Methylation Levels at Selected CpG Sites in the Factor VIII and FGFR3 Genes, in Mature Female and Male Germ Cells: Implications for Male-Driven Evolution

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Summary

Transitional mutations at CpG dinucleotides account for approximately a third of all point mutations. These mutations probably arise through spontaneous deamination of 5-methylcytosine. Studies of CpG mutation rates in disease-linked genes, such as factor VIII and FGFR3, have indicated that they more frequently originate in male than in female germ cells. It has been speculated that these sex-biased mutation rates might be a consequence of sex-specific methylation differences between the female and the male germ lines. Using the bisulfite-based genomic-sequencing method, we investigated the methylation status of the human factor VIII and FGFR3 genes in mature male and female germ cells. With the exception of a single CpG, both genes were found to be equally and highly methylated in oocytes and spermatocytes. Whereas these observations strongly support the notion that DNA methylation is the major determining factor for recurrent CpG germ-line mutations in patients with hemophilia and achondroplasia, the higher mutation rate in the male germ line is apparently not a simple reflection of sex-specific methylation differences.

Introduction

The involvement of cytosine methylation in the regulation of gene expression and as an epigenetic modification marking imprinted genes is well established (Bird 1987; Laird and Jaenisch 1994; Olek and Walter 1997; Wutz 1997). Methylated cytosines are predominantly found within CpG dinucleotides (Bird 1980; Gruenbaum et al. 1981). 5-Methylcytosines are prone to spontaneous deamination on hydrolytic attack, yielding the natural base thymine, and therefore are inherently more difficult to repair. As a result of the hypermutability of 5-methylcytosine, CpG dinucleotides are underrepresented in the human genome, by a factor of four to five (Schorderet and Gartler 1992). The remaining 5-methylcytosines place a heavy mutational burden on the human genome. Approximately 37% of all germ-line mutations responsible for genetic diseases are localized to CpG dinucleotides (Cooper and Youssoufian 1988).

The parental origin of germ-line mutations is not equally distributed between the two sexes; Haldane (1947) first reported a higher male than female rate for the origin of mutations in hemophilia families. Recently, molecular identification of underlying mutations in many diseases has amply confirmed a sex bias in germ-line mutations. Most studies of the sex ratio for point mutations in hemophilia A and B have shown a greater number of mutations in males (Rosendaal et al. 1990; Ketterling et al. 1993; Oldenburg et al. 1993; Becker et al. 1996; Sommer and Ketterling 1996). This effect is even more pronounced with respect to point mutations at CpG sites (Ketterling et al. 1993), which account for 40% of all point mutations in hemophilia (Pattinson et al. 1990). Studies of the X-linked zink-finger-protein gene (ZFX)—and its homologous gene ZFY—on the Y chromosome have estimated a male:female mutation ratio of 6.5 (Shimmin et al. 1993). In birds, in which males are homogametic (i.e., ZZ) and females are heterogametic (i.e., WZ), a male:female mutation ratio of 5.2 was found for the Z-linked CHD gene (chromo-helicase DNA–binding protein), compared with its homologue on the W chromosome (Ellegren and Fridolfsson 1997).

There are three major explanations for the observed bias in the origin of mutation: (i) If germ-line mutations are assumed to be largely the results of replication errors, then the higher number of mitotic cell divisions in sper-

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matocytes, relative to oocytes, might explain the higher mutability of spermatocytes (Vogel and Motulsky 1997). (ii) Differences in the level of CpG methylation, between male and female germ cells, might result in sex-specific mutation patterns (Driscol and Migeon 1990; Crow 1997; Sapienza 1996). (iii) A combination of both mechanisms—namely, the fixation of an elevated mutation rate at 5-methylated CpGs, via replication, could be responsible.

