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Genetics

# DNA methylation stabilizes X chromosome inactivation in eutherians but not in marsupials: Evidence for multistep maintenance of mammalian X dosage compensation

(CpG clusters/glucose-6-phosphate dehydrogenase/hypoxanthine phosphoribosyltransferase/*Didelphis virginiana*/nuclease sensitivity)

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**ABSTRACT** **In marsupials and eutherian mammals, X chromosome dosage compensation is achieved by inactivating one X chromosome in female cells; however, in marsupials, the inactive X chromosome is always paternal, and some genes on the chromosome are partially expressed. To define the role of DNA methylation in maintenance of X chromosome inactivity, we examined loci for glucose-6-phosphate dehydrogenase and hypoxanthine phosphoribosyltransferase in a North American marsupial, the opossum *Didelphis virginiana*, by using genomic hybridization probes cloned from this species. We find that these marsupial genes are like their eutherian counterparts, with respect to sex differences in methylation of nuclease-insensitive (nonregulatory) chromatin. However, with respect to methylation of the nuclease-hypersensitive (regulatory) chromatin of the glucose-6-phosphate dehydrogenase locus, the opossum gene differs from those of eutherians, as the 5′ cluster of CpG dinucleotides is hypomethylated in the paternal as well as the maternal gene. Despite hypomethylation of the 5′ CpG cluster, the paternal allele, identified by an enzyme variant, is at best partially expressed; therefore, factors other than methylation are responsible for repression. In light of these results, it seems that the role of DNA methylation in eutherian X dosage compensation is to "lock in" the process initiated by such factors. Because of similarities between dosage compensation in marsupials and trophectoderm derivatives of eutherians, we propose that differences in timing of developmental events—rather than differences in the basic mechanisms of X inactivation—account for features of dosage compensation that differ among mammals.**

Compensation for the sex differences in dosage of X chromosomes occurs in many species. In *Drosophila*, the expression of X-linked genes is equalized by enhancing transcription of these genes in the male. The mechanism in mammals is unique, as it results in inactivating one X chromosome in female cells. Although X chromosome inactivation (XCI) occurs in both eutherian mammals and marsupials, specific features of the differentiation event are not the same. Most prominent are differences in parental origin of the inactive X chromosomes and in stability of inactivation. In the eutherian embryo proper, XCI occurs randomly, so that either paternal or maternal X may be the inactive chromosome (reviewed in ref. 1). Furthermore, from studies of "housekeeping" enzymes (2), it seems that, once inactivated, the chromosome remains silent in most tissues. In contrast, XCI in marsupials preferentially silences the paternal X; segregation of electrophoretic variants of glucose-6-phosphate dehydrogenase (G6PD) (3, 4), phosphoglycerate kinase A (PGK1) (5, 6), and α-galactosidase A (GLA) (7) indicates that the maternal allele is always expressed. Further, the paternal allele is not stably repressed: whereas the paternal *GLA* allele is silent in all tissues of an Australian marsupial (7), the paternal *G6PD* allele is expressed in some cultured fibroblasts (8), and the paternal *PGK1* allele is weakly expressed in all cultured fibroblasts (9) and in many tissues (10). A similar instability has been observed in the North American opossum (*Didelphis virginiana*): the paternal *G6PD* allele can be expressed, but in many tissues, activity is barely if at all detectable (11).

On the other hand, the special features of XCI in marsupials are also found in extraembryonic tissues of some eutherian mammals. Preferential inactivation of the paternal X has been observed in trophectoderm and primitive endoderm of rodents (12, 13), whereas either X may be inactive in the corresponding human tissues (14, 15). Further, the inactive X is frequently expressed in human trophectoderm derivatives (chorionic villi) (15, 16).

Methylation of the dinucleotide CpG plays a role in XCI, as shown by DNA-mediated transfection (17) and 5-azacytidine-induced reactivation of the hypoxanthine phosphoribosyltransferase (HPRT) locus (18). Although not an exclusive feature of the X chromosome (19), CpGs are "clustered" in the 5′ region of several X-linked housekeeping genes (20–23). Methylation of these 5′ clusters is associated with inactivity of *HPRT* (21, 24, 25), *G6PD* (D. Toniolo, G. Martini, B.R.M., and R. Dono, unpublished data), and *PGK1* (22, 26). Further, the methylation status of clustered CpGs 3′ to *G6PD* is correlated with expression of that locus (27, 28). When alleles on the silent X chromosome are reactivated either spontaneously, as in chorionic villi (15, 16), or by 5-azacytidine, not only does the relevant CpG cluster become less methylated, but the chromatin in the region of the cluster acquires nuclease hypersensitivity (29). Hence, in the absence of DNA methylation within the cluster, the silence of alleles on the inactive X chromosome is not well maintained.

