THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 30, Issue of July 25, pp. 18896–18904, 1997
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Printed in U.S.A.

Functional Analysis of *in Vivo* and *in Organello* Footprinting of HeLa Cell Mitochondrial DNA in Relationship to ATP and Ethidium Bromide Effects on Transcription*

(Received for publication, December 23, 1996, and in revised form, April 14, 1997)

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***In vivo* and *in organello* footprinting techniques based on methylation interference have been utilized to investigate protein-DNA interactions in the transcription initiation and rDNA transcription termination regions of human mitochondrial DNA (mtDNA) in functionally active mitochondria. In particular, the changes in methylation reactivity of these regions in response to treatment of the organelles with ATP or ethidium bromide, which affects differentially the rates of mitochondrial rRNA and mRNA synthesis, have been analyzed. Two major sites of protein-DNA interactions have been identified in the main control region of mtDNA, both *in vivo* and *in organello*, which correspond to the regions of the light-strand promoter and heavy-strand rRNA-specific promoter. The *in organello* footprinting of the latter showed ATP- and ethidium bromide-dependent modifications that could be correlated with changes in the rate of rRNA but not of mRNA synthesis. By contrast, no ATP effects were observed on the *in organello* footprinting pattern of the termination region and on *in vitro* transcription termination, strongly suggesting that ATP control of rRNA synthesis occurs at the initiation level. Several methylation interference sites were found upstream of the whole H-strand transcription unit, pointing to possible protein-DNA interactions related to the activity of this unit. *In vivo* footprinting of the rDNA transcription termination region of human mtDNA has revealed a very strong protection pattern, indicating a high degree of occupancy of the termination site by mitochondrial transcription termination factor (~80%).**

One of the hallmarks of the compact gene organization of mammalian mtDNA is the location of most of the regulatory *cis* elements in a restricted segment adjacent to the D-loop, which contains the promoters for the transcription of the heavy (H)[1]- and light (L)-strands and the origin of replication for the H-strand (1). Mapping and kinetic analysis of *in vivo* synthesized H-strand transcripts in HeLa cells (2–5) and *in organello* studies (6–8) have indicated that the mechanism of H-strand transcription involves the activity of two overlapping, independently controlled transcription units starting at closely located sites in the main control region, one covering the rDNA region and the other the whole H-strand (9). The differential activity of the two H-strand transcription units and an H-strand transcription attenuation event at the 3′-end of the 16 S rRNA gene account for the fact that the rRNA genes and adjacent tRNA genes are transcribed to a 20 to 50 times higher rate than the downstream protein coding and tRNA genes (10). A central role in the attenuation phenomenon mentioned above is played by the mitochondrial transcription termination factor (mTERF), a DNA-binding protein that protects a 28-base pair DNA segment immediately adjacent and downstream of the 16 S rRNA/$tRNA^{Leu(UUR)}$ boundary (11–13), which comprises a tridecamer sequence critical for directing accurate termination (14).

Protein factors that bind to mtDNA in the main control region (15, 16) and at the rDNA transcription termination site (11–13) have been identified, and their interactions with DNA have been investigated by DNase I protection footprinting assays using purified components (11, 15) and, more recently, by applying on isolated organelles (17–20) and on intact cells (21, 22) the footprinting method of methylation interference (23, 24).

Previous work carried out in our laboratory with a highly efficient RNA-synthesizing system utilizing isolated HeLa cell mitochondria (6–8) has shown that, in this system, the relative transcription rates of rRNA and mRNA can be modulated independently by ATP (8) and by the intercalating drug EtBr (6). In the present work, the changes in the relative rates of rRNA and mRNA synthesis produced in response to ATP or EtBr have been correlated with the patterns of protein-DNA interactions in the transcription initiation and rDNA transcription termination regions of mtDNA, as detected by analysis of methylation interference in cultured human cells or in organelles isolated from HeLa cells. In particular, the significant ATP- and EtBr-related modifications observed in the footprint of the rRNA-specific promoter, which were specifically correlated with changes in the rate of rRNA synthesis, have given support to the model of two independently controlled overlapping H-strand transcription units (9). Furthermore, no significant ATP effect on rDNA transcription termination has been detected.

EXPERIMENTAL PROCEDURES

Cells and Culture Media—HeLa S3 cells were grown in suspension in high phosphate-containing Dulbecco's modified Eagle's medium with 5% calf serum. The human cell line 143B.TK$^-$ (25) was grown on solid substrate in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Dimethyl Sulfate (DMS) Treatment of mtDNA in Intact Cells—Samples of 10^7 to 10^8 HeLa S3 cells or 3×10^6 143B.TK$^-$ cells were routinely

* This work was supported by National Institutes of Health Grant GM-11726 (to G. A.) and scholarships from the Spanish Ministry of Education (to V. M. and P. F.-S.). 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.

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[1] The abbreviations used are: H, heavy; L, light; DMS, dimethyl sulfate; mTERF, mitochondrial transcription termination factor; mTFA, mitochondrial transcription factor A; EtBr, ethidium bromide; MOPS, 4-morpholinepropanesulfonic acid.

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used. The methylation reaction, cell lysis, and total nucleic acid extraction were carried out as described previously (21). The yield of total nucleic acids was 0.4 to 4.0 mg from each HeLa cell sample and ~200 μg from each 143B.TK$^-$ cell sample.

In Organello Methylation of mtDNA and RNA Synthesis—The mitochondrial fraction was isolated from 1.5 to 3.0 $\times$ 10^8 HeLa cells, essentially as described previously (8), resuspended in incubation buffer (40 mM Tris-HCl, pH 7.4, 25 mM NaCl, 5 mM $MgCl_2$, 10% glycerol) and washed three times; the final pellet was then resuspended, at ~2 mg/ml, in incubation buffer containing 1 mg/ml bovine serum albumin, 10 mM NaH_2PO_4, 2.5 mM pyruvate, and the desired ATP or EtBr concentration. For each incubation condition tested, two 500-μl samples of the mitochondrial suspension were transferred into Eppendorf tubes, and to one of them, destined for analysis of RNA synthesis, 10 μCi of [α-^{32}P]UTP (400 Ci/mmol) was added. One of the two tubes was incubated at 37 °C under shaking for 20 min and the other for 30 min, and then DMS treatment or, respectively, RNA extraction was performed on the two samples, as detailed below. In particular, for DMS treatment, 26 μl of a freshly prepared 2% DMS solution in water was added to each mitochondrial suspension incubated for 20 min and allowed to react for 2 min at 37 °C, and then 900 μl of ice-cold phosphate-buffered saline was added, and the mitochondrial fraction was pelleted at 12,000 $\times$ *g* for 1 min. The DMS-treated mitochondrial fraction was washed three times by centrifugation and resuspension in 1 ml of ice-cold phosphate-buffered saline, resuspended in 400 μl of proteinase K buffer (10 mM Tris-HCl, pH 7.5 (25 °C), 0.2 M NaCl, 0.1% SDS, 0.1 mg/ml proteinase K) by vortexing, and incubated at room temperature for 30 min. The sample was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and the nucleic acids were ethanol-precipitated as previously detailed for *in vivo* DMS-treated samples (21). For each set of *in organello* footprinting experiments, a sample of the mitochondrial fraction was treated identically, except for the omission of DMS treatment, and DNA extracted from it for subsequent *in vitro* DMS treatment.

For analysis of the *in organello* transcription products, after the 30 min incubation, the mitochondrial samples were pelleted at 12,000 $\times$ *g* for 1 min, washed, dissolved in proteinase K buffer, and digested for 10 min at 37 °C. The samples were then phenol-extracted and ethanol-precipitated; the RNA was electrophoresed on a 2.2 M formaldehyde, 1.4% agarose gel using MOPS buffer (20 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0, at 25 °C), and the RNA bands were quantified, after drying the gel, using a PhosphorImager screen and ImageQuant software (Molecular Dynamics).

