5-Azadeoxycytidine-induced Chromatin Remodeling of the Inactive X-linked HPRT Gene Promoter Occurs prior to Transcription Factor Binding and Gene Reactivation*

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During the process of 5-aza-2'-deoxycytidine (5aCdr)-induced reactivation of the X-linked human hypoxanthine phosphoribosyltransferase (HPRT) gene on the inactive X chromosome, acquisition of a nucleo-sensitive chromatin conformation in the 5' region occurs before the appearance of HPRT mRNA. In vivo footprinting experiments reported here show that the 5aCdr-induced change in HPRT chromatin structure precedes the appearance of three footprints in the intermediate 5' flanking region that are characteristic of the active HPRT allele. These and other data suggest the following sequence of events that lead to the reactivation of the HPRT gene after 5aCdr treatment: (a) hemi-demethylation of the promoter, (b) an "opening" of chromatin structure detectable as increased nuclease sensitivity, (c) transcription factor binding to the promoter, (d) assembly of the transcription complex, and (e) synthesis of HPRT RNA. This sequence of events supports the view that inactive X-linked genes are silenced by a repressive chromatin structure that prevents the binding of transcriptional activators to the promoter.

A unique system of differential gene expression in mammals is established during female embryogenesis by X chromosome inactivation (1, 2). The inactivation of one X chromosome within each female somatic nucleus generates a transcriptionally active and inactive allele of most X-linked genes and results in dosage compensation for X-linked genes between males and females. A variety of molecular mechanisms have been implicated in regulating the initiation, spreading, and maintenance of X inactivation (1–7). The involvement of DNA methylation in this process has been established by studies using methyl-sensitive restriction enzymes (8–10), DNA-mediated demethylation in this process has been established by studies using methyl-sensitive restriction enzymes (8–10), DNA-mediated demethylation and alteration of chromatin structure (11–13), genomic sequencing (14–16), and the DNA demethylating agent 5-aza-2'-deoxycytidine (5aCdr). All of these studies support the notion that hypermethylation of the 5' CpG island is involved in the transcriptional silencing of these genes on the inactive X chromosome.

The ability to demethylate and reactivate individual genes on the human inactive X chromosome in rodent-human somatic cell hybrids by treatment with 5-aza-2'-deoxycytidine (5aCdr) (6, 20) suggests that transcriptional regulation of X-linked genes by X chromosome inactivation involves some measure of local control either at the level of individual genes or at the level of chromatin domains. Reactivation of inactive X-linked genes such as the hypoxanthine phosphoribosyltransferase (HPRT) and phosphoglycerate kinase (PGK-1) genes after 5-aza-2'-deoxycytidine or 5aCdr treatment is associated with both a change in chromatin structure from a nuclease-inaccessible to a nuclease-accessible conformation and a reduction in DNA methylation levels in the 5' CpG island (17, 21).

In previous studies, Sasaki et al. (22) assayed four parameters during 5aCdr reactivation of the human HPRT gene in a hamster-human somatic cell hybrid cell line (X8–6T2) containing the inactive human X chromosome. The parameters examined were HPRT mRNA levels and three properties of the 5' region, including hemi- and symmetrical demethylation of DNA, and Msp1 nucleosome sensitivity of chromatin. Hemi-demethylation and Msp1 sensitivity were detectable 6 h after the addition of 5aCdr and reached maximum levels at 24 h, whereas symmetrical demethylation and HPRT mRNA levels became detectable at 24 h and reached maximum levels 48 h after exposure to 5aCdr. Thus, the initial events during reactivation of the HPRT gene by 5aCdr treatment are the hemi-demethylation and alteration of chromatin structure in the promoter region, followed by symmetrical demethylation and transcription of the gene. A similar sequence of events is reported for 5aCdr-mediated reactivation of the mouse APRT gene (23). The major question we address here is whether the binding of transcription factors to the promoter region upon 5aCdr reactivation is correlated with the early change in chromatin structure or with actual transcription of the gene (i.e., appearance of mRNA).

