
Methylation pattern of mouse mitochondrial DNA

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ABSTRACT

The pattern of methylation of mouse mitochondrial DNA (mtDNA) was studied using several techniques. By employing a sensitive analytical procedure it was possible to show that this DNA contains the modified base 5-methylcytosine ($m^5\text{Cyt}$). This residue occurred exclusively at the dinucleotide sequence CpG at a frequency of 3 to 5%. The pattern of methylation was further investigated by determining the state of methylation of several MspI (HpaII) sites. Different sites were found to be methylated to a different extent, implying that methylation of mtDNA is nonrandom. Based on the known base composition and nucleotide sequence of mouse mtDNA, the dinucleotide sequence CpG was found to be underrepresented in this DNA.

The features of mtDNA methylation (CpG methylation, partial methylation of specific sites and CpG underrepresentation) are also characteristic of vertebrate nuclear DNA. This resemblance may reflect functional relationship between the mitochondrial and nuclear genomes.

INTRODUCTION

Modified bases occur, with very few exceptions, in the DNA of all prokaryotes and eukaryotes (1). In *Escherichia coli* DNA both 6-methyladenine ($m^6\text{Ade}$) and 5-methylcytosine ($m^5\text{Cyt}$) are found (2), in GATC and $\text{CC}^{\text{A}}\text{GG}$ sequences respectively (3). In higher eukaryotes, $m^5\text{Cyt}$ is the only methylated base (4) and is present in the dinucleotide sequence CpG (5). In plant DNA where high levels of $m^5\text{Cyt}$ are found (6), many of the methylations are also found in the dinucleotide sequence CpG (7-10). In addition, methylated cytosine is also found in a variety of other cytosine-containing dinucleotides all of which, however, are part of the basic trinucleotide CXG (11). The biological role of DNA methylation is now becoming clear. In *E. coli*, methylation is probably involved in protecting the DNA from the action of specific nucleases. Such is the case with the restriction-modification system where it was shown that methylated bases are playing a role (12,13). In addition, other findings (14,15) suggest that methylation may serve as an indirect signal for processes involving single stranded breaks in DNA (e.g. mismatch

repair, genetic recombination). As it is suggested that methylation protects DNA from single stranded breaks (14), a limited number of transiently unmethylated sites over a high methylation background, may serve as a signal for initiating these processes (15). In higher eukaryotes, methylation is correlated with gene expression (16). Analysis of the state of methylation of different genes revealed that in many instances hypomethylation of specific sites in a gene coincides with the initiation of its expression (17).

Although it is an established fact that mitochondria of all organisms contain DNA (18), whose nucleotide sequence in several species is partly or completely known (19-23), the occurrence of modified bases in this DNA is still in dispute. Thus, m⁵Cyt has not been detected in mitochondrial DNA (mtDNA) from Paramecium aurelia (24), HeLa cells and Xenopus laevis (25). The internal cytosine residue in the sequence CCGG of mtDNA from different strains of yeast, Neurospora crassa, rat and calf, was also found to be unmethylated (26). On the other hand, varying amounts of m⁵Cyt were observed in mtDNA from mouse, hamster, rat, calf, fish and birds (27-30). In none of these cases, however, has the distribution of this methylated residue among the different cytosine containing dinucleotides been determined. The increasing amount of data pointing to the role of m⁵Cyt in gene expression on one hand, and the unclear situation concerning the existence of this residue in mtDNA on the other hand, prompted us to examine in detail the state of methylation of mouse mtDNA.

MATERIALS AND METHODS

Strains and culturing conditions. E. coli K12 JCS183 (31) was used for cloning. Ltk^{-aprt} mouse cells, a derivative of clone D (32) were used as a source for mitochondrial DNA, and were maintained in Dulbecco modified Eagle's medium supplemented with 10% (vol/vol) new born calf serum.

Enzymes and chemicals. Restriction enzymes Bam HI, Taq I, Cla I, Xho I, Msp I and Hpa II, DNA polymerase and T4 DNA ligase were purchased from New England Biolabs. Conditions used for digesting DNA with restriction enzymes were those recommended by the manufacturer. Excess quantities of enzymes (20U/μg) were used in Msp I and Hpa II digestions. The restriction pattern was not changed even when higher quantities of Hpa II (60U/μg) were used, thus excluding the possibility of incomplete digestion.