Here we present the first detailed comparison of the methylation maps of nonclustered CpG dinucleotides within the factor VIII and FGFR3 genes in human male and human female germ cells. These two genes were selected to as a means to address the correlation between sex-specific mutation rates and the methylation status of male and female germ cells, because they show high rates of CpG mutation and paternal bias for the origin of mutations. Our results for the factor VIII and FGFR3 genes provide evidence that these CpG sites are highly methylated in both male and female germ cells.

Material and Methods

Sample Preparation

Ejaculates from 10 donors were pooled. DNA was extracted according to the method of Gill et al. (1985): The samples containing mature sperm cells, epithelial cells, and lymphocytes were washed with lysis solution containing SDS and proteinase K. The released DNA was discarded, and the unlysed mature sperm-cell pellet was treated with DTT, SDS, and proteinase K. DNA from the supernatant was ethanol precipitated and dissolved in Tris-EDTA (TE) buffer. Nonfertilized metaphase I or GV-phase oocytes from women undergoing in vitro fertilization were collected. Oocytes were purified briefly with hyaluronidase enzyme, to remove cumulus cells. Residual adhering cells were removed by repetitive pipetting in a micropipette. Purified oocytes were stored in 1 × PBS at −20°C until use. For PCR amplification, a pool of $\geqslant$40 oocytes were used in each reaction. A pool of 23 first polar bodies was obtained by laser-assisted microdissection of the zona pellucida and picking of the polar bodies by means of a micropipette (Montag et al. 1998).

Bisulfite Modification of DNA

Modification of the oocyte DNA and of the isolated chromosomal sperm DNA, by sodium bisulfite, was performed according to the method of Olek et al. (1996). Approximately 5 μl of oocyte pool suspended in 1 × PBS was mixed with 2 vol of 2% low melting point (LMP) agarose prepared in 1 × PBS. A 10-μl portion of this mixture was pipetted into 300 μl of cold mineral oil laid over 800 μl of lysis solution (10 mM Tris-HCl, 10 mM EDTA, 1% SDS, 20 mg proteinase K/ml). After overnight incubation at 50°C, agarose beads were treated with para-methyl-sulfonyl-fluorid (PMSF) to inactivate proteinase K, were washed several times with TE buffer (pH 9), were equilibrated against *Eco*RI restriction-enzyme buffer, and were digested overnight. DNA was denatured by equilibration against 0.3 M NaOH and 0.1 M NaOH. Individual beads were overlaid with mineral oil and were boiled for 15 min to ensure complete separation of individual DNA strands. The tube was put on ice, to resolidify the agarose beads. Isolated sperm DNA was digested with *Eco*RI, was incubated with 0.3 M NAOH for 15 min at 50°C, and subsequently was mixed with 2% LMP agarose to form agarose beads.

Individual agarose beads containing sperm DNA or oocyte DNA were incubated at 50°C for 4 h in the dark, in 100 μl of 2.5 M sodium metabisulfite, 125 mM hydroquinone, pH 5.0. Beads were then washed with 1 ml of TE solution (six times, 15 min each), which was followed by desulfonation in 500 μl of 0.2 M NaOH (twice, 15 min each). The reactions were neutralized by HCl, and the beads were washed with TE followed by a washing with double-distilled H_2O.

PCR Reaction

Each PCR target region was amplified in two rounds of PCR, by means of two nested pairs of primers, to achieve best sensitivity and maximum specificity. All PCRs were done in 100-μl vol containing 200 mM each dNTP, 2.25 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 units of *Taq* polymerase, and 25 pmol of each primer (table 1). PCRs were performed in a Biometra Trioblock-Thermocycler, with the following program: 94°C for 5 min; 35 cycles of 92°C for 45 s, 55°–68°C (depending on the pair of primers used; see table 1) for 1 min 15 s, and 72°C for 1 min 45 s; and a final extension step at 72°C for 10 min.

