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Genetics

Interindividual concordance of methylation profiles in human genes for tumor necrosis factors α and β

(genomic sequencing/5-methyldeoxycytidine/methylation patterns/tumor necrosis factor α expression)

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ABSTRACT **The DNA in mammalian genomes is characterized by complex patterns of DNA methylation that reflect the states of all genetic activities of that genome. The modified nucleotide 5-methyldeoxycytidine (^{5}mdC) can affect the interactions of specific proteins with DNA sequence motifs. The most extensively studied effect of sequence-specific methylations is that of the long-term silencing of eukaryotic (mammalian) promoters. We have initiated studies on the methylation status of parts of the human genome to view patterns of DNA methylation as indicators for genetic activities. In this report, analyses using both restriction enzyme–Southern blotting and the very precise genomic sequencing technique have been done. The genes for tumor necrosis factors (TNF) α and β—in particular, their 5′-upstream and promoter regions—have been investigated in DNA isolated from human lymphocytes, granulocytes, and sperm. The results are characterized by a remarkable interindividual concordance of DNA methylation in specific human cell types. The patterns are identical in the DNA from one cell type for different individuals even of different genetic origins but different in the DNA from different cell types. As an example, in the DNA from human granulocytes of 15 different individuals (ages 20–48 yr, both sexes), ^{5}mdC residues have been localized by the genomic sequencing technique in three identical sequence positions in the 5′-upstream region and in one downstream position of the gene encoding TNF-α. The promoter of this gene is free of ^{5}mdC, and TNF-α is expressed in human granulocytes. The TNF-β promoter is methylated in granulocytes from 9 different individuals, and TNF-β is not expressed. In human lymphocytes, the main source of TNF-β, the TNF-β promoter is free of ^{5}mdC residues. All 5′-CG-3′ sites studied in the TNF-α and -β genes are methylated in DNA from human sperm. In human cell lines HL-60, Jurkat, and RPMI 1788, the extent of DNA methylation in TNF-α and -β genes has also been studied.**

Sequence-specific promoter methylation can inactivate or strongly inhibit eukaryotic (mammalian) promoters (for reviews, see refs. 1–6). Adenovirus promoters have been used as models to study the mechanism of promoter inactivation (7–15). The development of *de novo* patterns of DNA methylation in transformed cell lines is characterized by the ordered, nonrandom spreading of methylation (16–18).

By using restriction enzyme and Southern blotting analyses (19), as well as the very exact genomic sequencing procedure (17, 20, 21), we have studied human DNA methylation in segments and in the promoter regions of the tumor necrosis factor (TNF) genes α (cachectin) and β (lymphotoxin) (22–29). Depending on the tissue origin of human DNA, highly specific patterns of methylation exist without interindividual variance. In established human hematopoietic cell lines a higher variability in patterns of methylation has been seen. Evidence for inheritance of methylation patterns and for variation in the methylation of allelic sites has been presented (30).

MATERIALS AND METHODS

Cells, Cell Lines, and Extraction of DNA. (*i*) Fresh blood or buffy coats were obtained by venipuncture from healthy human volunteers and diluted with RPMI 1640 medium (31). Subsequently, lymphocytes and granulocytes were fractionated on a Ficoll-Paque gradient. Granulocytes were recovered from the pellet and freed from contaminating erythrocytes by lysing the latter in 0.83% NH_4Cl. The resulting granulocyte preparation was >95% pure, as determined in a Pappenheim-stained (32) cell smear. The lymphocyte preparation was >90% lymphocytes, 5–10% monocytes, and <3% granulocytes. (*ii*) Human cell lines HL-60, a human promyelocytic leukemia cell line; Jurkat, a T-cell line; and RPMI 1788, an IgM-secreting B-lymphoblastoid cell line, were propagated in RPMI 1640 medium (GIBCO–BRL) supplemented with 10–20% fetal bovine serum. (*iii*) Human sperm cells were washed, and the DNA was extracted after dithiothreitol treatment (33). The nuclear DNA from the different cell preparations was extracted by standard protocols (34).

