Chromatin differences between active and inactive X chromosomes revealed by genomic footprinting of permeabilized cells using DNase I and ligation-mediated PCR

Gerd P. Pfeifer and Arthur D. Riggs

Beckman Research Institute of the City of Hope, Department of Biology, Duarte, California 91010 USA

Ligation-mediated polymerase chain reaction (LMPCR) provides adequate sensitivity for nucleotide-level analysis of single-copy genes. Here, we report that chromatin structure can be studied by enzyme treatment of permeabilized cells followed by LMPCR. DNase I treatment of lysolecithin-permeabilized cells was found to give very clear footprints and to show differences between active and inactive X chromosomes (Xa and Xi, respectively) at the human X-linked phosphoglycerate kinase (PGK-1) locus. Beginning 380 bp upstream and continuing 70 bp downstream of the major transcription start site of PGK-1, we analyzed both strands of this promoter and CpG island and discovered the following: (1) The transcriptionally active Xa in permeabilized cells has several upstream regions that are almost completely protected on both strands from DNase I nicking. (2) Nuclei isolated in polyamine-containing buffers lack these footprints, suggesting that data from isolated nuclei can be flawed; other buffers are less disruptive. (3) The Xa has no detectable footprints at the transcription start and HIP1 consensus sequence. (4) The heterochromatic and transcriptionally inactive Xi has no footprints but has two regions showing increased DNase I sensitivity at 10-bp intervals, suggesting that the DNA is wrapped on the surface of a particle; one nucleosome-sized particle seems to be positioned over the transcription start site and another is centered ~260 bp upstream. (5) Potassium permanganate and micrococcal nuclease (MNase) studies indicate no melted or otherwise unusual DNA structures in the region analyzed, and MNase, unlike restriction endonuclease *MspI*, does cut within the positioned particles on the Xi. Results are discussed in the context of X chromosome inactivation and the maintenance of protein and DNA methylation differences between euchromatin and facultative heterochromatin at CpG islands.

[**Key Words:** X chromosome inactivation; chromatin structure; nuclei; nucleosomes; CpG island; DNA methylation]

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The interaction of DNA elements with their cognate regulatory proteins is usually determined by *in vitro* footprint analysis and gel mobility-shift experiments done using nuclear extracts. But is the DNA-binding activity of factors isolated from nuclei meaningful *in vivo*? Do isolated nuclei and nuclear extracts accurately represent the true *in vivo* situation? To answer these questions, DNA-protein contacts can be studied at single-nucleotide resolution in living cells by *in vivo* footprinting on the basis of genomic sequencing (Church and Gilbert 1984; Ephrussi et al. 1985; Becker et al. 1987; Saluz and Jost 1987). However, this method has been technically challenging because of the complexity of the mammalian genome; and, despite the importance of this approach, <10 mammalian genes have been analyzed since the technique was introduced 7 years ago. Recently, genomic sequencing (Pfeifer et al. 1989) and *in vivo* foot-

printing (Mueller and Wold 1989) techniques have been developed that overcome most of the previous problems. These new procedures attain much improved sensitivity and specificity by using a ligation-mediated polymerase chain reaction (LMPCR) to amplify the component DNA fragments of the sequence ladder. Genomic sequencing studies done with dimethylsulfate (DMS), a small alkylating agent, have already indicated that some DNA-binding factors, though present in nuclear extracts, do not bind *in vivo* (Becker et al. 1987; Mueller and Wold 1989; Pfeifer et al. 1990a). Agents in addition to DMS for determining *in vivo* chromatin structure and protein binding should be useful. UV footprinting can be done (Wang and Becker 1988; Pfeifer et al. 1991), but only some sequences are informative, so more general agents and procedures are needed.

As a step in this direction, Zhang and Gralla (1989)

recently demonstrated that SV40 minichromosomes in infected cells can be studied by DNase I and micrococcal nuclease (MNase) treatment of lysolecithin-permeabilized cells. Cells permeabilized by lysolecithin remain intact, display very efficient DNA replication and transcription activities (Miller et al. 1978; Contreras and Fiers 1981), and should maintain normal nuclear structure better than isolated nuclei. Zhang and Gralla (1989) also treated intact cells with potassium permanganate (KMnO_4) to probe for single-stranded regions in the SV40 minichromosomes, and they identified a region at the transcription start site that is apparently melted in vivo. We adapted LMPCR-aided genomic sequencing for studies of permeabilized cells (Tanguay et al. 1990), and we report here the first detailed analysis of a single-copy gene by the Zhang and Gralla procedures.

