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Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome: Implications for X-chromosome inactivation

(dosage compensation/"housekeeping" genes/5-azacytidine/X-chromosome reactivation/mouse–human hybrids)

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ABSTRACT **To explore the role of DNA methylation in maintaining dosage compensation of X chromosome-linked genes and in regulating the transcriptional activity of "housekeeping" genes, we characterized DNA methylation of active, inactive, and derepressed alleles at the locus for hypoxanthine phosphoribosyltransferase (*HPRT*) on the human X chromosome. The methylation of *Hpa* II and *Hha* I sites in *HPRT* alleles on the active X chromosome was the same in all tissues. The consensus pattern includes hypomethylation of 5′ clustered sites and extensive methylation of the 3′ sequence. The striking feature of methylation of inactive X-chromosome alleles is nonuniformity and less extensive hypomethylation of the 5′ cluster. Analysis of *HPRT* alleles reactivated in response to 5-azacytidine showed at least partial restoration of the consensus pattern. These observations indicate that methylation of housekeeping genes on the X chromosome is the same as that of autosomal ones and that the overall pattern and methylation of multiple sites within a cluster may cooperate to facilitate transcription. Furthermore, the fidelity of methylation of the active allele and the extensive drift in methylation of the inactive allele suggest that mechanisms involved in X-chromosome dosage compensation may be directed at the active rather than inactive X chromosome.**

DNA methylation not only has been implicated as an important determinant of gene activity (1) but also has been considered to have a primary role in compensating for sex differences in the dosage of X chromosome-linked (X-linked) genes (2, 3). Evidence suggests that DNA methylation is involved in regulation of the hypoxanthine phosphoribosyltransferase (*HPRT*) locus. The cytosine analog 5-azacytidine (5-azaC) induces the localized derepression of inactive *HPRT* alleles (4–6), presumably by inducing demethylation at sites within or near the locus. DNA purified from these derepressed alleles or "reactivants" is competent to transfer *HPRT* activity to recipient cells, whereas DNA from the inactive alleles is not (6–8).

Yet this evidence does not show any direct role for DNA methylation in dosage compensation—i.e., patterns of methylation exclusively concerned with maintaining inactivity of the silent X chromosome. Loci on the X chromosome, like those on other chromosomes, represent an array of developmentally regulated, hormone-responsive, and constitutively expressed ("housekeeping") genes (9). If there are special features of the X chromosome relevant to dosage compensation, then they must be superimposed on regulatory features common to all chromosomes. Therefore, demethylation may induce reexpression of *HPRT* by directly affecting transcription of the locus, rather than by reversing some developmental program specifically associated with dosage compensation.

The *HPRT* locus is extraordinarily useful for exploring the role of DNA methylation in maintaining the silence of the inactive X chromosome and in transcriptional regulation. The inactive X chromosome provides the means to examine the locus in an inactive state, not usually possible with housekeeping genes. In addition, active and inactive alleles can be examined within the same cell, so that methylation differences between tissues do not obscure relevant differences. The ability to separate the inactive X chromosome from its active homologue in hybrids also facilitates the analysis. Finally, selection in hypoxanthine/aminopterin/thymidine makes it possible to obtain rare cells in which the locus on the inactive X chromosome is expressed.

Our observations of DNA methylation around *HPRT* reveal a significant difference between active and inactive alleles. We have identified a consensus pattern for active alleles that includes extensive hypomethylation at the 5′ end and extensive methylation throughout the rest of the gene. In contrast, inactive alleles have a variety of methylation patterns. This differential methylation of homologous loci most likely reflects transcriptional differences between the two alleles, rather than special features of X-chromosome inactivation and suggests that maintenance of X-chromosome dosage compensation is mediated by the same mechanisms that determine transcriptional activity of autosomal genes.