Because the inactive state is so poorly maintained in marsupials, we thought that DNA methylation might not play a role in silencing the paternal X chromosome in these species. Therefore, to better define the role of DNA methylation in XCI, we undertook studies of the North American marsupial *D. virginiana* (the opossum), looking for CpG clusters in the genome as a whole, and for specific ones 5′ to the X-linked housekeeping gene *G6PD*. Using genomic probes for opossum *G6PD* and *HPRT* (30), we compared methylation of alleles on maternal and paternal X chromosomes. We also examined the endonuclease sensitivity of chromatin at the *G6PD* locus, as well as the relationship

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Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase; PGK1, phosphoglycerate kinase A; *G6PD*, *HPRT*, and *PGK1*, genes encoding G6PD, HPRT, and PGK1, respectively; XCI, X chromosome inactivation.

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between methylation status of the CpG cluster and expression of the locus in opossum fibroblasts. We propose a model that might account for the salient differences in dosage compensation between marsupials and eutherians.

## MATERIALS AND METHODS

**Cells and Tissues.** A fibroblast line, established from the ear pinna of a female opossum heterozygous for a rare G6PD variant, was transformed with simian virus 40; the transformed line is designated SV080. Liver, heart, and brain tissues from a litter of pouch young and liver tissue from an adult opossum were obtained immediately postmortem, frozen, and maintained at −70°C.

**Opossum Genomic Probes.** Cloned genomic fragments of opossum *G6PD* (λ-OPUS 1 or 2; ref 30) were subcloned into pUC18 or pEMBL18, recovered by electrophoresis in low-melting-point agarose gel, and radiolabeled by random-primer synthesis as described (30). A 1.5-kilobase (kb) *Hin*dIII fragment 5′ to exon 4 of opossum *HPRT* from cloned probe λ-HOP 1 (30) was isolated by low-melting-gel electrophoresis and radiolabeled without further purification; it contains no coding sequence, as it does not hybridize to human HPRT cDNA.

**Preparation of DNA.** DNA was purified from fibroblasts (31) or frozen tissues (30).

**End-Labeling of *Msp* I and *Hpa* II Fragments.** High molecular weight genomic DNA (1 μg) was digested with restriction endonuclease *Msp* I or *Hpa* II and end-labeled with [$^{32}$P]-dCTP by a modification of the method of Cooper *et al.* (32). The labeled DNA was separated from unincorporated [$^{32}$P]-dCTP by double ethanol precipitation in the presence of mussel glycogen (50 μg/ml) and 2.5 M $NH_4OAc$, resuspended in 10 mM Tris·HCl, pH 8.0/1 mM EDTA, and then fractionated in a 1.2% agarose gel. Wet gels were autoradiographed for 15–30 min at 22°C, using an intensifying screen.

**Restriction Enzyme Digests and Hybridization of Genomic DNA.** High molecular weight DNA was digested, electrophoresed, blotted, and probed as described (30). Filters were washed (30) with 15 mM NaCl/1.5 mM sodium citrate, pH 7, at 65°C (two washes, each 15 min). For studies of tiny fragments described in Fig. 5, high molecular weight DNA (30 μg) was digested first with *Pst* I and then with *Hpa* II or *Msp* I, fractionated in a 2.5% NuSieve agarose (FMC, Rockland, ME)/1.0% BRL agarose (Bethesda Research Laboratories) gel, and immobilized on nylon membranes by UV crosslinkage.

**Preparation of Chromatin.** Nuclei purified from SV080 fibroblasts were incubated with *Msp* I for 30 min at 37°C as described (29). (See Fig. 6.)

## RESULTS

**Tiny *Hpa* II Fragments in the Marsupial Genome.** If there are unmethylated CpG clusters in the marsupial DNA, we expect very small [<200-base-pair (bp)] fragments in DNA digested by *Hpa* II (sensitive to CpG methylation) as well as in DNA digested with *Msp* I (the methyl-insensitive isoschizomer). Fig. 1 shows that the DNA of opossums, like human DNA, has a large number of tiny *Hpa* II fragments, compatible with clustering of nonmethylated CpG dinucleotides. As expected if CpG clusters are present throughout the genome, there are no obvious sex differences in *Hpa* II digests.

