In vivo footprint and methylation analysis by PCR-aided genomic sequencing: comparison of active and inactive X chromosomal DNA at the CpG island and promoter of human PGK-1

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The promoter region of the X-linked human phosphoglycerate kinase-1 (PGK-1) gene is a CpG island, similar to those often found near autosomal genes. We used ligation-mediated polymerase chain reaction (LMPCR) for a genomic sequencing study in which 450 bp of the human PGK-1 promoter region was analyzed for the presence of in vivo protein footprints and cytosine methylation at all CpG sites. A technique was devised to selectively visualize the DNA of the inactive X chromosome (Xi), even in the presence of the active X chromosome (Xa). We found that the human Xa in both normal male lymphocytes and hamster–human hybrids is completely unmethylated at all 120 CpG sites. In contrast, 118 of the CpG sites are methylated on the human Xi in hamster–human hybrids. The Xi in normal female lymphocytes is also highly methylated, but some GCG or CGC trinucleotides partially escape methylation; all other CpGs are fully methylated. In vivo footprinting studies with dimethylsulfate (DMS) revealed eight regions of apparent protein–DNA contacts on the Xa. Four of the footprints contained the consensus sequence of the binding site for transcription factor Spl. The other regions include potential binding sites for transcription factors ATF, NF1, and a CCAAT-binding protein. The Xi did not show any specifically protected sequences, and with the exception of four hyperreactive sites, the in vivo DMS reactivity profile of XI DNA was very similar to that of purified, linear Xi DNA. The implications of these findings with regard to the maintenance of methylation-free islands, X chromosome inactivation, and the chromatin structure of facultative heterochromatin are discussed.

[Key Words: X chromosome inactivation; in vivo footprinting; DNA methylation; transcription factors]

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Information about cytosine methylation patterns and protein–DNA contacts is lost upon cloning but can be obtained by genomic sequencing [Church and Gilbert 1984; Saluz and Jost 1987]. Genomic sequencing has recently been considerably improved by use of a ligation-mediated polymerase chain reaction [LMPCR] to add specificity and to exponentially amplify sequence ladder fragments [Mueller and Wold 1989; Pfeifer et al. 1989]. Here, we report the use of LMPCR-aided genomic sequencing for a study of the CpG island and promoter at the 5’ end of the human X-linked phosphoglycerate kinase-1 (PGK-1) gene. CpG-rich islands are found at the 5’ ends of many genes, especially housekeeping genes [Bird 1986, 1987], and studies using methylation-sensitive restriction endonucleases, such as HpaII, have shown that these islands are usually not methylated at the analyzable sites. It was the lack of methylation at HpaII sites that led to the discovery of CpG islands [Cooper et al. 1983]. A long-standing question has been concerned with how these islands are maintained methylation free, because most [70–80%] of HpaII sites are methylated in mammals.

In contrast to autosomal CpG islands, several on the inactive X chromosome were found to be highly methylated at HpaII sites [Wolf et al. 1984; Yen et al. 1984; Keith et al. 1986; Toniolo et al. 1988]. X chromosome inactivation is a gene-silencing mechanism, unique to mammals, that affects almost all genes of a whole chromosome [for review, see Gartler and Riggs 1983; Grant and Chapman 1988]. One of the two chromosomes in female cells becomes genetically silent in the early embryo at about the time of implantation. The inactive X chromosome [Xi] is late-replicating and stays relatively condensed during interphase, whereas the active X chromosome [Xa] is indistinguishable from autosomes. Thus, the X chromosome is facultatively heterochromatic, capable of being euchromatic in male cells and heterochromatic in female cells. The inactive state is somatically heritable and is an example of stable cell memory, which is a necessary part of normal mamma-
lian development (Riggs 1989). There is rather convincing evidence that DNA methylation is important for the maintenance of X chromosome inactivation (for review, see Monk 1986; Grant and Chapman 1988; Lyon 1988; Riggs 1990). Some of the critical methylation sites involved in the maintenance of X chromosome inactivation may be found in CpG islands, because sex-specific island methylation is correlated with stability of the inactive state (Kaslow and Migeon 1987; Migeon et al. 1989).

