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Multiple Protein-Binding Sites in the TAS-Region of Human and Rat Mitochondrial DNA

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To study the molecular mechanisms responsible for the regulation of mitochondrial DNA copy number, *in vivo* and *in organello* dimethyl sulfate footprinting experiments in human fibroblasts and rat liver mitochondria were carried out. By this approach we identified in both species two specific protein binding sites in the 3′ region of the displacement loop of mitochondrial DNA. One site contains the TAS-D element of human and rat mitochondrial DNA; the other covers TAS-C and TAS-B in human, whereas in rat it comprises part of TAS-B. We suggest that the protected sequences might be the site of action of protein factors involved in the premature termination of mitochondrial DNA heavy-strand synthesis.

Mammalian mitochondrial DNA (mtDNA) replication is an unidirectional and asymmetric process. It starts with the heavy strand (H-strand) synthesis from an origin (Ori-H), located in the main non coding region (D-loop region) of the genome, utilizing as primer an RNA transcribed from the light strand (L-strand) promoter (for review see ref. 1-2). L-strand synthesis starts from a separate origin, only after 2/3 of the H-strand have been completed. Nearly 95% of the H-strand initiation events terminate at sites placed 500-1000 bp downstream the initiation site, depending on the vertebrate species. Nascent chains remain annealed to the supercoiled template and form a triplex structure known as *d*isplacement loop (D-loop).

The regulation of mtDNA synthesis is an important event in mitochondrial metabolism: highly oxidative tissues such as the heart muscle contain a higher number of mtDNA molecules than glycolitic type II fibers of skeletal muscles (3). Studies on rabbit skeletal muscle showed that the mitochondrial gene induction in response to enhanced contractile activity may take place by increasing the gene dosage levels (4). Moreover alterations of human mtDNA replication have been associated to some mitochondrial pathologies. In particular, a mendelian inherited disease caused by the almost complete depletion of mtDNA (5) might depend on mutations of nuclear-encoded mitochondrial proteins involved in mtDNA synthesis.

Around the D-loop termination sites several conserved sequences of 15-16 bp were found in vertebrates (6-11). These sequences, known as *t*ermination *a*ssociated *s*equences (TAS), might constitute *cis*-elements for termination of mtDNA synthesis. Madsen et al. (12) detected in bovine mitochondria a 48 kDa DNA-binding protein, able to bind specifically a single TAS element. This protein might be involved in the regulation of mtDNA copy number as modulation of mitochondrial replication may take place at the sites of premature termination of H-DNA synthesis. Since variable numbers of TAS elements, exceeding the number of the D-loop termini, were identified in different organisms (6-11), as first step in the investigation of the structure and function of the TAS-binding protein we performed a study addressed to detect DNA-protein interactions at the 3′ end of the D-loop of human and rat mtDNA. Here, by means of *in vivo* and *in organello* dimethyl sulfate (DMS) footprinting, we report specific protein-DNA interactions in two sites located in the TAS region of human and rat mtDNA. One of these sites contains the TAS-D element of human and rat mtDNA, whereas the other covers partially TAS-C and TAS-B in human, and TAS-B in rat.

MATERIALS AND METHODS

In vivo footprinting with dimethyl sulfate on human fibroblasts. Human fibroblasts were grown in 175 cm^2 flask in Eagle's minimal essential medium (MEM) with Earle's salts (Seromed) and 2.2 g/l $NaHCO_3$, supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Medium was exchanged 2 hours before DMS addition. For DMS treatment about 2×10^6 of 70-80% confluent cells were incubated at 37°C for 2 min in 15 ml of prewarmed medium containing 0.1% DMS at 37°C. After removing the medium, cells were washed three times with cold

