Expression of the G6PD locus on the human X chromosome is associated with demethylation of three CpG islands within 100 kb of DNA

Daniela Toniolo[1,3], Giuseppe Martini[1], Barbara R.Migeon[2] and Rosanna Dono[1]

[1]Istituto Internazionale di Genetica e Biofisica, CNR, Naples, Italy and [2]Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD, USA

[3]Present address: Istituto di Genetica Biochimica ed Evoluzionistica, CNR, Via Abbiategrasso 207, 27100 Pavia, Italy

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We have previously reported that expression of the G6PD locus is correlated with the methylation status of two islands of CpG dinucleotides which are 3' to the locus and in the 5' region of two adjacent genes of unknown function, P3 and GdX. We have now examined the methylation of a third CpG island in the promoter region of the G6PD gene itself in DNA from males, females and reactivants that express G6PD on the inactive X chromosome. Our results show that expression of the G6PD gene is associated with concordant demethylation of all three CpG islands in this 100-kb region of DNA.
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Introduction

Dosage compensation of X-linked genes in mammals is achieved through the inactivation of one of the two X chromosomes in each female cell (Lyon, 1972). The inactivation event is random and only one chromosome remains active in the somatic cells of the adult (reviewed in Gartler and Riggs, 1983). Once established, X inactivation is clonally inherited and is stably maintained through cell division with the exception of the trophoectoderm where alleles on the inactive X are frequently expressed (Kratzer et al., 1983; Migeon et al., 1985). Little is known of the mechanism(s) that initiate X chromosome inactivation, but DNA methylation has a role in the maintenance of X chromosome inactivation, through cell division. DNA from the inactive chromosome cannot complement an HPRT mutation in DNA-mediated gene transfer experiments, suggesting that modification of the DNA alone is sufficient to silence the HPRT gene (Liskay and Evans, 1980). Treatment with cytosine analogs that inhibit DNA methylation results in reactivation of genes on the inactive X chromosome. The DNA isolated from the reactivants is rendered capable of complementing an HPRT mutation after DNA-mediated cell transformation (Venolia et al., 1982).

Although active and inactive X chromosomes are not differentially methylated along the entire chromosome (Wolf and Migeon, 1982; Lindsay et al., 1985; Ruta Cullen et al., 1986), the patterns of methylation do differ from some loci on the active and the inactive X. Furthermore, some of the differential methylation is correlated with gene expression.

Specifically, differential methylation patterns have been observed at the HPRT locus of humans (Wolf et al., 1984a; Yen et al., 1984) and mouse (Lock et al., 1986) and at the human PGK locus (Riggs et al., 1985).

We have previously reported that expression of the G6PD locus is correlated with the methylation status of two CpG islands located 3' to the G6PD locus on the human X chromosome; these islands were methylated on the inactive X but unmethylated on the active X (Toniolo et al., 1984) and demethylated when the locus on the inactive X is re-expressed (Wolf et al., 1984b). The elucidation of the structure of the G6PD gene and subsequent studies have shown that these CpG islands are 40 kb downstream from the 3' end of the G6PD gene (Martini et al., 1986), and each is at the 5' end of neighboring genes. These genes (GdX and P3) are transcribed in many tissues (Toniolo et al., 1988; M.Alcalay and D.Toniolo, in preparation). Using cloned DNA from the human G6PD locus, we have found yet another CpG island in the 5' region of this gene. Examination of the methylation of this CpG cluster and of the two others on active and inactive X chromosomes and in reactivants that express G6PD on the inactive X shows that funcitonal methylation at the G6PD locus resides within the CpG islands. Furthermore, our observations indicate that re-expression of the G6PD gene is associated with demethylation of all three CpG islands.