The region we have analyzed, the transcription start and upstream region of human phosphoglycerate kinase-1 (PGK-1), is of interest for several reasons. The PGK-1 gene is X-linked, is subject to strong transcriptional silencing by X chromosome inactivation, and is located near the X chromosome inactivation center (Brown et al. 1991). An intriguing aspect of this ubiquitously expressed gene is that in female cells the promoter on the inactive X chromosome (Xi) remains stably silenced in the face of factors obviously sufficient for good expression from the active X chromosome (Xa) present in the same nucleus. What renders the Xi immune to these factors? The stable, somatically heritable maintenance of differentiated states is of central importance for vertebrate development. However, little is known about the required cell memory mechanisms (see Riggs 1989, 1990a), although methylated CpG sites are somatically heritable entities (Pfeifer et al. 1990b) and a strong case can be made for DNA cytosine methylation being involved as an important component of X chromosome inactivation. The transcription start sites for PGK-1 are located near the center of what appears to be a typical CpG-rich island. Although this promoter is very GC-rich and lacks a TATA box, it can be rather efficient, as evidenced by transient expression studies in HeLa cells (J. Singer-Sam, J. LeBon, and A. Riggs, unpubl.), where it compares favorably with the SV40 promoter. Although CpG islands are characteristically unmethylated (Bird 1986), and the PGK-1 island on the Xa is unmethylated, the Xi is methylated at 119 of 121 CpG dinucleotides analyzed in this region (Pfeifer et al. 1990a,b). Our earlier in vivo footprinting studies, done by DMS treatment of intact cells (Pfeifer et al. 1990a), indicated that the PGK-1 promoter on the Xa was protected at several sites, apparently by transcription factors. Surprisingly, the DMS reactivity of this region on the Xi was very similar to that of naked DNA. The enzyme footprinting work reported here confirms these DMS studies, but protection from the large 31-kD DNase I enzyme is much more obvious and complete, with larger and additional footprints being seen. The reactivity profile of the Xi is much different from the Xa, with the Xi apparently having two positioned nucleosomes or nucleosome-like structures. The procedures reported here should be generally useful

for determining many details of chromatin structure. Single-copy genes can now be analyzed in a minimally perturbed state by enzymes and other agents.

Results

PCR-aided amplification of nuclease and KMnO_4 -derived fragments

We employed LMPCR to obtain the necessary sensitivity and specificity to study single-copy genes. The first step of LMPCR is preparation of DNA with 5' phosphorylated ends or nicks, such as those provided by limited DNase I digestion. Next, after denaturation, a gene-specific oligonucleotide (primer 1) is used for a primer extension reaction, generating molecules having a blunt end on one side. Linkers are ligated to the blunt ends, and the linker-ligated molecules are then amplified by an exponential PCR reaction done using the longer oligonucleotide of the linker (linker-primer) and a second, nested, gene-specific primer (primer 2). This method amplifies all molecules that have undergone complete primer extension and linker ligation. Electrophoretic separation of the amplified fragments on DNA sequencing gels gives high-quality sequence ladders that can be visualized by hybridization with an appropriate probe. However, to obtain adequate sensitivity for single-copy genes, LMPCR must be efficient. DNase I treatment of DNA conveniently provides 5' phosphorylated ends, but the 3' ends are free hydroxyl groups. We observed that priming by these genomic 3' ends during the primer extension and/or PCR steps of LMPCR significantly reduces overall efficiency and gives a background smear. To reduce such interference, the 3' ends were blocked by addition of a dideoxynucleotide (Tanguay et al. 1990). DNA molecules produced by MNase have 3' phosphates and 5' hydroxyls and work well in LMPCR after 5' phosphorylation by T4 polynucleotide kinase. Piperidine cleavage of KMnO_4 -treated DNA leaves DNA with both 5' and 3' phosphates and thus is ideal for LMPCR.

Figure 1 shows the location and orientation of the eight primer sets that were used in this study to analyze both strands of the PGK-1 promoter, which is part of a CpG island and is very GC rich. Figure 2 shows genomic footprinting data obtained by DNase I treatment of naked DNA, cells, and nuclei. Prior work and the data shown in Figures 2–7 have established that although all

Figure 1. Oligonucleotide primer sets used for LMPCR-aided analysis of the 5' region of human PGK-1. The curved arrow marks the major transcription start site. The solid arrows show the position and orientation of the primers.

Figure 2. DNase I analysis of the PGK-1 promoter on the lower strand between nucleotide -30 and -170 , using primer set A. (Lanes 1–3) Maxam–Gilbert-cleaved DNA (G+A, T+C, C) from HeLa cells; (lanes 4–8) active X chromosomal DNA (X α) from Y162-11C cells; (lanes 9–15) inactive X chromosomal DNA (Xi) from X86T2 cells. DNase I-treated naked DNA controls are in lanes 4 and 5 (X α DNA) and 9 and 10 (Xi DNA). (Lanes 6 and 7 and 11–13) DNA from permeabilized cells treated with DNase I. DNA from nuclei treated with DNase I is shown in lanes 8, 14, and 15. The DNase I concentrations were 0.8 μ g/ml (lanes 4 and 9), 1.6 μ g/ml (lanes 5 and 10), 10 μ g/ml (lanes 8 and 14), 30 μ g/ml (lane 15), 25 μ g/ml (lanes 6 and 11), and 50 μ g/ml (lanes 7, 12, and 13). Footprints on the X α are indicated by the open boxes.

molecules do not amplify with identical efficiencies, they amplify with reproducible relative efficiencies and thus reproducible ladders are obtained as long as 1 μ g or more of genomic template DNA is used per lane and PCR cycles are kept relatively low (Pfeifer et al. 1989, 1990a). We always compare each lane with an appropriate naked DNA control. Also, the nylon membranes are usually stripped and rehybridized consecutively to reveal two different DNA ladders. This often provides a convenient control to show that the effects seen, for example, in a footprint region, are not a reflection of gel electrophoresis or transfer artifacts.