Cloning of PCR Products and DNA Sequencing

The desired PCR products were cloned in pCR II-TA cloning vector, by means of the original TA Cloning Kit from Invitrogen. Positive clones were selected and identified by PCR. Approximately 20 positive clones from each region were sequenced by means of Thermo Sequenase™ and DYEnamic ET primers (-40 M13 forward-labeled primer) from Amersham (ABI Prism 373 automated sequencer). Individual clones were identified by comparison of the positions of residual ($\ll$1%) unmodified or non-CpG methylated positions within the individual sequences.

Table 1

Primers Used in PCR Amplifications for Regions A–D (Factor VIII Gene) and Region E (FGFR3 Gene)

Region and Exon (Size; Annealing Temperature)	Primer Names (Sequences)
A:	
Exons 8 and 9:	
External (581 bp; 60°C)	BiF8/9F12 (acc ttg ggt ata tta tat tgt tgt tga aga gg)
	BiF8/9R12 (ctc tcc aaa ctt ttt ctt ctt acc taa cct)
Nested (506 bp; 65°C)	BiF8/9F13 (tgt tgt tga aga gga gga ttg gga tta tgt)
	BiF8/9R13 (tat atc tcc aac ttc ccc ata aaa taa aaa tc)
Exon 9:	
Nested (180 bp; 60°C)	BiF8/9F16 (aga agt tat aaa agt taa tat ttg aat aat gg)
	BiF8/9R13: (same as above)
B:	
Exon 14:	
External (617 bp; 50°C)	BiF8/14F5 (tag taa tgg gtt ttt tgg tta ttt gga t)
	BiF8/14R6 (taa aaa act act cat ccc ata atc cca a)
Nested (561 bp; 55°C)	BiF8/14F6 (agg gag ttt ttt tta ggg aat aga ggg a)
	BiF8/14R5 (taa tcc caa aac ctc tcc act aca aca a)
C:	
Exons 17 and 18:	
External (616 bp; 60°C)	BiF8/18F14 (att tgt ttt gtt ttt tat tat ttt tga tga gat)
	BiF8/18R14 (taa tca cta att ata ttc cca ata cct aaa cc)
Nested (490 bp; 62°C)	BiF8/18F15 (tat tga aaa tat gga aag aaa ttg tag ggt)
	BiF8/18R15 (caa aca aac tca tac cta aat aaa aat tat aca)
D:	
Exon 23U:	
External (570 bp; 63°C)	BiF8/23F3 (gta ttt tga att gtt ttt agg tga tgt tgt t)
	BiF8/23R4 (ctc aaa ata act aaa aca att aat cac cct a)
Nested (460 bp; 60°C)	BiF8/23F4 (att atg ttg gag tag tag agt tta aat ggt t)
	BiF8/23R3 (act taa cac tta cta ttt aat cac aac cca t)
Exon 23L:	
External (546 bp; 63°C)	BiF8/23F1 (tag tta gtt att tta ttt atg gtt gag gga a)
	BiF8/23R1 (att acc ctc aaa taa tac tac cta ctc cta a)
Nested (507 bp; 55°C)	BiF8/23F2 (tta ttt tat tta tgg ttg agg gaa gaa gga t)
	BiF8/23R2 (cta taa acc ata ata aaa taa caa aac cca a)
E:	
Exon 10:	
External (470 bp; 60°C)	BiFGFR3/10F1 (ttt ttt tag ggt ttg gtt ttt tag att tat)
	BiFGFR3/10R1 (aca aaa aaa act cac aca aca caa aac)
Nested (308 bp; 60°C)	BiFGFR3/10F2 (ggt ttt ggg tta ggg gaa ttt atg gga)
	BiFGFR3/10R2 (aac aaa caa ctc aaa acc taa tat cta ctt)

Statistical Analysis

χ^2 Tests were applied for statistical analysis. The equation used was as follows: $\chi^2 = (ad - bc)2n/[(a + b)(a + c)(b + d)(d + c)]$, where a and b are the number of methylated clones at sites 1 and 2, respectively, whereas c and d are the number of unmethylated clones at sites 1 and 2, respectively, and n is the total number of clones for sites 1 and 2.