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1 × PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and lysed by incubating for 150 min at 50°C in 3 ml of RSB (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 25 mM EDTA) containing 1% SDS and 1 mg/ml of proteinase K (Boehringer). After addition of 0.5 M NaCl, the nucleic acids were purified by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) followed by two ether extractions and precipitated with 2.5 volumes of ethanol. After centrifugation the pellet was washed twice with 75% ethanol, suspended in 400 μl of water and treated with 0.1 μg/μl of RNase A in 500 μl of TE (10 mM Tris-HCl pH 8, 1 mM EDTA) for 30 min at 37°C. DNA was phenol-extracted as before and precipitated with 2.5 volumes of ethanol in the presence of 0.2 M NaCl. One hundred μg of *in vivo* DMS-treated DNA were suspended in 200 μl of 1M piperidine and incubated for 30 min at 90°C. Then they were chilled on ice and passed through a Sephadex G-50 (Pharmacia-LKB) spin column; the column eluate was lyophilized, washed twice with water and finally suspended in water at a concentration of 1 μg/μl. Control samples of naked DNA (protein-free) were obtained extracting the nucleic acids from the same amount of cells as above. One hundred μg of DNA were suspended in 200 μl of 37°C prewarmed TE and treated with 0.1% DMS for 2 min at 37°C. The reaction was blocked by adding 50 μl of cold 1.5 M sodium acetate pH 7.4, 1 M 2-mercaptoethanol, and DNA was ethanol-precipitated. The pellet was lyophilized and suspended in 200 μl of 1 M piperidine and incubated for 30 min at 90°C. After Sephadex G-50 separation the DNA was suspended in water at a concentration of 1 μg/μl. In most experiments piperidine treatment was omitted since preliminary tests showed that the primer extension effectively terminates in correspondence of the modified bases; in this case the DNA pellets were suspended in 100 μl of TE, purified by spin-column chromatography and recovered in water.

In organello footprinting with dimethyl sulfate on rat liver mitochondria. Rat liver mitochondria were prepared by differential centrifugation (13). Mitochondrial pellets (4 mg of proteins) were suspended in 500 μl of 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K_2HPO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris-HCl pH 7.4, 1 mg/ml bovine serum albumin. In these conditions the integrity of mitochondrial membranes was preserved, as shown by the observation that mitochondria supported RNA and DNA synthesis for a long time (14-16). Mitochondria were preincubated for 20 min at 37°C and then DMS was added to a final concentration of 0.1% for 2 min at 37°C. Immediately after the incubation the samples were placed on ice and 900 μl of PBS were added. The mitochondria were pelleted by centrifuging for 1 min at 12,000 × *g*. After two additional washes with PBS, the pellets were suspended in 400 μl of lysis buffer (200 mM NaCl, 1% SDS, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.2 mg/ml proteinase K) and incubated for 30 min at 37°C. Then DNA was isolated by two extractions with phenol, two with phenol/chloroform/isoamyl alcohol (25:24:1), one with chloroform, and then ethanol-precipitated. The pellets were dried, suspended in 100 μl of 1 M piperidine and incubated for 30 min at 90°C. Then they were chilled on ice and passed through a Sephadex G-50 (Pharmacia-LKB) spin column; the column eluates were lyophilized, washed twice with water and finally suspended in 35 μl of water. Control samples of naked DNA (protein-free) were obtained extracting the nucleic acids from the same amount of mitochondria as above. Then the DNA pellets were suspended in 100 μl of TE, preheated for 2 min at 37°C and treated with 0.1% DMS for 2 min at 37°C. The reaction was blocked by adding 25 μl of 1.5 M sodium acetate pH 7.4, 1 M 2-mercaptoethanol and then the DNA was ethanol-precipitated. The pellets were lyophilized, suspended in 100 μl of 1 M piperidine and incubated for 30 min at 90°C. Sephadex G-50 separation was performed as above and DNA was recovered in 35 μl of water. As for human mtDNA in most experiments piperidine treatment was omitted.