MATERIALS AND METHODS

Source of DNA. DNA was purified (10) from blood, placentas, lymphoblasts, and fibroblasts including clones (11) from males and females. Hybrid cells with active X chromosomes were derived from matings with mouse A9 cells and human fibroblasts. Hybrids with inactive X chromosomes were obtained from two matings, Gl × A9 (11) and MF × A9 (12), by back selection in 6-thioguanine (12). Hybrids with reactivated *HPRT* alleles were obtained from a 6-thioguanine-derived clone of Gl × A9 by induction with 2 μM 5-azaC followed by selection in hypoxanthine/aminopterin/thymidine (13).

Restriction Enzyme Analysis and Southern Blotting. Digestions were for 16 hr under conditions suggested by the manufacturer (Bethesda Research Laboratories) with 10 units per μg of DNA for methyl-sensitive enzymes. Electrophoresis, transfer to nitrocellulose, and hybridization with nick-translated probes have been described (10, 14).

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; X-linked, X chromosome-linked; 5-azaC, 5-azacytidine; bp, base pair(s); kb, kilobase(s); IVS, intervening sequence; G6PD, glucose-6-phosphate dehydrogenase.

Restriction Site Mapping of Cloned DNA Fragments. *Hpa* II and *Hha* I restriction sites were determined from DNA sequence analysis of cloned fragments (ref. 15; unpublished results) in the −1.9- to 0-kilobase (kb) region (Fig. 1) and in the 0- to +9.5-kb region from partial digestion (16) of the 9.5-kb *Bam*HI–*Eco*RI fragment.

RESULTS

Characterization of the *HPRT* Locus and Probe. Fig. 1 shows a map of the *HPRT* locus. At the 5′ end of the gene is the first exon of ≈150 base pairs (bp), followed by an intron of at least 9.5 kb (intervening sequence I, IVS I). The initial methionine codon begins 23 bp in front of the first known splice site. A striking feature of this gene is the CpG cluster at the 5′ end. There are at least 12 *Hpa* II and 15 *Hha* I sites in the 1000 bases preceding IVS I and at least 11 *Hpa* II and 8 *Hha* I sites in the initial 9.5 kb of the intron.

Because there are sequences homologous to human HPRT cDNA on human autosomes and in the mouse genome (17), we chose to use an intron probe. The 1.7-kb *Bam*HI–*Pst* I intron fragment pPB1.7 (ref. 14; Fig. 1) hybridizes with only the human X-linked alleles (see below). This *Bam*HI site, 40 bp within IVS I, is used as the reference point (0 kb). Restriction sites 3′ to the reference site are expressed in terms of positive-kilobase distances and those 5′ as negative-kilobase distances.

