THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 42, Issue of October 20, pp. 25020–25027, 1995

*Printed in U.S.A.*

# Identification by *in Organello* Footprinting of Protein Contact Sites and of Single-stranded DNA Sequences in the Regulatory Region of Rat Mitochondrial DNA

PROTEIN BINDING SITES AND SINGLE-STRANDED DNA REGIONS IN ISOLATED RAT LIVER MITOCHONDRIA*

(Received for publication, March 27, 1995, and in revised form, August 14, 1995)

**Palmiro Cantatore‡, Luciana Daddabbo, Flavio Fracasso, and Maria Nicola Gadaleta**

*From the Department of Biochemistry and Molecular Biology, University of Bari and Centro Studi sui Mitocondri e Metabolismo Energetico, CNR, via Orabona 4A, 70126, Bari Italy*

**Footprinting studies with the purine-modifying reagent dimethyl sulfate and with the single-stranded DNA probing reagent potassium permanganate were carried out in isolated mitochondria from rat liver. Dimethyl sulfate footprinting allowed the detection of protein-DNA interactions within the rat analogues of the human binding sites for the transcription termination factor mTERF and for the transcription activating factor mtTFA. Although mTERF contacts were localized only at the boundary between the 16S rRNA/tRNA$^{Leu}_{UUR}$ genes, multiple mtTFA contacts were detected. Contact sites were located in the light and the heavy strand promoters and, in agreement with *in vitro* footprinting data on human mitochondria, between the conserved sequence blocks (CSB) 1 and 2 and inside CSB-1. Potassium permanganate footprinting allowed detection of a 25-base pair region entirely contained in CSB-1 in which both strands were permanganate-reactive. No permanganate reactivity was associated with the other regions of the D-loop, including CSB-2 and -3, and with the mTERF contact site. We hypothesize that the single-stranded DNA at CSB-1 may be due to a profound helix distortion induced by mtTFA binding or be associated with a RNA polymerase pause site. In any case the location in CSB-1 of the 3′ end of the most abundant replication primer and of the 5′ end of the prominent D-loop DNA suggests that protein-induced DNA conformational changes play an important role in directing the transition from transcription to replication in mammalian mitochondria.**

The majority of mammalian mitochondrial (mt)[1] DNA molecules possess a triple helix structure called D-loop due to the displacement of the parental H-strand by short nascent H-strand chains. The D-loop region, that is the main noncoding region and the most variable part of vertebrate mt genomes, contains the origin of H-strand DNA replication ($O_H$) and the promoters for H and L-strand transcription (for review see Refs. 1–3). The two promoters (HSP and LSP) hold the binding sites for the transcription factor mtTFA (4), which activates H- and L-strand *in vitro* transcription. The H-strand transcripts initiate in two points. One, $I_{HR}$, located few bases upstream of the tRNA$^{Phe}$ gene, is responsible for the synthesis of the two rRNAs 16S and 12S and of 2 tRNAs; the other, $I_{HT}$, located near the 5′ end of the 12S rRNA gene, directs the synthesis of 12 mRNAs and of 12 tRNAs (5–7). The L-strand is entirely transcribed as a single transcription unit, starting from a point that in humans is located 216 bp upstream of $O_H$. It has a low informational content, because it directs only the synthesis of the primer for H-strand replication, of 1 mRNA and of 8 tRNAs. The contrast between the complete L-strand transcription and its low informational content is not yet fully understood. Moreover, despite many studies in different laboratories, the molecular mechanisms that regulate the production of the mature H- and L-strand transcripts are not yet completely known. They likely involve protein factors and nucleolytic enzymes. In particular, the termination of the H-strand ribosomal transcription unit depends on a mt termination factor, mTERF, that binds to a sequence located few bases downstream of the 5′ end of the tRNA$^{Leu}_{UUR}$ gene (8), whereas the primary transcript processing seems to be mediated by a RNase P-like endonuclease, which should recognize the cloverleaf structure of the tRNA genes that separate most of the rRNA and mRNA genes (9). Furthermore, the mapping of the nascent human mt transcripts had suggested that also transcriptional pausing plays a role in regulating the expression of mt genes (5).

As far as L-transcripts are concerned, studies on the biosynthetic mechanism of human and mouse H-strand replication primers (10, 11) showed the existence of several L-strand transcripts initiating from the L-strand promoter and terminating in the D-loop region at three conserved sequence blocks (CSB),[1] CSB-1, CSB-2, and CSB-3. The 3′ termini of these three RNA species were joined with the 5′ termini of nascent H-strand DNA chains, and the prominent replication initiation site was located at the 5′ end of CSB-1. Clayton's group (10) hypothesized that the 3′ ends of the RNA primers were generated by post-transcriptional processing of the primary transcript. Later it was found that a nuclear RNA-containing endonuclease (RNase MRP), presumably involved in the maturation of cytoplasmic rRNA (12, 13) and likely also present at a low concentration in mitochondria (14–16), was able to cleave *in vitro* the L-strand primary transcript at some of the sites found *in vivo* (17–20).

Here, in order to elucidate some of the aspects concerning the regulation of mt transcription and replication, we used an experimental approach aiming to obtain evidence about the physiological role of some of these protein factors. In particular

* This work was supported in part by grants from Ministero dell'Universita e della Ricerca Scientifica e Tecnologica (40% and 60%) and from the Progetto Finalizzato "Ingegneria Genetica" CNR. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 39-80-5443378; Fax: 39-80-5443317; E-mail: e103pl10@area.ba.cnr.it.

[1] The abbreviations used are: mt, mitochondrial; CSB, conserved sequence block; DMS, dimethyl sulfate; LSP, light strand promoter; HSP, heavy strand promoter; bp, base pair.

we used an *in vivo*-like environment, made by rat liver isolated mitochondria, to investigate by means of *in organello* footprinting with dimethyl sulfate (DMS) and potassium permanganate ($KMnO_4$) the occurrence of protein binding sites and of single-stranded DNA structures in the regulatory region and in the transcription termination region of rat mt DNA. By these techniques we were able to detect, within the mitochondrion, multiple protein-DNA interactions and, for the first time, a single-stranded-DNA region located within CSB-1. The data obtained with this approach suggest that protein-induced mt DNA conformational changes play an important role in defining the 5′ ends of the prominent H-DNA nascent chains.

## MATERIALS AND METHODS

*In Organello Footprinting with Dimethyl Sulfate*—Rat liver mitochondria were prepared by differential centrifugation (21). 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 glutammate, 2.5 mM malate, 10 mM Tris-HCl, pH 7.4, and 1 mg/ml bovine serum albumin. In these conditions the integrity of mt membranes was preserved, as shown by the observation that mitochondria supported RNA and DNA synthesis for a long time (22–24). 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 phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na(PO_4)$ pH 7.4, and 1.4 mM $K(PO_4)$, pH 7.4) were added. The mitochondria were pelleted by centrifuging for 1 min at 12,000 × *g*. After two additional washes with phosphate-buffered saline, the pellets were suspended in 400 μl of lysis buffer (200 mM NaCl, 0.1% SDS, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.2 mg/ml proteinase K) and incubated for 30 min at 37 °C. DNA was isolated by two extractions with phenol, two with phenol/chloroform/isoamyl alcohol (25:24:1) and one with chloroform, and then DNA was 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 buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), 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 and treated with 100 μl of 1 M piperidine for 30 min at 90 °C. Sephadex separation was performed as above, and the final samples were recovered in 35 μl of water. In most experiments the piperidine treatment of samples and controls was omitted because preliminary tests showed that the primer extension effectively terminated in correspondence of modified bases; in this case the DNA pellets were suspended in 100 μl of TE buffer, purified by spin column chromatography, and recovered in 35 μl of water. To set up the footprinting conditions and to check the fidelity of mt DNA methylation pattern, preliminary control experiments, using as template a recombinant plasmid containing the DNA region under investigation, were carried out. 2 μg of the pFF28 plasmid containing a rat mt DNA insert of 712 bp from position 15719 to position 132 (21) were combined in a final volume of 100 μl with 3 mM $MgCl_2$, 100 mM KCl, 0.2 mM dithiothreitol, 30 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA. After preheating for 2 min at 37 °C, DMS was added to a final concentration of 0.1%, and the samples were incubated for 5 min at 37 °C. The reactions were blocked by adding 200 μl of cold 3 M ammonium acetate, 1 M 2-mercaptoethanol, 20 mM EDTA, and 250 μg/ml tRNA. The DNA was recovered by ethanol precipitation and processed as for DMS-treated mt DNA.

