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Hemimethylation and Hypersensitivity Are Early Events in Transcriptional Reactivation of Human Inactive X-Linked Genes in a Hamster × Human Somatic Cell Hybrid

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Reactivation of the hypoxanthine phosphoribosyltransferase (HPRT) gene on an inactive human X chromosome in a somatic cell hybrid was analyzed following exposure to 5-aza-2′-deoxycytidine. Hemimethylation and chromatin hypersensitivity in the 5′ CpG island appeared by 6 h after exposure and continued to increase for 24 h in an exponentially growing cell culture. These results imply that the conformation of inactive chromatin requires a symmetrically methylated 5′ G+C-rich promoter region. In addition, quantitative analysis of the time course patterns suggest that chromatin sensitivity changes may depend on strand-specific demethylation. Symmetrically demethylated DNA was first detected at 24 h and continued to increase until 48 h. HPRT mRNA was first detected at 24 h and increased in a biphasic pattern until 48 h. These results suggest that hemimethylation permits nuclease attack but not transcription factor binding, which requires symmetrically demethylated DNA. We also show that in G_1-arrested cells, 5-aza-2′-deoxycytidine has no effect on methylation, chromatin conformation, or transcription. We conclude that reactivation of the HPRT gene present on the inactive X chromosome of a somatic cell hybrid involves the initial events of DNA hemimethylation and chromatin hypersensitivity at the 5′ CpG island, followed by symmetrical demethylation and transcriptional reactivation.

All species with XY systems of sex determination exhibit dosage compensation, a mechanism which brings about the general equivalence in expression of the X-linked genes in males and females. In mammals, the most common dosage compensation system involves X-chromosome inactivation, a developmentally controlled mechanism in which one of the two X chromosomes in the female is inactivated (23).

A unique molecular feature of the stable maintenance of X-chromosome inactivation in human somatic cells is hypermethylation of the 5′ G+C-rich promoter regions of inactive X-linked housekeeping genes (18, 37, 41, 43). Molecular analysis of such genes reactivated by 5-azacytidine (5aC) shows that their 5′ G+C-rich promoter regions have changed from a methylated and nuclease-insensitive state to a demethylated and nuclease-hypersensitive state (13, 14, 20, 21, 25, 38, 42). The hypomethylation and nuclease hypersensitivity of 5′ G+C-rich promoter regions may be characteristic of all actively transcribed housekeeping genes (3, 19, 40). These results are generally supportive of the idea that X-chromosome inactivation is controlled at the transcriptional level and provide strong support for a repressive role of methylation in X-chromosome inactivation. Maintenance of inactivation by DNA methylation is also attractive because methylation and expression patterns are both inherited by daughter cells after DNA replication (29). However, it still remains to be shown whether DNA methylation is a primary and causative event in this transcriptional repression or whether it merely plays a secondary and fail-safe role (2, 22).

The reactivation experiments referred to above were based on material analyzed many cell generations after exposure to 5aC. Therefore, the relative timing and importance of events characterizing a switch from an inactive to active state is unclear. It is possible that 5aC has an effect on cells other than through demethylation (34). In this study, we have analyzed the hypoxanthine phosphoribosyltransferase (HPRT) gene for demethylation, nuclease sensitivity of chromatin, and transcriptional activity in the period immediately following exposure of an exponentially growing culture to a cytosine analog, 5-aza-2′-deoxycytidine (5aCdr). An analysis of sites in the first intron, in which methylation patterns are known to be associated with gene expression (41, 43), indicates that hemimethylation and an increase in nuclease sensitivity of chromatin occur within 6 h of exposure, possibly by a strand-specific mechanism. Demethylation of both strands and appearance of transcription are not detectable until 24 h after exposure. We also show, using G_1-arrested cells, that 5aCdr has no effect on demethylation, nuclease sensitivity, or transcription initiation in nonreplicating cells. These results indicate that DNA demethylation plays a primary role in the switch from an inactive to an active state of X-linked housekeeping genes in somatic cell hybrids.

MATERIALS AND METHODS

Cell lines. X8-6T2 is a hamster × human hybrid cell line containing an inactive human X chromosome (8, 9). The parental Chinese hamster ovary (CHO) cell line, CHOYH21, is deficient for HPRT and glucose 6-phosphate dehydrogenase (G6PD) expression (31), and these genes are not reactivated by 5aCdr treatment (33a). The human parental line is wild type for HPRT, G6PD, and other genes analyzed in this study. The CHO cell line, which is wild type for the HPRT and G6PD genes, was used as a control in reverse transcriptase (RT)-polymerase chain reaction (PCR) experi-

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ments. Y162-11c is a hamster × human somatic cell hybrid line containing an active human X chromosome (13).