DNase I footprints on the X α and a comparison of naked DNA, permeabilized cells, and nuclei

All experiments reported here have been done using Chinese hamster–human hybrid cells containing either a

human X α (cell line Y162-11C) or a human Xi (cell line X86T2). The human X chromosomes in these hybrid cells have been shown to stably maintain activity states, methylation patterns, and DMS footprints very similar to normal human lymphocytes (Hansen et al. 1988; Pfeifer et al. 1990a,b).

Figure 2 shows a DNase I analysis using primer set A. Information about nicking of the lower strand was obtained between nucleotide -170 and -30 relative to the major transcription start site. The DNase I digestion pattern of protein-free purified DNA (lanes 4 and 5) can be compared with the digestion pattern of DNA in lysolecithin-permeabilized cells (lanes 6 and 7). The sequence ladders seen are reproducible, even when different DNase I concentrations are used (cf., e.g., lanes 4 and 5). However, the ladders are very different between naked DNA and permeabilized cells. DNA from permeabilized cells treated with DNase I shows some large gaps with almost no fragments at all, even at sites that are strongly cut in naked DNA. Such strong footprints mean that all molecules are hyporeactive, and this implies that percent occupation of that site by the transcription factor is high in all cells. Three different footprints are apparent in the region shown. The top of the gel shows a 31-bp protected region (nucleotide -32 to -63) that contains three consensus sequences (GC boxes) for transcription factor Sp1. The footprint in the middle of the gel (nucleotide -84 to -115) contains the sequence CCAAT and probably is occupied by a member of the family of CCAAT-binding proteins (McKnight et al. 1989). The protected area spanning the CCAAT sequence is interrupted on both strands by a site showing increased reactivity relative to protein-free DNA. Such hyper-reactive sites are often seen at the ends of footprints, so another protein (dotted box in Fig. 2) may bind immediately 3' to the CCAAT-binding protein. A large footprint is very obvious at the lower part of the gel (nucleotides -160 and -123). The dimensions of this footprint were previously difficult to ascertain by DMS footprinting (Pfeifer et al. 1990a), as only a few of the guanines showed differential reactivity when compared with naked DNA. This region contains two sequences with homology to the binding site of transcription factor NF1 (TGGCA-binding protein; Nilsson et al. 1989 and references therein). The boundaries of this footprint were resolved more clearly with a primer set located farther upstream (Fig. 3). Figure 4, lanes 7 and 8, shows the DNase I reactivity patterns of the upper strand between nucleotide -40 and -190 . Here, the GC-box footprint is located at the bottom of the gel, the CCAAT footprint is in the middle, and the NF1-like footprint is at the top of the gel. These results illustrate that both strands are highly protected from DNase I at the footprint sites. Figure 8A [below] shows a summary of these results.

Our initial DNase I footprinting experiments done on nuclei isolated by a common procedure (see Kornberg et al. 1989) gave very different results. The footprints seen in permeabilized cells (Fig. 2, lanes 6 and 7; Fig. 3, lanes 7 and 8) were almost totally absent in nuclei (Fig. 2, lane 8; Fig. 3, lanes 9 and 10), which showed reactivity very

Figure 3. DNase I analysis of the lower strand of the Xa between nucleotide -70 and -230 using primer set G. (Lanes 1–4) Maxam–Gilbert-cleaved DNA (G, G + A, T + C, C) from HeLa cells. Naked DNA controls are in lanes 5 and 6. DNA from DNase I-treated permeabilized cells is shown in lanes 7 and 8. DNA from DNase I-treated nuclei is shown in lanes 9 and 10. The DNase I concentrations were 0.8 μ g/ml (lane 5), 1.6 μ g/ml (lane 6), 25 μ g/ml (lane 7), 50 μ g/ml (lane 8), 10 μ g/ml (lane 9), and 30 μ g/ml (lane 10). Footprints showing strong protection are indicated by a box with a solid border; a region of weaker protection is marked by a box with a dotted border.

similar to naked DNA. These results establish that some nuclear isolation conditions cause detachment of transcription factors from the DNA and prompted us to investigate other nuclear isolation conditions. We found that buffers that do not contain the polyamines spermine and spermidine retain the footprint pattern characteristic of permeabilized cells, although with reduced clarity (Fig. 5, lanes 3 and 4). Nonionic detergents such as NP-40 are apparently rather innocuous for the factors on the Xa. Mechanical disruption of the cells by Dounce homogenization gave footprints of inferior quality (Fig. 5, lane 5). Cell permeabilization, which can be used for both monolayer and suspension cells (Contreras and Fiers 1981), seems to be the simplest technique for reliably detecting DNase I footprints.

The Xa does not contain a footprint near the transcription initiation site, and the overall reactivity of this region is similar to that of naked DNA. The PGK-1 promoter contains no TATA box, but there is a consensus sequence at the transcription start for the binding of HIP1, a protein proposed to play a role in transcriptional initiation of GC-rich promoters (Means and Farnham 1990). However, we have seen no footprint over the HIP1 consensus sequence, either by DMS (Pfeifer et al. 1990a) or by DNase I footprinting (data not shown), so this site

is probably not highly occupied in vivo. The Xa does show several DNase I hypersensitive sites near the transcription initiation site (Fig. 8A, below), and the pattern of hypersensitive sites is different from that on the Xi.