Analysis by in vivo footprinting shows that the promoters of the active HPRT (24) and PGK-1 (7, 15) alleles are bound by transcription factors, whereas the promoters of the correspondingly inactive alleles are devoid of these factors. On the active human HPRT allele, in vivo footprints are associated with each of five potential Sp1 binding sites, a potential AP2 binding site, and a region near the multiple transcription initiation sites.

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(24). For both the HPRT and PGK-1 genes, no evidence has been found for the binding of sequence-specific repressors to the promoters of the inactive alleles. Furthermore, there is no evidence for the interaction of methylated DNA-binding proteins (25) with the 5′ regions of these genes on the inactive X chromosome. These in vivo footprinting studies indicate that a major component of transcriptional silencing on the inactive X chromosome is the exclusion of transcription factors from promoter regions.

To determine the timing of transcription factor binding during 5aCdr-induced reactivation of the human HPRT gene on the inactive X chromosome, we have performed dimethyl sulfate (DMS) in vivo footprinting on X8–6T2 cells at various times after initiating 5aCdr treatment. We now demonstrate that the binding of transcription factors to three sites in the promoter region correlates with the appearance of HPRT mRNA rather than with the preceding change in nucleosome sensitivity of chromatin. Thus, the remodeling of chromatin structure during 5aCdr reactivation of the HPRT gene on the inactive X chromosome precedes and thus does not require the binding of at least three sequence-specific transcription factors to the promoter region.

EXPERIMENTAL PROCEDURES

Cell Lines—DNA samples were prepared from cultures of cell lines described previously (24, 26). Briefly, GM00468 (National Institute of General Medical Sciences Human Genetic Mutant Cell Repository) is a normal diploid human male fibroblast cell line containing an active X chromosome. Cell line 4.12 (generously provided by David Ledbetter) is a hamster-human somatic hybrid cell containing only the active human X chromosome in the HPRT-deficient hamster cell line RJK88; RJK88 carries a deletion of the endogenous hamster HPRT gene. GM06318 is also a human-rodent somatic hybrid cell containing an active human X chromosome. Cell lines X8–6T2 and 8121 are hamster-human somatic hybrids containing an inactive human X chromosome (20, 27, 28). 5aCdr Treatment—X8–6T2 cells were treated with 5aCdr as described previously by Sasaki et al. (22). Briefly, cells were treated with 0.4 μg/ml 5aCdr in growth medium (RPMI 1640 medium with 10% fetal bovine serum and 40 μg/ml gentamicin) for 24 h. Cells were then washed with phosphate-buffered saline and returned to normal medium.

MspI Treatment of Chromatin—Assaying chromatin structure changes during 5aCdr reactivation was performed by nuclease digestion of isolated nuclei as described by Sasaki et al. using the restriction enzyme MspI (22). Briefly, nuclei were isolated from X8–6T2 cells 0, 12, 24, 32, 48, and 60 h after initiating treatment with 5aCdr. Nuclei from each time point were treated with 0, 200, and 600 units/ml MspI, and genomic DNA was isolated and digested further as before and then subjected to Southern blot analysis with human HPRT hybridization probe PB1.7. The approximate positions of the clustered MspI sites, the PB1.7 hybridization probe, the 1.6-kb MspI-PstI sub-band, and the transcription factor binding sites are shown in Fig. 1.

