Evidence That Silencing of the HPRT Promoter by DNA Methylation Is Mediated by Critical CpG Sites*

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The strong correlation between promoter hypermethylation and gene silencing suggests that promoter methylation represses transcription. To identify methylation sites that may be critical for maintaining repression of the human HPRT gene, we treated human/hamster hybrid cells containing an inactive human X chromosome with the DNA demethylating agent 5-azadeoxycytidine (5aCdr), and we then examined the high resolution methylation pattern of the HPRT promoter in single cell-derived lines. Reactivation of HPRT correlated with complete promoter demethylation. In contrast, the 61 5aCdr-treated clones that failed to reactivate HPRT exhibited sporadic promoter demethylation. However, three specific CpG sites remained methylated in all unreactivated clones, suggesting these sites may be critical for maintaining transcriptional silencing of the HPRT gene. Re-treatment of partially demethylated (and unreactivated) clones with a second round of 5aCdr did not increase the frequency of HPRT reactivation. This is consistent with mechanisms of methylation-mediated repression requiring methylation at specific critical sites and argues against models invoking overall levels or a threshold of promoter methylation. Treatment of cells with the histone deacetylase inhibitor, trichostatin A, failed to reactivate HPRT on the inactive X chromosome, even when the promoter was partially demethylated by 5aCdr treatment, suggesting that transcriptional repression by DNA methylation is unlikely to depend upon a trichostatin A-sensitive histone deacetylase.

In mammals, DNA methylation at CpG dinucleotides in the 5' region of genes is frequently associated with transcriptional silencing (1), particularly in housekeeping genes on the inactive X chromosome. Numerous studies suggest that this association between promoter hypermethylation and transcriptional repression has a functional basis. For instance, individual loci on the inactive human X chromosome in human/hamster hybrid cell lines may be reactivated using DNA-demethylating agents such as 5-azacytidine (2, 3) and 5-azadeoxycytidine, which inhibit the maintenance methyltransferase and incorporate into newly synthesized DNA (4), resulting in a failure to maintain methylation patterns during growth. Likewise, in vitro methylation of various promoter constructs results in inhibition of transcription in transient expression assays (5–9). However, despite significant evidence that DNA methylation represses transcription, specific mechanisms of this repression are only now becoming apparent.

Recent reports suggest that DNA methylation mediates transcriptional repression indirectly, via binding of the methylated DNA-binding protein MeCP2, which in turn recruits histone deacetylases that modify the local chromatin structure (10, 11). However, additional mechanisms may also act to repress transcription by methylation, such as direct inhibition of transcription factor binding to its cognate site in DNA. Indeed, methylation of the binding sites of several transcription factors has been shown to alter the affinity of the factor for its binding site (reviewed by Tate et al. (12)).

Whether transcriptional repression relies on methylation at specific critical CpGs or on the overall level of promoter methylation remains unclear. Several studies indicate that methylation of CpG dinucleotides in the vicinity of the transcriptional initiation site(s) of genes is important for gene silencing (13–17). Therefore, perturbation of the methylation pattern in this region may identify specific CpG sites whose methylation is required to maintain silencing and provide insight into mechanisms(s) by which methylation mediates transcriptional repression.

The X-linked human hypoxanthine phosphoribosyltransferase (HPRT)1 gene exhibits strong differential methylation in the promoter region on the active and inactive X chromosomes. High resolution methylation analysis by ligation-mediated PCR (LMPCR)-assisted genomic sequencing indicates that the promoter on the active X chromosome is unmethylated, whereas the promoter on the inactive X chromosome is methylated at most, but not all, CpG dinucleotides (18). The promoter methylation of the inactive HPRT allele, like that of other X-linked housekeeping genes, is unusual in that it occurs in a CpG island, which is typically unmethylated in autosomes. DNA-demethylating agents such as 5-azacytidine (5aCdr) can alter the methylation pattern of the inactive HPRT allele and can reactivate the genetic on the inactive X chromosome in rodent/human hybrid cell lines (2, 3, 19). Cis-acting regulatory elements in the HPRT promoter region have been identified by dimethyl sulfate (DMS) in vivo footprinting (20).

By examining the altered methylation patterns of the HPRT promoter in single cell-derived clones from 5aCdr-treated cells, we have identified three specific CpG dinucleotides in the pro-