*In Organello Footprinting with Potassium Permanganate*—Mitochondrial pellets (2 mg of proteins) were suspended in 200 μl of 1 mM ATP, 10 mg/ml bovine serum albumin, 20 mM $NaH_2PO_4$, 25 mM pyruvate, and 10% glycerol and incubated for 20 min at 37 °C. Following this, permanganate was added to mitochondria at 37 °C to a concentration of 10 mM for 4 min. The mitochondria were immediately transferred to ice-chilled Eppendorf tubes and spun for 5 min at 12,000 × *g*. The pellets were suspended in 400 μl of lysis buffer (200 mM NaCl, 0.1% SDS, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.2 mg/ml proteinase K) and incubated for 30 min at 37 °C. The nucleic acids, extracted as described above, were suspended in 55 μl of water and passed through a 1-ml Sephadex G-50 spin column. Column eluates were lyophilized twice and collected in 35 μl of water. Control samples were obtained by extracting the nucleic acids from the same amount of mitochondria as above. The DNA pellets were suspended in 17.5 μl of 3 mM $MgCl_2$, 100 mM KCl, 0.2 mM dithiothreitol, 30 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA, preheated for 2 min at 37 °C, and then permanganate was added to a final concentration of 10 mM for 4 min. The reaction was blocked by adding 2 μl of 14.7 M 2-mercaptoethanol, chilling on ice, and then adding 6 μl of 0.2 M EDTA and 27 μl of water. The samples were passed through a 1-ml Sephadex G-50 spin column, lyophilized twice, and finally collected in 35 μl of water. Also in this case preliminary experiments were carried out with 2 μg of pFF28 DNA; the plasmid DNA was treated with permanganate following the same protocol reported for controls. In most experiments, before carrying out the primer extension, mt DNAs from test and control samples were digested with the restriction enzyme *Bgl*I, which cuts at positions 15932 and 12650, allowing the primers to anneal to a linear template of 13,017 bp (25).

*Primer Extension Analysis of DMS and $KMnO_4$-treated Samples*—The entire 35-μl sample was used for primer extension analysis. The reaction mixture contained also 10 μl of 10 × *Taq* polymerase buffer (100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, and 500 mM KCl), 4 μl of 5 mM dNTP mix, 1 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) for 2 min at 94 °C; then 15 cycles followed (1 min at 94 °C, 2 min at the primer hybridization temperature, and 3 min at 72 °C). The reactions were ended by denaturing for 2 min at 94 °C, annealing for 2 min, and final extension 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 and 5 mM EDTA. The pellets were washed with 70% ethanol, dried down, suspended in 6 μl of 98% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue, and resolved in a 6% polyacrylamide/7 M urea sequencing gel (0.2 mm × 40 cm). Probe labeling was carried out with [$\gamma$-$^{32}P$]ATP and polynucleotide kinase as reported by Sambrook *et al.* (26).

*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 Gel-Scan-XL Evaluation software. In some experiments, to account for any loading difference, control and test scans were normalized using bands, located in regions lacking any observable footprints, and placed just above or just below the reactive areas. When the intensities of the bands in the open complexes were much higher than those in the reference bands, light and dark exposures of the same gel were scanned. Relative levels of protection or hypersensitivity were then determined using the normalized values. 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

Several reports have shown that isolated mitochondria from different mammalian sources are able to synthesize and process mt RNA in a way that closely resembles the *in vivo* process (21–24, 27–29). Here, in order to detect within the mitochondria protein binding sites and single-stranded DNA regions, we carried out DNA footprinting in an *in vivo*-like environment made of isolated mitochondria from rat liver. In a typical experiment, freshly purified rat liver mitochondria were treated with the modifying agent, and the mt DNA was isolated. Control samples were obtained by a similar treatment of naked mt DNA (protein-free) purified from the same amount of mitochondria used for *in organello* footprinting. Mitochondrial DNA, isolated from test and control samples, was subjected to primer extension with *Taq* DNA polymerase in the presence of a 5′ end-labeled oligonucleotide, and the reaction products were resolved on a polyacrylamide sequencing gel. The sites of DMS-altered reactivity or permanganate sensitivity were dependent on the organelle integrity, because they were not detected either in mitochondria stored at −80 °C, or in organelles whose permeability was altered by incubation in hypotonic media

(experiments not shown). The results here reported were obtained in at least five different independent experiments on different individuals; each different sample of mitochondria displayed the same *in organello* footprinting pattern.

*DMS Footprinting in Isolated Rat Liver Mitochondria*—Dimethyl sulfate, a molecule that acts methylating DNA at N7 and N3 guanine and adenine residues, respectively (30), has been used to map contact sites between proteins and DNA. When bound to specific DNA residues, proteins can decrease or intensify purine reactivity to methylation with respect to naked DNA (31). DMS readily permeates cellular and nuclear membranes, allowing the analysis of *in vivo* protein-DNA interactions. Recently Ghivizzani *et al.* (32, 33) have used DMS footprinting to detect mt DNA-protein interactions in isolated bovine and human mitochondria, thus showing that DMS may cross also mt membranes. To set up the footprinting conditions in isolated rat liver mitochondria, the first experiment was carried out to detect the bases that contact the rat analogue of the human mt termination factor (mTERF). Such region was located by *in vitro* DNaseI footprinting a few bases downstream of the 5′ end of the $tRNA^{Leu}_{UUR}$ gene (8). Fig. 1 shows that the L-strand and H-strand primers ND1 (position 2761–2740) and 16S (position 2542–2563) detected a region of 15 bp (position 2659–2673) containing hypermethylated and undermethylated bases; almost all the involved residues (7 on the L-strand and 3 on the H-strand) were comprised in the conserved tridecamer sequence block (8, 34), which contains the binding site for mTERF. To estimate the protein occupancy at each region of DMS-altered reactivity, the relative level of methylation protection was measured; it can be considered equivalent to the percentage of the sites bound continuously by the protein or to the percentage of the time in which all the available sites were bound. Within the mTERF binding domain, the level of DMS protection on the L-strand was about 82%, a value that suggested that *in organello* as *in vitro* (8) mTERF binds the mt DNA with high affinity.

