S mtDNA is positioned close to these critical regulatory sequences, suggesting a role for the triplex DNA structure in the regulation of mtDNA transcription or replication. However, experimental methods for direct assessment of the function of the mitochondrial D-loop are currently limited. Mutational analyses would be informative but must await the development of improved methods for mitochondrial transformation of vertebrate cells (21). Currently, no system for in vitro synthesis of the D-loop form of mtDNA exists. Nevertheless, in this study we pose several questions concerning mammalian mtDNA structure that are approachable by available methods but not answered fully by the available literature. Is the proportion of triplex (D-loop) to duplex forms of mtDNA invariant among different tissues of the same species or within the same tissue in different species? If substantial variation among species or among tissues occurs, is this variation random or correlated with physiological and biochemical properties of the tissue with respect to respiratory capacity and expression of mitochondrial genes?

In this study we determined that the relative proportion of duplex and triplex forms of mtDNA in mammalian striated muscles is not random but is strikingly different in tissues adapted for high rates of respiratory activity in comparison with tissues adapted for anaerobic glycolysis. Furthermore, the prevalence of the D-loop form is correlated with a number of biochemical markers that reflect rates of mtDNA replication and transcription within these tissues.

With respect to the functional significance of the triplex mtDNA form, this study indicates that the D-loop form is probably not transcribed at greater efficiency than the duplex form of mtDNA. However, results of this study raise the hypothesis that mtDNA structure may influence the relative frequency of partial versus full-length transcription of the heavy strand.

MATERIALS AND METHODS

Preparation of cell and tissue samples. Mouse C2 myogenic cells were propagated and induced to differentiate as described previously (12). Tissues from adult animals were rinsed free of blood in sterile saline and either frozen in

* Corresponding author.
† Present address: The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8573.

liquid nitrogen and stored at −70°C for enzymatic and RNA analyses or chilled on ice and used immediately for DNA analyses. As described previously (32–34), miniature pulse generators were implanted in adult New Zealand White rabbits, and stimulating electrodes were placed adjacent to the common peroneal nerve of one hind limb. Stimulation at 6 to 10 Hz resulted in continuous contractions of the anterior compartment muscles of the operated limb and was continued for 21 days.

Mitochondrial matrix enzyme activities. We measured the maximum catalytic capacity of citrate synthase and DNA polymerase γ relative to total cellular proteins as markers for differences in the fractional mass of mitochondria among various muscle tissues. Enzymatic assays of citrate synthase were used as an internal control for the recovery and integrity of mitochondrial matrix proteins and DNA following cell fractionation procedures. Citrate synthase was assayed as described by Srere (29). Assays of DNA polymerase γ were performed as described by Yamaguchi et al. (35). In all muscle extracts, DNA polymerase γ activity was linear over at least a fivefold range of protein concentrations, and specific activities were monitored within this range. Activity increased linearly with time up to 30 min of incubation and was not increased by concentrations of dTTP or primer-template ratios that exceeded those used in the standard assay. All detectable activity was inhibited by 0.3 mM *N*-ethylmaleimide or 5 μM dideoxythymidine triphosphate, indicating the specificity of the reaction for DNA polymerase γ, and the absence of contaminating activity of DNA polymerase α or β (35). Enzyme activities were calculated relative to protein concentration, with bovine serum albumin (BSA) as a standard, as described previously (33, 34).

Determination of mtDNA copy number. DNA was prepared from cells and tissues without fractionation of mitochondria and analyzed as previously described (32). DNA concentrations were estimated from A_{260} measurements, and equal amounts were blotted directly onto Nytran filters in a vacuum manifold apparatus. The filters were probed with a plasmid (pMM26) which contains an 8,349-bp *Bam*HI fragment of the mouse mitochondrial genome that spans the D-loop region and includes coding regions for 12S and 16S rRNA, cytochrome *b*, and several NADH dehydrogenase subunits. The plasmid was internally labeled by nick translation with [α-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear) to a specific activity of 0.5×10^7 to 2×10^7 cpm/μg of DNA. Filters were hybridized and washed as described previously (32). Autoradiograms were scanned with a Bio-Rad model 620 videodensitometer, and relative hybridization signals were calculated.

Cell fractionation and mtDNA extraction. Cell fractions enriched in mitochondria were prepared by limited protease digestion, homogenization, and differential centrifugation by the method described by Hutson (19). Mitochondrial pellets were suspended in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1 mM EDTA and lysed in 0.1% sodium dodecyl sulfate (SDS). DNA was collected by ethanol precipitation after serial phenol-chloroform extractions.

Southern analysis for determination of the proportion of triplex and simple circular forms of mtDNA. DNA samples from cell fractions enriched in mitochondria were subjected to electrophoresis in a 1.2% agarose–Tris acetate gel and transferred onto Nytran filters by blotting as described previously (32). Samples were run in parallel, with and without heat denaturation (100°C for 5 min), prior to electrophoresis. Filters were baked at 80°C for 2 h and prehybridized for 4 h at 50°C in 6× SSC–0.5% SDS–5× Denhardt solution–100 μg of denatured salmon sperm DNA per ml. A 27-base single-stranded oligonucleotide probe (Fig. 1) was designed from a conserved sequence within the D-loop region of mammalian mitochondrial genomes and synthesized with a MilliGene 7500 DNA synthesizer. The oligonucleotide was end labeled with polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear) to a specific activity of 1×10^6 to 6×10^6 cpm/pmol and added to the prehybridization mix. Filters were rinsed in 6× SSC–0.1% SDS at room temperature, washed two times in the same solution for 30 min at 60°C, and exposed to Kodak XAR-5 film for 4 to 24 h without an intensifying screen.

