Complete concordance between glucose-6-phosphate dehydrogenase activity and hypomethylation of 3' CpG clusters: implications for X chromosome dosage compensation

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ABSTRACT

To explore the molecular basis of X chromosome inactivation, we have examined the human locus for glucose-6-phosphate dehydrogenase (G6PD) in various human tissues. Studies of DNA from males and females and from somatic cell hybrids with active or inactive X chromosomes, show that two remarkably dense clusters of CpG dinucleotides in the 3' coding sequences are hypomethylated in active G6PD genes but extensively methylated in inactive ones. Reacquisition of G6PD activity, either spontaneous or induced by 5-azacytidine, is accompanied by demethylation of both clusters; however, the clusters remain methylated in reactivants that express HPRT but not G6PD. Our observations implicate these 3' CpG clusters in the transcription of G6PD and in maintenance of dosage compensation for X linked housekeeping genes.

INTRODUCTION

It has been suggested that DNA methylation is responsible for the inactivity of X chromosomes in somatic cells of mammalian females (reviewed in reference 1) and, therefore, is a key factor in achieving dosage compensation for X linked genes (2). Reactivation of alleles on inactive X chromosomes by 5-azacytidine (5-azaC), an inhibitor of methylation (3), lends support to this hypothesis. We have been examining DNA methylation of X chromosomal genes to determine the role it plays in X dosage compensation. Studies of X-linked genes provide one of the best means to examine the relationship between methylation and expression of the locus. Active and inactive versions of the gene can be compared within the same cell. Furthermore, in this way one can observe the inactive version of genes that are constitutively expressed, not usually possible with autosomal genes. Our observations of the X linked hypoxanthine phosphoribosyl transferase (HPRT) locus have revealed that active and inactive genes differ in

their patterns of DNA methylation (4; 5). The most significant difference involves the dense cluster of CpG dinucleotides within the promoter region; This cluster is invariably hypomethylated in active HPRT genes, but extensively methylated in inactive ones (4).

Two clusters of CpG dinucleotides that are remarkably similar to the 5' HPRT cluster, have been found in the 3' region of the glucose-6-phosphate dehydrogenase (G6PD) locus (6) in the Xq28-qter segment of the human X chromosome (7,8). Our observations of active, inactive and reactivated G6PD alleles in a variety of tissues suggest that 3' CpG clusters are important in the regulation of the G6PD locus and consequently, in the maintenance of X chromosome dosage compensation.

MATERIAL AND METHODS

1. Cells and Tissues

Blood and placenta were obtained from normal males and females as previously described (9). Fibroblasts and lymphoblasts from normal males and females were derived from cultures that had been stored in liquid nitrogen. Hybrid cells were derived from 3 matings: Clone81xA9 (10) G1xA9 (4,7) and ExA9 (human dermal fibroblasts of 46 X isodicentric X karyotype, (11). Reactivants included the 6TG^R 7E1-2 and 7E1-4 hybrids previously described (10) that contain the late replicating X from human fibroblast clone81, but not the active X. The G6PD locus on the inactive X chromosome in the hybrids is expressed at approximately half activity. The other reactivants were induced from the ExA9 hybrids having the isodicentric inactive X (but no active X) by treatment with 5×10^{-6} M 5-azaC (3). Reactivant clones 24 and 34 expressed both human HPRT and G6PD while clones 11 and 20 had only human HPRT.

2. Isolation and Analysis of DNA

Methods for purifying DNA from cells and tissues, plasmid purification, nick-translation and Southern analysis were standard and have been described previously (17).

3. Restriction Enzyme Digestion

Complete digests were carried out according to manufacturer's instructions using 10 u/ug DNA for methyl sensitive enzymes, 3-5 u/ug DNA for the rest, and overnight incubations.

Partial digests with HpaII were obtained by adding different amounts of enzyme ranging from 0 - 5 u/ug DNA to a series of samples of DNA that had been completely digested with a methyl insensitive restriction enzyme. Specimens were incubated for 30' at 37°C. The reactions were stopped by adding EDTA to 10 mM.