Quantitation of hybridization signals in Southern blots was performed by PhosphorImager analysis as described previously. The radioactivity in each 794-bp HPRT RT-PCR product was quantitated by PhosphorImager analysis for each 5aCdr-treated sample (at 0, 12, 24, 32, 48, and 60 h) using 0.5 μg of RNA in the RT-PCR reaction and averaging the results of duplicate RT-PCR reactions. The relative levels of human HPRT mRNA in representative samples on the Southern blot were calculated in Fig. 6 as follows: percentage maximal = 100 × (units 5aCdr – background/micrograms of RNA)/(units GM06318 – background/micrograms of RNA). Units 5aCdr is the PhosphorImager units of the 794-bp HPRT RT-PCR product, background is the average PhosphorImager units at seven separate points on the autoradiogram that did not contain experimental samples, micrograms of RNA is the micrograms of RNA used in the RT-PCR reaction (0.5 μg of RNA was used for all calculations), and units GM06318 is the number of PhosphorImager units of the GM06318 sample after the total RNA for the GM06318 sample was normalized to that of the experimental samples according to the amount of MUC2 RT-PCR product. PHRT values for the GM06318 sample were considered to represent 100% reactivation.

Preparation of DNA: In Vitro DMS Treatment and DNA Isolation—DMS treatment of cells, DNA purification, and piperidine cleavage of DMS-treated DNA were performed essentially as described previously (24, 29). 5aCdr-treated cells were footprinted in vivo by treatment with 0.2% DMS for 5 min in RPMI growth medium.

Ligation-mediated PCR (LMPCR) and Detection of in Vivo Footprints—LMPCR was carried out essentially as described by Hornstra and Yang (24, 26, 29). For LMPCR, primer set E was used to analyze the upper strand encompassing the −91 footprint, and primer set C was used to analyze the upper strand in the region of the GC boxes, as described previously (21) in vivo footprinting of the human HPRT gene (24). The position of the LMPCR primers (and the regions they analyze) and the location of the footprinted sites used in this study are shown in Fig. 1. Reaction conditions for first-strand synthesis, ligation, and PCR amplification were identical to those described previously (26).

Subsequent gel electrophoresis and electrophoretic blotting were carried out as described previously, using a 5% Long Ranger gel (AT Biochem) substituted for the standard polyacrylamide DNA sequencing gel (24, 229).
The major 1.6-kb fragment (MspI were digested with MspI) reached maximal sensitivity to digestion with MspI after initiating treatment with 5aCdr. Thus, the major chromatin structure changes in the HPRT gene 5’ region during 5aCdr-induced Reactivation of the HPRT Gene—Discreet reactivation of the HPRT gene, treated and untreated X8–6T2 nuclei were digested with MspI as described previously by Sasaki et al. (22). As shown in Fig. 2, sensitivity of chromatin in the 5’ region of the human HPRT gene was monitored for changes in chromatin structure, appearance of human HPRT mRNA, and binding of transcription factors to the human HPRT gene promoter in 5aCdr-treated cells can be readily detected by the increased accessibility of MspI sites in chromatin to cleavage by MspI, occurring within 12–24 h of exposure to 5aCdr. These results are similar to those reported by Sasaki et al. (22) in which maximal sensitivity of chromatin to MspI digestion was achieved by 24 h after initiating 5aCdr treatment.

Appearance of Human HPRT mRNA during 5aCdr-induced Reactivation of the HPRT Gene—The appearance of human HPRT mRNA in the same populations of 5aCdr-treated X8–6T2 cells was assayed by RT-PCR of total RNA. Fig. 3 shows Southern blot analysis of human HPRT RT-PCR products amplified from total RNA of 5aCdr-treated samples using a radio-labeled human HPRT cDNA hybridization probe. Human HPRT mRNA first became detectable at 24 h after the addition of 5aCdr and reached maximal levels at 60 h, when the experiment was terminated. Thus, detectable HPRT mRNA levels did not begin to appear until the chromatin structure of the 5’ region had nearly reached its maximal sensitivity to MspI, an observation similar to that of Sasaki et al. (22).