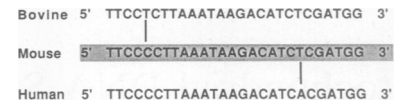

FIG. 1. Oligonucleotide probe used in Southern analysis to determine ratio of duplex to triplex mtDNA and in primer extension analysis to map 7S mtDNA start site. A conserved nucleotide sequence from the light strand of the D-loop region is shown for three mammalian mitochondrial genomes. The sequences correspond to the bases 15830 to 15857 from mouse (5), 16311 to 16338 from bovine (2), and 16544 to 1 from human (1). Solid lines connect the only nonidentical bases. The 27-base sequence from the mouse mitochondrial genome (shaded) was used as the single-stranded oligonucleotide probe for Southern analysis and primer extension assays.

Heat denaturation released the 7S mtDNA from the double-stranded mtDNA, as shown in Fig. 2 by the appearance of a hybridizing band which corresponded to the predicted size of the 7S mtDNA (D-loop) strand. The 7S mtDNA band was sensitive to S1 nuclease treatment and resistant to exonuclease III after the heat denaturation step. The heat denaturation step also altered the mobility of the full-length mtDNA species, as shown in Fig. 2. Although the conformational structure represented within these different full-length mtDNA species was not assessed by additional studies, identical bands were detected by probing with a mouse

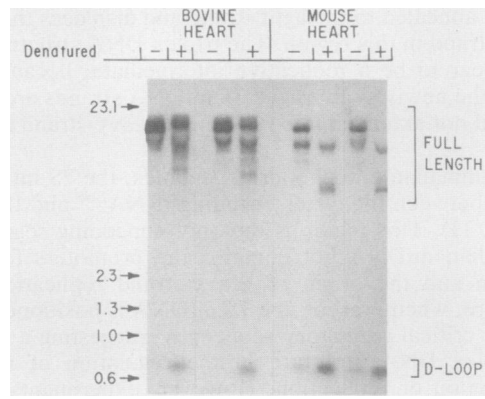

FIG. 2. Southern analysis demonstrates the relative abundance of duplex and triplex mtDNAs in bovine and murine cardiac muscle samples. Two examples for each species are shown. From left to right, pairwise lanes were loaded with 5 and 6 μg (bovine) and 4 and 2 μg (mouse) of DNA, extracted from the mitochondrial pellet as described in the text. Some samples (lanes +) were heat-denatured prior to electrophoresis. The hybridizing band between the 600- and 1,000-base molecular size markers (left, in kilobases) is the 7S mtDNA (D-loop). The ratio of D-loop to full-length hybridization signals was used to calculate the fraction of triplex mtDNA.

mtDNA probe (pMM26). Because a single recognition site is present on the 7S mtDNA and the heavy-strand mtDNA, the fraction of mitochondrial genomes originally present in triplex form was determined from the ratio of D-loop to full-length hybridization signals after denaturation. This value was independent of variations in DNA load and was determined from two different nonsaturating autoradiograms of each sample scanned with a Bio-Rad model 620 videodensitometer.

Mapping 5′ ends of 7S mtDNA. Nucleic acid samples were prepared as described for the Southern analysis. Samples were heated for 150 s at 90°C to release the 7S mtDNA. Primer extension reactions were performed with 5 U of Klenow fragment (Boehringer Mannheim Biochemicals) as described by King and Low (22), except that the primer was the end-labeled oligonucleotide (10^5 cpm) shown in Fig. 1, the annealing temperature was 50°C, and products were resolved on a 4% polyacrylamide–8 M urea gel. In early experiments, RNase was added after the heat denaturation step to exclude the possible extension of an RNA-DNA hybrid. Since RNase treatment did not alter the results, all subsequent reactions were performed without RNase treatment.

S1 nuclease analyses were also performed as described by King and Low (22). A 5′-end-labeled single-stranded probe was prepared by hybridization and extension of the end-labeled oligonucleotide probe shown in Fig. 1 on a denatured template of pMM26. A uniform 3′ end was created by *Ava*II endonuclease digestion. The labeled probe was purified on a denaturing polyacrylamide gel, and 10^4 cpm was annealed to the DNA prepared and denatured as described in the primer extension methods. After treatment with 300 U of S1 nuclease for 30 min at 37°C, the products were resolved on a 4% polyacrylamide–8 M urea gel as described above.

Mitochondrial mRNA and rRNA analysis. RNA was prepared by a modification of the guanididium isothiocyanate-CsCl method (17). Tissue samples were placed in GI buffer (4 M guanidinium isothiocyanate, 50 mM Tris hydrochloride [pH 7.4], 10 mM EDTA, 2% *N*-lauroylsarcosine, 1% β-mercaptoethanol), immediately homogenized, and sheared through a 22-guage needle. The homogenate was layered over a cushion of 5.7 M CsCl and centrifuged in a TI 641 rotor at 35,000 × *g* for 16 h at 18°C. RNA was extracted from C2 cells in a similar manner. The RNA pellet was precipitated twice in ethanol, and RNA concentrations were determined from the A_{260}. Northern (RNA) blots were prepared on Nytran filters and hybridized to internally labeled pMM26 as previously described (32). The relative abundance of 12S rRNA, 16S rRNA, and cytochrome *b* mRNA was determined by densitometry.

