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DNA methylation in the promoter of ribosomal RNA genes in human cells as determined by genomic sequencing

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Abstract Many RNA polymerase II- or III-transcribed genes are inactive when their promoter is methylated at critical CpG dinucleotides. We have applied the genomic sequencing method and a direct DNA blotting technique to analyze the extent of DNA methylation in the 5′-CpG-3′ rich promoter region of the RNA polymerase I-transcribed ribosomal RNA genes (rDNA) in DNA from primary human cells, primary human tumor cells and human cell lines. In none of the analyzed primary human cells and primary human tumor cells was the DNA in the rDNA promoter region found to be detectably methylated. In contrast, in some of the cell lines this promoter is methylated in all 5′-CpG-3′ dinucleotides in the majority of the approximately 200 ribosomal RNA gene copies. In actively growing cells, rDNA gene activity is a prerequisite for cell viability. The high levels of DNA methylation in the promoter region of rDNA in the human cell lines raise questions on the role of promoter methylation in these RNA polymerase I-transcribed genes. It is, however, conceivable that a subset of the about 200 rDNA copies per haploid genome have escaped methylation and account for the rRNA synthesis in these cell lines. Alternatively, complete 5′-CpG-3′ promoter methylation may be compatible with promoter activity as demonstrated for certain viral genomes.

Key words: Genomic sequencing; DNA methylation; RNA polymerase I; Ribosomal RNA genes; rDNA

1. Introduction

DNA methylation entails long-term inactivation of mammalian RNA polymerase II- and III-transcribed genes (for reviews, see [1,2]). Since the three different eukaryotic RNA polymerases share certain protein components and transcription factors [3–7], DNA methylation might also have a function in the control of RNA polymerase I-transcribed genes. We have analyzed deoxycytidine methylation in the promoter region of the RNA polymerase I-transcribed ribosomal RNA genes in different primary human cells, in primary human tumor cells, and in human cell lines.

Since the promoter region of the human ribosomal RNA genes is extraordinarily rich in 5′-CpG-3′ dinucleotides, methylation-sensitive restriction endonucleases are not useful in precisely determining the extent of DNA methylation in this DNA segment. We have, therefore, chosen the genomic sequencing technique to assess the methylation status of each deoxycytidine in a region of approximately 350 base pairs (bp) in the rDNA promoter. The multiple copy nature and the high degree of sequence conservation in the ribosomal RNA genes, which are located on human chromosomes 13, 14, 15, 21, and 22, have facilitated this analysis [8].

2. Materials and methods

2.1. Primary cells, cell lines and extraction of DNA

The derivation of the primary human cells and human cell lines have been described in detail [9,10]. Briefly, DEV is a human Hodgkin lymphoma cell line, Jurkat and CEM are T cell lines, 660 is a B-cell leukemia line, and KB and HeLa are epithelial cell lines established from human tumors.

The extraction of cellular DNA followed standard protocols [10,11].

2.2. Genomic sequencing

Plasmid pBE2Δ carrying a fragment of the human rRNA gene encompassing nucleotides −513 to +696 was obtained from M. Muramatsu [12]. Cellular DNA was cut with the restriction endonuclease *Bst*EII, which cleaved 80 bp downstream from the start site of transcriptional initiation in the RNA polymerase I-transcribed ribosomal genes (Fig. 1).

The original genomic sequencing method [13] was applied. Hydrazine, which distinguished between methylated and unmethylated deoxycytidines (C), and the deoxyguanosine-specific dimethylsulfate (DMS) were allowed to react with 1–2 μg of genomic DNA at 20°C for 10 and 4 min, respectively. Upon chemical cleavage of the reaction products with 1 M piperidine at 90°C, the DNAs were ethanol precipitated and lyophilized twice. DNA fragments were subsequently separated by electrophoresis on 0.19 mm thin, 8% polyacrylamide gels in TBE (0.1 M Tris, 0.77 M borate, 25 mM EDTA, pH 8.3) containing 8 M urea. During electrophoresis at 1750 V and 13 mA, DNA fragments were directly blotted onto uncharged nylon membranes in a transfer APPARATUS (MWG Biotech, Constance, Germany). The running speed of the supporting belt was 8 cm/h. At the end of electrophoresis, the DNA was fixed to the nylon membrane by irradiation with 254 nm UV light for 3 min at a distance of 20 cm.

