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Studies on Functional Role of DNA Methylation within the *FXYD5-COX7A1* Region of Human Chromosome 19

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Abstract—We used the Rapid Identification of Genomic Splits technique to get a detailed methylation landscape of a 1-megabase-long human genome region (*FXYD5-COX7A1*, chromosome 19) in normal and tumor lung tissues and in the A549 lung cancer cell line. All three samples were characterized by an essentially uneven density of unmethylated sites along the fragment. Strikingly enough, the distribution of hypomethylated regions did not correlate with gene locations within the fragment. We also demonstrated that the methylation pattern of this long genomic DNA fragment was rather stable and practically unchanged in human lung cancer tissue as compared with its normal counterpart. On the other hand, the methylation landscape obtained for the A549 cell line (human lung carcinoma) in the *USF2-MAG* locus showed clear differences from that of the tissues mentioned above. A comparative analysis of transcriptional activity of the genes in this region demonstrated the general absence of direct correlation between methylation and expression, although some data suggest a possible role of methylation in the regulation of *MAG* expression through *cis*-regulatory elements. In total, our data provide new evidence for the necessity of revising currently prevailing views on the functional significance of methyl groups in genomic DNA.

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Key words: methylation profile, regulation of gene expression, *MAG*, *HAMP*

Methylation of mammalian genomic DNA is widely accepted as a process used to regulate the cellular expression pattern during cell functioning [1]. Methylation affects expression by modifying certain CpG sites within DNA *cis*-regulatory regions or due to the modification of extended genomic regions associated with chromatin conformational changes. Several mechanisms have been proposed for methylation-induced gene repression, but the actual mechanisms determining whether or not a certain DNA stretch will take part in gene expression regulation in a certain cell type is still rather far from being clear [2-7]. The data on the dependence of expression patterns of various genomic regions on their methylation status in various cellular contexts could help to construct more comprehensive landscapes describing and predicting the involvement of methylation in regulatory circuits. Numerous reports on whole-genome methylation analysis (reviewed in [8]), as a rule, lack detailed analysis of shorter, megabase (Mbase) long, genomic segments that could shed light on dynamics of tissue and environment dependent multi-layer regulatory systems because these segments still contain quite a number of genes and *cis*-regulatory sites. Such a detailed view could provide models for better understanding of genome functioning in general.

Earlier, we developed a method for determining DNA large-scale methylation landscapes for extended genomic regions that allows one to construct high density maps of unmethylated CpGs for predetermined genomic loci regardless of whether or not the CpG dinucleotides are located within CpG islands [9, 10]. Here we used the developed method to analyze the distribution of methylated residues within a 1 Mbase long *FXYD5-COX7A1* locus of human chromosome 19 for normal and cancerous lung tissue (non-small cell lung carcinoma), as well as for the A549 lung cancer cell line. The analysis allowed us to detect a gene-containing region of about 30 kb that was hypermethylated in the cell line genome as compared to the tissues. We also performed a detailed analysis of the transcriptional activity of the *MAG* and

Abbreviations: 5-aza-dC, 5-azadeoxycytidine; T_m, melting temperature; TSA, trichostatin A.

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HAMP genes located in this region and studied the chromatin structure of the gene promoter regions in normal and cancerous samples. According to the data obtained, the *HAMP* gene is actively transcribed provided that DNA is demethylated and histone deacetylation is inhibited. In the case of the *MAG* gene, methylation is supposed to act through inactivation of *cis*-regulatory element(s).

MATERIALS AND METHODS

Basic protocols. Growth and transformation of *Escherichia coli* cells, preparation of plasmid and cosmid DNAs, PAGE and agarose gel electrophoresis, as well as other standard manipulations were performed as described [11]. Genomic DNA samples were isolated using a Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's recommendations. Oligonucleotides were synthesized using an ASM-102U DNA synthesizer (Biosset Ltd., Russia). Cosmid clones of the *FXYD5-COX7A1* locus were a kind gift of Lawrence Livermore National Laboratory, USA.

Database searches and computer analysis. Nucleotide sequences were analyzed using the National Center for Biotechnology information (http://www.ncbi.nlm.nih.gov) and the Draft Human Genome Browser (http://genome.ucsc.edu/).

Tissue samples of non-small cell lung carcinoma (stage II according to TNM classification, UICC) and adjacent non-neoplastic lung tissue ("normal" tissue surrounding tumor) were obtained at the Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences. According to histology data, the "normal" sample did not contain tumor cells. Samples were obtained at the time of surgery and immediately snap frozen in liquid nitrogen.