**Sex Differences in Methylation of the Body of Housekeeping Genes Are Unrelated to Expression.** We examined tissues from five males and four females and fibroblasts from a female (SV080) for methylation patterns in the body of *G6PD*. The *Hha* I sites shown in Fig. 2 were determined from restriction digests of cloned DNA, and all except sites 2 and 3a were confirmed by DNA sequencing. It is likely that *Hha* I site 3a (Fig. 2, lane b), not present in the cloned DNA, is

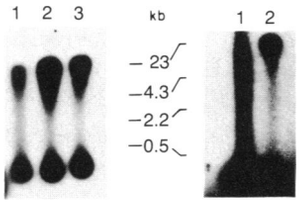


FIG. 1. DNA blot hybridization showing tiny *Hpa* II fragments in the marsupial genome. (*Left*) *Hpa* II-digested female human DNA (lane 1) and female and male opossum DNA (lanes 2 and 3, respectively). (*Right*) Female opossum DNA digested with *Msp* I (lane 1) or *Hpa* II (lane 2). Autoradiography was for 30 min. Fragment sizes of *Hin*dIII-digested bacteriophage λDNA are indicated.

polymorphic, as it was found in the opossum that did not originate from an inbred colony. In liver DNA from females, one of the two *G6PD* alleles is like the active gene in males, being fully methylated at *Hha* I sites 3–5; the other allele is unmethylated to some degree, at *Hha* I sites 1–5 (Fig. 2). The pattern was identical in heart and brain DNA (data not shown). As the female-specific bands are less intense than the others, in some cells the paternal X must be methylated at these sites. *Hpa* II sites in this region are also fully methylated in males, whereas in females, one allele is often less methylated (data not shown).

To look for functional differences in this region, we compared methylation patterns in liver, heart, and brain—

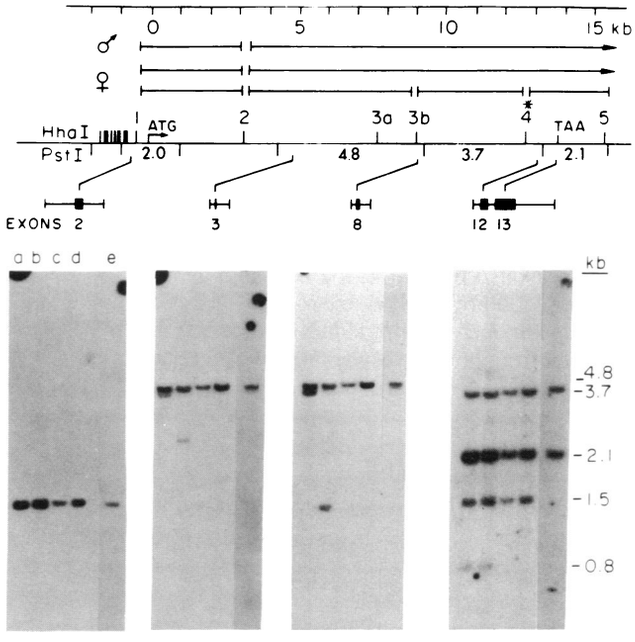


FIG. 2. Blot hybridization of opossum DNA showing female-specific fragments in the body of the gene. Map of the *G6PD* locus shows *Pst* I and *Hha* I sites (*Hha* I sites outside the 5′ cluster are numbered 1–5) and origin of genomic probe for each blot. Black boxes show exons, numbered to correspond with the human homologues (23). Interrupted lines above the map show sites that are unmethylated. Site 2 was shown to be unmethylated in both male and female DNA, based on *Bgl* II/*Hha* I digests (data not shown). Asterisk denotes the female-specific fragments. Samples were *Pst* I/*Hha* I digests of liver DNA from two females (lanes a and b) and two males (lanes c and d) and of SV080 fibroblast DNA (lane e). The blot was first hybridized with the exon 2 probe (autoradiograph at left). Without removal of the exon 2 probe, the filter was rehybridized with the exon 12/13 probe (autoradiograph at right), so that the dosing 1.5-kb fragment (arrow) serves as internal control. The filter was then sequentially washed and reprobed with exon 3 and exon 8 probes (center autoradiographs).