Primer extension analysis of DMS-treated samples. Half of the methylated human DNA (about 50 μg of total DNA) and the entire sample of DNA extracted from 4 mg of rat mitochondrial proteins (35 μl) were used for primer extension analysis. The reaction mixture contained also 10 μl of 10 × Taq buffer (100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl), 4 μl of 5 mM dNTP mix, 1-3 pmol of [^{32}P] 5′ end labeled primer (see figure legends for primer identification), 2.5 units of Taq DNA polymerase (Boehringer-Mannheim) and water to a final volume of 100 μl. Samples were covered with mineral oil and first heated in a DNA Thermal Cycler (Perkin Elmer mod. 480) for 2 min at 94°C; then 15 cycles followed (1 min at 94°C, 2 min at the primer hybridization temperature, 3 min at 72°C). The reactions were ended by denaturing for 2 min at 94°C, annealing for 2 min and finally extending for 10 min at 72°C. Reaction products were separated from mineral oil and DNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol in the presence of 1 M ammonium acetate, 5 mM EDTA. The pellets were washed with 70% ethanol, dried, suspended in 6 μl of 98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue and resolved on a 6% polyacrylamide-7M urea sequencing gel (0.2 mm × 40 cm). Primer labeling was carried out with [γ-^{32}P]ATP and polynucleotide kinase as reported by Sambrook et al. (17).

Footprint quantitation. The gels were exposed with intensifying screens for different lengths of time. The desired lanes were scanned densitometrically by using a LKB-Pharmacia Ultroscan-XL Laser densitometer equipped with a Gel-Scan-XL Evaluation Software. Mean differences between control and test greater than 30% (twice the standard deviation of the mean difference among unaffected nucleotides) were reported as being footprinted.

RESULTS AND DISCUSSION

The purpose of this work was to detect, with the approach of DMS footprinting, protein binding sites at the D-loop 3′ end of human and rat mtDNA. DMS is a small molecule that methylates DNA at the N7 guanine and N3 adenine residues respectively (18). When bound to DNA residues, proteins can decrease or intensify purine reactivity to methylation with respect to naked DNA. Protection from methylation is due to protein-DNA contacts at the level of major groove that inhibit the access of DMS. DMS hypersensitivity may result from protein-induced hydrophobic pockets or from local conformational changes in DNA which favor DMS reaction (19, 20). As DMS readily crosses cellular, nuclear and mitochondrial membranes, protein-DNA interactions may be analyzed in intact cells and organelles (21-29). In a typical experiment, human fibroblasts or rat liver mitochondria were treated with the modifying agent and the DNA was isolated. Control samples were obtained treating with DMS naked DNA (protein-free) purified from the same amount of human fibroblasts or from the same amount of rat liver mitochondrial proteins. DNA isolated from test and control samples was subjected to linear amplification in the presence of Taq DNA polymerase and of suitable 5′ end-labeled oligonucleotides; the reaction products were resolved on a polyacrylamide sequencing gel. The results here reported were obtained in at least five independent experiments; the pattern obtained from each different sample of human fibroblasts or rat liver mitochondria was the same.

Figure 1 shows the result of DMS *in vivo* footprinting on human fibroblasts. By using the primer *H-TAS-For*