RESULTS

G6PD Map and Probes

Figure 1 shows the 3' region of the human G6PD locus near the poly A addition site. Sequences 5' to this region have not yet been cloned. The map is based on restriction enzyme and sequence analysis of recombinant phage obtained by screening a genomic library with a G6PD cDNA clone (6,12). A notable feature of this region of the gene are two clusters of CpG dinucleotides at approximately 2.5 kb (A-1 cluster) and 6.5 kb (A-2 cluster) 5' from the poly A addition site. For ease in describing the results, HpaII sites are numbered beginning with the most 5' site that has been identified in cloned fragments.

The probes used in this study are shown in Figure 1. The pGD1.4 probe hybridizes to mature G6PD mRNA. Both pGD3 and pGD1.4 are highly conserved as they hybridize to mouse genomic DNA under stringent conditions, and pGD3 has been used to regionally map the single locus on both human and murine X chromosomes by *in situ* hybridization (8).

CpG Clusters in Active G6PD Alleles of Males are Extensively Unmethylated

Methylation of CpG dinucleotides in DNA from blood, placenta, lymphoblasts and fibroblasts has been determined by cleavage with the methyl sensitive enzyme, HpaII. Less extensive analyses with HhaI are not discussed in detail, but produced similar results. The position of unmethylated HpaII sites has been defined by subsequent digestions with other restriction enzymes and Southern analysis using pGD3 and/or pGD1.4 probes.

The results shown in Figure 2 and summarized in Figure 1B confirm previous studies of male leukocytes (6) and extend observations to DNA from other tissues. The HpaII sites that flank both clusters in active G6PD alleles, are consistently

Figure 1. Map of the 3' coding region of human G6PD showing location of A-1 and A-2 clusters and summary of methylation pattern.

A. The position of sites for methyl sensitive enzymes are indicated. The relevant sites for other enzymes are also shown (P, PstI; S, SstI; Bg, BglI; RI, EcoRI). The dark lines represent the two probes used in this study. The poly A addition site is arrowed.

B. Diagram of representative fragments obtained in HpaII digests of DNA from active X (X^a), inactive X (X^i) and reactivated X. Dots indicate regions where variable methylation was found (see text). Slashes indicate the boundaries of the regions analysed.

(*) All inactive genes in placenta are unmethylated at sites H14 or H15 so that patterns of methylation 5' of those sites can not be examined.

(+) Only one female lymphoblast line was analysed.

unmethylated as shown by the following: HpaII/PstI digests probed with pGD1.4, show that site H36 at the 3' end of the A-1 cluster is open (0.9 kb fragment, Figure 2A lanes 1-4); HpaII/SstI digests probed with pGD3 show that the 5' end of the cluster (site H18) is also unmethylated (data not shown); and HpaII/PstI digests probed with pGD3 indicate that one or the other of sites H14 or H15 at the 3' end of the A-2 cluster

Figure 2. Southern blots showing differential methylation of clusters in males (♂) and females (♀) and tissue specific differences.

HpaII/PstI digested DNA from fibroblasts (lanes 1 and 5) lymphoblasts (lanes 2 and 6), blood (lanes 3 and 7) and placenta (lanes 4 and 8) probed with A) pGD1.4 and B) pGD3

Note: the 5' end of the 0.9 kb fragment is site H36, as shown by the 1.7 kb fragment in HpaII/BglI digests (See Figures 3C and 5A). The relative weaker intensity of the fragments from the inactive X may reflect heterogeneity of inactive X methylation in fibroblasts (See Figures 4,5, and text), whereas in placenta it is attributable at least in part, to hypomethylation of these clusters on the inactive X (See text).

is always unmethylated (2.2 and 2.0 kb fragments, Figure 2B, lanes 1-4).

