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Genetics

Differential methylation of hypoxanthine phosphoribosyltransferase genes on active and inactive human X chromosomes

(X inactivation/5-azacytidine/mouse–human hybrid cell/Southern blotting)

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ABSTRACT **Previous theoretical considerations and some experimental data have suggested a role for DNA methylation in the maintenance of mammalian X chromosome inactivation. The isolation of specific X-encoded sequences makes it possible to investigate this hypothesis directly. We have used cloned fragments of the human hypoxanthine phosphoribosyltransferase (*HPRT*) gene and methylation-sensitive restriction enzymes to study methylation patterns in genomic DNA of individuals with different numbers of X chromosomes and in somatic cell hybrid lines containing human X chromosomes that are either active or inactive or have been reactivated by treatment with 5-azacytidine. The results of these analyses show that there is hypomethylation of active X chromosomes relative to inactive X chromosomes in the 5′ region of this gene. In the middle region of the gene, however, a site that is consistently undermethylated on inactive X chromosomes was identified. Taken together, the data suggest that the overall pattern of methylation, rather than methylation of specific sites, plays a role in the maintenance of X chromosome inactivation.**

Early in mammalian embryogenesis, one of the two X chromosomes in all female somatic cells is functionally inactivated (1). This leads to heterochromatinization and late replication of the inactive chromosome and failure of detectable expression of X-encoded alleles at most, but not all, loci on the inactive X. X inactivation is a random process in which either the maternally or paternally derived X can be inactivated; however, once established, X inactivation is genetically fixed for any given cell and its progeny (2).

The molecular mechanism resulting in this highly heritable alteration of gene expression is not known, although there is evidence that direct modification of DNA molecules is involved. A number of years ago, it was proposed that methylation of specific cytosine residues could account for alterations in the pattern of gene expression that could be transmitted to cellular progeny faithfully (3, 4). Riggs (5) suggested that such DNA methylation could theoretically account for many of the biological observations regarding X chromosome inactivation, but no direct evidence was available to support this concept. Recently, however, the technique of DNA-mediated gene transfer has been used to show that a hypoxanthine phosphoribosyltransferase (*HPRT*) gene on an active X chromosome, but not one on an inactive X, is competent in transferring the $HPRT^+$ phenotype to $HPRT^-$ recipient cells (6, 7). This functional distinction between active and inactive X chromosomes is apparently true for cultured cells and somatic tissues but not for yolk sac endoderm derived from mouse embryos (8). Also, it has been shown that the potent methylation inhibitor, 5-azacytidine, can reactivate *HPRT* and other genes on an inactive human X chromosome in somatic cell hybrids (9, 10). After 5-azacytidine-induced reactivation, previously incompetent *HPRT* genes regain their ability to function in a gene transfer assay (10, 11). All of the above observations support the role of methylation of cytosine residues (the only known base modification of mammalian DNA) in the control, or more accurately, in the maintenance of X chromosome inactivation in female somatic cells. It is likely that other factors also play a role in the initiation and, perhaps, in the persistence of this process as well.

We now report the use of several cloned human *HPRT* probes to directly investigate methylation patterns at this locus. Using restriction enzymes that are sensitive to methylation at their recognition sites and DNA containing *HPRT* sequences in various states of activity, we are able to demonstrate that there is a difference in the level of methylation around the *HPRT* gene on active and inactive human X chromosomes.

MATERIALS AND METHOD

Cell Lines. The cell lines used were originally derived from the fusion of human fibroblasts with $HPRT^-$ mouse A9 cells. Both 25-3 and 25-8 contain an active human X and 26RD has an inactive X chromosome (12, 13). 26RD-1e was derived from 26RD after spontaneous reactivation of human *HPRT*. All the rest were isolated after 5-azacytidine treatment of 26RD or another cell line with an inactive X (9, 12, 14). In T-3 and T-9, none of the known enzyme gene loci from the human X chromosome were reactivated. 26RD-1a, -1b, and -1c; III-a-24; IV-22; IV-33; 2BR-2; and 2BR-10 were selected for reactivation of human *HPRT*. In addition to HPRT, 26RD-1c expressed human glucose-6-phosphate dehydrogenase; 26RD-1b, III-a-24, and IV-33 expressed human α-galactosidase A; and IV-22 expressed both. 2BR-2 and 2BR-10 retained multiple copies of the X chromosome per cell (average 3.5), suggesting partial reactivation of the human *HPRT* gene (10).