In general, numerous studies have established that there is a good correlation between methylation of regulatory regions and transcriptional silence (for review, see Razin and Riggs 1980; Doerfler 1983; Riggs and Jones 1983; Cedar 1988; Grünwald and Pfeifer 1989), but the mechanisms involved in the suppression of gene expression by DNA methylation are largely unknown. Interference by cytosine methylation with the binding of transcription factors is one possibility, as the binding of some transcription factors is eliminated or reduced by CpG methylation (Becker et al. 1987; Kovesdi et al. 1987; Watt and Molloy 1988; Hermann et al. 1989; Iguchi-Ariga and Schaffner 1989). However, SP1 and CTF bind to their recognition sites irrespective of methylation (Harrington et al. 1988; Höller et al. 1988; Bennett et al. 1989). Suppression of transcription and/or the formation of inactive chromatin (Cedar 1988) by methylation could be brought about also by binding of a protein(s) that interacts with methylated DNA, independent of sequence. One such protein identified in human placenta shows a limited sequence specificity (Huang et al. 1984; Wang et al. 1986). Another protein recently identified by Bird and co-workers has no obvious sequence specificity but requires clusters of methylated CpGs (Meehan et al. 1989). Such a protein would be a candidate for binding to methylated CpG islands on the Xi. However, prior to this study there was no direct evidence that proteins can bind differentially to DNA regions on the Xa and the Xi. Here, we report on the presence of specific in vivo protein–DNA contacts within a CpG island on Xa and Xi. In addition, the complete strand-specific DNA methylation pattern of a CpG island has been determined.

Results

Methylation analysis and selective visualization of the Xi in female cells

Chemical DNA sequencing distinguishes methylated from unmethylated cytosines because, at high salt concentrations, the base-modifying agent hydrazine does not react with 5-methylcytosine, thus, a gap appears in the C lane of a sequence ladder (Church and Gilbert 1984). The LMPCR procedure used here is outlined in Figure 1A. This procedure preserves methylation information because the chemical sequencing reactions are done before the PCR amplification (Mueller and Wold 1989; Pfeifer et al. 1989). Briefly, the first step is base-specific chemical cleavage of DNA at either G, G + A, T + C, or C, generating 5′-phosphorylated molecules. Next, primer extension of a gene-specific oligonucleotide (primer 1) generates molecules that have a blunt end on one side. Linkers are ligated to the blunt ends, and an exponential PCR amplification of the linker-ligated fragments is done by using the longer oligonucleotide of the linker (linker-primer) and a second gene-specific primer (primer 2). After performing 15–18 amplification cycles, the DNA fragments are separated on a sequencing gel, electroblotted onto nylon membranes, and hybridized with a gene-specific probe to visualize the sequence ladder. By rehybridization, several gene-specific ladders can be sequentially visualized from one sequencing gel (Pfeifer et al. 1989).

To analyze both strands of a 450-bp region at the 5′ end of the human PGK-1 gene, we have used eight different oligonucleotide primer sets (Fig. 1B), often using two sets simultaneously. Representative sequencing data are shown in Figure 2. The relative band intensities are very reproducible if adequate amounts of DNA are used. For this region and primer sets, ~1 μg/lane is sufficient (for a discussion of statistical factors, see Pfeifer et al. 1989). Sites of potential methylation (CpG) are indicated in Figure 2. In lanes 5 and 9 (Fig. 2), which show DNA from an Xi, methylation is indicated at several sites by the band intensity dropping to the background level. Methylated sites are not seen in other lanes, including lane 11 (Fig. 2), which shows cloned DNA. For bands appearing weak in the exposure shown, longer exposures were used, methylation information was obtained at every C.

Direct analysis of DNA from female human diploid cells does not give easily interpretable results, because both Xa and Xi are present. To selectively reveal Xi DNA in female cells, we took advantage of the known
differential methylation state of the HpaII sites in this region on the Xa and Xi in female blood cells [Keith et al. 1986]. For example, using a quantitative methylation assay, Steigerwald et al. [1990] found that the HpaII site at position +23 in this region is >98% unmethylated on the Xa in normal male lymphocytes and 50% methylated in the Xa/Xi mixture in normal female lymphocytes. The strategy outlined in Figure 3 simplifies the sequence ladders and may be generally applicable for allele- or imprint-specific methylation differences. Cleavage of DNA with HpaII cuts the 5' region from the Xa to small fragments. The LMPCR primers were selected so as to be close to HpaII sites or to contain a HpaII site within the primer sequence. Two of the primer sets could not be arranged to be near HpaII sites. In this case, the proximity of other methylation-sensitive restriction enzyme sites [HhaI for primer set I and FspI for primer set C] was exploited. After cutting female DNA with the respective methylation-sensitive restriction enzyme, the first primer extension reaction proceeds only to the site of the restriction cut in DNA derived from the Xa. DNA from the Xi remains uncut, and the primer extension and amplification reactions proceed over the whole sequence 3' to the cut [Fig. 3]. Male lymphocyte DNA is completely removed from the sequencing gel by prior cutting with HpaII [Fig. 2, lane 10]. However, the sequence ladder is retained above the HpaII cut in female [e.g., Fig. 2, lane 9; Fig. 4, lanes 8 and 9]. The only likely interpretation is that the ladder above the HpaII site comes from Xi.