Results

The G6PD gene is 18 kb long and consists of 13 exons (Martini et al., 1986). We obtained a complete map of the HpaII sites and a partial map of the HhaI sites of the G6PD locus from the nucleotide sequence and from fine restriction mapping of regions that had not been sequenced (Figure 1). This analysis identified 59 HpaII sites in 28 kb of DNA. In the 5' region of the gene, spanning 1 kb upstream from the main mRNA initiation point to the first intron, we found a cluster of CpG dinucleotides containing 21 HpaII sites (H5 – H24) and 18 HhaI sites (Hh1 – Hh18). The sequence in this region shows that this island fits the definition of Bird (1986) as the C + G content is >65% and the CpGs equal the GpCs. Despite the high C + G content of the G6PD gene along its total length (Persico et al., 1986), very few HpaII or HhaI sites were found in the large second intron, and no other cluster of CpG was found in the coding region, in the 3' end of the gene or in the 3' flanking DNA.

The G6PD CpG island is unmethylated on the active X

Filters containing single and double digests of male DNA prepared from leukocytes were hybridized with the single copy probes shown in Figure 1. Figure 2A shows HpaII – BamHI digests of DNAs hybridized with probes for the 5' region of the gene (pTb4-21 and pTb4-24). Digests of male DNAs (XY) hybridized with pTb4-21 show only one band of ~0.6 kb, which corresponds to the digestion
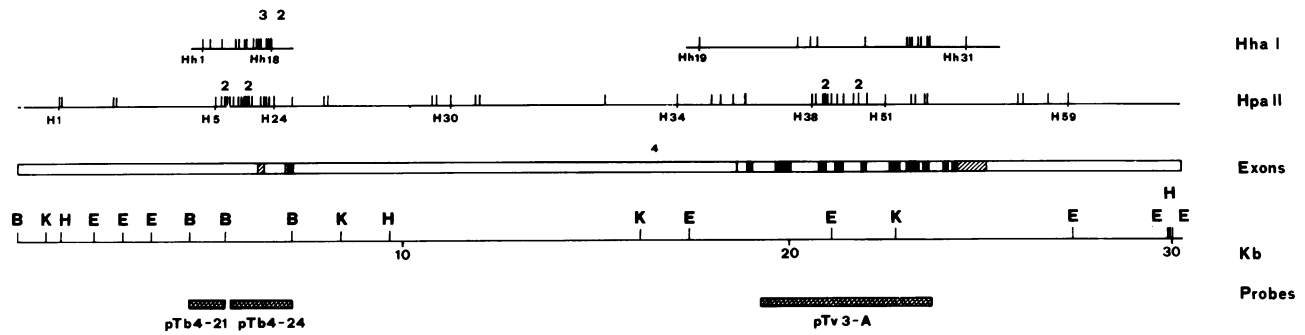
Fig. 1. Map of the *Hpa*II and *Hha*I sites of the human Gd gene and flanking regions. Probes are shown as cross-etched segments. Coding regions are black and etched segments are non-coding. A restriction map of the *Bam*HI (B), *Kpn*I (K), *Hin*dIII (H) and *Eco*RI (E) site is also shown. Vertical bars indicate the positions of the *Hpa*II or *Hha*I sites.
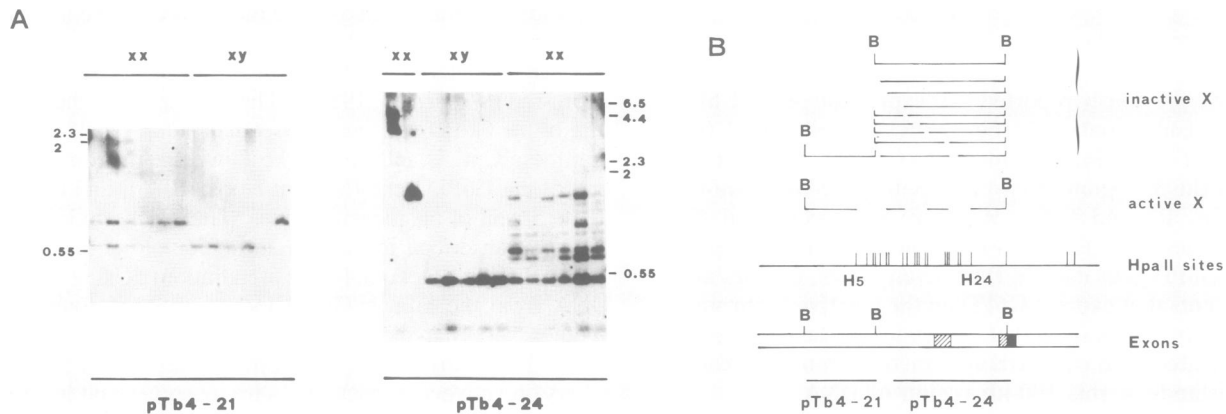
Fig. 2. Panel A: Southern blots of male (XY) and female (XX) leukocyte DNAs hybridized with 5′ probes of the G6PD gene. All digestions are *Hpa*II−*Bam*HI except the male in **lane 12**, which was digested only with *Bam*HI, and the females in **lanes 13** and **14**, digested with *Hpa*II and *Bam*HI respectively. Probes are indicated at the bottom. **Panel B**: Schematic representation of the restriction fragments resulting from the digestions of active and inactive X chromosomes shown in panel A. Below is an enlargement of the *Hpa*II restriction map at the 5′ end of G6PD of Figure 1.