DNA Isolation and Southern Blot Analysis. High molecular weight DNA was isolated from human placenta, sperm, leukocytes, and tissue culture cells according to published procedures (15–18). Restriction enzyme digestion was carried out for 6–18 hr in a volume of 50 μl containing 10 μg of DNA and 20–30 units of enzyme. Completeness of digestion was monitored by using marker DNA coincubated with the samples. Southern blotting and hybridization were carried out by standard methods (19, 20) except that, when probe I was used, heat-denatured human repetitive DNA ($C_0t \leq 40$) at 40 μg/ml was added to the hybridization mixture.

RESULTS

Methylation Near the First *HPRT* Exon. *HPRT* genomic clones have been isolated from several human libraries by

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; kb, kilobase(s); bp, base pair(s).

using human and mouse *HPRT* cDNA probes (unpublished observations). Preliminary data suggest that, like the mouse HPRT gene (21), the human gene has nine exons and spans more than 30 kilobases (kb). The structure of the region around the first exon, which consists of at least 90 base pairs (bp) of 5′ untranslated sequence plus coding information for the first nine amino acids of mature HPRT, is shown in Fig. 1*A*. This region was contained in an originally isolated λ clone λ4X5, which was later subcloned in pUC8 to give clones pλ4X5-BR1.8 and pλ4X5-BP1.6 (Fig. 1*A*). Probe I was a 0.5-kb *Ava* II/*Msp* I fragment of pλ4X5-BR1.8 that was relatively free of repetitive sequences. Probes II, III, and IV were a 0.6-kb *Msp* I fragment, a 0.7-kb *Msp* I/*Xho* I fragment, and a 1.1-kb *Ava* I fragment of pλ4X5-BP1.6, respectively, and are derived from the first intron. They contain only single-copy sequences. All four of these probes failed to hybridize with mouse DNA. To study the methylation level around exon I, four methylation-sensitive restriction enzymes, *Hpa* II (C-C-G-G), *Hha* I (G-C-G-C), *Ava* I (C-Py-C-G-Pu-G), and *Sma* I (C-C-C-G-G-G) were used. All of these enzymes will not cut their respective sites when the internal cytidine is methylated (22). *Msp* I recognizes the same sequence as *Hpa* II but its activity is not affected by methylation of the internal cytidine. There are relatively frequent *Msp* I and *Hha* I sites within exon I and the sequences immediately 5′ to it. These have been numbered as indicated in Fig. 1*A*.

When cellular DNA was digested with *Hpa* II/*Pst* I and hybridized with probe I (Fig. 2*A*), an unmethylated *Msp* I site (M-1) gave a 0.67-kb fragment. The methylation status of site M-2 could be studied only on those molecules on which M-1 was methylated, and the status of site M-3 could be assessed only on those molecules on which M-1 and M-2 were methylated. When M-1 was methylated and M-2 was not, a 0.88-kb fragment resulted. A 1.15-kb fragment was derived from sites M-3 or M-4, which were not methylated. These