The analyses of protein-DNA contacts in the regulatory region are reported in Figs. 2 and 3. The L-strand and the H-strand probes P-REV1 (position 51–30) and D-rat-viv2 (position 16108–16129) detected two regions of altered DMS reactivity. The first region contained hypermethylated or undermethylated bases from 16197 to 16211; nine of the modified residues were located on the L-strand, and three were on the H-strand. The second region displayed a lower level of altered DMS reactivity; it encompassed 29 bp, from position 16252 to 16280. Also in this case both strands were involved, but the H-strand was slightly more affected than the other. Two more regions of altered DMS reactivity (Figs. 2 and 4) were detected with the probes D-REV2 (position 16107–15986) and D-rat-viv1 (position 15955–15976). They comprise bases contained between CSB-1 and CSB-2 (from 16042 to 16064) and bases contained within CSB-1 (from 16018 to 16034). DMS-altered activity was not detected in the bases located in CSB-2 and downstream of CSB-1 (results not shown). The region comprised between CSB-1 and CSB-2 showed a level of occupancy of at least 40%, with nucleotides 16050 and 16051 showing a DMS protection of about 90%, whereas CSB-1 was associated with a DMS protection level of about 50%. Although in most of the cases the altered methylation pattern concerned the purines, in some cases pyrimidine residues were also affected. In particular two T residues at positions 16018 and 16019 showed DMS hyper-reactivity (Fig. 4). It has been reported (35, 36) that DMS may methylate cytosine and to a minor extent thymidine when these bases are in a single-stranded DNA. This is the case for T-16018 and T-16019 that, as shown by permanganate footprinting reported below (see Fig. 6), are in a single-stranded DNA.

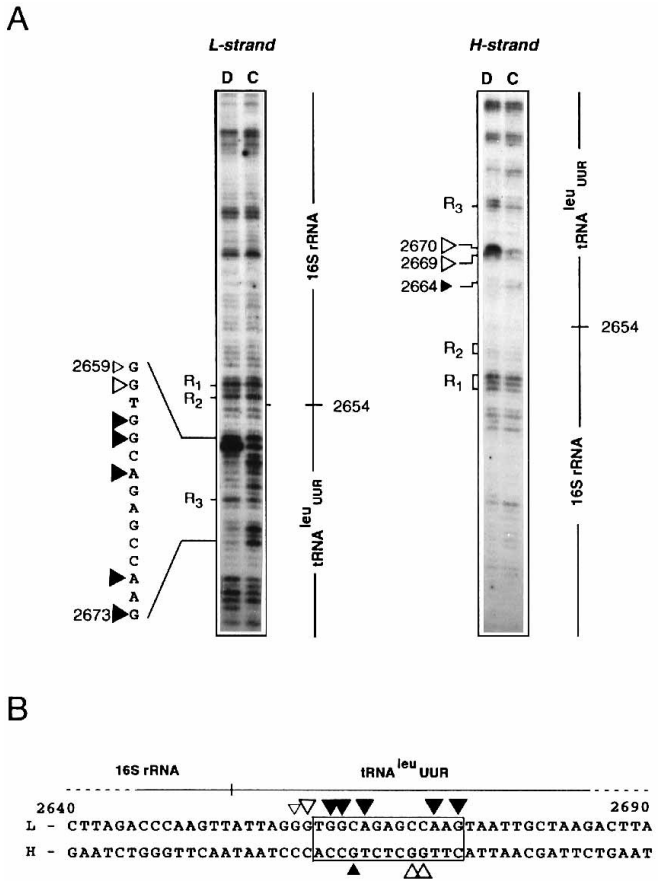


FIG. 1. **In organello DMS footprinting at the termination site of ribosomal gene transcription unit.** *A*, DNA from DMS-treated (*D*) and untreated (*C*) mitochondria was prepared as described in the text. L-strand and H-strand probes were the oligos ND1 (position 2761–2740) and 16S (position 2542–2563), respectively. Probe sequences were: ND1, 5′ GATTAGGAGTGTTAGGATATTA 3′, and 16S, 5′ CCCAGTTACGAAAGGACAAGAG 3′. Rat mt DNA positions (25) were deduced from control G-ladder and by sequencing reactions run in preliminary experiments (not shown). Sites of *in organello* methylation hypersensitivity are indicated by *open triangles*, and *filled triangles* indicate sites of methylation protection. The *size* of the *triangles* is roughly proportional to the amount of modification; *larger triangles* indicate at least a 3-fold difference between normalized values of test and control samples. The bands $R_1$, $R_2$, and $R_3$ in each panel serve as reference for normalization. The position of the 16S rRNA/$tRNA^{Leu}_{UUR}$ boundary is shown. *B*, sequence positions of the footprinted bases. The *triangles* indicate the sites of altered DMS reactivity as deduced from *A*. The rat analogue of the human tridecamer transcription termination site (34) is *boxed*. The junction between 16S rRNA and $tRNA^{Leu}_{UUR}$ genes is indicated. H-strand transcription proceeds from *left* to *right*.

To obtain information relative to the significance of these DNA-protein interactions in rat liver, sequence homology studies were performed. Fig. 5*A* shows that Rat I, a sequence of 21 bp (from 16196 to 16216) containing the first block of reactive bases, was homologous to the mtTFA binding site of human, mouse, and bovine LSP (4, 32, 33, 37), whereas (Fig. 5*B*) the second block of reactive bases contained a region, Rat II (extending from 16247 to 16268), displaying a significative homology with the mtTFA binding site of HSP. Therefore the DMS-modified bases of Rat I and Rat II are probably due to contacts with the rat analogue of human factor mtTFA, which stimulates mt transcription interacting with DNA sequences located upstream of the transcription initiation sites (4). The similarity of the sequence Rat III, located between CSB-1 and CSB-2 (from 16041 to 16064) with the mtTFA-footprinted regions in LSP and HSP, shown in Fig. 5*C*, suggests that the altered

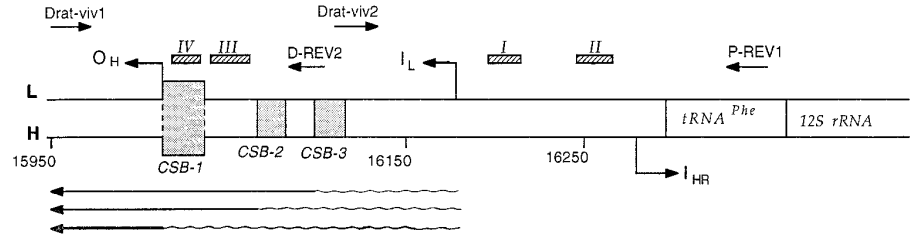


FIG. 2. **Schematic diagram summarizing the results obtained by DMS and $KMnO_4$ in organello footprinting in the regulatory region of rat mt DNA.** The positions and the orientation of the oligonucleotide probes are shown at the *top* of the figure; their precise positions are reported in the legends of the following figures. *Hatched boxes* (*I, II, III* and *IV*) indicate the protein contact sites as deduced from the DMS footprinting experiments reported in Figs. 3 and 4. Permanganate reactivity at CSB-1 (see Fig. 6) is indicated by displacing both strands. In the case of DMS footprinting, probes PREV-1 and D-rat-viv-2 were used to detect regions I and II (see Fig. 3). Regions III and IV (see Fig. 4) were found with probes D-REV2 and D-rat-viv-1. The permanganate reactive region in CSB-1 (see Fig. 6) was detected with the probes P-REV1, D-REV2, and D-rat-viv-1. The *bottom part* of the diagram shows the presumptive approximate positions of primer RNAs and of the H-DNAs in rat as deduced from the mapping of such species in human and mouse (10, 11). *Wavy* and *solid lines* indicate the primer RNAs and the H-DNAs, respectively. *Bold lines* show the most prominent primer and H-DNA species. The numbers refer to the genomic position of rat mt DNA (25). *H*, H-strand; *L*, L-strand; *CSB*, conserved sequence block; *CSB-1*, 16012–16037; *CSB-2*, 16068–16084; *CSB-3*, 16101–16118. $O_H$ is the main H-strand replication origin, which in rat is located at position 16011 (63). $I_L$ and $I_{HR}$ are the L and H-strand initiation sites that in rat are located around position 16178 and 16183, respectively (63).[2]