of the *Bam*HI site and of the *Hpa*II site H5. In the hybridization of the same filter with the probe pTb4-24, two fragments can be seen in male DNAs (XY): the larger band of 0.5 kb is the *Hpa*II−*Bam*HI fragment corresponding to digestion of the *Hpa*II site H24, the smaller band(s) come from cleavage of the *Hpa*II sites H11, H12, H18 and H19 in the cluster. A schematic representation of the fragments resulting from the digestions shown in Figure 2A is in Figure 2B, with an enlargement of the restriction map of the 5′ region of the gene. In summary, in these and other experiments (not shown) we observed four unmethylated *Hpa*II sites on the active X chromosome (only three sites, H5, H24 and H28, are shown in Figure 1), resulting in an 8-kb (from an *Hpa*II site outside the map to H5) and a 4-kb *Hpa*II fragment (H24−H28). Two of the unmethylated sites (site H5 and H24) border the CpG island described above. The analysis of the *Hpa*II cluster is difficult, because the sites are very close together. However, the *Hpa*II sites in the cluster, which upon digestion produce fragments detectable by Southern blotting, are predominantly unmethylated. The size of the other fragments produced by *Hpa*II digestion of that region is below the threshold of detection by Southern blotting.

The G6PD CpG island is methylated on the inactive X

DNA samples from leukocytes of several females were run side by side with male DNA: in blots probed with pTb4-21 or pTb4-24 we observed all the restriction fragments seen
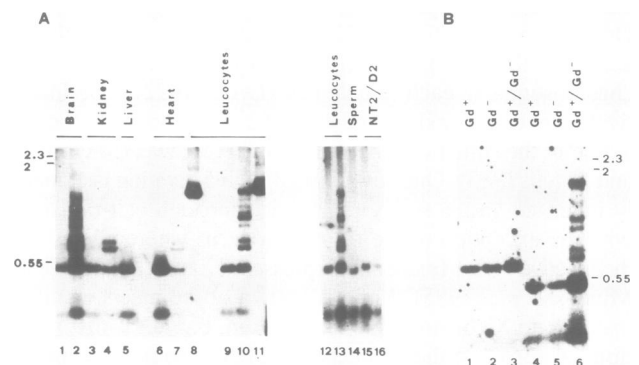
Fig. 3. Panel A: Southern blot of male and female DNAs from various tissues, hybridized to probe pTb4-24. NT2/D2 is a human male teratocarcinoma cell line, before (lane 15) and after (lane 16) retinoic acid-induced differentiation. Digestions are *Hpa*II/*Bam*HI in all lanes except lanes 8 and 11 (only *Bam*HI); lanes 1, 3, 5, 6, 8, 9, 12, 14, 15, 16 correspond to male DNA; the rest to female DNA. **Panel B**: Southern blot of DNA from a female heterozygous for G6PD deficiency (lanes 3 and 6), her two sons (lanes 1, 2, 4 and 5). Digestions are: lanes 1−3, *Hha*I−*Bam*HI; lanes 4−6, *Hpa*II−*Bam*HI. The probe is pTb4-24.