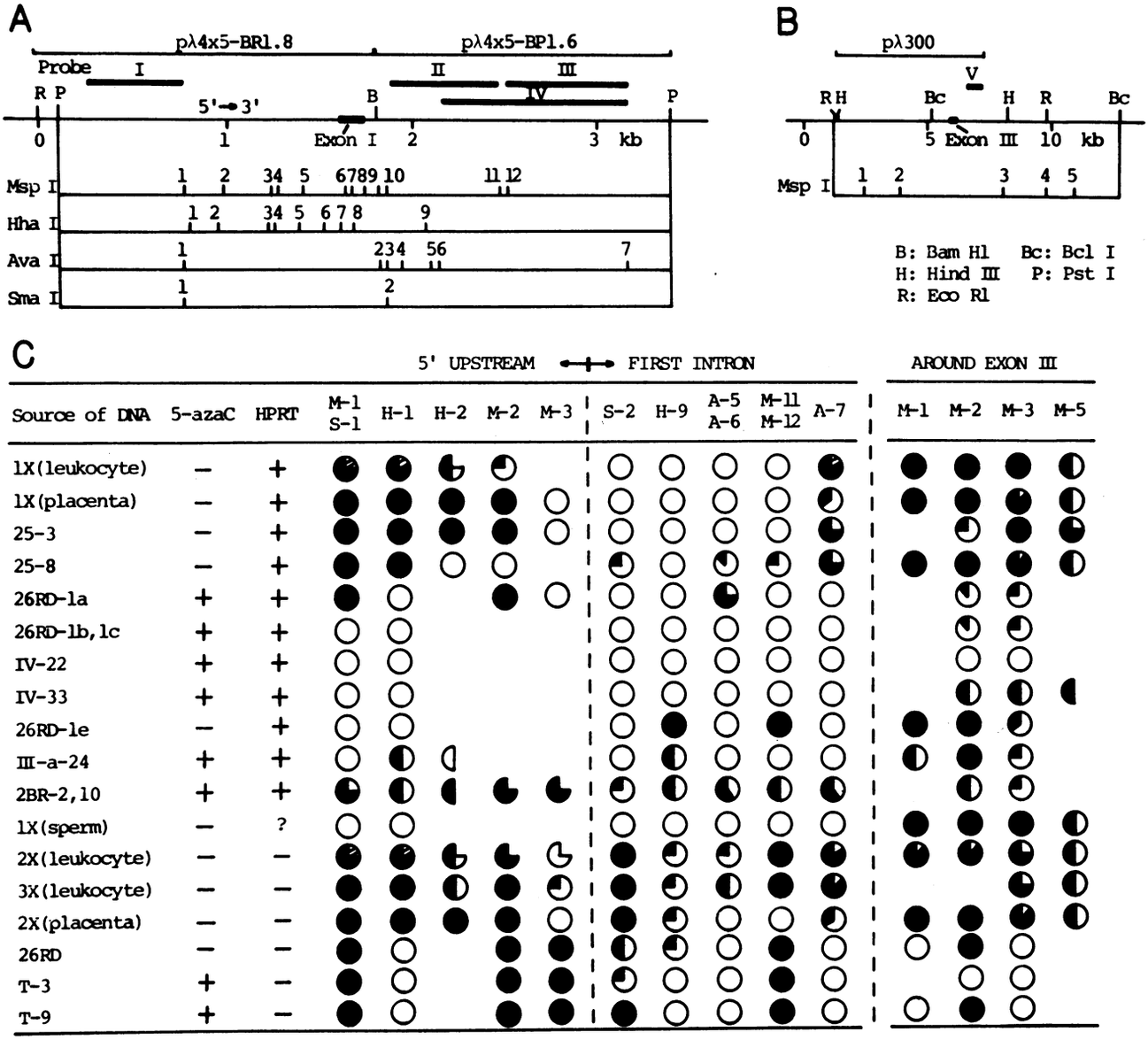

FIG. 1. Restriction maps around exon I (*A*) and exon III (*B*) of the human *HPRT* gene. The restriction sites are numbered sequentially from the 5′ end of the map. Also indicated are the regions from which probes were isolated. (*C*) Methylation patterns of the restriction sites. Whether the cell lines have been treated with 5-azacytidine and the status of expression of the *HPRT* gene on each of these X chromosomes are also shown. ●, No detectable cleavage; ○, no methylation; ◐, variation between individuals (e.g., some samples showed no detectable cleavage while others showed 25% cleavage); ◔, ◖, methylation status is unknown in 25% and 50% of the molecules, respectively. The methylation levels in female DNA represent only the inactive X chromosomes, assuming that the level of methylation on the active X chromosome is the same as that in the male DNA.

