J Hum Genet (2002) 47:239–242

ORIGINAL ARTICLE

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The CpG island in intron 22 of the factor VIII gene is predominantly methylated on the X chromosome of human males

Received: December 4, 2001 / Accepted: February 12, 2002

Abstract The inactivation of one of the two X chromosomes in females is a random process associated with methylation principally in CpG islands. The methylation status of a CpG island in intron 22 of the human factor VIII (FVIII) gene was investigated using a novel practical approach. Genomic DNA from men and women was digested with various methylation-sensitive (MS) restriction enzymes, the recognition sequences of which occurred at least once in the FVIII CpG island. Long distance-polymerase chain reaction (LD-PCR) was then used to amplify the island. Successful amplification indicated that the island was methylated and the absence of a PCR product indicated that at least one restriction site was unmethylated. To analyze the relative methylation status of the extragenic and intragenic copies of the island, we used Southern blot with MS restriction enzymes. The MS LD-PCR patterns obtained from male and female DNA samples indicated that at least some copies of the intragenic CG island were fully methylated at all sites investigated. Additionally, the island showed consistent differences among individuals. Southern blot studies using female DNA showed partial resistance to MS digestion for the intragenic and extragenic CpG island homologs. Our observations indicate that this CpG island is predominantly methylated on the X chromosome of males and suggest that its methylation pattern does not correlate with X inactivation of females. This prevents the use of this island coupled with DNA polymorphisms for investigation of X-chromosome inactivation.

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Key words Intron 22 · Factor VIII gene · *Xba*I restriction fragment length polymorphism · CpG island · X-chromosome inactivation · Methylation

Introduction

Dosage compensation in humans is achieved through random inactivation of one of the two X chromosomes in the cells of normal women (Lyon 1961). This inactivation occurs early in mammalian embryogenesis, affects all regions of the X chromosome with only few exceptions (for example, Brown and Williard 1990), and results in individuals who are cellular mosaics with either the maternal or paternal X chromosome inactivated.

DNA methylation of cytosine residues has been implicated in the inactivation process. Studies on X-linked housekeeping genes have revealed CpG dinucleotide clusters located at the 5′ regions. These "CpG islands" were found to be consistently unmethylated on the active X chromosomes and methylated on the X-inactive, and this pattern is highly conserved among different individuals and tissues (Maestrini et al. 1992). Outside these CpG islands, CpG sites are scarce and are generally found to be methylated on the X-active as well as the inactive X chromosome (Bird 1986).

Determination of nonrandom X inactivation is of potential value in explaining the symptomatic status of carriers of X-linked recessive disorders (Puck and Willard 1998) or in the investigation of clonality in proliferative disorders (Vogelstein et al. 1985). Among the approaches used to assess X inactivation, analysis of a polymorphism linked to the recognition sequence for a methylation-sensitive restriction enzyme (MSRE) contained within a CpG island, the methylation status of which correlates with X activation/inactivation, has proven valuable.

The human factor VIII (FVIII) gene is X linked and contains, within intron 22, a CpG island that promotes constitutive expression of two genes, F8A and F8B (Levinson et al. 1990, 1992). Recently, Peters and Ross (2001) have

isolated a 40-KDa protein encoded by the open reading frame F8A. They indicate that the protein has an important role in the function of normal huntingtin and is a candidate for involvement in the aberrant nuclear localization of mutant huntingtin found in degenerating neurons in Huntington's disease. The island neighbors an *Xba*I restriction fragment length polymorphism (RFLP) and a recently found *Msp*I RFLP (Bowen et al. 2000). The region constituting the CpG island, *Xba*I RFLP, *Msp*I RFLP, and F8A gene, is duplicated twice extragenically on chromosome X (Levinson et al. 1990).