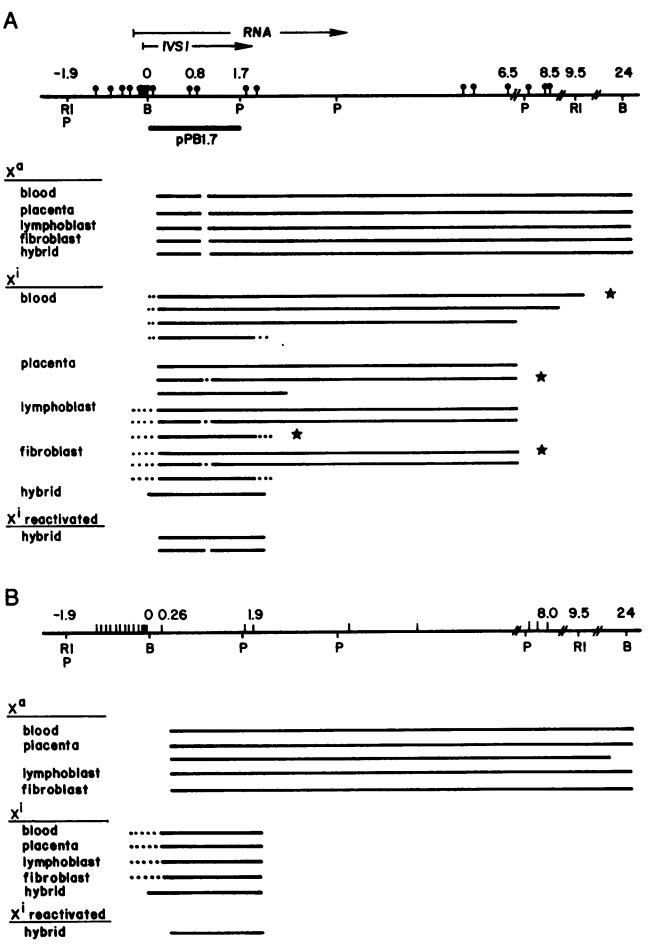

FIG. 1. Methylation of active and inactive *HPRT* at *Hpa* II (*A*) and *Hha* I (*B*) sites. •, *Hpa* II; |, *Hha* I; RI, *Eco*RI; B, *Bam*HI; and P, *Pst* I; X^i and X^a, inactive and active X chromosomes. The hybridization probe is shown by the bar below the map in *A*. The lines represent *Hpa* II (*A*) and *Hha* I (*B*) fragments from complete digests of genomic DNA; the predominant X^i fragment in populations studied is indicated by a star. The ends of some X^i fragments are represented by dots to indicate that the specific unmethylated site within clusters may not be the same for all chromosomes (see Fig. 3 and text).

Sex Differences in *HPRT* Methylation. DNA from leukocytes obtained from males, digested with *Bam*HI and the methyl-sensitive enzyme *Hpa* II, and probed with pPB1.7 produced two fragments (24 kb and 0.8 kb; Fig. 2). DNA from females yielded the same bands, but in addition, bands at 6.5 and ≈2.0 kb. These additional bands were derived from inactive *HPRT* alleles as shown below.

The Active *HPRT* Allele Has a Consistent Pattern of DNA Methylation. DNA from a variety of tissues and cultured cells from males was digested with *Hpa* II (18 males) or *Hha* I (7 males). The position of unmethylated sites was defined by subsequent cleavages with *Eco*RI, *Bam*HI, *Pst* I, and combinations thereof. Results of some of these analyses are presented in Figs. 2 and 3 and summarized in Fig. 1. The 0.8-kb *Hpa* II fragment was the product of an unmethylated *Hpa* II site very close to 0 kb and another site at +0.8 kb. Fragments larger than 24 kb indicate that all *Hpa* II sites in the region extending more than 24 kb distal to the +0.8-kb site were methylated (Fig. 2). Methylation of *Hha* I sites was similar; the 24-kb *Hha* I/*Bam*HI fragments resulted from an unmethylated site at +0.26 kb and methylation of all sites to +24 kb (Fig. 3 *C* and *D*).

That the pattern for the active X chromosome in females is the same as that in males is clear from studies of fibroblasts from a female, heterozygous for an *HPRT*/*Bam*HI restriction fragment-length polymorphism as well as for electrophoretic variants of glucose-6-phosphate dehydrogenase (*G6PD A*/*G6PD B*). The polymorphism, resulting from a novel *Bam*HI site at +12 kb (17) was syntenic with the *G6PD B* allele. In digests of DNA from clonal cultures expressing *G6PD B*, the largest *Hpa* II/*Bam*HI fragment was 11.2 kb rather than 24 kb; in digests from clonal cultures expressing *G6PD A*, the largest band was 24 kb (Fig. 4). These are the results expected if methylation of the active X in females is the same as in males.

The striking feature of active alleles was the uniformity of methylation in all tissues, cultured cells (Figs. 3 and 4), and interspecies hybrids (Fig. 5). *Hpa* II and *Hha* I sites near the 5′ end of the gene were unmethylated, while all distal sites for at least 24 kb were methylated. Exceptions to this consensus pattern occurred occasionally and are discussed below.