tissues that barely, if at all, express paternal G6PD (11)—with that in fibroblasts (SV080) that clearly express the paternal allele (Fig. 3*B* lane 1). The fibroblasts, cultured from the ear pinna of a female opossum heterozygous for a rare electrophoretic variant of G6PD (G6PD-A), had a normal diploid (22,XX) karyotype, with one late-replicating X chromosome (Fig. 3*A*). Although easily discriminated from its late-replicating homologue, the maternal X has several late-replicating bands. Because there are only two X chromosomes, the predominance of the G6PD-A homodimer relative to the G6PD-B homodimer (Fig. 3B) in all clones derived from this culture means that whereas the *G6PD-A* allele (on the maternal X) is fully expressed, the *G6PD-B* allele (on the paternal X) is partially expressed. The only detectable difference in methylation of the paternal allele in tissues and SV080 fibroblasts involves *Hha* I site 4 (see asterisk in Fig. 2). As the site is paradoxically methylated in the expressing cell, this variation most likely reflects *de novo* DNA methylation that occurs in cultured cells (33) (see *Discussion*).

We also observed sex differences in methylation of the body of the X-linked *HPRT* gene, which codes for the enzyme (30). Using an intron fragment from the opossum gene to examine the region near exon 4, we found no *Msp* I or *Pst* I polymorphisms, and a consistent pattern in *Hpa* II/*Pst* I digests of DNA from liver (Fig. 4) and from heart and brain (data not shown). Fragments common to both sexes most likely come from the active (maternal) X, whereas those found exclusively in females originate from the paternal X. Because the female-specific fragments shown in Fig. 4 are smaller, the paternal gene must be relatively unmethylated in this region of the gene.

**No Sex Differences in the CpG Cluster at the 5′ End of *G6PD*.** The sequence 5′ to exon 2 of the *G6PD* locus is G+C-rich (>60%) with no suppression of CpG dinucleotides; i.e. no. of CpG = no. of GpC (30). In addition, within a 2-kb region, there are 10 *Msp* I/*Hpa* II sites (Fig. 5) and 11 *Hha* I sites (Fig. 2), typical of a CpG cluster (19). We examined this cluster to determine if the striking sex difference seen in methylation of the analogous human *G6PD* CpG cluster (D. Toniolo, G. Martini, B.R.M., and R. Dono, unpublished data) was also present in the opossum gene.

The pRIK probe hybridizes to the 2-kb *Pst* I fragment that includes exon 2 and the 5′ CpG cluster. *Msp* I/*Pst* I digests of DNA from both sexes yield four fragments totaling 1.45 kb (only two of these are visible in Fig. 5, lanes 8 and 9); therefore, as expected from location of *Msp* I sites determined from restriction maps and sequence of the pRIK clone, the remaining 550 bp are cleaved into fragments <200 bp long. If the CpG cluster in opossum *G6PD* were like its human counterpart, it would be extensively methylated on the paternal X but unmethylated on the maternal X. Clearly, this is not the case, as *Hpa* II/*Pst* I digests (lanes 1–7) yielded no fully methylated (2-kb) fragments in DNA of females, and fragments are no larger in females than males. Fig. 5 shows that all bands from females have counterparts in males. The presence of fragments >580 bp indicates that, in males as well as females, this region can be partially methylated. In addition, as the sum of fragment lengths is >2 kb, methylation of this region must be heterogeneous. When this filter

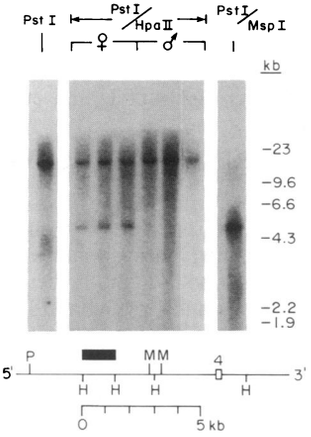


FIG. 4. Blot hybridization of liver DNA showing unmethylated female-specific fragments 5′ to exon 4 of opossum *HPRT*. Map shows location of the *Pst* I (P), *Msp* I (M), and *Hin*dIII (H) sites in the region of exon 4 (open box) and the 1.5-kb *Hin*dIII intron probe (black box). Female DNA was used for *Pst* I and *Pst* I/*Msp* I digests.