Results

Using the sodium bisulfite–conversion method, we analyzed the methylation rate of selected CpG dinucleotides of the factor VIII and FGFR3 genes, in human mature spermatocytes and mature oocytes. By this method all nonmethylated cytosine nucleotides are converted to uracil, whereas the methylated cytosines remain unconverted under the experimental condition (Frommer et al. 1992; Olek et al. 1996). After amplification, cloning, and sequencing of PCR fragments obtained from sodium bisulfite–treated chromosomal DNA, methylation was determined by the presence of nonconverted cytosines in the sequencing profile.

In mature spermatocytes, all of the 33 CpG sites investigated were found to be highly methylated (fig. 1). a total of 25 CpG sites in regions A, C, and D of the factor VIII gene, including one intronic CpG site, and in region E of the FGFR3 gene were almost completely methylated, with overall methylation levels of 95%–100%. In region B of the factor VIII gene, which con-

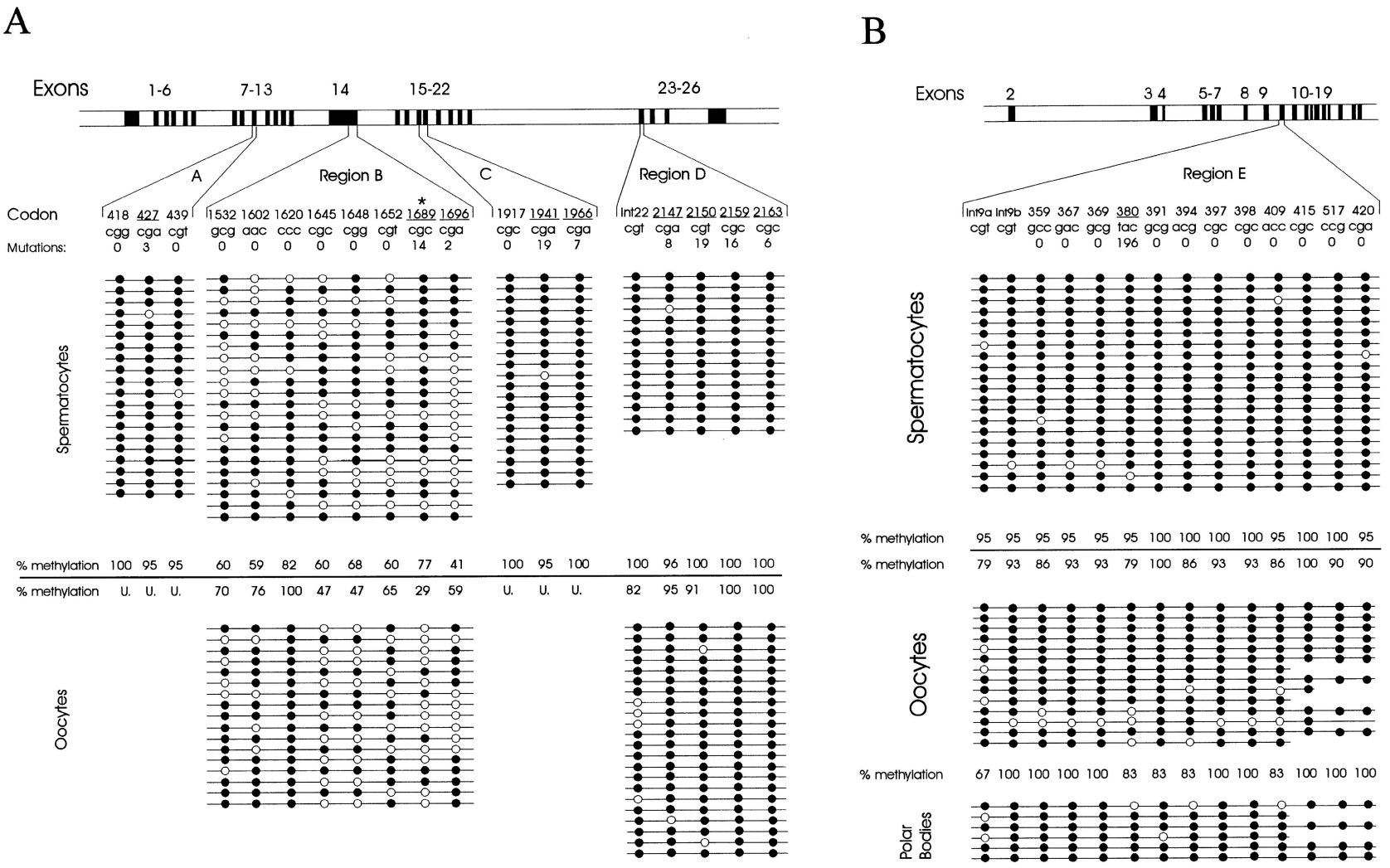

Figure 1 Methylation profiles of clones from selected regions of the factor VIII and FGFR3 genes. Shown at the tops of the panels are the exon/intron structures of the genes, for the regions analyzed. Exons are represented as blackened bars. Gene regions examined are labeled "A"–"E" and have been enlarged. The no. of mutations reported for each codon is indicated. Bisulfite sequences obtained from individual cloned fragments are depicted as horizontal lines with individual CpGs represented by unblackened circles (for unmethylated) and blackened circles (for methylated). Compilation of results for the factor VIII (*A*) and FGFR3 gene (*B*) genes is shown. The percentages of methylated clones and of unmethylated clones are listed along the line separating sperm data and egg data.

tains eight CpG sites, the situation is somewhat different. All CpGs exhibit a heterogeneous and less prevalent methylation level, 41%–82%. Methylation in this region is not only significantly lower than that in regions A, C, or D ($P = .027$ to $P < .001$), but it also shows a mosaic distribution on individual chromosomes. However, although the distribution of methylated and nonmethylated CpGs appears to be highly mosaic, all chromosomes show at least 40% overall methylation.