In Vitro DMS Treatment of Naked DNA—The DNA methylation was carried out on pellets containing about 200 μg of total nucleic acids deriving from non-DMS-treated cells or 5–10 μg of nucleic acids from untreated mitochondrial fractions as described elsewhere (21).

Piperidine Cleavage of DNA—This was performed on pellets containing approximately 200 μg of total nucleic acids from DMS-treated cells or 5–10 μg of total nucleic acids from DMS-treated mitochondrial fractions and on equivalent samples of *in vitro* DMS-treated total nucleic acids from cells or isolated mitochondria, as described previously (21).

Primer Extension of DMS-treated DNA—The following oligodeoxynucleotides, designated according to the numbering system of the Cambridge sequence (26), were used for primer extension of DMS-treated DNA in the assay as follows: L315, 5′-CGCTTCTGGCCACAG-CAC-3′; L476, 5′-CTAATCTCATCAATACAACC-3′; H495, 5′-GGTTG-TATTGATGAGATTAG-3′; and H719, 5′-CTCACTGGAACGGGGATG-3′ for the fingerprinting of the initiation region; and L3064, 5′-ATCT-GAGTTCAGACCGG-3′; and H3360, 5′-TGCCATTGCGATTAGAATGG-3′ for the fingerprinting of the rDNA transcription termination region. The oligodeoxynucleotides were 5′-end-labeled with [γ-^{32}P]ATP as detailed previously (21).

Five μg of DMS-treated and piperidine-cleaved total DNA from cells (containing approximately 0.005 pmol of mtDNA) or 0.2–0.4 μg of similarly treated DNA from the isolated mitochondrial fractions were used as a template for primer extension analysis. Polymerase chain reaction amplification and electrophoresis of the products through 6% polyacrylamide, 7.7 M urea sequencing gels in Tris borate/EDTA buffer were carried out as described previously (21).

DNA Binding Assays—The DNA binding activity of mTERF was determined by mobility shift assays using the double-stranded 44-mer oligodeoxynucleotide probe described previously (11). A constant amount of mitochondrial lysate protein was incubated with 10 fmol of probe in the presence of the indicated concentrations of ATP, and the samples were then run on a native 5% polyacrylamide gel, as detailed elsewhere (27).

In Vitro Transcription Termination and S1 Protection Assays—The pTER plasmid, used as a template for the transcription termination assays, has been previously described (11), as has been the clone BSAND, utilized to synthesize the RNA probe employed in the S1 protection assays (12, 27). The transcription termination reactions and the S1 protection assays were performed as detailed previously (12, 27); quantification of the S1-resistant products was carried out by laser densitometry of the autoradiogram.

RESULTS

Synthesis of RNA in Isolated HeLa Cell Mitochondria—Mitochondria isolated from HeLa cells were incubated in the presence of [α-^{32}P]UTP in the appropriate buffer, as detailed under "Experimental Procedures." It has been previously shown that HeLa cell mitochondria, under these conditions, are able to carry out RNA synthesis in a way closely resembling the *in vivo* process (6–8). In particular, transcription has been shown to start at the two H-strand initiation sites (H_1 for the rDNA transcription unit, and H_2 for the whole H-strand transcription unit) and at the single L-strand initiation site, and the transcripts have been shown to be processed in a way reproducing the *in vivo* patterns; however, the relative rates of rRNA synthesis and polyadenylation are somewhat reduced in this *in vitro* system, as compared with the *in vivo* situation (7).

To determine the optimum time of DMS treatment for *in organello* footprinting during mitochondria incubation, time course and time interval pulse-labeling experiments were carried out. Fig. 1*a* shows the electrophoretic patterns in an agarose-formaldehyde gel of RNA labeled with [α-^{32}P]UTP in isolated HeLa cell mitochondria, in the presence of 1 mM ATP, after different times of incubation or after different time interval pulses. Fig. 1, *b* and *c*, shows the radioactivity incorporated into total mitochondrial RNA, measured as overall signal from gel-separated RNA bands scanned with a PhosphorImager, in the continuous incorporation and, respectively, time interval pulse-labeling experiments. From Fig. 1*b* it appears that the accumulation of label into total mitochondrial RNA proceeds at a fairly constant rate for 30 min at 37 °C. On the other hand, the time interval pulse-labeling experiments, which tend to exclude a possible role in the observed kinetics of changes in RNA turnover rate during *in vitro* incubation, indicate that the maximum rate of incorporation of radioactivity occurs between 10 and 30 min after the beginning of incubation and suggest a fairly constant rate of incorporation up to 45 min. On the basis of these experiments, DMS treatment for *in organello* footprinting was carried out 20 min from the start of incubation, and the synthesized RNA was isolated at 30 min.

In Vivo and in Organello Footprinting of the L-Strand and H-Strand Transcription Promoter Regions of HeLa Cell and 143B.TK$^-$ Cell mtDNA—Fig. 2*a* shows the *in vivo* methylation patterns of the H-strand in the L-strand transcription promoter region of HeLa cell and 143B.TK$^-$ cell mtDNA and the *in organello* methylation pattern of the H-strand in the same region of HeLa cell mtDNA (*lanes E*). By comparing these patterns with those generated by DMS treatment of naked DNA isolated from total cells or mitochondria (*lanes N*), one recognizes regions of altered reactivity to DMS. In particular, in the region around the L-strand initiation site (at position ~407), one H-strand residue (nucleotide 409), exhibits hypermethylation in the *in vivo* footprinting pattern, whereas five H-strand residues (nucleotides 401, 403, 408, 409, and 410) exhibit methylation protection or hypermethylation in the *in organello* footprinting pattern. In the region spanning nucleotides 418 to 447 upstream of the L-strand transcription initiation site, which corresponds to the binding site of the mitochondrial transcription factor A (mTFA) (15), three H-strand residues (nucleotides 427, 437, and 438) (Fig. 2*a*) and one L-strand residue (nucleotide 429) (not shown) exhibit methylation protection, and one L-strand residue (nucleotide 443) (not shown) hypermethylation in the *in vivo* footprinting pattern. In

