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Insights into X chromosome inactivation from studies of species variation, DNA methylation and replication, and vice versa

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Summary

I am indebted to Mary Lyon as her X-inactivation hypothesis stimulated my mentor, Barton Childs, and in turn, myself, to think about the consequences of X-inactivation in heterozygous females. I often reread her original papers setting forth the single active X hypothesis, and still marvel at the concise and compelling exposition of the hypothesis and the logical predictions which seemed prophetic at my first reading, and have survived the test of time.

My contribution to this Festschrift reviews evidence derived from studies of DNA methylation, species variation and DNA replication that reveals an important role for methylated CpG islands and suggests a role for late DNA replication in propagating X inactivation from one cell to its progeny. These studies also show that X inactivation is a powerful research tool for identifying the factors which program and maintain developmental processes.

1. Introduction

For reasons not well understood, in organisms with the XX/XY and XX/X0 types of sex determination the sex difference in the numbers of X chromosomes has necessitated mechanisms to equalize the dosage of at least some X-linked genes. There are many ways to accomplish this, and compensatory mechanisms range from upping the transcriptional output of the single X in males to silencing one X chromosome in females. However, only mammals compensate by X inactivation. As both marsupials and placental mammals uniquely use X inactivation, the basic mechanism should be the same for all mammals. Therefore, features of X inactivation that differ among mammals are merely variations on a theme.

2. Species variation gives clues to dosage compensation

The molecular basis for events that initiate X inactivation is elusive, occurring at a stage not easily accessible for study, and there are no recognized mutations which interfere with the process. However, the analysis of features of inactivation that differ among mammals has revealed some molecular mechanisms. Such variable features as the choice of inactive X (random or paternal) and stability of inactivation can also differ among tissues of a single mammals. Distinctive features of X inactivation in marsupials include preferential inactivation of the paternal X chromosome and that the paternal allele (on the inactive chromosome) is often expressed along with the maternal one (Cooper *et al.* 1971; Johnston & Robinson, 1985; Samollow *et al.* 1987; Migeon *et al.* 1989). Yet, paternal inactivation also occurs in placental mammals, specifically in some cells of extraembryonic tissues (Frels *et al.* 1980; Takagi & Sasaki, 1975; Harrison, 1988). And the leaky expression of repressed alleles has been observed in the human placenta; unlike cells derived from the embryo proper, which rarely (if ever) derepress silent alleles (Migeon *et al.* 1982*b*; Wolf & Migeon, 1982) many cells cultured from human chorionic villi express both glucose-6-phosphate dehydrogenase (G6PD) alleles (Johnston *et al.* 1978; Migeon *et al.* 1989).

3. Developmental regulation of dosage compensation

We have suggested that variable features of dosage compensation result from differences in the timing of this developmental event. Such temporal differences are influenced by the number of cell generations preceding inactivation, and coincidence with other developmental pathways that interact in some way with X inactivation (Kaslow & Migeon, 1987; Migeon

et al. 1989). Spermatogenesis and implantation of the blastocyst are examples of such interacting pathways. Clearly, the time that inactivation is initiated in the mouse differs among cell lineages, occurring earlier in trophectoderm and endoderm than in the primitive ectoderm which gives rise to the embryo proper (Monk & Harper, 1979), and there is evidence that inactivation in marsupials occurs before the partitioning of the blastocyst into embryonic and extraembryonic regions (Johnston & Robinson, 1987). The occurrence of paternal X inactivation both in marsupials and in extraembryonic tissues of the mouse may be a consequence of the relatively early onset of inactivation in these tissues. Although some genes on the paternal X, like *HPRT*, are expressed very early (Chapman, 1985), others whose activity might be needed to maintain activity of the chromosome may only be expressed later. (The issue of chromosome activation is discussed by Grant & Chapman, 1988 and Lyon & Rastan, 1984.) Perhaps such genes are silenced during spermatogenesis and in the earliest differentiating cell lineages are derepressed only after dosage compensation is initiated. In lineages which initiate inactivation later, either X chromosome (paternal or maternal) can serve as the active X, and inactivation would be random. Although purely hypothetical, such a scenario illustrates at least one way in which differential timing could influence the choice of inactive chromosome.