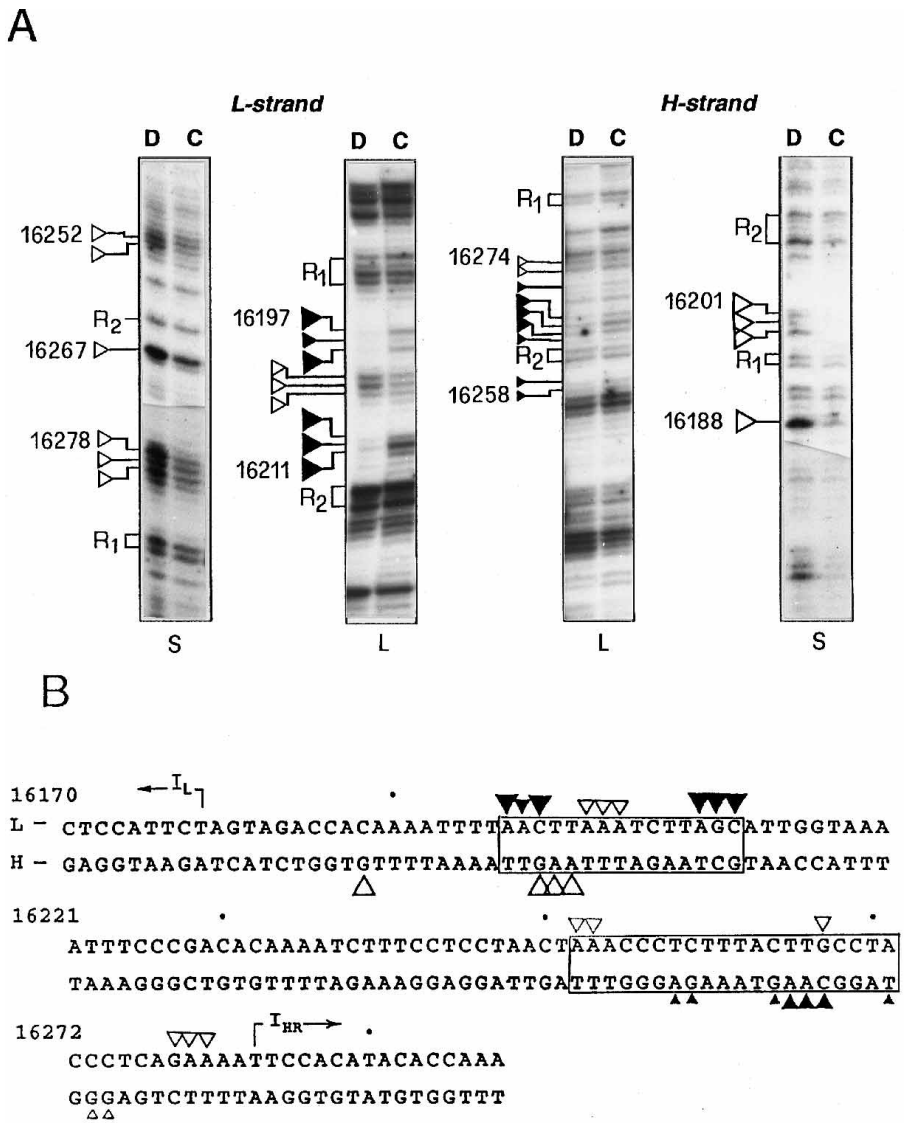


FIG. 3. ***In organello* DMS footprinting near the H- and L-strand promoters.** *A*, L-strand and H-strand probes were the oligos P-REV1 (position 51–30) and D-rat-viv-2 (position 16108–16129), respectively. Primer sequences were: P-REV1, 5′ GAATCCATCTAAGCATTTTC-AG 3′, and D-rat-viv-2, 5′ CCCCAAAAA-CATTAAAGCAAGA 3′. The bands $R_1$ and $R_2$ in each panel serve as reference for normalization. Symbols indicating the site and the extent of altered methylation reactivity are the same as in Fig. 1. For each primer a short (*S*) and a long (*L*) gel run are shown. *D*, DMS treated mitochondria; *C*, control. *B*, genomic position of the bases with altered methylation. The *triangles* indicate the sites of altered DMS reactivity as deduced from *A*. $I_L$ and $I_{HR}$ are the initiation sites of L and H transcripts. The two *boxed* regions are the putative binding sites of rat mtTFA at LSP and HSP.

reactivity of this region is due to contacts with the same protein. This hypothesis is supported by the capacity of mtTFA to bind *in vitro* an analogous region of human mt DNA (4, 33). The bases located within CSB-1 (Fig. 2, *block IV*, and Fig. 3*B*) do not share significative homology with the other footprinted regions. However footprinting analysis in human mt DNA (33) showed that mtTFA was able to bind nonhomologous sequences *in vitro*, including those contained in CSB-1. These data, which were confirmed by *in organello* footprinting (33), let us to

[2] P. Cantatore, L. Daddabbo, F. Fracasso, and M. N. Gadaleta, unpublished observations.