**Methylation differences between Xa and Xi**

Active X chromosomal DNA was studied in HeLa cells, male human lymphocytes, and Chinese hamster-human hybrid cells containing a human Xa [cell line Y162-11C]. Figures 2 and 4 show results obtained with primer sets D and H. By determining relative band intensities between adjacent bands in the same sequence ladder and then comparing with cloned, unmethylated DNA, it can be seen that DNA from male lymphocytes is not detectably methylated at any of the CpG dinucleotides [Fig. 2, lanes 7 and 8; Fig. 4, lanes 6 and 7], and the same is true for DNA from hybrid cells containing human Xa [Fig. 2, lane 6; Fig. 4, lane 5]. HeLa cell DNA also is unmethylated in this region [Figs. 2 and 4], consistent with this cell line having only Xa. Methylation data for the whole region are summarized in Figure 5. DNA from the Xa is not detectably methylated at any of the 120 CpG dinucleotides in the analyzed region.

In contrast, human Xi in Chinese hamster-human hybrid cells [X86T2] has a very high level of methylation [Fig. 5C]. Only one CpG is not detectably methylated

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**Figure 2.** Methylation analysis with primer set D. Arrows indicate the position of CpG dinucleotides. Lanes 1–3 are sequencing controls (G, G + A, T + C) from HeLa cells. Lanes 4–11 are C-specific reactions: (lane 4) HeLa cells, (lane 5) hybrid cells containing human Xa, (lane 6) hybrid cells containing human Xa, (lane 7) male lymphocytes, (lane 8) female lymphocytes after HpaII cleavage to remove Xa DNA, (lane 9) male lymphocytes after HpaII cleavage, (lane 11) cloned DNA.

**Figure 3.** Outline of the method used to separate Xa DNA from Xi for genomic sequencing and in vivo footprinting.
Xa ladder, because fully methylated sites are found in the same lane of the sequencing gel. Therefore, we conclude that the Xi in female lymphocytes contains some sites that are only partially methylated. This is also consistent with results obtained by Southern blotting that indicated partial demethylation of some HhaI sites and a Nal site (D. Keith, unpubl., and data not shown). The majority (75%) of CpG sites on lymphocyte Xi are fully methylated. The 25% of the CpG sites in female lymphocytes that are only partially methylated on the Xi (Fig. 5B) are all located in the trinucleotide CGG or its complement CGC. However, not all of these trinucleotides escape full methylation, so there seem to be some additional sequence requirements. For example, many of the partially methylated CGGs are in a very high G + C sequence context. In most cases, a sequence of five or more adjacent G/C base pairs with a central CGC or CGG trinucleotide does not become completely methylated.

In vivo footprinting of Xa and Xi

By using the same combination of primers, we have in

Figure 4. DNA methylation analysis with primer set H. Arrows indicate the position of CpG dinucleotides. Xa DNA was removed from female DNA by HpaII digestion at position −67. Cloned DNA was used as an unmethylated control. (Lanes 1 and 2) Sequencing controls (G, G + A) obtained from HeLa DNA: (lanes 3–12) C-specific reactions. Some bands seen at G positions in the C lanes are side reactions of hydrazine with G. They do not affect methylcytosine analysis. (Lane 3) HeLa cells; (lane 4) hybrid cells containing a human Xi; (lane 5) hybrid cells containing a human Xa; (lanes 6 and 7) male lymphocytes; (lanes 8 and 9) female lymphocytes after HpaII cleavage; (lane 10) male lymphocytes after HpaII cleavage; (lane 11) female lymphocytes without HpaII cleavage; (lane 12) cloned DNA.