Cell cultures. The human lung carcinoma cell line A549 (CCL-185) was grown in RPMI-1640 : DMEM/F12 (1 : 1) medium supplemented with 10% fetal calf serum (Gibco BRL, USA). Cells were seeded at low density ($2 \cdot 10^5$ cell per flask (25 cm^2)) 24 h before addition of 5-azadeoxycytidine (5-aza-dC) and then treated for 72 h with 5 μM 5-aza-dC (Sigma, USA) at each 24 h medium change. In TSA (trichostatin A, histone deacetylase inhibitor) experiments, cells were treated with 200 nM TSA (Sigma) for 13 h before the end of the 72-h cultivation. For a combined drug treatment, cells were incubated with 5-aza-dC for 59 h and then for an additional 13 h after addition of TSA (72 h in total).

RIDGES (Rapid Identification of Genomic Splits) was performed as described [10] starting from 0.5 μg of genomic DNA.

Bisulfite sequencing. Primers specific to bisulfite modified DNA were designed using MethPrimer software (http://itsa.ucsf.edu/~urolab/methprimer/) (see table). Bisulfite modification was done according to [12]. Agarose beads were directly used in two steps of PCR. The first and second reactions were performed for 35 and 25 cycles (at 95°C for 20 sec, at T_m (°C) for 20 sec, 72°C for 40 sec; for T_m see the table), respectively, in a 25 μl volume containing 0.2 μM of each primer and 1 U Taq DNA Polymerase Recombinant (Gibco BRL). The PCR products were cloned and sequenced.

RNA isolation and cDNA synthesis. Total RNA was isolated using an RNeasy Mini RNA purification kit (Qiagen, USA). All RNA samples were further treated with DNase I to remove residual DNA. The cDNA synthesis was performed according to the manufacturer's protocol using random hexamer primers (Promega, USA), with (+RT) or without (−RT) addition of PowerScript II reverse transcriptase (Clontech, USA). The efficiency of cDNA synthesis was equal in all preparations, as verified by RT-PCR with *GAPDH* gene-specific primers (table).

Real-time PCR was performed using an EVA green RT-PCR kit (Sintol, Russia) with 10 ng of the total cDNA sample or genomic DNA sample as templates. Primers (table) were used at final concentrations of 0.2 μM each. The reactions were performed in a 25 μl volume as follows: preincubation at 95°C for 10 min followed by 45 cycles at 95°C for 30 sec, at T_m (table) for 30 sec, and at 72°C for 1 min. All real-time experiments were done in triplicates. The expression level of each gene was normalized using *GAPDH* as a reference gene. At the end of the amplification, a dissociation curve was plotted to confirm the specificity of the product. To exclude contamination by genomic DNA, (−RT) experiments were done in parallel.

Southern blot hybridization. The A549 cell line genomic DNA samples (5 μg) were digested with HpaII and MspI restriction enzymes (Fermentas, Lithuania) as recommended by the manufacturer. The DNA fragments obtained were separated in a 0.8% agarose gel and blotted to Hybond N (Amersham, UK) membranes. Prehybridization was performed for 2 h at 68°C in 0.5 M Na_2HPO_4, 7% SDS, pH 7.5, hybridization buffer. The radioactively labeled probe was prepared by the random primed labeling method (Prime-a-Gene Labeling System; Promega), denatured, and added to the hybridization mix. The samples were hybridized for 16 h at 68°C, and the filters were washed with 40 mM Na_2HPO_4, 1% SDS, pH 7.5, washing solution at the same temperature and subsequently autoradiographed overnight.

Chromatin immunoprecipitation was performed according to [13]. The cells in the logarithmic growth phase ($\sim 3 \cdot 10^8$) were fixed with 1% formaldehyde for 10 min at 37°C, then washed with phosphate-buffered saline (PBS) containing 1 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride) and protease inhibitor cocktail (aprotinin, leupeptin, and antipain; 10 μg/ml final concentration of each component; Sigma), harvest-