A 587 bp *Eco*RI-*Bst*EII fragment encompassing the rDNA promoter and the first 80 bp of the external transcribed spacer of the ribosomal RNA genes was cloned into the *Eco*RI and *Bam*HI cloning sites of phage M13mp18 or M13mp19 DNA (Fig. 1). After the annealing of appropriate primers, single-stranded DNA probes were generated using the Klenow fragment [14] of the *E. coli* DNA polymerase [15] and 125–250 μCi of α[^{32}P]dCTP (6000 Ci/mmol). Primer rDNA 1, bottom, had the nucleotide sequence 5′-GTCACCGGTAGGCCAGAGCC-3′. After the labeling reaction, the product was cleaved with *Hin*fI. Primer rDNA 2, top, had the sequence 5′-TTCGCTCCGAGTCGGCATTT-3′. After the labeling reaction, the product was cleaved with *Bam*HI in the polylinker sequence (Fig. 1). Single-stranded probes were purified by polyacrylamide gel electrophoresis under denaturing conditions, followed by electroelution. Hybridization was performed as described [13].

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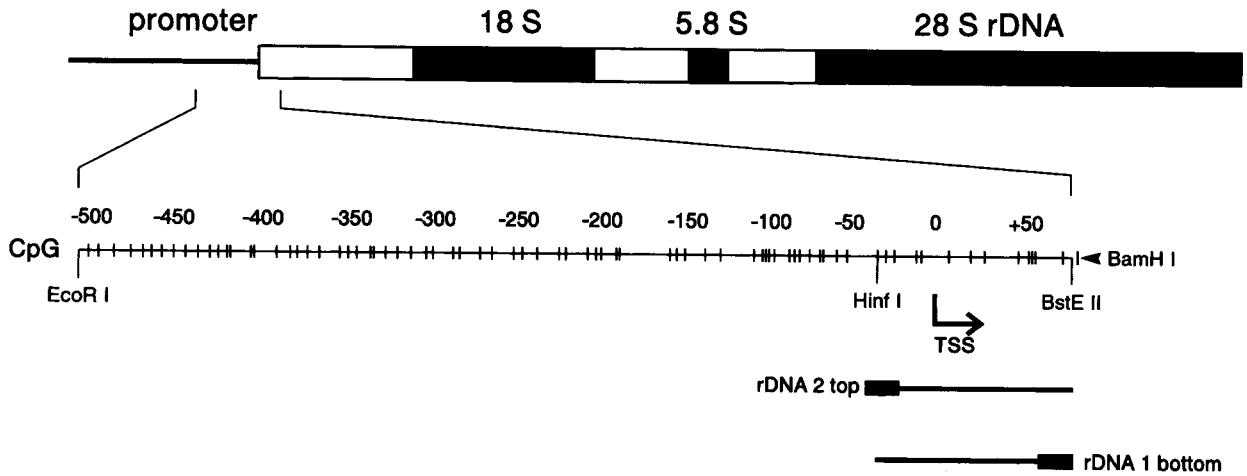

Fig. 1. Map of the human RNA polymerase I-transcribed ribosomal RNA genes with the locations of some of the restriction sites and all 5′-CpG-3′ dinucleotides (vertical bars). The genomic sequencing strategy is also explained. Genomic DNA was cleaved with *Bst*EII and subjected to the chemical sequencing reactions (for details see Section 2). Oligodeoxyribonucleotide primers rDNA 2, top, and rDNA 1, bottom, were used for the synthesis of ^{32}P-labeled single-stranded DNA probes (solid line). TSS: transcription start site. The *Bam*HI site was located outside the ribosomal RNA gene in the polycloning site.

3. Results and discussion

An autoradiogram of a genomic sequencing experiment of the bottom strand of the promoter region of the ribosomal RNA genes is shown in Fig. 2. In lanes 1–8, the G sequencing ladders for DNAs from different human cell lines and primary human cells are presented. In lanes 9–16, the C reaction products of DNA from the same cell types are analyzed. All deoxycytidines in the 5′-CpG-3′ positions are indicated by arrowheads.