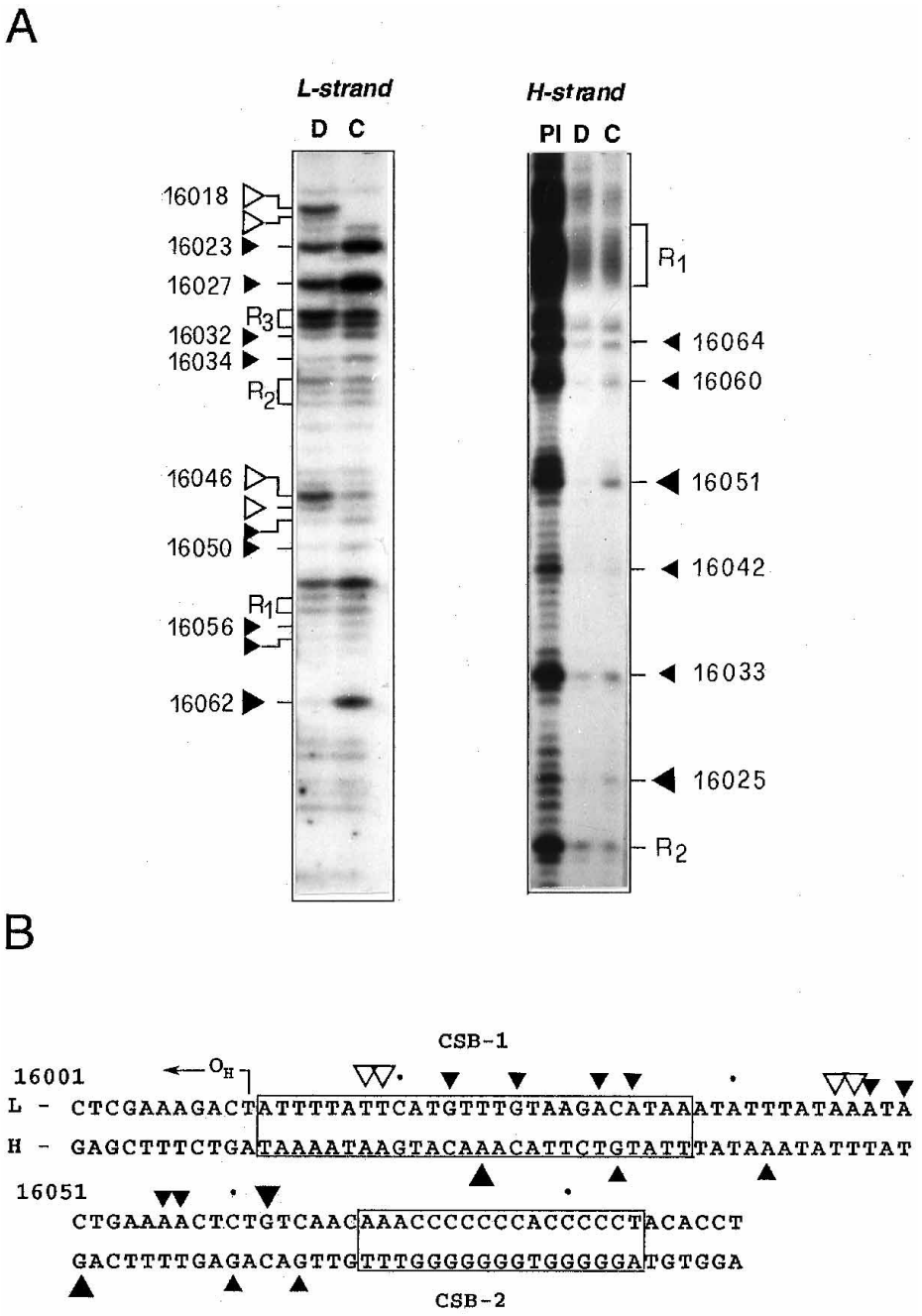


FIG. 4. ***In organello* DMS footprinting near the replication origin of rat liver mt DNA.** *A*, L-strand and H-strand probes were the oligos D-REV2 (position 16107–15986) and D-rat-viv1 (position 15955–15976), respectively. The sequences were: D-REV2, 5′ TTTGGCATTGAAGTTTCAGGTG 3′, and D-rat-viv1, 5′ CCTGTGGAACCTTTTAGTTAAG 3′. Symbols used to indicate the sites and the extent of altered DMS reactivity are as in Fig. 1. The bands $R_1$, $R_2$, and $R_3$ in each panel serve as reference for normalization. *D*, DMS-treated mitochondria; *C*, control; *Pl*, recombinant plasmid pFF28 (it contains a rat mt DNA insert of 712 bp comprising position 15719–132 (21)) treated *in vitro* with DMS. *B*, genomic position of the bases with altered DMS reactivity as deduced from *A*. The positions of the H-strand replication origin ($O_H$) and of CSB-1–2 are shown.

ascribe also the DMS-altered reactivity of CSB-1 to the rat analogue of human mtTFA.

*Permanganate Footprinting in Rat Liver Mitochondria*—Although DMS is extremely useful in detecting the occupancy of DNA sites by proteins, it is less valuable as a probe for detecting the structural changes associated with the various steps of transcription. Potassium permanganate, a small molecule that preferentially oxidizes single-stranded pyrimidine residues at their 5=6 double bond, (38) has been used *in vitro* and *in vivo* to detect distortions in double-stranded DNA (39) and to identify single-stranded DNA regions associated with open complexes with RNA polymerase (40–43) or with RNA polymerase pause sites (44, 45). The reagent is able to penetrate bacterial cells and eukaryotic nuclei (44, 46, 47), thus suggesting that it may also cross mt membranes. Figs. 2 and 6 report the results of permanganate footprinting in isolated rat liver mitochondria using probes encompassing the mt DNA regulatory region. Panel I shows that the L-strand probe P-REV1 identified a region, contained in CSB-1, that has a strong permanganate hyper-reactivity. No signals were detected in CSB-2 and CSB-3. To carefully map the hyper-reactive bases, the D-REV2 probe closer to CSB-1 was used. Panel II shows that on the L-strand the reactive region extends from position 16013 to 16035, which comprise bases all contained in CSB-1. The H-strand probe D-rat-viv1 detected hyper-reactive bases situated in the same region (from 16016 to 16037), indicating that almost all the Ts contained in both strands of CSB-1 are permanganate-sensitive and that in isolated rat mitochondria, this 25-bp block has a single-stranded configuration. Two bases different from pyrimidines (A-16034 on the L-strand and A-16016 on the H-strand) also showed permanganate hyper-reactivity; this was probably due to their 5′ position with respect to reactive T residues (46, 48).

To test the presence of single-stranded DNA regions in other

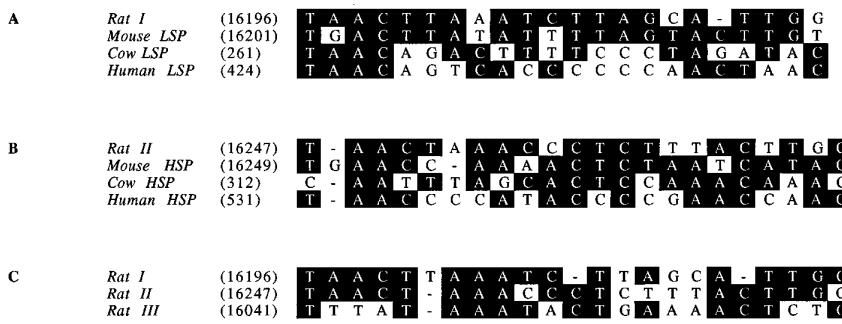


FIG. 5. **Sequence alignment of DMS footprinted regions.** *A*, alignment of rat footprinted region near $I_L$ with the mtTFA binding sites at the LSP of human, mouse, and bovine mt DNA (33, 35). *B*, alignment of rat footprinted region near $I_{HR}$ with mtTFA binding sites at the HSP of human, mouse, and bovine mt DNAs. *C*, alignment of DMS footprinted bases in the noncoding region of rat mt DNA. The *numbers* in parentheses (human, bovine, and mouse genomic positions are according to Anderson *et al.* (70, 71) and Bibb *et al.* (72)) refer to the position of the first nucleotide of the sequence.