Primers used in the experiments

Primer	Primer sequence (5′ to 3′)	T_m, °C
	Primers used for bisulfite sequencing	
HAMP-Out-for	AAGTTTAATAGTTATGGGGTT	57
HAMP-Out-rev	CCTTACATCTAAAACTCCTAA	57
HAMP-Int-for	GGAGTTATTTTTGGGGTG	57
HAMP-Int-rev	CACCCACCTACTACCTCA	57
MAG-pr-out-for	TTGGTAAGTGGGGGTTT	56
MAG-pr-out-rev	CAAACCACACACTCCTACAT	56
MAG-pr-int-for	GTTTAGATTTTGGAAGGTAG	56
MAG-pr-int-rev	CACACACTCCTACATCCTAA	56
	Primers used for real-time RT-PCR	
MAG-Q-1-for	CAACCCTCCCGTCCTGT	63
MAG-Q-1-rev	CAGCCTCCTCTCAGATCCCA	63
HAMP-RT-for-Q	CAGCCTGACCAGTGGCTC	62
HAMP-RT-rev-Q	GGTGTCTCGCCTCCTTC	62
GAPDH-Q-for	TGGCACCGTCAAGGCTG	63
GAPDH-Q-rev	CAGAGGGGGCAGAGATGATG	63
	Primers used for ChIP analysis	
MAG-For2 -ChIP-Q	TGCAGAAGCAACTGAGTCCAAGT	66
MAG-Rev2 -ChIP-Q	CATCCCCAAAGCAGCCCCAA	66
HAMP-For2-ChIP-Q	CGCCACCACCTTCTTGGAAATGA	66
HAMP-Rev2-ChIP-Q	TGCCATCGTGCCGTCTGT	66
GAPDH-For2-ChIP-Q	CTCAGTCGTTCCCAAAGTCC	60
GAPDH-Rev2-ChIP-Q	CGTAAAACCGCTAGTAGCCG	60

ed, pelleted at 400*g* for 4 min, resuspended in 200 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, and 10 mM EDTA), cooled for 10 min on ice, and then sonicated with a Cole-Parmer (USA) CP750 ultrasonic processor (20 cycles for 3 sec each with 10 sec intervals). Cell debris were removed in a microcentrifuge (10 min, 16,000*g*, 4°C), and the supernatant was diluted 10-fold with 16.7 mM Tris-HCl, pH 8.0, 16.7 mM NaCl, 1.2 mM EDTA, 1% Triton X-100, 0.01% SDS, 1 mM PMSF, and 1 μl/ml of the protease inhibitor cocktail. At this stage, aliquots were taken to be used as a positive control (input chromatin). The cell lysates were precleared by incubation with protein A-Sepharose beads and then incubated with 1.5 μg of anti-Ac-K9H3 rabbit polyclonal antibodies (Abcam, GB) or control anti-thaumatin rabbit polyclonal antibodies overnight at 4°C with rotation. DNA–protein complexes were collected using protein A-Sepharose beads, washed, and eluted from the antibodies by two incubations for 15 min in elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature. Then 5 M NaCl was added up to 0.2 M, and cross-links were reversed by sequential addition of RNase A and proteinase K, followed by incubation at 65°C for 4 h. The samples were extracted twice with phenol–chloroform and precipitated with ethanol overnight in the presence of 20 μg glycogen as a carrier. DNA fragments were collected by centrifugation, resuspended in water, and real-time PCR was performed.

RESULTS

Distribution of unmethylated sites within the *FXYD5-COX7A1* locus of human chromosome 19 in normal and tumor lung tissues and in the A549 cell line. The distribution of unmethylated CpG sites as parts of CCGG restriction sites within the 1 Mbase *FXYD5-COX7A1* locus of human chromosome 19 was studied by the RIDGES method [9]. Specimens of tumor lung tissue (squamous lung cancer), normal lung tissue adjacent to the tumor,