DNase, micrococcal nuclease and spleen phosphodiesterase were from Sigma, calf intestinal alkaline phosphatase was from Boehringer. The four [α ³²P]-labelled deoxyribonucleoside triphosphates(3000 Ci/mmol) were purchased from Amersham.

Preparation of DNA. mtDNA was prepared from isolated mitochondria according to Bogenhagen & Clayton (33) using the "one step" procedure and replacing Mg^{+2} with Ca^{+2} in the homogenization buffer. Plasmid DNA was purified according to Holmes & Quigly (34).

Cloning of mitochondrial fragments. Clones A, B and C, carrying the 8.35kb, 6.89kb and 0.71kb Bam HI fragments of mouse mtDNA (see Fig. 2) respectively, were obtained after ligating Bam HI restricted mouse mtDNA to Bam HI and alkaline phosphatase treated pBR322 and transformation (35) of *E. coli* K12 JC5183 with the ligated mixture. Clones D and E, carrying a 1079bp and 1773bp Taq I fragments respectively (see Fig. 2), were obtained by ligating each of the two purified Taq I fragments to Cla I and alkaline phosphatase treated pBR322.

All the clones were analyzed by several restriction enzymes and hybridization to [$\alpha^{32}P$] labelled mouse mtDNA and were found to contain the intact presumed mitochondrial fragments (data not shown).

Hybridization. Mouse mtDNA was restricted as indicated and electrophoresed on agarose (Seakem) gels. DNA fragments were transferred to nitrocellulose paper (36) and hybridized to probes labelled by nick translation (37) with [$\alpha^{32}P$]dCTP to a level of $2-3 \times 10^8$ cpm/ μ g. Hybridization was performed at 42°C for 15hr in a solution containing 50% formamide/0.75M sodium chloride/0.075M trisodium citrate/50mM sodium phosphate pH 6.5/ 75 μ g/ml sheared and denatured salmon sperm DNA/10% Dextran sulfate/0.02% each of polyvinylpyrrolidone, bovine serum albumin and ficoll/7ng/ml of heat denatured [$\alpha^{32}P$]-labelled probe. Blots were then washed once at room temperature for 10 minutes with 50% formamide/2xSSC/0.1% SDS, three times at room temperature for 20 minutes each with 2xSSC/0.1% SDS and twice at 50°C for 15 minutes each in 0.1xSSC/0.1% SDS. The air dried sheets were then exposed to X-Ray film (Curix RP2, Agfa) for autoradiography.

Assay of m⁵Cyt in CpX containing sequences. The degree of methylation of CpX containing sequences was determined by an extension (38) of the standard nearest neighbour analysis (39). DNA samples (1-2 μ g) were nicked with DNase I (0.7 μ g/ml) for 15 min at 37°C. The 3' ends of the nicks were end labelled separately with each of the four [$\alpha^{32}P$] labelled deoxyribonucleoside triphosphates using *E. coli* DNA polymerase I (9 units) in a 50 μ l mixture containing 50mM Tris-HCl (pH 7.4), 50mM CaCl₂ and 1.4mM mercaptoethanol. The reaction was performed at 15°C for 30 min and terminated by the addition of EDTA to a final concentration of 10mM. Unreacted labelled dXTP was removed by application to Sephadex G-50 (Pharmacia) mini-columns. The labelled DNA in the

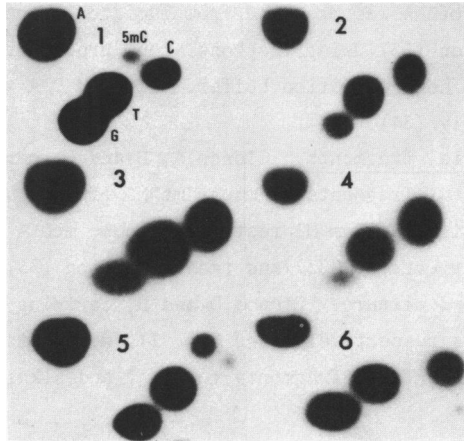


Fig. 1. Analysis of $m^5\text{Cyt}$ at CpX sequences of mouse mtDNA. Analysis was performed as described under Materials and Methods using [$\alpha^{32}\text{P}$] labelled 1) dGTP; 2) dTTP; 3) dATP and 4) dCTP. Mouse nuclear DNA (5) and *Drosophila melanogaster* sperm DNA (6) served as controls of highly methylated and unmethylated DNA at CpG sequences respectively. A, G, T, C and 5mC correspond to dAMP, dGMP, dTMP, dCMP and 5-methyl dCMP respectively.