Isolation of Cellular RNA. Granulocytes were purified >99%. Granulocytes or the cells from established cell lines were kept in culture for 5 hr in the absence or presence of 8 nM PMA (phorbol 12-myristate 13-acetate). Total cellular RNA was extracted as described (35).

Genomic DNA Sequencing. The method of Church and Gilbert (20) was applied (17, 18, 21). For analysis of the TNF-α promoter (Figs. 1*a* and 3*a*), the DNA was cleaved with *Stu* I and *Bgl* I. The appropriate promoter fragments were isolated by zone velocity sedimentation in 5–20% sucrose gradients (36, 37) and were subsequently cleaved with *Bst*XI or *Sty* I. For analysis of the TNF-β promoter (Figs. 1*b* and 4*a*), the initial cleavage was with *Pvu* II or *Bam*HI. After fragment selection, the DNA was cut with *Hin*fI.

The cytidine reaction for DNA sequencing (38) with hydrazine proceeded at 20°C (TNF-β) or at 25°C (TNF-α) for 27 min, the guanosine reaction with dimethyl sulfate was at 20°C for 4 min. Cleavage was effected by 1 M piperidine treatment for 30 min at 90°C. DNA fragments were separated by electrophoresis on 6% polyacrylamide gels containing 8 M urea and were then electrotransferred to a GeneScreen*Plus* membrane. The TNF-α or -β fragments were cloned into M13 (mp18 or mp19) DNA. Single-stranded probes were gener-

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Abbreviations: ^{5}mdC, 5-methyldeoxycytidine; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate.

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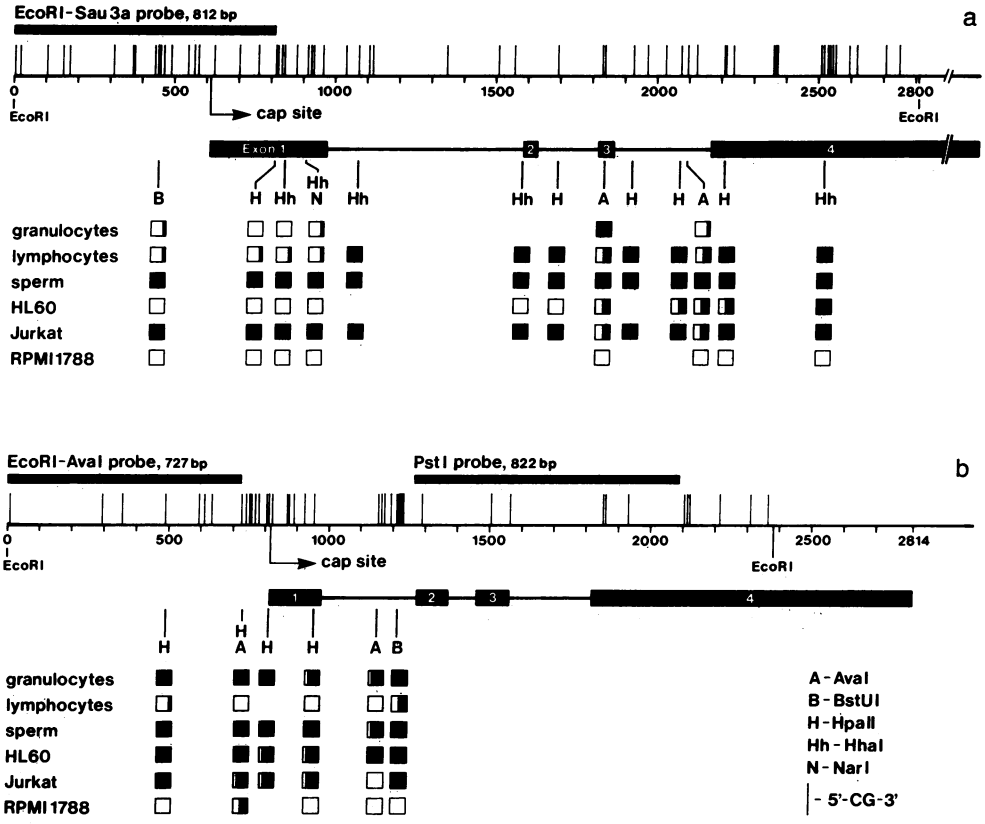