in males. However, we also observed larger fragments, indicating that sites H5 and H24 can be methylated in females. Examples are the *Hpa*II−*Bam*HI digests of female DNA (XX) in Figure 2A. The relative intensity of these female-specific fragments compared to those that are common to
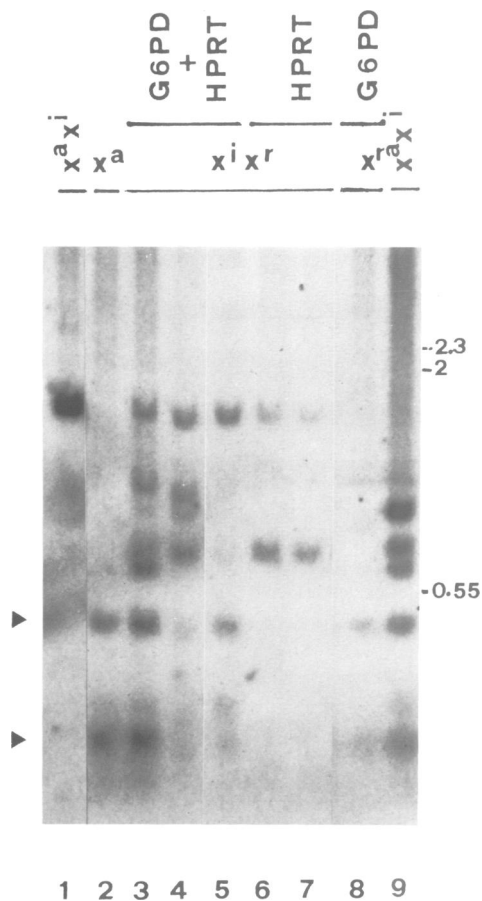
Fig. 4. Southern blots of DNAs from males (x^a), females ($x^a x^i$) and reactivants ($x^i x^r$ or x^r) probed with the G6PD probe pTB4-24. Digestions: lane 1, *Bam*HI; lanes 2−9, *Hpa*II−*Bam*HI. DNAs are from: female (lanes 1 and 9) and male (lane 2) leukocytes; mouse−human hybrids expressing human G6PD as well as HPRT (lanes 3−5); mouse−human hybrid expressing human HPRT and PGK, but not G6PD from the inactive X chromosome (lane 6); mouse−human hybrid with reactivated human HPRT locus, but not G6PD (lane 7); mouse−human hybrid derived from clone 81 (lane 8).

both sexes is consistent with the hypothesis that larger fragments come from the inactive X, and represent extensive methylation of the *Hpa*II sites within the island. The variety of larger fragments indicates that methylation of the *Hpa*II sites in the island on the inactive X is rather heterogeneous. Evidence that these differences are not due to a common polymorphism stems from studies of a female, heterozygous for G6PD deficiency (Gd+/Gd−), and her two sons, one of them Gd+, the other Gd−. Whereas the mother's DNA shows the female pattern we have just described, the DNAs from the two sons carrying the two different G6PD alleles yield the same male pattern (Figure 3B, lanes 4−6).

Analysis of the *Hha*I sites gave similar results. *Hha*I site Hh1, at the 5' border of the island (Figure 1), is unmethylated in male DNA, and methylated in ~50% of female DNA (not shown). At the 3' border of the island there are seven *Hha*I sites in <100 bp of DNA and it is impossible to determine the methylation status of each site or to detect any change in methylation of just one or a few of them (Figure 3B, lanes 1−3).