effluent was digested to deoxyribonucleoside-3'-monophosphates (40) and the digest was chromatographed in two dimensions (38). The chromatograms were autoradiographed and the spots corresponding to cytosine and 5-methylcytosine were scraped and counted. The percentage of cytosine methylation was then calculated.

RESULTS

Level and sequence specificity of mtDNA methylation. To determine the amount of $m^5\text{Cyt}$ residues in mtDNA and establish its distribution we have used a modification of the nearest neighbour analysis. By this method the state of methylation of the CpG, CpC, CpT and CpA dinucleotide sequences can be estimated. The limit of sensitivity of this analysis is 0.1% enabling the detection of one methylated cytosine residue in 1000 of each of the four cytosine containing dinucleotides. As can be seen in Fig. 1, mtDNA contains $m^5\text{Cyt}$ exclusively at the CpG sequence. Using different preparations of mtDNA, 3-5% of the CpG sequences were found to be methylated in their cytosine residues. The same level of methylation was observed in mtDNA preparations purified by agarose gel electrophoresis, thus excluding the possibility of any nuclear DNA contamination. This value is equivalent to 17 to 29 $m^5\text{Cyt}$ residues per molecule, which is in good agreement with the value previously reported for L-cell mtDNA

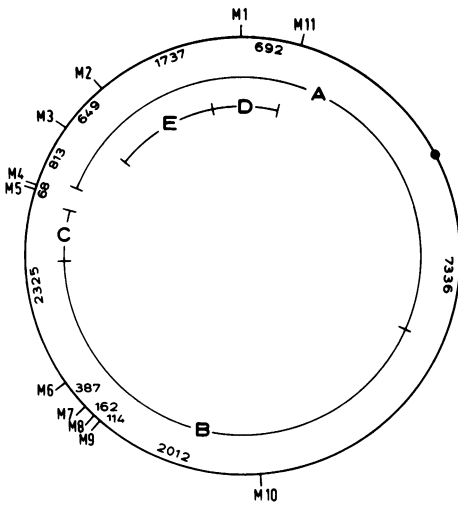


Fig. 2. MspI (HpaII) restriction map of mouse mtDNA. Sites were determined according to the known nucleotide sequence of mtDNA (20) using a computer programme written by Dr. S. Ben-Sasson. Mouse mtDNA contains 16295 bp and M1-M11 correspond to sites at positions 133; 1870; 2519; 3332; 3400; 5725; 6112; 6274; 6388; 8400 and 15736 respectively. Fragment sizes in base pairs are indicated along the inner circumference. Also indicated (●) is the single XhoI site at position 13551. A-E indicate mitochondrial fragments used as probes in this study. A-C correspond to the 8.35, 6.89 and 0.71kb BamHI fragments respectively. D and E correspond respectively to a 1079 and 1773 base pair TaqI fragments.

by Nass (27). It should be pointed out that such low levels of $m^5\text{Cyt}$ could not have been detected by most of the procedures used before. For example, the method used by David (25) for quantitation of $m^5\text{Cyt}$ in *X. laevis* and HeLa cells mtDNA could not detect less than 30 $m^5\text{Cyt}$ residues per molecule.