The degree of stability of inactivation may also depend on the timing of the X inactivation event, as inactivation is less stable in trophectoderm and other early differentiating tissues than in primitive ectoderm which differentiates later; stability could be related to the time when *de novo* DNA methylation patterns are established in the various tissues (see below).

4. DNA methylation is one factor responsible for silencing X-linked genes

It seems likely that many steps are involved in X dosage compensation, and numerous factors must be involved in initiating and maintaining the silence of the inactive X. Yet, molecular studies comparing the two X chromosomes have not detected obvious differences in DNA content or X-specific regulatory sequences. However, they have revealed differences in replication and methylation of X-linked genes. The methylation status of CpG dinucleotides clearly has a role in dosage compensation. Methylation patterns are heritable from cell to cell, maintained by a maintenance methylase (reviewed by Riggs, 1989). These methyltransferases recognize hemimethylated sites formed after DNA replication and convert newly incorporated cytosine to 5-methylcytosine; unmethylated sites on the template strand remain unmethylated as the methylating enzyme(s) prefers hemimethylated sites. Although DNA methylation has been long considered the most likely candidate to silence an X chromosome (reviewed by Gartler & Riggs, 1983), substance for this suggestion comes from evidence that DNA from the inactive X chromosome does not function in DNA-mediated transfection (Liskay & Evans, 1980) and that demethylating agents induce the expression of genes on the inactive X (Mohandas *et al.* 1981). The most compelling evidence that DNA methylation plays a role comes from observations of methylation of X-linked housekeeping genes, namely *HPRT*, *PGK1*, *G6PD*, and neighbouring genes *P3* and *GDX*. CpGs clustered in the promotor regions of constitutively expressed genes, (CpG islands) are the only regions of X-linked housekeeping genes where methylation status is correlated with gene expression. Thus, methylation of CpG islands seems to be the only DNA methylation of consequence for dosage compensation. The CG rich islands on the inactive X are usually methylated, whereas those on the active X are not (Wolf *et al.* 1984*a*, *b*: Yen *et al.* 1984; Wolf & Migeon, 1985; Keith *et al.* 1986; Toniolo *et al.* 1988), and the island is demethylated when the locus reactivates (either spontaneously or induced by 5-azacytidine) (Wolf *et al.* 1984 *a*, *b*; Toniolo *et al.* 1988). Spontaneous reactivation of the *G6PD* locus, extremely rare in most human cells, occurs frequently in cells cultured from human chorionic villi, and is associated with undermethylation of CpG islands in that tissue (Migeon *et al.* 1985).

5. The role of DNA methylation in dosage compensation is to stabilize inactivation

Methylation analysis of X-linked housekeeping genes shows not only that DNA methylation has a role in silencing genes on the inactive X chromosome, but also that the role is to maintain (rather than initiate) the silence of the inactive gene. *De novo* methylation of the 5′CpG island in the murine *HPRT* gene is seen for the first time at least 3 days after inactivation of the locus is thought to occur (Lock *et al.* 1987). Furthermore, the 5′CpG island in the paternal (inactive) *G6PD* allele in the marsupial, *D. virginiana*, is unmethylated (Kaslow & Migeon, 1987), and it is likely that the lack of methylation accounts for the leaky expression of this allele. Although not accessible for study, the CpG island in the opossum's paternal *HPRT* allele probably also is unmethylated as it often spontaneously reactivates in cultured cells (Migeon *et al.* 1989), in striking contract to the reactivation of this locus in human cells (Migeon *et al.* 1982*b*, 1988).