DNase I studies of the Xi and evidence for wrapped DNA

Even as free DNA, the PGK-1 promoter region is different between the Xa and Xi because of heavy cytosine methylation on the Xi (Pfeifer et al. 1990a,b). The DNase I digestion pattern of methylated Xi DNA is significantly different from that of unmethylated Xa DNA (Fig. 2, cf. lanes 4 and 5 with 9 and 10; Fig. 4, cf. lanes 5 and 6 with 9 and 10), with enhanced cutting on the 5' side of methylcytosine. This effect has been observed previously at methylated *Hha*I sites (G^mCGC) by Fox (1986), who concluded that a two- to threefold increase in DNase I reactivity was caused by an alteration of the precise orientation of the phosphodiester bond on the 5' side. For these

Figure 4. DNase I analysis of the upper strand between nucleotide -40 and -190 using primer set H. (Lanes 1–8) Xa DNA; (lanes 9–12) Xi DNA. Lanes 1–4 are sequencing controls (G, G + A, T + C, C) done with HeLa cell DNA. Naked DNA controls are in lanes 5 and 6 (Xa DNA) and 9 and 10 (Xi DNA). DNA from DNase I-treated permeabilized cells is shown in lanes 7, 8, 11, and 12. The DNase I concentrations were 0.8 μ g/ml (lanes 5 and 9), 1.6 μ g/ml (lanes 6 and 10), 25 μ g/ml (lanes 7 and 11), and 50 μ g/ml (lanes 8 and 12). Footprints on the Xa are indicated by boxes.

Figure 5. DNase I analysis of the Xa in isolated nuclei. A naked DNA control is shown in lane 1 (0.8 µg/ml of DNase I); DNA from lysolecithin-permeabilized Y162-11C cells (Xa) treated with 50 µg/ml of DNase I is shown in lane 2. Shown are results of DNase I digestion of nuclei isolated (see Materials and methods) either in buffer A without polyamines (lane 3) or in buffer B (lane 4). (Lane 5) Results obtained with nuclei isolated by Dounce homogenization in buffer B without NP-40.

reasons, naked DNA controls should always be obtained from the same cell type as the *in situ*-treated sample.

In addition to these methylation-induced differences, the footprints specific for the Xa are definitely not seen on the Xi (see Figs. 2 and 4). No sequence on the Xi is protected from DNase I for >3 bp. Although the sequence ladders seen for the Xi are similar overall to naked DNA, there are very clear differences. For example, there are several DNase I hypersensitive sites in treated cells that appear to alternate with protected sites over the region shown in the lower part of Figure 4, lanes 11 and 12 (nucleotide -40 to -100). These periodic patterns are even more obvious in the autoradiogram shown in Figure 6. This region includes upper strand sequences from nucleotide -262 to -338. Clusters of DNase I hyper-reactive sites occur at intervals of ~10 bp and are separated by hyporeactive nucleotides. Figure 7 illustrates that obviously periodic patterns extend over relatively large regions. Primer set J allowed analysis of the upper strand over almost 160 bp from nucleotide -187 to -340, and here periodic DNase I hyper-reactive sites are seen over a region of ~130–140 nucleotides. This

periodic pattern of clustered sites on the upper strand ends upstream of -330 and downstream of about -200, although the boundaries are not sharp (Figs. 4, 6, and 7, and data not shown). Densitometric analysis of the autoradiogram of Figure 6 has shown that the relative intensities of bands from treated-cell DNA and naked DNA differ by over an order of magnitude, suggesting that the majority of molecules have this feature. Figure 6B, lanes 7 and 8 shows data for the region near the transcription initiation site. Here also periodic DNase I hypersensitive sites are apparent on the Xi. Combined data from analysis with primer sets H (Fig. 4) and F (Fig. 6B) indicate that the periodicity extends over a region of ~140 bp. Figure 8B summarizes the hyper- and hyporeactivities seen on the Xi. Periodic nicking is not seen for naked DNA, and the reactivity profile of DNA upstream, downstream, and in between the regions showing clustered and spaced hyper-reactive sites is not greatly different from naked DNA. Thus, the cutting pattern in treated cells strongly suggests that the DNA helix is wrapped around a protein surface. The ~10-bp periodicities are most prominent around nucleotides -260 and -20, suggesting two positioned factors, one of which is over the transcription start sites. It appears that the 10-bp periodicity is slightly interrupted near the transcription initiation site. The positioned factors are resistant to disruption by nuclear isolation in the presence of polyamines (see Fig. 7) and are thus quite different from the putative transcription factors seen on the Xa. The size of

Figure 6. DNase I analysis of the Xi in X86T2 cells. (A) Analysis of the upper strand between nucleotide -262 and -338 using primer set D. (B) Analysis of the upper strand between nucleotide +55 and -27 using primer set F. Lanes 1–4 are sequencing controls (G, G+A, T+C, C) with DNA from HeLa cells. Naked DNA controls are in lanes 5 and 6. DNA from DNase I-treated permeabilized cells is shown in lanes 7 and 8. The DNase I concentrations were 0.8 µg/ml (lane 5), 1.6 µg/ml (lane 6), 25 µg/ml (lane 7), and 50 µg/ml (lane 8).