Cell cultures and 5aCdr treatment. Cells were routinely cultured at 37°C in RPMI 1640 (GIBCO) medium containing 10% fetal bovine serum and 40 μg of gentamicin per ml in a 5% CO_2 humidified atmosphere. For G_1 arrest experiments (36), cells grown in complete medium were washed twice with phosphate-buffered saline (PBS) and then cultured in isoleucine-free RPMI 1640 (GIBCO) medium containing 10% dialyzed fetal bovine serum (HyClone Laboratories). For 5aCdr treatment, about 2×10^6 cells in 177-cm^2 culture dishes were exposed to 5aCdr (0.4 μg/ml of medium) for 24 h and then washed with PBS and returned to normal medium. Y162-11c cells were routinely cultured in HAT medium (normal medium supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine).

Extraction and analysis of RNA. Total cellular RNA was isolated from cell monolayers by the acid guanidinium thiocyanate-phenol-chloroform extraction method (6), with slight modifications. Cells were washed four times with cold PBS and lysed directly in the same dish by adding 4 ml of denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. The lysate was transferred to a 15-ml polypropylene tube to which the following were added: 0.4 ml of 2 M sodium acetate (pH 4.0), 4 ml of phenol saturated with diethyl pyrocarbonate (DEPC)-treated water, and 0.8 ml of chloroform. The mixture was shaken vigorously for 10 s and chilled on ice for 15 min. The sample was centrifuged at 10,000 × *g* for 20 min at 4°C. After centrifugation, the upper aqueous phase was transferred to a new polypropylene tube, mixed with 4 ml of isopropanol, and placed at −20°C for at least 1 h. The mixture was centrifuged at 10,000 × *g* for 20 min at 4°C, and then the supernatant was discarded. The RNA pellet was dissolved in 0.5 ml of DEPC-treated water in a 1.5-ml microcentrifuge tube, and 50 μl of 2 M sodium acetate (pH 4.0) was added. The RNA was precipitated with 0.55 ml of isopropanol. The final RNA pellet was dissolved in DEPC-treated water.

For RT-PCR analysis, first-strand cDNA was generated from 0.5 μg of total RNA. Samples were incubated at 90°C for 5 min and chilled on ice for 2 min. A concentrated reaction buffer was added for cDNA synthesis that resulted in a final reaction volume of 20 μl containing 1 mM each deoxynucleoside triphosphate, 20 U of RNasin, 7.5 μM random hexamer, 200 U of Moloney murine leukemia virus RT (Bethesda Research Laboratories), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. After 10 min at room temperature, the mixture was incubated for 1 h at 37°C, 69.5 μl of water was added, and the sample was heated to 95°C for 5 min and quickly chilled on ice. For PCR, the sample of heat-treated cDNA was combined with 10.5 μl of buffer containing 74.1 mM Tris-HCl (pH 8.3), 370 mM KCl, 11.1 mM $MgCl_2$, 7.4 mg of gelatin per ml, 20 pmol of each primer, and 2.5 U of AmpliTaq DNA polymerase. PCR parameters after a 2-min initial denaturation at 94°C were as follows: 27 cycles of 1 min at 94°C, 30 s at 59°C, and 1.5 min at 72°C, followed by a final elongation of 4 min at 72°C. The following primers were used for specific human cDNAs: for HPRT, HPRT-1 (TCCTCCTGA GCAGTCAGC [exon 1]) and HPRT-3 (GGCGATGTCAAT-AGGACTC [exon 9]); for phosphoglycerate kinase 1 (PGK-1), PGK1-R3 (TCGGCTCCCTCGTTGACCGAA [exon 1]) and PGK1-R4 (CAGCTGGGTTGGCACAGGCTT [exon 4]); for G6PD, PD9 (TGCAGGCCAACAATGTGGTCCT [exon 9]) and PD13R (AGGAATGTGCAGCTGAGGTCAA [exon 13]); and for *MIC2*, XMIC2 (ACCCAGTGCTGGGGA TGACTTT) and XMIC2R (CTCTCCATGTCCACCTC CCCT). Amplified products were electrophoresed on 1% agarose gels and examined by staining with 0.5 μg of ethidium bromide per ml or Southern hybridization, using the human HPRT cDNA probe (16). Hybridization probes were ^{32}P radiolabeled by the random hexamer priming method (10).