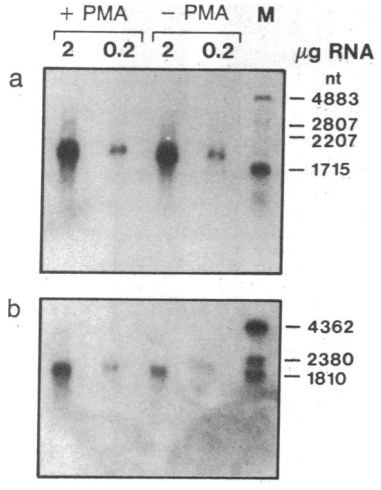
FIG. 1. Restriction and 5'-CG-3' maps of the TNF-α and -β genes and levels of DNA methylation of these genes in different human cell types and cell lines. Maps of the coding and upstream regions as well as of the introns of the human TNF-α (*a*) and TNF-β (*b*) genes are based on published sequences (26–28). Vertical lines on the maps designate the 5'-CG-3' sequences; numbers refer to nucleotides in the sequence. Restriction sites and DNA from different human cell types or cell lines are designated. Open squares denote nonmethylated 5'-CG-3' sequences, partly filled squares denote partly methylated sequences, and filled squares denote completely methylated sites. Map locations of the hybridization probes are indicated by alignment.

ated by using Klenow polymerase (39) and 250–500 μCi of [α-^{32}P]dCTP (6000 Ci/mmol; 1 Ci = 37 GBq).

Other Methods of Molecular Biology. Restriction analyses, Southern (19) and RNA transfer methods (40), and DNA–DNA and DNA–RNA hybridization experiments (41) followed published protocols.

RESULTS AND DISCUSSION

The Tissue-Specific Methylation Patterns of the TNF-α and -β Genes. These genes are closely linked on chromosome 6 (22, 28) and are differentially expressed in cells of the hematopoietic system. Moreover, the upstream and promoter sequences as well as the intron and exon sequences are rich in 5'-CG-3' dinucleotides (26–28). The 5'-CG-3' methylation-sensitive restriction endonucleases *Hpa* II/*Msp* I (5'-CCGG-3'), *Hha* I (5'-GCGC-3'), *Ava* I (5'-CYCGRG-3'), and *Xho* I (5'-CTCGAG-3') were used to survey the extent of DNA methylation in the human TNF-α and -β genes in DNA from lymphocytes, granulocytes, sperm (haploid cells of >99% purity), and from the human cell lines HL-60, Jurkat,



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	TNF α			TNF β		
	−PMA	+ PMA	Promoter Methylation	− PMA	+ PMA	Promoter Methylation
Granulocytes	+ +	+ +	-	-	-	+
HL 60	+	+ + +	-	-	-	+
Jurkat	-	-	+	(+)	+	(+)
RPMI 1788	+	+ +	-	+	+ + +	-

FIG. 2. Expression of the TNF-α gene in human granulocytes. Total cellular RNA (0.2 or 2 μg) from human granulocytes was prepared from untreated or PMA-treated (8 nM, 5 hr) granulocytes. The RNA was electrophoresed and transferred to a nitrocellulose filter as described. The 2.8-kilobase *Eco*RI fragment of the TNF-α gene (*a*) (map, compare with Fig. 1*a*) or the pBR322-cloned rat β-actin gene (*b*) was ^{32}P-labeled by random oligonucleotide priming (42) and used as hybridization probe. The *Sac* I, *Eco*RI, *Bgl* I, and *Rsa* I fragments of the TNF-α gene-pAT153 clone (*a*) or the *Eco*RI and *Bgl* I fragments of the TNF-α gene-pBR322 clone (*b*) were alkali-denatured and coelectrophoresed as size markers (lane M). Fragment and estimated RNA lengths were indicated in nucleotides (nt). (*c*) Summary of data on transcription and on levels of methylation of the TNF-α and, for comparison, of the TNF-β genes in human granulocytes (ref. 46; this report) and in cell lines HL-60, Jurkat, and RPMI 1788 (refs. 43 and 44 and this report). The symbols − and (+), +, ++, and +++ refer to the absence or to different estimated levels of TNF-α and TNF-β or of promoter methylation, respectively. In the Jurkat cell line, methylation of the TNF-β promoter seems heterogeneous (+).