Active *HPRT* Alleles Are Extensively Unmethylated in the 5′ CpG Cluster. Because all active *HPRT* alleles were unmethylated at 0 kb, complete digests with methyl-sensitive en-

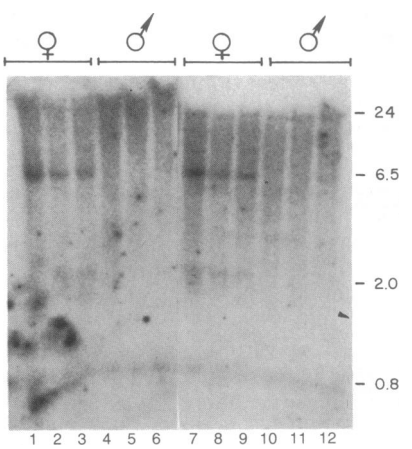

FIG. 2. Sex differences in the pattern of *HPRT* methylation. DNA from the blood of males and females was digested with *Hpa* II (lanes 1–6) or *Hpa* II/*Bam*HI (lanes 7–12). Note that the >24-kb *Hpa* II fragments are reduced to 24 kb when cleaved with *Bam*HI, while other bands remain the same.

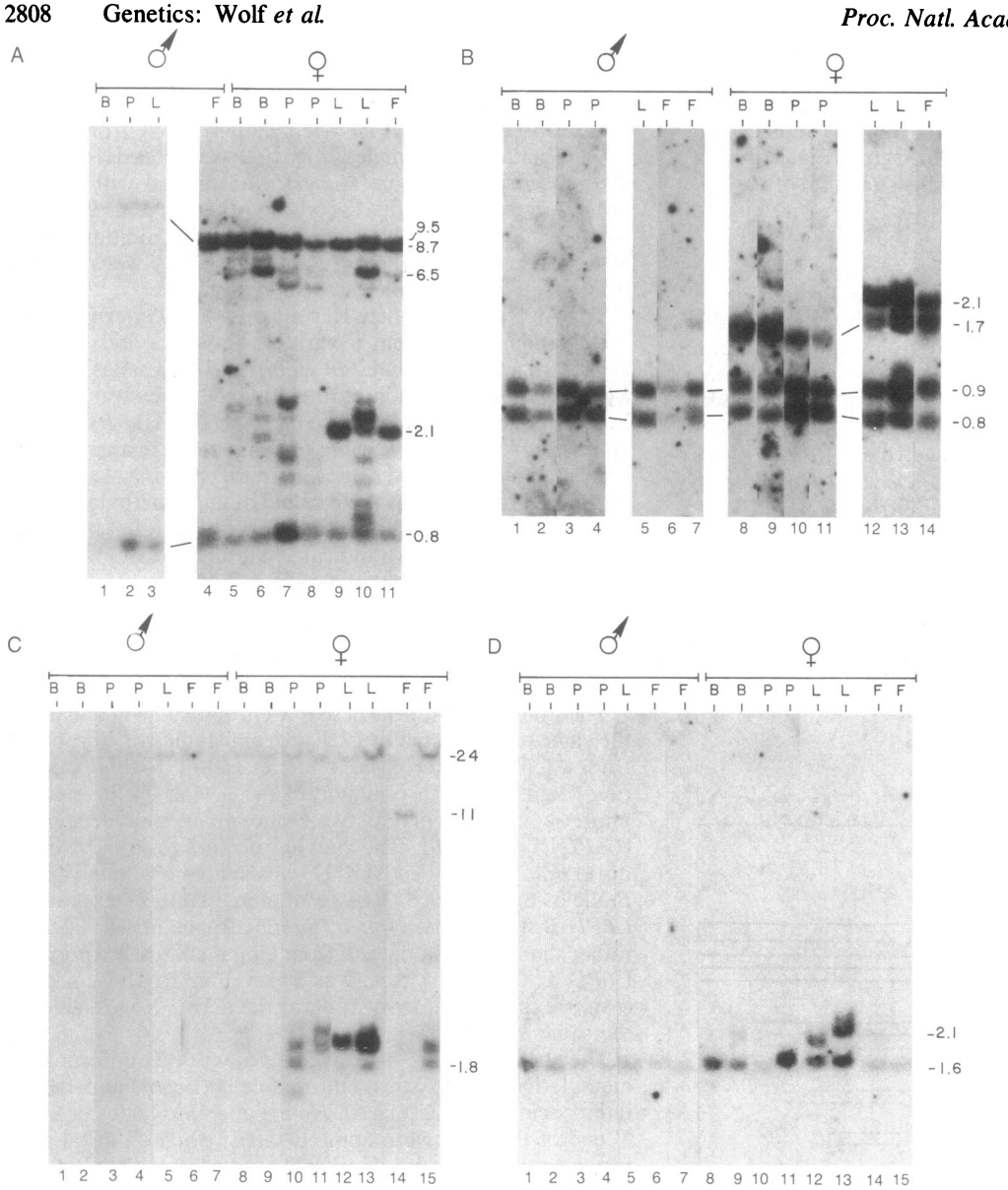

FIG. 3. *HPRT* methylation in a variety of tissues from males and females showing differences in location of unmethylated sites on inactive and active X chromosomes. B, blood; P, placenta; F, fibroblasts; L, lymphoblasts. (*A*) *Hpa* II/*Eco*RI/*Bam*HI. (*B*) *Hpa* II/*Pst* I. (*C*) *Hha* I/*Bam*HI. (*D*) *Hha* I/*Pst* I. The 5′ end of the 0.8-kb fragment is close to the *Bam*HI reference site because: (*i*) the fragment is not cut by *Bam*HI (Fig. 2); (*ii*) the 24-kb fragment is reduced to 0.9 kb by *Pst* I, and (*iii*) the *Bam*HI–*Pst* I probe is 1.7 kb. Some inactive X chromosomes must be unmethylated in the +0.8-kb region. In placenta the intensity of the 1.7-kb *Hpa* II/*Pst* I band does not equal the sum of the 0.8- and 0.9-kb bands.

zymes could not reveal the extent of hypomethylation within the 5′ cluster. To determine this, DNA from males, completely digested with *Pst* I, was partially digested with *Hpa* II (Fig. 6 *Left*) or *Hha* I. The array of bands that resulted indicates that many sites within the cluster were unmethylated in active alleles.