Localization of $m^5\text{Cyt}$ along the mtDNA molecule. As the sequence of mouse mtDNA is known (20), it is possible to determine precisely the position of the methylated sites. A common method to determine the state of methylation of CpG sequences is by using CpG restriction enzymes whose activities are affected by the state of methylation of the nucleotide sequence they recognize. The isoschizomeric pair of restriction enzymes, MspI and HpaII, recognize the sequence CCGG, the latter however will not cut if the internal cytosine in this sequence is methylated (41). Thus, by comparing the patterns of a DNA digested with each of the enzymes, the state of methylation of the DNA at these sites can be determined. Mouse mtDNA contains 287 CpG containing sites, eleven of them are MspI (HpaII) sites (see Fig. 2). If the methyl groups found in mtDNA are randomly distributed between all the CpG containing sites, less than one Msp I (HpaII) site is expected to be methylated. In this case, differences between patterns of Msp I and Hpa II digested mtDNA will be hard to detect. As it is shown in Fig. 3, Msp I and Hpa II digests of mouse mtDNA yield identical fragments and of the expected sizes. This result indicates that the CCGG sites in mouse mtDNA are probably not preferentially methylated. However, as ethidium bromide staining is not sufficiently sensitive for de-

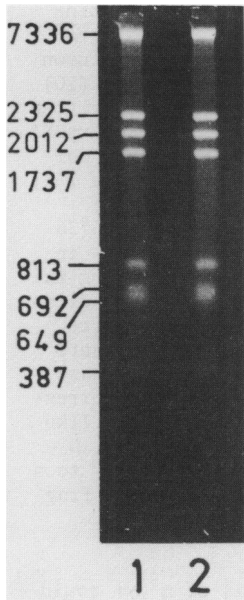


Fig. 3. Comparison of ethidium bromide stained Msp I and Hpa II digests of mtDNA. Mouse mt DNA (1 μ g) was digested with Msp I (lane 1) or Hpa II (lane 2) under conditions described in Materials and Methods and fragments were separated on 1.2% agarose gel. Fragment sizes in base pairs are indicated on the left, determined by reference to ϕ X174 RF DNA / Hae III and λ DNA / Hind III markers.

tecting possible differences between Msp I and Hpa II digests of low methylated DNA, radioactive labelled mitochondrial probes were used instead.

When a mixture of probes A and B (see Fig. 2) were used for analyzing XhoI/MspI and XhoI/HpaII digested mtDNA, two fragments of an approximate size of 6.8kb and 2.7kb were present in the XhoI/HpaII digest, while missing in the XhoI/MspI digest (Fig. 4, lane 2 vs 1). When the same blot was hybridized to probe D, these two bands were not observed and another 2.4kb fragment could be seen in the XhoI/HpaII digest (Fig. 4, lane 4). From the fact that the 6.8kb and 2.7kb fragments did not hybridize to probe D, it can be concluded that these fragments are not the result of methylation at either MspI (HpaII) sites M11, M1 or M2. From the remaining XhoI/MspI fragments (see Fig. 2) it can be calculated that methylation of site M10 or of sites M3 to M9 will yield fragments similar in size to the 6.8kb fragment. The 2.7kb fragment can be a result of methylation of site M6 or of sites M7, M8 and M9. In order to distinguish between these possibilities, Xho/MspI and Xho/HpaII digests were hybridized to probe C which carries a mitochondrial fragment located within the 2325bp MspI fragment (see Fig. 2). If the appearance of each of the bands is a result of methylation of a single site, the 2.7kb fragment will hybridize to probe C, while the 6.8kb fragment will not. If, on the other hand, the fragments are the result of methylation of multiple sites,