Extraction and analysis of total cellular DNA. After being washed with cold PBS, cells were lysed directly in the 177-cm^2 culture dish by addition of 4 ml of a buffer containing 40 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 20 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS). The lysates were first treated with 0.1 mg of RNase A per ml at 37°C for 15 min, incubated with 0.5 mg of proteinase K per ml at 55°C for 60 min, and then extracted with phenol and chloroform. Total cellular DNA (20 μg) was digested for 4 h at 37°C with 60 U of either *Hpa*II or *Nci*I in a 50-μl reaction mixture containing buffers supplied by the manufacturer. A second digestion was subsequently carried out with 60 U of *Pst*I for an additional 4 h at 37°C. Reactions were stopped by adding EDTA, SDS, and NaCl to final concentrations of 20 mM, 0.5%, and 200 mM, respectively. DNA was then precipitated by ethanol and redissolved in electrophoresis loading buffer. For Southern hybridization, UV-cross-linked membranes were prehybridized with a buffer containing 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.5% SDS, and 0.5 mg of denatured salmon testes DNA per ml at 42°C for 6 h and then hybridized with radiolabeled probe for 24 h at 42°C in a buffer containing 50% formamide, 6× SSC, 1× Denhardt's solution, 0.5% SDS, and 0.5 mg of denatured salmon testes DNA per ml. The final wash of filters was with 0.1× SSC–0.1% SDS at 65°C. The hybridization probe was PB1.7, which is specific for the human HPRT gene (15, 41).

Nuclease sensitivity assay of chromatin. Nuclear isolation was performed essentially as described previously (13, 30). Cell layers were washed four times with cold PBS and harvested in PBS by scraping with a rubber spatula. The harvested cells were centrifuged at 450 × *g* for 5 min at 4°C; pellets were resuspended in 10 ml and brought up to 50 ml with ice-cold RSB buffer (5 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.6], 10 mM NaCl) plus 0.5% Nonidet P-40 and then centrifuged at 450 × *g* for 3 min at 4°C. After three cycles of this procedure with RSB containing Nonidet P-40, the final pellet was resuspended in RSB without Nonidet P-40 and adjusted to 11.6 A_{260} units/ml. *Msp*I was added to a final concentration of 0.35 U per μg of DNA (200 U/ml), and the sample was incubated for 20 min at 37°C. The treatment of isolated nuclei with *Msp*I was terminated by adding EDTA, SDS, and proteinase K, and then DNA was extracted from the treated nuclei as described previously (13).

Quantitation of methylation, chromatin sensitivity, and mRNA level changes. Densitometric analysis of autoradiograms in the linear range, as determined by dilution experiments, was carried out with a Quick Scan instrument (Helena Laboratories). Values for untreated and treated X8-6T2 were compared with values for similarly prepared materials from Y162-11c, and the final values for X8-6T2 are reported as percentages of Y162-11c values.

RESULTS

Specific mRNA levels of active, inactive, and reactivated X-linked genes. To determine the expression patterns of X-linked genes in hamster × human somatic cell hybrids

containing either an inactive (X8-6T2) or active (Y162-11c) human X chromosome, mRNAs were assayed by the RT-PCR method. Figure 1A shows that mRNAs specific for human HPRT, PGK-1, and G6PD genes were detected only in Y162-11c, whereas mRNA specific for *MIC2*, a gene known to escape X-chromosome inactivation, was detected in X8-6T2 as well as in Y162-11c. No signals for any of these RNAs were detected in wild-type CHO cells, demonstrating that the primers used for PCR are human gene specific. These results indicate that the X8-6T2 cell line does not express mRNAs for the human HPRT, PGK-1, or G6PD gene. These genes are known to be subject to X-chromosome inactivation.

To analyze early events in the process of demethylation-induced reactivation of X-linked genes, we examined the human HPRT gene following treatment with 5aCdr. As shown in Fig. 1B, human HPRT mRNA became detectable in X8-6T2 at 24 h after initiation of treatment in exponentially growing cells at a level about 0.3% of that in Y162-11c. The HPRT mRNA levels increased to about 3% at 36 h and 14% at 42 h, reached a maximal level of 33% at 48 h, and maintained this level from 48 to 96 h. The input level of RNA was exactly the same for all time points. As a control, the mRNA levels of the *MIC2* gene, which escapes X inactivation, were determined under the same conditions and with the identical material used in the HPRT experiment. No apparent change in *MIC2* mRNA level was detected following 5aCdr treatment (Fig. 1C).

Demethylation of both DNA strands in the 5′ CpG island of the HPRT gene following 5aCdr treatment. To determine the time course of demethylation in X8-6T2 after 5aCdr treatment, we examined the methylation status in the 5′ CpG island of the human HPRT gene in X8-6T2 at various times after 5aCdr treatment. Using the methylation-sensitive restriction enzyme *Hpa*II and hybridization with a probe specific for the human HPRT gene (PB1.7; Fig. 2A), we determined the methylation status at several sites in the CpG island known to be correlated with gene expression. As shown in Fig. 2B, *Hpa*II digestion of Y162-11c DNA resulted in two detectable fragments of 0.64 kb (*Hpa*II 6-*Hpa*II 7) and 0.87 kb (*Hpa*II 8-*Pst*I). This result indicates that these *Hpa*II sites in the CpG island of the active human X chromosome in Y162-11c are unmethylated because *Hpa*II cleaves DNA only when both DNA strands of the recognition site are unmethylated. In contrast, the same digestion of untreated X8-6T2 DNA resulted in a 1.57-kb fragment (*Hpa*II 6-*Pst*I), which indicates that sites *Hpa*II 7 and 8 are methylated on the inactive X chromosome, whereas site *Hpa*II 6 is unmethylated.