Figure 7. DNase I analysis of the Xi in X86T2 cells. The upper strand between nucleotide -187 and -340 was analyzed using primer set J. Lanes 1–3 are sequencing controls (G + A, T + C, C) with DNA from HeLa cells. Naked DNA controls are in lanes 4 and 5. DNA from lysolecithin-permeabilized cells treated with DNase I is shown in lanes 6 and 7. DNA from DNase I-treated nuclei is shown in lanes 8 and 9. The DNase I concentrations were 0.8 $\mu\text{g/ml}$ (lane 4), 1.6 $\mu\text{g/ml}$ (lane 5), 25 $\mu\text{g/ml}$ (lane 6), 50 $\mu\text{g/ml}$ (lane 7), 10 $\mu\text{g/ml}$ (lane 8), and 30 $\mu\text{g/ml}$ (lane 9). The arrows indicate nucleotides that are more reactive to DNase I in nuclei and permeabilized cells than in protein-free DNA.

each wrapped region is about that expected for nucleosomes.

Figure 8, A and B, shows a summary of our DNase I footprinting data for both strands of the Xa and the Xi. Data for the lower strand of the Xi were obtained for the whole region by use of primer sets C, G, A, and E. As for the upper strand, periodic DNase I hyperreactive sites on the Xi were also apparent for the lower strand. However, the reactivity differences were generally about two- to threefold smaller than for the respective upper strand sequences.

MNase and KMnO₄ studies

Only DNase I data are shown, but we have found that MNase and KMnO₄ give equally clear ladders. MNase has been used extensively for chromatin structure analysis because this enzyme shows a strong preference for single-stranded DNA and also preferentially cuts between nucleosomes. Because our DNase I studies suggested the presence of positioned nucleosomes on the Xi, we attempted to confirm their presence by MNase experiments. Because the PGK-1 promoter is very GC-rich and MNase shows a marked preference for AT-rich DNA, the region was cut relatively infrequently. We

also have found that MNase ladders are not as reproducible as DNase I ladders, with some MNase bands decreasing with increased enzyme treatment. No regions of preferential cutting suggestive of melted DNA have been seen. Interestingly, by LMPCR analysis the putative internucleosomal regions were not preferentially sensitive, and regions within the putative nucleosomes cores were cut (data not shown).

KMnO₄ can be used as a DNA-modifying agent on intact cells and shows a strong preference for reaction with pyrimidines in single-stranded DNA (Rubin and Schmid 1980; Hayatsu and Ukita 1967). Using this reagent, Zhang and Gralla (1989) saw evidence for melted DNA over about a 30-bp span immediately upstream of the transcription start site of SV40. Previous studies in prokaryotes have also suggested some melting at start sites (Sasse-Dwight and Gralla 1989). A comparison between naked and in vivo KMnO₄-treated DNA for a region spanning the transcription start site of PGK-1 on the Xa revealed no footprints, and there were only minor differences between naked DNA and treated cells (data not shown). This result argues against the presence of stable melted DNA but certainly does not exclude transient melting of the region by components of the transcription machinery.

Discussion

Chromatin structure analysis of a single-copy gene and a comparison of permeabilized cells and isolated nuclei

We have used cells treated with three agents—DNase I, MNase, and KMnO₄—to examine chromatin structure at nucleotide-level resolution at the CpG island and promoter of the human PGK-1 gene. DNase I, a relatively large 31-kD protein, has proved to be a particularly good reagent for chromatin analysis at nucleotide resolution, giving clear and unambiguous footprint patterns in treated cells. Xa-specific footprints obtained by DNase I treatment of lysolecithin-permeabilized cells confirm our earlier DMS footprinting results obtained using intact cells (Pfeifer et al. 1990a), and this fact makes it very likely that the results obtained by both methods reflect the true in vivo situation. However, following Zhang and Gralla (1989), perhaps the term *in situ* should be used when referring to permeabilized cells. Often, *in vivo* has been used very loosely, in the literature, even referring to studies on isolated nuclei. Our study indicates that care should be taken in the interpretation of studies on isolated nuclei. For example, spermine and spermidine are often used in the nuclear isolation buffers to keep nuclei from swelling and rupturing (Kornberg et al. 1989). However, we find that these polyamines cause loss of transcription factors from the Xa promoter, leaving it with a reactivity similar to naked DNA. Thus, it seems that small variations in conditions during nuclei isolation may have significant impact on chromatin structure. Even if transcription-factor binding can be maintained during nuclear isolation, there may be changes reflecting

Figure 8. Summary of DNase I footprint data. (A) The active promoter and unmethylated CpG island on the Xa in Y162-11C cells. Strong footprints with almost complete protection from nicking are indicated by solid bars; weaker footprints are shown by hatched bars. (B) The inactive and methylated CpG island on the Xi in X86T2 cells. No strong footprints are seen on the Xi. For both Xa and Xi, protection of specific nucleotides on the upper or lower strand is indicated by arrows pointing down. Hyper-reactive sites are marked by arrows pointing up. The length of the arrow varies to indicate reactivity differences. Unmarked nucleotides in cells react similarly to those in naked DNA. The major transcription initiation site is indicated by the curved, horizontal arrow in A.

other aspects of nucleoprotein structure (Zhang and Gralla 1990).