and RPMI 1788. Detailed restriction and genetic maps for the TNF-α (Fig. 1*a*) and -β (Fig. 1*b*) genes as well as the results of the restriction analyses are presented in Fig. 1. It is apparent that the TNF-α segment analyzed contains 60 5′-CG-3′ sequences of which 13 (22%) are accessible to restriction analyses. In the TNF-α and -β genes in human sperm all the 5′-CG-3′ sites that can be analyzed by restriction endonucleases are methylated; the same holds true for most of the sites in DNA from granulocytes in the TNF-β gene. In DNA from lymphocytes several of the 5′-CG-3′ sequences amenable to restriction analysis are methylated in the TNF-α gene. In the TNF-β gene very few sites are methylated. In both genes the promoter region seems hypomethylated in DNA from lymphocytes. DNA from gran-

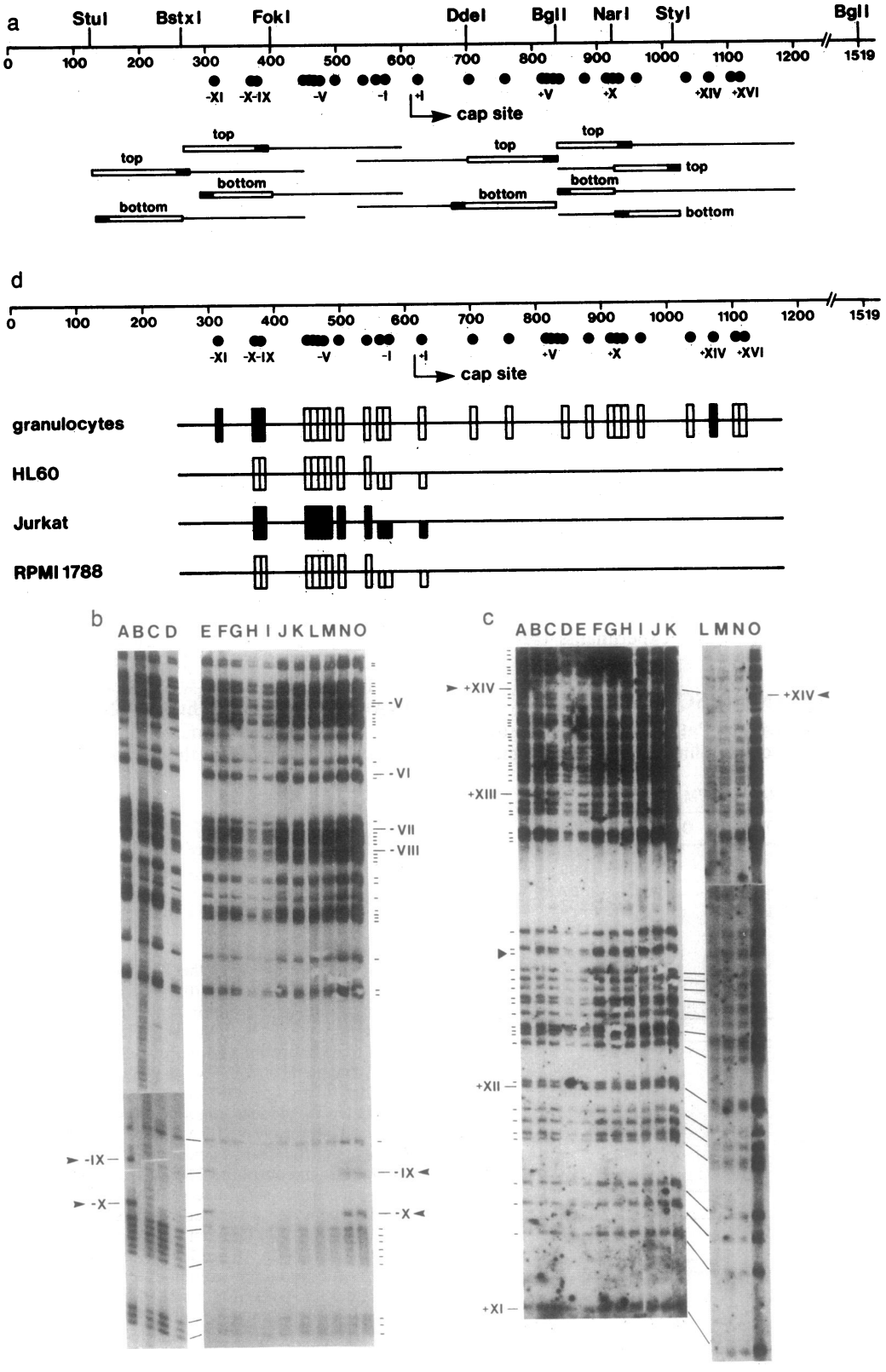
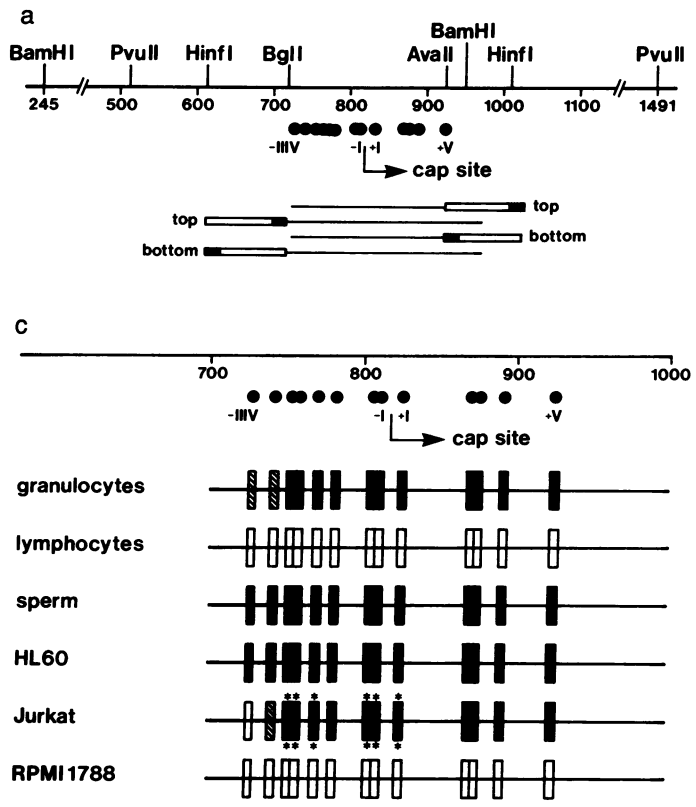