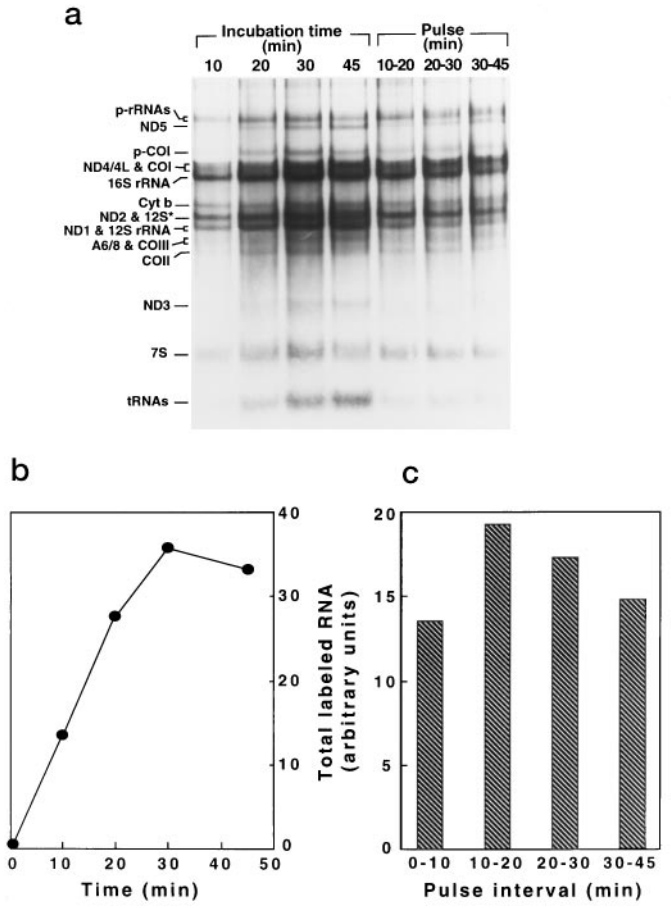

FIG. 1. **Labeling of RNA in isolated HeLa cell mitochondria during continuous exposure or after different time interval exposures to [α-^{32}P]UTP.** *a*, autoradiogram of formaldehyde-agarose gel displaying RNA samples labeled for different times or after different time interval pulses. *b* and *c*, quantification of total radioactivity incorporated into RNA, as determined from the overall signal obtained by PhosphorImager scanning of the individual bands in the gel in the continuous exposure experiment (*b*) or in the different time interval pulse experiment (*c*). *p-rRNAs*, rRNA precursors (4); *12 S**, 12 S rRNA precursor (7); *7 S*, 7 S RNA; *pCOI*, COI mRNA precursor (43); *COI, COII,* and *COIII*, mRNAs for cytochrome *c* oxidase subunits; *ND1, ND2, ND3, ND4, ND4L,* and *ND5*, mRNAs for subunits of the respiratory chain NADH dehydrogenase; *Cyt b*, mRNA for apocytochrome *b*; *A6* and *A8*, mRNAs for subunits ATPase 6 and ATPase 8 of the H^+-ATPase.

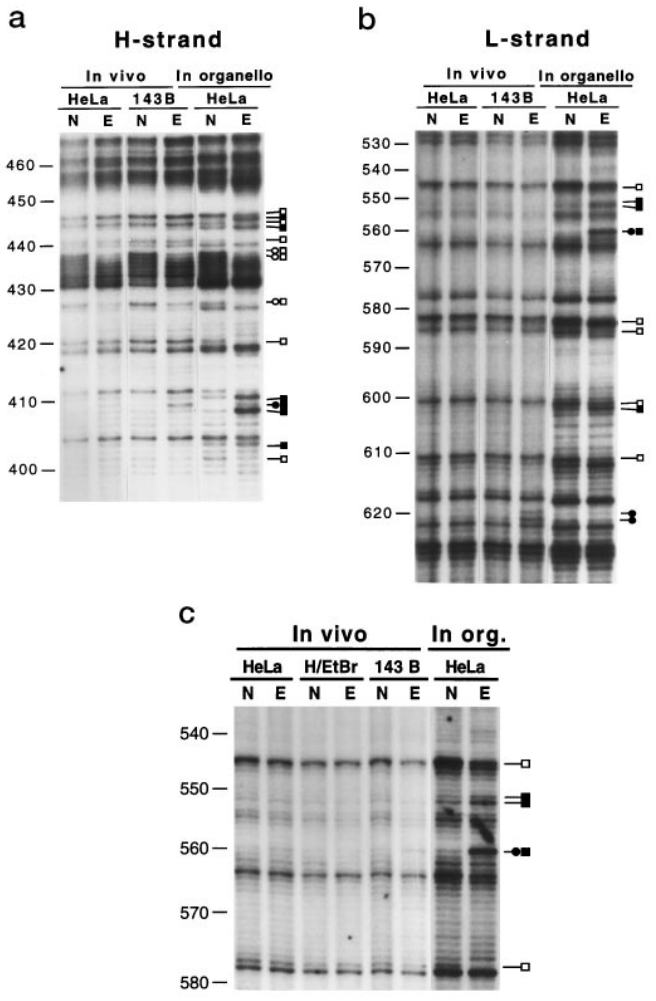

FIG. 2. ***In vivo* and *in organello* footprinting patterns of human mtDNA L-strand (*a*) and H-strand (*b* and *c*) transcription promoter regions.** *c* shows the same samples of *b*, after a longer electrophoretic run, as well as the primer extension products of *in vivo* DMS-treated total DNA from HeLa cells pretreated overnight with 0.125 μM EtBr (*H/EtBr*). ○, *in vivo* protected bands; ●, *in vivo* hypermethylated bands; □, *in organello* protected bands; ■, *in organello* hypermethylated bands. *N*, naked DNA; *E*, experimental DNA.

the *in organello* footprinting pattern, eight H-strand residues (420, 427, 437, 438, 441, 444, 445, 446, and 447) (Fig. 2*a*) and two L-strand residues (429 and 443) (not shown) exhibit methylation protection or hypermethylation in the same region. In both the sequence immediately surrounding the L-strand transcription initiation site and in the mTFA binding domain there is a reasonable correspondence between the residues exhibiting protection or hypermethylation in the *in vivo* and in the *in organello* footprinting (~30 and 50% correspondence of residues showing methylation interference in the two regions). Particularly noteworthy is the partial correspondence of hypermethylated nucleotides *in vivo* and *in organello* immediately upstream of the L-strand transcription initiation site.

Fig. 2*b* shows the *in vivo* methylation patterns of the L-strand in the regions of the H1 and H2 H-strand transcription initiation sites of HeLa cell and 143B.TK$^-$ cell mtDNA and the *in organello* methylation pattern of the L-strand in the same regions of HeLa cell mtDNA. The *in vivo* and *in organello* methylation interference patterns in the L-strand of the region of the rRNA-specific H-strand transcription initiation site H1 (at position ~559) and of the adjacent regions is shown with a greater resolution in Fig. 2*c* after a longer electrophoretic run of the same samples used in the experiment of Fig. 2*b*. In particular, in the segment immediately surrounding the H1 initiation site, one L-strand residue (nucleotide 560) exhibits a hypermethylation in the *in vivo* footprinting pattern (Fig. 2, *b* and *c*); in the *in organello* footprinting pattern, the same residue exhibits a much stronger hypermethylation (Fig. 2, *b* and *c*), whereas one H-strand residue (nucleotide 557) shows methylation protection (not shown). In the mtDNA segment spanning nucleotides 524–546 upstream of the H_1 transcription initiation site, which corresponds to the mTFA binding site (15), a single L-strand residue (nucleotide 545) exhibits methylation protection in the *in organello* footprinting pattern (Fig. 2, *b* and *c*). Within the segment between the mTFA binding site and the H_1 initiation site, two additional L-strand residues (nucleotides 551 and 552) exhibit hypermethylation in the *in organello* pattern (Fig. 2, *b* and *c*). Furthermore, in the segment immediately downstream of the H_1 start site, three L-strand residues (nucleotides 577, 583, and 586) (Fig. 2, *b* and *c*) and two H-strand residues (nucleotides 568 and 575) (not shown) exhibit methylation protection in the *in organello* pattern.

The protection or hypermethylation phenomena described above appear to occur at positions of known protein-DNA in-