The pattern of methylation of the G6PD gene in leukocytes

was also found in cultured cells and other tissues (Figure 3A), including human sperm and a male teratocarcinoma cell line before and after differentiation.
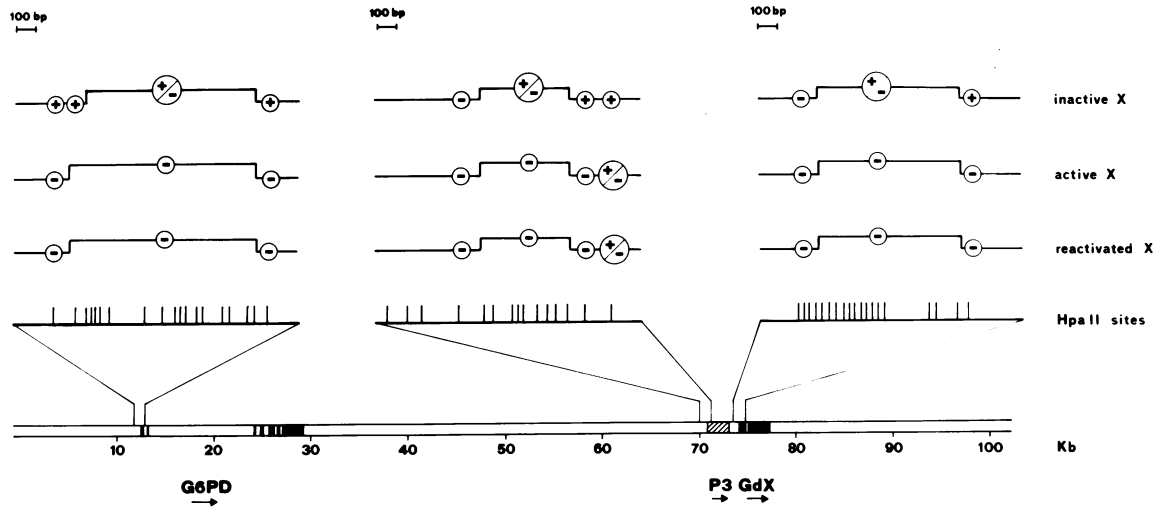
Methylation of the body of the gene

The same analysis described above was performed with a third probe, pTV-3A, which corresponds to most of the G6PD coding exons and the 3' end (Figure 1). This probe hybridizes to a major 8-kb *Hpa*II fragment and two *Hha*II fragments of 8 kb and >10 kb (data not shown). The probe also hybridizes to several minor and smaller bands. We observed no differences in methylation of *Hha*I and *Hpa*II sites in male and female DNAs.

Methylation of the G6PD gene in reactivants

To assess the functional importance of methylation at the G6PD locus, we analyzed G6PD alleles on inactive X that had been reactivated. Among the reactivants analyzed, two were mouse−human hybrids derived from clone 81, a normal diploid clone of human fibroblasts with spontaneous reactivation of the G6PD locus (Migeon *et al.*, 1982). A second group of reactivants analyzed consisted of mouse−human hybrids retaining only the inactive human X chromosome, which after treatment with 5-azacytidine (5-AzaC) expressed human G6PD as well as human HPRT: in these hybrids the inactive X is an isodicentric X chromosome with two copies of the G6PD gene and it was not known *a priori* if one or both loci were reactivated (Wolf *et al.*, 1984a,b). From this group we analyzed five independent clones, and subclones cultured for different lengths of time. Finally we also examined seven independent reactivants derived from mouse−human hybrids treated with 5-AzaC, which expressed HPRT: two of these reactivants expressed PGK but none expressed G6PD (Wolf *et al.*, 1984a,b; Wolf and Migeon, 1985).

Figure 4 shows the analysis of some of the reactivants, using the G6PD probe pTb4-24. None of the hybrids that re-expressed HPRT but not G6PD (two are in lanes 6 and 7 in Figure 4) demethylated site H24, or sites H11, H12, H18 and H19 within the G6PD 5' cluster, although relevant demethylation had occurred at the HPRT locus (Wolf *et al.*, 1984a; Wolf and Migeon, 1985). In the *Hpa*II−*Bam*HI digests in Figure 4 this is shown by the lack of the 0.5-kb and 0.1-kb bands, characteristic of active X digests (one is in lane 2). The hybrids derived from reactivant clone 81 (one example is given in lane 8) had the same pattern of *Hpa*II fragments as the active genes: the presence of the 0.5-kb *Hpa*II−*Bam*HI band indicates that site H24 is unmethylated. It is also likely that many *Hpa*II sites within the cluster on the reactivated X are unmethylated because in this hybrid, which only has the reactivated X, we see the small fragments that correspond to cleavage of sites H11, H12, H18 and H19 (bands around 0.1 kb in Figure 4). In all the hybrids where expression of the G6PD locus on the inactive isodicentric X had been induced by 5-AzaC (examples are in Figure 4, lanes 3−6) we observed demethylation within the cluster. The pattern is complicated, because it seems that only one of the two G6PD loci on the isodicentric X has been reactivated; however, bands corresponding to demethylation of site H24 (0.5-kb *Hpa*II−*Bam*HI band) and of sites H11, H12, H18 and H19 (0.1-kb *Hpa*II bands) within the cluster are detectable.