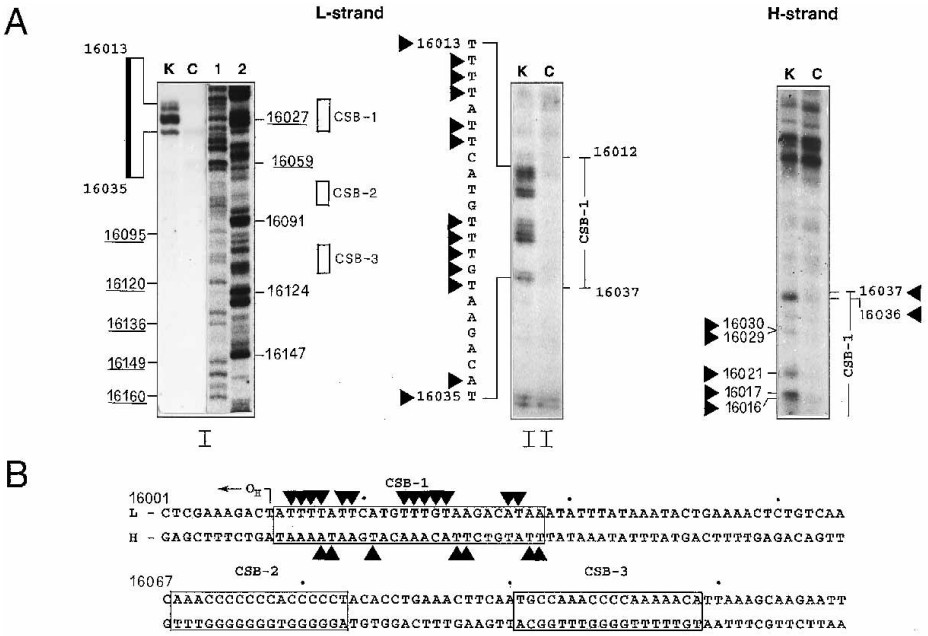


FIG. 6. ***In organello* permanganate footprinting near the replication origin of rat mt DNA.** *A*, L-strand probes were the oligos P-REV1 and D-REV2; H-strand probe was the oligo D-rat-viv1. *Panel I* shows the P-REV1 extension products of undigested mt DNA extracted from $KMnO_4$-treated (*K*) and untreated(*C*) mitochondria. In *lanes 1* and *2* are reported the extension products of the plasmid pFF28 (21). The plasmid was first treated with DMS (*lane 1*) and $KMnO_4$ (*lane 2*) as described under "Materials and Methods," digested with *Bgl*I, and then subjected to primer extension with *Taq* DNA polymerase and labeled P-REV1 primer. Mitochondrial genomic positions refer to bands in *lanes 1* (*underlined*) and *2* (*not underlined*). In the other two panels mt DNA from test (*K*) and control (*C*) was first digested with *Bgl*I and then subjected to primer extension with D-REV2 and D-rat-viv1, respectively. Sites of *in organello* permanganate hyper-reactivity are indicated by *triangles*. The positions of CSB-1 (16012–16037), CSB-2 (16068–16084), and CSB-3 (16101–16118) are indicated. *B*, sequence positions of the permanganate reactive bases. The *triangles* indicate the sites of hyper-reactivity as deduced from *A*. The positions of CSB-1 to CSB-3) and of H-strand replication origin ($O_H$) are indicated.

locations and in particular at a site where protein-dependent transcription termination takes place, we tested the permanganate hypersensitivity of the region containing the 16S rRNA/$tRNA^{Leu}{}_{UUR}$ boundary. This is the site where the ribosomal transcription unit ends due to the interaction with the termination factor mTERF (8). Fig. 7 shows the absence of any significative signal due to permanganate hyper-reactive bases. These data were quite surprising in the light of a recent report (49) showing that mTERF binding induced DNA bending. The lack of permanganate sensitivity in the presence of DNA bending might be explained by hypothesizing that the mt DNA complexed with mTERF assumes a stacked configuration that prevents the thymidine oxidation by permanganate (50). Moreover, this result implies that the single-stranded DNA regions functioning as pause sites are not required for termination, so that mTERF alone is able to generate, probably by a physical blockage mechanism, the end of transcription.

## DISCUSSION

*In Organello DMS Footprinting Reveals the Contact Sites for DNA-binding Proteins*—The dimethyl sulfate footprinting experiments in isolated rat liver mitochondria, reported in Figs. 1–5, enabled the detection of multiple protein-DNA interactions. The bases with altered methylation at the 16S rRNA/$tRNA^{Leu}{}_{UUR}$ boundary are involved in the binding with the termination factor mTERF, whereas the altered methylation observed at multiple sites of the D-loop region depends on the binding with a single protein factor: the transcription activating factor mtTFA. This is the first time that the contact sites with these DNA-binding proteins have been identified in the rat.

The mtTFA binding to the different portions of the D-loop is related to the multiple roles of this factor in mt transcription and replication. The binding that takes place at LSP and HSP

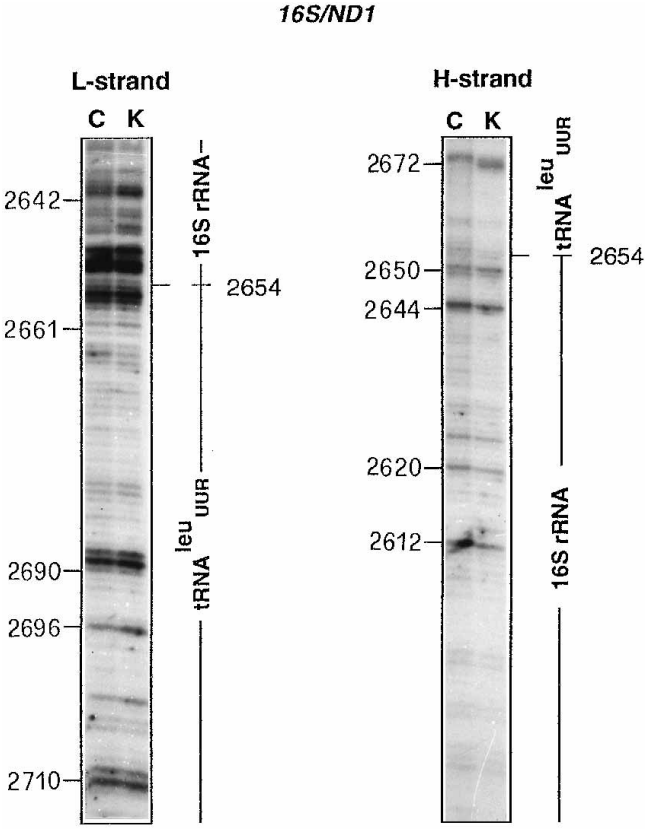


FIG. 7. ***In organello* permanganate footprinting at the boundary between 16S rRNA/tRNA$^{Leu}$UUR genes.** Probes used were the oligos ND1 and 16S (see Fig. 1). The position of the gene boundary is indicated. *C*, control; *K*, permanganate-treated mitochondria. The *numbers* at the *left* of each *lane* indicate the genomic positions of some bands, as deduced by control T+C ladder (not shown).

serves for stimulating mt transcription (4, 51). It occurs with different efficiencies ranging from 82% in LSP (Fig. 2, block I) to 45–50% in HSP (Fig. 2, block II); this is in agreement with both *in vitro* binding experiments with human mtTFA (4) and with the higher rate of *in vivo* mt transcription of the L-strand compared with that of H (3, 5). It is likely that variations in the level of mtTFA within the mitochondria may regulate promoter selection such that at low concentrations L-strand transcription, which is linked to H-DNA replication, would predominate, whereas at higher mtTFA levels HSP transcription would also take place. The recent finding of a NRF-1 binding site in the human mtTFA gene (52) is a first evidence of the modulation of mtTFA gene expression. The mtTFA contacts with LSP and HSP are quite asymmetric; at LSP most of the purines located on the L-strand contact mtTFA, whereas on the H-strand only 3 purine residues exhibit DMS hyper-reactivity. Although both H- and L-strand promoters may function bidirectionally *in vitro* and *in vivo* (53), the transcription of the respective main coding strand largely predominates. The substantial asymmetric binding of mtTFA to LSP and HSP found *in organello* and *in vitro* (4) may represent the mechanism used for the substantial unidirectionality of the two promoters.

Recent *in organello* and *in vitro* footprinting experiments of Ghivizzani *et al.* (33) showed that human mtTFA, in addition to binding LSP and HSP, is able to bind the entire regulatory region of mt DNA in a phased arrangement, contributing to specific packaging of mt DNA control region *in vivo*. It has been suggested that phased mtTFA binding might be required for facilitating mt transcription (33, 54) or RNA processing (17). The data reported in this paper confirm this view because DMS-altered reactivity, presumably due to the rat analogue of human mtTFA, was found in the region between CSB-1 and CSB-2 and within CSB-1.