Because the HpaII sites flanking both clusters are not methylated, partial HpaII digests were used to determine if sites within the clusters were also unmethylated. If many HpaII sites were open, then partial digestion with HpaII should produce multiple fragments. The extent of methylation in the A-1 cluster was determined from DNA that had been completely digested with PstI or BglI, but only partially digested with HpaII, using pGD1.4 as the probe. The A-2 cluster was analyzed similarly but with complete PstI digestion and the pGD3 probe. In these partial digests (Figure 3), we observed an array of fragments, reflecting many unmethylated HpaII sites in the A-1 cluster of active genes from blood, placenta and lymphoblasts, (Figure 3A, lanes 3 and 9; Figure 3C, lanes 2 and 8, respectively). Analysis of the A-2 cluster also revealed multiple bands (Figure

Figure 3. Southern blots showing extent of methylation within A-1 and A-2 clusters.

Lanes 1-6 and 7-12 contain aliquots of DNA incubated with increasing amounts of HpaII (ranging from 0 - 5 u/ug DNA, see Methods).

A. Partial HpaII, complete PstI digest from blood, probed with pGD1.4; B. Same digest, probed with pGD3; C. Partial HpaII, complete BglI digest of lymphoblasts, probed with pGD1.4. The size (kb) and position of λ phage HindIII fragments, included as molecular weight markers, are indicated on the right.

3B, lanes 3 and 10).

Although both clusters in active G6PD alleles are always extensively hypomethylated there are some tissue specific differences in patterns with respect to the extent of methylation

or position of methylated sites within each cluster. Site H15 was frequently methylated in blood, but was often unmethylated in placenta and in cultured cells (Cf. relative intensity of 2.0 and 2.2 kb fragments, Figure 2B, lanes 1-4). Furthermore, differences in the extent of hypomethylation within the A-2 cluster are also apparent as the multiple bands produced by partial HpaII digests are more distinct in blood than in placenta (Figure 3B). On the other hand, tissue specific patterns did not vary among individuals.

Methylation of CpG Clusters in Inactive Alleles is Extensive

HpaII digested DNA from females had all of the fragments observed in males, but additional ones as well. Based on studies of mouse-human hybrid cells (see below), the extra fragments are derived from inactive alleles on the silent X. In these inactive genes the A-1 and A-2 clusters are extensively methylated (Figures 2, 3C, 4 and summarized in 1B). Methylation within the A-1 cluster is evident from the fragments larger than 0.9 kb in HpaII/PstI digests of blood and cultured cells (Figure 2A, lanes 5-7), probed with pGD1.4. However, inactive genes of placental origin frequently appear to be less methylated than in other tissues. For example the band greater than 0.9kb in Figure 2A, lane 8 is barely visible (see below). That the A-2 cluster is methylated was evident from the array of bands larger than 2.0 kb in HpaII/PstI digests probed with pGD3 (Figure 2B, lanes 5-8).

Methylation of Inactive Alleles is Heterogeneous

Although extensively methylated, the inactive genes are methylated in a variety of ways. For example in HpaII/PstI digests probed with pGD3 or pGD1.4, there are multiple bands derived from the inactive G6PD gene (Figure 2). Because each gene should produce no more than one HpaII fragment (See Figure 1), multiple bands indicate that all inactive genes are not methylated the same way.

The relative intensity of bands from the inactive X differs considerably from one tissue to another, so that inactive genes, like active ones show tissue specific methylation of the 3' cluster region (Figures 2-4). Moreover in some tissues the relative intensity of fragments derived from active and inactive

Figure 4. Southern blots showing variable methylation of inactive G6PD alleles.

HpaII/EcoRI digests of (A) blood and (B) placenta, and HhaI digest of (C) blood and (D) placenta, probed with pGD3. The size and position of λ phage HindIII fragments are indicated.

X chromosomes is unequal; too few inactive X fragments are detected (Figures 2A and B, lanes 5 and 8). This may reflect heterogeneous methylation of inactive chromosomes, i.e., the signal is distributed among many fragments and therefore can not be detected. Alternatively, some inactive genes may mimic active ones, i.e., having an open site at the end of a cluster, but extensive methylation within the cluster. Or in some cells the clusters may, in fact, be relatively hypomethylated. The latter is the case for placenta,

Figure 5. Partial HpaII digests of inactive, active and reactivated G6PD alleles, showing extent of methylation within clusters.