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1 The abbreviations used are: HPRT, human hypoxanthine phosphoribosyltransferase; TSA, trichostatin A; 5aCdr, 5-azadeoxycytidine; LMPCR, ligation-mediated PCR; DMS, dimethyl sulfate; DMEM, Dulbecco’s modified Eagle’s medium; HAT, hypoxanthine/aminopterin/thymidine; PCR, polymerase chain reaction; RT, reverse transcriptase; bp, base pair.
moter region whose methylation is highly correlated with maintaining transcriptional repression of the HPRT gene on the inactive X chromosome. Consistent with the requirement for methylation of specific critical CpG sites, we find no correlation between the level of pre-existing demethylation and the reactivation frequency of the gene when clonal lines that have undergone partial demethylation of the promoter are re-treated with 5aCdr. We also find that the inactive HPRT allele appears to be insensitive to reactivation by the histone deacetylase inhibitor trichostatin A (TSA), and we also show that this resistance to TSA reactivation cannot be overcome by partial demethylation of the promoter region. Furthermore, we present evidence for de novo methylation of the HPRT promoter upon 5aCdr treatment, and we discuss its potential relevance to transcriptional silencing by DNA methylation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—8121 (20) and X8-6T2 (21) are human/hamster somatic cell hybrids containing an inactive human X chromosome. They were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1 x 10^{-6} M 6-mercaptopurine. Sigma (Sigma, 1 x 10^{-6} M 6-mercaptopurine) is a human/hamster hybrid containing an active human X chromosome. 4.12 cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1 x HAT (hypoxanthine/aminopterin/thymidine; Life Technologies, Inc.) supplement. All cells were maintained in culture at 37 °C in 5% CO2.

5-Azacytidine (5aCdr) Treatment and Isolation of Single Cell-derived Clones—8121 cells were grown to approximately 80% confluence, switched to medium lacking 6-thioguanine, and treated for 24–48 h with 0.5–2.0 μg/ml 5aCdr. The cells were then washed with phosphate-buffered saline, tryspinized, counted by hemocytometer, and serially diluted. Cells were plated at a density of 10–10^5 cells per 100-mm plate in non-selective medium (without HAT or 6-thioguanine) or 1 x 10^4 cells per 100-mm plate in HAT-supplemented medium. After 2–4 weeks, well isolated single cell-derived colonies were isolated with cloning rings and individually expanded in 24-well plates and then in individual culture flasks. Fifty six clones isolated from 5aCdr-treated cells grown without selection and four clones isolated from 5aCdr-treated cells grown under HAT selection were expanded for further analysis.

5-Azacytidine Reactivation Studies—Cells were grown to 80% confluence in DMEM with 10% fetal bovine serum and 25 μg/ml genticamin and then treated with 1.0 μg/ml 5aCdr for 24 h. The cells were allowed to recover for 24 h in medium without 5aCdr, plated in duplicate at a density of 20,000 cells per 150-mm plate in HAT medium, as well as 1,000 cells per 150-mm plate in non-selective medium (to normalize at a density of 20,000 cells per 150-mm plate in HAT medium, as well as 1,000 cells per 150-mm plate in non-selective medium (to normalize for plating efficiency), and incubated at 37 °C for 10 days. Single cell-derived cells in each dish were stained with Coomassie Blue for 2–5 min and counted. The 5aCdr-induced reactivation frequency for each cell line was normalized for plating efficiency by calculating the average number of HAT-resistant colonies in each plate/average number of colonies on each non-selected plate x 20). The overall reactivation frequency of all of the clones together was calculated as ((total number of HAT-resistant colonies)/(total number of colonies on all non-selected plates x 20)).

**Trichostatin A (TSA) Treatment of Cells**—Cells were grown to 50% confluence and then switched to medium supplemented with 300 ng/ml TSA. Cells were lysed, and RNA was isolated (as described below) after 0, 12, 24, and 48 h of TSA treatment.

**Genomic DNA Preparation**—Cells in monolayers were washed once with phosphate-buffered saline and then lysed and incubated overnight at room temperature in 10 ml of DNA lysis buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 25 mM EDTA, 0.5% SDS, 300 μg/ml proteinase K). The lysate was extracted once with Tris-ethylated phenol (pH 7.0), twice with phenol/chloroform and once with chloroform. The genomic DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate, spotted out, washed briefly with 70% ethanol, air-dried, and then resuspended in TE (10 mM Tris (pH 8.0), 1 mM EDTA) at approximately 1 μg/ml and stored at 4 °C.

**RNA Preparation**—RNA was prepared using the Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions and suspended in 100 μl of diethyl pyrocatecholate-treated H2O. RNA concentration was determined by spectrophotometry and adjusted to 1 μg/ml in diethyl pyrocatecholate-treated H2O, and the RNA was stored at -20 °C.