The use of long distance-polymerase chain reaction (LD-PCR) has been described for genotyping the intragenic *Xba*I RFLP (De Brasi et al. 1999) and *Msp*I RFLP (Bowen et al. 2000). We proposed that these RFLPs may permit analysis of X inactivation in heterozygous females if any MSRE site presents consistent methylation only on the X-inactive. The proposed analysis involved three stages: first, digestion of genomic DNA with an MSRE for which sites were present in the region to be amplified by LD-PCR; second, LD-PCR; and finally, *Xba*I or *Msp*I genotyping of the LD-PCR product. Unmethylated targets would not be available for amplification following MSRE digestion, but methylated targets would. Random (nonskewed) X inactivation would result in both *Xba*I alleles or both *Msp*I alleles (depending on which RFLP is genotyped) being represented in the amplification products (a heterozygous RFLP result). With skewed X inactivation, one specific allele would predominate.

Assuming an identical methylation pattern on the X chromosome of males to that of the X-active from females and using the methylation-sensitive LD-PCR approach described earlier, we investigated the methylation status of the CpG island in intron 22 of the FVIII gene to improve our understanding of its relationship with X inactivation, and to assess the value of this approach as a tool to study X lyonization in human females.

Materials and methods

Methylation-sensitive LD-PCR

Leukocyte genomic DNA (500 ng) from male and female samples was digested to completion with eight different MSREs (Table 1) using 10 U enzyme (added in two pulses of 5 U) per μg of genomic DNA, overnight (more than 16 hr), and the conditions recommended by the manufacturer (Promega, Buenos Aires, Argentina and New England Biolabs, Cardiff, UK). This was followed by heat inactivation (60°C for 30 min), ethanol precipitation, resuspension in water, and then LD-PCR as previously described (De Brasi et al. 1999).

Methylation-sensitive Southern blots

Genomic DNA (5–8 μg) extracted from peripheral blood leukocytes was digested with *Bcl*I or *Xba*I + *Kpn*I with or without an MSRE (Table 1). Where necessary, ethanol precipitation was used to permit changes in restriction conditions (e.g., for double and triple digests). The digests were electrophoresed and Southern blotted, and the blots were probed with the 0.9-kb *Eco*RI-*Sac*I fragment from plasmid p482.6 (F8A probe) (Chan et al. 1989).

Results

LD-PCR of genomic DNA predigested with an MSRE is expected to yield a product only if all MSRE sites in the amplified region are methylated. The anticipated result was therefore that a PCR product would not be obtained in male samples, but would be in female samples. However, an LD-PCR product was obtained from both male and female DNA samples digested with seven of the eight MSREs studied and was not obtained from either with *Hpa*II (Fig. 1 and Table 1).

Because partial digestion of MSRE was of particular concern, the following control experiments were performed: genomic DNA was amplified using LD-PCR without MSRE pretreatment and the LD-PCR products (unmethylated by origin) were digested with each MSRE and then amplified in a second round of LD-PCR. No amplification products were obtained, indicating that, in absence of methylated restriction sites, MSREs digest to completion the DNA substrate, preventing any further PCR amplification. In addition, these control experiments exclude the presence of MSRE site polymorphisms as a cause for false positive PCR amplification.

The LD-PCR results, which specifically related to the CpG island within intron 22 of the FVIII gene and not to the two extragenic copies, therefore strongly indicated that the intragenic island is fully methylated at least in some leukocytes in men as well as in women. This was an unex-

Table 1. Methylation-sensitive assays on male and female DNA samples

MSRE[a]	Number of recognition sites in the template[a]	Fully methylated DNA samples/total samples analyzed LD-PCR (Southern blot)	
		Males XY	Females XX
*Fsp*I	1	2/2	2/2
*Sac*II	1	3/4	4/4 (4/4)
*Xho*I	3	2/3	2/4 (0/2)
*Bss*HII	5	1/2	1/2 (1/2)
*Sma*I	5	3/3	3/3 (4/4)
*Nae*I	6	2/2	2/2
*Nar*I	6	1/2	1/2
*Hpa*II	38	0/2[b]	0/2 (0/2)[b]

MSRE, Methylation-sensitive restriction enzyme; LD-PCR, long distance-polymerase chain reaction

[a] The nucleotide sequence used for restriction mapping and selection of MSREs was GenBank entry X86012 (Naylor et al. 1995)

[b] Results in agreement with Southern blot experiments from Levinson et al. (1990)