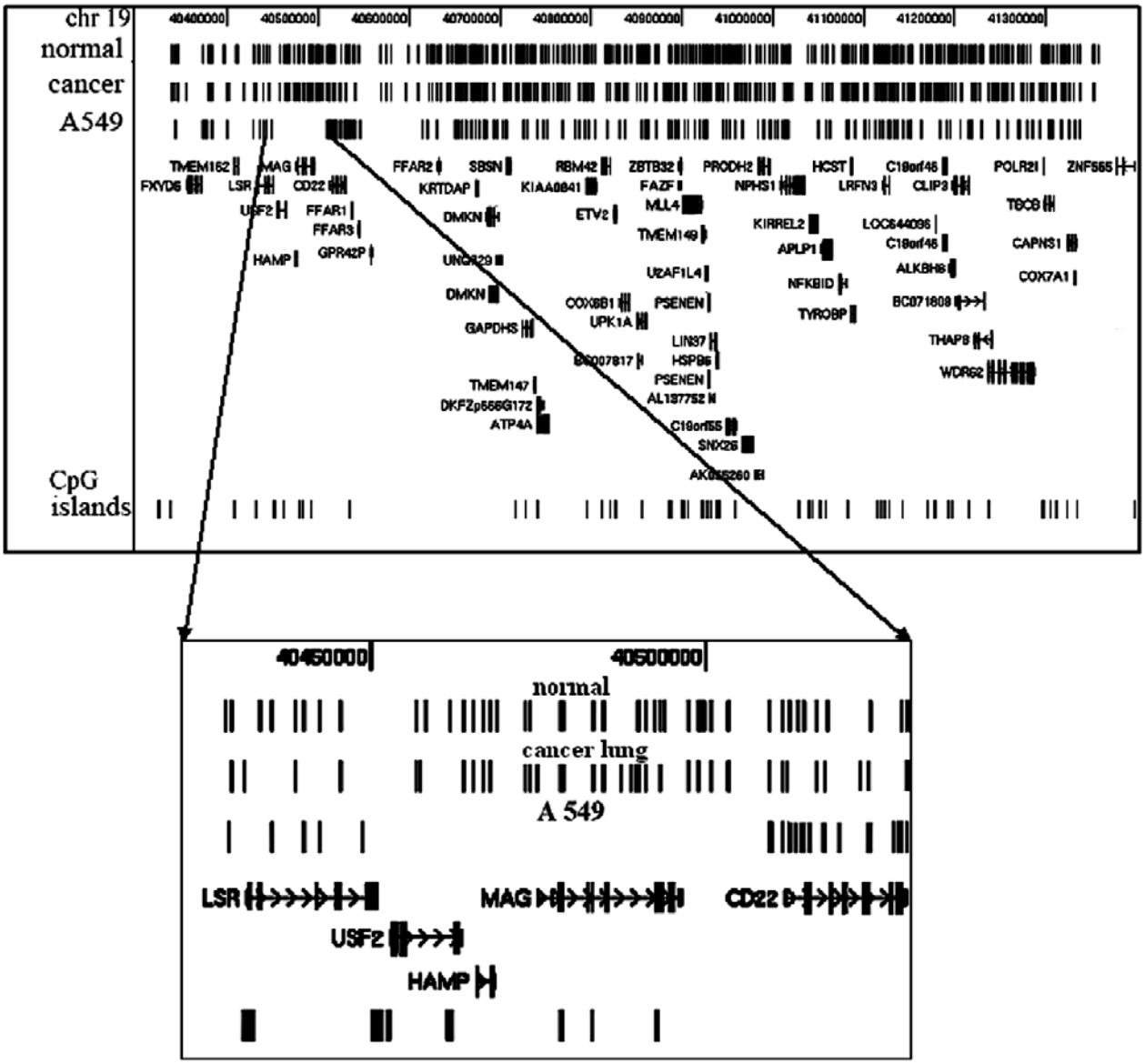

Fig. 1. Distribution of unmethylated CCGG sites within the *FXYD5-COX7A1* locus in normal and tumor lung tissue and in the A549 cell line.

and of the A549 cell line were analyzed. The analysis allowed us to map 429, 352, and 165 unmethylated sites for normal tissue, tumor tissue, and the A549 cell line, respectively. Based on the clone repetition in the libraries of unmethylated fragments, it was concluded that the number of the sites revealed is sufficient for a statistically reliable evaluation of the methylation density changes trend across the locus. Figure 1 presents the mapping data obtained using the UCSC Human Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Another way of data representation (Fig. 2), the density map of unmethylated sites, allows one to make a correction for uneven distribution of CCGG sites in the genome.

The methylation patterns in normal and tumor lung tissues were generally similar as judged from the distribution of unmethylated sites. In spite of the common opinion that gene coding sequences tend to be hypomethylated [14], the results demonstrate that hypomethylated DNA stretches in normal and tumor tissue can be located in both gene-rich and gene-poor (e.g. between the *FFAR3* and *FFAR2* genes; 0.31-0.35 interval in Fig. 2) regions. On the other hand, there were also hypermethylated gene-containing regions, e.g. 0.67-0.7 interval in Fig. 2. The gene-poor region may however contain unknown coding sequences; at least it was recently shown to contain transcription factor binding sites [15].