Inactive *HPRT* Alleles Have a Variety of Methylation Patterns. Because the methylation of active *HPRT* alleles was consistent, methylation patterns of the inactive locus could be inferred from digests of DNA from females; bands not present in digests of DNA from males were presumably of inactive X origin. Methylation patterns from mouse–human hybrids containing only the human inactive X chromosome substantiate this inference.

Results with samples from females, 27 digested with *Hpa* II and 8 with *Hha* I, are summarized in Fig. 1. In contrast to the active X chromosome, there was a variety of methylation patterns for *HPRT* alleles on the inactive X chromosome. At sites where the pattern in DNAs from males was rigorously maintained, methylation in DNA from females was inconsistent, so that absence of a consensus pattern was the rule. This diversity occurred not only from one tissue to another but also between cells of the same tissue (Figs. 2 and 3).

Hpa II sites within the +0.8-kb to +24-kb region were frequently unmethylated on the inactive X chromosome. A prominent band in females was due to an unmethylated *Hpa* II site at +6.5 kb. Other common bands resulted from unmethylated sites at approximately +2 kb, while smaller bands reflected open sites within the pPB1.7 sequence. However, some inactive loci could be as extensively methylated as active ones, as a 9.5-kb band in leukocytes from females (Fig. 3*A*) resulted from an unmethylated site near 0 kb and methylation of all distal *Hpa* II sites for at least 9.5 kb.

Unlike active alleles, *Hpa* II sites in the +0.8-kb region of most inactive alleles were methylated, based on the presence of bands ranging from 1.7 to 2.1 kb in *Hpa* II/*Pst* I digests from females (Fig. 3*B*, lanes 8–14). However, this region could be unmethylated, as the intensity of bands in some DNA from females was inappropriate (Fig. 3*B*, lanes 10 and 11).