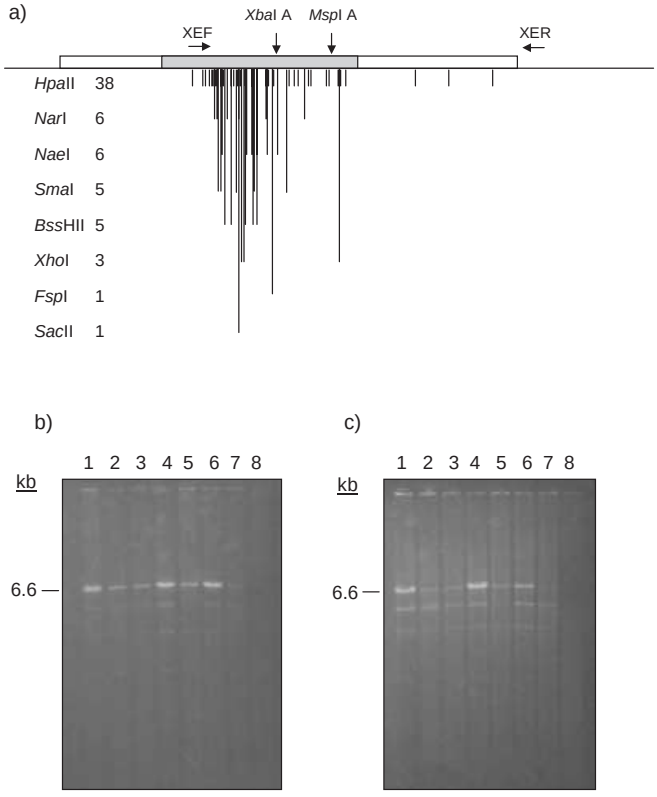

Fig. 1a–c. Methylation-sensitive long distance-polymerase chain reaction (LD-PCR). **a** Schematic showing the intron 22 CpG island (*gray box*) of the human factor VIII (FVIII) gene, the primers for LD-PCR (*XEF* and *XER*), the positions of the polymorphic loci *Xba*I A and *Msp*I A, and the distribution of relevant methylation-sensitive restriction sites (*vertical lines*). **b** and **c** Agarose gel electrophoresis of LD-PCR products from methylation-sensitive restriction enzyme (MSRE)-digested genomic DNA samples of one male **b** and one female **c**. *Lane 1*, no pretreated DNA; *lanes 2–8*, respectively, DNA pretreated with the following enzymes: *Sma*I, *Sac*II, *Fsp*I, *Xho*I, *Nae*I, *Nar*I, *Bss*HII. The 6.6-kb band identifies the specific LD-PCR product obtained from genomic DNA that has resisted digestion by the relevant MSRE

