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In vitro methylation of nuclear respiratory factor-1 binding site suppresses the promoter activity of mitochondrial transcription factor A ☆

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Abstract

DNA methylation on CpG dinucleotides inactivates the expression of the many genes. The decreased amount of mitochondrial DNA (mtDNA) has been suggested to be an important indicator of mitochondrial biogenesis and the pathogenesis of many human diseases. Since mitochondria transcription factor A (Tfam) is a key molecule to regulate mtDNA replication and its promoter contains many CpG dinucleotides, potential methylation sites, we investigated whether the site-specific methylation would modulate the Tfam promoter-driven transcriptional activity in vitro. The luciferase reporters ligated to Tfam promoter (pGL2-Tfam2378) were in vitro methylated by *Sss*I (CG), *Hpa*II (CCGG), or *Hha*I (GCGC) methylase and luciferase activities were monitored after transient transfection of HepG2 cells. The *Sss*I or *Hpa*II methylation of pGL2-Tfam2378 or the SV40 promoter-luciferase plasmid (pGL2-Control) decreased the luciferase activities to less than 10% of the unmethylated plasmids, indicating that this inactivation by *Sss*I and *Hpa*II methylation might not be specific for the Tfam promoter. In contrast, *Hha*I methylation of pGL2-Tfam2378 suppressed the promoter activity to 24.4%, without affecting the control vector. There are two *Hha*I sites in the nuclear respiratory factor-1 (NRF-1) binding site of the Tfam promoter, whereas *Hpa*II sites are present out of the NRF-1 region. It was concluded that the methylation on the NRF-1 sites might be a route for silencing Tfam promoter resulting in decrease of mitochondrial biogenesis.
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Many regions of the mammalian genome are methylated at one or more CpG dinucleotides epigenetically, contributing to repression of transcription [1]. The CpG sites are usually located in the vicinity of genes and are often found near the promoters of widely expressed genes. They are observed in various cancers, parental imprinting, X chromosome inactivation, tissue-specific gene expression, and ageing [2–6]. The methylation of DNA would suppress the gene transcription via a direct interference on binding of transcription factors [7], indirect inhibition by methyl-DNA binding proteins [8], or chromosomal instability/deformation [9].

Mitochondrion is a major energy generating organelle in cells and has its own mitochondrial DNA (mtDNA). The mutation or depletion of mtDNA results in mitochondrial dysfunction, which may be involved in pathogenesis of many human diseases. In our laboratory, we have suggested that the quantitative changes in mtDNA copy number may be one of the causes for diabetic development [10,11]. The nuclear encoded mitochondrial transcription factor A (mtTFA, Tfam) is an important control point of mitochondrial biogenesis since the Tfam is essential to activate transcription of mtDNA and generate a RNA primer for replication of mtDNA [12]. The promoter regions of Tfam have been characterized in human, rat, and mouse [13–15].

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The sequence analyses reveal that there are three DNA binding motifs in its proximal region, nuclear respiratory factor 1, 2 (NRF-1, 2), and Sp1. The elimination of NRF-1 or NRF-2 binding by their mutation reduced the promoter activities in cultured cells, providing that NRF binding sites were apparently the major determinant of Tfam promoter activity [16].

Another distinct characteristic of the human Tfam promoter is that there are 67 potential CpG methylation loci between −2246 and +132 bp of the promoter, suggesting that cytosine methylation may play a role in regulation of Tfam expression. In the present study, we examined if in vitro methylation of Tfam promoter modulated the Tfam promoter-driven transcription using luciferase reporter assay. The site-specific methylation of the NRF-1 binding sites in Tfam promoter down-regulated the Tfam expression in HepG2 cells. This result suggests that methylation of the Tfam promoter in mammalian cells may silence the Tfam expression and contribute to decrease the mtDNA copy number, mitochondrial biogenesis.

Materials and methods

Cell culture. HepG2 (ATCC HB-8065) human hepatoblastoma cells were grown in minimal essential medium (MEM, Gibco-BRL, Rockville, MD) supplemented with non-essential amino acids, 100 U/ml penicillin, 10 μg/ml streptomycin, and 10% FBS.

Vector constructs. The human Tfam promoter reporter vectors (pGL2-hTfam2378) were constructed by PCR using pBluescript-Tfam promoter containing −2246/+132 bp (a generous gift from Dr. Kagawa, Jichi University, GenBank Accession No. X64269) as a template [13]. The transcription initiation site was numbered as +1 and the start codon for translation begins at +133. The oligonucleotide primer sets containing *Xho*I and *Hin*dIII sites (5′-aaa tac tcg agg cca atg gtg gga gta ttt aca-3′ and 5′-aaa taa agc ttc gct ccg gtg gat gag gca-3′) were used for amplification of the 2378 bp Tfam promoter of −2246 to +132 region by PCR at 95 °C 30 s, 60 °C 30 s, and 72 °C 1 min, for 30 cycles. After digestion with *Xho*I and *Hin*dIII, the 2378 bp PCR fragment was ligated to pGL2-Basic vector (Promega, Madison, WI, USA).

