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Genome Res. 1996 6: 114-123
Access the most recent version at doi:10.1101/gr.6.2.114

RESEARCH

Methylation Analysis of a Marsupial X-linked CpG Island by Bisulfite Genomic Sequencing

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Paternal X chromosome inactivation occurs in rodent extraembryonic membranes and in all tissues of marsupials. Methylation of CpG islands occurs on the inactive X in eutherians and is considered to be a stabilizing mechanism. The only previous study of a marsupial X-linked CpG island was of the *G6PD* gene of the Virginia opossum, in which the paternally derived allele is not completely repressed. We have cloned the 5′ end of the *G6PD* gene from an Australian marsupial, the common wallaroo, and sequenced the associated CpG island. The paternally derived *G6PD* allele is completely repressed in tissues of this species. Methylation analysis using *Hpa*II and *Cfo*I restriction enzymes and bisulfite genomic sequencing of 47 CpG dinucleotides in a 613-bp region reveals hypomethylation of male and female DNA from tissues, cultured fibroblasts (in which the paternal allele is partially expressed) and sperm. This suggests that methylation of CpG islands is not required for maintenance of X inactivation in marsupials even where repression of the paternal allele is complete.

Dosage compensation by X chromosome inactivation occurs in both eutherian (placental) and metatherian (marsupial) mammals. In eutherians inactivation of either X chromosome occurs at random in female embryos and is stably maintained. In the extraembryonic endoderm of rodents the paternally derived X chromosome is inactivated (West et al. 1977). The paternally derived X chromosome is always preferentially inactivated in marsupials (Sharman 1971).

X inactivation in marsupials is less complete than in eutherians. In marsupials, X inactivation occurs in a tissue-specific fashion, and the activity state of paternally derived alleles varies for different genes and in different species. For example, in the Virginia opossum (*Didelphis virginiana*), a North American marsupial, there is detectable activity of the paternal allele of glucose-6-phosphate dehydrogenase (*G6PD*) in all tissues studied (VandeBerg et al. 1987). In contrast, subspecies crosses between the Australian common wallaroo and euro (*Macropus robustus robustus* and *Macropus robustus erubescens*) show no detectable activity of the paternally derived *G6PD* allele in any adult tissue except cultured fibroblasts (VandeBerg et al. 1987).

Methylation of cytosines at CpG dinucleotides within CpG islands occurs on the inactive X chromosome in eutherians. Early evidence for a direct role of DNA methylation in X inactivation was obtained from studies in which 5-azacytidine was shown to cause reactivation of previously inactive hypoxanthine phosphoribosyltransferase (*HPRT*) alleles in cultured human–mouse somatic cell hybrids and mouse embryonal carcinoma cell lines (for review, see Singer-Sam and Riggs 1993). Studies utilizing methylation-sensitive restriction enzymes have demonstrated that CpG islands on the inactive X are more highly methylated than those on the active X at the housekeeping loci *HPRT*, phosphoglycerate kinase 1 (*PGK-1*), and *G6PD* (Singer-Sam and Riggs 1993). Methylation analysis using 13 probes that detected X-linked CpG islands showed that, except at two putative pseudoautosomal loci, the majority of rare cutter restriction enzyme sites were methylated on the inactive but not the active X (Norris et al. 1991). The investigators concluded that methylation of CpG islands is a general feature of X inactivation.

It has been suggested that methylation of critical sites, rather than the overall pattern of methylation, may be important in maintaining X inactivation (Hansen et al. 1988). Single informative restriction sites that are always methylated on the inactive but not the active X have been described in the CpG islands of the *Pgk-1* and *G6pd* genes (Singer-Sam et al. 1990; Toniolo et al. 1991). The *Hpa*II site in the *Pgk-1* promoter re-

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6:114–123 ©1996 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/96 $5.00

gion becomes methylated in the preimplantation embryo, beginning at ~3.5 days postconception (dpc), whereas the *G6pd Hpa*II site becomes methylated postimplantation at 5.5 dpc (Grant et al. 1992). Methylation of the critical sites on the inactive allele occurs in both the embryonic lineages, where X inactivation is random, and in the extraembryonic endoderm, where paternal X inactivation occurs. In the germ line the critical sites remain unmethylated in both males and females throughout development (Grant et al. 1992).

More detailed analysis of methylation patterns has been possible using genomic sequencing methods that do not rely on the presence of restriction enzyme sites and can therefore be used to determine the methylation status of many more CpG dinucleotides (Grigg and Clark 1994). The CpG islands associated with the human and mouse *PGK*-1/*Pgk-1* genes have been studied using a chemical cleavage-based genomic sequencing protocol (Pfeiffer et al. 1990; Tommasi et al. 1993). The human *PGK*-1 gene is methylated on the inactive X chromosome at 61 of 62 CpGs (Pfeiffer et al. 1990). In contrast, the *Pgk-1* gene in mice is methylated to a much lower extent, and only the single potentially critical site is fully methylated on the inactive X (Tommasi et al. 1993). The human *HPRT* gene has been analyzed both by the chemical cleavage method of genomic sequencing and by the more recently described bisulfite genomic sequencing technique (Frommer et al. 1992; Clark et al. 1994). Analysis with both methods demonstrated that the CpG island region is highly methylated on the inactive X (Hornstra and Yang 1994; Park and Chapman 1994); however, the bisulfite-based technique provided determination that the degree of methylation is heterogeneous within tissues (Park and Chapman 1994).