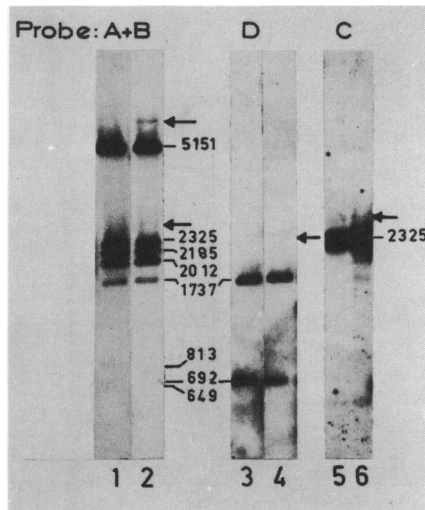


Fig. 4. Comparison of Msp I and Hpa II digests of mtDNA by hybridization with different mitochondrial probes. mtDNA (0.1 μ g) was digested with Xho I and Msp I (lanes 1,3,5) or Xho I and Hpa II (lanes 2,4,6) as described. The digests were electrophoresed on 0.8% agarose gels, blotted and hybridized to nick translated 32 P-labelled probes A+B (lanes 1+2), D (lanes 3+4) or C (lanes 5+6). The same blot was used for hybridization with probes A+B and D. The sizes of fragments obtained from unmethylated molecules is indicated in base pairs. Arrows indicate fragments observed in HpaII digests resulting from methylation of sites as discussed in the text. The sizes of these fragments are: 6.8kb and 2.7kb (in lane 2), 2.4kb (in lane 4) and 2.7kb (in lane 6).

then the 6.8kb fragment will hybridize to probe C and the 2.7kb fragment will not. As can be seen in Fig. 4 (lane 6) the former is the case. Namely, the 6.8kb fragment is a result of a methylation of site M10 and the 2.7kb fragment is a result of methylation of site M6.

The 2.4kb fragment which hybridized to probe D (Fig. 4, lane 4) can result from methylation of site M1 (resulting in a 2.43kb fragment from site M1 to site M2) or of site M2 (resulting in a 2.39kb fragment from site M1 to M3). To distinguish between these two possibilities, MspI and HpaII digested mtDNA was further cleaved with TaqI and analyzed by hybridization to two different probes (D and E) each carrying mitochondrial fragments containing one of the two MspI (HpaII) sites in question. Probe D carries a 1079bp TaqI fragment containing site M1 and probe E carries a 1773bp TaqI fragment containing site M2 (see Fig. 2). The results of this experiment are presented in Fig. 5. When probed with D, the 2.4kb fragment observed in the HpaII digest (lane 2) is cut by TaqI (lane 5) yielding a 1079bp fragment which could result only if

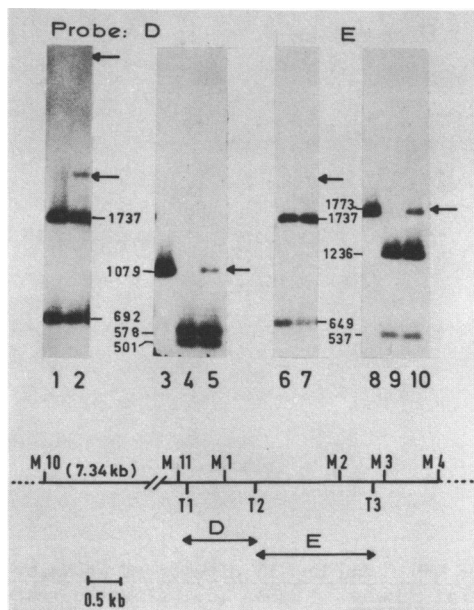


Fig. 5. The state of methylation of *MspI* (*HpaII*) sites M1, M2 and M11. mtDNA was analyzed as described in the legend to Fig. 4. The autoradiograms presented are of the same blot hybridized separately with probe D and probe E. Lanes correspond to mtDNA digested with: *MspI* (1,6); *HpaII* (2,7); *TaqI* (3,8); *MspI* + *TaqI* (4,9) and *HpaII* + *TaqI* (5,10). Indicated with an arrow are the 8.0kb and 2.4kb (lane 2), 1079bp (lane 5), 2.4kb (lane 7) and 1773bp (lane 10) fragments observed in *HpaII* digests of mtDNA resulting from methylation of sites discussed in the text. The *MspI/HpaII* (M) and *TaqI* (T) restriction sites in the mtDNA region analyzed and the *TaqI* probes used are presented at the lower part of the figure. T1, T2 and T3 are at positions 15850, 634 and 2407 respectively.

site M1 was methylated. When the same blot was hybridized to probe E, the 2.4kb fragment (lane 7) was cut by *TaqI* to a 1773bp fragment (lane 10) indicating that site M2 was methylated. An additional 8.0kb fragment was observed in the *HpaII* digest when probed with D (Fig. 5, lane 2). As this fragment did not hybridize to probe E (lane 7) and as no additional fragments were observed after further digestion with *TaqI* and hybridization with probe D (lane 5), it can be concluded that the 8.0kb fragment resulted from methylation of site M11. In contrast to *MspI* (*HpaII*) sites M1, M2, M6, M10 and M11, which were found to be partially methylated, site M3 was found to be unmethylated. Methylation of this site will yield after *HpaII* digestion a 1.46kb fragment, which should hybridize both with probe A and probe E (see Fig. 2). As such a frag-

ment was not observed (Fig. 4, lane 2 and Fig. 5, lane 7), one can conclude that this site is unmethylated.