(position −260). This same site is also not methylated on the Xi in female lymphocytes (Fig. 5B). One site (position −257) in the human—hamster hybrid cells is partially methylated, however, all other sites (a total of 116) are not detectably unmethylated, that is, the band seen is not greater than the background level for that position. Xi DNA from female lymphocytes shows mostly fully methylated sites [Fig. 5B], but, some sites appear to be only partially (40 to 80%) methylated [Figs. 2, 4, and 5]. This partial methylation cannot be explained by incomplete digestion and thus, incomplete removal of the Xa ladder, because fully methylated sites are found in the same lane of the sequencing gel. Therefore, we conclude that the Xi in female lymphocytes contains some
vestigated in vivo protein–DNA interactions by dimethylsulfate (DMS) footprinting. DMS reacts with the N7 position of guanosines, enabling later cleavage by pyrimidine. The ability of DMS to penetrate cell membranes has allowed its use for true in vivo footprinting experiments [Ephrussi et al. 1985]. Proteins in contact with DNA either decrease accessibility of specific guanosines to DMS (protection) or, often at the edges of a footprint, increase reactivity (hyperreactivity).

In vivo footprints on active X chromosome DNA was studied in HeLa cells, male human lymphocytes, and hamster–human hybrid cells (Y162-11C) containing the human Xa. The Xi was analyzed first for protein footprints in hybrid cells containing a human Xi (X86T2) and then in normal female lymphocytes by selective removal of Xa DNA, as was done for methylation analysis. Figure 6 shows differential in vivo footprints on the Xa and Xi. There is a region of strong protein–DNA interaction at nucleotide position −40 to −65 relative to the major transcription initiation site. This region (footprints I and II, see Fig. 9, below) contains two Sp1 consensus sequences [Briggs et al. 1986]. These sequences are protected in vivo and are separated by a hyperactive guanosine on the upper strand (Figs. 6 and 7) and a hyperactive adenosine on the lower strand (not shown). Therefore, we assume that two proteins (possibly Sp1) are binding to this region. These in vivo footprints are observed only on the Xa, and they are identical between HeLa cells, male human lymphocytes, and hamster–human hybrid cells containing a human Xa. There is no in vivo protein–DNA contact in this region on the Xi in hybrid cells containing a human Xi and on the Xi in female lymphocytes. Figure 6, lanes 12 and 13, shows data from an experiment in which Xa DNA was removed from the sequence ladder by prior HpaII digestion at position +23. The band just above the footprints (position −67; Fig. 6) is a HpaII site and thus probably is due to <100% complete restriction digestion. It does not
affect the interpretation of the footprints because Xα-specific footprints disappear in female DNA after HpaII treatment. Without removal of the Xα from female DNA by HpaII cutting, an intermediate footprint reflecting a mixture of the Xα and Xi was obtained (Fig. 6, lane 15). These results show that transcription factors bind to this region selectively on an Xα in female cells, as they do in cells containing Xα only. A more detailed resolution of the −40 to −65 footprint is shown in Figure 7, in which we used primer set H. Xα and Xi DNA are not separated in this region [−40 to −65] in DNA from female cells, because the HpaII site is located farther downstream. There is an additional region of protein–DNA interaction near nucleotide position −140 to −155 on Xα, which possibly extends to a region near −115 [footprint IV, see Fig. 9]. Figure 8 shows another footprint on DNA from the Xα between nucleotides −240 and −258 [footprint VI, see Fig. 9]. This region contains another Sp1 consensus sequence.

The DMS reactivity profiles of Xi DNA in normal female lymphocytes and X86T2 cells look very similar to the purified DNA that is used as a control (e.g., Fig. 6, lane 9; Fig. 7, lane 10). It should be noted that the DMS reactivity of naked DNA is slightly different between highly methylated and unmethylated DNA (cf. DNA from X86T2 and Y162-11C; Fig. 8, lanes 6 and 7). Therefore, both controls of naked DNA were always included. No specific protection of guanosines were seen on the Xi in the whole region investigated. However, there are some guanosines that are hyperreactive with DMS on the Xi in vivo (Figs. 7 and 10, below). This hyperreactivity is not accompanied by a specific protection in the neighborhood, as seen for Xα footprints. In two of four cases, two immediately adjacent guanosines in the sequence GpG were found to be hyperreactive (Fig. 9).

A summary of the in vivo footprinting data is shown in Figure 9. One of the footprints located around nucleotide position −360 [footprint VIII, Fig. 9] on the Xα is seen only in HeLa cells [data not shown]. It corresponds to a region that is specifically protected in vitro by a HeLa cell extract (Yang et al. 1988). Another protected region [footprint VI] is detected only in lymphocytes but not in HeLa or hybrid cells [Fig. 10]. Again, this footprint is seen only on the Xα.