Unlike active alleles, all sites within the 0- to −0.4-kb region were methylated in many inactive X chromosomes: *Hpa* II/*Pst* I digests from lymphoblasts and fibroblasts yielded a prominent 2.1-kb band (Fig. 3*B*, lanes 12–14). The 1.7-kb fragment in *Hpa* II/*Pst* I digests of DNA from all female tissues indicates that at least one site was unmethylated in some inactive alleles near 0 kb. However, partial *Hpa* II digests of inactive X chromosome DNA produced a single

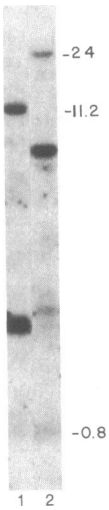

FIG. 4. *Hpa* II/*Bam*HI cleavage showing that the active X chromosome in females is methylated like that in males. DNA is from fibroblast clones heterozygous for *G6PD* and the *Bam*HI restriction fragment-length polymorphisms. Lanes: 1, clone expressing *G6PD B*; 2, clone expressing *G6PD A*. The *G6PD B* allele is on the same chromosome as the *HPRT* restriction fragment-length polymorphism, so that the larger active X chromosome fragment is 11.2 kb in lane 1 but 24 kb in lane 2.

sharp band (Fig. 6 *Center*). Therefore, when unmethylated in the 5′ region, inactive alleles are unmethylated at only one or a few sites.

Methylation of *Hha* I sites is analogous to that for *Hpa* II. Partial *Hha* I digests revealed that the 5′ CpG cluster is extensively methylated. In addition, there were female-specific bands at 1.8, 2.0, and 2.3 kb, reflecting some unmethylated sites near 0 and at +1.9 kb (Fig. 3 *C* and *D*).

Origin of the Variable Methylation of Inactive Alleles. Fragments of inactive X-chromosome origin produced by *Hpa* II or *Hha* I were frequently multiple, and several were larger than the probe, indicating that there are subpopulations of inactive X chromosomes, each with a different pattern of methylation. It is likely that some of the variety reflects the heterogeneous cell composition of tissues. However, at least part is due to *de novo* changes in DNA methylation. Clonal populations of lymphoblasts and fibroblasts, derived from single cells, also produce multiple bands (Fig. 4; ref. 13).

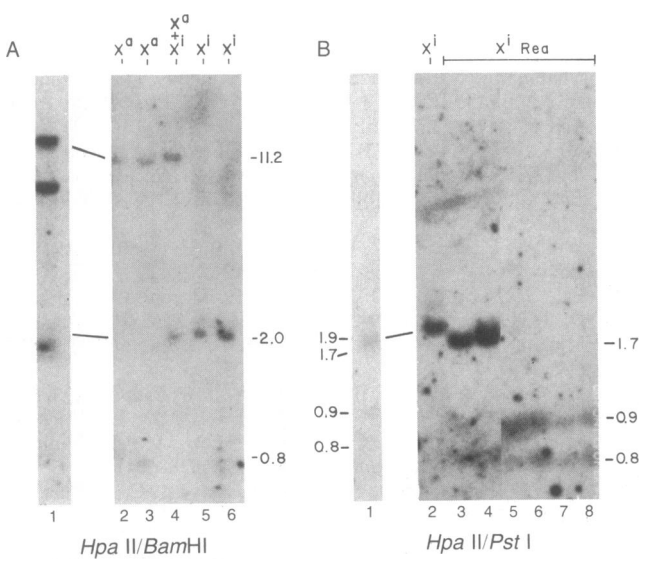

FIG. 5. Methylation pattern of active, inactive, and reactivated human *HPRT* alleles in mouse–human hybrid cells. (*A*) *Hpa* II/*Bam*HI digests of DNA from MF × A9 hybrids retaining the active X chromosome (X^a) (lanes 2 and 3), both an active and an inactive X chromosome (lane 4), and only the inactive X chromosome (X^i) (lanes 5 and 6). Lane 1 shows DNA from the parent human fibroblast clone. The X^a fragment is 11.2 kb rather than 24 because of the restriction fragment-length polymorphism. Note the absence of mouse-specific bands. (*B*) *Hpa* II/*Pst* I digests of the human parental fibroblast (lane 1), Gl × A9 hybrid retaining an inactive X chromosome (lane 2), and six independent reactivants induced by 5-azaC (lanes 3–8).