FIG. 3. (*Legend appears at the bottom of the opposite page.*)



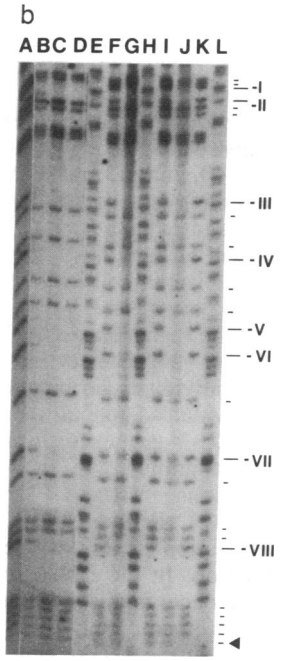


FIG. 4. Genomic sequencing in TNF-β gene. (*a*) Organization and symbols are as described in the legend for Fig. 3*a*. (*b*) Lanes contain the reaction products of the bottom strand of the following DNAs. Lanes: A and K, cytidine reaction of TNF-β gene plasmid control; L, guanosine reaction of same control; B, cytidine reaction of Jurkat cell line; C and D, cytidine reaction of sperm DNA from different individuals; E, guanosine reaction of lymphocyte DNA; F, cytidine reaction of lymphocyte DNA from individual 1; G, cytidine reaction of granulocyte DNA from individual 1; H, I, and J, equivalent samples from individual 2. (*c*) Summary of the genomic sequencing data. Asterisks indicate heterogeneous methylation; hatched bars represent partly methylated sites.