Differences between the Xa and the Xi

X chromosome inactivation, which is essential for normal mammalian development, has been reviewed recently (Grant and Chapman 1988; Riggs 1990b). Much remains to be learned, but higher-order chromatin structure seems likely to be an important aspect of this complicated, chromosome-wide phenomenon. Our DNase I footprinting results provide new information about both the in situ chromatin structure of the PGK-1 promoter and differences between the Xa and the Xi. Figure 8 summarizes DNase I results on a sequence diagram, and Fig-

ure 9 shows a schematic representation. The Xa has four regions that are almost completely protected from DNase I attack on both strands, and an additional region around nucleotide -210 that is less well protected. Previously, a DMS footprint at nucleotide -210 was not seen in HeLa cells or in the Xa-containing hybrid cell (Y162-11C) but was seen in normal human lymphocytes (Pfeifer et al. 1990a). These results suggest that the amount of this factor or its binding efficiency may vary according to cell type. Hyper-reactive sites, defined as sites relatively more sensitive in permeabilized cells than in naked DNA, are also seen, often at the edge of a footprint. Hyper-reactivity not only indicates accessibility but also changes in the precise orientation of the phosphodiester bonds relative to the minor groove. The

Figure 9. Schematic summary of in situ DNase I footprinting data for PGK-1. (A) Footprints on the active promoter and unmethylated CpG island of the Xa. The arrow indicates the position of the major transcription start site and all numbers are relative to this site. (B) The inactive promoter and CpG island of the Xi. The Xi has no footprints. Instead, the rounded rectangles represent nucleosome-sized particles around which the DNA is wrapped. The marks on the DNA strand represent methylated CpG sites.

regions between the footprints react normally with DNase I, giving a cutting pattern very similar to naked DNA. These regions of normal reactivity are shown as unmarked sequences in Figure 8. In the schematic representation (Fig. 9), we have shown as double those sites with multiple consensus sequences. The exact number of binding proteins is not yet known. Nevertheless, the picture that emerges for this CpG island and promoter on the transcriptionally active Xa is alternating regions of relatively free, DNase I accessible DNA and DNA well protected on both strands by protein factors. The transcription start site is not obviously melted (our KMnO_4 results) or complexed with proteins, but, as free DNA, could be accessible to transient binding by RNA polymerase and other factors.

The heterochromatic and transcriptionally inactive Xi is very different, having no footprints such as those seen on the Xa. Instead, one sees periodic DNase I hypersensitive sites spaced at ~ 10 -bp intervals over two nucleosome-size regions. The DNA probably is wrapped, perhaps around two positioned nucleosomes, which are separated by a region with DNase I reactivity similar to naked DNA. Previously, DMS studies established that the reactivity of the whole PGK-1 promoter on the Xi was only slightly, if at all, different from that of protein-free DNA (Pfeifer et al. 1990a). This finding, though inconsistent with the presence of tight binding proteins, is compatible with the presence of nucleosomes, since it is known that nucleosomes do not alter DMS reactivity (McGhee and Felsenfeld 1979; Moyer et al. 1989). MNase has often been used to localize nucleosomes, and we used this agent in an attempt to confirm the presence of positioned nucleosomes. However, although bulk DNA showed evidence for core particle-size DNA, our LMPCR results (data not shown) indicate cutting within the wrapped regions. McGhee and Felsenfeld (1983) have reported intranucleosomal cutting by MNase, so this result does not argue strongly against the presence of normal nucleosomes. Also, Zhang and Gralla (1989) ob-

served cutting by MNase within a putative nucleosome positioned just downstream of the cap site in their studies with SV40 minichromosomes. Apparently, MNase data are not always decisively informative at the nucleotide-resolution level. Hansen et al. (1988) have done restriction endonuclease digestion studies on isolated nuclei to probe the Xi-specific chromatin structure in this region. No cutting after treatment with *MspI* was seen, and we notice that all *MspI* sites are located within or in very close proximity to the two regions showing periodic DNase I cutting. Restriction enzymes do not cut within positioned nucleosomes (Archer et al. 1991). Thus, the data obtained with restriction enzyme digestion are compatible with two positioned nucleosomes. A puzzling result that we have obtained is strand asymmetry with regard to DNase I cutting within the nucleosome-like particles. We offer no explanation for this feature; however, it has been seen previously for reconstituted nucleosomes (Simpson and Stafford 1983; Ramsay et al. 1984). In future work it will be of interest to examine a larger region to see whether the same or different nucleosome-like particles are present flanking the region analyzed. The LMPCR-aided procedures described here have the potential for such studies, because several sequence ladders can be produced simultaneously and then visualized sequentially. Eventually it may be possible to get information on the higher-order 30-nm fiber that may be present at least on the Xi. Current data are intriguing in this regard because most models for the 30-nm fiber suggest large reactivity differences between the inside and outside of the solenoid with respect to accessibility by a 31-kD enzyme. Such differences were not apparent in this study.

The maintenance of X chromosome inactivation and chromatin activity states

Available data on the chromatin structure of the PGK-1 promoter on the Xa and Xi can be summarized as fol-

lows: On the Xa, the whole CpG island is completely unmethylated at cytosines, does not contain positioned nucleosomes, and is covered by a number of transcription factors. On the Xi, the CpG island is methylated at almost every CpG, does not contain any transcription factors, and is wrapped around two positioned nucleosomes or nucleosome-like particles. These differences between sequence-identical promoters are stably maintained as a somatically heritable trait, because an Xi is replicated without activation. How is this differentiation maintained with high fidelity through countless cell divisions? Why does the promoter on the Xi never pick up transcription factors and become activated, and why is the active promoter immune to silencing?