Fig. 5. Schematic representation of methylation and demethylation of *Hpa*II sites at the three CpG clusters of the G6PD region. Arrows below each gene indicate the direction of transcription: Blacked areas are exons, the etched area corresponds to the P3 gene and it shows that the gene structure has not been fully determined. (+) sign above *Hpa*II sites indicates methylation, (−) sign indicates demethylation.

Coordinate demethylation of CpG clusters in the 100-kb region

We re-examined the methylation status of the CpG clusters 3′ to the G6PD gene (Toniolo *et al.*, 1984) in the same DNA from the reactivant clones and subclones analyzed with probes for the 5′ end of the G6PD gene. Probe pGd3 was used for the cluster at the 5′ end of the P3 gene, and probe pGd1.4 for the 5′ end of the GdX gene (Toniolo *et al.*, 1984). As in the previous analysis, in DNAs from hybrids with at least one active G6PD gene both clusters are unmethylated, whereas they remain methylated in DNAs from hybrids where the G6PD remains inactive (data not shown). Figure 5 summarizes the methylation status of these sites in these three clusters on inactive, active and reactivated X chromosomes.

Non-functional methylation in the body of G6PD

In contrast to the situation in the region of the clustered CpGs, all 5-AzaC reactivants, irrespective of whether the locus was expressed, were highly under-methylated in the body of the G6PD gene: no differences were detected between reactivants that re-expressed only HPRT or both HPRT and G6PD (data not shown).

Methylation outside the G6PD gene

Using four single-copy DNA fragments (M.D'Urso *et al.*, in preparation) to probe the regions surrounding the G6PD, P3 and GdX genes, we compared male and female DNA digested with *Hpa*II and *Hha*I. With the probes used we were able to study methylation of a region of ~90 kb, corresponding to the whole DNA cloned, with the exception of a region of 10−15 kb immediately downstream from the G6PD gene. No differences in methylation were found with any of the four probes (data not shown).

Search for CpG clusters

To determine whether additional clusters were present in the 100 kb of DNA we have cloned, we digested all the genomic subclones in this region (Figure 6A) with the enzyme *Hpa*II to look for the large number of tiny fragments expected from CpG clusters. Most of the DNA analyzed had no *Hpa*II fragments smaller than 45 bp (Figure 6B, lanes 1, 4, 7, 8,

A

B
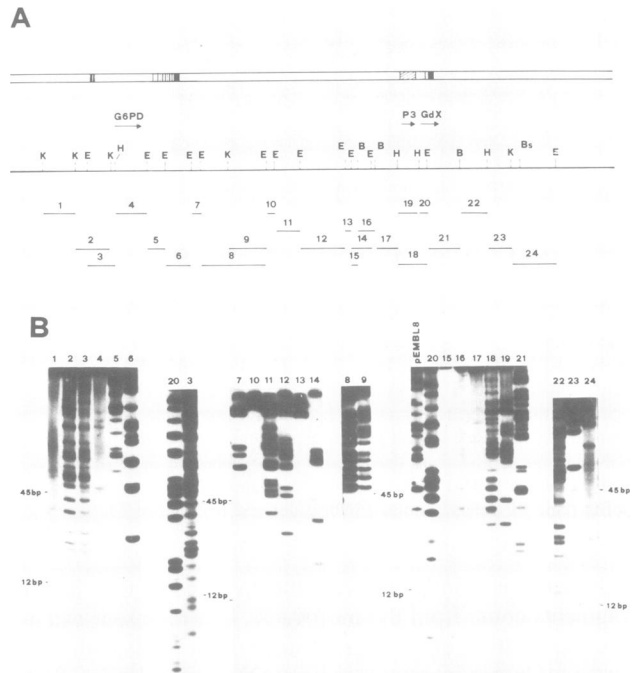
Fig. 6. Search for CpG clusters. **Panel A**: a restriction map of the region. The genomic DNA fragments analyzed are numbered from 1 to 24 and shown below the map. Restriction enzyme symbols are as in Figure 1; Bs = *Bst*EII. **Panel B**: the *Hpa*II fragments derived from the genomic DNA subclones shown in panel A. The genomic DNA fragments (numbered above the lanes) were digested with *Hpa*II, end-labelled as described in Materials and methods, fractionated in 20% acrylamide gels in TEB and autoradiographed. Molecular size markers are: 45 bp, xylene cyanol dye; 12 bp, bromophenol blue dye.