In vitro methylation. The pGL2-hTfam2378 plasmids (10 μg) were in vitro methylated with *Sss*I methylase, *Hha*I methylase, or *Hpa*II methylase (New England BioLabs, Beverly, MA, USA). The *Sss*I methylation, which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence (5′-CG-3′), was performed at 10 mM Tris, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 160 μM *S*-adenosylmethionine at 37 °C for 1 h. The *Hha*I and *Hpa*II methylations, which modify the internal cytosine residue with the following sequences; 5′-GCGC-3′, 5′-CCGG-3′, respectively, were performed at 50 mM Tris, pH 7.5, 10 mM EDTA, 5 mM of 2-mercaptoethanol, and 80 μM *S*-adenosylmethionine at 37 °C for 1 h. The procedure for mock-methylation reaction was identical to the one for DNA methylation except no *S*-adenosylmethionine was included. The plasmids after methylation reaction were purified by phenol extraction and checked for the degree of methylation, which was determined by analyzing the band patterns on gel electrophoresis after digestion of the purified plasmids with *Sss*I, *Hha*I, or *Hpa*II restriction enzymes. The completely methylated plasmids were utilized for transient transfection assays.

Transient transfection and luciferase assay. Transient transfections were performed by the calcium phosphate precipitation method as described [17]. Exponentially growing HepG2 cells (4×10^5 cells/well) in 6-well plate were transfected with the indicated plasmid (methylated or unmethylated, 1 μg) and pcDNA3.1/LacZ (1 μg) by calcium phosphate co-precipitation method. After 4 h incubation of calcium-DNA precipitates, the cells were washed with PBS and incubated with MEM (10% FBS and antibiotics) for 48 h. Cells were harvested with Luciferase Assay lysis buffer (Promega) and the cell extracts were assayed for luciferase activity by using luciferase assay kit (Promega, Madison, WI, USA) and a luminometer (Berthold, Badwildbad, Germany). The transfection efficiencies were normalized by the β-galactosidase activity of the co-transfected β-galactosidase expression vector, pcDNA3.1/LacZ (Invitrogene, Carlsbad, CA, USA). All transfections were carried out at least three independent experiments in duplicate and data are expressed as mean values with standard errors.

Results and discussion

We performed in vitro methylation of human Tfam promoter reporter vector (pGL2-hTfam2378) to methylate cytosine using one of the site-specific M.*Sss*I (CG), M.*Hha*I (GCGC), and M.*Hpa*II (CCGG) methylases. Each methylation site is designated as lollipop as shown in a simplified schematic overview of the Tfam promoter region (Fig. 1). Sixty-seven site-specific methylation sites of CpG dinucleotides (M.*Sss*I), three sites of M.*Hha*I, and nine sites of M.*Hpa*II are located in the promoter region. There are two sites for M.*Hha*I and one site for M.*Hpa*II inside the NRF-1 and NRF-2 binding sites, respectively. We digested the pGL2-hTfam2378 with *Sss*I, *Hha*I, or *Hpa*II restriction enzyme and analyzed by gel electrophoresis to confirm a completion of in vitro methylation of the Tfam promoter. The restriction enzymes digested none of the methylated vectors, whereas they fragmented the unmethylated vectors, indicating that the vectors were completely methylated by each methylase in vitro (Fig. 2).

Next, the methylated Tfam promoters were transiently transfected in HepG2 cells. The methylation by M.*Hha*I, M.*Sss*I, or M.*Hpa*II decreased the luciferase activities of pGL2-hTfam2378 to 24.4%, 1.1%, and 10.2% of the unmethylated ones, respectively (Fig. 3). To exclude the possibility that the methylation of the regions except the Tfam promoter reduced the reporter activities, the pGL2-Control plasmid bearing SV40 promoter was used as control since the SV40 early promoter does not contain any methylation CpG site. The methylation of the pGL2-Control by M.*Sss*I or M.*Hpa*II also decreased the luciferase activity to 4.9% and 21.8% of the mock methylation, respectively. These results indicated that the M.*Sss*I- or M.*Hpa*II-methylation of the vector itself, as well as the Tfam promoter, would decrease the transcriptional activities of pGL2-hTfam2378. However, the activity of the M.*Hha*I-methylated pGL2-Control plasmid was almost same as the unmethylated control (Fig. 3), implying that the M.*Hha*I methylation reduced the Tfam promoter-driven transcription specifically without affecting the vector.