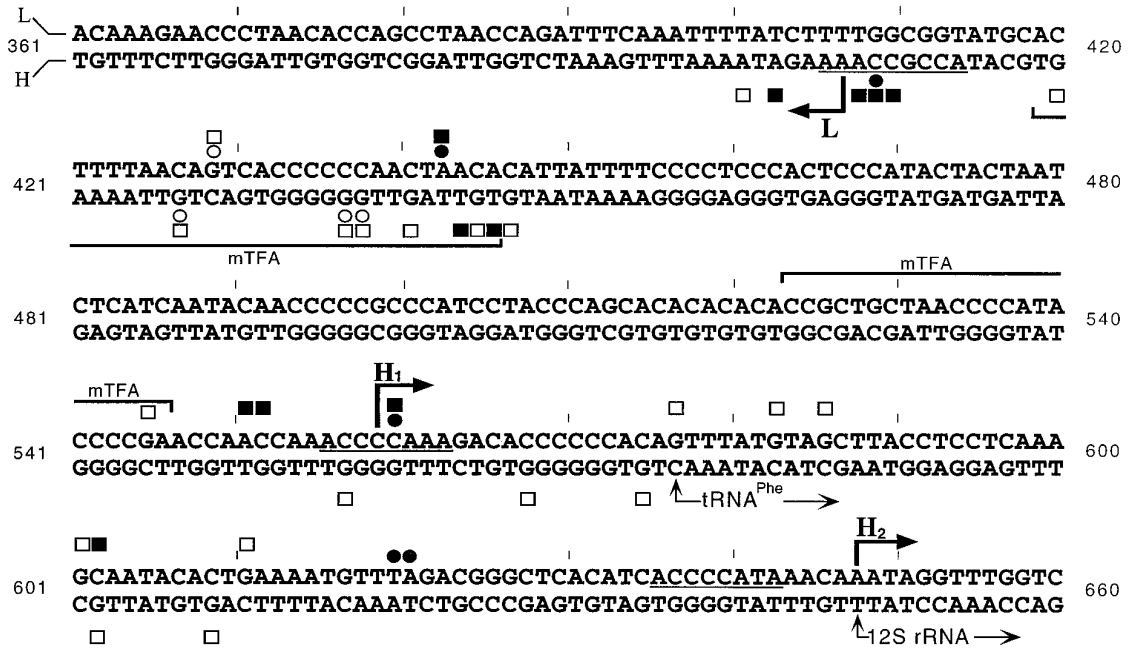

FIG. 3. **Summary of *in vivo* and *in organello* methylation interference patterns of human mtDNA L-strand and H-strand transcription promoter regions.** *Symbols* are as in Fig. 2. *L*, L-strand; H_1 and H_2, H-strand transcription start sites; *underlined* are the octanucleotide motifs at or near these sites.

teractions (like those involving mTFA) or at potential sites of similar interactions (involving RNA polymerase or transcription factor(s)) at or near the RNA synthesis start positions. An additional pattern of altered methylation reactivity was observed in the L-strand in the segment spanning nucleotides 601–621 within the $tRNA^{Phe}$ gene, located about 40 nucleotides downstream of the H_1 transcription initiation site at position ~559. This mtDNA segment lies 27 nucleotides upstream of the second H-strand transcription initiation site (H_2 in Fig. 3) near the 5′-end of the 12 S rRNA gene. In particular, two L-strand residues (nucleotides 620 and 621) are hypermethylated in the *in vivo* footprinting (Fig. 2*b*), and three L-strand residues (nucleotides 601, 602, and 611) (Fig. 2*b*) and two H-strand residues (nucleotides 602 and 609) (not shown) exhibit altered methylation reactivity in the *in organello* pattern. Fig. 3 summarizes the *in organello* and *in vivo* methylation reactivity pattern of the L-strand and H-strand transcription promoter regions.

Effect of ATP and EtBr on in Vivo and/or in Organello Footprinting of the Promoter Regions—It has been previously shown that the ATP requirements for rRNA synthesis and mtDNA L-strand transcription in isolated HeLa mitochondria are markedly different from those for mRNA synthesis (8). To investigate whether ATP had any effect on the *in organello* footprinting patterns, which could be correlated with changes in transcription, isolated HeLa cell mitochondria were incubated in different concentrations of ATP, ranging from 0 to 10 mM, and DMS treatment was performed 20 min after the beginning of incubation. With due account for some differences in the amount of sample loaded, no significant change in the *in organello* footprinting pattern corresponding to the H-strand (Fig. 4*a*) or L-strand (not shown) of the L-strand promoter was observed in mtDNA from mitochondria incubated in different ATP concentrations. By contrast, a clear-cut ATP-dependent change was detected in the footprinting pattern of the H-strand rDNA-specific promoter. In particular, the hypermethylated L560 band exhibited a marked increase in intensity after addition of ≥0.25 mM ATP to the medium, reaching a maximum between 1 and 5 mM, and declining at higher ATP concentrations (Fig. 4*b*). Since the L560 band showed the most evident change in intensity in relationship to ATP concentration, it was utilized as an indicator of modifications in putative protein-DNA interactions in subsequent experiments. It should be noticed that the *in organello* methylation patterns obtained in the presence of 1 mM ATP for the L-strand of the rDNA-specific H-strand promoter and the H-strand of the L-strand promoter, which are shown in Fig. 4, *a* and *b*, are substantially identical to those observed in the independent experiment illustrated in Fig. 2, *a* and *b*, indicating the reproducibility of the in *organello* patterns.

Fig. 4*c* shows the patterns in a formaldehyde-agarose gel of RNA labeled in isolated HeLa cell mitochondria in the presence of different concentrations of ATP. It is clear that, in the absence of added ATP, the mRNA species are labeled to a substantial extent, whereas there is only marginal labeling of the rRNA species; by contrast, addition of ATP at ≥0.25 mM strongly stimulates rRNA synthesis, which reaches a maximum (including the rRNA precursors) at about 1 mM, with only a moderate increase in mRNA synthesis. Fig. 4*d* correlates the ATP-related changes in intensity of the L560 band with the variations of the *in organello* rate of synthesis of 16 S rRNA and mitochondrial mRNAs (ND5, ND4/4L, COI, Cytb, A6/8, COIII, and COII). It is clear that the changes in level of methylation of the L560 band follow much more closely the modifications in rate of synthesis *in organello* of rRNA than the changes in mRNA synthesis.

It has been previously shown (6) that, in isolated HeLa cell mitochondria, intercalating drugs, like EtBr, inhibit preferentially rRNA synthesis over mRNA synthesis. Such behavior is clearly shown in the experiment illustrated in Fig. 4*e*. To investigate the possible occurrence of EtBr-induced changes in the *in organello* footprinting patterns of the L-strand and H-strand transcription promoter regions, isolated HeLa cell mitochondria were incubated in the appropriate buffer containing 1 mM ATP in the presence of different concentrations of the drug and then DMS-treated 20 min after the beginning of incubation. A progressive decrease in methylation interference of the H-strand footprinting pattern of the L-strand transcrip-