6. Genes that escape inactivation lack methylated CpG islands

Most X-linked genes of placental mammals seem subject to dosage compensation. However, some loci on the inactive X are expressed. Such genes are usually within or in the vicinity of the pseudo-autosomal region of the X chromosome (distal short

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arm) and have functional or once functional homologues on the Y chromosome. *MIC2* (Goodfellow *et al.* 1988), steroid sulfatase (*STS*) (Migeon *et al.* 1982*a*) and *ZFX* (Schneider-Gadicke *et al.* 1989) are three genes shown to be expressed on both X chromosomes. Another locus (*TSA1*) that is expressed on the inactive X (Brown & Willard, 1989) is located in the proximal short arm (at Xq11). These genes may not truly 'escape' inactivation as in no case is the evidence compelling that these genes are expressed to the same degree on active and inactive X; in fact, the *STS* allele on the inactive X is clearly not as active as its homologue (Migeon *et al.* 1982*a*). That these alleles are not fully expressed is probably attributable to the immediate environment of the locus (discussed below) and may reflect the evolutionary history of these genes (discussed by Lyon 1989). One must also consider the possibility that some of the genes shown to be leaky by assays of cultured cells or somatic cell hybrids, may not in fact be expressed *in vivo*. The observed activity may have been induced or enhanced by cell culture conditions; that is, the locus may have been derepressed *in vitro*, like paternal *G6PD* and *HPRT* loci in marsupials (Migeon *et al.* 1989), presumably, because silence of the locus has not been locked in by DNA methylation. Only two of these loci (*MIC2* and *STS*) have been extensively studied, and neither has a methylated CpG island; there are 5′CpG islands in the *MIC2* genes but these are unmethylated in both alleles (Goodfellow *et al.* 1988), like the marsupial *G6PD* genes and CpG islands in autosomal genes. There are no CpG islands within the *STS* locus (Shapiro, L. J., personal communication, 1990) and differences in methylation of alleles on active and inactive X have not been reported. Therefore, what seems to distinguish silent genes from leaky ones is that fully repressed loci have methylated 5′CpG clusters.

7. DNA methylation stabilizes inactivation of X chromosomal domains

Reactivation of loci on the inactive X usually occurs piecemeal so that some genes are expressed, whereas others are not (Kahan & DeMars, 1975; Lester *et al.* 1982; Mohandas *et al.* 1981, 1984). The frequency varies considerably among mammalian cells, perhaps related to species origin, nature of the gene and expressing tissue, effect of growth *in vitro*, and for housekeeping genes, the methylation status of the CpG island. In most cases, the reactivated gene is not fully expressed, and the chromosome remains late replicating (Mohandas *et al.* 1984; Migeon *et al.* 1985). When demethylating agents are used to induce reactivation, more than one locus may be reactivated and the chromosome is extensively demethylated (Toniolo & Migeon, unpublished observations). There is some evidence that reactivation of closely linked genes may occur coordinately, as CpG islands in 3 genes located within a 100 kb segment of DNA have concordant patterns of methylation (Toniolo *et al.* 1988); all three are demethylated when one of them is expressed. Furthermore, expressibility of an X-linked locus seems to be determined autonomously. That the *STS* locus on the inactive X continues to be expressed even when the relevant region of the chromosome is translocated to the vicinity of genes that are fully repressed (Mohandas *et al.* 1987) suggests that the active or repressed state is influenced by chromatin flanking the locus (its domain or buffer zone). When chick or mouse autosomal genes are transfected into the mouse inactive X chromosome (Goldman *et al.* 1988; Krumlauf *et al.* 1986), the transferred gene functions autonomously if enough of the locus is transferred – the flanking sequences serve to maintain the status quo either by means of cis acting inactivators or by buffering the effects of the new genetic environment. In this respect, genes on the mammalian X resemble dosage-compensated Drosophila genes when they are transposed from X to autosomes (reviewed by Lucchesi & Manning, 1987; Gutierrez *et al.* 1989); all the information needed for compensation (hypertranscription) of the locus seems to be contained within the few kilobases flanking the locus. Although the genetic content of the new environment no doubt influences expressibility and modulates the level of expression, at least part of the dosage compensation machinery seems to reside in sequences within or close to the locus.

Such results might be expected if inactivation were maintained regionally through the loop structure of DNA; the numbers of CG rich islands correspond roughly to the numbers of loops of 30-nm DNA fibres approximately 100 kb in length anchored to the nuclear matrix. As these loops may be the units of transcription and replication (reviewed by Goldman, 1988), they might be the units of X inactivation. The methylation of CpG islands could maintain the silence of the DNA within the loop if the islands were situated at the sites where these loops are attached to the nuclear scaffold. In fact, studies of scaffold organization of these islands indicate that these and other CG rich sequences are specifically excluded from the nuclear scaffold, and there are no differences in attachment sites for active and inactive X (Beggs & Migeon, 1989). Even though not attached to the scaffold, CpG islands may influence the transcription and/or replication of genes within these DNA loops, so that loop structure of DNA may still serve to define the domain of inactivated DNA.