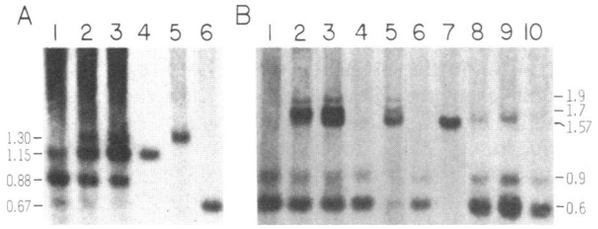

FIG. 2. Southern blot analysis of *Hpa* II/*Pst* I digestion of various DNAs. (*A*) Hybridization with probe I. (*B*) Hybridization with probe II. Lanes: 1, one X (leukocyte); 2, two X (leukocyte); 3, three X (leukocyte); 4, 25-3; 5, 26RD; 6, 26RD-1e (*A*) or 26RD-1c (*B*); 7, 26RD-1e; 8, one X (placenta); 9, two X (placenta); 10, one X (sperm).

sites were too close to one another to be resolved. The results are summarized in Fig. 1*C*.

By a similar strategy, probe II was used to assess the methylation status of sites M-9 through M-12 (Fig. 2*B*). Male leukocyte DNA gave a 0.6-kb fragment, which indicated that both M-9,10 and M-11,12 were not methylated. (M-9 and M-10, as well as M-11 and M-12 were too close to be distinguished.) The 0.9-kb fragment was very likely derived from contamination of probe II with probe III. In addition to the 0.6-kb fragment, female leukocyte DNA has several closely clustered bands around 1.5 kb. These bands are derived from methylated M-11, M-12 sites and unmethylated M-10, M-9, or M-8 sites because they were absent in digests with *Hpa* II alone. Southern blots using probe III further confirmed these interpretations. The relative intensities of the 0.6-kb and 1.5-kb bands showed that in females with two and three X chromosomes, 58% and 70% of the M-11,12 sites were methylated, respectively. These results imply that M-11,12 sites are unmethylated on active X DNA and methylated on inactive X DNA in leukocytes.

The methylation levels of *Hha* I sites H-1, H-2, and H-3 were studied by digesting the DNA with *Hha* I/*Pst* I and then hybridizing with probe I and that of H-9 was studied with probe IV (data not shown). There is no obvious correlation between methylation of these *Hha* I sites and *HPRT* activity in the DNA samples studied (Fig. 1*C*).

Digestion of male leukocyte DNA with *Sma* I/*Pst* I and hybridization with probe IV gave one predominant 1.5-kb fragment (Fig. 3*A*). The faint 3.3-kb fragment contributed only 5% of the total band intensity. This indicates that the *Sma* I site, S-2, is mainly unmethylated. In female leukocyte and placental DNA, the appearance of 2.6-kb and 3.3-kb fragments suggested that S-2 sites in these cells are partially methylated. This was also true for DNA isolated from 26RD and T-9, which contain inactive *HPRT* genes. 25-3 and IV-22, which express *HPRT*, gave only the 1.5-kb fragment. It should be pointed out that S-2 and M-10 represent the same site. The clustering of M-8 and M-9 to M-10 made the analysis of methylation levels at M-10 difficult when *Hpa* II/*Pst* I was used.

The methylation levels of two additional sites were studied using the restriction enzyme *Ava* I. As can be seen from the recognition sequences, *Ava* I sites should be either *Sma* I sites or *Xho* I sites. Using the λ clones, we identified A-1 as S-1, A-3 as S-2, and A-7 as an *Xho* I site. However, A-2, A-4, A-5, and A-6 were not cleaved by either *Sma* I or *Xho* I. When human DNA was used, A-6 was cut by *Ava* I, indicating that the internal cytidine is not methylated, while it again could not be cleaved by either *Sma* I or *Xho* I. It is possible that the activity of *Sma* I and *Xho* I, but not that of *Ava* I, is affected by methylation of nucleotides other than the internal cytidine in the recognition sequence. The results of studies using *Ava* I/*Pst* I digestion and probe IV are shown in Fig. 3*B*. The 1.1-kb fragment is derived from unmethylated