GlxA9 hybrid with inactive X (lanes 1-5), ExA9 hybrid with isodicentric inactive X (lanes 6-11), Clone81xA9 hybrid with active X (lanes 12-17), and Clone81xA9 hybrid, (6TG 7E1-4), with reactivated X (lanes 18-22). Size (kb) of genomic fragments is indicated on the right. (Conditions for partial digests are the same as in Figure 3 Legend, except that Lane 18 in Figure 5A contains DNA digested with 0.01 unit HpaII/ug DNA).

A. Partial HpaII/complete BglI, pGD 1.4, probing A-1 cluster.
B. Partial HpaII/complete PstI, pGD3, probing A-2 cluster.

as cells expressing both G6PD alleles have been observed in chorionic villi (Migeon, B.R., Wolf, S.F., Axelman, J., Kaslow, D.C. and Schmidt, M., manuscript submitted).

Furthermore, individuals vary considerably in methylation of inactive genes within the same tissue. This heterogeneity is readily apparent in HpaII/EcoRI digests of blood (Figure 4A) and in HhaI digests of placenta (Figure 4D). Variability, although more subtle, is also found in other digests of blood and placenta (Figures 4B and C). The multiple bands might reflect the heterogeneity of cell populations within the tissue; however, as tissue composition among individuals is relatively similar, the

Figure 6. Cellulose acetate electrophoresis showing G6PD phenotype of reactivants. B, G6PD B; A, G6PD A; M, G6PD mouse (A9). Unlabeled intermediate bands are heterodimers.

Reactivants:

Clone81 fibroblasts (lane 2);

Clone81xA9 hybrid HAT 7E1, with both human X chromosomes (lane 5);

Clone81xA9 hybrid (6TG 7E1-4), with only the reactivated locus (lane 6).

ExA9 clone 24 (lane 10) and clone 34 (lane 13) reexpressing G6PD B.

ExA9 clone 20 expressing human PGK and HPRT, but no human G6PD (lane 9).

Controls:

G6PD B (lane 3), G6PD A (lane 7), G6PD AB (lanes 4, 11, 14) G6PD mouse (lanes 8, 12). G6PD AB 69 XXY fibroblasts (lane 1), showing the AB heterodimer.

extent of variability in the size and intensity of bands among individuals makes it likely that inactive X chromosomes are not identically methylated, even within the same type of cell.

Methylation of Active and Inactive Alleles Separated in Mouse-Human Hybrid Cells

Mouse-human hybrids provide the means to separate the active and inactive chromosomes from female cells and thus confirm the inferences based on sex differences in methylation patterns. The array of bands seen in partial HpaII digests of hybrids containing the active X from a female cell (Figure 5, lanes 12-17) shows that the A-1 and A-2 clusters in active genes from females are as extensively hypomethylated as those from males. Complete HpaII digests of DNA from an additional three hybrids retaining other active X chromosomes, also produced fragments similar to those in males.

Figure 7. Southern blot of reactivant Clone81 and derivative hybrids showing demethylation of HpaII sites

A. HpaII digests probed with pGD3. Fibroblast clone with active G6PD B allele, but inactive G6PD A (lane 1). Clone81 of same origin as clone in lane 1, but with both alleles active (lane 2). Clone81xA9 hybrid, (HAT 7E1) retaining both active and inactive loci (lane 3), two derivative 6TG hybrids (7E1-2, 7E1-4) with only the reactivated locus (lanes 4, 5) and another derivative 6TG hybrid (7E1-3) with no human X chromosome (lane 6). Note: From HpaII/SstI digests it is clear that the 3.2 and 3.0 kb fragments in lanes 1-5 result from unmethylated sites at H14 or H15 and H18 and that the 3.5 kb fragment in lane 1 results from methylation of H15 to H35. Thus the reactivated allele in clone81 differs from the same allele when inactive (lane 1) and resembles active genes.