8. Maintaining inactivity of tissue specific genes

DNA methylation has a more tangible role in silencing housekeeping genes than those that are not ubiquitously expressed. Consistent sex differences in methylation have not been observed at X-linked loci which lack CpG islands, i.e., blood clotting factor IX (*F9*)

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(Ruta Cullen *et al.* 1986) and ornithine transcarbamylase (*OTC*) (Hannibal *et al.* 1986; Mullins *et al.* 1987). Methylation of *F9* differs from one tissue to another, but both alleles are methylated alike, even in liver which is the expressing tissue (Ruta Cullen *et al.* 1986). Such genes have TATA and CAT boxes, need special tissue-specific factors for expression, and are expressed only in a limited number of tissues. Although not differentially methylated, the alleles on the inactive X are obviously severely repressed; the clinical phenotype of females heterozygous for hemophilia and OTC deficiency indicate that these X-linked tissue-specific loci are subject to X inactivation. However, they may reactivate more easily than housekeeping genes; the age-related instability of *OTC* loci in the mouse (Wareham *et al.* 1987) contrasts with the stability of the *HPRT* locus in human cells (Migeon *et al.* 1988), and may reflect the absence of CpG clusters in tissue specific genes. On the other hand, although the promotor regions of these tissue-specific genes lack CpG islands, their activity might be affected by the methylation status of CpG islands in the vicinity. In any event, the mechanism(s) responsible for inactivating and maintaining the silence of tissue-specific genes remains to be discovered.

9. Lack of DNA methylation in germ cells permits reversal of X inactiva...

Reversal of X inactivation is developmentally programmed and occurs spontaneously only in oogonia during their ontogeny (Gartler *et al.* 1975). One X chromosome in precursors of germ cells undergoes inactivation and is derepressed only after the germ cells arrive in the ovary, coincident with the onset of meiosis in the mouse (Kratzer & Chapman, 1981). The events which reverse the process are unknown, but like X inactivation, it seems to be a global event as the whole chromosome is reprogrammed, and becomes early replicating. There are models for reversal in cultured cells: Reversal can be induced in cells cultured from human chorionic villi by fusion with mouse fibroblasts (Migeon *et al.* 1986), perhaps facilitated by the instability of inactivation in these trophectoderm derivatives. Reversal of inactivation has also been induced in mouse cells by fusion with teratocarcinoma stem cells (Takagi *et al.* 1983).

Germ cells seem uniquely permissive for reversal of the process, and recent evidence suggests that lack of DNA methylation in these cells plays a role. Based on end labelling of mouse genomic DNA, Monk and colleagues (1987) suggested that murine germ cells are markedly hypomethylated. Sanford *et al.* (1985, 1987) found that reiterated DNA sequences were hypomethylated in mouse oocytes. Observations of single copy genes and specific CpGs (HpaII sites) have recently been possible by examining human female germ cells from fetal ovaries of 8–21 wks gestation (Driscoll & Migeon, 1989, 1990). At this stage the ovary contains the greatest numbers of germ cells; some are proliferating (in mitosis), many no longer synthesize DNA as they are undergoing meiosis, and some of these are arrested in diplotene. We found that all of the 57 CpGs (CCGG sites) examined in seven single copy genes (six X-linked and one autosomal locus) were unmethylated in female germ cells. As both chromosomes are active in meiotic germ cells, it is not surprising that CpG islands were unmethylated. However, the methylation status of non clustered CpGs (CpGs outside the islands) was unexpected as these sites which are usually extensively methylated in active genes in somatic tissues were not methylated. What remains to be determined is if the germ cells remain unmethylated throughout their ontogeny or if demethylation plays a role in this process. In any event, the methylation imprint in meiotic female germ cells is erased each generation. It is especially significant that in sperm and adult testes, consisting predominantly of male meiotic germ cells, these non clustered CpGs are methylated as they are in somatic tissues (Driscoll & Migeon, 1990); the striking difference between the methylation status of female and male germ cells furnishes a molecular basis for observations that genes contributed by egg and sperm are not functionally equivalent (Barton *et al.* 1984; Cattanach & Kirk, 1985). The subject of parental imprinting has been discussed by Lyon (1989) and Lyon & Rastan (1984), who suggest that the maternal X imprint might protect the chromosome against subsequent inactivation. The absence of DNA methylation imprint in the oocyte may be ultimately responsible for giving the maternal X chromosome an edge in remaining the active X in the earliest differentiating tissues. In addition, there is little doubt that lack of relevant DNA methylation, and therefore non-fixation of the inactive state in female germ cells facilitates reversal of X inactivation in these cells.