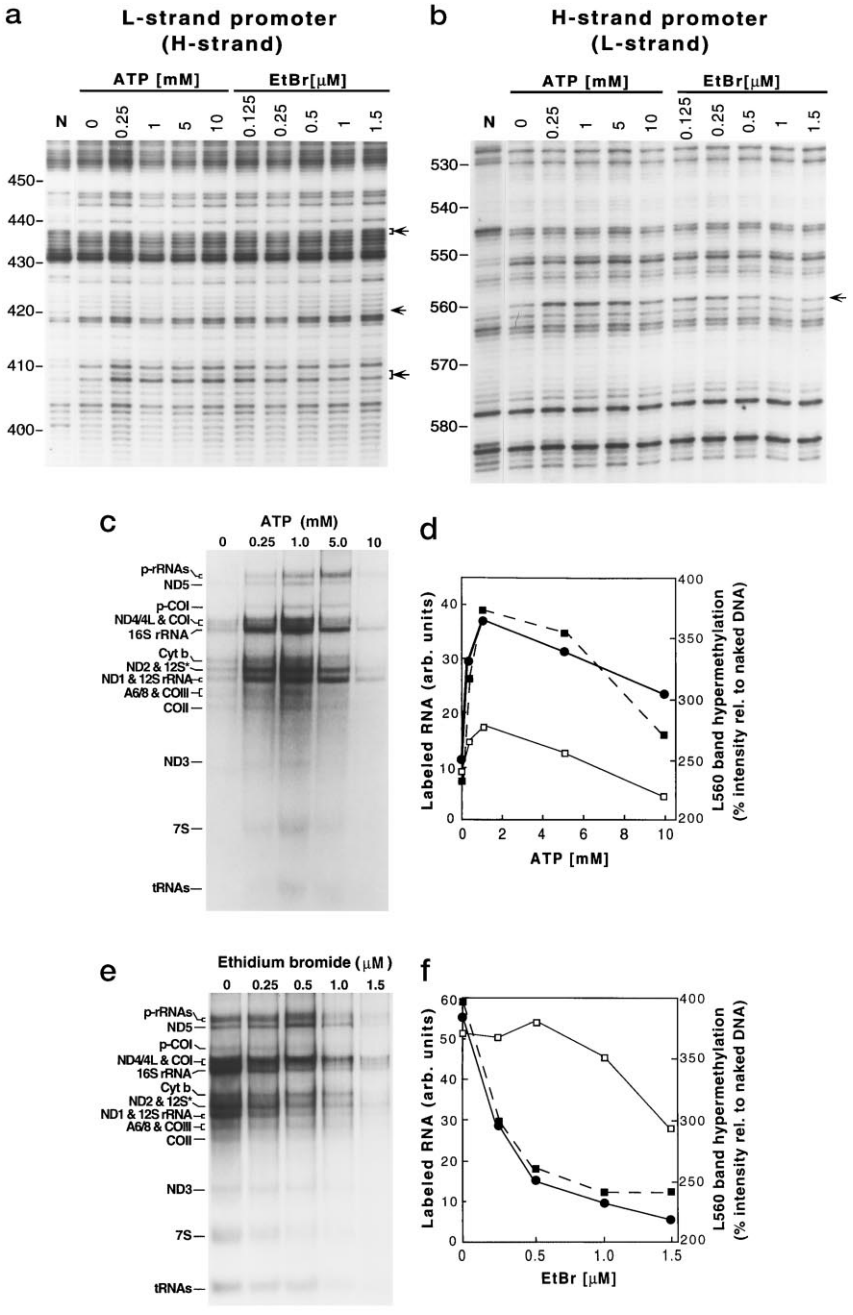

FIG. 4. **Effects of increasing concentrations of ATP and EtBr on the *in organello* footprinting patterns of the H-strand of the L-strand promoter region and of the L-strand of the H-strand promoter region, and on RNA synthesis during incubation of isolated HeLa cell mitochondria.** *a* and *b*, footprinting patterns of H-strand of L-strand promoter region (*a*) and of L-strand of H-strand promoter region (*b*) (*N*, naked DNA). *c* and *e*, autoradiograms of formaldehyde-agarose gels displaying RNA samples labeled in isolated mitochondria for 30 min in the presence of the indicated concentrations of ATP (*c*) or EtBr (*e*). *Symbols* are as in Fig. 1. *d* and *f*, quantification by PhosphorImager analysis of the L560-hypermethylated band (from *b*), expressed as percent relative to the value for the naked DNA control, and of the 16 S rRNA band and mRNA bands (ND5, ND4/4L, COI, Cytb, A6/8, COIII, and COII) (from *c* and *e*), expressed in arbitrary units. ■, L560 band; ●, 16 S rRNA; □, mRNAs.

tion promoter (at the sites indicated by *arrows* in Fig. 4*a*) and of the L-strand footprinting pattern of the same promoter (not shown) was observed with increasing EtBr concentrations. In the H-strand transcription promoter region, the hypermethylated band L560 again exhibited the most pronounced changes, in relationship to the EtBr concentration, decreasing progressively in intensity with increasing doses of the drug (Fig. 4*b*). That these changes reflected a phenomenon occurring *in vivo* was shown by the finding that a clear decrease in the hypermethylation of L560 was also observed by *in vivo* footprinting in HeLa cells exposed to 0.125 μM EtBr (Fig. 2*c*). Fig. 4*f* correlates the changes in intensity of the L560 band in the footprinting patterns obtained from isolated HeLa cell mitochondria incubated in the presence of different EtBr concentrations with the variations in labeling of 16 S rRNA and mRNAs (ND5, ND4/4L, COI, Cytb, A6/8, COIII, and COII) synthesized *in organello* under the same conditions. It is apparent that the intensity of the L560 band and the amount of labeled 16 S rRNA decrease in a parallel way over the range of EtBr concentrations utilized, whereas the labeling of the mRNAs remains essentially constant in the presence of EtBr up to 1 μM and decreases moderately at 1.5 μM.

In Vivo and in Organello Footprinting of the Mitochondrial rDNA Transcription Termination Region of HeLa and 143B.TK⁻ Cells and Effects of EtBr—Fig. 5, *a* and *b* (panels labeled *HeLa*), shows the *in vivo* methylation patterns of the L-strand and, respectively, H-strand of the HeLa cell mitochondrial rDNA transcription termination region, which is the site of interaction of mTERF with DNA (11, 12). The mtDNA segment extending from nucleotide 3235 to nucleotide 3250, which is entirely comprised within the region protected by mTERF from DNase I digestion, exhibits several sites of methylation protection or hypermethylation in the *in vivo* footprinting pattern. In particular, three L-strand residues (nucleotides 3235, 3236, and 3244) are hypermethylated, and five L-strand residues (nucleotides 3238, 3239, 3242, 3248, and 3249) and three

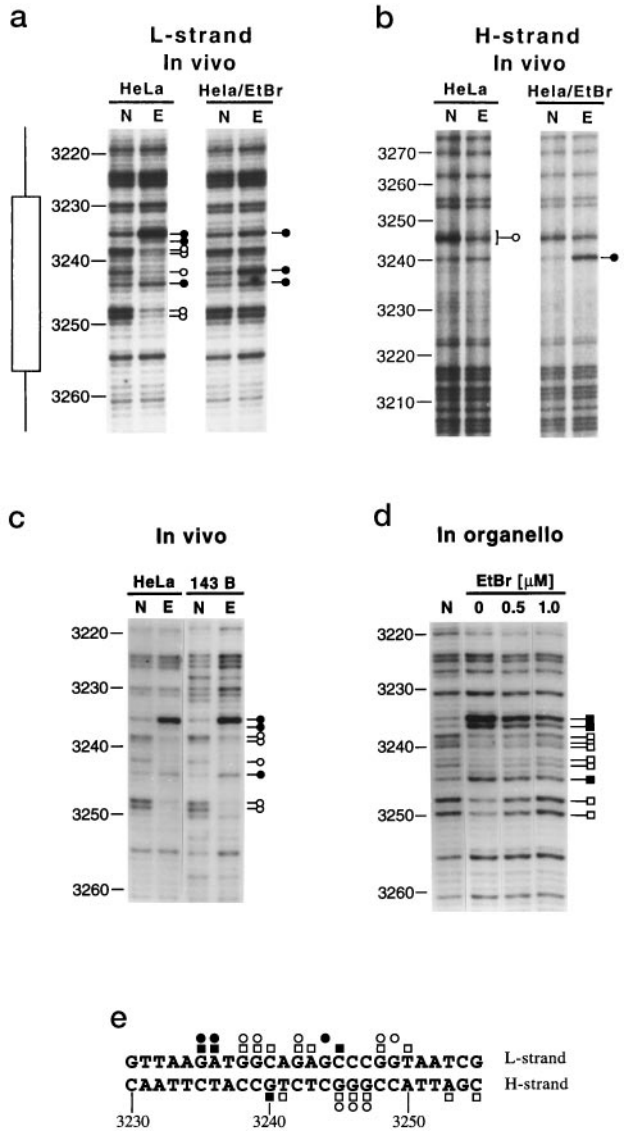

FIG. 5. ***In vivo* and *in organello* footprinting of the HeLa cell mitochondrial rDNA transcription termination region and effects of EtBr.** *a* and *b*, *in vivo* methylation interference patterns of the L-strand (*a*) and H-strand (*b*) of the termination region from untreated HeLa cells (*HeLa*) or from cells treated overnight with 0.125 μM EtBr (*HeLa/EtBr*). *c*, *in vivo* methylation interference patterns of the L-strand of the termination region in a repeat independent experiment with HeLa cells as in *a* and in an experiment with 143B.TK⁻ cells. *d*, effects of increasing concentrations of EtBr on the *in organello* footprinting pattern of the L-strand of the termination region from HeLa cells. The *symbols* represent the methylation interference pattern of the sample not treated with EtBr. *e*, summary of *in vivo* (from *a* and *b*) and *in organello* (from the pattern for 0 EtBr in *d* and the pattern for 1 mM ATP in Fig. 6*b*) methylation interference patterns of L-strand and H-strand of the mitochondrial rDNA transcription termination region. *Symbols* are as in Fig. 2.