Information on overall DMS reactivity of the Xi relative to Xα is also contained in our data. A small reduction in reactivity of Xi DNA is seen, and this may be significant because in female lymphocytes, the ratio of band intensities in the ladder above and below the relevant HpaII site can be measured. Thus, there is an internal control. Comparative densitometry of the data in Figure 6 [lanes 12 and 15] indicates that the band intensities above the HpaII site are 30% more reduced than the expected 50%. It can be concluded that DMS reactivity is not dramatically different between Xα and Xi islands, as might have been the case if the Xi were covered by a sequence-nonspecific, but tight, binding protein.

Discussion

Methylation of CpG islands

Our results extend the presently available data on DNA methylation in CpG islands to all CpG dinucleotides in the PGK-1 promoter region. Not only are restriction sites unmethylated on Xα, but so is each of a total of 120 analyzed CpG dinucleotides. In dramatic contrast, the same region on the Xi is very highly methylated. Despite reports suggesting some methylation at sites other than CpG in mammals (Crowther et al. 1989), we have not observed any methylation in dinucleotides other than CpG. Furthermore, we have not seen hemimethylated sites, the occurrence of which may be limited to certain critical gene regions in the course of gene activation (Saluz et al. 1986). A human Xi in a Chinese hamster background retains a high level of methylation, indicating that the methylation system is most likely highly conserved. Surprisingly, one CpG dinucleotide at position −260 escapes methylation completely in the hybrids and in normal female lymphocytes. There is no protein footprint on the Xi in this region. The only obvious sequence peculiarity is a stretch of six Cs in close proximity to the unmethylated site. Methylation of some sites on the Xi in female lymphocytes is partial, suggesting either less efficient maintenance than in hybrid cells or a mixture of cell types with specific methyl-
Complete methylation that we see is not correlated with methylation profile at a specific site can be stably maintained in tissues and cell lines (Turker et al. 1989). The trinucleotides; thus, there may be a bias of DNA methylation and HeLa cell extracts (Yang et al. 1988) revealed, for the Spl consensus sequences, only the -360 footprint. Another protected region observed in the in vitro study and centered at position -130 is also seen in vivo (footprints III and IV); however, the most prominent reactivity differences are seen at the left-most boundary (-140 to -155). The region of footprint IV shows a significant homology to a high-affinity NF1-binding site (Nilsson et al. 1989). Two guanosines of the CCAAT element at position -108 are also protected (footprint III). The PGK-1 promoter contains no TATA box, and the region near the transcription initiation sites shows no obvious sequence-specific protein–DNA interactions detectable by in vivo DMS footprinting. Because a consensus sequence for HIP1 (Means and Farnham 1990) is at the transcription start point of PGK-1, it should be noted that our in vivo DMS studies have not revealed a footprint for HIP1. This new factor has been proposed to be a general factor for G + C-rich promoters and to bind near the transcription start point (Means and Farnham 1990). Footprint VII, which spans only a few nucleotides, contains a consensus sequence (CGTCA) for the transcription factor ATF (Lee et al. 1989). Footprint V, which is seen only in lymphocytes, shows no obvious sequence similarity to any known binding sequences of transcription regulatory proteins.

Factors bind selectively to Xa

We have identified in vivo eight different footprints in the CpG island of PGK-1 on Xa, suggesting binding of sequence-specific proteins. No footprints are seen on Xi in hybrids and female cells. This result emphasizes that the mere presence of a transcription factor in the cell does not necessarily mean that it actually binds to its recognition sequence in vivo (see also Becker et al. 1987; Mueller and Wold 1989). By sequence analysis, the investigated region has five potential binding sites for transcription factor Spl. Three of these sites show a footprint in all cell types that contain an Xa (footprints I, II, and VI, Fig. 9). One footprint at -360 (footprint VIII) is seen only in HeLa cells, and one Spl consensus sequence at -30 is not protected at all in any cell type, indicating that potential Spl-binding sites can be selectively occupied in vivo. Earlier work using in vitro foot-
Six of the eight sequences of human PGK-1 identified by the presence of in vivo footprints [I, II, III, IV, VI, and VII] show a high degree of homology to mouse sequences that are similarly spaced in the promoter of mouse PGK-1 [Adra et al. 1987]. Also of significance is that the same in vivo footprint patterns are seen in human cells and in hamster–human hybrid cells, suggesting again, as for methylation, a high degree of evolutionary conservation.