9, 10, 13, 15, 23, 24). Some had between one and three fragments in this same range (Figure 6B, lanes 5, 6, 11, 12, 14, 16, 17) and had a digestion pattern similar to the plasmid pEMBL8, used as a control. Fragments corresponding to the CpG clusters at the 5′ region of G6PD, P3 and GdX genes had more than three fragments smaller than 45 bp (lanes 2, 3, 20, 18, 19, 21). No other region gave a similar digestion pattern, with the exception of a subclone (Figure 6B, lane 22) from a region 20 kb downstream from

the GdX gene. More detailed analysis by partial digestion mapping confirmed the clustered *Hpa*II sites in this region, but showed the absence of clustered *Hha*I sites (data not shown). We consider this cluster of *Hpa*II sites an 'atypical' CpG cluster, because of the lack of *Hha*I sites. This region appears fully methylated in leukocyte DNA form both sexes (data not shown).

Discussion

The methylation patterns which we observed at the G6PD locus using methylation-sensitive restriction enzymes show that only in the region of clustered CpG dinucleotides is differential methylation correlated with gene expression; methylation in this region is associated with inactivity of the locus. Although silence of the locus is associated with extensive methylation of the island, some sites within the inactive cluster may be unmethylated and the status varies from chromosome to chromosome. On the other hand, most sites within the 5' G6PD island need to be unmethylated for the locus to be expressed.

Comparing active and inactive G6PD alleles in different tissues allowed us to discriminate between tissue-specific variations and functional differences in methylation of the locus. Further evidence that demethylation of the island is related to function of the locus comes from studies of the reactivants. Re-expression of the G6PD locus on the inactive X is always associated with demethylation of the island. In contrast, the body of the gene was extensively demethylated in AzaC-treated G6PD reactivants as well as in reactivants where only the HPRT gene had been reactivated. Furthermore, our inability to identify differences in methylation of non-transcribed sequences in this region of the X chromosome confirms previous observations, using random probes (Lindsay *et al.*, 1985; Wolf and Migeon, 1982) or tissue-specific genes (Ruta Cullen *et al.*, 1986), that isolated CpGs outside the islands are not differentially methylated.

Reactivation of G6PD is associated with demethylation of regional CpG clusters

In this and in a previous paper (Toniolo *et al.*, 1984) we have characterized three CpG islands within 100 kb of DNA in the region of the G6PD locus (Figure 5): each island is in the 5' region of a gene that is costitutively expressed (Toniolo *et al.*, 1988; M.Alcalay and D.Toniolo, in preparation). As we were unable to identify additional *bona fide* CpG islands in this region, it seems that also CpG islands on the X chromosome are predominantly located at the 5' end of genes.

Our analysis of reactivants shows that the demethylation event(s) associated with re-expression of the G6PD locus affects not only the CpG island in the 5' region of the G6PD gene, but also the CpG islands at neighboring P3 and GdX loci. We have previously reported that reactivation of the G6PD locus is highly correlated with demethylation of P3 and GdX islands (Toniolo *et al.*, 1984; Wolf *et al.*, 1984b) Now we show that this is true for the G6PD island as well. Whereas all three islands remain fully methylated in all of the seven independent reactivants that do not express G6PD, their demethylation is strictly associated with the acquisition of G6PD activity (Figures 4 and 5). The coordinate methylation status of the three CpG islands in the region of the G6PD locus and the correlation with the function of the G6PD locus provides support for the hypothesis that X

chromosome inactivation operates on a regional basis rather than locus by locus (Riggs *et al.*, 1985).