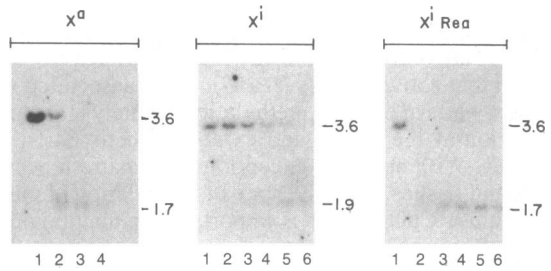

FIG. 6. Active *HPRT* alleles are extensively hypomethylated at their 5′ end. (*Left*) 46 XY lymphoblasts. (*Center*) Gl × A9 hybrid with only an inactive X chromosome (X^i). (*Right*) A derivative reactivant induced by 5-azaC. DNAs were digested to completion with *Pst* I. Aliquots were subsequently digested for 30 min with *Hpa* II (0, 0.03, 0.1, 0.3, 1, and 10 units/μg of DNA in lanes 1–6, respectively). Blots were probed with pPB 1.7. Note that ≈1.9-kb bands are always sharp for the inactive chromosome (*Center*) but are initially broad for the active chromosome (X^a) and the reactivant (X^i Rea).

Despite extensive variation, DNA methylation of inactive *HPRT* alleles had some tissue specificity. The 9.5-kb band in *Hpa* II/*Eco*RI digests was found only in blood, whereas the 5.7-kb band, resulting from unmethylated sites at +0.8 kb and +6.5 kb, was prominent only in placenta (Fig. 3*A*). Similarly, the 2.2-kb *Hpa* II fragment resulting from open sites at −0.4 kb and +1.8 kb was prominent only in cultured cells (Fig. 3*A*, lanes 9–11).

Methylation Patterns of *HPRT* Alleles in Hybrids and Reactivants. Interspecies hybrid cells provided the means to separate inactive from active X chromosomes. Methylation of active *HPRT* alleles in these hybrids was the same as that in human tissues (Fig. 5*A*). Methylation of inactive alleles in the two independent hybrids (*Gl* × *A9* and *MF* × *A9*) with only the inactive X chromosome was the same as a pattern observed in cells from females [open at −0.2- and +2.0-kb *Hpa* II sites but methylated at all sites between (Fig. 5)].

To assess the biological significance of the consensus methylation pattern, we analyzed hybrid clones that had derepressed *HPRT* alleles on the human inactive X chromosome (Fig. 5*B*). Reactivants were derived from the Gl × A9 hybrid by treatment with 5-azaC, and two were spontaneous (from control dishes). Each reactivant had an intact human X chromosome and expressed human HPRT but not human G6PD or phosphoglycerate kinase. Methylation of *Hpa* II sites was determined for 11 independent reactivants; 6 were demethylated at 0.0- and +0.8-kb *Hpa* II sites, resembling active *HPRT* alleles, whereas 5 were demethylated at 0.0 but not at +0.8 kb.

The hybrid parent and six of the derivative reactivants were also examined for methylation of *Hha* I sites. The inactive allele of the parent was unmethylated at −0.3 kb and +1.9 kb, producing a fragment like that in female cells; the fragment was smaller in each reactivant, indicating that the +0.26-kb site that is open on active alleles had been demethylated.

Unlike the parent hybrid (Fig. 6 *Center*), in all of the reactivants examined for extent of demethylation of the 5′ cluster (three with *Hpa* II and two with *Hha* I) we observed many bands, indicating that multiple sites in the cluster had been demethylated (Fig. 6 *Right*).

Exceptions to the Consensus Pattern. Not all features of the consensus pattern were invariably associated with activity of the locus in cultured cells. A few active X chromosomes were methylated in the +0.8-kb region (Fig. 3*B*, lanes 6 and 7; Fig. 5*B*, lane 4), while a few were less extensively methylated in the +0.8-kb to +24-kb region. However, no deviation from the consensus pattern at the 5′ end of the gene was observed.