The distributions of unmethylated sites in normal and tumor tissue were found to be very similar, suggesting that the methylation of the locus under study practically does not change due to tumor progression in the given

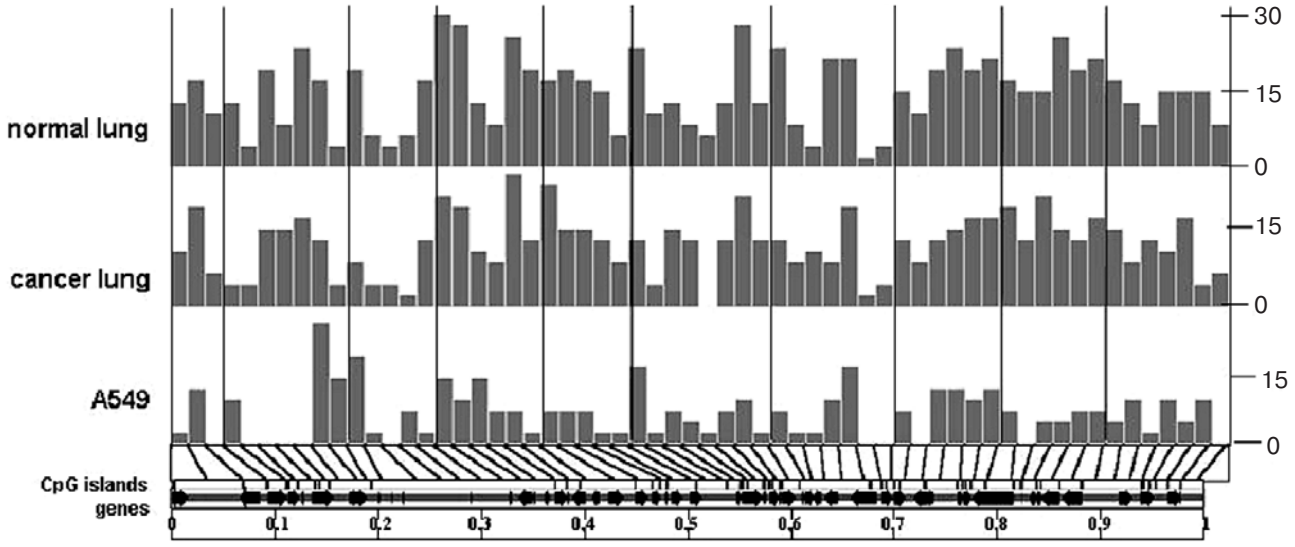

Fig. 2. Density map of unmethylated CCGG sites within the *FXYD5-COX7A1* locus. The data are normalized to equal (30) number of CCGG sites. The number of unmethylated CCGG sites per 30 CCGG sites is given on the Y axis.

patient. In spite of the common notion that carcinogenesis is always accompanied by epigenetic changes [16, 17], some recent reports demonstrate that methylation changes in tumorogenesis are not necessarily characteristic of the whole genome [18-20]. It looks more likely that significant methylation changes are mostly characteristic of the genes that take part in carcinogenesis and affect only certain rather short genomic regions. The persistence of the methylation pattern of the *FXYD5-COX7A1* locus under study can be explained if this locus contains no genes directly implicated in the development of a given tumor.

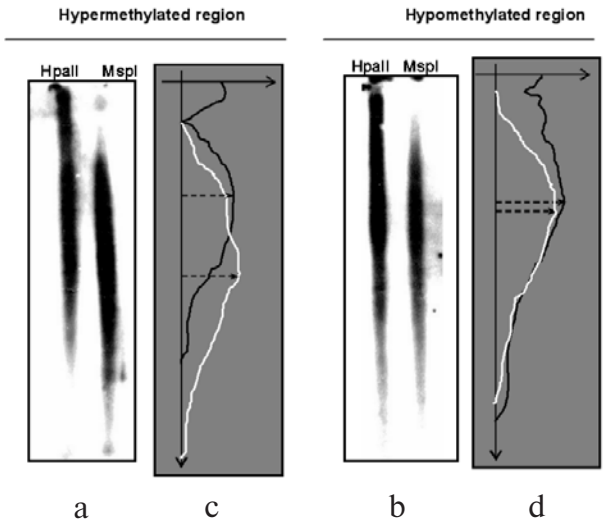

Fig. 3. Southern hybridization. Electrophoretically separated fragments of A549 cell line DNA digested with HpaII or MspI, hybridized with radioactively labeled cosmids harboring a fragment of the hypermethylated (a) or hypomethylated (b) genomic region of the A549 cell line. Plots (c) and (d) present the intensity of the hybridization signal (Y axis) for fragments of different lengths (X axis). Black and white lines correspond to the hybridization density for DNA restricted with HpaII and MspI, respectively. Dotted arrows indicate the positions of hybridization density maximums.