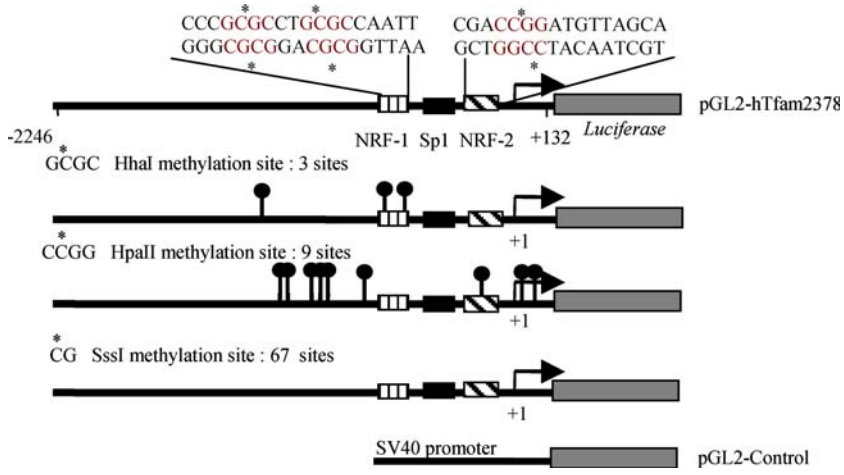

Fig. 1. Schemes of the human Tfam reporter constructs and pGL2-Control vector used for the study. The potential methylation sites of the human Tfam promoter in pGL2-hTfam2378 are shown. The pGL2-hTfam2378 construct contains the human Tfam promoter from −2246 to +132 bp ligated to luciferase. The transcription initiation site is depicted as the bent arrows and indicated as +1. Boxes represent three transcription factor-binding sites for NRF-1, Sp1, and NRF-2 and the sequences of NRF-1 and NRF-2 binding sites are shown. The locations of methylation sites by *Hha*I, *Hpa*II, and *Sss*I methylases are indicated as lollipop, together with the recognition sequences of the methylases. Asterisk (*) indicates the position of cytosine that is methylated by each methylase. Note that there are two sites for *Hha*I methylase in NRF-1 binding sites and one site for *Hpa*II methylase in NRF-2 binding site. The pGL2-Control vector containing SV40 promoter ahead of luciferase gene was utilized for normalization of vector methylation.

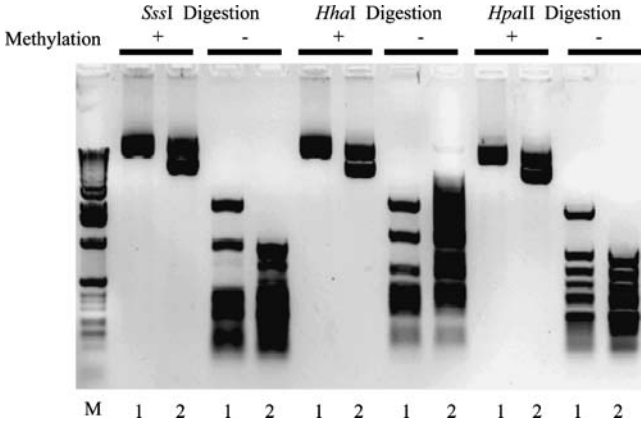

Fig. 2. The band pattern of pGL2-hTfam2378 and pGL2-Control plasmids after in vitro methylation, which was generated by *Sss*I, *Hha*I, or *Hpa*II restriction. A completion of methylation was confirmed by restriction digestion. The pGL2-Control (lane 1) or pGL2-hTfam2378 (lane 2) was methylated using M.*Sss*I (CG), M.*Hha*I (GCGC), or M.*Hpa*II (CCGG) methylases (methylation +). The mock methylation reactions were performed in the same conditions as methylation except the absence of *S*-adenosylmethionine (methylation −). The plasmids were digested with each corresponding restriction enzyme and analyzed on agarose gel electrophoresis. Lane M is the 1 kb DNA size marker.

The results also demonstrated the stability of the *Hha*I-methylated plasmids because the pGL2-Control plasmid contained 25 *Hha*I sites in the region except SV40 promoter. In addition, the repressed Tfam promoter activities by methylation were not restored to the unmethylated control even at 72 h after transfection (data not shown), suggesting that methylation sites were not demethylated in transfected cells at least during our experiments. It was concluded that the methylation of NRF-1 binding sites might silence the Tfam promoter activity since two M.*Hha*I methylation sites were found in NRF-1 binding sites of the Tfam promoter.

Functional NRF-1 recognition sites (Py**GCGC**A Py**GCGC**Pu) were also found in several nuclear DNA-encoded mitochondrial proteins including the subunits of several respiratory proteins and the RNA moiety of mitochondrial RNA processing endonuclease (MRP RNA) [18]. Table 1 summarizes the NRF-1 recognition sites of them. The presence of GCGC M.*Hha*I methylation sites in functional NRF-1 binding sites of these genes implies that DNA methylation may modulate the function of NRF-1, consequently the mitochondrial biogenesis, respiratory chain function [18], and coordination of the nuclear and mitochondrial genetic systems.