Only one marsupial CpG island has been examined previously for methylation. No differential methylation was detected at *Hpa*II or *Cfo*I restriction sites in the 5′ CpG island of the *G6PD* gene of *D. virginiana* (Kaslow and Migeon 1987). The investigators proposed that the lack of DNA methylation on the inactive X in marsupials accounts for the less complete form of X chromosome inactivation. They suggested that DNA methylation in CpG islands is a stabilizing mechanism in eutherians that is not present in marsupials. Although the conclusions of Kaslow and Migeon (1987) have been accepted widely, promoter methylation has not been studied in any other loci or in any species with complete repression of the paternal allele. In addition, many potential sites of methylation are not within restriction sites. Since the existence of critical sites has been postulated in humans and mice (Hansen et al. 1988), and given the comparative hypomethylation of mouse inactive X CpG islands, it is possible that only one or a few CpG sites are responsible for maintaining X inactivation in marsupials.

The full cDNA for *G6PD* from *M. r. robustus* has been cloned recently (Loebel et al. 1995), including the 5′ untranslated region. This region shows characteristics of a CpG island, as do the corresponding regions of the eutherian *G6PD* genes. We have used the *M. r. robustus G6PD* cDNA to isolate a genomic λ clone containing the 5′ end of the *G6PD* gene to study the methylation status of the CpG island on the active and inactive X chromosomes using the bisulfite-mediated genomic sequencing protocol.

RESULTS

Cloning of the 5′ CpG Island

One positive clone (λ12B4) was obtained after screening 5×10^5 plaques from the unamplified λ genomic library using a PCR product amplified from exons 2–5 of *M. r. robustus G6PD* cDNA (Loebel et al. 1995). A clone containing exons 1 and 2 of the *M. r. robustus G6PD* cDNA (Loebel et al. 1995) hybridized to a 1.1-kb *Bgl*II–*Hin*dIII fragment and a 4.2-kb *Bgl*II fragment within λ12B4 (Fig. 1). The 4.2-kb *Bgl*II fragment was also detected by an oligonucleotide probe specific to exon 2. Clones containing the 1.1-kb *Bgl*II–*Hin*dIII fragment (pBH1.1) were sequenced, revealing a region with the characteristics of a CpG island. Analysis of the sequence with CPGPLOT determined that a CpG island of ~500–600 bp was present within this clone (Fig. 2). Restriction mapping and partial sequencing of subclone pBH1.7, containing the 1.7-kb *Bgl*II–*Hin*dIII fragment immediately upstream of pBH1.1, revealed a region of ~300 bp with CpG island-like characteristics contiguous with the 5′ end of pBH1.1. The CpG island appears to be divided into two parts, the majority contained within pBH1.1 and separated from a second, smaller region farther upstream, within the pBH1.7 subclone, by a region of lower G + C content. Figure 2 demonstrates the clear bimodality of the CpG island.

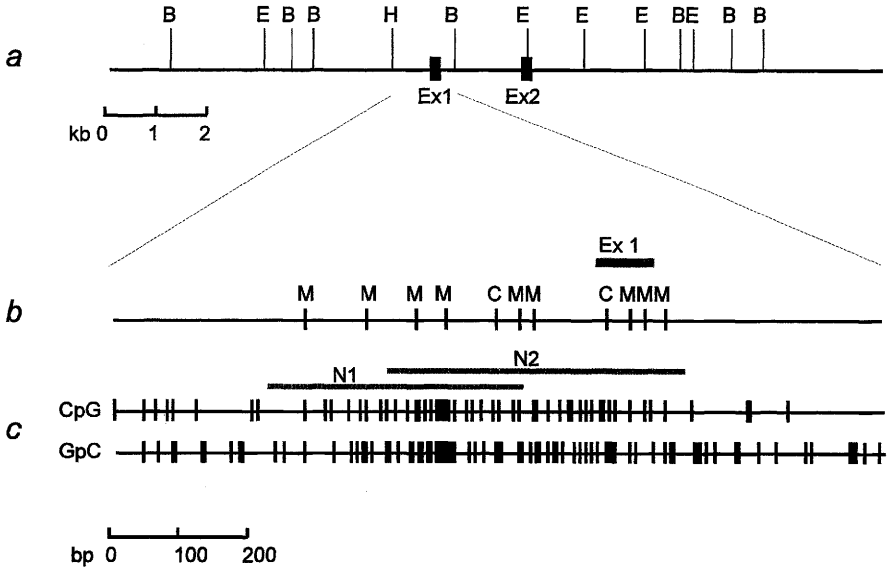

Figure 1 (*a*) Restriction map of λ12B4 showing locations of restriction sites for *Bgl*II (B), *Eco*RI (E), and *Hin*dIII (H). Locations of exons 1 and 2 are shown by shaded boxes. (*b*) Map of all *Msp*I–*Hpa*II sites (M) and *Cfo*I (C) sites in the 1.1-kb *Bgl*II–*Hin*dIII fragment containing exon 1. The bar above the restriction map indicates the location of exon 1, at the 3′ end of the CpG island. (*c*) Map of all CpG and GpC dinucleotides in the 1.1-kb *Bgl*II–*Hin*dIII fragment. Bars show the regions included in genomic sequencing analysis. (N1) The region amplified by nested primer set 1; (N2) the region amplified by nested primer set 2.