The binding of mtTFA to CSB-1 probably underlies a further function of this factor. In fact, contrary to the rest of the other binding sites whose sequence among mammalian species is highly diverging (37, 55), CSB-1 is universally conserved among vertebrates (55–57). Moreover, it contains the transition site between the most abundant replication primer and the prominent D-loop DNA (55, 58, 59). Therefore, it is likely that the binding of mtTFA to CSB-1 might have a rather different role with respect to that hypothesized for the other regions of the D-loop. The most likely possibility is that the mtTFA-CSB-1 complex may be part of a recognition signal for the transition from L-RNA to H-DNA chains. The presence of a single-stranded DNA in this region (see below) reinforces the hypothesis of a peculiar role for the mtTFA-CSB-1 complex.

*The Significance of the CSB-1 Permanganate-reactive Region*—In the experiments reported here, the permanganate footprinting technique was applied for the first time to isolated mitochondria. By using two probes spanning the control region from tRNA$^{Phe}$ to downstream of CSB-1, a very strong signal associated with a sequence entirely contained in CSB-1 was detected on both strands. No significative reactivity was found in the other CSBs or in the bases contained between them (Figs. 3 and 6). The permanganate reactivity of CSB-1 could be due to DNA distortions or be associated with a single-stranded structure possibly functioning as RNA-polymerase pause site. The first hypothesis is based on the binding of mtTFA to CSB-1 (Figs. 2 and 4) (33) and on the observation that mtTFA is able to induce wrapping and bending (54) of human mt DNA. The lack of permanganate sensitivity of CSB-2 and CSB-3 and in the bases between them exclude the possibility that the permanganate sensitivity of CSB-1 may be due to generalized mtTFA-induced distortions that in such a case should have been present also at other sites ot mtTFA binding. The permanganate sensitivity of CSB-1 might be then associated with a peculiar mtTFA binding, and this, as underlined above, might have a specific role in directing the RNA-DNA transition at the prominent replication origin.

Few cases of DNA distortions induced by DNA-binding proteins have been reported. They include the RAP-1 protein, whose binding to *Saccharomyces cerevisiae* telomers induces an unusual permanganate reactivity of the C-rich strand (60), the Epstein-Barr virus DNA replication origin binding protein EBNA1 (61), and the TATA box-binding protein, whose binding *in vivo* to the coding strand of the promoter of the phosphoenolpyruvate carboxykinase gene produces an altered permanganate activity on the opposite strand (62). It is interesting to observe that although the distortions induced by these proteins (60–62) concern a limited stretch of DNA, the extended permanganate reactivity of CSB-1 (13 reactive bases on the L-strand and 7 on the H-strand in a stretch of 25 bp) would create a profound structural change that should involve more than one helical turn.

An alternative explanation of the permanganate reactivity at CSB-1 is based on the presence among nascent human transcripts of many paused RNA molecules (5) and based on the location within CSB-1 of the multiple 3′ ends of RNAs originating from the L-strand initiation site (10–11, 21, 63). These data suggest that the single-stranded DNA at CSB-1 may be associated with a RNA-polymerase pause site. The existence of a transcription pause site in CSB-1 might have implications for the mechanism of replication primer formation. It could be hypothesized that the formation of the 3′ end of the longest and presumably more active replication primer (7S RNA), which terminates in CSB-1, might take place by transcriptional paus-

ing and would not necessarily require the action of a nuclease activity. On the contrary, the lack of permanganate-reactive bases at CSB-2 and CSB-3 would require the presence of one or more nuclease activities needed to create the 3′ ends of the primers terminating at these sites. The existence of a transcription pause site at CSB-1 might also help to explain the regulation mechanism of L-strand transcription. Although the L-strand is transcribed at a higher rate than the H-strand (5), its products are not more abundant than the H-strand coded RNAs (64). This could depend either on a lower stability of the L-strand polycistronic transcript (65) or on the existence of a pause site at CSB-1. According to the latter hypothesis the L-strand transcription would start at a high rate, producing the replication primer; then the RNA-polymerase should meet the pause site from where only a minority of the polymerase molecules should escape to synthesize the L-strand coded products. Transcriptional regulation at steps following the initiation is a well established mode of controlling gene expression, not only in prokaryotes (66) but also in eukaryotes. Recently, Giardina *et al.* (44) showed by *in vivo* permanganate footprinting that pausing occurred after few bases from the transcription initiation site of two *Drosophyla* heat shock genes. Krumm *et al.* (45) reported that the block to transcriptional elongation of the human c-*myc* gene, which occurs near the end of the first exon, was determined by promoter proximal pausing.

The two explanations of permanganate reactivity at CSB-1 are not necessarily in contrast with each other because both envisage a role for single-stranded DNA at CSB-1 in the transition from transcription to replication. The overall data suggest that the mtTFA binding at CSB-1 and the strong and specific permanganate sensitivity of this region are probably both part of the same signal that regulates the transition from RNA primers to nascent H-DNA chains. Moreover the results reported in this paper emphasize the potential of *in organello* footprinting with chemical reagents in analyzing at a molecular level the changes in mt DNA structure that are linked to transcriptional or replicative activity of the organelle. This technique will represent a useful approach for studying the changes in mt structure and expression observed in different physiological and pathological conditions in an *in vivo*-like environment, such as during aging or in mitochondrial diseases (67–69).

*Acknowledgments*—We thank Drs. A. Lezza, M. Roberti, P. Loguercio Polosa, and V. Petruzzella for the helpful discussion and for critical reading of the manuscript.