Binding of Transcription Factors during 5aCdr-induced Reactivation of the HPRT Gene—The binding of transcription factors to the 5’ region during reactivation of the inactive HPRT gene by 5aCdr treatment was assayed by LMPCR in vivo footprinting (24, 26, 29). In previous studies, an in vivo DMS footprint in the 5’ region of the human HPRT gene was detected at position −91 (relative to the translation start site) on the active human X chromosome (24); this footprint was not detected on the inactive HPRT allele. The identical footprint was also observed in 5-azacytidine-treated cells that were hypoxanthine-, aminopterin-, and thymidine-containing medium-selected for reactivation of the human HPRT gene on the inactive X chromosome (24). This footprint is characterized by a band of strongly enhanced autoradiographic intensity at position −91 in the guanine-specific DNA sequencing ladder (as compared with the intensity of the same band in the transcriptionally active allele or in naked DNA), indicative of a very DMS-reactive guanine residue on the active allele due to the binding of a transcription factor in vivo.
The figure shows an autoradiogram of in vivo footprint analysis of the HPRT GC box region after treatment of X8–6T2 cells with 5aCdr using LMPCR primer C (24). Lane 1, no 5aCdr treatment; lane 2, 12 h after initiating 5aCdr treatment; lane 3, 24 h after initiating 5aCdr treatment; lane 4, 32 h after initiating 5aCdr treatment; lane 5, 48 h after initiating 5aCdr treatment; lane 6, 60 h after initiating 5aCdr treatment; lane 7, 4,12 cells; lane 8, GM00468 cells; lane 9, 8121 cells. Arrows indicate positions of enhanced in vivo DMS reactivity (in vivo footprints) on the active HPRT allele, and numbers indicate nucleotide position relative to the translation start site.

The data also indicate that the binding of transcription factors at positions −198 and −210 (most likely Sp1) occurs late in the process of 5aCdr-mediated reactivation (well after maximal levels of nuclease sensitivity have been achieved at 24–32 h) and correlates more closely in time with active transcription of the HPRT gene rather than alteration in the chromatin structure of the HPRT locus.

In contrast, the site of enhanced DMS reactivity at position −163 in the GC box region does not exhibit a clear increase in intensity (relative to the adjacent band at position −164) during the course of 5aCdr reactivation (data not shown). This site is not as strongly footprinted in cells that express HPRT fully (24), and the percentage of reactivated cells at any of the time points examined after 5aCdr treatment is relatively low. This is also true for the AP2 site and the remaining Sp1 sites. Therefore, the inability to demonstrate clear evidence of these footprints during 5aCdr reactivation most likely reflects limitations on the sensitivity of the in vivo footprinting assay at these sites.

Summary of Nuclease Sensitivity, HPRT mRNA, and Transcription Factor Binding—A graphical summary of the events following 5aCdr treatment of the inactive X hybrid is shown in Fig. 6, in which chromatin structure (nuclease sensitivity), transcription factor binding at positions −91, −198, and −210, and HPRT mRNA levels are plotted as a function of time after initiating 5aCdr treatment. The appearance of the −91, −198, and −210 footprints are correlated with the appearance of HPRT mRNA rather than with the earlier change in chromatin structure. This change in chromatin structure, therefore, does not require binding of a factor(s) to the −91 region, a region that is near the multiple sites of transcription initiation and in a location similar to regions previously reported to be critical for silencing other genes by DNA methylation (33, 34).
5-Azadeoxycytidine Reactivation of the HPRT Gene

data demonstrate that chromatin remodeling in response to 5aCdr treatment does not require transcription factor binding to multiple transcription factor binding sites in the HPRT promoter region.

DISCUSSION

The 5aCdr-induced reactivation of the inactive X-linked HPRT gene involves an initial hemi-demethylation of the promoter region that is associated with a change in chromatin from a nuclease-resistant to a nuclease-sensitive structure. After the alteration in chromatin structure, symmetrical demethylation occurs and HPRT mRNA appears (22). We show here that transcription factor binding to at least three sites in the promoter region is correlated with the appearance of HPRT mRNA rather than with the preceding remodeling of chromatin structure.