ulocytes is strikingly hypomethylated in the TNF-α gene and its promoter region. The TNF-α gene seems undermethylated in cell lines HL-60 and RPMI 1788 and heavily methylated in the Jurkat cell line (Fig. 1*a*). The TNF-β gene is hypomethylated only in cell line RPMI 1788 (Fig. 1*b*).

Transcription of TNF-α and -β Genes in Human Granulocytes and in Human Cell Lines. Total cellular RNA from human granulocytes has been investigated for TNF-α-specific RNA sequences. The results of RNA transfer and hybridization experiments (Fig. 2) document that TNF-α-specific RNA is transcribed in human granulocytes (Fig. 2*a*). Treatment of human granulocytes with PMA has no effect on the transcription of the TNF-α gene. The results of RNA hybridization experiments on the same filter to a ^{32}P-labeled β-actin probe ascertain that equivalent amounts of RNA have been applied to each lane on the agarose gel (Fig. 2*b*). In contrast, the TNF-β gene is not transcribed as stable mRNA in peripheral blood granulocytes (data not shown). Fig. 2*c* summarizes data on expression of the TNF-α and -β genes in primary human granulocytes and in the human cell lines HL-60, Jurkat, and RPMI 1788 without and after stimulation by PMA (43, 44); this table also juxtaposes the data on DNA methylation. In cell line Jurkat, the TNF-α promoter is methylated and is not transcribed even after PMA stimulation.

Genomic Sequencing Analyses in the Promoter and Upstream Regions of the TNF-α Gene. This investigation has been performed for the promoter and the 5′-upstream regions of the TNF-α gene in human granulocytes in sequences comprising some 800 nucleotides. The maps in Fig. 3*a* outline the sequencing strategies, and the autoradiograms present some of the genomic sequencing data (Fig. 3 *b* and *c*). In 15 different individuals exactly the same three deoxycytidine residues in positions −IX to −XI are methylated in the 5′-upstream region of the TNF-α gene (Fig. 3*b*). Eleven of these individuals were of central European origin, and 4 individuals were of Chinese and of African origins. The data in Fig. 3*b* and results not shown demonstrate that the deoxycytidine residues in positions −IX and −X are also methylated in the DNA from granulocytes of individuals of Chinese or of African origin. The data in the control lanes (Fig. 3*b*) have been derived from cytidine reactions with the control plasmids containing the TNF-α gene. A fourth deoxycytidine in position +XIV (first intron) is also methylated in all 15 individuals (Fig. 3*c*), as shown for 9 individuals of European origin, one individual of Chinese origin, and two individuals of African origin. All the genomic sequencing data in the TNF-α gene have been summarized in Fig. 3*d*. The results show a surprisingly high concordance in the location of 5-methyldeoxycytidine (^{5}mdC) residues in the regulatory