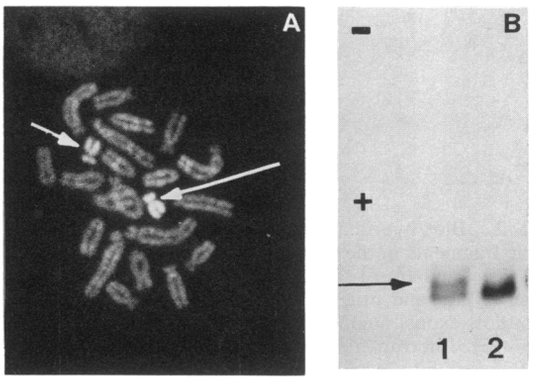


FIG. 3. Karyotype (*A*) and G6PD phenotype (*B*) of SV080 fibroblasts. (*A*) Metaphase (BrdUrd/acridine orange) showing 22 chromosomes, including late-replicating X chromosome (long arrow) and relatively earlier-replicating homologue (short arrow). (*B*) Cellulose acetate electrophoresis (15), showing the predominant maternal G6PD-A homodimer with paternal allele encoding G6PD-B expressed (lane 1) and not expressed (lane 2). The AB heterodimer (arrow) shows that the paternal allele is expressed; as expression is only partial, the B homodimer is barely detectable.

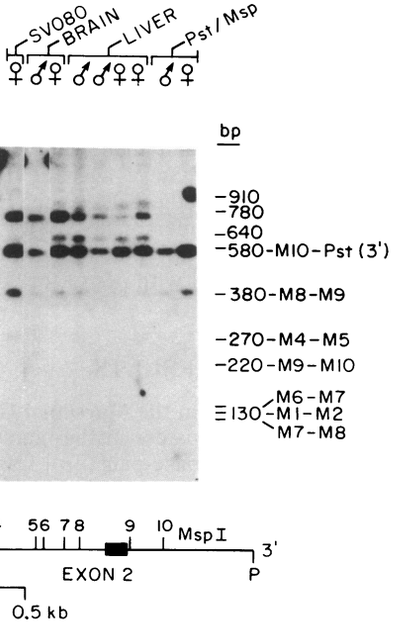


FIG. 5. Blot hybridization showing lack of differential methylation of the 5′ *G6PD* CpG cluster. Liver DNA digested with *Pst* I/*Msp* I (lanes 8 and 9) and liver, brain, and SV080 DNA digested with *Pst* I/*Hpa* II (lanes 1–7) were size-fractionated in NuSieve agarose. Blots were probed with pRIK, a 2-kb *Pst* I fragment that includes exon 2 (black box) and *Msp* I sites, numbered M1–M10 (deduced from sequence). The most likely origin of *Pst* I/*Msp* I fragments is indicated. Fragment sizes were estimated from $\phi$X174 DNA (*Hae* III) markers.

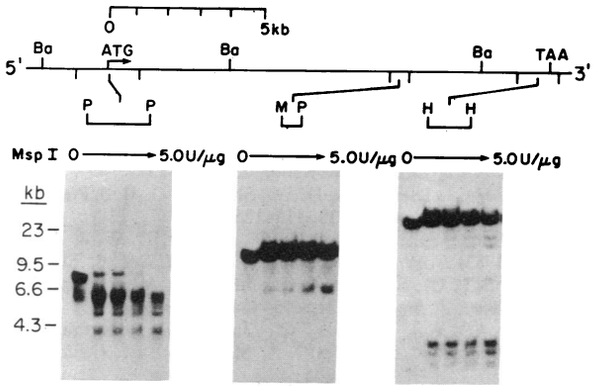


FIG. 6. Nuclease hypersensitivity of G6PD loci on active and inactive X chromosomes. Map shows *Bam*HI (Ba) sites and the *Hha* I (H), *Pst* I (P), and *Msp* I (M) sites that delineate the three probes (shown below the map). Nuclei from SV080 were digested with increasing amounts of *Msp* I [0, 0.5, 1.0, 2.5, or 5.0 units (U)/μg of DNA]. DNA purified from these nuclei was restricted with *Bam*HI, fractionated in a 1.0% agarose gel, and transferred to nylon membranes. The filter was sequentially hybridized with each probe. *Hin*dIII-digested λDNA provided markers.

was reprobed with a 1.1-kb *Pst* I–*Xba* I subfragment of pRIK (30) that excludes *Msp* I sites 9 and 10, not all of the larger *Hpa* II–*Pst* I fragments disappeared (data not shown), indicating that some variability comes from sites 5′ to exon 2.