Primer extension analyses were also performed to determine the relative abundance of 12S rRNA and cytochrome *b* mRNA in murine cells and tissues. A 36-base antisense oligonucleotide homologous to mouse 12S mt rRNA (5′-TACCCTCTCCTTAAATTTTAAGTAAATGTTTAAGGG-3′) was extended with reverse transcriptase to produce a full-length extension product of 110 bases. Similarly, a different 36-base antisense oligonucleotide homologous to mouse cytochrome *b* mRNA (5′-TGGATGGGGCAGGTAG GTCAATGAATGAATGAGTGG-3′) produced a full-length extension produce of 79 bases. The single-stranded oligonucleotides were synthesized with a MilliGene 7500 DNA synthesizer, purified on a denaturing acrylamide gel, and end labeled as described earlier. Oligonucleotides (10^5 cpm) were added to 0.5 to 5 μg of RNA for hybridization overnight at 30°C in 80% formamide–400 mM NaCl–1 mM EDTA–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4]. The samples were precipitated with ethanol and suspended in 50 mM Tris (pH 8.0)–6 mM $MgCl_2$–40 mM KCl–10 mM dithiothreitol–50 μM each dNTP–100 μg of BSA per ml. Reactions were performed at 42°C for 60 min after the addition of 40 U of reverse transcriptase (Moloney murine leukemia virus; Boehringer Mannheim Biochemicals). After phenol-chloroform extraction and ethanol precipitation, the final products were resolved on an 8% polyacrylamide–8 M urea gel.

TABLE 1. Relative mitochondrial enzyme activity, triplex mtDNA, and mtDNA content in striated muscles[a]

Species and tissue	Sp act		Triplex (D-loop) mtDNA (% of total)	Relative mtDNA/ total cellular DNA ratio[b]
	Citrate synthase (μmol/min/mg of protein)	DNA polymerase γ (nmol/min/mg of protein)		
Rabbit				
TA	99 ± 3 (6)	0.7 ± 0.3 (6)	1.0 ± 0.4 (4)	1 (6)
TA, stimulated[c]	254 ± 4 (6)	2.4 ± 0.8 (6)	3 (2)	4.1 (6)
Heart	516 ± 11 (6)	4.9 ± 2.7 (6)	40 ± 8 (4)	5.8 (6)
Mouse				
C2 myotubes	ND[d]	ND	5 (2)	1 (2)
Heart	1,060 ± 130 (6)	5.5 ± 2.2 (4)	65 ± 11 (4)	4 (2)
Bovine heart	451 ± 130 (6)	2.9 ± 0.7 (4)	12 ± 2 (4)	ND

[a] Data are expressed as means ± SD; the number of tissues examined is shown in parentheses. SD was not calculated for fewer than four tissue samples.
[b] Relative to TA for rabbit tissues and to C2 myotubes for mouse tissues.
[c] Electrically paced skeletal muscle.
[d] ND, Not determined.

RESULTS

Variation in mtDNA structure in hearts of large and small mammals. Murine and bovine hearts differed markedly in the relative proportion of triplex and duplex forms of mtDNA (Fig. 2 and Table 1). In association with a 2.4-fold-higher specific activity of citrate synthase, a 1.9-fold-higher specific activity of DNA polymerase γ and a 2.0-fold-greater abundance of mtDNA relative to nuclear DNA, 65% ± 11% of the murine heart mitochondrial genomes were present in the triplex form, compared with 12% ± 2% in the bovine heart (mean ± SD, $n = 4$).

Variation in mtDNA structure in specialized striated muscles of the same species. In the rabbit, cardiac muscle was enriched in the triplex form of mtDNA in comparison to the glycolytic tibialis anterior (TA) skeletal muscle (Fig. 3A). Relative to the TA, cardiac muscle expressed 5.2-fold- and 6.4-fold-higher specific activities of citrate synthase and DNA polymerase γ, respectively, and had a 5.8-fold greater abundance of mtDNA (Table 1). In association with these differences, the rabbit heart maintained 40% ± 8% ($n = 4$) of its mtDNA in the triplex form, compared with only 1% ± 0.4% ($n = 4$) in the TA. Primer extension studies (Fig. 4) confirmed the quantitative differences discerned by Southern blot analyses and indicated that the major start sites for the 7S mtDNA strand were not appreciably different in these two tissues.

In another comparison of specialized cells from the same species, we examined the fraction of mitochondrial genomes present in the triplex form in murine C2 skeletal myocytes grown in culture under conditions in which contractile