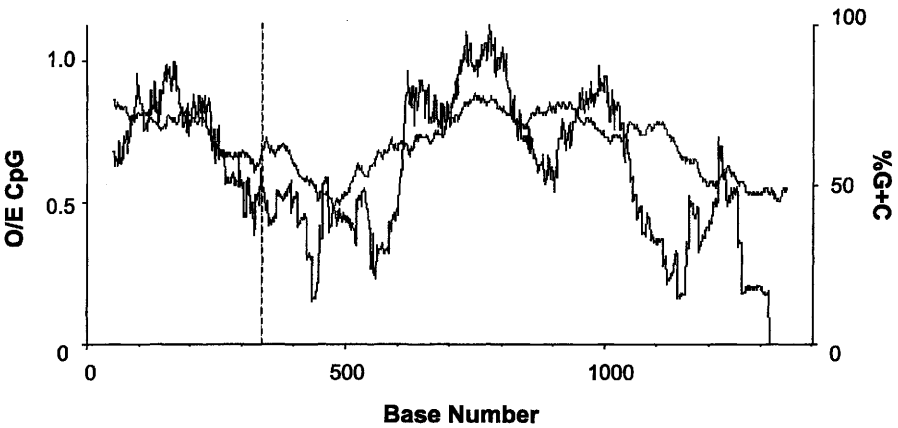

Figure 2 Plot of ratio of observed/expected CpG frequency (solid line) and percent G + C (shaded line) of the two CpG island regions. Analysis was performed using the program CPGPLOT, based on the methods of Gardiner-Garden and Frommer (1987) and accessed through the Australian National Genome Information Service (ANGIS). CpG islands are defined by the CPGPLOT program as having a moving average of observed over expected CpG > 0.6 and a moving average of percent G + C > 0.5. Average values are plotted using a 100-bp window moving in 1-bp increments. The broken vertical line indicates the *Hin*dIII site dividing the clones pBH1.7 and pBH1.1.

Exon 1 was located by comparison to the cDNA sequence (Loebel et al. 1995; see Fig. 1). The G + C content and observed/expected CpG ratio fell below those expected for a typical CpG island immediately after the intron–exon boundary.

Methylation Analysis by Southern Blotting

Initially, the methylation status of the CpG island was analyzed using the pBH1.1 clone as a probe against Southern blots of genomic DNA from liver, kidney, spleen, brain, lung, and heart tissues of male and female *M. r. robustus*. DNA was digested initially with *Bgl*II and *Hin*dIII, followed by either *Msp*I (restriction site CCGG, not sensitive to cytosine methylation), *Hpa*II (restriction site also CCGG, sensitive to methylation of the internal cytosine), or *Cfo*I (restriction site GCGC, sensitive to methylation of the internal cytosine). Probing of *Hpa*II-digested DNA revealed two major bands in all male and female tissues corresponding to the largest *Msp*I fragments predicted from the sequence (Fig. 3A). No larger fragments corresponding to methylated DNA were visible. Many of the smallest fragments predicted from DNA sequencing were not visible on autoradiographs. Two bands corresponding to the largest *Cfo*I fragments could be visualized (Fig. 3B), but the third smaller fragment was not visible.