REFERENCES

1. Cantatore, P., and Saccone, C. (1987) *Int. Rev. Cytol.* **108,** 149–208
2. Attardi, G., and Schatz, G. (1988) *Annu. Rev. Cell Biol.* **4,** 289–333
3. Clayton, D. A. (1991) *Annu. Rev. Cell Biol.* **7,** 453–478
4. Fisher, R. P., Topper, J. N., and Clayton, D. A. (1987) *Cell* **50,** 247–258
5. Cantatore, P., and Attardi, G. (1980) *Nucleic Acids Res.* **8,** 2605–2624
6. Montoya, J., Christianson, T., Levens, D., Rabinowitz, M., and Attardi, G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7195–7199
7. Montoya, J., Gaines, G. L., and Attardi, G. (1983) *Cell* **34,** 151–159
8. Kruse, B., Narasimhan, N., and Attardi, G. (1989) *Cell* **58,** 391–397
9. Doersen, C.-J., Guerrier-Takada, C., Altman, S., and Attardi, G. (1985) *J. Biol. Chem.* **260,** 5942–5949
10. Chang, D. D., and Clayton, D. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 351–355
11. Chang, D. D., Hauswirth, W. W., and Clayton, D. A. (1985) *EMBO J.* **4,** 1559–1567
12. Schmitt, M. E., and Clayton, D. A. (1993) *Mol. Cell. Biol.* **13,** 7935–7941
13. Chu, S., Archer, R. H., Zengel, J. M., and Lindhal, L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 659–663
14. Kiss, T., and Filipowicz, W. (1992) *Cell* **70,** 11–16
15. Topper, J. N., Bennett, J. L., and Clayton, D. A. (1992) *Cell* **70,** 16–20
16. Li, K., Smagula, C. S., Parsons, W. J., Richardson, J. A., Gonzalez, M., Hagler, H. K., and Williams, R. S. (1994) *J. Cell Biol.* **124,** 871–882
17. Chang, D. D., and Clayton, D. A. (1987) *EMBO J.* **6,** 409–417
18. Chang, D. D., and Clayton, D. A. (1987) *Science* **235,** 1178–1184
19. Topper, J. N., and Clayton, D. A. (1990) *Nucleic Acids Res.* **18,** 793–799
20. Karwan, R., Bennett, J. L., and Clayton, D. A. (1991) *Genes & Dev.* **5,** 1264–1276
21. Cantatore, P., Loguercio Polosa, P., Mustich, A., and Gadaleta, M. N. (1988) *Curr. Genet.* **14,** 477–482
22. Enriquez, J. A., Lopez-Perez, M. J., and Montoya, J. (1991) *FEBS Lett.* **280,** 32–36
23. Enriquez, J. A., Perez-Martos, A., Fernandez-Silva, P., Lopez-Perez, M. J., and Montoya, J., (1992) *FEBS Lett.* **304,** 285–288
24. Enriquez, J. A., Ramos, J., Perez-Martos. A., Lopez-Perez, M., and Montoya, J. (1994) *Nucleic Acids Res.* **22,** 1861–1865
25. Gadaleta, G., Pepe, G., De Candia, G., Quagliariello, C., Sbisà, E., and Saccone, C., (1989) *J. Mol. Evol.* **28,** 497–516
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 11.31–11.33
27. Gaines, G., and Attardi, G. (1984) *Mol. Cell. Biol.* **4,** 1605–1617
28. Gaines, G., and Attardi, G. (1984) *J. Mol. Biol.* **172,** 451–466
29. Fernandez-Silva, P., Petruzzella, V., Fracasso, F., Gadaleta, M. N., and Cantatore, P. (1991) *Biochem. Biophys. Res. Commun.* **176,** 645–653
30. Lawley, P. D., and Brookes, P. (1963) *Biochem. J.* **89,** 127–138
31. Ogata, R. T., and Gilbert, W. (1979) *J. Mol. Biol.* **132,** 709–728
32. Ghivizzani, S. C., Madsen, C. S., and Hauswirth, W. W. (1993) *J. Biol. Chem.* **268,** 8675–8682
33. Ghivizzani, S. C., Madsen, C. S., Nelen, M. R., Ammini, C. V., and Hauswirth, W. W. (1994) *Mol. Cell. Biol.* **14,** 7717–7730
34. Christianson, T. W., and Clayton, D. A. (1988) *Mol. Cell. Biol.* **8,** 4502–4509
35. Kirkegaard, K., Buc, H., Spassky, H., and Wang, J. C. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 2544–2548
36. Lawley, P. D. (1966) *Prog. Nucleic Acid Res. Mol. Biol.* **5,** 89–132
37. Fisher, R. P., Parisi, M. A., and Clayton, D. A. (1989) *Genes & Dev.* **3,** 2202–2217
38. Hayatsu, H., and Ukita, T. (1967) *Biochem. Biophys. Res. Commun.* **29,** 556–561
39. Borowiec, J. A., Zhang, L., Sasse-Dwight, S., and Gralla, J. D. (1987) *J. Mol. Biol.* **196,** 101–111
40. Sasse-Dwight, S., and Gralla, J. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8934–8938
41. Kainz, M., and Roberts, J. (1992) *Science* **255,** 838–841
42. Wang, W., Carey, M., and Gralla, J. D. (1992) *Science* **255,** 450–453
43. Giardina, C., and Lis, J. T. (1993) *Science* **261,** 759–762
44. Giardina, C., Perez-Riba, M., and Lis, J. T. (1992) *Genes & Dev.* **6,** 2190–2200
45. Krumm, A., Meulia, T., Brunvand, M., and Groudine, M. (1992) *Genes & Dev.* **6,** 2201–2213
46. Sasse-Dwight, S., and Gralla, J. D. (1989) *J. Biol. Chem.* **264,** 8074–8081
47. Sasse-Dwight, S., and Gralla, J. D. (1991) *Methods Enzymol.* **208,** 146–168
48. Borowiec, J. A., and Gralla, J. D. (1986) *Biochemistry* **25,** 5051–5057
49. Shang, J., and Clayton, D. A. (1994) *J. Biol. Chem.* **269,** 29112–29120
50. Mukhopadyay, G., Carr, K., Kaguni, J. M., and Chattoraj, D. (1993) *EMBO J.* **12,** 4547–4554
51. Topper, J. N., and Clayton, D. A. (1989) *Mol. Cell. Biol.* **9,** 1200–1211
52. Virbasius, J. V., and Scarpulla, R. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1309–1313
53. Chang, D. D., Hixon, J. E., and Clayton, D. A. (1986) *Mol. Cell. Biol.* **6,** 294–301
54. Fisher, R. P., Lisowsky, T., Parisi, M. A., and Clayton, D. A. (1992) *J. Biol. Chem.* **267,** 3358–3367
55. Saccone, C., Pesole, G., and Sbisà, E. (1991) *J. Mol. Evol.* **33,** 83–91
56. Cairns. S. S., and Bogenhagen, D. F. (1986) *J. Biol. Chem.* **261,** 8481–8487
57. Desjardins P., and Morais, R. (1990) *J. Mol. Biol.* **212,** 599–634
58. Ghivizzani, S. C., Mackay, S. L. D., Madsen, C. S., Laipis, P. J., and Hauswirth, W. W. (1993) *J. Mol. Evol.* **37,** 36–47
59. King, T. C., and Low, R. L. (1987) *J. Biol. Chem.* **262,** 6204–6213
60. Gilson, E., Roberge, M., Giraldo, R., Rhodes, D., and Gasser, S. M. (1993) *J. Mol. Biol.* **231,** 293–310
61. Hsieh, D. J., Camiolo, S. M., and Yates, J. L. (1993) *EMBO J.* **12,** 4933–4944
62. Faber, S., O'Brien, R. M., Imai, E., Granner, D. K., and Chalkley, R. (1993) *J. Biol. Chem.* **268,** 24976–24985
63. Sbisà, E., Nardelli, M., Tanzariello, F., Tullo, A., and Saccone, C. (1990) *Curr. Genet.* **17,** 247–253
64. Attardi, G., Cantatore, P., Chomyn, A., Crews, S., Gelfand, R., Merkel, C., Montoya, J., and Ojala, D., (1982) in *Mitochondrial Genes* (Slonimski, P., Borst, P., and Attardi, G., eds) pp. 51–71, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
65. Gelfand, R., and Attardi, G. (1981) *Mol. Cell. Biol.* **1,** 497–511
66. Yanofsky, C. (1988) *J. Biol. Chem.* **263,** 609–612
67. Gadaleta, M. N., Petruzzella, V., Renis, M., Fracasso, F., and Cantatore, P. (1990) *Eur. J. Biochem.* **187,** 501–506
68. Wallace, D. C. (1992) *Science* **256,** 628–632
69. Di Mauro, S., and Wallace, D. C. (eds) (1993) in *Mitochondrial DNA in Human Pathology*, Raven Press, New York
70. Anderson, S., Bankier, A. T., Barrel, B. G., de Bruijin, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G., (1981) *Nature* **290,** 457–465
71. Anderson, S., de Bruijin, M. H. L., Coulson, A. R., Eperon, I. C., Sanger, F., and Young, I. G., (1982) *J. Mol. Biol.* **156,** 683–717
72. Bibb, M. J., Van Etten, R. A., Wright, C. T., Walberg, M. W., and Clayton, D. A. (1981) *Cell* **26,** 167–180

# Identification by *in Organello* Footprinting of Protein Contact Sites and of Single-stranded DNA Sequences in the Regulatory Region of Rat Mitochondrial DNA: PROTEIN BINDING SITES AND SINGLE-STRANDED DNA REGIONS IN ISOLATED RAT LIVER MITOCHONDRIA

Palmiro Cantatore, Luciana Daddabbo, Flavio Fracasso and Maria Nicola Gadaleta

*J. Biol. Chem. 1995, 270:25020-25027.*
*doi: 10.1074/jbc.270.42.25020*