DISCUSSION

Relationship of Methylation Patterns to *HPRT* Activity. We observed that activity at the *HPRT* locus is associated with a consistent pattern of DNA methylation, namely an extensive open region at the 5′ end and extensive methylation of all other sites. With only rare exceptions, this pattern occurs in all cells and tissues. That the state of methylation of multiple sites within a CpG cluster is important for maintaining the activity of housekeeping genes is an appealing hypothesis. Activity dependent upon the kind of cooperativity that clusters provide would be less subject to chance reversal. Moreover, the extensive methylation associated with activity at the *HPRT* locus suggests that, paradoxically, methylated sites as well as unmethylated sites are important determinants of gene activity. The over-all pattern of methylation may facilitate interactions between the correct initiation site and the transcriptional machinery, while inhibiting nonfunctional associations. Thus, an open 5′ cluster may be essential to initiation of transcription, whereas 3′ methylation may enhance the efficiency with which it is carried out.

Based on methylation patterns in reactivants, restoration of the full-consensus pattern is not essential for reexpression. None of the reactivants was fully methylated at all the 3′ *Hpa* II and *Hha* I sites. Nevertheless, it is significant that 55% of the reactivants become demethylated at the +0.8 kb *Hpa* II site, and all of those tested were open at the +0.26 *Hha* I site, suggesting that this region within the IVS I has some functional importance. Furthermore, reexpressed alleles were extensively unmethylated within the 5′ cluster.

Relationship of Methylation Pattern to *HPRT* Inactivity. The most consistent feature of inactive *HPRT* alleles is the absence of a consensus methylation pattern. Much of the variability is explained by *de novo* changes in methylation but is compounded by tissue-specific methylation. Because tissue-specific patterns occur exclusively in inactive alleles, it is unlikely that they are functionally significant. Perhaps they reflect tissue-specific differences in the degree of methylating activity or in chromatin.

The lack of a consensus pattern for alleles that are not expressed suggests that transcriptional inactivity of housekeeping genes is a passive, rather than active, process.

Relationship to X Chromosome Inactivation. We found no features of the methylation of the locus on the inactive X chromosome that can be attributed to a special mechanism responsible for maintaining dosage compensation. The number of *Hpa* II and *Hha* I sites is large, but the distribution is not unusual, as 5′ clusters characterize housekeeping genes on autosomes as well (18). The 5′ hypomethylation and extensive 3′ methylation pattern also has been observed for autosomal genes (1, 18, 19). Furthermore, the drift in methylation patterns of the locus on the inactive X chromosome is reminiscent of that associated with inactive autosomal loci (20). Because *HPRT* is transcriptionally active on the active X chromosome and inactive on the silent chromosome, the differences in methylation of the two loci probably reflect transcriptional regulation. Therefore, it is likely that reexpression associated with demethylation is mediated by affecting transcription directly and not by altering special features of inactive X chromosomes.

The mechanism for dosage compensation of X-linked genes in mammalian cells is complex (21) and involves initiation of a process that results in the inactivity of one chromosome and the activity of the other. Maintenance of this dimorphic state seems to be mediated by localized events because derepression affects some loci and not others on the same chromosome (refs. 6 and 12; this paper). It is not known if the initiation of dosage compensation is achieved by maintaining activity of a single X chromosome or turning off the activity of the other, nor if the transmission of the dimorphic state from one cell to its progeny is accomplished by propagating activity or inactivity or both. Our observations shed some light on these questions. The striking similarity between methylation of active genes on X chromosomes and autosomes and the absence of unique features of inactive X chromosome methylation, imply that transmission of X chromosome dosage compensation is achieved by maintaining the activity of a single active X chromosome in each cell. As a corollary, the inactive X chromosome is silent because its activity has not been maintained. This kind of passive process would explain the diversity in methylation patterns of the inactive X chromosome that we observe. Any random change in pattern would have little functional significance and so need not be eliminated. Furthermore, the similarities in methylation of X chromosomal and autosomal genes suggest that the molecular basis for the propagation of a single active X chromosome is the same as that for the regulation of autosomes. At the *HPRT* locus, this is achieved by maintaining the activity of the allele on the active X chromosome. If this hypothesis is supported by observations of other X-linked loci, then what remains to be explained is the chromosomal event that results in the coordinate regulation of multiple loci on only a single X chromosome.

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