**Heterochromatin structure and the Xi**

The Xi has the cytological features and genetic silence typical of heterochromatin, yet the Xi is of particular interest because in female cells a chromosome with indistinguishable base sequence (inbred mice) is maintained euchromatic. What is the difference between euchromatin and facultative heterochromatin? The LMPCR procedure, in conjunction with HpaII treatment, has allowed us to compare facultative heterochromatin and euchromatin in vivo for the first time. We find that the DMS reactivity profile of the Xi is very similar to torsion-free, protein-free DNA. The DMS reaction profile of the PGK-1 promoter region in supercoiled plasmid DNA is distinguishably different (G.P. Pfeifer et al., unpubl.), and the eight protein footprints apparent on the Xa are definitely not seen on the Xi. At first contemplation, these results seem to be at odds with the cytologically condensed state of the Xi. One might have expected that the heterochromatinic Xi would have, as do some satellite DNAs [Levinger and Varshavsky 1982] and Drosophila heterochromatin [James and Elgin 1986; Reuter et al. 1990], a unique DNA-binding factor or set of bound factors that would give an Xi-specific footprint pattern. However, heterochromatin has been reported to have less nonhistone proteins than euchromatin (Comings et al. 1977). Moreover, with an important exception that will be discussed below, several nuclease digestion studies on isolated nuclei have indicated that general nucleosome accessibility of the Xi is not much different from that of the Xa and bulk chromatin [Lin and Chinault 1988; Antequera et al. 1989]. If nucleosome accessibility is a reflection of higher order chromatin structure, these results suggest only small global differences in structure between euchromatin and facultative heterochromatin.

Our studies do not yet provide much direct information about nucleosome structure and phasing, because it is known that the DMS reactivity profile of DNA in nucleosomes is not very different from free DNA, the N7 of guanine is as solvent and (DMSI accessible in nucleosomal DNA as it is free of protein [McGhee and Felsenfeld 1979; Wang and Becker 1988]. The only difference reported is an enhanced reactivity at approximately nucleotide 62 from the 5' end of the nucleosome [McGhee and Felsenfeld 1979]. The only feature of Xi DNA that we observe to be significantly different from protein-free DNA is enhanced DMS reactivity of Gs at positions +4, 5, −87, −122, 123, and −218. These sites of enhanced reactivity could possibly be a reflection of phased nucleosomes, but the spacing between the sites is not obviously consistent with this notion. Hyperreactive sites could also be caused by altered DNA conformation in vivo [Z-DNA, bent DNA, etc.], which could be influenced directly or indirectly by the high level of methylation.

We cannot rule out that methylation alone is sufficient in vivo for maintenance of the inactive state. The simplest explanation would be that transcription factors do not bind to DNA that is completely methylated. However, Sp1 binding was shown to be not methylation sensitive or only weakly methylation sensitive in vitro [Harrington et al. 1988; Holler et al. 1988; Ben-Hattar et al. 1989]. Meehan et al. [1989] have identified a protein (MeCP) that binds preferentially to clusters of 15 or more methylated CpGs. MeCP binding at methylated CCGG sites inhibits the action of MspI, a methylation-insensitive restriction endonuclease. Studies on isolated nuclei by Hansen et al. [1988] have shown that the methylated human PGK-1 promoter on the Xi is >100-fold less sensitive to MspI digestion than the unmethylated region is on the Xa. Antequera et al. [1989] obtained similar results for mouse HPRT. Thus, this line of evidence indicates that the methylated PGK-1 island on the Xi should bind MeCP, but we observe no protein footprints in vivo. Because MeCP is not sequence-specific, one possible explanation for this result might have been tight but random binding of MeCP. Present evidence, however, is against random, tight interactions. Tight binding should give a strongly decreased DMS reactivity throughout the methylated CpG island, but this is not seen. MeCP requires 15 CpGs for binding; thus, interactions at each site are likely to be weak. Like histones, MeCP binding may not greatly affect DMS accessibility to guanine N7 in the major groove of the DNA helix.

Our current working hypothesis continues to be that the methylated PGK-1 island on the Xi is packaged in nucleosomes associated with MeCP, as suggested by Meehan et al. [1989]. This packaging would preclude the binding of transcription factors, including Sp1. Studies with agents other than DMS, such as DNases that probe nucleosomal structure, will be required to test this model. Because random binding is rather likely, these studies will need to be quantitative, measuring, for example, band intensities above and below HpaII sites as we were able to do here for DMS studies.