**HPRT mRNA by Reverse Transcriptase-PCR (RT-PCR)**—HPRT mRNA was assayed using a protocol modified from Sasaki et al. (19). Briefly, 1 μg of RNA in 6 μl of diethyl pyrocatecholate-treated H2O was denatured for 5 min at 95 °C and then placed on ice for 2 min. The reverse transcription (RT) reaction was then performed in a final volume of 20 μl containing 10 units of RNasin (Promega), 7.5 μl random hexamers, 1 mM dNTPs, 10 mM dithiothreitol, 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 200 units of murine leukemia virus reverse transcriptase (Promega) and was incubated for 10 min at 20 °C and then 1 h at 37 °C. PCR amplification was performed on 10 μl of the RT products in a final volume of 100 μl containing 20 mM Tris (pH 8.4), 50 mM KCl, 0.2 mM dNTPs, 1.75 mM MgCl2, 60 pmol each of the HPRT-specific primers HPRT1 (5’-TCTCCTCCTGAGCAGTCCAGC-3′) and HPRT3 (5’-GGGCTAGTCAATAGGACTCTC-3′), 7.5 μl of each primer, and 1 μl of the HPRT-specific primers HPRT1 (5’-TCTCCTCCTGAGCAGTCCAGC-3′) and HPRT3 (5’-GGGCTAGTCAATAGGACTCTC-3′), 7.5 μl of each of the human M12C gene (M12C)-specific primers XMIC2 (5’-ACCAGTGCTGGAGATGGTTTT-3′) and XMIC2R (CTCTCAGTCCACTCTCCCTT-3′), and 10 units of Taq polymerase (Life Technologies, Inc.). The PCR cycling conditions were 2 min at 94 °C, 30 cycles of 1 min at 94 °C, 30 s at 59 °C, and 90 s at 70 °C; 4 min at 72 °C; hold at 4 °C. 10 μl of the PCR reaction was loaded onto a 1% agarose gel, run at 100 V for 2 h, stained in ethidium bromide, and visualized by UV fluorescence.

**Identification of Critical CpG Dinucleotides**—Analysis of the methylation status of each CpG dinucleotide within the 5’ region of the HPRT gene was accomplished using the cytosine-specific Maxam-Gilbert genomic sequencing reaction (22) followed by gene-specific amplification using ligation-mediated PCR (LMPCR) (23). 75 μg of genomic DNA were treated with 60% hydrazine in 1.5 mM NaCl for 10 min at 20 °C. The reaction was stopped with 200 μl of hydrazine stop solution (0.1 M sodium acetate (pH 5.5), 0.1 mM EDTA), and then precipitated in ethanol in a dry ice bath. The pellet was reprecipitated with sodium acetate and washed briefly with 75% ethanol and dried under vacuum. The sample was resuspended in 90 μl of H2O, and 10 μl of pipedine was added. Pipidene cleavage of modified cytosines was performed at 95 °C for 30 min. 1 μl of H2O was added, and the sample was allowed to dry overnight under vacuum to remove the pipedidene. The resulting chemically cleaved DNA was resuspended at a final concentration of 1 μg/ml in H2O. Fragments representing cleavages at unmethylated cytosines within the HPRT promoter were amplified by LMPCR.

**LMPCR Amplification of Piperidine-treated DNA**—The LMPCR amplification of fragments representing cleavages within the HPRT promoter was performed essentially as described previously (18) except that the PCR step was carried out using Taq polymerase (20) instead of Vent polymerase. For the methylation analysis, 2 μg of hydrazine-treated DNA was amplified using the E and M primer sets as described previously (20). The E1 and E2 primers, 5’-AGCTGCTCAC- CACGAGCG-3′ and 5’-CCAGGGCTGCGGTCTGCAATAA-3′, respectively, were used to examine upper strand methylation, whereas the M1 and M2 primers, 5’-GAATAGGACTAGTGGTGG-3′ and 5’-GGACC- TTCGGCTTCTCTGGAGA-3′, respectively, were used to examine the lower strand. These two LMPCR primer sets cover the region from approximately positions 235 to 10 relative to the translation start site on both strands and include the entire minimal promoter (24). LMPCR products were size-fractionated on a 5% acrylamide gel DNA sequencing gel, electrotransferred to a nylon membrane, and hybridized with an appropriate 32P-labeled probe as described previously (25).

**RESULTS**

**Identification of Critical CpG Dinucleotides**—To identify specific CpG sites within the promoter region whose methylation may be critical for maintaining transcriptional repression of the HPRT gene on the inactive X chromosome, we treated 8121 cells (containing an inactive human X chromosome) with the demethylating agent 5aCdr and isolated both HAT-selected (HAT selects for the HPRT gene on the inactive X chromosome) and non-selected single cell-derived clones. If critical CpG sites exist within the HPRT promoter region of the cell, these sites should remain methylated after exposure to 5aCdr in all clones that fail to reactivate the HPRT gene (and, conversely, undergo demethylation in all clones that do reactivate). Each of 4 HAT-selected and 62 non-selected clones were screened for expression of HPRT...
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Fig. 1. RT-PCR analysis of HPRT mRNA expression. A, representative RT-PCR analysis of HAT-selected and non-selected single cell-derived clonal lines from 5aCdr-treated 8121 cells. The lane designations indicate the name of each clone examined. 8121 is the parental human/hamster hybrid cell line containing an inactive human X chromosome. 4.12 is a human/hamster hybrid that contains an active human X chromosome. HPRT indicates the position of the expected HPRT-specific RT-PCR product. MIC2 indicates the expected location of the MIC2-specific RT-PCR product. B, sensitivity of the RT-PCR assay to detect HPRT mRNA. Total RNA from 4.12 cells (wild type) was serially diluted with total RNA from 8121 cells, and the RNA mixture was subjected to RT-PCR analysis. Percentages from 0–100% indicate the portion of the total RNA that is derived from 4.12 cells. HPRT and MIC2 represent the expected location of the respective RT-PCR products.