FIG. 3 (*on opposite page*). Genomic sequencing in the TNF-α gene. (*a*) Restriction map and sequencing strategy. The published nucleotide sequence (26–28) was used for the construction of this map, and nucleotide numbers were from this sequence. Roman numerals refer to upstream (−) and downstream (+) 5′-CG-3′ sequences (●) relative to the site of transcriptional initiation (bent arrow, at position 615); these numerals were also used in (*b–d*) and relate to the same nucleotides. Map locations of the ^{32}P-labeled hybridization probes on the top or bottom strand are included. Oligonucleotide primers (▬) used for the synthesis of ^{32}P-labeled probes (▭) and the region for which the nucleotide sequence was genomically determined (—) are designated. Methylation at sites +II and +III was readable within the probe region. Restriction sites not relevant for probe constructions were omitted. (*b* and *c*) Autoradiograms of genomic sequencing gels: top strand (*b*) between 5′-CG-3′ positions −V to −X and bottom strand (*c*) between positions +XI to +XIV (first intron) were genomically sequenced. Lanes of *b*: A, E, N, O, plasmid controls; B, DNA from Chinese individual; C and F–M, DNA from Europeans; D, DNA from African. Lanes of *c*: A, K, and O, plasmid controls; B–J, DNA from 9 Europeans; L, DNA from Chinese individual; M and N, DNA from individuals of African origin. The ^{5}mdC residues in positions −IX, −X, and +XIV are indicated by arrowheads. The filled triangle points to a CCAGG sequence in the dcm$^+$ host-grown control plasmid. (*d*) Summary of genomic sequencing data. Map indicates nucleotide numbers and the site of transcriptional initiation (bent arrow) as well as upstream (−) and downstream (+) 5′-CG-3′ sequences (roman numerals). □, Unmethylated 5′-CG-3′ sequences; ■, methylated sites. Symbols above line, top strand; symbols below line, bottom strand.

region of the TNF-α gene (genomic sequencing data, Fig. 3) and in the gene itself (restriction analyses, Fig. 1*a* and genomic sequencing data, Fig. 3) in DNA from granulocytes in 15 different individuals of diverse ethnic origins. The promoter region of the TNF-α gene is unmethylated in all 5′-CG-3′ residues in granulocyte DNA (Fig. 3). The presence of the ^{5}mdC residues in the upstream region of the TNF-α gene in granulocyte DNA is apparently compatible with the transcription of this gene in granulocytes (Fig. 2*a*). Fig. 3*d* also summarizes the results of the genomic sequencing experiments in the promoter and upstream regions of the TNF-α gene in human cell lines HL-60, Jurkat, and RPMI 1788 (data not shown). Gene expression inversely correlates with promoter methylation (Figs. 2*c* and 3*d*).

State of Methylation in the 5′ Region of the TNF-β Gene. Patterns of DNA methylation in established cell lines can differ significantly from those in living organisms. We initiated a study on DNA methylation in the region spanning the cap site of the TNF-β gene involving ≈200 nucleotides in DNA from granulocytes from nine individuals, in partially purified lymphocytes from five individuals, and in sperm from six different individuals. All 5′-CG-3′ sequences in this region are methylated in the DNA from granulocytes of nine different individuals, except for positions −VII and −VIII, which are partly methylated (Fig. 4*c*). Perhaps there is heterogeneity in the level of methylation in this segment among different cells in the population. In the DNA from human lymphocytes, all 5′-CG-3′ sequences in this region are unmethylated, but these sequences are completely methylated in DNA from sperm. Expression of the TNF-β gene in human lymphocytes has been shown (44, 45).

The same region has also been investigated in three human cell lines—HL-60, Jurkat, or RPMI 1788. The TNF-β gene is expressed in cell lines Jurkat and RPMI 1788 (Fig. 2*c*) but is not expressed in HL-60 cells (refs. 43 and 44 and this report). The expression of the TNF-β gene can be stimulated in Jurkat and RPMI 1788 cells by PMA (Fig. 2*c*). Strong methylation appears in all 5′-CG-3′ sequences in the 200-nucleotide region surrounding the cap site of the TNF-β gene in cell line HL-60 (Fig. 4*c*), in which the TNF-β gene is not transcribed. The same region is completely unmethylated in cell line RPMI 1788, in which the TNF-β gene is actively transcribed. In the Jurkat cell line, which expresses the TNF-β gene weakly, many 5′-CG-3′ sequences are methylated in the analyzed region, except for a few sites upstream (Fig. 4*c*).

We conclude that in the regions of the human genome investigated here, a surprising concordance of DNA methylation at the nucleotide level exists between different individuals. Patterns of DNA methylation are, of course, different in the DNAs from cells of different tissues. Whether this concordance in human DNA methylation extends to other parts of the human genome requires further investigation. Moreover, our results on the high degree of stability of cell type-specific methylation patterns will be of interest in relation to their genetic control.

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