The possibility that the minor bands shown in Figs. 4 and 5 are the result of unmethylated sites cut by HpaII at a low rate was tested by following the kinetics of appearance of the 2.4kb fragment. The intensity of this band remained the same after 30, 60, 90 and 120 minutes of digestion, thus ruling out the above mentioned possibility.

The extent of methylation of the various MspI (HpaII) sites was estimated by densitometric tracings of Southern-transfer autoradiographs. Sites were found to be methylated to a different extent. Thus, approximately 1% of sites M6 and M11 were found to be methylated whereas between 6 to 10% of sites M1, M2 and M10 were methylated. The state of methylation of the remaining MspI (HpaII) sites (4,5,7,8 and 9) could not be determined as the fragments resulting from methylation of these sites were either too small for analysis or similar in size to fragments obtained after HpaII digestion of unmethylated sites.

XhoI and HhaI are also sensitive to methylation of cytosine at its recognition sequences (CT*CGAG, G*CGC respectively (42)). Mouse mtDNA contains a single XhoI site at position 13551. Methylation of this site should yield after MspI/XhoI digestion (Fig. 4, lane 1) a 7.34kb fragment. As such a band is not observed, it can be concluded that this site is unmethylated. HhaI cuts mouse mtDNA at positions 2382 and 2604 producing a 222bp and 16,073bp fragments. These two bands were observed when a HhaI digest of mtDNA was hybridized with probe A, indicating that these two sites are not fully methylated. Due to the proximity of these two sites, it was however not feasible to determine with confidence whether these sites are completely unmethylated or partially methylated.

DISCUSSION

While the basic features of DNA methylation, both in prokaryotes and eukaryotes, are pretty well characterized, the situation concerning mtDNA is unclear. While it is accepted that m⁶Ade is absent from this molecule, the occurrence of m⁵Cyt is controversial. The reported results vary from no methylation through low to high levels of methylation. Although species specificity could account for these variations, the lack of a sensitive and reproducible procedure for assessing m⁵Cyt might have contributed in part to these discrepancies. In view of recent findings (43-49), suggesting integration of mtDNA sequences into the nuclear genome, and the increasing amount of

evidence correlating methylation with various aspects of gene expression, the elucidation of the mtDNA methylation pattern seemed of obvious importance. Mouse mtDNA was chosen for that purpose as the gene organization and the complete nucleotide sequence of this molecule is known (20).

By employing a sensitive analytical method we were able to detect m⁵Cyt in mtDNA. The methylated cytosine appeared exclusively in the dinucleotide sequence CpG at an extremely low extent of 3 to 5%. The pattern of methylation of mouse mtDNA was further examined by determining the state of methylation of several CpG containing sites. The conclusion that can be drawn from this type of analysis is that different sites seem to show different levels of methylation, implying that the methylation of mtDNA is non random. Although no direct experiments were designed to verify the existence of molecules methylated at more than one HpaII site, the absence of unaccounted for hybridizable bands in our blots may indicate that such molecules are not abundant. Many of the HpaII fragments of such molecules differ in size from those of unmethylated species and therefore, if present, should have been observed. In a recent report (50) fully methylated mtDNA molecules (at CCGG sites) were observed in mortal human fibroblasts. Such molecules were not observed in this study. The reasons for the difference in results are still obscure.