Following treatment of X8-6T2 with 5aCdr, no change in the *Hpa*II digestion pattern was detected until after 12 h. At 24 h, the two fragments resulting from cleavage at sites *Hpa*II 7 and 8 (0.64 and 0.87 kb) appeared at a level which indicates that approximately 12% of X8-6T2 cells were unmethylated at these sites. The cleaved fractions increased to approximately 24% at 36 h and 44% at 48 h and remained at this level at 72 and 96 h. These results indicate that HPRT gene inactivity correlates with methylation in the 5′ CpG island and that the increase in mRNA level following treatment with 5aCdr parallels the demethylation of both DNA strands in this region.

Change in chromatin sensitivity at the 5′ CpG island following 5aCdr treatment. Chromatin sensitivity to nuclease digestion in the 5′ CpG island was studied by *Msp*I treatment of isolated nuclei. As shown in Fig. 3, active chromatin isolated from Y162-11c was extremely sensitive to *Msp*I

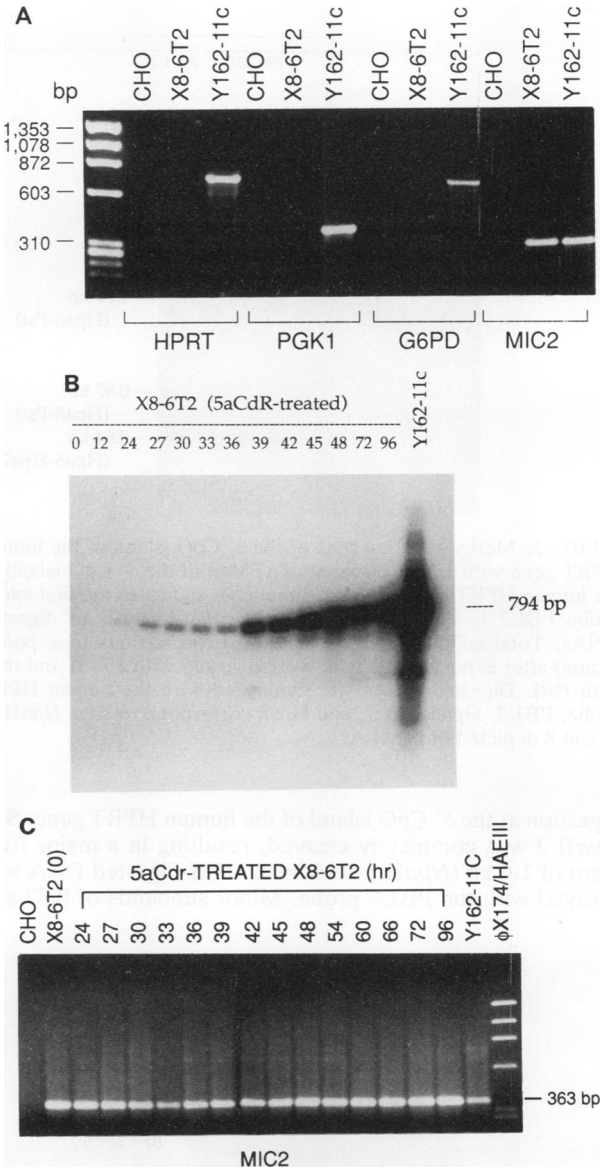

FIG. 1. Expression patterns of specific mRNAs of active, inactive, and reactivated human X-linked genes. First-strand cDNA was reverse transcribed from 0.5 μg of total cellular RNA with random hexamers and amplified by 27 cycles of PCR with specific primers. (A) Differential expression of inactive (X8-6T2) and active (Y162-11c) human X chromosomes. RT-PCR products were detected by staining with ethidium bromide. RT-PCR products for HPRT, PGK-1, G6PD, and *MIC2* are 794, 397, 757, and 363 bp, respectively, in size. (B) mRNA levels of the human HPRT gene in untreated Y162-11c and in X8-6T2 following exposure to 5aCdr. X8-6T2 cells were treated with 5aCdr during the initial 24 h. Specific mRNA was amplified by RT-PCR and analyzed by hybridization of a Southern blot with a human HPRT cDNA probe. Numbers above the lanes indicate times (hours) after initiation of treatment. (C) mRNA levels of the human *MIC2* gene in untreated Y162-11c and X8-6T2 following exposure to 5aCdr. X8-6T2 RNA samples were from the experiment described for panel B. RT-PCR products were detected by staining with ethidium bromide.