From this sequence of events, we propose that the change in chromatin structure of the 5' region as a result of 5aCdr treatment does not require transcription factor binding in the immediate promoter region (footprints associated with the -91, -198, and -210 sites). Reactivation of HPRT apparently requires a 5aCdr-induced remodeling of chromatin structure such that DNA binding sites in the promoter region become accessible to transcriptional activators. The binding of these activators, which are known to be present in the nucleus before 5aCdr treatment (and are bound to the active HPRT allele in female cells), would then affect further changes in chromatin structure of the promoter region that potentiate transcriptional activity (e.g., an alteration in the nucleosomal structure). The primary mechanism by which DNA methylation maintains the silence of the HPRT gene on the inactive X chromosome may therefore involve a role in organizing or stabilizing chromatin into a conformation that prevents the accessibility of transcriptional activators or otherwise precludes their binding to DNA.

Although we were only able to analyze three of the seven sequence-specific footprints characteristic of the transcriptionally active HPRT gene (24), reports by Martinez-Balbas et al. (35) and Lee and Garrard (36) that nuclease hypersensitivity is independent of transcription factor binding suggests that if it were possible to analyze the remaining footprints, these would also appear after the induction of chromatin remodeling by 5aCdr treatment. It is possible that DNA-binding proteins not detected by our earlier DMS in vivo footprinting studies of the promoter region of the active and inactive HPRT alleles could be responsible for remodeling of chromatin structure in response to 5aCdr. However, recent DNase I in vivo footprinting of the human HPRT gene on the active and inactive X chromosome shows no evidence for sequence-specific DNA-protein interactions on the upper strand between positions -77 and -227 on the inactive allele, nor does it show additional footprints in regions not previously footprinted by DMS on the active allele (24).2 These DNase I studies are consistent with our original findings that only the GC boxes, AP2 site, and the transcription initiation region are bound by factors on the active HPRT allele and that no DNA-binding proteins are found on the inactive allele in this region (including methyl DNA-binding proteins). The same conclusions were reached by Pfeifer and Riggs (7) and Pfeifer et al. (15) in similar in vivo footprinting studies of the X-linked human PGK-1 gene using DMS and Dnase I.

It has been postulated that DNA methylation affects transcription by directly altering the interaction of DNA-binding regulatory proteins with their binding sites (37) or by altering chromatin structure and secondarily altering sequence-specific DNA-protein interactions (38, 39). Recent studies of high-resolution methylation patterns in the human HPRT gene and the human and mouse PGK-1 gene 5' regions suggest that direct, methylation-induced alteration of sequence-specific DNA-protein interactions in the promoter region is unlikely to be the primary mechanism by which X-linked genes are silenced by DNA methylation. In these studies methylation patterns in the 5' region of the human and mouse PGK-1 genes (15, 16, 34) and human and mouse HPRT genes (14, 40) reveal no strict correlation between methylated CpG dinucleotides and binding sites for transcriptional activators and no discernible conserved pattern of CpG methylation among the inactive alleles of these genes (other than a generally higher level of methylation compared with the active allele). In particular, the methylation pattern seen in the mouse PGK-1 gene makes it unlikely that DNA methylation functions by directly modifying individual interactions between transcriptional activators and their DNA binding sites in the immediate promoter because only a single CpG dinucleotide is fully methylated on the inactive X chromosome (16). The results we report here support the concept that DNA methylation and demethylation primarily affect chromatin structure and secondarily (as a result of changes in chromatin structure) influence sequence-specific DNA-protein interactions of the promoter region within native chromatin.

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5-Azadeoxycytidine Reactivation of the HPRT Gene

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5-Azadeoxycytidine-induced Chromatin Remodeling of the Inactive X-linked HPRT Gene Promoter Occurs prior to Transcription Factor Binding and Gene Reactivation

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