B. HpaII/PstI digests probed with pGD1.4. GlxA9 hybrid with human active X (lane 1), ExA9 hybrid with human inactive X (lane 2), Clone81xA9 hybrid with active X (lane 3), Clone81xA9 hybrid 6TG 7E1-4 with reactivated X (lane 4), and mouse A9 (lane 5).

The faint bands at 4.4 and 0.9 kb are from homologous mouse sequences (M).

The inactive X was also examined in two hybrids, each with a different inactive human X chromosome. One (ExA9) contained an isodicentric X chromosome with two inactive loci. All three inactive alleles were extensively methylated in both clusters (Figure 5, lanes 1-11). Some of the inactive X chromosomes were methylated differently than others (Figure 5, lane 11), so that the variability observed in female cells is also present in hybrids.

Figure 8. Analysis of the A-1 cluster using partial HpaII digests of DNA from 5-azaC induced HPRT reactants.

Lanes 1-5 contain DNA with increasing amounts of HpaII, (see Figure 3). M, homologous mouse sequences.

A. ExA9 Clone 24 also expressing G6PD B, HpaII/BglI.

B. ExA9 Clone 20, not expressing human G6PD, HpaII/PstI.

Methylation Patterns of Reactivated G6PD Alleles

To assess the functional importance of methylation in the A-1 and A-2 clusters, we analyzed G6PD alleles that had been reactivated. In one case, the reactivation event occurred spontaneously in an otherwise normal clone of human fibroblasts (Clone81, 10). In these cells the G6PD A allele on the inactive X had approximately half wild type activity (Figure 6, lane 2), but neither the PgK locus (10) nor the HPRT locus (4) is expressed. The mouse-human hybrids retaining the relevant X chromosome (Figure 6, lanes 4 and 5) have been described (10). Figure 7 shows that in reactant Clone81 (Figure 7A, lane 2) and derivative hybrids, (Figure 7A, lanes 5 and 6; 7B, lane 4) the HpaII sites flanking the A-1 cluster had been demethylated. Partial HpaII digests of DNA from one of these hybrids (6TG 7E1-4), revealed that both clusters were extensively unmethylated (Figure 5, lanes 18-22).

G6PD reactants induced by 5-azaC were also examined. The two reactants (ExA9 clones 24 and 34) were derived from hybrid cells retaining only the inactive X chromosome, by treatment with 5-azaC and selection in HAT medium. They were identified by screening for the presence of human G6PD (Figure 6, lanes 10 and 13). Because the human inactive X chromosome in these hybrids was an isodicentric X with two copies of the G6PD locus, it is not certain if one or both of the loci had been reactiva-

ted. Nevertheless, the A-1 cluster in both G6PD reactivants had been extensively demethylated (one is shown in Figure 8A) and at least several sites in the A-2 cluster had been demethylated (data not shown). As controls, we examined 2 sibling reactivants- (Clones 11 and 20), and 4 independent reactivants derived from GlxA9 hybrids in which 5-azaC had reactivated HPRT but not G6PD (one is shown in Figure 8B). In each of them the inactive G6PD alleles were methylated throughout the clusters, although relevant demethylation had occurred at the HPRT locus (4 and unpublished results).

DISCUSSION

Hypomethylation of Clusters is Correlated with Expression

Although hypomethylation has been associated with expression of many genes, the correlation is not absolute, nor has a causal relationship been clearly established (reviewed in reference 14). The discordant observations may be attributable to the heterogeneous nature of the loci and tissues in which they were examined. We have studied the relationship between DNA methylation and expression of a gene that is constitutively transcribed by RNA polymerase II. The ability to examine inactive versions of this housekeeping gene, i.e. inactive alleles on the silent X chromosome, has revealed features of the methylation pattern that are associated with activity of the locus. By comparing active and inactive genes within the same cell, functional differences have been discriminated from those that reflect tissue specific variation. Studies of reactivants that reexpress the gene on the silent X, indicate that the features of the pattern presumed important for expression of the locus, are indeed functional, as they directly correlate with reacquisition of activity. Because the two CpG clusters in the 3' coding region are always hypomethylated in active G6PD genes, largely methylated in inactive genes, and demethylated in reactivated genes, hypomethylation of these clusters is clearly a significant component of the mechanisms that maintain the activity of the G6PD locus.