At the same time, the A549 cell line shows a methylation pattern of the *FXYD5-COX7A1* locus different from that in the normal and tumor lung tissues. The most appreciable difference is the presence of three specific and neighboring DNA regions of about 30 kb each (Fig. 1). Two of them, bordered by the *USF2-MAG* and *FFAR3-AK128099* genes, respectively, are hypermethylated, while the region between the *CD22-FFAR1* genes is hypomethylated. The difference in the methylation level between the *USF2-MAG* and *CD22-FFAR1* regions was confirmed by Southern hybridization (Fig. 3). To independently confirm the hypermethylation status of CpGs within the *USF2-MAG* region, we additionally performed bisulfite sequencing of fragments of promoter regions (Fig. 4).

Correlation of transcription, chromatin structure, and methylation in promoter regions of the *MAG* and *HAMP* genes in the A549 cell line. There is much evidence of the correlation between transcriptional activity of a gene and methylation of its promoter, enhancer, or other regulatory elements [21]. It is also known that the methylation levels of some extended genomic regions are associated with the transcription of the genes located within these regions (Long Range Epigenetic Silencing) [7, 22]. Therefore, it was interesting to analyze the transcription of the genes within the *USF2-MAG* region hypomethylated in the normal and tumor lung tissues but hypermethylated in the A549 cell line. According to RT-PCR data, these genes were transcribed in none of the three samples.