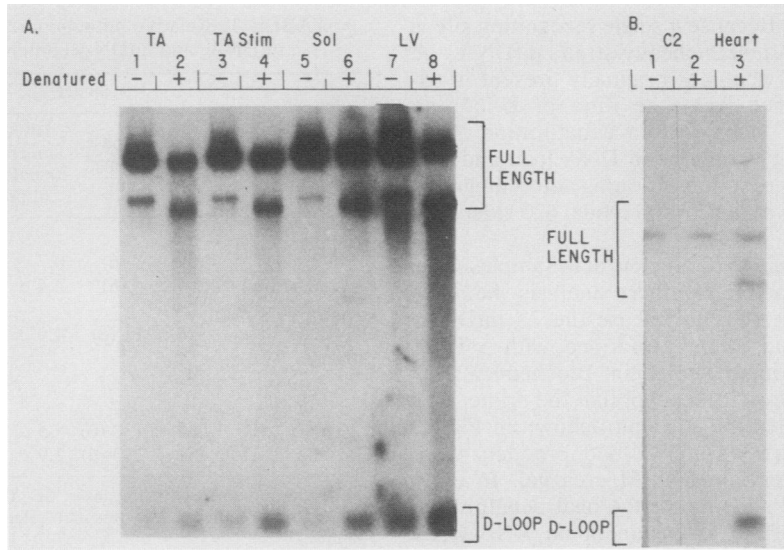

FIG. 3. Southern analysis demonstrates the relative abundance of duplex and triplex mtDNA in specialized muscle tissues. (A) Adult rabbit muscles. Pairwise lanes were loaded with DNA extracted from the glycolytic TA muscle (1.5 μg), electrically paced TA muscle (TA Stim; 1.0 μg), oxidative soleus skeletal muscle (Sol; 0.8 μg), or cardiac left ventricle (LV; 1.0 μg). Some samples (lanes +) were heat-denatured prior to electrophoresis. (B) Mouse muscles. Lanes were loaded with DNA extracted from cultured C2 skeletal myotubes (1.0 μg) or mouse heart (1.0 μg).

activity (and hence demand for oxidative metabolism) is minimal compared with the fraction in the highly oxidative murine heart (Fig. 3B). The murine heart had fourfold more mtDNA than C2 cells (Table 1). Whereas 65% of murine heart mtDNA exhibited the triplex form, only 5% of the mtDNA of C2 cells was in this form. Primer extension studies (Fig. 5A) revealed no variation in start sites for the 7S mtDNA strand in C2 versus cardiac myocytes, and S1 nuclease protection assays confirmed the position of the major start site of the 7S mtDNA strand (Fig. 5B).

Effects of nerve stimulation on mtDNA structure. We examined the effect of 21 days of nerve stimulation on mtDNA structure in the rabbit TA skeletal muscle. As described in previous reports from this laboratory, this physiological stimulus induces expression of genes encoding myoglobin and mitochondrial proteins and increases the abundance of mtRNA and mtDNA (32–34). This stimulus also increased the proportion of triplex to duplex forms of mtDNA from 1 to 3% (Fig. 3A and Table 1). This latter value was comparable to that found in the oxidative soleus skeletal muscle. Primer extension studies (Fig. 4) confirmed the quantitative difference in the proportion of triplex to duplex mtDNA forms but revealed no significant difference in the start sites of the 7S mtDNA.

Abundance of rRNA and mRNA transcripts. We have previously reported that mtDNA copy number and the abundance of mtRNA vary in direct proportion in rabbit striated muscles of differing respiratory activities (32, 34). Our current results indicate that these specialized muscle subtypes differ markedly in the proportion of D-loop versus

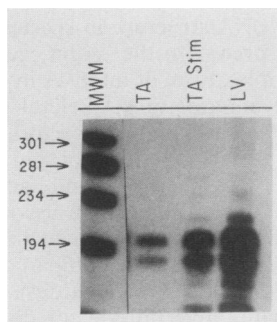

FIG. 4. 7S mtDNA start site analysis in rabbit striated muscles. The abbreviations designating the sources of template mtDNA from specialized rabbit muscles (TA, 5.6 μg; TA Stim, 2.7 μg; and LV, 1.0 μg) are described in the legend to Fig. 3. Two major start sites, located 190 and 200 bases from the conserved nucleotide sequence (primer), were identified. The two bands had approximately the same relative intensity in all three muscle samples. Size markers (lane MWM) are shown in bases.

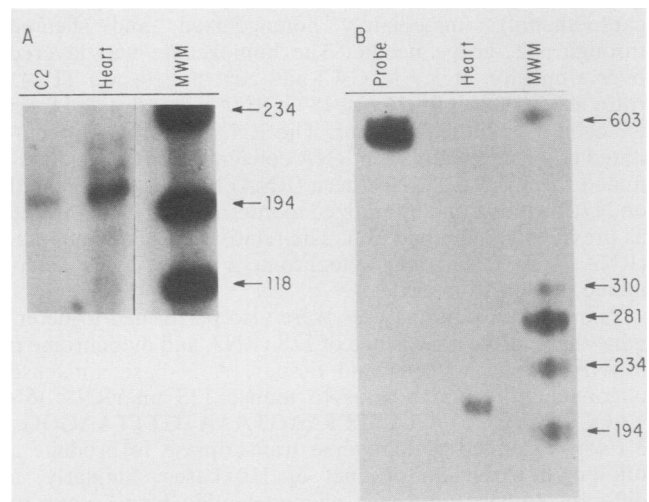

FIG. 5. 7S mtDNA start site analysis in murine muscles. (A) Primer extension analysis. Template DNA was extracted from mouse C2 skeletal myotubes (20 μg of DNA) and mouse heart (2 μg). One major extension product of 198 bases was identified, and this corresponded to the major 5′ end of the mouse 7S mtDNA identified by Doda et al. (13). (B) S1 nuclease analysis. 7S mtDNA from mouse heart protected a fragment of approximately 198 bases corresponding to the 5′ terminus demonstrated by primer extension. Size markers (lanes MWM) are shown in bases.