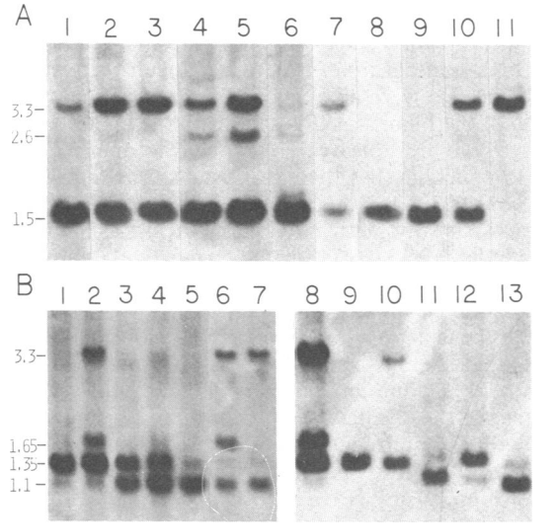

FIG. 3. Southern blot analysis. (*A*) Digestion with *Sma* I/*Pst* I and hybridization with probe IV. Lanes: 1, one X (leukocyte); 2, two X (leukocyte); 3, three X (leukocyte); 4, one X (placenta); 5, two X (placenta); 6, one X (sperm); 7, 26RD; 8, 25-3; 9, IV-22; 10, 2BR-10; 11, T-9. (*B*) Digestion with *Ava* I/*Pst* I and hybridization with probe IV. Lanes: 1, one X (leukocyte); 2, two X (leukocyte); 3, one X (placenta); 4, two X (placenta); 5, one X (sperm); 6, 2BR-2; 7, 2BR-10; 8, three X (leukocyte); 9, 25-3; 10, 25-8; 11, 26RD; 12, 26RD-1a; 13, 26RD-1b.

A-6 (or A-5) and A-7 while the 2.35-kb fragment is derived from methylated A-7. This assignment was confirmed using *Ava* I alone or using *Xho* I/*Bam*HI digestion (data not shown). There is no apparent correlation between methylation at either site A-6 (or A-5) or site A-7 and *HPRT* activity.

Methylation Near the Third *HPRT* Exon. Early in our studies of methylation of the human *HPRT* gene, the mouse cDNA clone pHPT4 was used as a probe (23). There is >90% homology between human and mouse cDNA coding sequences (24). When human leukocyte DNAs were digested with *Hpa* II/*Bcl* I and blotted with ^{32}P-labeled pHPT4 insert (Fig. 4*A*), there was a 2.9-kb fragment that was present only in the female samples and whose intensity increased with the number of X chromosomes in the cells. When *Bcl* I was replaced with *Eco*RI, there was again a fragment (6.7 kb) that was present only in females and that showed a dosage effect (data not shown). In Southern blots of restriction digests of human DNA with *Eco*RI, *Hin*dIII, *Msp* I, *Bcl* I, or a combination thereof, there was always a prominent band that constituted >50% of the total hybridization. By analyz-

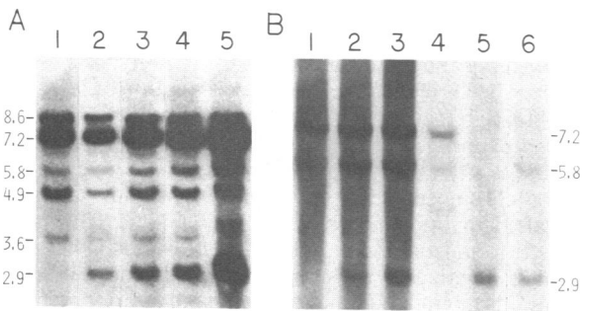

FIG. 4. Southern blot analysis around exon III of the human *HPRT* gene. (*A*) Digestion with *Hpa* II/*Bcl* I and hybridization with pHPT4. Lanes: 1, one X (leukocyte); 2, half amount of two X (leukocyte) DNA; 3, two X (leukocyte); 4, three X (leukocyte); 5, five X (leukocyte). (*B*) Digestion with *Hpa* II/*Bcl* I and hybridization with probe V. Lanes: 1, one X (leukocyte); 2, two X (leukocyte); 3, three X (leukocyte); 4, 25-3; 5, 26RD; 6, 26RD-1e.