H-strand residues (nucleotides 3245, 3246, and 3247) exhibit methylation protection. Fig. 5*c* shows the *in vivo* footprinting pattern of the L-strand of the termination region in HeLa cells, in a repeat independent methylation experiment, and in 143B.TK⁻cells. It is apparent that the *in vivo* footprinting pattern is quite reproducible.

To analyze the effect of EtBr on the *in vivo* footprinting of the mitochondrial rDNA transcription termination region, HeLa cells were pretreated overnight with the drug at 0.125 μM. As shown in Fig. 5, *a* and *b* (panels labeled *HeLa/EtBr*), the methylation interference pattern of the binding site of mTERF changed dramatically under these conditions. Among the L-strand residues which were affected in their methylation in the absence of the drug (Fig. 5*a, HeLa*), only three exhibited methylation interference in the ethidium bromide-treated cells. In particular, L-strand residues 3235 and 3244 remained hypermethylated, and the protected L-strand nucleotide 3242 became hypermethylated (Fig. 5*a, HeLa/EtBr*). The protection of H-strand residues 3245, 3246, and 3247 (Fig. 5*b, HeLa*) all but disappeared in the presence of EtBr. By contrast, the H-strand residue 3240 became strongly hypermethylated (Fig. 5*b, HeLa/EtBr*). It appears that the *in vivo* methylation interference pattern of the rDNA transcription termination region, after treatment of the cells with a low concentration of EtBr, became in general more similar to the pattern obtained with naked DNA. This effect pointed to a decrease, and probably a modification, in the binding of mTERF to mtDNA, which presumably caused a reduction in its transcription termination activity.

To investigate the effect of EtBr on the in organello footprinting of the HeLa cell rDNA transcription termination region, isolated organelles were exposed to various concentrations of the drug. Fig. 5*d* shows the *in organello* methylation patterns of the L-strand of that region. The naked DNA methylation pattern in the segment between nucleotides 3230 and 3250 shows a general similarity to that obtained for DNA extracted from whole cells (Fig. 5, *a* and *c*). However, there are some differences in the identity, type of methylation interference, and relative abundance of the methylated residues, as compared with the pattern obtained for naked DNA extracted from whole cells. These differences are possibly related to changes in the structure of mtDNA (conversion of supercoiled to open circular form?), that may have occurred during the isolation and incubation of the organelles and thereby affected the methylation efficiency of DMS or possibly the pausing of *Taq* polymerase (20). Despite these differences in the reference DNA pattern, the *in organello* methylation interference pattern obtained after incubation of the organelles in the absence of EtBr (*i.e.* under the standard conditions for in *organello* footprinting) (Fig. 5*d*) shows a substantial similarity to the *in vivo* pattern, with ~50% correspondence of the residues exhibiting protection or hypermethylation. Likewise, the footprinting pattern obtained in the absence of EtBr for the H-strand of the termination region is reasonably similar to the *in vivo* pattern (not shown).

Fig. 5*e* summarizes the *in vivo* DMS reactivity pattern, as well as the *in organello* footprinting pattern, of the rDNA transcription termination region. The percentage of protection at several nucleotides, after correction for the difference in loading of the lanes, ranged from 60% (nucleotide 3242) to 82% (nucleotides 3248 and 3250).

After exposure of the isolated mitochondria to 0.5 and 1.0 μM EtBr, there is a progressive decrease in the hypermethylation of L-strand residues 3235, 3236, and 3245 and a progressive decrease in the protection of L-strand residues 3238, 3242, 3243, 3248, and 3250 (Fig. 5*d*). As a result of these changes, the methylation pattern of the L-strand tends to become more similar to that of the naked DNA, as already observed for the *in vivo* footprinting in the presence of EtBr (Fig. 5*a*). This tendency, therefore, also points to a decreased binding of mTERF to mtDNA in the presence of EtBr.

Lack of ATP Effects on in Organello Footprinting of HeLa Cell Mitochondrial rDNA Transcription Termination Region— The experiments described in a previous section, which correlated the effects of ATP on the *in organello* footprinting of the H-strand transcription promoter region with those on the synthesis of RNA in isolated organelles (Fig. 4*d*), did not exclude the possibility that ATP acted at the level of termination of

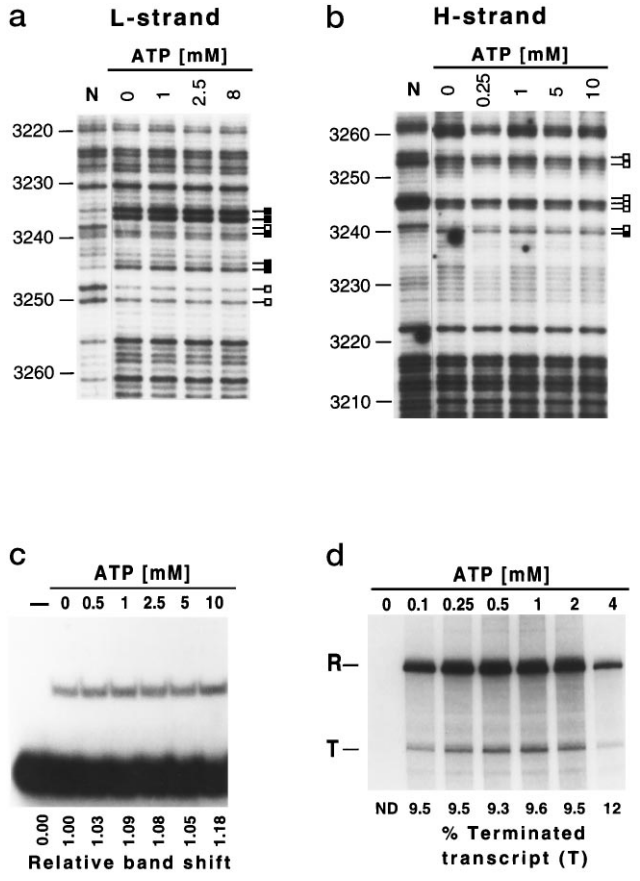

FIG. 6. **ATP effects on *in organello* footprinting of HeLa cell mitochondrial rDNA transcription termination region and on *in vitro* mTERF binding to DNA and termination promoting activity.** *a* and *b*, *in organello* methylation interference patterns of L-strand (*a*) and H-strand (*b*) of HeLa cell mtDNA in the presence of different concentrations of ATP (*N*, naked DNA). *Symbols* as in Fig. 2. *c*, effect of ATP on *in vitro* mTERF binding to DNA (—, no mTERF present). *d*, transcription termination *in vitro* (*R*, run-off transcripts; *T*, terminated transcripts; *ND*, not detectable).

mitochondrial rDNA transcription. To obtain evidence on this possibility, the ATP effects on *in organello* footprinting of the termination region were analyzed. Fig. 6, *a* and *b*, illustrates the *in organello* methylation patterns of the L-strand and, respectively, H-strand of the termination region after incubation of the organelles in the presence of various concentrations of ATP. It should be noted that the L-strand pattern obtained in the presence of 1 mM ATP (*i.e.* under our standard conditions of *in organello* footprinting) is almost identical to that observed in the independent methylation experiment in the absence of EtBr shown in Fig. 5*d*, pointing again to the reproducibility of the *in organello* methylation interference pattern. The most significant result of the experiments shown in Fig. 6, *a* and *b*, is the absolute identity of the methylation patterns obtained for both the L- and H-strands after incubation of the organelles in the presence of concentrations of ATP varying between 0 and 8–10 mM. These observations argue against the possibility of an ATP effect at the level of transcription termination.