**How is the methylation difference between Xa and Xi maintained?**

Our results dramatically illustrate that in human–hamster cell lines, all of the 120 potentially methylatable sites in the CpG island of PGK-1 are maintained without apparent methylation on the Xa. We believe that 10% methylation would have been detectable at most sites. In a separate study, LMPCR was used to focus on the methylation of certain HpaII and NarI sites (positions +23 and −343) [Steigerwald et al. 1990]. In that study, which used restriction-cut fragments to obtain methylation information, the experiment was designed to give a positive signal from a methylated site (longer frag-
ment), as well as from an unmethylated site [shorter fragment].

These experiments showed no detectable methylation on Xa and no detectable undermethylation on the Xi, and it was concluded that the methylation differential was >50-fold. There has not been selection for PGK-1 activity in the cell lines used for this study, yet two alternate states of the CpG island [fully unmethylated, or fully methylated] have been stably maintained for >30 cell generations since the last cell cloning, even in a heterologous hamster–human system. How is this accomplished? High cooperativity is often invoked to explain the low abundance of intermediate states, in this case, the absence of partially methylated islands. Cooperativity may be part of the story, but patchwork methylation can be observed on the Xi in clones derived from azacytidine-treated cells [G.P. Pfeifer, S.D. Steigerwald, R.S. Hansen, S.M. Gartler, and A.D. Riggs, unpubl.], thus, cooperativity seems limited. In addition, simple cooperativity seems inadequate for long-term stability such as is seen for X inactivation. The reasons are at least twofold. First, on the Xa, occasional de novo methylation events in a population of cells should cause a drift toward methylation, especially because gene silencing and de novo methylation at HpaII sites at autosomal CpG islands does occur in tissue culture [Reno et al. 1989; Jones et al. 1990]. Second, in a growing population, failure to maintain a methylated site with 100% efficiency results in dilution and eventual complete loss of the methylated site, unless dilution is counteracted by significant de novo methylation. Rather than relying on perfect maintenance efficiency, a more dynamic system with an error-correction mechanism seems more likely. Whatever the complete mechanism, one component could be that the proteins [large protein complex!] observed on the Xa are crucially involved in keeping this region methylation free by inhibiting both de novo and maintenance activity of DNA methyltransferase, which is a bulky 190-kD protein [Pfeifer and Drahovsky 1986].

Methods

Cells and cell lines

Chinese hamster hybrid cells containing either a human Xa (Y162-11C) or Xi [X86T2] [Hansen et al. 1988] were kindly provided by R.S. Hansen and S.M. Gartler [Seattle, WA]. HeLa cells were strain S315 obtained from G. Attardi [Pasadena, CA]. Human lymphocytes were obtained from heparinized blood of strain 315 obtained from G. Attardi [Pasadena, CA) or Xi (X86T2) [Hansen et al. 1988] were kindly provided by R.S. Hansen, S.M. Gartler, and A.D. Riggs, unpubl.].

In vivo footprinting

In vivo DMS footprinting was performed on cultured cells (5 × 10^6 to 5 × 10^7) by replacing the culture medium with medium containing 10% fetal calf serum and 0.1% DMS [freshly prepared and incubating at room temperature for 5 min. Other concentrations of DMS and various incubation times have revealed the same footprints. The DMS-containing medium was quickly removed, and cells were washed with medium without DMS and detached by trypsin treatment. The cell suspension was diluted 10-fold by adding ice-cold phosphate-buffered saline (PBS), and cells were collected by centrifugation. Lymphocytes were resuspended in RPMI medium containing 5% fetal calf serum, DMS was added to a final concentration of 0.1%, and cells were incubated for 10 min at room temperature. The reaction was quenched by adding a 10-fold volume of ice-cold PBS followed by centrifugation. After washing with ice-cold PBS, nuclei were prepared [Wijnholds et al. 1988], and DNA was purified [300 μg/ml proteinase K, 20 mM Tris-HCl [pH 8], 20 mM NaCl, 20 mM EDTA, 1% SDS for 3 hr at 37°C]. After phenol/chloroform extraction, the DNA was precipitated in ethanol and redissolved in 10 mM Tris-HCl [pH 7.8], 1 mM EDTA. The viscosity of the DNA solution was reduced by digestion with EcoRI, which does not cut within the region to be analyzed. After restriction digestion, DNA was extracted once with phenol/chloroform and ethanol-precipitated. The DNA was dissolved in 1 M piperidine [Sigma], and the strand scission reaction was performed in sealed tubes at 95°C for 30 min. The fragments were precipitated in 0.3 M sodium acetate with 2.5 volumes of ethanol at −70°C. After centrifugation, pellets were washed twice in 80% ethanol. Residual traces of piperidine were removed by drying the sample overnight in a Speedvac concentrator. At this stage, it is usually useful to analyze the DNA on an agarose gel. For optimum molecule usage, the average fragment size length should be <500 nucleotides. If this is not the case, the in vivo DMS treatment has been relatively inefficient, and more DNA has to be used as starting material in the Sequenase reaction (see below). To obtain similar band intensities in individual lanes of the sequencing gel, approximately equal amounts of DNA [1−3 μg as estimated from the gel] were processed for the Sequenase reaction, as described below. Reducing this amount of DNA may result in occasional missing of certain bands, and this could be mistaken as a footprint [Mueller and Wold 1989; Pfeifer et al. 1989].