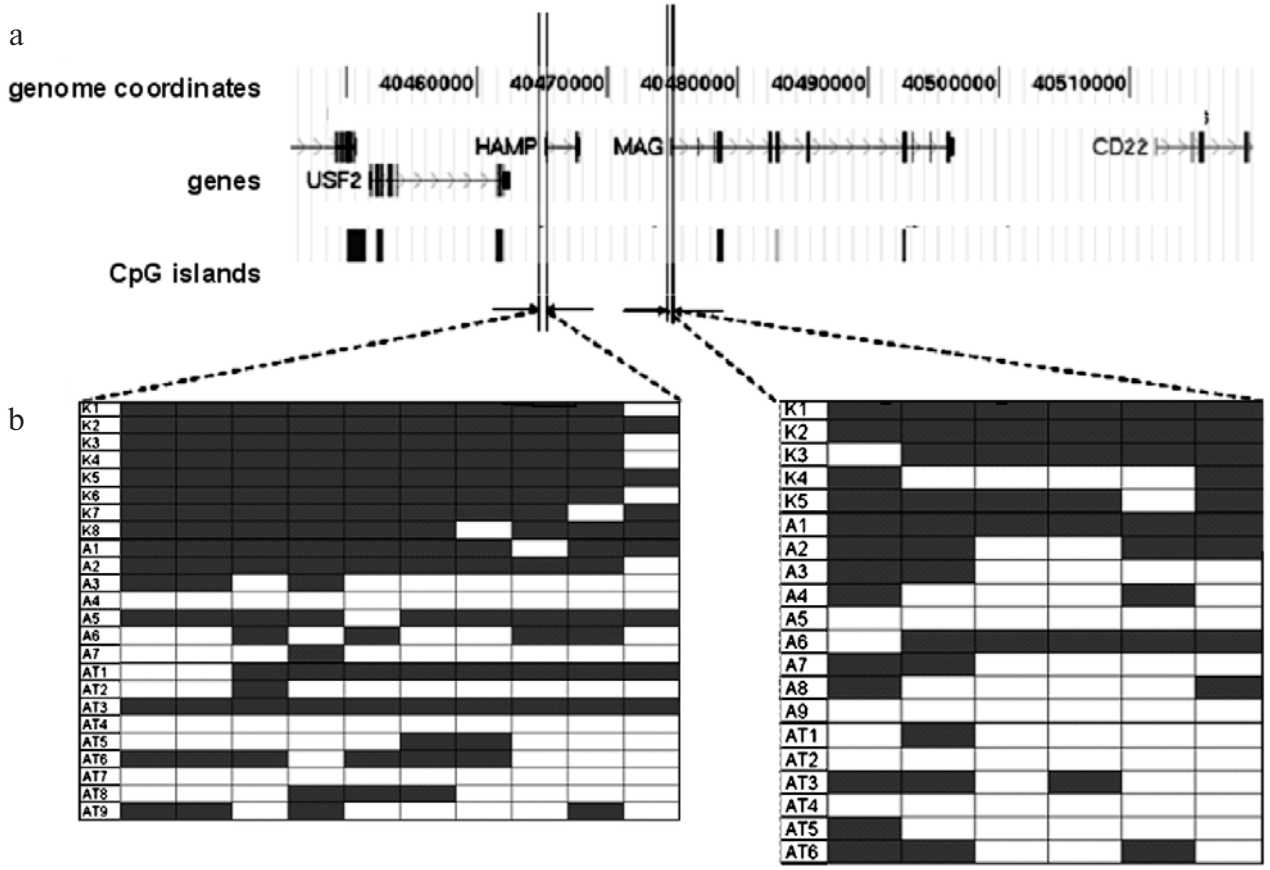

Fig. 4. Bisulfite sequencing of the promoter regions of the *HAMP* and *MAG* genes in the A549 cell line. a) Schematic representation of the genes and positions of CpG islands. b) Sequencing results for fragments of the *HAMP* and *MAG* gene promoter regions. Black and white squares designate methylated and unmethylated CpGs, respectively. K, A, and AT designate results for the A549 cell line grown under usual conditions (K), in the presence of 5-aza-dC (A), and in the presence of both 5-aza-dC and TSA (AT).

A treatment of the A549 line with 5-aza-dC led to decrease of DNA methylation and, in particular, of the promoter regions of the *HAMP* and *MAG* genes that was confirmed by bisulfite sequencing (Fig. 4). However, this treatment failed to stimulate transcription of the genes (Fig. 5). The data suggest the lack of correlation between the transcription of the *MAG* and *HAMP* genes and the methylation status of both their promoter regions and the extended locus where these genes reside.

In recent years, numerous studies revealed a correlation between DNA methylation pattern and various histone modifications (the so-called histone code) jointly involved in gene transcription regulation [23-25]. In particular, acetylated at lysine residues histones H3 and H4 are known to be associated with transcriptionally active genes [26]. To find a correlation between the methylation status, transcriptional activity of the *MAG* and *HAMP* genes, and histone acetylation within the *USF2-MAG* region in the A549 cell line, we analyzed the acetylation level of histone H3 lysine 9 (Ac-K9H3) in this region. To this end, we used chromatin immunoprecipitation with antibodies to Ac-K9H3, and the acetylation level was estimated from the quantity of the first exon fragments in the DNA fraction isolated by immunoprecipitation. Figure 5 presents the quantity of Ac-K9H3 associated with the genes within the hypermethylated region of A549 DNA. Treatment of cells with 5-aza-dC led to an increase in the histone H3 acetylation level for *MAG* (2-fold) and *HAMP* (1.5-fold).

When the cell line was grown in the presence of the histone deacetylase inhibitor TSA, the K9H3 acetylation level increased 1.6- and 1.2-fold for *MAG* and *HAMP*, respectively, as compared to A549 control cells. The transcription of *MAG* and *HAMP* genes was detected but the transcription level was still very low. A combined treatment of the cells with 5-aza-dC and TSA resulted in a greater increase in the Ac-K9H3 level (4-6-fold relative to control), the levels for the two genes being almost equal. Thus, the transcription of the *MAG* and *HAMP* genes was activated only by a combined action of two agents: a DNA demethylating agent and an agent that facilitates histone acetylation.