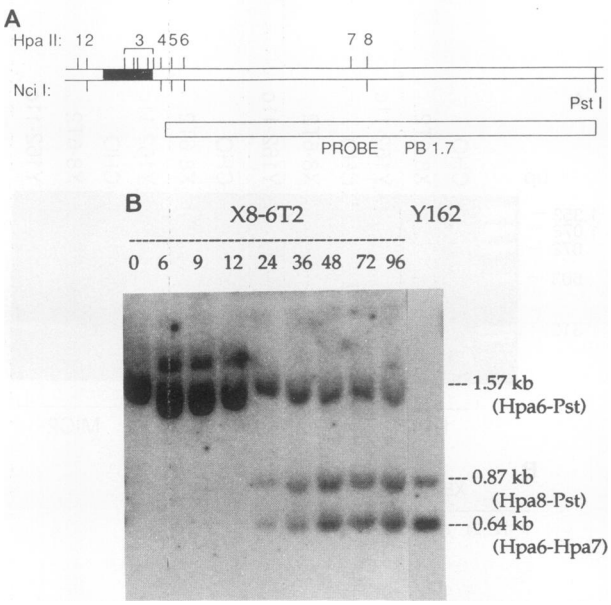

FIG. 2. Methylation analysis of the 5′ CpG island of the human HPRT gene with *Hpa*II digestion. (A) Map of the 5′ CpG island of the human HPRT gene. The solid rectangle indicates the first exon. Probe PB1.7 is shown below the map. (B) Analysis of digested DNAs. Total cellular DNAs from X8-6T2 at various time points (hours) after exposure to 5aCdr were digested with *Hpa*II and then with *Pst*I. Digested DNAs were analyzed with the human HPRT probe, PB1.7. Hpa6, Hpa7, and Hpa8 correspond to sites *Hpa*II 6, 7, and 8 depicted in panel A.

digestion at the 5′ CpG island of the human HPRT gene. Site *Hpa*II 3 was completely cleaved, resulting in a major fragment of 1.6 kb (*Hpa*II 3-*Pst*I) when *Pst*I-digested DNA was assayed with the PB1.7 probe. Minor subbands of 0.87 and

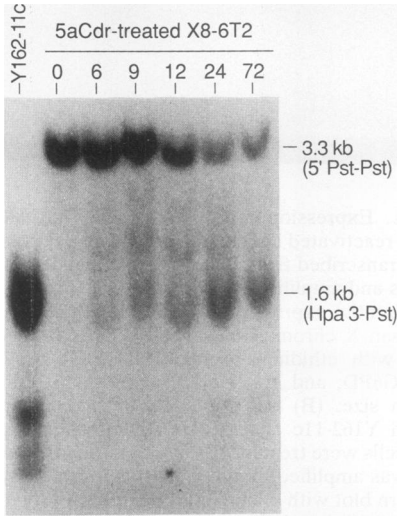

FIG. 3. Chromatin sensitivity to *Msp*I digestion. Nuclei isolated from Y162-11c, X8-6T2, and 5aCdr-treated X8-6T2 were digested with 0.35 U of *Msp*I per μg of DNA for 20 min. DNAs were extracted from the treated nuclei, cleaved with *Pst*I, and then analyzed with probe PB1.7. 5′ Pst-Pst corresponds to the *Pst*I site located 5′ to the CpG island. Times (hours) after initiation of treatment are indicated above the lanes.

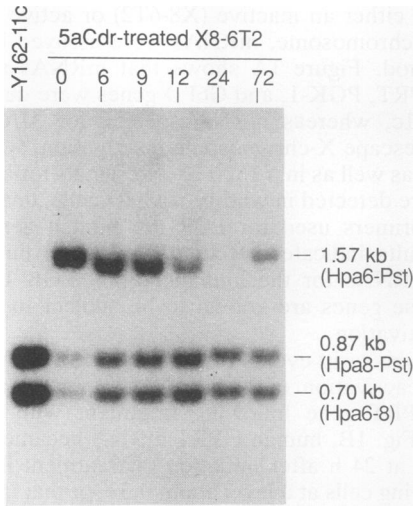

FIG. 4. Hemimethylation analysis at the 5′ CpG island of the human HPRT gene. Total DNAs from Y162-11c and from X8-6T2 at various time points (hours) following 5aCdr treatment were digested with *Nci*I and subsequently with *Pst*I and then analyzed with probe PB1.7. *Nci*I can cleave sites indicated in Fig. 2A when the sites are symmetrically unmethylated or either strand is hemimethylated.

0.70 kb were also seen, indicating the presence of minor *Msp*I-sensitive sites at sites *Hpa*II 8 and 6. Chromatin isolated from untreated X8-6T2 cells showed a marked resistance to *Msp*I digestion at the 5′ CpG island of the human HPRT gene. Only 3% of the parent fragment was cleaved after *Msp*I treatment of isolated nuclei. However, a change in chromatin sensitivity to *Msp*I occurred at the HPRT gene 6 h after 5aCdr treatment. At 6 h, the fraction cleaved at site *Hpa*II 3 increased to about 12%. The cleaved fraction increased further to 20% at 12 h and to 53% at 24 h, where it remained for the duration of the experiment (72 h). This time course of chromatin sensitivity following 5aCdr treatment indicates that the change to an open chromatin conformation at the HPRT gene appears, and reaches a maximal level, before the appearances of symmetrical demethylation and HPRT mRNA.