Lack of ATP Effects on in Vitro mTERF Binding to DNA and Transcription Termination—Further evidence against a role of ATP in transcription termination was provided by experiments in which the effects of ATP on *in vitro* mTERF binding to DNA (Fig. 6*c*) and on *in vitro* transcription termination (Fig. 6*d*) were analyzed. As shown in Fig. 6*c*, mTERF binding to DNA, as measured by band shift assays, was only marginally increased in the presence of ATP concentrations from 0.5 to 5.0 mM, as compared with no ATP, and slightly more at 10 mM ATP. As illustrated in Fig. 6*d*, the proportion of terminated transcripts in an *in vitro* reaction remained about 10% over the whole range of ATP concentrations from 0.1 to 4 mM. Concentrations higher than 4 mM inhibited almost completely the transcription activity in our *in vitro* system (42).

DISCUSSION

Technical Aspects—DMS is known to methylate guanine residues at the N-7 position and, with a lower frequency, adenine at positions N-3 and N-7. It can also methylate adenine at position N-1 and cytosine at position N-3 in single-stranded DNA (28). Thymine can also be methylated at N-3 in single-stranded DNA, but, being a much weaker base than cytosine, its alkylation would be expected to occur in alkaline solution; however, there are reports of alkylation of thymine at neutral pH (28).

In the present work, although the majority of methylation reactivity changes were observed at purine sites, a few pyrimidines also showed an altered methylation pattern, in particular, in almost all cases, a hypermethylation. The methylation of cytosine residues *in vivo* or *in organello* could be explained by the occurrence of single-stranded DNA segments at those sites as a result of helical distortions caused by protein binding (20, 29–31) or by RNA polymerase pauses (32, 33). However, since piperidine does not cleave effectively the DNA chains at the methylated cytosine sites, it is likely that *Taq* polymerase pauses created fragments ending at those sites. Effective termination of primer extension by *Taq* polymerase at modified bases has been recently demonstrated (20). In the case of thymine, it seems possible that the alkaline environment of the mitochondrial matrix would favor the methylation of this base. In previous work on *in organello* footprinting of mammalian mtDNA by DMS interference, evidence was reported for the presence of methylated thymine residues in single-stranded mtDNA segments (20), as well as for methylated cytosine residues (18).

In the present study, the *in organello* footprinting patterns of the transcription initiation and rDNA transcription termination regions of human mtDNA showed a substantial similarity to the *in vivo* footprinting patterns, as concerns the location of the mtDNA segments exhibiting methylation interference. These included, in particular, the L-strand and H_1 H-strand transcription start sites, the mtDNA regions upstream of the L-strand and of the H_2 H-strand transcription start sites, and the transcription termination region. However, in each segment, there were differences in the identity, type of methylation interference, and relative abundance of residues exhibiting protection or hypermethylation. The reason for these differences is not known, but they were quite reproducible in independent methylation experiments. They probably reflect small variations in the protein-DNA contact sites due to DNA or protein conformational changes. These may occur during the isolation or incubation of mitochondria as a result of modifications in the internal environment of the organelles or of the presence of additional components interacting with DNA or with the DNA-binding factors (11, 34). Despite these variations, it is reasonable to assume that the fundamental protein-DNA interactions underlying the methylation reactivity of mtDNA in isolated organelles reflect those occurring *in vivo*. This conclusion is supported by the close similarity between the RNA synthesis pattern obtained, under the same conditions utilized for *in organello* footprinting, in mitochondria isolated from HeLa cells, and the *in vivo* pattern (6–8, and the present work), and by the comparable EtBr effects on the methylation interference patterns *in vivo* and *in organello* in the mtDNA transcription initiation and termination regions.

Evidence for Protein-DNA Interactions at the L-strand and

H_1 H-strand Transcription Start Sites—In previous work, the *in organello* footprinting patterns, as determined by methylation interference, of the promoter regions of mtDNA have been analyzed in mitochondria from bovine brain (17), human placenta (18), and rat liver (20). Methylation reactivity changes were observed in the mtDNA region upstream of the L-strand transcription start site and other changes, although considerably weaker, in the region upstream of the H_1 H-strand transcription start site; these regions correspond to the mTFA binding domains (15). The observations reported here on the *in vivo* and *in organello* footprinting of the L-strand promoter region agree with the above-mentioned findings. By contrast, almost no nucleotide with altered methylation reactivity was found in the mTFA binding region of the H_1 H-strand promoter. However, it should be noted that the present results are consistent with the evidence indicating a much lower affinity of mTFA for binding *in vitro* at the H_1 H-strand promoter, as compared with the L-strand promoter (15).

A significant observation made in the present work is the presence of hypermethylated nucleotides, both *in vivo* and *in organello*, at or very near the L-strand and the H_1 H-strand transcription start sites. It is interesting that, at both sites, the hypermethylated nucleotides occur in the non-template strand. In previous *in organello* footprinting studies, a weak hypermethylation of the non-template strand had also been observed at the L-strand and H_1 H-strand transcription initiation sites in human placenta mtDNA (18). No such DMS hypersensitivity near the RNA start sites had, on the contrary, been reported in the *in organello* footprinting analysis of bovine brain (17) and rat liver mtDNA (20). A possible explanation of this discrepancy is the difference in nucleotide context of the transcription start sites in human *versus* bovine or rat mtDNA. Earlier investigations utilizing deletion analysis and site-specific mutagenesis had identified sequence elements absolutely necessary for transcription around the transcription start sites in human mtDNA (35, 36). It should be mentioned, in this connection, that the L-strand and H_1 H-strand transcription start sites are surrounded by the octanucleotide consensus sequence ACC–CCAAA (*underlined* in Fig. 3) (35, 37) and that the homologous sites in *Xenopus laevis* mtDNA are also surrounded by an octanucleotide consensus sequence, although different (38). These findings had suggested possible protein-DNA interactions at these sites. However, the present observations provide the first clear evidence of local DNA conformational changes or increases in reagent concentration in hydrophobic pockets, due to protein-DNA interactions, at these sites (39). Since the *in vivo* and *in organello* hypermethylated residues at both the L-strand and the H_1 H-strand transcription start sites were exclusively (*in vivo*) or mostly (*in organello*) Cs, it is possible that these sites contain single-stranded DNA segments as a result of helical distortions caused by protein binding to DNA, a situation required for transcription activity.

Correlation of L-560 DMS Reactivity with ATP- and EtBr-dependent Changes in Rate of 16 S rRNA Synthesis in Isolated Mitochondria—The main observation reported in the present work is that the changes in degree of *in organello* hypermethylation of L-560 at the H_1 H-strand transcription initiation site detected in response to either ATP or EtBr followed closely the variation in rate of 16 S rRNA synthesis but not the variation in rate of mRNA synthesis. This observation supported the earlier strong evidence indicating that rRNA and mRNA synthesis are independently controlled in mammalian mitochondria (7, 8, 40). Furthermore, in view of the lack of influence of ATP on transcription termination, which has been observed in the present work, the results obtained on the ATP effects corroborated the idea that the regulation by ATP of rRNA synthesis occurs mainly, if not exclusively, at the level of initiation. It is particularly relevant to mention here that a specific requirement for ATP at an early step of *in vitro* transcription of the rDNA of human mtDNA has been previously documented (41). A regulatory role of ATP in mitochondrial RNA synthesis in mammalian cells has been recently proposed (42).