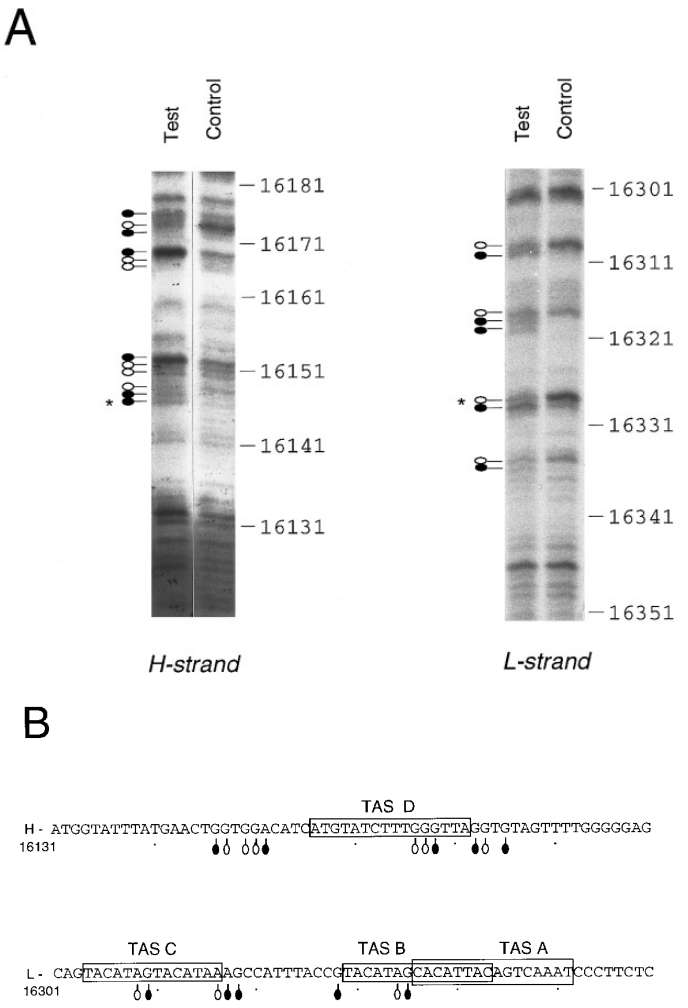

FIG. 1. *In vivo* DMS footprinting in the TAS region of human mtDNA. (A) Human fibroblasts were treated with DMS and total DNA was extracted (test lanes). Naked total DNA was also treated with DMS (control lanes). After piperidine cleavage, test and control DNA were subjected to linear amplification with Taq DNA polymerase in the presence of 5′ end-labeled oligonucleotides. Primers were *H-TAS-For* (L-16049-16070) and *H-TAS-Rev* (L-16417-16396) to visualize bases (on the H-strand and L-strand, respectively) in the TAS region of human mtDNA. Human mtDNA positions (36), shown at the right of each panel, were deduced from control G-ladder and from sequencing reactions run alongside (not shown). At the left of each panel the sites of methylation protection and hypersensitivity are indicated by open and filled circles, respectively. The asterisks indicate nucleotides other than purines reacting with DMS; they were not considered as footprinted in the summary (B). (B) Positions of the *in vivo* footprinted bases at the TAS region of human mtDNA. Filled and open circles indicate the sites of DMS altered reactivity as deduced from A. TAS elements, as deduced from the alignment of the TAS region of human, cow, and rat mtDNA (see Fig. 3), are boxed.

(Fig. 1A), bases on the H-strand showing altered DMS reactivity were detected between positions 16147-16176 (nucleotides 16147, 16148, 16150, 16151, 16152, 16167-16169, 16173, 16174, 16176). This region corresponds to the TAS element (TAS D) previously identified by Doda et al. (7) and it is located about 50 bp upstream the 3′ end of human mtDNA D-loop (nucleotides 16103-16105). The primer *H-TAS-Rev* detected another region with altered DMS reactivity. Affected bases span part of TAS-C and TAS-B and are comprised between positions 16309-16336 (nucleotides 16309,