Because the only fragments visible in both the *Hpa*II and *Cfo*I digests corre-

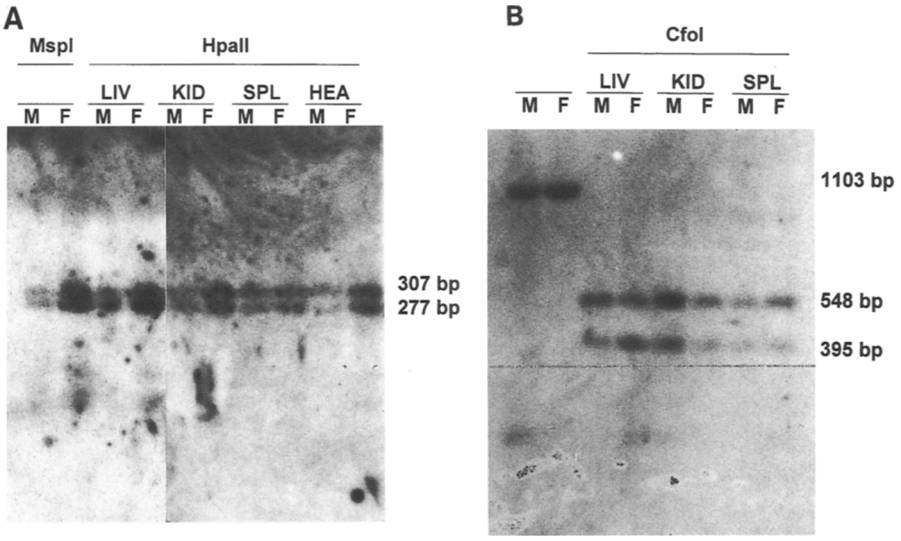

Figure 3 Autoradiographs of *M. r. robustus* genomic DNA from male (M) and female (F) tissues probed with pBH 1.1. DNA was initially digested with *Bgl*II and *Hin*dIII. (*A*) DNA was subsequently digested with *Msp*I or *Hpa*II and size fractionated on a 2% agarose gel. The largest fragments visible correspond to the 307-bp and 277-bp *Msp*I fragments predicted from DNA sequencing. (*B*) DNA was either subjected to no further digestion (lanes *1, 2*) or digested with *Cfo*I. Digested DNA was size fractionated in 1.5% agarose. The visible fragments correspond to the 548-bp and 395-bp fragments predicted from the DNA sequence. Tissues shown are liver (LIV), spleen (SPL), kidney (KID), and heart (HEA).

sponded to those that would be expected if no methylation were present in the region, it appeared that the CpG island was largely unmethylated. However, because a large number of *Hpa*II sites are present in this region, giving rise to small (some 50 bp or less) fragments, it could not be determined whether all *Hpa*II sites were unmethylated in all samples. Many CpG dinucleotides are not within restriction sites; therefore, it was possible that differential methylation was present but could not be detected by Southern blotting.

Bisulfite-mediated Genomic Sequencing

A region of 613 bp within the CpG island, beginning 460 bp upstream of exon 1 and continuing 69 bp into the first intron, was analyzed by bisulfite genomic sequencing in two overlapping regions (Fig. 1). Efficient conversion, as determined by PCR product yield and direct sequencing of products, was obtained most reliably by incubating the conversion reaction in a thermal cycler at 55°C, with a denaturing step (94°C for 1.5–3 min) every 3 hr. Sufficient amounts of PCR product for sequencing and cloning were obtained with nested PCR reactions, where 2–5 μl of the first round amplification was used as a template for a second 25-μl reaction with primers located internal to those used in the first-round amplification.

PCR-amplified products were sequenced directly to determine the average methylation status of individual CpG dinucleotides. A control conversion was performed on pBH1.1 DNA grown in bacterial strain DH5α, a *dcm*$^+$ host strain. The methylation status of three *Eco*RII sites within the region amplified by nested primer set 2 was determined. The internal cytosines within all three *Eco*RII sites appeared highly methylated. Two of these sites are shown in Figure 4. The product was cloned, and sequence obtained from nine clones. Two clones appeared largely unconverted; however, in other clones the only unconverted cytosines were within *Eco*RII sites. The three sites were methylated in 6/7, 6/7, and 5/7 clones. This showed that the region did not contain sequence that was intrinsically resistant to bisulfite conversion and acted as a control to check for plasmid contamination of the experimental reactions, because *Eco*RII sites were not expected to be methylated in mammalian genomic DNA.

Direct sequencing of amplified products revealed no detectable unconverted cytosines in tissues (liver, spleen, or brain), cultured cells, or sperm DNA (Fig. 5). PCR products amplified by both nested primer sets from male and female liver DNA were cloned, and 10 clones of each product were sequenced. In the region amplified by nested primer set 1, no unconverted cytosines were detected in either male or female DNA. A low level of apparent methylation of CpGs was observed in male and female samples in the region amplified by nested primer set 2 (Fig. 6). More unconverted cytosines were observed in female DNA than in male DNA, (seven methylated CpGs over 10 clones in female liver DNA, com-

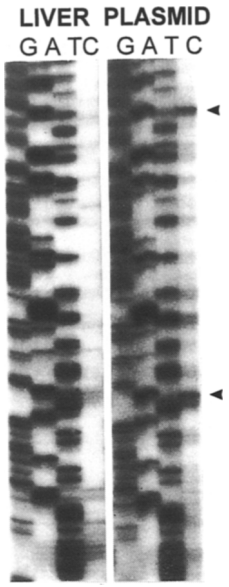

Figure 4 Comparison of methylation status of tissue and control plasmid DNA. Bisulfite converted female *M. r. robustus* liver and control pBH 1.1 plasmid DNA were amplified with nested primer set 2. Products were sequenced with the *fmol* cycle sequencing kit (Promega) following size fractionation on 1.5% agarose. Arrowheads point to cytosines within *Eco*RII sites that are highly methylated in plasmid but not liver DNA. The sequence of the region shown is CCCTCCTCCCTGAAGCGCAGGTGCGGAATCCCAGGTCTGGATCGGAGCCAAAGCCGGGGGAGGCAGGGAGGGCCGGGGCGGAGTCAGGGGCCAACGGGCTGGGCCAGGGGGCGGAGATGGGGA. *Eco*RII sites are underlined.

pared with one in male DNA). Of 10 clones, 5 contained at least one unconverted cytosine; however, individual cytosines remained unconverted in a maximum of 2/10 clones. A total of two unconverted cytosines outside CpG dinucleotides were detected in all 40 clones sequenced.