Earlier, two CTCF binding sites were mapped in the *MAG* gene [27], one of them located in the first intron and

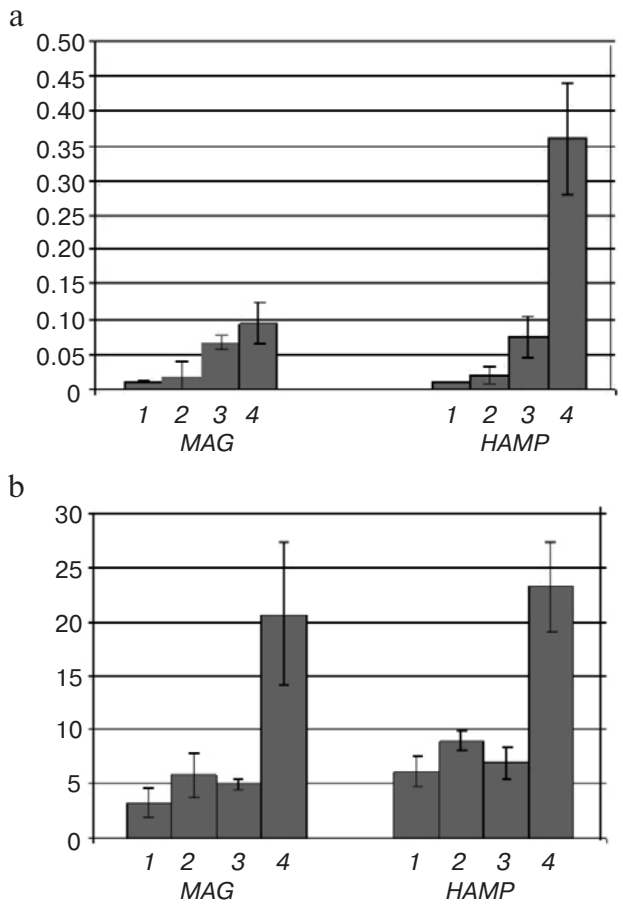

Fig. 5. Transcription level and K9H3 acetylation level for the *HAMP* and *MAG* genes in the A549 cell line grown under: *1*) usual conditions; *2*) in the presence of 5-aza-dC; *3*) in the presence of TSA; *4*) in the presence of both 5-aza-dC and TSA. a) Transcription level (relative to that of the *GAPDH* gene); b) relative K9H3 acetylation level.

the other in the third intron, close to a CpG island. It is known that methylation prevents binding of CTCF to DNA [28]. Therefore, it can be suggested that removal of methylation in the case of *MAG* (but not *HAMP*) enables the binding of CTCF to its sites within the *MAG* locus that further leads to transcriptional repression. A similar mechanism of transcriptional repression was reported, e.g. for the *hTERT* gene [29].

The *MAG* gene encodes a myelin-associated and very specialized glycoprotein expressed mainly in cells of the nervous system. It was demonstrated that the transcription was upregulated in the course of oligodendrocyte differentiation/maturation, and CpG sites in the promoter region were progressively demethylated, while methylation of CpG sites within the gene remained unchanged [30, 31]. These data and the data obtained here might suggest that the *MAG* gene is actively expressed when its promoter region is demethylated, whereas CpG sites within the gene remain methylated.

DISCUSSION

An analysis of the distribution of unmethylated CpG sites within the 1 Mbase *FXYD5-COX7A1* locus of chromosome 19 in the A549 cell line (lung carcinoma) revealed an anomalously hypermethylated gene-containing region of about 30 kb in length. At the same time, the methylation level of this same region in normal lung and in non-small lung cell carcinoma samples was lower. The region in question contains the *MAG* and *HAMP* genes whose expression is known to be highly tissue-specific. Practically undetectable transcription of these genes in all three specimens suggests the lack of correlation between the transcription and the methylation status of the locus harboring the genes. Moreover, a removal of methylation by growing the cell line in the presence of a demethylating agent did not lead to any considerable increase in the transcription level.

This observation is additional evidence for the necessity of revising currently prevailing views on the functional significance of methylation of genomic DNA. In our very first experiments on tissue-specific methylation maps of extended DNA regions, we analyzed the expression of genes located in differently methylated regions and found no clear correlation between changes in methylation and expression levels [9, 10].

The standard view on the functional role of CpG methylation in gene expression regulation suggests a correlation between DNA methylation and gene expression levels. Today this view is being questioned due to rapidly accumulating data (reviewed in [8]) in favor of a much more complicated interplay between DNA methylation and gene expression. This trend is largely due to a newly developed by us and other researchers technique [9, 10, 18, 19, 32-35] for large-scale analysis of DNA methylation density across long multigenic DNA regions. Methylation may be just a mean of defense against viruses [36], or may affect gene expression through controlling *cis*-regulatory regions. It could also gain other functions, the real essence of which is still not understood.

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