ing the relative intensities of the fragments and taking into account whether a band was derived from a sequence on the X chromosome or a presumed pseudogene sequence on an autosome, we were able to deduce a restriction map of the region of the human *HPRT* gene with the greatest sequence homology to the mouse cDNA probe (Fig. 1*B*). We were also able to conclude that the extra band in the female DNA is derived from an *Msp* I site (M-3) that is methylated in males but only partially methylated in females. Assuming that the unmethylated M-3 site is located on inactive X chromosomes only, we calculated that 20–40% of M-3 sites are unmethylated.

After the isolation of genomic *HPRT* clones, it became apparent that the region shown in Fig. 1*B* contained exon III of the gene. A λ clone containing a region with the same restriction map as the one deduced by Southern blotting was identified and a 0.6-kb fragment was isolated from a plasmid subclone, pλ300, for use as a probe (probe V) to better delineate methylation in this area. This fragment comes from the third intron of the human gene and does not hybridize with mouse DNA. *Hpa* II/*Bcl* I digestion of human leukocyte DNA again gave the 2.9-kb fragment only in female DNA (Fig. 4*B*). This confirmed the results obtained using pHPT4 as the probe. Furthermore, these studies were extended to the various hybrid cell lines, and the methylation levels of M-1, M-2, and M-5 in addition to M-3 were evaluated.

Correlation of Methylation and Expression. The results of our studies are summarized in Fig. 1*C*. For leukocyte and placenta DNA samples containing more than one X chromosome, a single active X was assumed to be present and data are presented only for the inactive chromosomes after comparison with the one X DNA pattern. In the sequence 5′ to exon I, an active gene is in general less methylated than an inactive gene. We could not find any specific restriction sites whose methylation was directly correlated with gene activity. However, the region accessible for study closest to exon I (M-3) was still 400 bp from the presumed transcription initiation site. The region immediately proximal to the gene could not be studied because of the presence of numerous *Msp* I sites and a substantial amount of repetitive sequence.

We identified two sites in the first intron (S-2 and M-11,12) that did show a good (although not perfect) correlation between methylation of the site and gene activity. That is to say, these sequences are unmethylated on active genes and methylated on inactive genes. Exceptions observed are that M-11,12 is unmethylated on the inactive X in placenta and methylated in 26RD-1e, which does express *HPRT*. Also, S-2 is partially methylated in 25-8 and 2BR-2,10, which have active *HPRT* genes, and in 26RD and T-3, which has an inactive X. The actual status of transcription of the *HPRT* gene in sperm is unclear although it is thought to be inactive. 2BR-2 and 2BR-10 contain more than one X chromosome and are $HPRT^+$. It is not known whether *HPRT* is expressed from one or all of the X chromosomes in these hybrids.

There is no rigidly consistent configuration of methylation that invariably correlates with gene expression in the region around exon III. However, site M-3, just downstream from exon III, is relatively more methylated on active than inactive X chromosomes in leukocyte DNA. This same site is fully demethylated in hybrids 26RD, T-3, and T-9 with inactive X chromosomes and is largely methylated in hybrids 25-3 and 25-8 with active X chromosomes. All of the spontaneous or 5-azacytidine-induced reactivated hybrids studied are at least partially methylated at this site with the exception of IV-22.