In the DNAs from primary human granulocytes (G) and spermatozoa (Sp), (Fig. 2, lanes 15 and 16, respectively), the intensities of the C bands in the 5′-CpG-3′ positions were identical when compared to the intensities of the C bands in non-5′-CpG-3′ positions within the same lanes. Thus, deoxycytidine methylation in 5′-CpG-3′ positions was not detectable by this method in the DNAs of these cells. Similar results were obtained with the DNAs from the human cell lines 660 and DEV (lanes 9 and 14, respectively), with DNA from primary human T lymphocytes, and with DNA from a chronic T cell leukemia (T-CCL), a chronic B cell leukemia (B-CLL), or a chronic myelocytic leukemia (CML) (data not shown and Table 1). Details of the isolation of human T cells and of the cells from the human leukemias have been published previously [16].

In contrast, in the DNAs from the human cell lines CEM, KB, HeLa, and Jurkat (Fig. 2, lanes 10, 11, 12, 13, respectively), a very marked decrease in the intensities of all C bands in the 5′-CpG-3′ positions was apparent when compared to the intensities of C bands in non-5′-CpG-3′ positions in the same lanes.

Densitometric comparisons of signal intensities between unmethylated non-5′CpG-3′ and partly methylated 5′-CpG-3′ positions revealed average reductions in band intensities due to C-residues in 5′-CpG-3′ positions to a level of about 15% for the DNA from cell line Jurkat, to a level of about 20% for the DNA from cell lines CEM, to a level of 50% for the DNA from the KB cell line and to a level of 35% for the DNA from the HeLa cell line. There was some variation between different 5′-CpG-3′ sequences in the same lanes. Hence, in the DNAs from cell lines Jurkat, CEM, KB, and HeLa about 85%, 80%, 50%, and 65% of the rDNA copies, respectively, were methylated in 5′-CpG-3′ dinucleotides (Table 1).

Identical results were obtained with the opposite DNA strand as hybridization probe (data not shown). The possibility of C-residue mutations being responsible for the observed differences in C signal intensities between 5′-CpG-3′ and non-5′-CpG-3′ positions in the DNA from some of the cell lines was excluded by performing DMS (G) reactions for both DNA strands in all analyzed DNA samples. For each C residue in one strand, a G residue was found in the corresponding position of the complementary strand in the segments analyzed. In these experiments, we never observed C

Table 1
Extent of 5′-CpG-3′ methylation in the promoter region of ribosomal RNA genes[a]

Source of DNA	Percent methylation[b] in 5′-CpG-3′ dinucleotides
Human cell lines	
Jurkat (T cell)	85
CEM (T cell)	80
HeLa (cervical cancer)	65
KB (oral cancer)	50
DEV (Hodgkin lymphoma)	0
660 (B cell)	0
Primary human cell types	
Primary human granulocytes	0
Primary human T lymphocytes	0
Human spermatozoa	0
Primary human tumor cells	
Chronic T cell leukemia T-CCL	0
Chronic myeloic leukemia CML	0
Chronic B cell leukemia B-CLL	0

[a]In this report, we examined only 350 bp in the 5′ upstream region of the ribosomal RNA genes. It was not known whether there were methylated sequences in regions located further upstream.
[b]These values are based on the densitometric evaluation of several autoradiograms and intensity comparisons between 5′-CpG-3′ located C signals with those in non-5′-CpG-3′ C positions.