mRNA by RT-PCR. The RT-PCR assay was performed on total RNA as a multiplex reaction using both HPRT-specific primers and human MIC2 (MIC2)-specific primers. Because MIC2 is an X-linked gene that escapes X inactivation, MIC2 expression serves as an internal positive control for the RT-PCR. As expected, RT-PCR analysis revealed that all 4 HAT-resistant clones had reactivated the HPRT gene (Fig. 1A). In addition, one of the 62 non-selected clones examined also had reactivated the HPRT gene following 5aCdr treatment. To determine the sensitivity of the RT-PCR assay, total RNA from 4.12 cells (which contain an active human X chromosome and express the HPRT gene) was serially diluted with total RNA from 8121 cells, and the resulting mixtures were subjected to RT-PCR. The results (Fig. 1B) indicate that the RT-PCR assay can readily detect as little as 0.2% of “wild type” (i.e. 4.12 cells) HPRT mRNA levels. All 5 clones that reactivated the HPRT gene after 5aCdr treatment exhibited full wild type HPRT mRNA levels, and all 61 clones that failed to reactivate the HPRT gene after 5aCdr treatment showed no detectable HPRT mRNA; no low or intermediate HPRT mRNA levels were observed for any of the clones examined in our RT-PCR assays.

All clones assayed for HPRT reactivation by RT-PCR were also subjected to high resolution DNA methylation analysis by LMPCR genomic sequencing (Figs. 2 and 3). The specific region of the HPRT promoter examined extends from approximately 140 bp upstream to 90 bp downstream of the two major transcription initiation sites identified by Kim et al. (25) and encompasses the entire minimal promoter (26) and all but one of the transcription factor binding sites previously identified by DMS in vivo footprinting (20). This region was chosen for analysis because previous studies suggest the region surrounding transcription initiation sites is crucial for transcriptional repression by DNA methylation (13–17). In the parental 8121 cell line, this region contains 20 symmetrically methylated CpG sites (where paired CpGs on the upper and lower strand count as a single site) and 12 unmethylated sites (18). Purified genomic DNA from each clone was subjected to the Maxam-Gilbert cytosine-specific sequencing reaction followed by ligase-mediated PCR (LMPCR). The cytosine-specific reaction uses hydrazine to modify cytosine nucleotides in DNA, but this modification is significantly inhibited by methylation at the 5-position of cytosine. The differential reactivity of hydrazine with cytosine versus 5-methylcytosine allows for examination of the methylation status of every cytosine (including CpG dinucleotides) within a region of interest (18, 23, 27). In this high resolution assay, demethylation at a specific cytosine results in the appearance of a new band (relative to a methylated sample) in the autoradiogram of the cytosine-specific sequencing ladder (Fig. 2). A site was scored as “demethylated” in 5aCdr-treated clones if a new cytosine-specific band was detected that was previously undetectable (i.e. methylated) at the same position in the cytosine-specific ladder from the 8121 parental cells. All sites designated as “methylated” showed no detectable band on the cytosine-specific sequencing ladder above background levels; therefore, a site in the 5aCdr-treated clones was considered demethylated if the associated band was detectable above background (Fig. 3).

All clones that reactivated the HPRT gene after 5aCdr treatment demethylated all CpGs in the region around the major transcription initiation sites of the HPRT gene (data not shown), a pattern identical to that of the active HPRT allele on the active X chromosome and consistent with previous 5aCdr reactivation studies of the HPRT gene (18). In addition, two HAT-selected 8121-derived clones that had spontaneously re-
activated the human HPRT gene in the absence of 5aCdr treatment also exhibited complete demethylation of all CpGs in the same region of the promoter (data not shown). These data suggest that the complete demethylation of this region of the HPRT promoter in transcriptionally reactivated clones is not simply an artifact of 5aCdr or HAT treatment and is a requirement for transcriptional activation of the gene.

Consistent with these results, clones that failed to reactivate the HPRT gene following 5aCdr treatment had promoters that remained relatively hypermethylated compared with the unmethylated active HPRT allele (Fig. 2). Among unreactivated clones, the extent and pattern of 5aCdr-induced demethylation at the HPRT promoter were quite variable, with as few as 0 and as many as 11 demethylated CpG sites out of a possible 20 methylated sites in the parental 8121 cells, with an overall frequency of 7.8% demethylation/site. Nevertheless, even the clone with 11 demethylated sites failed to express any detectable HPRT mRNA as determined by RT-PCR, suggesting that the repressive effect of methylation on transcription is not simply cumulative nor directly proportional to the number of methylated CpG sites.