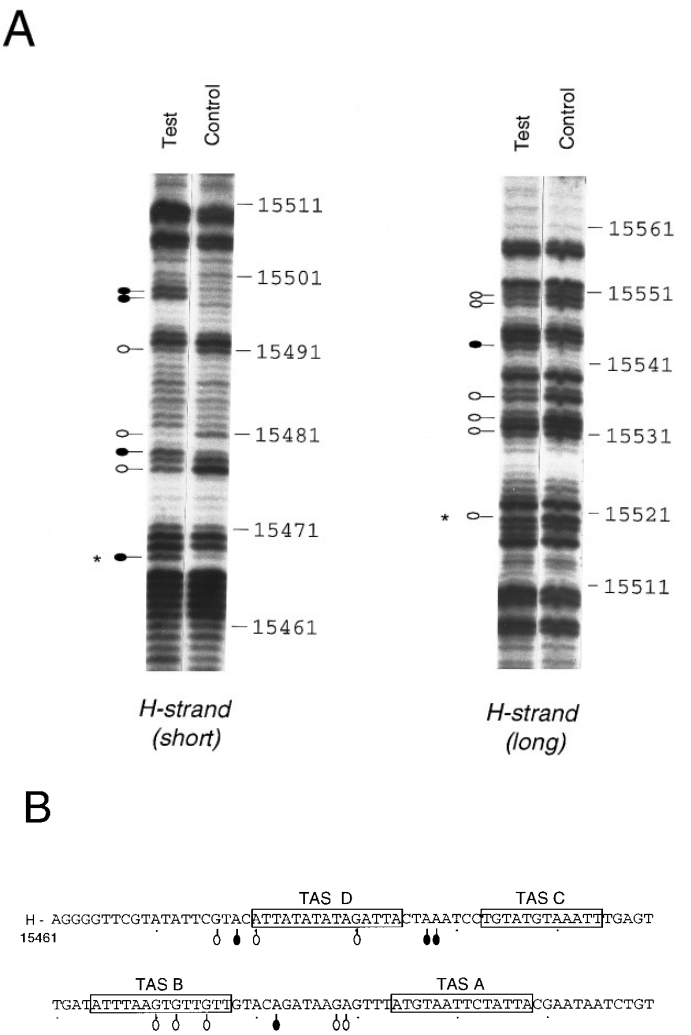

FIG. 2. *In organello* DMS footprinting in the TAS region of rat mtDNA. (A) DNA from DMS-treated (test) and untreated (control) mitochondria was prepared as described in the text. After piperidine cleavage, test and control DNA were subjected to linear amplification with Taq DNA polymerase in the presence of 5′ end-labeled *R-TAS-For* primer (L-15403-15423) to visualize bases (on the H-strand) in the TAS region of rat mtDNA. Short and long gel runs are shown. Rat mtDNA positions (37), shown at the right of each panel, were deduced from control G-ladder and from sequencing reactions run alongside (not shown). At the left of each panel the sites of methylation protection and hypersensitivity are indicated by open and filled circles respectively. The asterisks indicate nucleotides other than purines reacting with DMS; they were not considered as footprinted in the summary (B). (B) Positions of the *in organello* footprinted bases at the TAS region of rat mtDNA. Filled and open circles indicate the sites of DMS altered reactivity as deduced from A. TAS elements, as deduced from the alignment of the TAS region of human, cow, and rat mtDNA (see Fig. 3), are boxed.