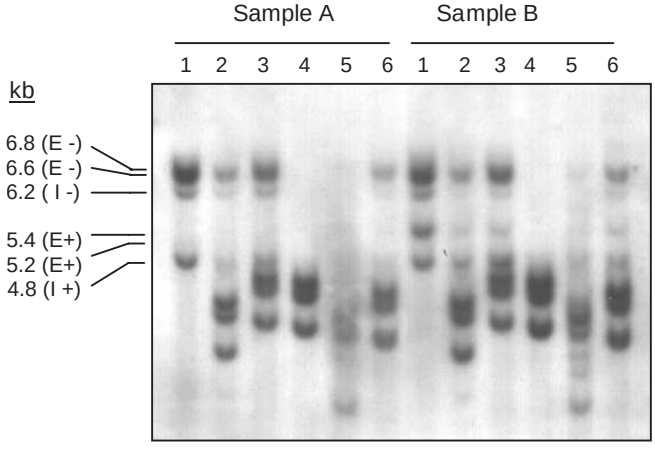

Fig. 2. Methylation-sensitive Southern blot using two female DNA samples. This Southern blot was based on the method designed by Chan et al. (1989) to investigate the multiple *Xba*I polymorphisms linked to the FVIII gene: *Xba*I + *Kpn*I digests were probed with a DNA segment from the upstream third of the CpG island (F8A probe). All six possible hybridization signals corresponding to the alleles of the multiple *Xba*I polymorphisms were indicated (*E* corresponds to extragenic alleles; *I*, to intragenic; and + or −, to the *Xba*I status). Sample A has the genotype *Xba*I [A B C] [+/− −/− −/−] and sample B has the genotype *Xba*I [A B C] [+/− +/_ −/−] (symbol _ indicates + or −). For both samples A and B, *lane 1* shows the results following digestion with *Xba*I + *Kpn*I, and *lanes 2–6* correspond to triple digests containing *Xba*I + *Kpn*I+ one MSRE as follows: *Sma*I (*lane 2*), *Sac*II (*lane 3*), *Hpa*II (*lane 4*), *Xho*I (*lane 5*), *Bss*HII (*lane 6*). Partial resistance to methylation-sensitive digestion is evident for all MSREs except *Hpa*II and *Xho*I

pected finding, a corollary of which is that in women, as in men, the active FVIII gene may be methylated at certain CpG sites contained in MSRE recognition sequences within the island.

The LD-PCR results additionally indicated that the methylation status of the island may differ among different individuals: an LD-PCR product was obtained with some DNAs, but not with others, despite repeated testing and controlling for PCR inhibition.

Only the MSRE *Hpa*II resulted in the absence of an LD-PCR product in all samples tested. This enzyme has an extremely large number of recognition sequences within the amplification target (Fig. 1, Table 1) and this may provide the explanation for the loss of amplification in women as well as in men: digestion at only one site is sufficient to prevent PCR.

Methylation-sensitive Southern blots were designed to investigate the methylation status of the intron 22 CpG island of the FVIII gene and the two extragenic copies of this island. *Xba*I + *Kpn*I were used to show heterozygosity for the intron 22 *Xba*I RFLP, and *Xba*I + *Kpn*I were used in conjunction with one MSRE to determine the methylation status of each discriminated allele. *Bcl*I was used to establish the intron 22 inversion genotype in informative carrier women. The results from two female DNA samples reveal some degree of resistance for methylation-sensitive digestion using *Sac*II, *Sma*I, or *Bss*HII as MSRE but none using *Hpa*II and *Xho*I (Fig. 2, Table 1). These Southern blot results confirm those obtained by methylation-sensitive LD-PCR except for *Xho*I (Table 1). This is probably due to the higher sensitivity of LD-PCR than Southern blot to detect small amounts of undigested DNA templates. The data obtained by Southern blot additionally indicate that, like the intragenic copies of the island, at least some of the extragenic copies show methylation of their CpG sites, preventing digestion with *Sac*II, *Sma*I, or *Bss*HII.

Discussion

The results of this study suggest that the CpG island in intron 22 of the human FVIII gene shows differences in methylation between individuals. This observation contrasts notably with the highly conserved methylation pattern between individuals for other CpG islands on the X chromosome (Maestrini et al. 1992). The methylation status of the intron 22 CpG island does not appear to reflect an active or inactive X chromosome as was previously sug-

gested (Levinson et al. 1990). The results exclude the CpG island of the FVIII gene as a suitable target for the investigation of nonrandom X inactivation using MSRE digestion followed by LD-PCR and RFLP analysis (De Brasi et al. 1999).

The FVIII gene intron 22 CpG island and its associated constitutive promoter show two interesting features: they are duplicated at least twice on Xq28 (Levinson et al. 1990) and they belong to a rare group of CpG islands longer than 3 kb (<1.2%) (Lander et al. 2001). It is possible that the presence of multiple copies of the island and the F8A gene explain the lack of correlation between methylation and X inactivation: for example, if the intragenic island is methylated such that the intragenic F8A is switched off, the extragenic copies of the island may be unmethylated so that the extragenic F8A is expressed from those loci. Expression of F8A from the extragenic copies has been demonstrated in hemophilia A patients with deletions of the entire FVIII gene (Levinson et al. 1990). Additionally, the length of this island and the large number of CpG dinucleotides within it make the identification of those sites (if any) that exhibit a correlation between methylation and X inactivation an extremely difficult task.

Acknowledgments This paper was supported by grants from the Alberto J. Roemmers Foundation, the René Barón Foundation, the Academia Nacional de Medicina, the Adolfo H. Aztiria Foundation, and the National Research Council (CONICET). We thank Dr. Eduardo Tizzano from the Saint Pau Hospital, Barcelona, Spain, for supplying the probe.

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