Since any methylation site(s) essential for transcriptional repression should remain methylated in all unreactivated clones, the methylation patterns of each of the 61 unreactivated HPRT clones were compared (Fig. 3). This analysis identified three CpGs at positions -48, -54, and -97 (relative to the translation initiation site) that remain methylated on both strands in all 61 unreactivated 5aCdr-treated clones. The site at position -97 is located immediately upstream of a major transcription initiation site, and the sites at positions -48 and -54 are located approximately 35-45 bp downstream of the two major transcription start sites (in the portion of the gene encoding the 5'-untranslated region). The perfect correlation between methylation at these three “critical” CpG sites and transcriptional repression of the HPRT gene in 61 independent clones suggests that methylation at these sites is necessary for maintaining repression of HPRT on the inactive X chromosome. In contrast, all other CpG sites examined in this region exhibited demethylation in at least one unreactivated clone, indicating that methylation at each of these sites is not essential for maintaining transcriptional repression.

**Statistical Analysis of the Demethylation Pattern in Unreactivated Clones**—The 5aCdr-induced demethylation pattern observed in the 61 unreactivated clones was subjected to statistical analysis to determine the likelihood that the three critical CpG sites escaped 5aCdr-induced demethylation simply by chance. To simplify the analysis, statistical analysis was performed using data only from the upper strand (which was informative at more sites than the lower strand). The null hypothesis $H_0$ is that the frequency of demethylation in the $m = 20$ methylation sites examined in each clone is in fact uniform, and the pattern observed is based on sampling error. We reject the null hypothesis if the number of sites that never demethylate, $X$, is too large. Let there be $r = 61$ clones, and at the kth clone $n_k$ of the sites has not been demethylated at least once. Then the $p$ value to reject the null hypothesis is calculated by the probability shown in Equation 1,

$$ p = P(X > x_0) \quad (\text{Eq. 1}) $$

where $x_0$ is the observed number of sites which never demethylated. Probability 1 can be computed recursively by letting $p(k,i)$ indicate the probability that there are still $i$ methylated sites never demethylated after $k$ clones have been observed. Then, under $H_0$ (Equation 2),

$$ p(k,i) = \sum_{j=0}^{n} p(k-1,j) \binom{n-j}{i} \binom{m-j}{n_k}, k = 2, \ldots, r \quad (\text{Eq. 2}) $$

With the initial condition (Equation 3)

$$ p(1,i) = \begin{cases} 1 & \text{if } i = n_k \\ 0 & \text{otherwise} \end{cases} \quad (\text{Eq. 3}) $$

The $p$ value (Equation 1) is shown in Equation 4,
Critical Methylation Sites for the Repression of HPRT

**TABLE I**

| Clone designation | No. of demethylated sites | Plating efficiency | Reactivation frequency | Normalized reactivation frequency |
|-------------------|--------------------------|--------------------|------------------------|-----------------------------------|
|                   |                          | No. of viable colonies/plate | % viable | No. of HAT resistant colonies/plate | % reactivated | |
| NS1A4 0           |                         | 278, 290            | 0.28       | 62, 56                                 | 0.30         | 1.0    |
| ID1 0             |                         | 320, 342            | 0.33       | 89, 79                                 | 0.42         | 1.3    |
| IIIA2 0           |                         | 394, 390            | 0.39       | 111, 119                               | 0.58         | 1.5    |
| HIC1 0            |                         | 520, 520            | 0.52       | 190, 174                               | 0.91         | 1.7    |
| NS2A1I 0          |                         | 370, 382            | 0.38       | 200, 190                               | 0.98         | 2.6    |
| IH7 1             |                         | 264, 225            | 0.24       | 113, 119                               | 0.58         | 2.4    |
| HIC2 3            |                         | 621, 584            | 0.60       | 119, 130                               | 0.62         | 1.0    |
| HIC1 3            |                         | 419, 383            | 0.40       | 118, 96                                | 0.54         | 1.3    |
| IVE1 4            |                         | 339, 321            | 0.33       | 82, 105                                | 0.47         | 1.4    |
| III1 5            |                         | 600, 552            | 0.58       | 125, 128                               | 0.63         | 1.1    |
| III 8             |                         | 375, 405            | 0.39       | 8, 11                                  | 0.05         | 0.1    |
| IIIA4 11          |                         | 800, 820            | 0.81       | 138, 160                               | 0.75         | 0.9    |
| S121 N/A          |                         | 475, 492            | 0.48       | 108, 134                               | 0.61         | 1.2    |
| X8–6T2a N/A       |                         | 1142, 1095a         | 0.45       | 501.1, 485b                            | 20.0         | 44.0   |

*Because it has a much higher reactivation frequency, both plating efficiency and reactivation frequency for X8–6T2 were determined by plating 2500 cells in duplicate in non-selective medium and HAT medium, respectively.

\[ p \text{ value} = \sum_{i=1}^{m} p(r,i) \]  
\[ p_0 = \frac{\text{total no. demethylations}}{\text{total no. sites examined}} = \frac{95}{17 \times 61} = 0.0916 \]  
\[ p_1 = 1 - \left(1 - (1 - 0.0315)^3\right) = 0.0106 \]
higher 5aCdr-induced reactivation frequency. However, if methylation of specific critical sites is more important for maintaining repression, the reactivation frequency should be largely unaffected by the increasing levels of pre-existing demethylation at non-critical sites. The results of this experiment are shown in Table I.