In view of the differences between regions B and D of the factor VIII gene in spermatocyte chromosomes, we analyzed the methylation levels of these regions and of region E of the FGFR3 gene, in chromosomes of mature oocytes. For regions B and D, 17 and 22 individual clones, respectively, were sequenced, which—when compared with the number of oocytes used as a starting material—represent ~20%–25% of the chromosomes analyzed (fig. 1). We therefore can exclude any significant bias during PCR or cloning, toward either methylated or nonmethylated fragments. Again, regions D and E displayed a rather homogeneous highly methylated profile on all chromosomes, whereas region B appeared to be highly mosaic. When these results were compared with those obtained for spermatocytes, 26 of the 27 CpG sites analyzed revealed a very similar overall methylation level, including the heterogeneously methylated region B in exon 14. Only a single CpG dinucleotide at codon 1689 was significantly less methylated in oocytes than in spermatocytes (29% vs. 77%; $P = .003$).

To test whether the methylation levels analyzed in the oocyte samples might represent differences in chromosomes derived from the egg versus those derived from the first polar body, we performed a bisulfite methylation analysis with a pool of 23 first polar bodies. Analysis of six clones of region E of the FGFR3 gene showed that the CpG dinucleotides of DNA from pooled first polar bodies were methylated at a level comparable to that observed in mature oocytes and spermatocytes (fig. 1). These results suggest that the methylation observed in oocytes is probably established prior to the first meiotic division.

At present, the hemophilia A database (Wacey et al. 1996) contains 272 mutations at CpGs, 95 of which are confined to CpGs analyzed in our study (fig. 1). When the overall methylation levels are compared with the occurrence of mutations, no obvious correlation between degree of methylation and frequency of mutations can be detected; that is, mutational hot spots apparently do not coincide with sites of preferential methylation. However, since, for most of these sites, such an effect might be masked by different phenotypes of the disease (e.g., by a possible founder effect of some mutations or regions within the factor VIII gene that bear less significance for protein function, or by a certain bias in the screening methods for mutations), we concentrated on the analysis of a subgroup of hot spots. All CpG mutations at these hot spots (codons 427, 1696, 1941, 1966, and 2163) create uniform nonsense mutations leading to phenotypically comparable cases of severe hemophilia. Yet, even in this group, no significant correlation between methylation levels and number of mutational events was observed.