**Nuclease Sensitivity of Active and Inactive X Chromatin.** In search of differences between the maternal and paternal X, we examined the endonuclease sensitivity of chromatin from the *G6PD* locus in opossum fibroblasts (Fig. 6). At very low concentrations of *Msp* I, both active and inactive X chromatins are hypersensitive to endonuclease digestion in the 5′ region of the gene. The cleavable (hypersensitive) *Msp* I sites and the CpG cluster map to the same region. In chromatin 3′ to the CpG cluster (in the body of the gene), cleavage requires much more enzyme (5 units of *Msp* I per μg of DNA), and approximately half of the *G6PD* loci are insensitive even in the presence of excess enzyme (30 units per μg of DNA) (data not shown).

## DISCUSSION

**Lack of Differential Methylation in Nuclease-Hypersensitive Chromatin.** We have identified a CpG cluster in the 5′ region of the opossum *G6PD* gene and shown that in both alleles, the chromatin of the cluster is nuclease-hypersensitive. Moreover, in contrast to eutherians, the patterns of *Hpa* II methylation in the cluster are similar in males and females, irrespective of the extent of transcriptional activity. The *G6PD* cluster is predominantly unmethylated whether the gene is active, partially expressed, or almost fully repressed.

**Sex Differences in Methylation Are Limited to Insensitive Chromatin Regions and Are Historical Rather Than Functional.** The only sex differences in methylation of opossum *G6PD* occur in the body of the gene, where the paternal allele is less methylated than the maternal one. Several lines of evidence suggest that such differential methylation is not functional: (*i*) differences occur in nuclease-insensitive regions; (*ii*) in eutherians, methylation outside of CpG clusters fails to show a consistent correlation with gene activity or inactivity (21, 24, 25, 27, 34); and (*iii*) methylation patterns in this region persist even when the silent locus is reactivated (15, 21) or the late-replicating chromosome becomes early-replicating (16). Most likely, sex differences in methylation of the body of the gene result from differences in secondary structure of these regions on the active and inactive X at the time of *de novo* methylation and are heritable vestiges of early embryonic events rather than regulatory in nature.

**Role of DNA Methylation in CpG Clusters Is to Stabilize XCI.** Our results clearly show that unstable inactivation of the paternal *G6PD* allele in the opossum is associated with lack of extensive DNA methylation within the 5′ CpG cluster. In this respect, the paternal X in this marsupial resembles the reactivated X chromosome in eutherians; hypomethylation of the relevant CpG clusters is associated with the poorly maintained inactivity of silent alleles in reactivants (21, 24, 25, 27) and human chorionic villi (15) and may explain why DNA from the inactive X in rodent extraembryonic tissues can transfer HPRT activity (35), and autosomal genes transposed to the inactive X can be expressed in these tissues (36). Because clustered CpG dinucleotides are unmethylated, or have been demethylated, in genes that do not "lock in" and stably maintain the inactive state, it seems that the factor that stabilizes XCI in eutherians is DNA methylation within the relevant cluster. Our observations of marsupials implicate DNA methylation of CpG clusters as the final step that "locks in" inactivity of X-linked housekeeping genes in eutherians.

**Other Factors Maintain Silence of the Inactive X.** We have shown that the 5′ *G6PD* cluster is not differentially methylated, even when the paternal allele is severely repressed. That the paternal allele remains silent, even though the cluster is unmethylated, is compelling evidence that mechanisms or factors other than DNA methylation within the cluster maintain transcriptional silence of the locus. These factors may be important for maintaining repression in eutherians as well, but may not be needed once the CpG cluster is methylated. Identifying such factors may be facilitated by the absence of functional DNA methylation in the opossum.

Comparison of the 5′ regions of human and mouse *HPRT*, human *G6PD* and *PGK1*, and opossum *G6PD* (30) reveals no highly conserved sequence other than the 5′ CpG cluster. As homology between X chromosomes of species as distant as human and opossum seems to be limited (30), the opossum model should facilitate the search for conserved X-specific sequences involved in XCI.