Hemimethylation correlates with the change in chromatin sensitivity to *Msp*I. The finding that chromatin sensitivity preceded symmetrical demethylation prompted us to study the appearance of hemimethylated DNA with respect to the time after 5aCdr treatment. The restriction enzyme *Nci*I recognizes CCCGG:CCGGG and can cleave the site when the CpG dinucleotide on one strand is methylated (either strand) or when both strands are unmethylated but not when both strands are methylated (26). Sites *Hpa*II 2, 4, 5, 6, and 8 are also *Nci*I sites (Fig. 2A). Figure 4 shows the *Nci*I-digested patterns of the 5′ CpG island in Y162-11c and X8-6T2 before and after 5aCdr treatment. Site *Hpa*II 6 was cleaved with *Nci*I both in Y162-11c and in untreated X8-6T2. However, site *Hpa*II 8 on the active HPRT gene in Y162-11c was completely cleaved with *Nci*I, resulting in fragments of 0.70 kb (*Hpa*II 6-*Hpa*II 8) and 0.87 kb (*Hpa*II 8-*Pst*I), while the same site on the inactive human HPRT gene in untreated X8-6T2 cells was cleaved to a level of only about 10%. Following treatment with 5aCdr, the *Nci*I-cleaved fraction of X8-6T2 DNA did not change in the interval from 0 to 3 h (data not shown) but showed an increase to about 32% at 6 h, to 68% at 12 h, and to 93% at 24 h when the treatment was

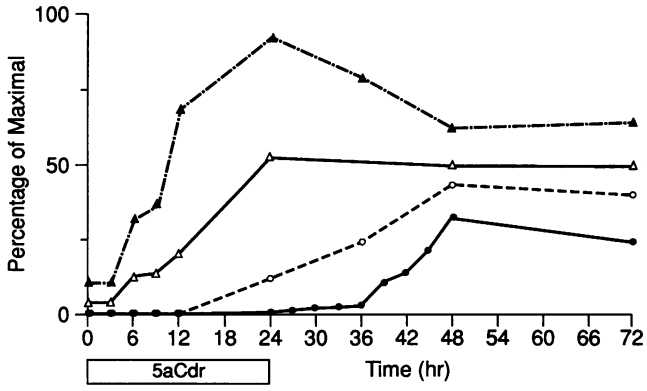

FIG. 5. Time courses of methylation states, chromatin sensitivity, and the mRNA level of the human HPRT gene in X8-6T2 following 5aCdr treatment in an exponentially growing culture. Data represent symmetrical demethylation (○), sum of either-strand demethylation (▲), *Msp*I-hypersensitive chromatin (△), and mRNA level (●).

terminated. After removing the 5aCdr, the *Nci*I-cleaved fraction decreased to about 63% at 48 h and then remained at this level until 96 h, when the experiment was terminated.

The time courses of hemimethylation (*Nci*I), symmetrical methylation (*Hpa*II), chromatin sensitivity (*Msp*I), and transcriptional activity following exposure to 5aCdr are summarized in Fig. 5. The increase in hemimethylation correlates with the appearance of chromatin sensitivity at the 5′ CpG island. These events precede the simultaneous appearances of symmetrical demethylation and mRNA.

Absence of change in methylation status, chromatin sensitivity, and mRNA level following 5aCdr treatment in G_1-arrested cells. There is convincing evidence that 5aC must be incorporated into DNA to inhibit the action of DNA methyltransferase (35) and to bring about reactivation of inactive X-linked housekeeping genes as observed many cell generations after 5aC exposure (17). However, there are no studies on the immediate effect of 5aC on methylation, chromatin sensitivity, and transcriptional reactivation as we have carried out here. Therefore, we felt that a G_1 arrest experiment was a necessary control for our log-phase culture studies. In G_1-arrested X8-6T2 before 5aCdr treatment, about 10% of the parental fraction (1.57 kb) was cleaved by *Nci*I at site *Hpa*II 8, producing the 0.87- and 0.70-kb fragments that are seen in exponentially growing X8-6T2 cells (Fig. 6A). In contrast to the exponential-phase culture, this *Nci*I-cleaved fraction did not increase in the G_1-arrested cells following 5aCdr exposure.

We also analyzed *Msp*I sensitivity of chromatin and mRNA level in these G_1-arrested cells (Fig. 6B and C). The results indicate that *Msp*I sensitivity of HPRT chromatin showed no change following 5aCdr treatment, nor was any HPRT mRNA detected in the G_1-arrested cells.