Base-specific DNA cleavage

Between 10 and 50 μg DNA, prepared as above, was used per base-specific reaction. This amount is sufficient for analysis with at least 10 different primer sets. For analysis of cytosine methylation, DNA was cleaved with hydrazine in the presence of 1.5 M NaCl [Maxam and Gilbert 1980]. Hydrazine cleavage of 30 μg genomic DNA for 20 min at 20°C resulted in an average fragment size of 100–200 nucleotides. DNA was piperidine-cleaved and precipitated as described above for in vivo footprinting. For the methylation studies, cloned DNA was used as an unmethylated control. Plasmid pSPT19.1, which contains the PGK-1 5′ region [Singer-Sam et al. 1984], was linearized with EcoRI and diluted to the single-copy level, with Micrococcus luteus DNA as a carrier. G, G + A, T + C, and C reactions were performed on HeLa DNA, and these were included on the sequencing and footprinting gels to provide position markers.

LMPCR

Gene-specific oligonucleotide primers were annealed to the piperidine-cleaved DNA fragments and extended to blunt ends by using Sequenase as described [Pfeifer et al. 1989]. A complete protocol for LMPCR-aided genomic sequencing is available upon request. The following oligonucleotide primer sets [see Fig. 1] were used for the Sequenase reaction [primers 1] and PCR amplification [primers 2]: For sequencing the lower strand, we used primer set A [primer A1, 5′-AAGTCGGGAAGGTTCCCTT, primer A2, 5′-AAGGTTCCTTGC-GGTTGCGCGC, nucleotides −238 to −208], primer set C [primer C1, 5′-ATCCAGGGTTGGGG, primer C2, 5′-GGTTGCGCGGTTGGTTGGC, nucleotides −436 to −406], primer set E [primer E1, 5′-TGTGGCCAA-

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TAGCGGCT, primer E2, 5'-AATAGCCGCTGCTACAGCAGGGCC, nucleotides -112 to -83, primer set G (primer G1, 5'-TCACTTCGCACGACG, primer G2, 5'-CGTCGGCAGCGCAATCCGGGATC), for sequencing the upper strand, we used primer set D (primer D1, 5'-TTTGTACGTCGCGAC, primer D2, 5'-ACGGCGCAGACCGCAAGAACCT, nucleotides -192 to -229), primer set F (primer F1, 5'-CGTCGGCAGTTGTCGGAC, primer F2, 5'-TCCAGCTACGTTGAGAACCCG, nucleotides +134 to +99), primer set H (primer H1, 5'-CCGGAGCTTGGTGAGAGAATGC, primer H2, 5'-CGTCGGCAGATTGTCGGAC, nucleotides +25 to -5), and primer set I (primer I1, 5'-CGGGTCGGCAGCGCTGT, primer J2, 5'-CATCGCGGTCGGCAGCGCTGT, nucleotides -120 to -137). Ligation of the oligonucleotide linker to the blunt-ended primer-extended molecules was done as described (Mueller and Wold 1989). After precipitation of the DNA, PGK-1-specific fragments were amplified with Taq polymerase by using the longer oligonucleotide of the linker and primers 2 as described (Pfeifer et al. 1989). Seventeen to 18 amplification cycles were performed. After amplification and precipitation of the DNA, the fragments were separated on sequencing gels (50 x 0.04 cm for foot-printing, 95 x 0.04 cm for 5-methylcytosine determination). The sequences were visualized by autoradiography after electroblotting to nylon membranes and hybridization with a single-stranded probe (Pfeifer et al. 1989). These hybridization probes did not overlap with the sequence of the primers used for PCR amplification. Primer sets D and F, and G and E, respectively, were included simultaneously in primer extension and amplification reactions. The sequence ladders for the individual primers were then visualized separately by rehybridization of the nylon membrane (Pfeifer et al. 1989).

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