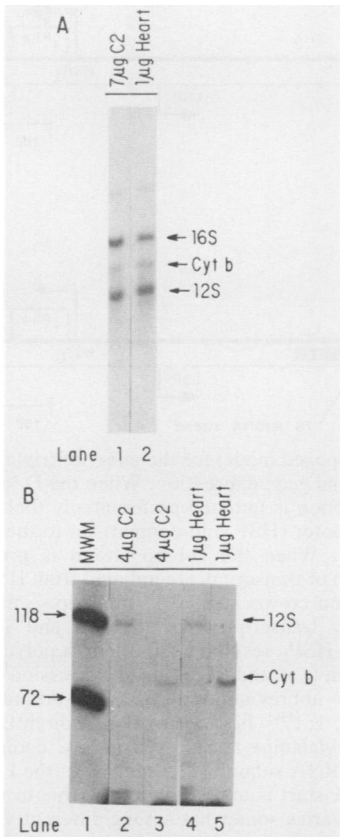

FIG. 6. RNA analysis demonstrates the relative abundance of mitochondrial cytochrome *b* mRNA and rRNAs in murine tissues. (A) Northern blots, with different total RNA loads, from C2 skeletal myotubes and mouse heart were probed with pMM26. The relative hybridization signal of mitochondrial cytochrome *b* (Cyt b) mRNA to 12S and 16S rRNAs was greater in lane 2 than in lane 1. (B) Primer extension analysis was performed with different quantities of template RNA, noted above each lane, from C2 myotubes and mouse heart. End-labeled probes were added for 12S rRNA (lanes 2 and 4) and cytochrome *b* (Cyt b) mRNA (lanes 3 and 5). Labeled ϕX174 molecular size markers are in lane 1 (sizes shown in bases).

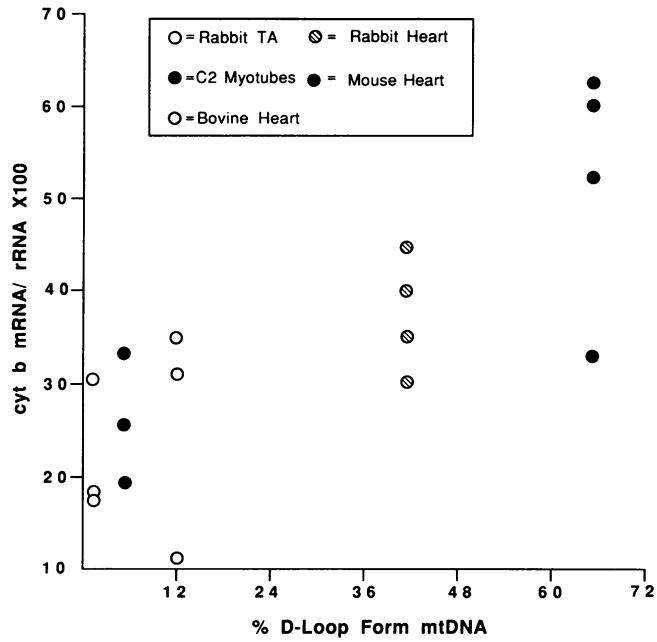

FIG. 7. Comparison of the ratio of mitochondrial mRNA (cytochrome *b*) to mitochondrial rRNA with the fraction of mtDNA present in the triplex (D-loop) form in mammalian striated muscle samples. Blots were prepared with RNA extracted from striated muscles and probed with pMM26. Hybridization signals for cytochrome *b* (cyt b), 12S rRNA, and 16S rRNA were quantified by laser densitometry, and the ratio of the cytochrome *b* signal to the mean of the two rRNA signals was calculated. Abbreviations are described in the legend to Fig. 3.

duplex mtDNA forms, suggesting that the triplex structure is not transcribed at a detectably different rate than the duplex form of mtDNA. The current analysis of murine C2 cells in comparison with mouse cardiac muscle also indicates that differences in the abundance of mtRNA are proportional to differences in mitochondrial genome copy number (Fig. 6 and Table 1) and unrelated to the proportion of D-loop forms.

However, we did observe differences in the relative abundance of cytochrome *b* mRNA and 12S or 16S mitochondrial rRNA subunits that correlated with the proportion of D-loop and duplex mitochondrial genomes. Northern and primer extension analyses revealed higher expression of cytochrome *b* mRNA relative to mt rRNA transcripts in mouse heart than in C2 cells (Fig. 6). When muscle subtypes of several species were compared in Northern blots, the same relationship was observed: tissues that maintained a higher fraction of D-loop forms tended to express a greater abundance of cytochrome *b* mRNA than of 12S and 16S rRNA subunits (summarized in Fig. 7).

DISCUSSION

The mitochondrial D-loop is an oddity among the forms of DNA encountered in living organisms. The proximity of the triplex region of mtDNA to the origin of heavy-strand replication and to the heavy-strand and light-strand promoters within the mitochondrial genome suggests that this structure may influence mtDNA replication and/or transcription (8, 10, 22), but its functional role remains obscure, and experimental approaches to define its function are currently limited. In this context, the correlation of naturally occurring or experimentally induced variations in mtDNA structure with other variables that reflect mtDNA replication and transcription are useful to generate hypotheses concerning the functional significance of the D-loop form of mitochondrial DNA.

Proliferating mouse L-cells maintain a high fraction of triplex mitochondrial genomes, but this fraction decreases and the duplex form predominates when they reach stationary phase (20). *Xenopus laevis* oocytes likewise increase the frequency of triplex mitochondrial genomes during the period of rapid mitochondrial biogenesis in their maturation (8). However, after partial hepatectomy in the rat, the proportion of triplex mtDNA forms was reported to decline in regenerating hepatocytes compared with the proportion in nonproliferating cells (20). Thus, observations made in immortalized cell lines or germ cells may not reflect relationships in somatic cells of intact organisms. Furthermore, biochemical events that regulate mtDNA structure in relation to the cell cycle may differ from those that operate in postmitotic myocytes.