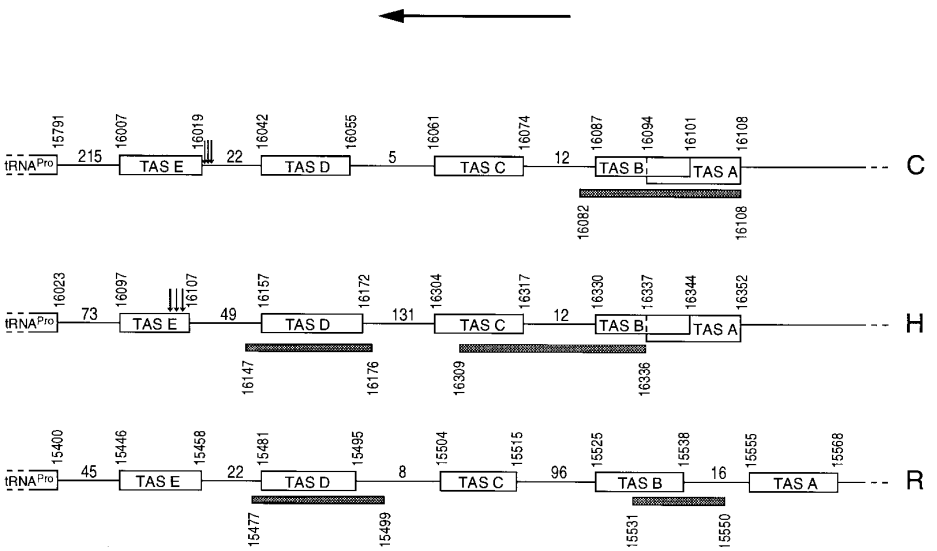

FIG. 3. Schematic diagram showing the position of protein binding sites in the TAS A-E region of human, rat, and cow mtDNA. The relative positions of the TAS elements have been deduced by a sequence alignment of the three genomes, as slightly modified from Sbisà *et al.* (11). Vertical numbers indicate the sequence positions (36-38); horizontal numbers indicate the distance (nucleotides) between TAS elements. Shadowed boxes below each diagram indicate the position of the *in vivo* footprinted regions, as deduced from experiments shown in Figs. 1 and 2 for rat and human and from Ref. 12 for cow. Arrows above human and cow diagrams show the positions of the 3′ end of the D-loop (7, 8). The horizontal arrow above the figure indicates the direction of H-strand synthesis.

16310, 16317-16319, 16329, 16335, 16336). The locations of the footprinted bases are summarized in Fig. 1B. DMS footprinting was performed also in isolated rat liver mitochondria (Fig. 2A). By using the labeled primer *R-TAS-For,* bases with altered DMS reactivity were detected between residues 15477-15499 which encompass TAS D (nucleotides 15477, 15479, 15481, 15491, 15498, 15499); additional modified residues (15531-15550) were observed which cover part of TAS B (nucleotides 15531, 15533, 15536, 15543, 15549, 15550). The positions of the footprinted bases are summarized in Fig. 2B.

To compare the location of the footprinted regions in human and rat mtDNA with that of cow, the sequences of the TAS regions of these organisms were aligned. Figure 3 shows that, except for some differences in the length of the regions among TASs, there is a good correspondence between TAS elements A-E. The only relevant difference concerns TAS-A and B that overlap in human and bovine whereas they are distinct in rat. From this alignment it results that one DMS footprinted region in human and rat is located in a similar position and corresponds to TAS-D. The second footprinted region partially covers TAS-C and TAS-B in human, whereas in rat it comprises only part of TAS-B. By comparing these data with those reported in bovine an important difference can be observed: whereas only one protein binding site was found in the TAS-region of cow mtDNA (12), two footprinted sequences are shown in the TAS-region of human and rat mtDNA, in corresponding positions. Of these two footprinted regions, the one partially covering TAS-B corresponds in part to the binding site of the bovine TAS-binding protein. The protected sequences in human and rat might interact with the same or with distinct proteins involved in the termination of the D-loop. Experiments addressed to identify the protein(s), by using the information obtained in this paper, are now in progress.

The mechanism by which the TAS-binding protein promotes the termination of the D-loop is still unknown. In bacteria the termination of DNA synthesis depends on the interaction between terminator sequences (*ter*) and a cognate terminator protein (*tus*), that recognizes *ter* sites and binds to them tightly (30). The arrest complex is thought to work by blocking the helicase-mediated unwinding of duplex DNA at the fork apex (31). According to this model the TAS-binding protein could act as *anti*-DNA-helicase. An ATP-dependent DNA helicase has been detected in mitochondria from bovine (32) and from sea urchin eggs (33). In the latter organism we also purified a DNA-binding protein that is able to bind specifically a sequence containing the 3′ end of the very short (about 80 nt) D-loop (34, 35). It is very likely that this protein, whose cDNA has been recently cloned (Loguercio Polosa et al. in preparation), is the homologue of the TAS-binding protein identified in mammals.

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