DISCUSSION

In this report we present the results of methylation analysis of DNA from male and female *M. r. robustus* using methylation-sensitive restriction enzymes and genomic sequencing analysis for 47 CpG dinucleotides within the CpG island in the 5′ region of the *G6PD* gene of *M. r. robustus*. The only previous study of methylation of a marsupial X-linked CpG island was in an American species that exhibits significant levels of expression of the paternal allele in all somatic tissues (Kaslow and Migeon 1987). The investigators found no evidence of sex-specific methylation differences in a CpG island 5′ to exon 2 of the *G6PD* gene of *D. virginiana*. This study extends those previous findings to a distantly related marsupial species with complete paternal repres-

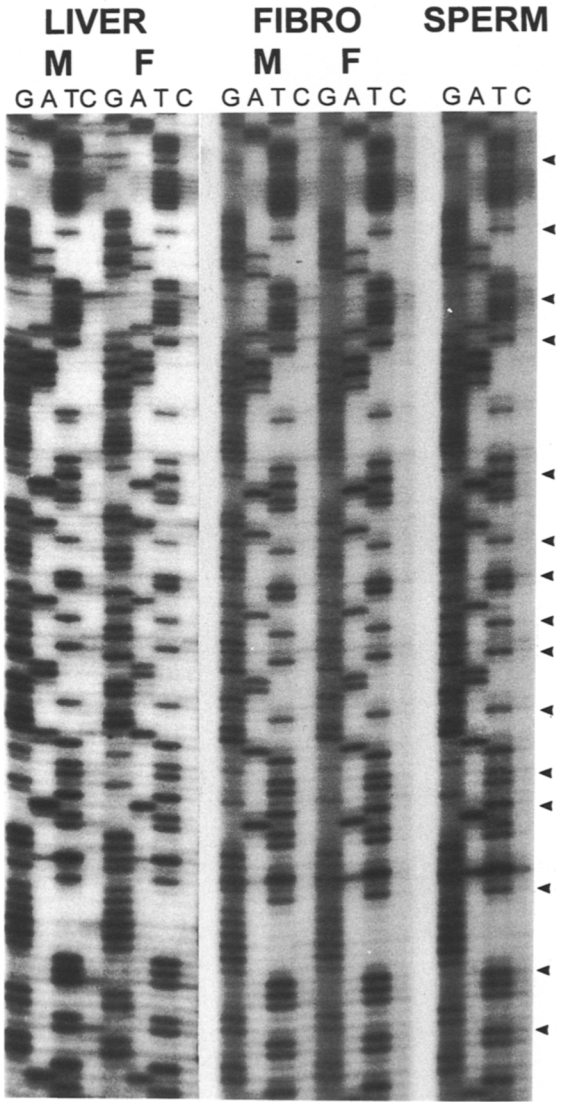

Figure 5 Methylation status of tissue (liver), cultured fibroblast, and sperm DNA. Bisulfite-treated DNA was amplified with with nested primer set 2, size fractionated, and sequenced with the *fmol* cycle sequencing system (Promega). Arrowheads point to CpG dinucleotides. Methylation was not detected in any tissue, fibroblast, or sperm sample. Faint bands are probably artifactual, as they do not necessarily correspond to cytosines in the original sequence and no non-CpG methylation was detected by sequencing clones. The sequence of the region shown in the figure is GGGGGGCGTGGCCACGTCGCAGGCGGAAGCGGCGAGCCGGGCGAGGCCACGTGGGGGTGGGAGAGAGCGATCTCGCTTGAGGAGGCGGGCCCTCCTCCCTGAAGCGCAGGTGCGGAA

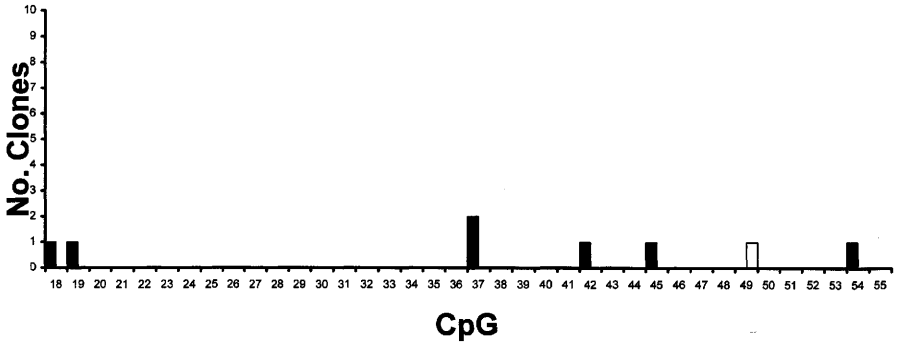

Figure 6 Methylation profile of CpG dinucleotides in the product amplified by nested primer set 2. Ten clones each of PCR products, amplified with nested primer set 2, from bisulfite converted male and female liver genomic DNA were sequenced. Solid bars represent female; open bars represent male.

sion of the *G6PD* gene in tissues but partial paternal expression in cultured fibroblasts. The use of a genomic sequencing technique in addition to Southern blotting has allowed a far greater number of CpG dinucleotides to be analyzed than would be possible using restriction enzymes alone.