The regulation of eukaryotic gene expression is a complicated process involving the interaction of a large number of transacting factors with specific *cis*-regulatory elements. DNA methylation may play a role in interfering with the binding of transacting factors to DNA and silencing transcription. Several transcription factors were found to be sensitive to the methylation status of their cognate binding sites. These include the E2F, CREB, AP2, cMyc/Myn, and NF-κB [19]. In contrast, methylation-independent binding was shown for Sp1, CTF, and YY1 [20]. Our in vitro methylation study demonstrated that NRF-1 might belong to methylation-sensitive transcription factors.

The methylation patterns are not inherited from parental gametes, but are established during development

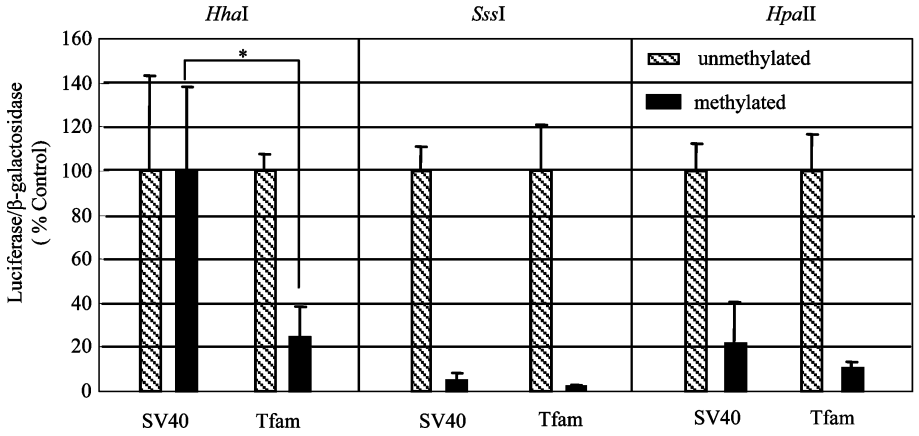

Fig. 3. Suppression of luciferase activities of pGL2-hTfam2378 by site-specific methylation in transiently transfected HepG2 cells. The methylated pGL2-hTfam2378 (Tfam) or pGL2-Control (SV40) was transfected into HepG2 cells along with the β-galactosidase expression vector, pcDNA3.1/LacZ. The cells were harvested 48 h after transfection and luciferase activities were measured. The luciferase activity was normalized by β-galactosidase activity. Values are means ± SD of three independent duplicate assays.

Table 1
Summary of NRF-1 recognitions sites in several human nuclear genes

Functional human NRF-1 binding sites	Sequence (Py**GCGC**APy**GCGC**Pu)	Location	References
Tfam	GG**C**GCAGG**C**GC**G**	−59/−70	[16]
Cytochrome *c*	CCAGCATG**C**GC**G**	−169/−158	[18]
Ubiquinone binding protein	T**G**C**G**CAGG **C**GCA	−53/−64	[18]
MRP RNA	C**G**C**G**CAC**G**C GCA	−293/−282	[18]
Eukaryotic initiation factor 2 α-subunit	TCCGCATG**C** GC**G**	−37/−26	[24]

The potential *Hha*I methylation sites are underlined and bold C indicates the position of methylated cytosine. The locations of NRF-1 sites are numbered from the transcription initiation site.

or carcinogenesis. Non-mutagenic environmental factors such as arsenic [21], nickel [22], and TCDD were often reported to cause changes in DNA methylation patterns contributing to tumor promotion. Moreover, dietary supplementation of methyl donor altered the methylation status of agouti gene promoter of offspring, suggesting the epigenetic effects of dietary nutrition [23]. To examine if the Tfam promoter is methylated in vivo, we isolated genomic DNAs from lymphocytes of some diabetic and normal subjects and sequenced the Tfam promoter after sodium bisulfite genomic modification. No methylated cytosine was found in the Tfam gene of normal and diabetic individuals (data not shown). Further study would be necessary to prove the in vivo role of DNA methylation in Tfam promoter regulation in the other tissues or the other diseased lymphocytes.

In conclusion, this is the first study demonstrating that the in vitro methylation of NRF-1 binding region strongly inhibited the activity of the Tfam promoter in transiently transfected HepG2 cells. We provided novel information that genomic DNA methylation would result in silencing the expressions of Tfam and possibly other NRF-1 responsive genes. The gene silencing by methylation, which might be obtained by environmental factors, would be one of the possible mechanisms of modulating mitochondria biogenesis and respiration.

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