Discussion

It is widely accepted that point mutations originate more often in male than in female germ cells, thereby providing a rationale for the concept of male-driven evolution (Shimmin et al. 1993; Becker et al. 1996; Ellegren and Fridolfsson 1997). At present, the role of sex-biased methylation in sex-specific mutation rates is being widely discussed. However, no direct proof for any of the proposed mechanisms has been provided until now (Sapienza 1996; Crow 1997).

To address this issue, we established a detailed methylation map of CpG dinucleotides within the factor VIII and FGFR3 genes in mature male and female gametes (fig. 1). These genes have been chosen because of the following reasons: The X-linked factor VIII gene displays a higher mutation rate in the male germ line, and CpG positions are affected in ~40% of all point mutations (Youssoufian et al. 1986; Pattinson et al. 1990). In the autosomal FGFR3 gene, a G→A transition within a CpG site at codon 380 is the cause of 95% of all cases of achondroplasia (Rousseau et al. 1994; Bellus et al. 1995). Moreover, a striking paternal-age effect has been described, suggesting an almost exclusive paternal origin of the causal mutation in achondroplasia (Rousseau et al. 1994; Orioli et al. 1995).

Our study presents the first detailed methylation profiles of human disease genes in mature germ cells. Analysis of 33 CpG sites in mature spermatocytes and of 27 CpG sites in mature oocytes revealed a high degree of similarity in methylation levels of male and female gametes.

This overall high level of methylation at CpG sites in germ cells supports the molecular link between methylation and hypermutability (Cooper and Youssoufian 1988). However, the observation of equal levels of methylation in the sexes contradicts conclusions drawn from previous studies. Driscoll and Migeon (1990) investigated the methylation pattern in single-copy genes of cell populations taken from human fetal ovaries and fetal testes. They found that, prior to the onset of meiosis, primordial germ cells were unmethylated in both sexes, and data on meiotic fetal ovary cells suggested that female germ cells remain unmethylated during oogenesis. In contrast, male germ cells became methylated postnatally, prior to or during the early stages of spermato-

genesis. However, Driscoll and Migeon provide no data on oocytes at the time of fertilization, and thus the possibility remains that mature germ cells are methylated de novo during late stages of their ontogeny. Our study is the first to address this subject in humans and clearly supports this hypothesis. Furthermore, It is in accordance with a study on murine mature oocytes and spermatocytes (Kafri et al. 1992), which showed CpG sites of single-copy genes to be methylated in mature germ cells from both sexes. In the case of the mouse, the onset of methylation occurs in both male and female germ cells during days 15.5 and 18.5 of development. Although the exact time at onset of germ-line–specific methylation remains to be determined in humans, our results on methylation in the first polar bodies indicate that, in humans, too, methylation may be established prior to the first meiotic division.

Overall, our results argue against previous models, which propose that the observed sex bias in de novo mutation rates might be a simple reflection of sex-specific methylation differences. It follows that the origin of sex-biased CpG mutation rates must be sought in either (i) the different time at onset of methylation in female versus male germ line, (ii) the different cellular environments of the female versus the male germ line, which may influence the rate of spontaneous deamination of methyl cytosines, or (iii) the vastly different cycles of DNA replications in female versus male germ cells. On the basis of our results, we favor the third explanation as being the most plausible. In human females, a constant number of divisions—that is, 24—occur during oogenesis, whereas the number of divisions during male gametogenesis reaches ~400 in a 30-year-old male and increases continuously with age (Crow 1997; Vogel and Motulsky 1997). As an explanation for the phenomenon of male-driven evolution, the greater number of mitotic divisions in the male germ line is more plausibly supported by our data than is the hypothesis of different methylation levels. Methylation definitely contributes to this effect, by creating hot spots for mutations at CpGs—mutations that, although occurring at equal rates in both germ lines, are, as a consequence of higher replication rates, fixed at a higher proportion in the male germ line.