**Hypothesis: Species Differences Are Attributable to Timing of Developmental Events.** To explain the paternal XCI in trophectoderm and primitive endoderm of rodents, yet random XCI in the fetus proper, Gartler and Riggs (37) proposed that earlier interactions between mechanisms that imprint the paternal X chromosome and those that initiate dosage compensation would favor inactivation of the paternal X chromosome; the loss of this imprint in tissues that differentiate a few cell generations later would permit randomization of the process. We propose that such differences in timing of developmental events are responsible for many features of XCI that differ among mammalian species (summarized in Fig. 7). For example, differences in the time between initiation of XCI and loss of the paternal X imprint might account for the paternal XCI in extra embryonic tissues of rodents, but random XCI in the analogous human tissues; a slight delay in the initiation of XCI in human trophectoderm relative to that of rodents might permit loss of the imprint and favor random inactivation. Moreover, earlier interactions between imprinting and XCI pathways might lead to paternal XCI in all tissues of the marsupial fetus. Although it is difficult to establish from available evidence, we suspect that XCI in marsupial tissues takes place earlier relative to eutherians, and that differentiation of trophectoderm derivatives in humans is a relatively late event with respect to the disappearance of the paternal X imprint.

In addition, we propose that species and tissue differences in the stability of XCI are also simply a matter of timing: unlike human extraembryonic membranes (16) and oocytes (38), where the process is unstable to the point of being reversible, tissues derived from the embryo proper undergo the additional step that "locks in" and maintains the silence of the inactive X; in tissues that differentiate relatively late

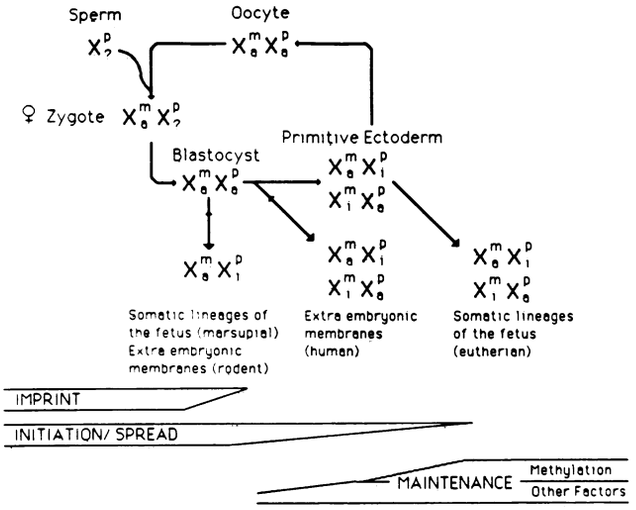


FIG. 7. Model for mammalian XCI. $X_a^m$, $X_i^m$, $X_a^p$, and $X_i^p$ denote active maternal X, inactive maternal X, active paternal X, and inactive paternal X, respectively. The state of transcriptional activity of the paternal X chromosome in sperm and zygote is not known ($X_?^p$). Lines with single arrowheads denote steps that are irreversible, whereas those with two arrowheads may be reversible. Tapered portions of the open bars indicate that the factor has a decreasing (or increasing) influence. The paternal X imprint responsible for paternal XCI may be independent of the dosage-compensation pathway. Not shown is the species variation that most likely exists in the relative timing of sequential steps in the developmental sequence; i.e., in one species, the interval between loss of imprint and onset of *de novo* methylation may be relatively long, but the interval between initiation of XCI and *de novo* methylation may be relatively short.

(39, 40), inactivation is remarkably stable (2). Our observations indicate that DNA methylation of 5′ CpG clusters contributes this stability and suggest that the *de novo* methylation events occur late, relative to the onset of inactivation. That DNA methylation is a late event in the inactivation process is supported by observations that sequences in the 5′ region of the *HPRT* allele on the inactive X in female rodent embryos do not become fully methylated until several days after inactivation is believed to occur (41). The striking undermethylation of CpG clusters in housekeeping genes on the inactive X observed in chorionic villi (15), the earliest human tissues to differentiate, may explain the reversibility of XCI in this tissue (16). We predict that the relevant CpG clusters are unmethylated in female germ cells, as these cells undergo reactivation spontaneously during their ontogeny.

Our studies of marsupials have revealed that the role of DNA methylation in mammalian XCI is more limited than previously suspected, and that other factors silence the inactive X chromosome. Further, the results suggest that the basic mechanism(s) for X dosage compensation is the same for all mammals, including marsupials, but that the features that differ among mammals result from differences in timing of developmental events in the dosage-compensation pathway and interacting developmental pathways.

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