10. Silence of housekeeping genes during spermatogenesis is not maintained by DNA methylation

There is evidence that a process reminiscent of X inactivation occurs during mammalian spermatogenesis. The X and Y chromosomes are isolated from other chromosomes in a sex vesicle, and some genes on the X chromosome have been shown to be repressed (evidence reviewed by Handel, 1987), presumably because of a need to silence some X-linked genetic activity during the production of male gametes. However, this inactivation of X linked genes does not in fact resemble X inactivation in female somatic cells. DNA from sperm can transfer HPRT activity in transfection studies (Venolia & Gartler, 1983) suggesting that it is not modified like the inactive X. Studies of adult male germ cells undergoing spermatogenesis as well as mature sperm show that methylation patterns of housekeeping genes in these

male germ cells are essentially the same as those in most somatic cells, and CpG islands in these genes are unmethylated to the same degree as in active genes of somatic tissues (Driscoll & Migeon, 1989). In male meiotic germ cells, the 5′CpG island in the *PGK-1* locus is also unmethylated. As this locus is inactivated in pachytene spermatocytes and sperm (McCarrey & Thomas, 1987), these observations show that genes can be repressed during spermatogenesis even if the CpG island is unmethylated; they provide further evidence of methylation-independent inactivation. Because such inactivation is not locked in by DNA methylation, it is not surprising that loci inactivated during spermatogenesis are subsequently reactivated.

11. Other factors inactivate genes on the silent X chromosome

There is no evidence that DNA methylation has anything to do with initiation of dosage compensation. In fact, *de novo* methylation of the 5′CpG island in the murine HPRT gene occurs much after inactivation of the locus is thought to occur (Lock *et al.* 1987). In addition, the *G6PD* locus on the opossum paternal (inactive) X is silent even when the CpG island is unmethylated (Kaslow & Migeon, 1987), suggesting that the gene must be repressed by other inhibitors of transcriptional activity. These inhibitory factors remain elusive, but their effects seem to be diluted out by cell culture (Migeon *et al.* 1989) as the expression of the paternal allele in cultured cells equals that of the maternal (active X) allele. One explanation for acquisition of activity in cell culture is that the locus was silenced *in vivo* by tissue factors which can not maintain silence of the locus through mitosis; when these factors are depleted by culture *in vitro*, the locus is derepressed. Many products of differentiated cells are lost in cultured cells (Gilbert & Migeon, 1977). There is evidence that transcription complexes are erased by the DNA replication fork (Wolffe & Brown, 1986), and inhibitor complexes also may not survive. Conceivably, inability of the inactivator to maintain silence of the gene during replication has necessitated some kind of memory device, and DNA methylation serves that purpose. The nature of unstable inactivating molecules remains to be elucidated, and genes, not locked in by DNA methylation provide an excellent model for such studies.