The *M. r. robustus* CpG island described here overlaps the start of transcription of the *G6PD* gene as determined by RACE (rapid amplification of cDNA ends)–PCR (Loebel et al. 1995). The characteristic high observed/expected CpG ratio ends in the first intron, within 100 bp of the exon–intron boundary. The sequence of the CpG island shows little conservation between species. The *M. r. robustus G6PD* CpG island is a typical CpG island, as in humans, but the corresponding region in the mouse has a much reduced G + C and CpG content (Toniolo et al. 1991). Regions have been identified that are highly conserved among human, mouse, and rat sequences (Toniolo et al. 1991; Rank et al. 1994). Only one of these regions was recognizable in *M. r. robustus*. A box 29 bp long, located 252 bp upstream of exon 1 (345 bp in mouse and 384 bp in human), is 62.2% homologous between the wallaroo and mouse sequences and 73.7% homologous between the wallaroo and human sequences (Fig. 7). The human and mouse CpG islands continue farther into the first intron than in *M. r. robustus G6PD*. The single *Hpa*II site that is always methylated on the inactive X in mouse and human occurs within the CpG island, 150 bp downstream of exon 1. In *M. r. robustus*, this region is relatively CpG poor, and not part of the CpG island.

The *M. r. robustus G6PD* CpG island appears to be divided into two sections. This bimodal distribution of CpG density may indicate that there is another unidentified gene farther upstream. However, CpG islands that are divided have been described previously. Gardiner-Garden and Frommer (1987) describe a CpG island associated with the mouse *int-1* that is highly fragmented and could be considered as three separate CpG islands. The CpG island associated with the mouse *G6pd* gene (Toniolo et al. 1991) has a generally lower CpG content and a distribution of CpG dinucleotides consisting of several small peaks.

Methylation analysis using methylation-sensitive restriction enzymes and Southern blotting resulted in detection of only fragments that resulted from complete digestion by *Hpa*II and *Cfo*I. However, smaller fragments predicted from the nucleotide sequence were not visible on resulting autoradiographs; therefore, it was only possible to directly determine the methylation status of the two *Cfo*I sites and two of nine *Hpa*II sites. Because no fragments larger than those that would be expected from complete digestion of the DNA by *Hpa*II or *Cfo*I were detected, it is reasonable to assume that there is no widespread methylation on either the active or inactive X chromosome in this region. However, in addition to the *Hpa*II sites that could not be directly tested, many CpG sites were not located within restriction sites.

We used the bisulfite genomic sequencing protocol of Frommer et al. (1992) to analyze the methylation status of all cytosines in the region of highest CpG density. Data are presented for the top strand of DNA only, because any functional DNA methylation must be symmetrical to be inherited stably. Direct cycle sequencing of PCR products amplified from bisulfite-treated DNA from tissues (in which inactivation of the paternally derived allele is complete), cultured fibroblasts (in which partial expression of the paternally derived allele is detectable), and sperm indicated hypomethylation of all cytosines.

These results are supported by the low level of methylation detected by sequencing cloned PCR products. The majority of CpG dinucleotides

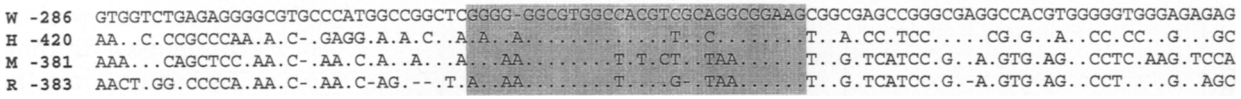

Figure 7 Region of conservation between wallaroo (*w*), human (*h*), mouse (*m*), and rat (*r*) upstream of exon 1. Dots indicate nucleotides identical to the wallaroo sequence; dashes represent gaps introduced to maximize homology. References for eutherian sequences are given in the text.

were completely unmethylated in all clones sequenced. Although a higher level of methylation was apparent in female liver DNA than in male DNA and half the clones derived from DNA from female liver contained at least one methylated CpG, it is unlikely that this is functional in maintaining X inactivation. If a critical site were present it would be methylated on 50% of the DNA strands and detected as bands of equal intensity in the T and C lanes when the amplified product was direct sequenced, or by presence of a C in 50% of clones. As the maximum number of clones in which any individual cytosine remained unconverted was 2/10 (Fig. 6), these sites are unlikely to be critical sites.

The differences in organization between the CpG islands at the 5′ ends of the eutherian (Toniolo et al. 1991) and wallaroo *G6PD* genes may be related to the different modes of control of *G6PD* expression from the inactive X. Because there are regions of high conservation between the human and mouse *G6PD* CpG islands (Toniolo et al. 1991; Rank et al. 1994) that are not present in the wallaroo, it is possible that they are involved in the maintenance of repression of the inactive allele by DNA methylation, a process that does not appear to occur in marsupials.