We found no correlation between pre-existing levels of stable demethylation in the promoter region and the 5aCdr-induced reactivation frequency of the HPRT gene. Most demethylated clones showed reactivation frequencies similar to that of the parental 8121 cells (no demethylated clone showed more than a 2-fold increase in reactivation frequency above 8121 cells), and the clone with the highest degree of pre-existing demethylation (IIA4) exhibited a normalized reactivation frequency slightly lower than 8121 cells. In fact, clones that showed the highest reactivation frequencies were ones carrying no demethylated sites (NS2A11) and ones carrying only a single demethylated site (IIH7), whereas the clone with the lowest reactivation frequency had 8 demethylated sites (IIIH1). Furthermore, the overall reactivation frequency of all partially demethylated clones re-treated with 5aCdr was 1.3% (calculated by (sum of all HAT-resistant colonies)/((20×sum of all viable colonies)), a level essentially identical to that of the 8121 parental cells.

This lack of correlation between overall levels of pre-existing demethylation and 5aCdr-induced reactivation frequencies is consistent with the existence of specific critical CpG sites whose methylation is required to maintain repression of the HPRT gene on the inactive X chromosome. Since all of the critical sites are methylated in all of the unreactivated clones, if reactivation requires demethylation of the critical sites, partial demethylation of non-critical sites would not be expected to affect subsequent 5aCdr-induced reactivation. On the other hand, these results are not consistent with models that invoke threshold levels of overall promoter methylation for maintaining repression of transcription. Since partially demethylated clones should be closer to the threshold level of demethylation required for reactivation of the gene, these clones should undergo reactivation at consistently higher frequencies after re-treatment with 5aCdr. Because data in Table I show this is not the case and that partial demethylation of non-critical sites has little effect on subsequent reactivation frequencies, methylation at non-critical sites seems to play a secondary role in maintaining transcriptional repression.

**Effects of Trichostatin A Treatment on HPRT Transcrip-**

**Fig. 3. Summary of methylation analysis of the HPRT gene 5' region in unreactivated clones.** The rows of squares immediately above and below the nucleotide sequence indicate the methylation pattern of the parental 8121 cell line. Open squares indicate unmethylated CpG dinucleotides; solid squares indicate methylated CpG dinucleotides, and half-filled squares indicate sites methylated in some cells but not others. ? indicates sites for which the methylation status could not be determined due to technical limitations of the genomic sequencing methodology. Only those unreactivated clones whose methylation state differed from the parental pattern at a given CpG site are scored as open or solid circles. Each open circle represents one unreactivated clone that exhibited demethylation at the indicated CpG site. Each solid circle represents one unreactivated clone that exhibited de novo methylation at the indicated CpG site. Numbers in parentheses indicate the number of clones that demonstrated de novo methylation at a given CpG site. In these cases of de novo methylation, the number of clones is scaled to half the number of methylation events at each site. ND indicates that the methylation status of the CpG site was not determined because it could not be resolved with the primer set used for that strand. The large vertical ovals indicate the location of the three critical methylation sites that remain methylated in all 61 unreactivated clones. Bolded italicized Gs within the sequence indicate the location of DMS in vivo footprints in the region (20). Large open rectangles indicate the location of GC boxes. The arrows indicate the location of the two major transcription initiation sites determined by Kim et al. (25), and the dashed line between the upper and lower strands of the nucleotide sequence indicates the region of multiple transcription initiation sites described by Patel et al. (26). All position numbers shown are relative to the translation start site (26).
Critical Methylation Sites for the Repression of HPRT

Recent reports (10, 11) have suggested that MECP2 may mediate transcriptional repression at methylated promoters by recruiting histone deacetylases. Therefore, it is possible that the three critical methylated sites in the HPRT promoter region may function on the inactive X chromosome by binding MeCP2 and recruiting histone deacetylases that, in turn, maintain the repressive chromatin structure of the promoter. However, in vivo footprinting studies of both the HPRT gene (20) and the human PGK-1 gene (PGK-1) on the active and inactive X chromosomes (29, 30) have not detected any evidence for stable binding of MeCP2 at methylated CpG dinucleotides on the inactive X chromosome (i.e. no in vivo footprints have been detected over methylated CpGs). Despite this caveat, MeCP2 may still play a role in repression of the HPRT locus since the absence of a footprint could indicate that either MECP2 binding is transient or that MECP2 simply does not footprint well.