Our current study indicates a consistent and direct rela-

tionship between the proportion of D-loop to duplex forms of mtDNA and respiratory capacity, mtDNA copy number, mtRNA content, and DNA polymerase γ activity of mammalian striated muscles. Muscle subtypes that are adapted for anaerobic metabolism (type IIb skeletal fibers) contain few D-loop forms (1% in the rabbit TA), whereas muscle subtypes adapted for high respiratory activity (cardiomyocytes) predominantly express the D-loop form (65% in the mouse heart). This relationship was consistent in systematic analyses across species (bovine versus rabbit versus mouse heart), within the same species (mouse cardiomyocytes versus C2 myotubes, and cardiac versus glycolytic versus oxidative skeletal muscles of adult rabbits), and within the same muscle placed under different physiological loads (motor nerve stimulation). These results indicate that mtDNA structure is under both developmental and physiological control.

Despite these marked variations in the proportion of D-loop forms in different muscle tissues, we could detect no major variation in the start sites of the 7S mtDNA strand. The dominant start site of the 7S strand was observed to vary with the growth state of cultured bovine epithelial cells (23) but appears to be unrelated to the respiratory capacity of mammalian striated muscles.

In the only previous study of mtDNA structure in striated muscles, Rajamanickam et al. (28) reported that aortic banding of rats acutely decreased the proportion of triplex mtDNA forms in the heart. This result appears to present an exception to the relationships noted in our current study, but features of the acute aortic banding model, such as cell injury, myocyte hypertrophy, and the absence of steady state, may perturb aspects of mitochondrial genomic structure and function that are encountered under physiological conditions. Other studies already cited illustrate that mtDNA structure can be modulated by factors such as the proliferative state of cells (8, 20, 23). Therefore, the relationships we describe in mammalian muscle tissues may be specific to postmitotic cells under resting conditions and should not be expected to be reflect relationships observed in all cell types under all conditions.

We considered the hypothesis that the D-loop form of mtDNA is more actively transcribed than the duplex form. A prediction of this hypothesis is that, after controlling for copy number of mtDNA, cells and tissues with a high proportion of triplex forms of mtDNA should express greater concentrations of mtRNA than cells in which the duplex form predominates. However, this prediction was not upheld in striated muscles. The glycolytic TA skeletal muscles of the rabbit (1% D-loop mtDNA) and cardiac muscle in the same species (40% D-loop forms) expressed equivalent ratios of mtRNA relative to mtDNA (32). Likewise, mouse C2 myotubes (5% D-loop mtDNA) and mouse cardiomyocytes (65% D-loop mtDNA) demonstrated an equivalent abundance of mtRNA and mtDNA. Thus, if the half-life of mtRNA is similar in these tissues, variation in mtDNA structure does not appear to alter the overall transcriptional efficiency of the mitochondrial genome.

We also considered the potential effects of mtDNA structure on a peculiar aspect of mtDNA heavy-strand transcription: the region encoding the 12S and 16S mt-rRNA subunits is transcribed at a greater rate than the downstream region that encodes mitochondrial mRNAs (16). In HeLa cells, Montoya and co-workers (26) have observed two independent heavy-strand transcription units, each initiated from distinct but closely approximated start sites (Fig. 8). Transcription initiated 20 to 40 nucleotides upstream of the

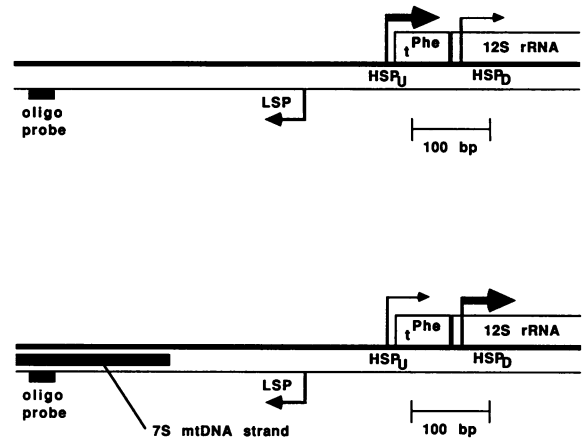

FIG. 8. Proposed model for the effect of triplex form of mtDNA on mitochondrial gene expression. When the D-loop form is absent (top), transcription is initiated preferentially (bold arrow) from the upstream promoter (HSP_U) in comparison to the downstream promoter (HSP_D). When the D-loop strand is present (bottom), a greater fraction of transcripts are initiated from HSP_D. As described by Montoya and co-workers (26), transcripts initiated from HSP_U terminate after transcription of the 12S and 16S rRNA genes. Initiation from HSP_D results in a full-length polycistronic transcript of the entire heavy strand, resulting in expression of both rRNA and mRNA. Other abbreviations: LSP, light-strand promoter; oligo probe, see Fig. 1; t^{Phe}, first coding region on mtDNA heavy strand encoding phenylalanine tRNA; 12S rRNA, coding region for 12S mitochondrial RNA subunit. The spacing of the LSP and HSPs and the 7S mtDNA start is approximate, relative to the length marker (100 bp), and varies somewhat among different vertebrate species. However, this general configuration is conserved.