Kaslow and Migeon (1987) explained the differences in X inactivation between marsupials and eutherians as the result of differential timing of developmental events. These investigators postulated that because random X chromosome inactivation occurs later in development, after the paternal imprint has been lost, and is stable, whereas paternal X inactivation in marsupials and eutherians is less stable, differences in methylation state and stability were attributable to differences in developmental timing. Paternal X inactivation, occurring earlier in development than random X inactivation, would not be stabilized by CpG methylation. The more recent finding that methylation of critical sites in the *G6pd* and *Pgk-1* genes occurs in both the embryonic and extraembryonic lineages at or close to the time of initiation of inactivation (Grant et al. 1992) suggests that the relationship between the mechanisms of paternal X inactivation in rodent extraembryonic membranes and marsupial tissues is more complex.

Timing of initiation of X inactivation may differ between marsupials and eutherians. In the mouse, both X chromosomes are potentially active initially, and initiation of X inactivation occurs during preimplantation development (Epstein et al. 1978). In marsupials, there is as yet no evidence for two active X chromosomes at any time in female development, in either somatic cells or oocytes up to the early dictyate stage (Johnston et al. 1985, 1994; Johnston and Robinson 1987), at which time reactivation of the previously inactive X has occurred in mice (Johnston 1981; Kratzer and Chapman 1981). Initiation of X inactivation may occur very early in the development of the marsupial embryo; however, data on gene expression and DNA replication patterns during cleavage divisions have so far proved impossible to obtain (Johnston et al. 1994).

Extensive methylation of the active but not the inactive X within coding and intronic regions has been observed consistently in marsupials in both *D. virginiana*, for *HPRT* and *G6PD* (Kaslow and Migeon 1987), and in *M. r. robustus* for *HPRT* (Piper et al. 1993) and *G6PD* (D.A.F. Loebel and P.G. Johnston, unpubl.). Evidence from in situ nick translation experiments suggest this is a chromosome-wide phenomenon (Loebel and Johnston 1993). There is limited evidence that this also occurs in eutherians (Lindsay et al. 1985; Viegas-Pequignot et al. 1988). It is as yet unknown whether this methylation pattern has a role in X chromosome inactivation, but the conservation between divergent taxa and its widespread nature suggests that it may have some function.

This study has confirmed the hypomethylation of CpG islands on the X chromosome of marsupials, demonstrating that methylation of CpG islands is probably not a factor in maintaining X chromosome inactivation marsupials. Cur-

rent understanding of the mechanisms of X inactivation in marsupials is very limited. Greater understanding of how X inactivation occurs in marsupials and its evolutionary relationship to eutherian X inactivation will only be obtained after more detailed examination of expression and methylation patterns of many more genes in several different species.

METHODS

Genomic DNA Preparation

High-molecular-weight DNA was isolated from liver, kidney, spleen, heart, lung, and brain tissue according to Sambrook et al. (1989). Sperm was obtained by cutting the cauda epididymis into small pieces in phosphate-buffered saline (PBS) and allowing the tissue fragments to settle (Venolia et al. 1984). Sperm DNA was isolated following the method of Gill et al. (1985), except that the concentration of SDS was increased to 4.5% and a total of 8 mg proteinase K was added to the overnight digestion. Fibroblasts were cultured in Iscove's medium supplemented with 12% fetal calf serum (Cooper et al. 1977). Cultured fibroblasts were detached from the surface of the culture vessel by incubation with trypsin and resuspended in PBS. DNA was isolated from cultured fibroblasts by a salting-out protocol according to Kunkel (1977).

Library Construction and Screening

A genomic library was constructed from female *M. r. robustus* liver DNA, partially digested with *Sau*3AI to produce optimally sized fragments. DNA was ligated into λGEM-12 vector (Promega) and packaged in Packagene (Promega) using the methylation-tolerant PMC128 *Escherichia coli* as a host strain (Doherty et al. 1993). Phage were plated out at a density of 1×10^4 plaques per plate. Duplicate lifts were made on nitrocellulose membranes (Schleicher & Schuell). The filters were screened with a PCR product containing exons 2–5 of the wallaroo *G6PD* cDNA (Loebel et al. 1995). Following electrophoresis in 2% agarose the electroeluted product was labeled with [α-^{32}P]dATP (Bresatec) by random priming (Boehringer). Overnight hybridization and washing were carried out at high stringency (65°C). After secondary screening, phage DNA was prepared according to Kaslow (1986).

Southern Blotting

DNA was transferred to Zeta-Probe nylon membrane (Bio-Rad) by alkaline capillary transfer according to the manufacturer's instructions. Blots of digested DNA from the single positive phage clone were probed with digoxigenin (Boehringer)-labeled oligonucleotides specific to exon 2, following the manufacturer's instructions, and also with a PCR product containing the 5′ end of the *M. r. robustus* *G6PD* cDNA cloned into the pGEM-T vector and labeled with [α-^{32}P]dATP (Loebel et al. 1995).