Since TSA has been shown to alleviate MECP2-mediated repression (10, 11), two independent human/hamster hybrid cell lines, 8121 and X8-6T2, each containing an inactive human X chromosome, were treated for 0–24 h with TSA to assess the role of histone deacetylase-mediated repression of the HPRT promoter. If HPRT repression by DNA methylation functions primarily via histone deacetylases, TSA treatment should re-activate the HPRT gene on the inactive X chromosome. Furthermore, because X8-6T2 reactivates at a significantly higher frequency than 8121, both spontaneously and upon 5aCdR treatment (see Table 1), it may be more responsive to TSA-induced reactivation. Results of this study are shown in Fig. 4A. While TSA treatment did appear to enhance the expression level of the control MIC2 gene (an X-linked gene which escapes inactivation), as has been previously observed in other gene systems (31, 32), it failed to re-activate the HPRT gene on the inactive X chromosome in both cell lines as determined by RT-PCR (Fig. 4A). The greater susceptibility of X8-6T2 cells to 5aCdR-mediated reactivation apparently does not confer a greater susceptibility to TSA-induced reactivation. These results are similar to that reported by Riggs et al. (33). Since these same cell lines can be reactivated by 5aCdR treatment, these TSA results are inconsistent with repression of the HPRT gene by the mechanism identified by Nan et al. (10) and Jones et al. (11). However, we have been unable to find a TSA-responsive gene to act as a direct positive control for TSA reactivation in the human/hamster hybrid cells. Nevertheless, indirect evidence, such as the growth inhibition of the cells by TSA treatment and increased MIC2 expression over the TSA treatment time course (Fig. 4A), are consistent with other studies that show that TSA inhibits cell proliferation (34) and increases the expression of active genes (31, 32), indicating that TSA is functioning in these cells.

Recently, Cameron et al. (35) have shown that high density methylation at the promoter may prevent TSA from reactivating genes even when histone deacetylases are involved in their transcriptional repression. Partial demethylation of the promoter can overcome this resistance to TSA-mediated reactivation, revealing a role for histone deacetylases in maintaining repression. To determine if the high density methylation of the HPRT promoter is preventing reactivation of the HPRT gene by TSA, each of the 12 partially and stably demethylated clonal lines shown in Table I above were treated with TSA for 0–48 h and assayed for HPRT expression by RT-PCR. Each of these single cell-derived clones from 8121 cells carried 0–11 CpG sites that were stably demethylated after 5aCdR treatment yet...
maintained stable repression of the HPRT gene. As shown in Fig. 4B, regardless of the level of pre-existing promoter demethylation (i.e. number of pre-existing demethylated CpGs), TSA treatment failed to reactivate the HPRT gene in any of the partially demethylated clones. This was true even for clone IIIA4 which is stably demethylated at 11 of 20 CpGs in the promoter. These results suggest that inhibition of histone deacetylation is not sufficient to overcome the repressive effects of DNA methylation, even in the context of reduced levels of promoter methylation. Because all of the demethylated sites in each of these TSA-treated clones are non-critical sites, these results are also consistent with a mechanism of DNA methylation-dependent repression involving specific critical sites of methylation.

**DISCUSSION**

We have examined the high resolution methylation pattern of the endogenous HPRT promoter region in 61 clonally derived lines that have undergone 5aCdr-induced demethylation but failed to reactivate the HPRT gene. Statistical analysis reveals a highly significant ($p = 0.00003$) correlation between maintenance of transcriptional repression and methylation at three specific CpG sites. In contrast, the other CpGs in the promoter region (outside these three critical sites) appear to be able to undergo demethylation without affecting repression of the gene, even when as many as 55% of the CpG sites in the promoter are demethylated (see clone IIIA4 in Table I). Furthermore, prior demethylation of the promoter region at non-critical CpG sites does not increase the frequency of HPRT gene reactivation after re-treatment of cells with a second round of 5aCdr. These data argue that three critical sites in the promoter region contribute disproportionately to the repression of the HPRT gene on the inactive X chromosome and that methylation of critical sites rather than the overall level of promoter methylation (or a certain threshold level of promoter methylation) is involved in maintaining transcriptional silencing.

None of the three critical sites of methylation fall within any of the transcription factor binding sites previously identified by DMS in vivo footprinting (20), although the critical site at −97 lies just upstream of a DMS footprinted region (see Fig. 9). The other two critical sites at positions −54 and −48 are located at least 21 bp from the nearest DMS in vivo footprinted region, and DNase I in vivo footprinting studies fail to detect footprints at or near these two sites on either the active or inactive X chromosome. Therefore, it is unlikely that a major mode of action for the three critical CpG sites involves interference with transcription factor binding upon methylation of these sites as proposed by Tate and Bird (12). Furthermore, Rincon Limas et al. (24) have shown that the region containing the three critical CpG sites is not required for maximal expression of the HPRT gene in transient expression assays. Thus, this region may be required more for repression of the gene on the inactive X chromosome than for activation or expression.