$tRNA^{Phe}$ gene yields a product that terminates after synthesis of the 12S and 16S rRNA subunits but no mRNA. A DNA-binding protein which directs termination of transcription after the 16S rRNA in vitro has recently been identified (25). Transcription initiated between the $tRNA^{Phe}$ and the 12S rRNA genes leads to a transcript of the entire heavy strand, including both rRNA and mRNA products (26). This alternative promoter selection may account for cell-specific differences in the abundance of mitochondrial mRNA transcripts and mitochondrial rRNA subunits observed by others (9).

Our observation that cells rich in triplex forms tend to express a greater abundance of cytochrome *b* mRNA than of mt rRNA subunits raises the possibility that the presence of a 7S mtDNA strand within the D-loop region could influence the frequency of transcriptional initiation at the downstream versus the upstream heavy-strand promoter and termination of transcription at the 3′ end of the 16S rRNA gene (Fig. 8). An alternative hypothesis that cannot be excluded is that formation of the D-loop and the regulation of heavy-strand transcription, though correlated, have no causal relationship. Each of these phenomena could result from variation in the abundance or functional activity of *trans*-acting proteins involved in mtDNA replication and transcription (15).

If the presence of the 7S mtDNA strand does influence promoter selection and the site of termination of transcription of the mtDNA heavy strand, what potential mechanisms account for this effect? The presence of the third strand and the resulting displacement of the native heavy strand may alter DNA conformation in the nearby promoter regions. Changes in DNA conformation could influence binding of *trans*-acting factors required for accurate and efficient initi-

ation of transcription (15, 27, 30). Certain proteins may preferentially recognize binding sites unique to triplex DNA (3, 18, 31). In addition, changes in negative superhelix density (8, 20) resulting from the presence of the third strand may influence transcription (4, 7, 14, 24).

In summary, the relative proportion of D-loop versus duplex forms of mtDNA in striated muscle tissues of several mammalian species demonstrates marked variation, ranging from 1% in glycolytic fast skeletal fibers to 65% in the mouse heart. The proportion of D-loop forms correlates directly with the respiratory activity of highly specialized muscle subtypes and with several markers that reflect the cellular requirements for mitochondrial biogenesis, including mtDNA copy number and the specific activity of DNA polymerase γ. There is no consistent relationship between the proportion of D-loop forms and overall transcriptional efficiency (assessed as the ratio of mtRNA to mtDNA in each tissue), but there is a tendency for tissues rich in D-loop forms to express products of complete (cytochrome *b* mRNA) versus incomplete (mt rRNA subunits) heavy-strand transcription. These results generate hypotheses regarding possible functions of the enigmatic mitochondrial D-loop that can be tested rigorously as technical abilities in this field advance.

ACKNOWLEDGMENTS

Brian H. Annex is a Merck Postdoctoral Fellow. R. Sanders Williams is an Established Investigator of the American Heart Association. This work was supported by Public Health Service grant HL-35639 from the National Institutes of Health.