DISCUSSION

We have studied methylation patterns in the vicinity of the first and third exons of human *HPRT* genes on active and inactive X chromosomes by using DNA from various human tissues, mouse–human hybrid cells, and methylation-sensitive restriction enzymes. In general, the 5′ region 400–800 bp upstream from the putative transcription start site was undermethylated on active X chromosomes as compared with inactive ones. We have identified two sites (S-2 and M-11,12) in the first intron that specifically show good correlation between methylation of the site and *HPRT* activity. The S-2 site is about 150 bp from the first exon–intron junction while M-11,12 is about 600 bp further downstream. H-9 and A-5,6, which are located between S-2 and M-11,12, do not show any specific methylation with regard to gene activity. This implies that, if these sequences in the first intron are involved in the control of *HPRT* activity, a long continuous stretch of DNA need not be coordinately methylated or demethylated. Site A-7, which is ≈1.5 kb downstream from the first exon–intron junction, also did not show any correlation between extent of methylation and expression. In the third intron, we identified an *Msp* I site that is less methylated on inactive genes than on active genes. Whether any of these sites are of necessity causally involved in the control of gene activity remains to be elucidated. A significant limitation of our method is that the restriction enzymes available detect only a small portion of the total cytidine residues in the genome. This analytic approach also depends on the distribution of the restriction sites and the location of single-copy restriction fragments that can be used as probes. This renders certain portions of the genome inaccessible.

Wolf and Migeon (25), using two single-copy X chromosome-specific DNA sequences as probes, were unable to detect any differences in methylation between male and female placental or fibroblast DNAs. However, it is not known whether their probes contained coding sequences that undergo X inactivation. The differences in the nature of the probes used may account for the variance in the results of the two studies. Wolf and Migeon felt that ubiquitous methylation was not involved in X inactivation and that there is variation in methylation at a given site even in clonally derived cell lines. Our results are in agreement with both of these conclusions and with cytologic observations using anti-5-methylcytosine antibodies that fail to discriminate between active and inactive X chromosomes (26).

Two other observations of interest may be derived from our results. It is generally believed that many genes in sperm cells are not transcriptionally active (27). It is also thought that the single X chromosome of males of many species (including *Drosophila*) undergoes X inactivation as an obligatory concomitant of spermatogenesis (28, 29). Recently, however, it has been shown that DNA from sperm of several mammalian species is competent in a DNA-mediated *HPRT* gene-transfer assay (30). Our results show that DNA from this source is extensively demethylated in the 5′ flanking and first intron regions and relatively methylated around the third exon including site M-3. This is a pattern of methylation consistent with that of expressed *HPRT* genes.

A second point of interest involves the mechanism of action of 5-azacytidine. This drug and related cytidine analogues function as potent inhibitors of cellular DNA methyltransferase activity but only after their incorporation into DNA (31). It is thought that these compounds bind the methylase irreversibly and inactivate it. An unanswered issue is whether the normal putative "maintenance" methylase acts in a processive fashion by binding to DNA or chromatin at fixed sites and scanning along a region of the genome acting on suitable substrate hemimethylated sites as they are encountered. Of the six hybrid cell lines studied after 5-azacytidine reactivation of *HPRT*, in four all detectable sites around exon I were unmethylated, while 26RD-1a and III-a-24 contained methylated sites intermixed with unmethylated sites. The *Msp* I sites associated with exon III in these cells were

either methylated or unmethylated. This indicates that 5-azacytidine treatment does not invariably result in long stretches of stable demethylation of DNA.

Most studies of DNA methylation and gene expression have focused on the 5′ end of functional genes and have shown an inverse correlation between the level of methylation and the activity of a gene, although no obvious changes were found in the case of the α(2)I chicken collagen gene (32). More recent observations suggest that control of expression of a variety of genes can be influenced by sequences 3′ to a gene (33) or by various transcriptional enhancers located 5′, 3′, or within a gene (34, 35). The effects of DNA and chromatin conformation on gene expression have also been studied extensively and the potential roles of methylation on DNA–protein interaction and B-to-Z transitions have been commented on (36). The results of gene transfer studies, 5-azacytidine-induced reactivation, and the direct molecular analysis of *HPRT* methylation patterns reported here support the concept of the likely importance of the overall configuration of methylation at several sites around structural genes in the maintenance of X chromosome inactivation.