The maintenance of gene expression on the Xa may be explained by a model in which transcription factors compete successfully with nucleosomes for binding to the unmethylated DNA immediately after replication (Svaren and Chalkley 1990). Once the region is occupied by these factors, nucleosomes as well as DNA methyltransferase would be excluded from this region (Pfeifer et al. 1990b). Exclusion of methyltransferase would aid stability by reducing de novo methylation and preventing de novo methylation mistakes from being propagated. With this model, the question becomes why do the transcription factors not bind to the Xi, even though they are obviously present in the same nucleus.

We suggest that one component of the answer is the temporal separation of replication; the Xi replicates in the second half of S phase (late S), whereas the Xa replicates in the first half of S phase (early S). Perhaps the higher-order chromatin structure that we have begun to analyze on the Xi can only form in the second half of S phase and, once formed, is stable until the next late S phase. This idea leads to an experimentally testable model for cell memory (Riggs 1990a).

Regardless of the detailed mechanism, key players for the maintenance of the inactive state are likely to be DNA methylation and nucleosome formation, as it has been shown that both cytosine methylation (Becker et al. 1987; Kovessi et al. 1987; Watt and Molloy 1988; Iguchi-Arigo and Schaffner 1989; Hermann et al. 1989; Comb et al. 1990) and nucleosomes (Piña et al. 1990; Wolffe 1990; Archer et al. 1991) interfere with the binding of transcription factors. However, DNA methylation does not prevent the binding of some factors. For example, Sp1 binding to the numerous GC-box sites in the PGK-1 region analyzed here should not be sensitive to methylation (Harrington et al. 1988; Höller et al. 1988). Definitive *in vitro* studies on the effect of methylation on factor binding to the sites identified in this study have not been done. One earlier *in vitro* study of PGK-1 (Yang et al. 1988), which showed no effect of methylation, illustrates the value of genomic sequencing. Both our *in vivo* (Pfeifer et al. 1990a) and *in situ* studies have confirmed the earlier *in vitro* footprinting and gel mobility-shift data. However, we have also found that the GC box at nucleotide -360, for which the best data were obtained by Yang et al. (1988), has *in vivo* footprints only in HeLa cells, the source of the nuclear extracts. Nucleo-

somes form on methylated DNA as they do on unmethylated DNA (Drew and McCall 1987), so there is no evidence supporting the idea that cytosine methylation by itself favors nucleosome formation. We cannot exclude the possibility that this may be different *in vivo*. Buschhausen et al. (1987) have shown that suppression of gene activity by methylation requires the formation of chromatin on injected DNA, and other studies support the notion that some sort of chromatin structure is required for methylation silencing (Cedar 1988).

Methylcytosine-binding proteins have been proposed to mediate the effect of methylation on chromatin formation (Wang et al. 1986; Meehan et al. 1989), but we have found no clear evidence for the presence of these proteins on the heavily methylated CpG island and promoter of the Xi, either by DMS or by DNase I footprinting. The methylated C-binding protein (MeCP) of Meehan et al. (1989) is thought to recognize methylation rather than nucleotide sequence, so weak and random binding of MeCP to this region of the Xi *in vivo* cannot be ruled out. Also, all results probably are consistent with the large 400- to 800-kD MeCP-containing complex (Meehan et al. 1989) being the particle(s) around which the Xi DNA is wrapped, and this might be an interesting hypothesis to guide future work.

Materials and methods

Cell lines

Chinese hamster-human hybrids containing either an active (cell line Y162-11C) or inactive human X chromosome (cell line X86T2) (Hansen et al. 1988) were kindly provided by R.S. Hansen and S.M. Gartler (University of Washington, Seattle). X86T2 cells were grown in RPMI 1640, medium, and Y162-11C cells were grown in the same medium supplemented with hypoxanthine, aminopterin, and thymidine (HAT).

Enzyme footprinting of permeabilized cells

For DNase I treatment, cells were grown as monolayers to ~80% confluency. Permeabilized cells (4×10^6) were prepared by treating monolayers with 0.05% lysolecithin (Sigma Chemical Co.) in solution 1 (150 mM sucrose, 80 mM KCl, 35 mM HEPES at pH 7.4, 5 mM K_2HPO_4 , 5 mM $MgCl_2$, 0.5 mM $CaCl_2$) for 1 min at 37°C (Miller et al. 1978). After removal of lysolecithin and washing with solution 1, the cells were treated with 25–50 µg/ml of DNase I (Boehringer Mannheim) in solution 2 (150 mM sucrose, 80 mM KCl, 35 mM HEPES at pH 7.4, 5 mM K_2HPO_4 , 5 mM $MgCl_2$, 2 mM $CaCl_2$) at room temperature for 5 min. The reaction was stopped, and cells were lysed by removal of the DNase I solution and the addition of 2.5 ml of stop solution (20 mM Tris-HCl at pH 8.0, 20 mM NaCl, 20 mM EDTA, 1% SDS, 600 µg/ml of proteinase K). The lysis mixture was diluted with 2.5 ml of 150 mM NaCl and 5 mM EDTA at pH 7.8, incubated for 3 hr at 37°C, phenol-chloroform-extracted, and ethanol-precipitated. RNA was removed by digestion with RNase A followed by phenol-chloroform extraction and ethanol precipitation.