Methylation Interference Upstream of the H_2 H-strand Transcription Start Site—Another significant finding made in the present work is the occurrence of *in vivo* hypermethylated nucleotides and *in organello* protected or hypermethylated residues in a ~20-base pair segment within the $tRNA^{Phe}$ gene, which was not previously analyzed by others for *in organello* footprinting and which is located 27 base pairs upstream of the second *in vivo* H-strand transcription start site (H_2 in Fig. 3). This site has been identified in this laboratory at the 5′-end of the 12 S rRNA gene (3, 9), whereas others have positioned it within the ACCCC at nucleotides 636–640 (37). It is particularly interesting that the latter assigned position for the second H-strand transcription start site lies within the octanucleotide ACCCCATA (*underlined* in Fig. 3), which exhibits a seven of eight nucleotide match to the consensus sequence mentioned above (36). The results obtained here, which point to protein-DNA interactions upstream of this transcription initiation site, provide support to the strong evidence from *in vivo* and *in organello* studies that indicate the existence of two overlapping, independently controlled H-strand transcription units in human mtDNA (3, 4, 6–9).

High in Vivo Occupancy of mTERF Binding Sites—The *in vivo* footprinting pattern of the mitochondrial rDNA transcription termination region observed in the present work in HeLa cells was nearly identical to the *in organello* footprinting pattern of the same region previously obtained from bovine brain (17) and rat liver (20). In this pattern, the nucleotides with altered reactivity are within the tridecamer sequence previously shown to be required for accurate transcription termination (14), with the exception of the two hypermethylated G residues which flank this tridecamer on the 5′-end side of the L-strand. In view of the fact that the tridecamer sequence is a recognition site for mTERF and is completely comprised within the DNA binding domain for this factor (11), it is reasonable to assume that the observed footprint of the rDNA transcription termination region is caused by the binding of mTERF. Remarkable is the pronounced asymmetry in the distribution between the two strands of the residues with altered DMS reactivity. These occur predominantly in the non-template L-strand, pointing to a higher affinity of mTERF for this strand. It is possible that this asymmetry is significant for the function of mTERF, in connection with the modulation of its activity that is required for the transcription of the downstream genes (11). The high proportion of *in vivo* occupancy of binding sites for mTERF in HeLa cell mtDNA, as estimated from the percentage of protection from methylation by DMS of the residues in the footprinting pattern (up to about 80% for several nucleotides), is in agreement with the observations made by *in organello* footprinting of the rDNA transcription termination region in bovine brain and rat liver mitochondria (17, 20).

Acknowledgments—We are very grateful to Giovanni Lesa for collaboration in the early stages of the investigations and to Carlo Ausenda for help in the experiments involving ethidium bromide treatment of HeLa cells. We also thank A. Drew and L. Tefo for expert technical assistance.

REFERENCES

1. Attardi, G., and Schatz, G. (1988) *Annu. Rev. Cell Biol.* **4,** 289–333
2. Cantatore, P., and Attardi, G. (1980) *Nucleic Acids Res.* **8,** 2605–2624
3. Montoya, J., Christianson, T., Levens, D., Rabinowitz, M., and Attardi, G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7195–7199

4. Montoya, J., Gaines, G., and Attardi, G. (1983) *Cell* **34,** 151–159
5. Yoza, B. K., and Bogenhagen, D. F. (1984) *J. Biol. Chem.* **259,** 3909–3915
6. Gaines, G., and Attardi, G. (1984) *J. Mol. Biol.* **172,** 451–466
7. Gaines, G., and Attardi, G. (1984) *Mol. Cell. Biol.* **4,** 1605–1617
8. Gaines, G., Rossi, C., and Attardi, G. (1987) *J. Biol. Chem.* **262,** 1907–1915
9. Chomyn, A., and Attardi, G. (1992) in *Molecular Mechanisms in Bioenergetics* (Ernster, L., ed) pp. 483–509, Elsevier Science Publishing Co., Inc., New York
10. Gelfand, R., and Attardi, G. (1981) *Mol. Cell. Biol.* **1,** 497–511
11. Kruse, B., Narasimhan, N., and Attardi, G. (1989) *Cell* **58,** 391–397
12. Daga, A., Micol, V., Hess, D., Aebersold, R., and Attardi, G. (1993) *J. Biol. Chem.* **268,** 8123–8130
13. Micol, V., Fernández-Silva, P., and Attardi, G. (1996) *Methods Enzymol.* **264,** 158–173
14. Christianson, T. W., and Clayton, D. A. (1988) *Mol. Cell. Biol.* **8,** 4502–4509
15. Fisher, R. P., Topper, J. N., and Clayton, D. A. (1987) *Cell* **50,** 247–258
16. Fisher, R. P., Parisi, M. A., and Clayton, D. A. (1989) *Genes Dev.* **3,** 2202–2217
17. Ghivizzani, S. C., Madsen, C. S., and Hauswirth, W. W. (1993) *J. Biol. Chem.* **268,** 8675–8682
18. Ghivizzani, S. C., Madsen, C. S., Nelen, M. R., Ammini, C. V., and Hauswirth, W. W. (1994) *Mol. Cell. Biol.* **14,** 7717–7730
19. Madsen, C. S., Ghivizzani, S. C., and Hauswirth, W. W. (1993) *Mol. Cell. Biol.* **13,** 2162–2171
20. Cantatore, P., Daddabbo, L., Fracasso, F., and Gadaleta, M. N. (1995) *J. Biol. Chem.* **270,** 25020–25027
21. Micol, V., Fernández-Silva, P., and Attardi, G. (1996) *Methods Enzymol.* **264,** 3–11
22. Ammini, C. V., Ghivizzani, S. C., Madsen, C. S., and Hauswirth, W. W. (1996) *Methods Enzymol.* **264,** 23–36
23. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 1991–1995
24. Ephrussi, A., Church, G. M., Tonegawa, S., and Gilbert, W. (1985) *Science* **227,** 134–137
25. King, M., and Attardi, G. (1989) *Science* **246,** 500–503
26. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijin, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Shreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981) *Nature* **290,** 457–465
27. Fernández-Silva, P., Micol, V., and Attardi, G. (1996) *Methods Enzymol.* **264,** 129–139
28. Lawley, P. D. (1966) *Prog. Nucleic Acid Res. Mol. Biol.* **5,** 89–131
29. Gilson, E., Roberge, M., Giraldo, R., Rhodes, D., and Gasser, S. M. (1993) *J. Mol. Biol.* **231,** 293–310
30. Hsieh, D. J., Camiolo, S. M., and Yates, J. L. (1993) *EMBO J.* **12,** 4933–4944
31. Faber, S., O'Brien, R. M., Imai, E., Granner, D. K., and Chalkey, R. (1993) *J. Biol. Chem.* **268,** 24976–24985
32. Krumm, A., Meulia, T., Brunvand, M., and Groudine, M. (1992) *Genes Dev.* **6,** 2201–2213
33. Giardina, C., Perez-Riba, M., and Lis, J. T. (1992) *Genes Dev.* **6,** 2190–2200
34. Fernández-Silva, P., Martínez-Azorin, F., Micol, V., and Attardi, G. (1997) *EMBO J.* **16,** 1066–1079
35. Chang, D. D., and Clayton, D. A. (1984) *Cell* **36,** 635–643
36. Hixson, J. E., and Clayton, D. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 2660–2664
37. Bogenhagen, D. F., Applegate, E. F., and Yoza, B. K. (1984) *Cell* **36,** 1105–1113
38. Bogenhagen, D. F., and Romanelli, M. F. (1988) *Mol. Cell. Biol.* **6,** 2917–2924
39. Ogata, R. T., and Gilbert, W. (1979) *J. Mol. Biol.* **132,** 709–728
40. Cantatore, P., Flagella, Z., Fracasso, F., Lezza, A. M. S., Gadaleta, M. N., and de Montalvo, A. (1987) *FEBS Lett.* **213,** 144–148
41. Narasimhan, N., and Attardi, G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4078–4082
42. Enríquez, J. A., Fernández-Silva, P., Perez-Martos, A., López-Pérez, M. J., and Montoya, J. (1996) *Eur. J. Biochem.* **237,** 601–610
43. Ojala, D., Merkel, C., Gelfand, R., and Attardi, G. (1980) *Cell* **22,** 393–403