In the hemophilia A database, the number of mutation events reported at the CpG sites investigated in this study is very heterogeneous, with a range of 0–19 (Wacey et al.1996). When these mutation frequencies are compared with the observed CpG methylation levels, no obvious correlation can be detected; that is, the methylation is not significantly higher at CpGs that exhibit a higher mutation rate. This becomes particularly obvious in a comparison of five mutational hot spots (at codons 427, 1696, 1941, 1966, and 2163), in which a C→T transition creates stop codons, which lead to a rather uniform and severe phenotype of the disease. Therefore, a simple scenario is not capable of explaining similar mutation rates at CpG codons with very different methylation levels within one of the sexes (fig. 1), an effect that has also been observed for p53 mutations in lung cancer cells (Denissenko et al. 1997). Since the efficiency of mutation fixation through DNA replication should be identical for all sites, differential methylation should be correlated with the mutation rate. We can invoke only three possible mechanisms for this discrepancy. First, the methylation status may not have any significant influence on the mutation rate. Given the host of available data supporting CpG mutability, this seems unlikely. Second, additional factors may influence the rate of deamination more or less site specifically. This may be mediated by preferential binding and interaction of exogenous chemicals with sequence-specific targets of DNA sequences, as is the preferential interaction of (+/−)anti–7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzopyrene (BPDE) to methylated CpG at codons 175, 248, and 273 of the p53 gene (Denissenko et al. 1997). However, how these adduct formations may influence the mutagenicity of theses methylated CpG sites is not known yet. Third—and, in our view, most convincingly—the efficiency of repair could be somewhat biased with regard to either sequence context or chromatin structural context of the affected sites.

The overall high methylation at all CpGs analyzed also raises the question of why a relatively high density of methylation is maintained in both genes, despite the fact that a considerable number of these CpG sites, in which a C→T transition would create only a conservative amino acid exchange, are evolutionarily maintained. This observation might indicate that a certain density of methylation must be maintained in both genes—to ensure either the structural integrity or the proper regulation of the genes.

One remarkable observation of our study concerns the significantly lower rate of methylation in region B (exon 14 of the factor VIII gene), compared with that in the other regions investigated. Such regional hypomethylation,which also has been reported for exon 25 of the retinoblastoma gene (Mancini et al. 1997), might be linked to specific features of DNA sequence and/or chromatin structure and may have some importance for transcription regulation of these genes. Exon 14 codes for the B domain, which constitutes ~40% of the mature factor VIII protein (Vehar et al. 1984; Toole et al. 1984). During activation by thrombin, the B-domain is completely removed from the protein (Kaufman 1992). Several studies have shown that the B-domain is not necessary for factor VIII function (Eaton et al. 1986; Toole et al. 1986). A recombinant B-domain–deleted factor VIII protein has already been successfully given to hemophilia A patients in a phase 3 clinical trial (Fijna-

vandraat et al. 1997). Although the function of the B-domain has not yet been elucidated, it is conceivable that both exon 14 (at the DNA level) and the B-domain (at the protein level) are involved in the regulation of factor VIII expression (Pittman et al. 1994). Further studies are needed to evaluate whether hypomethylation of exon 14 is in fact related to a regulatory role of this region in the expression of the factor VIII gene product.

In conclusion, the present study provides evidence for closely similar methylation rates at noncluster CpGs in mature germ cells of both sexes and thus implies that the greater number of mitotic divisions—rather than a sex-specific methylation effect—is the decisive factor that accounts for the higher mutability of the male germ line.

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