The Role of CpG Clusters in Transcription

Sequencing of the housekeeping genes, dihydrofolate reductase (DHFR) (15) and HPRT (16) has revealed unusual features of their

promoter regions; TATA and CAT sequences are absent or ectopic, whereas, dense clusters of CpG dinucleotides are present. Comparison of active and inactive HPRT alleles indicate that these 5' clusters have a role in regulating expression of that locus (4). From similar studies of G6PD alleles, we have shown that dense clusters of CpG dinucleotides in the 3' region upstream of the poly A addition site, play an important role in expression of this locus.

Our observations of HPRT and G6PD loci clearly demonstrate the concordance between hypomethylation of these clusters and expression of the relevant housekeeping gene. The nature and location of these clusters suggests several hypothesis regarding their function, that could be further explored: (1) The 3' clusters may be involved in proper termination. (2) CpG clusters may influence the architecture of the region. In fact, both 5' HPRT and 3' G6PD clusters are hypersensitive to nuclease digestion, but only if they are hypomethylated (manuscript in preparation). (3) These clusters may serve as enhancer sequences. Tracts of alternating purines and pyrimidines are present not only in the G6PD clusters, but also in the 5' clusters in the promoter regions of HPRT (26) and DHFR (15).

Although the precise relationship between these clusters and the transcriptional machinery is not known, the existence of clustered CpGs suggests that these dinucleotides may function cooperatively. Clustering of CpGs might ensure that de novo methylation has little effect as chance methylation of a few sites within the cluster is unlikely to silence the active G6PD gene.

Implications for X Inactivation

Because the inactive X chromosome at interphase is so distinctive, most models for dosage compensation assume that the silent X has special features that are responsible for its inactivity (reviewed in reference 1). We have been searching for a specific pattern of DNA methylation that might serve to maintain the silence of the inactive X chromosome, but have identified none. Our previous studies of methylation of active and inactive X chromosomes using random DNA probes did not reveal significant differences in methylation between the two chromosomes (17,18).

For both chromosomes, the pattern of methylation in a 22 kb region of DNA was variable, even within clonal populations of cells. Although, we have identified important differences in methylation of the homologous HPRT loci on active and inactive X chromosomes (4), it was the active genes that had the consensus pattern, namely hypomethylation in the 5' CpG cluster and extensive methylation distal to the promoter; the inactive genes had clusters that were methylated, but otherwise showed the same variability we had observed with the random probes. Because inactive HPRT genes lack a consensus pattern, we suggested that X dosage compensation was maintained by propagating the activity of the active X, rather than silencing the inactive one. Furthermore, because the consensus pattern of methylation that characterized active HPRT genes resembled those observed for the two autosomal housekeeping genes that have been analyzed (19), it seemed likely that expression of the locus on the active X was maintained in the same way as that of housekeeping genes on autosomes.

The 3' CpG clusters from active G6PD genes are also consistently hypomethylated. Furthermore, none of the features of G6PD methylation that we have identified can be attributed a special role in maintaining X chromosome inactivation. Although the 3' clusters of CpGs are unusual, studies to date do not preclude their presence in other housekeeping genes. Moreover, our studies of clone81 reactivants show that hypomethylation of these clusters is specific for expression of the G6PD locus; other loci on the inactive chromosome remain inactive and that chromosome remains late replicating (10, 20). In all the 5-azaC induced reactivants that were selected for HPRT activity, the HPRT cluster was hypomethylated; however, the 3' G6PD clusters were hypomethylated only in those that also expressed G6PD. Therefore, it seems that reexpression is controlled locus by locus rather than by some master switch. The reactivation event is invariably associated with acquisition of the methylation pattern that characterizes the gene on the active chromosome. These observations enhance the likelihood that X chromosome dosage compensation is transmitted from one cell to its progeny by maintaining transcriptional activity of the individual gene on the active X.

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