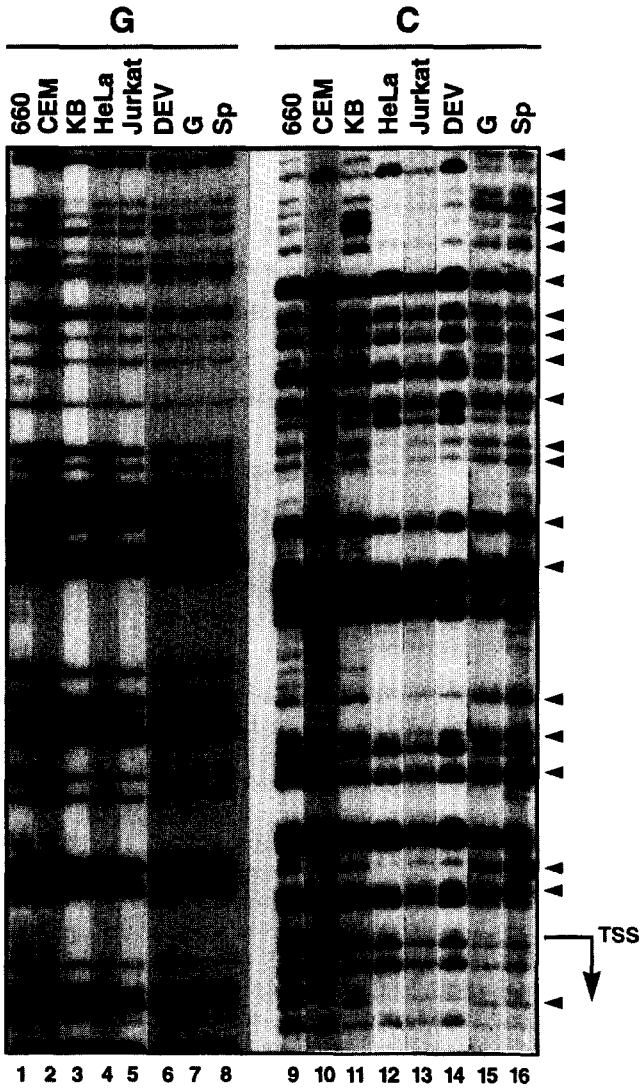

Fig. 2. Autoradiogram of a genomic sequencing experiment. The bottom strand between map positions −110 and +17 is shown. The arrowheads point to deoxycytidine residues in 5′-CpG-3′ positions. Lanes 1–8, DMS (G) reactions; lanes 9–16, hydrazine (C) reactions of different human DNA preparations whose sources were as follows: lanes 1 and 9, cell line 660; lanes 2 and 10, cell line CEM; lanes 3 and 11, cell line KB; lanes 4 and 12, cell line HeLa; lanes 5 and 13, cell line Jurkat; lanes 6 and 14, cell line DEV; lanes 7 and 15, human granulocytes (G); lanes 8 and 16, human spermatozoa (Sp). TSS: transcription start site.

residue methylations in positions other than in 5′-CpG-3′ dinucleotides. However, using this genomic sequencing technique, the methylation of an occasional C-residue in 5′-CpG-3′ or in non-5′-CpG-3′ positions in some of the 200 rDNA copies per haploid genome could not be excluded.

It is not possible to interpret these data in terms of rDNA function, as it is unknown whether promoter methylation of rDNA genes affects transcriptional activity of these multiple copy genes. The finding of 50–80% levels of rDNA promoter methylation in several fast growing cell lines might be reconciled with rDNA gene activity in cell culture as a fundamental prerequisite of cell viability. The ribosomal genes occur in multiple copies and the activity of some of these genes might suffice for cell growth in culture. By Southern blot analyses, we have excluded significant amplifications of the rDNA genes in the cell lines with high levels of rDNA methylation (data not shown).

Extrachromosomal ribosomal DNA from *Xenopus laevis* sperm has been found to be methylated at every 5′-CpG-3′ in the promoter region of rDNA. Upon injection into *Xenopus laevis* oocytes, methylated and unmethylated rDNA constructs have been transcribed equally well [17]. These findings suggest that complete 5′-CpG-3′ methylation of the rDNA promoter may not interfere with promoter activity. Perhaps, a specific subset of the 5′-CpG-3′ sites would lead to promoter inactivation, similarly to one of the frog virus 3 promoters which became inactivated only when the 5′-CCGG-3′ sequences were selectively and exclusively methylated [18]. Moreover, in wheat, the proportion of rDNA repeat units with methylated 5′-CCGG-3′ (*Hpa*II) sites has been found to be related to the number of rDNA genes in the cells. The total number of unmethylated 5′-CCGG-3′ sites in possibly actively transcribed rDNA is thought to be relatively constant between different genotypes [19].

We have to reserve judgement on the interpretation of our present data, until functional data on the effect of DNA methylation on the activity of the RNA polymerase I-transcribed rDNA genes in human cells and cell lines become available.

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