This suggests, at least in part, an indirect mechanism of methylation-mediated repression of the HPRT gene. Recent reports have indicated that repression by methylation involves MECP2 recruitment of histone deacetylases (10, 11), a mechanism that was shown to be TSA-sensitive. However, the HPRT gene on the inactive X chromosome appears to be resistant to reactivation by TSA, even when the promoter is partially (and stably) demethylated by 5aCdr treatment. This would suggest that methylation-mediated repression of the HPRT gene on the inactive X chromosome could act by either of two possible mechanisms. One possibility is that silencing of the HPRT gene does not involve a histone deacetylase complex; however, anti-bodies against acetylated histone H4 clearly show that the inactive X chromosome is hypoacetylated (36). Alternatively, repression of the HPRT gene by methylation could involve one or more histone deacetylases that are TSA-resistant, similar to those reported in yeast (37). It is also possible that the dynamic turnover of deacetylated histones on the inactive X chromosome is so slow, or histone acetylation occurs at such a low level on the inactive X, that inhibiting histone deacetylases does not lead to significant hyperacetylation of the HPRT gene.

Although transcriptional repression of the HPRT gene may require methylation at specific critical sites, reactivation appears to require complete demethylation of the promoter. The association between complete promoter demethylation and transcriptional reactivation is not specific to 5aCdr-induced reactivation because it also occurs in spontaneous reactivants. Complete promoter demethylation is also seen in the X-linked human phosphoglycerate kinase gene (hPGK-1) upon 5aCdr-mediated reactivation (13). Together with the fact that the promoters of other X-linked housekeeping genes on the active X chromosome are also generally unmethylated (16, 27, 30, 38), these observations argue that the absence of methylation in the promoter in vivo is essential for transcription of the HPRT gene and other X-linked housekeeping genes.

Since demethylation of all 20 CpG sites in the promoter region in a single cell appears to be required for reactivation of the HPRT gene, the average overall 5aCdr-induced demethylation frequency of 7.8% at each CpG is too low by itself to account for the observed reactivation frequency of the 8121 parental cells (see Table I). Therefore, in order to achieve full promoter demethylation and the HPRT reactivation frequency we observed for 8121 cells, active demethylation of the promoter region must occur following a crucial initial 5aCdr-induced demethylation event. Such demethylase activities have been reported recently (39, 40). We propose that the crucial event that triggers active demethylation of the promoter (and subsequent gene reactivation) is the stable demethylation of one of the three critical sites. Thus, the reason we never detected one of the critical sites in an unmethylated state in unreactivated clones is because demethylation of a single critical site resulted in active global promoter demethylation and gene reactivation.

Alternatively, the actual 5aCdr-induced demethylation frequency may be significantly higher than what is observed in the stable cell lines due to active remethylation of the promoter after 5aCdr is removed. The high frequency of de novo methylation observed at certain CpG sites in the HPRT promoter of unreactivated clones suggests that active remethylation of the promoter does occur in these cells. This remethylation activity could account for the discrepancy between the overall stable demethylation frequency we observe and the reactivation frequency of the 8121 cell line. Since the critical methylation sites are always methylated in cell lines that fail to reactivate the HPRT gene, these critical sites may either be relatively resistant to demethylation or have a propensity to remethylate. This tendency to maintain the methylated status of the critical sites might act to prevent full promoter demethylation and reactivation of the HPRT gene.

Although unlikely, we cannot formally exclude the possibility that methylation-mediated repression does not occur at the promoter at all and is instead mediated by a distant site. In this case, the critical sites in the HPRT promoter may remain methylated simply because they have a strong pre-disposition to de novo methylation during growth after 5aCdr-induced demethylation. However, this scenario is extremely unlikely in light of the substantial evidence that methylation at the promoter mediates transcriptional repression and that complete

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promoter demethylation appears to be required to reactivate the HPRT gene.

Overall, these studies suggest that transcriptional repression by methylation is a complex process involving non-equivalent methylation sites that appear to repress transcription by indirect mechanisms. The complete demethylation apparently necessary for reactivation and the evidence for de novo methylation further suggest that methylation itself is dynamic, a product of intrinsic active methylation and demethylation processes.

Experiments to examine further the function of these three critical CpG sites include transient expression assays using constructs differentially methylated at the critical and non-critical sites to determine if methylation of the critical sites is necessary and sufficient to repress promoter activity. In addition, to assess the role of chromatin structure in mediating the function of these three critical sites, these expression constructs could be pre-assembled into chromatin prior to transcription as described by Buschhausen et al. (5).

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