To reduce interference by genomic 3' ends produced by DNase I, the ends were blocked by addition of a dideoxynucleotide (Tanguay et al. 1990). After DNase I treatment, DNA (10 µg in 50 µl) was denatured and incubated with 5 units of Se-

quenase 2.0 [U.S. Biochemical], 5 μ M ddNTPs in 40 mM Tris-HCl (pH 7.7), 25 mM NaCl, and 6.7 mM MgCl₂ for 20 min at 37°C. The DNA was then denatured again and incubated with 30 units of terminal transferase (BRL) at 37°C for 30 min in the same reaction mixture supplemented to 200 mM potassium cacodylate (pH 7.0), 1 mM 2-mercaptoethanol. After phenol-chloroform extraction, DNA fragments were precipitated at room temperature by addition of ammonium acetate to a final concentration of 2 M and then two volumes of ethanol. DNA amounts and cleavage frequencies were estimated by use of 1.6% alkaline agarose gels. DNase I cleavage of permeabilized cells gave a broad distribution of fragment sizes with an average fragment size of ~600 nucleotides for the experiments reported. No uncut, high-molecular-weight DNA was apparent, indicating that the majority of cells were rendered permeable. Between 1 and 3 μ g of DNA was used in the first primer-extension reaction of the LMPCR procedure [Pfeifer et al. 1989, 1990a].

Naked DNA controls, which are essential for interpreting the in situ or chromatin-derived data, were prepared by digestion of 40 μ g of purified DNA from Y162-11C cells or X86T2 cells with 0.8–1.6 μ g/ml of DNase I for 10 min at room temperature.

For micrococcal nuclease treatment, cells were grown and permeabilized as described for DNase I treatment. After permeabilization, the cells were treated with 20–50 units of MNase (Worthington Biochemicals) in 150 mM sucrose, 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 2 mM CaCl₂ for 5–10 min at room temperature. The reaction was stopped, cells were lysed, and DNA was prepared as described above for DNase I. Because MNase digestion of DNA does not produce 5' phosphate groups, the fragment ends were phosphorylated with T4 polynucleotide kinase. This was done by incubating 3 μ g of DNA in 60 mM Tris-HCl (pH 7.7), 10 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM EDTA, and 100 μ M ATP with 10 units of T4 polynucleotide kinase (New England Biolabs) for 60 min at 37°C. T4 polynucleotide kinase was inactivated at 70°C, and the samples were used directly in the Sequenase step of the LMPCR procedure (see below), taking care to keep the final buffer conditions standard.

Preparation and DNase I digestion of nuclei

Nuclei were prepared by treatment of cells with buffer A (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris-Cl at pH 8, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.25% NP-40) for 5 min at 0°C, followed by centrifugation at 1000g at 4°C. Other nuclear isolation conditions included buffer A without polyamines, buffer B (15 mM Tris-Cl at pH 7.4, 15 mM NaCl, 60 mM KCl, 0.5 mM dithiothreitol, 5 mM MgCl₂, 0.3% NP-40), and buffer B without NP-40. For DNase I digestion, nuclei were resuspended in 800 μ l of solution 2 containing 10 μ g/ml of DNase I and incubated at 37°C for 1 min. The reaction was stopped by addition of 800 μ l of stop solution, and DNA was isolated as described above.

KMnO₄ treatment of intact cells

Solutions of KMnO₄ (5–20 mM) in phosphate-buffered saline (PBS) were added to cell monolayers after removal of the medium and washing in PBS. The cells were treated with KMnO₄ for 2 min at 20°C, the solution was removed, and the reaction was quenched by adding 1 M 2-mercaptoethanol in 150 mM NaCl, 5 mM EDTA. One volume of stop solution was added, and purification was done as for permeabilized cells. After purification, the DNA was broken at the sites of oxidized bases by heating in 1 M piperidine for 30 min at 90°C. Naked DNA was treated with KMnO₄ under the same conditions as described for

cells, and DNA was precipitated and then cleaved with piperidine. After piperidine treatment, the DNA was ethanol-precipitated and dried overnight in a SpeedVac concentrator to remove all traces of piperidine. The piperidine-cleaved fragments were used directly for LMPCR.

LMPCR

The oligonucleotides used for LMPCR are shown schematically in Figure 1, and the sequences have been published [Pfeifer et al. 1990a]. A detailed protocol for the entire procedure, which has been described earlier [Mueller and Wold 1989; Pfeifer et al. 1989, 1990a], is available on request. Primer 1 of each set was used for primer extension by Sequenase, and primer 2 was used for PCR amplification by *Taq* polymerase for 19 cycles. Amplified DNA fragments were separated on sequencing gels, and the sequences were visualized by autoradiography after electroblotting to nylon membranes and hybridization with a single-stranded probe [Pfeifer et al. 1989]. We used electroblotting and hybridization instead of directly extending a ³²P-labeled primer followed by gel electrophoresis. Hybridization introduces an additional level of specificity if a probe is used that does not overlap the amplification primer sequences. We have also found that longer single-stranded probes provide a stronger, clearer signal than end-labeled oligonucleotides. Single-stranded probes can be made easily by repeated run-off synthesis from any cloned template using a single primer and *Taq* polymerase [Stürzl and Roth 1990].

Primer sets A and C, G and E, or D and F were combined for simultaneous primer extension and amplification in the same reaction. The two sequence ladders were then visualized separately by hybridization with a specific probe, stripping, and rehybridization of the nylon membrane with a second probe [Pfeifer et al. 1989].

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G P Pfeifer and A D Riggs

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