DISCUSSION

Our analysis relies on the use of 5aCdr to induce reactivation of genes on the inactive X chromosome. The mechanism by which 5aCdr is assumed to induce reactivation is by incorporation of the analog into replicating DNA and subsequent demethylation through inhibition of the maintenance methylase (17, 35). The kinetics of appearance of reactivated genes following 5aCdr exposure supports this interpretation (9, 17). The time course patterns that we observed for demethylation of either strand versus both strands and our study of G_1-arrested cells provide additional support for this model of 5aCdr action. Within 6 h of exposure of an exponentially growing culture to 5aCdr, we can detect a significant increase in hemimethylation and an accompanying increase in chromatin sensitivity, as determined by *Msp*I and DNase I digestion (DNase I digestion data not shown). We are not able to separate these two events by time and probably will not be able to do so, as

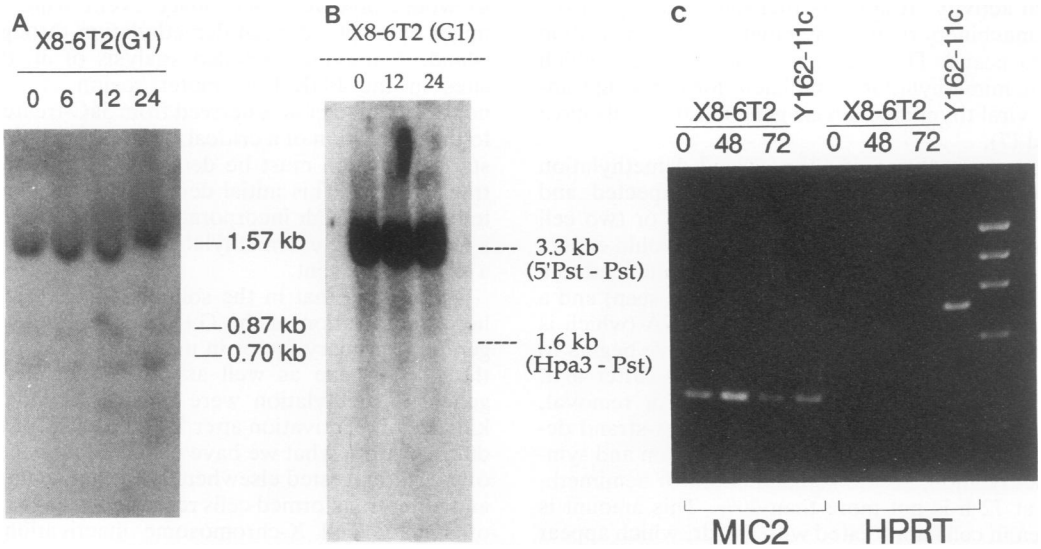

FIG. 6. Absence of change in methylation status, chromatin sensitivity, and mRNA level following 5aCdr treatment in G_1-arrested cells. X8-6T2 cells were arrested at the G_1 stage by culturing in isoleucine-free medium and then examined following 5aCdr treatment in the same medium. Shown are the patterns over time of hemimethylation (*Nci*I digestion) of total cellular DNA (A), chromatin sensitivity to *Msp*I digestion (B), and mRNA levels of the *MIC2* and HPRT genes (C). Probe PB1.7 was used in panels A and B. Ethidium bromide staining was used in panel C to analyze total cellular RNAs from G_1-arrested Y162-11c and G_1-arrested X8-6T2 at various points following 5aCdr treatment.

Weintraub (39) has shown that the establishment of a DNase I-hypersensitive chromatin conformation state may require only a few minutes.

It has been suggested to us that the instability of 5aCdr might cause nicking at the site of incorporation, thereby changing the superhelical tension in the looped domain and inducing chromatin hypersensitivity. If this were so, we would expect chromatin sensitivity to appear at many sites and precede the increase in hemimethylation. Also, the maximal level of chromatin sensitivity should be equal to the maximal level of hemimethylation because hemimethylation results from the incorporation of 5aCdr into either strand. Because neither of these predictions were supported by our data (Fig. 5), we think that hemimethylation is primary to chromatin hypersensitivity.

The maximal level of chromatin sensitivity induction is distinctly lower than that of demethylation in either strand (Fig. 5). It seems possible that the difference between these levels reflects a mechanism in which switching of chromatin conformation from an insensitive to hypersensitive state is dependent on demethylation of a specific strand rather than either strand, though we lack definitive evidence for this hypothesis. A similar association of specific-strand demethylation with a nuclease-sensitive chromatin conformation has been reported previously for the chicken vitellogenin gene (33). Such observations could be explained by the loss of a DNA-binding protein which binds to symmetrically methylated DNA but not to hemimethylated or unmethylated DNA (1, 4, 24). This mechanism may be involved in the regulation of the chicken vitellogenin gene (33) but is apparently not used for X inactivation of the human PGK-1 gene (27, 28).