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1. Lyon, M. F. (1961) *Nature (London)* **190,** 372–373.
2. Gartler, S. M. & Andina, R. J. (1976) *Adv. Hum. Genet.* **7,** 99–140.
3. Holliday, R. & Pugh, J. E. (1975) *Science* **187,** 226–232.
4. Sager, R. & Kitchin, R. (1975) *Science* **189,** 426–432.
5. Riggs, A. D. (1975) *Cytogenet. Cell Genet.* **14,** 9–25.
6. Liskay, R. M. & Evans, R. J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4895–4898.
7. Chapman, V. M., Kratzer, P. G., Siracusa, L. D., Quarantillo, B. A., Evans, R. & Liskay, R. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5357–5361.
8. Kratzer, P. G., Chapman, V. M., Lambert, H., Evans, R. E. & Liskay, R. M. (1983) *Cell* **33,** 37–42.
9. Mohandas, T., Sparkes, R. S. & Shapiro, L. J. (1981) *Science* **211,** 393–396.
10. Lester, S. C., Korn, N. J. & DeMars, R. (1982) *Somatic Cell Genet.* **8,** 265–284.
11. Venolia, L., Gartler, S. M., Wassman, E. R., Yen, P., Mohandas, T. & Shapiro, L. J. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2352–2354.
12. Mohandas, T., Shapiro, L. J., Sparkes, R. S. & Sparkes, M. C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5779–5783.
13. Mohandas, T., Sparkes, R. S., Hellkuhl, B., Grzeschik, K. H. & Shapiro, L. J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6759–6763.
14. Mohandas, T., Sparkes, R. S. & Shapiro, L. J. (1982) *Am. J. Hum. Genet.* **34,** 811–817.
15. Blin, N. & Stafford, D. W. (1976) *Nucleic Acids Res.* **3,** 2303–2308.
16. Borenfreund, E., Fitt, E. & Bendich, A. (1961) *Nature (London)* **191,** 1375–1377.
17. Geever, R. F., Wilson, L. B., Nallaseth, F. S., Milner, P. F., Bittner, M. & Wilson, J. T. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5081–5085.
18. Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. & Axel, R. (1979) *Cell* **16,** 777–785.
19. Southern, E. (1975) *J. Mol. Biol.* **98,** 503–517.
20. Chinault, A. C. & Carbon, J. (1979) *Gene* **5,** 111–126.
21. Melton, D. W., Konecki, D. S., Brennand, J. & Caskey, C. T. (1984) *Proc. Natl. Acad. Sci. USA*, in press.
22. McClelland, M. (1981) *Nucleic Acids Res.* **9,** 5859–5866.
23. Yen, P., Marsh, B., Mohandas, T., Patel, P., Melton, D. & Shapiro, L. J. (1983) *J. Cell. Biochem. Suppl.* **7A,** 149 (abstr.).
24. Konecki, D. S., Brennand, J., Fuscoe, J. C., Caskey, C. T. & Chinault, A. C. (1982) *Nucleic Acids Res.* **10,** 6763–6775.
25. Wolf, S. F. & Migeon, B. F. (1982) *Nature (London)* **295,** 667–671.
26. Miller, D. A., Okamoto, E., Erlanger, B. F. & Miller, O. J. (1982) *Cytogenet. Cell Genet.* **33,** 345–349.
27. Erickson, R. P., Kramer, J. M., Rittenhouse, J. & Salkeld, A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6086–6090.
28. Lifschytz, E. & Lindsley, D. L. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 182–186.
29. Gordon, J. W. & Ruddle, F. H. (1981) *Science* **211,** 1265–1271.
30. Venolia, L. & Gartler, S. M. (1983) *Somatic Cell Genet.* **9,** 617–627.
31. Santi, D. V., Garrett, C. E. & Barr, P. J. (1983) *Cell* **33,** 9–10.
32. McKeon, C., Ohkubo, H., Pastan, I. & de Crombrugghe, B. (1982) *Cell* **29,** 203–210.
33. Osley, M. A. & Hereford, L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7689–7693.
34. Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M. P. & Chambon, P. (1981) *Nucleic Acids Res.* **9,** 6047–6068.
35. Gillies, S. D., Morrison, S. L., Oi, V. T. & Tonegawa, S. (1983) *Cell* **33,** 717–728.
36. Behe, M. & Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1619–1623.