LITERATURE CITED

1. **Anderson, S., A. T. Bankier, B. G. Barrell, M. H. L. de Brujin, A. R. Coulson, J. Drouin, I. C. Eperon, D. P. Nierlich, B. A. Roe, F. Sanger, P. H. Schreier, A. J. H. Smith, R. Staden, and I. G. Young.** 1981. Sequence and organization of the human mitochondrial genome. Nature (London) **290**:457–465.
2. **Anderson, S., M. H. L de Brujin, A. R. Coulson, I. C. Eperon, F. Sanger, and I. G. Young.** 1982. Complete sequence of bovine mitochondrial DNA: conserved features of the mammalian mitochondrial genome. J. Mol. Biol. **156**:683–717.
3. **Barat, M., and B. Mignote.** 1981. A DNA-binding protein from *Xenopus laevis* oocyte mitochondria. Choromosoma (Berlin) **82**:583–593.
4. **Barat-Gueride, M., C. Dufresne, and D. Rickwood.** 1989. Effect of DNA conformation on the transcription of mitochondrial DNA. Eur. J. Biochem. **183**:297–302.
5. **Bibb, M. J., R. A. Van Etten, C. T. Wright, M. W. Walberg, and D. A. Clayton.** 1981. Sequence and gene organization of mouse mitochondrial DNA. Cell **26**:167–180.
6. **Bogenhagen, D., and D. A. Clayton.** 1978. Mechanism of mitochondrial DNA replication in mouse L-cells: kinetics of synthesis and turnover of the initiation sequence. J. Mol. Biol. **119**:49–68.
7. **Buzan, J. M., and R. L. Low.** 1988. Preference of human mitochondrial RNA polymerase for superhelical templates with mitochondrial promoters. Biochem. Biophys. Res. Commun. **152**:22–29.
8. **Callen, J. C., M. Tourte, N. Dennebouy, and J. C. Mounolou.** 1983. Changes in D-loop frequency and superhelicity among the mitochondrial DNA molecules in relation to organelle biogenesis in oocytes of *Xenopus laevis*. Exp. Cell Res. **143**:115–125.
9. **Canatatore, P., Z. Flagella, G. Pesole, M. N. Gadaleta, and C. Saccone.** 1983. Regulation of mitochondrial transcription in rat liver: determination of mitochondrial transcript concentration in adult and developing rat, p. 127–140. *In* R. J. Schweyen, K. Wolf, and F. Kaudewitz (ed.), Mitochondria 1983: nucleomitochondrial interactions. Walter de Gruyter, Berlin.
10. **Chang, D. D., R. P. Fisher, and D. A. Clayton.** 1987. Roles for a promoter and RNA processing in the synthesis of mitochondrial displacement-loop strands. Biochim. Biophys. Acta **909**: 85–91.
11. **Clayton, D. A.** 1984. Transcription of the mammalian mitochondrial genome. Annu. Rev. Biochem. **53**:573–594.
12. **Devlin, B. H., F. C. Wefald, W. E. Kraus, T. S. Bernard, and R. S. Williams.** 1989. Identification of a muscle-specific enhancer within the 5′ flanking region of the human myoglobin gene. J. Biol. Chem. **264**:13896–13901.
13. **Doda, J. N., C. T. Wright, and D. A. Clayton.** 1981. Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences. Proc. Natl. Acad. Sci. USA **78**:6116–6120.
14. **Ehrlich, R., A. Larousse, M.-A. Jacquet, M. Marin, and C. Reiss.** 1985. *In vitro* transcription initiation from three different *Escherichia coli* promoters: effect of supercoiling. Eur. J. Biochem. **148**:293–298.
15. **Fisher, R. P., J. N. Topper, and D. A. Clayton.** 1987. Promoter selection in human mitochondria involves binding of a transcription factor to orientation-independent upstream regulatory elements. Cell **50**:247–258.
16. **Gelfand, R., and G. Attardi.** 1981. Synthesis and turnover of mitochondrial ribonucleic acid in HeLa cells: the mature ribosomal and messenger ribonucleic acid species are metabolically unstable. Mol. Cell. Biol. **1**:497–511.
17. **Glisin, V., R. Crkvenjakov, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**: 2633–2637.
18. **Herendeen, D. R., G. A. Kassavetis, J. Barry, B. M. Alberts, and E. P. Geiduschek.** 1989. Enhancement of bacteriophage T4 late transcription by components of the T4 DNA replication apparatus. Science **245**:952–958.
19. **Hutson, S. M.** 1986. Branched chain α-keto acid oxidative decarboxylation in skeletal muscle mitochondria. J. Biol. Chem. **261**:4420–4425.
20. **Kasamatsu, H., and J. Vinograd.** 1974. Replication of circular DNA in eukaryotic cells. Ann. Rev. Biochem. **43**:695–719.
21. **King, M. P., and G. Attardi.** 1988. Injection of mitochondria in human cells leads to a rapid replacement of the endogenous mitochondrial DNA. Cell **52**:811–819.
22. **King, T. C., and R. L. Low.** 1987. Mapping of control elements in the displacement loop region of bovine mitochondrial DNA. J. Biol. Chem. **262**:6204–6213.
23. **King, T. C., and R. L. Low.** 1987. Mitochondrial DNA displacement loop structure depends on growth state in bovine cells. J. Biol. Chem. **262**:6214–6220.
24. **Kmiec, E. B., M. Ryoji, and A. Worcel.** 1986. Gyration is required for 5S RNA transcription from a chromatin template. Proc. Natl. Acad. Sci. USA **83**:1305–1309.
25. **Kruse, B., N. Narasimhan, and G. Attardi.** 1989. Termination of transcription in human mitochondria: identification and purification of a DNA binding protein factor that promotes termination. Cell **58**:391–397.
26. **Montoya, J., T. Christianson, D. Levens, M. Rabinowitz, and G. Attardi.** 1982. Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA. Proc. Natl. Acad. Sci. USA **79**:7195–7199.
27. **Pepe, G., G. Gadaleta, G. Palazzo, and C. Saccone.** 1989. Sequence-dependent DNA curvature: conformational signal present in the main regulatory region of the rat mitochondrial genome. Nucleic Acids Res. **17**:8803–8819.
28. **Rajamanickam, C., S. Merten, B. Kwiatkowska-Patzer, C.-H. Chuant, R. Zak, and M. Rabinowitz.** 1979. Changes in mitochondrial DNA in cardiac hypertrophy in the rat. Circulat. Res. **45**:505–515.
29. **Srere, P. A.** 1969. Citrate synthase. Methods Enzymol. **13**:3–11.
30. **Topper, J. N., and D. A. Clayton.** 1989. Identification of transcriptional regulatory elements in human mitochondrial DNA by linker substitution analysis. Mol. Cell. Biol. **9**:1200–1211.
31. **Van Tuyle, G. C., and P. A. Pavco.** 1985. The rat liver mitochondrial DNA-protein complex: displaced single strands of

replicative intermediates are protein coated. J. Cell Biol. **100:** 251–257.

32. **Williams, R. S.** 1986. Mitochondrial gene expression in mammalian striated muscle. J. Biol. Chem. **261:**12390–12394.
33. **Williams, R. S., M. Garcia-Moll, J. Mellor, S. Salmons, and W. Harlan.** 1987. Adaptation of skeletal muscle to increased contractile activity. J. Biol. Chem. **262:**2764–2767.
34. **Williams, R. S., S. Salmons, E. A. Newsholme, R. E. Kaufman, and J. Mellor.** 1986. Regulation of nuclear and mitochondrial gene expression by contractile activity in skeletal muscle. J. Biol. Chem. **261:**376–380.
35. **Yamaguchi, M., A. Matsukage, and T. Takahashi.** 1980. Chick embryo DNA polymerase γ: purification and structural analysis of nearly homogeneous enzyme. J. Biol. Chem. **255:**7002–7009.