Alternatively, demethylation could directly alter chromatin conformation or permit binding of specific proteins which induce a nuclease-sensitive chromatin conformation. Evidence for this mechanism has also been observed for the vitellogenin gene (32) and may also occur in the human PGK-1 gene (12a). Regardless of the mechanisms involved, it is clear that hemimethylation, and perhaps demethylation of a specific strand, permits attack of nucleases but not transcriptional activity. It appears that full assembly of the transcription machinery requires symmetrical demethylation of the 5′ CpG island. Data similar to these results which indicate that hemimethylation is sufficient for blocking transcription of a viral thymidine kinase gene in rat L cells have been reported (7).

The time course analyses of either-strand demethylation and symmetrical demethylation gave both expected and unexpected results (Fig. 5). In a period of one or two cell generations after removal of 5aCdr at 24 h, we would expect new maintenance methylase to be synthesized and lead to a plateau of symmetrical demethylation (which is seen) and a complete disappearance of hemimethylated DNA (which is not seen). The either-strand demethylation curve begins to drop rapidly after 24 h but then appears to plateau after 48 h. Even at 72 h, three cell generations after 5aCdr removal, some hemimethylated DNA remains. The either-strand demethylation curve measures both hemimethylation and symmetrical demethylation, so the actual amount of hemimethylated DNA at 72 h is not more than 20%. This amount is twice that seen in cells not treated with 5aCdr, which appear to maintain hemimethylated DNA at a level of about 10%. Similar reports of the maintenance of hemimethylated sites have been made by others (32, 33).

Our RT-PCR analysis shows that inactivated genes (HPRT, PGK-1, and G6PD) on the inactive X chromosome are not detectably transcribed. Amplification of diluted total RNA from Y162-11c reveals that the mRNA level of the human HPRT gene in untreated X8-6T2 is less than 0.1% of the level in Y162-11c (data not shown). These results confirm and extend two previous reports indicating that X inactivation involves control by mRNA level (5, 12).

After 5aCdr exposure, the level of HPRT mRNA increases gradually from 24 to 36 h and then increases dramatically between 36 and 48 h. This biphasic pattern in the mRNA time course is quite different from those of demethylation (hemimethylation and symmetrical methylation) and of chromatin conformation (Fig. 5). The mRNA level is determined by the proportion of cells reactivated, the transcriptional rate, and the degradation rate of the mRNA. The approach to mRNA equilibrium level is determined by the rate at which cells are reactivated and whether reactivation involves a single step or multiple steps. If reactivation involves a single step (i.e., full transcription follows initial symmetrical demethylation), a rapid approach to equilibrium is expected, with the steepness of the curve depending on the synchrony of the reactivated population. However, if reactivation involves more than one step (i.e., the initial transcription rate is slow but increases as a result of secondary events), then a stepwise approach to equilibrium is expected. This, in fact, appears to be what we have observed. It is not likely that this mRNA curve reflects a two-step recovery from 5aCdr toxicity; that is, the growth rate is markedly inhibited at first, after which the cells undergo recovery and assume a more rapid growth rate. If this were so, the demethylation effects would show a similar response, which they do not. Furthermore, our studies of doubling times following 5aCdr exposure show a rather modest change (the normal doubling time for X8-6T2 is 16 h, and it is extended to 17.5 h in the period following 5aCdr treatment).

The initial reactivation event may involve demethylation of both strands in a critical promoter region leading to limited transcription. The slow and linear rate of mRNA increase from 24 to 36 h would reflect the asynchrony of the cell population. The rapid increase in mRNA level (36 to 48 h) would involve a secondary event which we speculate might be a spreading of demethylation throughout the CpG island. A previous detailed analysis of all the methylated sites in the PGK-1 promoter region of reactivated and nonreactivated clones derived from 5aC-treated cells (28) led to the suggestion of a critical region around the transcription start site which must be demethylated for the initiation of transcription. This initial demethylation, which is assumed to be due to 5aCdr incorporation, would then be followed by a more extensive demethylation throughout the promoter as a secondary event.

We believe that in the somatic cell hybrids under study here, methylation of the G+C-rich promoter of the HPRT gene is a primary factor in maintaining the inactive state of the HPRT gene as well as other X-linked housekeeping genes. If methylation were only a secondary factor, the kinetics of reactivation after 5aCdr treatment would be quite different from what we have observed. It is possible, as one of us has suggested elsewhere (11), that somatic cell hybrids and other transformed cells represent a stage in the ontogeny of somatic cell X-chromosome inactivation in which an inactivation control mechanism has been turned off or lost. The existence of a redundant control mechanism for the maintenance of inactivation will require experiments with normal cells, a study which may now be possible with the methodology described here.

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