The main features of mtDNA methylation described hitherto (CpG methylation, partial methylation at specific sites) are similar to that of vertebrate DNA. It was therefore of interest to see whether underrepresentation of the dinucleotide sequence CpG, which is typical for vertebrate DNA and correlated to the level of methylation (51), also occurs in mtDNA. As summarized in Table 1, the frequency of CpG in mtDNA is 60% of the expected one. This is a relatively low underrepresentation compared to the one observed in mammalian DNA, where the frequency of CpG is only 25% of the one expected. However, this underrepresentation was shown (53) to be asymmetrically distributed, being only 50% in the coding region. As the entire genetic information of mtDNA, except for that in the two regions of the origins of replication, is serving a defined coding function, it can be concluded that the observed underrepresentation of CpG in mtDNA is yet another feature of similarity with eukaryotic DNA.

The level of methylation observed in mtDNA is 15 to 25 fold lower than that of mammalian DNA. This difference may be however actually smaller if methylation of nuclear DNA, like CpG underrepresentation, is asymmetrically distributed. Secondly, the work described here and by others was done on mtDNA isolated from cells grown under optimal conditions, or from actively

Table 1. Sequence specificity and extent of methylation of mouse mtDNA.

Source of DNA	Dinucleotide	Methylation (%) ^a	Dinucleotide Frequency (%) ^b	
			expected	observed
L-cells Mitochondria	CpG	3	3.00	1.76
	CpT	< 0.1	5.62	6.18
	CpC	< 0.1	3.73	4.60
	CpA	< 0.1	5.97	5.82
Mouse Liver	CpG	75	4.3	1.1
<i>Drosophila melanogaster</i> sperm	CpG	< 0.1	4.0	3.8

a) Extent of methylation was determined from the chromatogram shown in Fig. 1 as described in Materials and Methods.

b) The expected and observed values of the four different cytosine containing dinucleotides in mtDNA were calculated according to the base composition of each of the strands and the nucleotide sequence of mtDNA (20) respectively. The expected frequencies of CpG in mouse liver and *Drosophila melanogaster* DNAs were calculated according to the base composition of these DNAs (6) and the observed frequencies according to nearest neighbor data (51,52).

metabolizing tissues. In both cases, one may assume that the maximal activity of the mitochondrion was needed, a fact which might explain the observed low level of methylation. Supporting this assumption is the observation that the amount of methylated mtDNA molecules in mortal human fibroblast changes while grown *in vitro* (50). More direct experiments are needed, however, to prove this point.

The occurrence of methylated cytosine residues in mtDNA raises the question of how is this methylation achieved and what is its biological significance. As far as the first question is concerned, mitochondrial associated methylase activity, differing from other cellular DNA methylase activities, has been observed before in mouse L-cells and BHK cells (27). On the other hand, in lieu of the similarities in the methylation pattern of nuclear and mitochondrial DNA, the possibility that the nuclear methylase is responsible for methylating mtDNA cannot be excluded. There is, however, no experimental data supporting this possibility.

What is the biological role of mtDNA methylation? The similarities in the methylation pattern of mtDNA and nuclear DNA may indicate a functional relationship at the DNA level between the mitochondrial genome and the nuclear genome. Such a relationship exists in several systems, where it was shown that movement of genes occurs, or has occurred, between the mitochondrion and

the nucleus (43-49). If this phenomenon is of any biological significance, it stands to reason that the incoming DNA should have some properties which will enable its successful functioning in the nucleus. The pattern of methylation may be one of the parameters affecting the success of this process.

Another tempting possibility is that methylation may control the expression of the mitochondrial genome by affecting its transcription. In human mtDNA, two transcription events take place (54,55). One event leads to the synthesis of the bulk rRNA, the other to the synthesis of polycistronic RNA corresponding to almost the entire genome. Both events start at the rDNA region, at positions about 100bp apart. The mouse mitochondrial genome, whose organization is very similar to that of the human mitochondrial genome, is transcribed probably in the same manner (20,56,57). With this mode of transcription, the expression of the entire mitochondrial genome can be controlled by affecting one site on the DNA. A methylation event occurring in the rDNA region may serve this function. It should be noted that sites M1 and M2, which were shown to be methylated to a much higher extent than the other sites, are located within the rDNA region, downstream the presumed positions of the two promoters. These two sites or other CpG containing sites in this vicinity might therefore serve as sites of controlling the expression of the mitochondrial genome.

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Nucleic Acids Research

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