The lack of CpG islands within the factor IX locus and of differential methylation between active and inactive factor IX loci recently reported by Ruta Cullen *et al.* (1986) not only suggest that functional methylation is limited to CpG islands, but also lend support to the notion of regional control. The absence of detectable differences in methylation to explain the differential expression of the locus led these investigators to propose that maintenance of the inactive state for this tissue-specific gene was achieved by differential methylation outside the locus, presumably mediated by a CpG island in the vicinity of the gene: the fact that CpG islands occur frequently in the genome, perhaps as often as every 100–200 kb makes them ideal mediators of regional control. As suggested by Riggs *et al.* (1985), these clustered CpGs, although not specific to the X chromosome, may have taken on a special function with respect to maintaining the inactivity of X chromosome genes. Recent studies of Kaslow and Migeon (1987) of the 5' CpG island in the marsupial G6PD gene provide evidence that methylation of CpG islands contributes stability to the complex process of inactivating the locus.

A better understanding of the extent to which the CpG clusters are methylated and of their distribution along the X chromosome should help to clarify if their role in maintaining inactivity is limited to housekeeping genes.

Materials and methods

Cells
The cells used for these studies were mouse–human hybrids in which loci on the human inactive X chromosome had been reactivated, either spontaneously or induced by 5-AzaC (see text for specific details).

Probes
The DNA probes used in these studies were subclones of genomic DNA in pUC18 or pEMBL8: the probes corresponding to the G6PD genes are shown in Figure 1; the probes corresponding to the P3 and GdX gene are pGd3 and pGd1.4, respectively, and have been described in Toniolo *et al.* (1984).

Mapping of the HpaII and HhaI sites
Most the *Hpa*II and *Hha*I sites were identified from the nucleotide sequence (Martini *et al.*, 1986; Persico *et al.*, 1986). The remaining sites were mapped by partial digestion (Maniatis *et al.*, 1982). In brief, appropriate DNA fragments were terminally labelled with [γ-^{32}P]ATP and polynucleotide kinase, and digested to completion with a first restriction enzyme. After gel purification and electroelution the resulting fragments were partially digested with the enzymes *Hpa*II and *Hha*I (0.1 U of enzyme/μg of DNA for 1'–15'). The digestion products were fractionated on 5% polyacrylamide gels in TEB buffer and the gels were exposed to Kodak SO282 radiographic films.

Southern blot analysis
Human blood leukocytes were prepared from freshly drawn blood as previously described (Toniolo *et al.*, 1984). Samples of DNA from human tissues were provided by Dr G.Battistuzzi (Battistuzzi *et al.*, 1985) and Dr S.Metafora. DNA extraction from human leukocytes, human tissues and tissue culture cells was performed as described by Toniolo *et al.* (1984). Digestions with restriction enzymes were carried out under the conditions recommended by the supplier. DNA was fractionated on agarose gel (1% or 0.8% agarose) in TEB buffer (Maniatis *et al.*, 1982). Blotting was on nylon filters (AMF CUNO), and filters were hybridized at 65°C for 20–24 h in 5 × SSPE, 2 × Denhardt's, 1% SDS, 100 μg/ml herring sperm DNA with nick-translated probes and washed at 65°C in 2 × SSC and 0.2 × SSC.

Analysis of CpG clusters
The genomic subclones were digested with the appropriate restriction enzyme, fragmented in agarose or acrylamide gels and the DNA insert was

prepared by electroelution (Maniatis *et al.*, 1982). About 10 pmol of each fragment was digested with *Hpa*II or *Hha*I, dephosphorylated and labeled with [γ-^{32}P]ATP by polynucleotidekinase (Maniatis *et al.*, 1982). Fragments were fractionated in 20% polyacrylamide gels in TEB buffer. Gels were exposed overnight to radiographic films (Kodak SO282).

Acknowledgements

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