12. Relationships between DNA methylation, DNA replication and transcriptional activity

There is considerable evidence showing a relationship between the time a gene is replicated and its transcriptional activity (reviewed by Holmquist, 1987). Goldman (1988) has proposed that transcriptionally competent domains begin and end their replication during the first half of the S phase, while transcriptionally incompetent domains begin and complete replication during the last half of S. This is based on studies that show that constitutively expressed genes are usually early replicating, whereas genes with tissue specific activity are early replicating in the expressing tissue, but may be late replicating in tissues where they are not expressed (Goldman *et al.* 1984). Late replication has been the most obvious feature that distinguishes the inactive X from the active X and autosomes. Until recently, the fact that the inactive X was late replicating was based entirely on cytogenetic observations of replication of chromosomal bands. Utilizing BrdU sensitive restriction of DNA and differences in the size of DNA fragments from active and inactive X, we found that the *HPRT* locus on the inactive X replicates during the last half of S in contrast with its homolog that replicates during the first half of S (Schmidt & Migeon, 1990). Studies of an X chromosome whose *HPRT* locus had been reactivated by 5-azacytidine indicated that the reactivated gene has become early replicating. In this case the reactivation event was associated with a cytologically visible change in replication of the q26 band (Schmidt *et al.* 1985). Whether a switch to early replication is essential for derepression of a locus remains to be determined. Nor is it clear how demethylation of CpG islands predisposes to changes in the time the locus replicates. It is inviting to speculate that asynchronous replication of the chromosome to be inactivated serves to isolate this chromosome from all the others, thus making it inaccessible to transcription factors that may be available only briefly during a cell cycle. Supporting this hypothesis is the fact that the region in which genes on the inactive chromosome are expressed (Xp22) replicates relatively early. Further studies are needed to determine if replication changes precede the inactivation event, or are merely the consequence of the aggregation of many inactive genes. In any event, Holmquist (1987) has proposed that late replication of any locus could be a repression mechanism and it is conceivable that late replication of a locus on the inactive X (impeding accessibility to specific transcription factors available only in early S) might be another mechanism to perpetuate inactivity of the locus during cell proliferation.

13. Epilogue

Among the important questions for developmental biologists is the role of DNA methylation in cell differentiation. The human genome is extensively methylated, and as a consequence, we have a high frequency of detrimental mutations attributable to CpG dinucleotides (Cooper & Youssoufian, 1988). The function of DNA methylation in our genome has been elusive for a variety of reasons. Although there is abundant evidence that methylation differs in expressed and non-expressed genes, methylation studies of genes with limited tissue expression in most cases has necessitated comparisons of methylation patterns

in separate tissues. As a consequence it has been difficult to determine which sites of differential methylation are functional and which reflect tissue idiosynchronies unrelated to function. Furthermore, that differentiation of a complex organism like the fruit fly occurs without the benefit of DNA methylation has fostered the belief that the role of DNA methylation, if there were one, would be trivial. However, such a view is difficult to reconcile with the price mammals pay for an extensively methylated genome in terms of high mutation rate.

The models used to study X dosage compensation have proved to be useful for the methylation analysis of genes that are active in all tissues; the inactive X provides the means to examine such housekeeping genes in an inactive state. The ability to compare active and inactive genes within the same cell has distinguished methylation that is functional from that which is irrelevant noise. Reactivated genes have shown which differential methylation is essential for expression, and has suggested that a switch to early replication might be needed for reexpression of the locus. Such studies have not only revealed the role of DNA methylation in X inactivation, but have also provided insights as to how DNA methylation functions in cell differentiation. In the process, we have learned a good deal about the regulation of housekeeping genes. These studies have implicated CpG islands as regulators of housekeeping genes, and helped make the distinction that not all genes are created equal...and that constitutively expressed genes have different transcriptional mechanisms than genes that are not ubiquitously expressed.

Studies of methylation in X-linked tissue specific genes show that the tissue specific patterns of methylation, although most likely needed, are not sufficient for function, as the inactive locus has identical patterns as the active one. It is clear from studies of marsupials and mouse embryos that DNA methylation is not the inactivator, but serves to lock in inactivation so that the phenotype (whether active or inactive) is stably transmitted to cell progeny (through mitosis), and there are suggestions that the inactivator needs to be replenished during each cell cycle. In fact, based on these observations, Riggs (1989) has suggested that the greater numbers of cell divisions in the mammalian embryo than occurring in the development of the fly has necessitated cell memory mediated by DNA methylation in mammals, but not in flies. Studies of X inactivation in oocytes have contributed compelling evidence for sex differences in methylation during gametogenesis that implicate DNA methylation in parental imprinting of endogenous genes. One expects that further studies of X chromosome inactivation will provide many additional insights into the nature of factors operating during mammalian embryogenesis.

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