For methylation-sensitive restriction enzyme analysis of the CpG island, DNA from *M. r. robustus* was first digested with *Bgl*II and *Hin*dIII followed by *Msp*I, *Hpa*II, or *Cfo*I. Agarose gels (1.5% or 2%) were blotted and probed with pBH1.1, a subcloned fragment from the λ clone containing the CpG island region.

Bisulfite Conversion of DNA

Bisulfite genomic sequencing was performed according to Clark et al. (1994). DNA (1 μg) from male and female tissues, cultured fibroblasts, and sperm was digested for 6–8 hr with *Bgl*II in a volume of 10 μl. A control conversion was also performed on 500 ng of pBH1.1 plasmid DNA, grown in DH5α, and digested with *Hin*dIII. DNA was denatured with 0.3 M NaOH in a volume of 20 μl at 37°C for 15 min. Sodium metabisulfite and hydroquinone were added to final concentrations of 1.7 M (3.4M bisulfite) and 0.5 mM, respectively, in a total volume of 250 μl. The reaction mixture was incubated in a thermal cycler (MJ Research) for 15 hr at 55°C with a 3-min denaturing step at 94°C every 3 hr. Following the bisulfite reaction, the DNA was desalted using Gene-Clean (Bio 101) or Bresa-Clean (Bresatec). Three volumes of NaI solution and 5 μl of Glass milk (Gene-Clean) or silica (Bresa-Clean) were added to the reaction. After washing three times with NEW wash, the DNA was eluted into 45 μl H_2O. Desulfonation of the deaminated cytosines was carried out by adding 5 μl of 3 M NaOH and incubating at 37°C for 15 min. The converted DNA was precipitated and resuspended in 100 μl of 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA (pH8).

Polymerase Chain Reaction

Amplification by PCR was carried out on 2–5 μl of converted DNA in a reaction volume of 25 μl. Of this reaction, 2–10 μl was used directly in a nested reaction with internal primers. Two nested pairs of primers designed from the bisulfite reacted sequence were used. Nested primer combinations used were as follows:

Nested set 1:		
External	GGS1(forward)	TTTAGATATTAAGTTATTTTTTAGAGTATTTG
	GGS4(reverse)	CTAACCCAACCCTTTAACCCCAAACTC
Internal	GGS2(forward)	GAAGGATTTTTTTTGTTATTTTAAGTGTTT
	GGS3(reverse)	ACTTTAACTCCCATCCAAACCTAAAATTCC
Nested set 2:		
External	GGS2(forward)	As above
	GGS8 (reverse)	ACTTAAATAAAAAAAATCAAAAAACCTAA
Internal	GGS6(forward)	GGGGTTTTTGGTTAGAGATGGGAGATT
	GGS7(reverse)	ATCTATTAAAATAAAAAACTCAAAACAAAA

Each reaction contained 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1 μM each primer, and 1.25 units of *Taq* polymerase in 1× reaction buffer (Promega). Cycling conditions were an initial denaturation at 94°C

for 2 min followed by five cycles of 94°C for 1 min, 50°C for 2 min, 72°C for 3 min, then 30 cycles of 94°C for 0.5 min, 50°C for 2 min, 72°C for 1.5 min, with a final extension of 6 min at 72°C. Reactions were incubated in a MJ Research thermal cycler, in thin-walled microtiter plates (MJ Research).

PCR products were size-fractionated in 1.5% agarose, the slice of gel containing the product excised and the DNA purified with Gene-Clean or Bresa-Clean as described above. Products were resuspended in 10–20 μl H_2O and either sequenced directly to obtain an average methylation pattern or cloned into pGEM-T vector (Promega) and sequenced to determine the methylation status of individual DNA strands. Cloning and direct sequencing were performed on aliquots of the same PCR reaction.

Sequencing

Direct sequencing was carried out on 1–3 μl of PCR product using the *fmol* sequencing system (Promega), with ^{32}P end-labeled primers identical to the internal primers used in the nested PCR reaction.

Double-stranded plasmid DNA was prepared from overnight bacterial cultures by alkaline lysis (Sambrook et al. 1989) and sequenced using T7 polymerase (Sequenase, U.S. Biochemical). M13/pUC forward and reverse sequencing primers or specific primers for direct sequencing were used.

ACKNOWLEDGMENTS

We thank J. Doherty for kindly providing the bacterial strain PMC128; S. Clark and J. Harrison for assistance with genomic sequencing; and D. Cooper, J. Donald, M. Frommer, K. Humphrey, C. Metcalfe, and C. Watson for comments on draft versions of this paper. We also thank R. Claassens, S. McLeod, and R. Moore for assistance with care and handling of the animals. This research was supported by grants from the Australian Research Council and Macquarie University Research Grants scheme to P.G.J. D.A.F.L. is the recipient of an Australian Postgraduate Research Award. Sequence data described in this paper have been deposited with GenBank and assigned accession number U34997.

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Received October 3, 1995; accepted in revised form February 1, 1996.