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Demethylation of Specific Sites in the 5′ Region of the Inactive X-Linked Human Phosphoglycerate Kinase Gene Correlates with the Appearance of Nuclease Sensitivity and Gene Expression

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X8/6T2, a hamster-human hybrid cell line which contains an inactive human X chromosome, was treated with 5-azacytidine and selected for derepression of hypoxanthine-guanine phosphoribosyltransferase. Clones were examined for coreactivation of the phosphoglycerate kinase gene (*Pgk*). Of 68 of these hybrids, approximately 20% expressed measurable human phosphoglycerate kinase (PGK) activity. A 600-base-pair region of the *Pgk* 5′ CpG cluster was examined for the methylation status of eight CCGG sites (site 1 being 5′-most) in a number of PGK-negative and PGK-positive cell lines. The inactive X chromosome is normally methylated at all eight sites, and this was also true for the majority of X8/6T2 cells. However, several PGK-negative hybrids were demethylated in the site 3 to site 6 region. PGK activity correlated with demethylation at both sites 6 and 7. The data for PGK-positive and -negative hybrids indicate that demethylation at or near site 7 was necessary for reactivation of *Pgk*. Chromatin sensitivity to *Msp*I digestion in the nuclei of male lymphoblastoid cells and several PGK-positive and PGK-negative hybrids was examined. PGK-positive cell lines were hypersensitive to digestion, while PGK-negative hybrids were resistant. Cleavage at sites 6 and 7 was observed in all PGK-positive cell lines at each *Msp*I concentration examined. Sites 7 and 8 were less accessible to digestion than site 6. Cleavage in the site 2 to site 5 region was observable at the lowest *Msp*I concentration. In most PGK-positive hybrids, a nonspecific endogenous nuclease detected the presence of a hypersensitive region spanning at least 450 base pairs, bounded at the 3′ end near *Hpa*II site 6. Nuclease hypersensitivity appears to be related to promoter activity, because sites 7 and 8 are in transcribed regions of the gene. These data indicate that specific sites within the CpG cluster have a dominant controlling influence over the *Pgk* promoter conformation and the transcriptional activation of *Pgk*.

Dosage compensation of X-linked genes in mammalian females is achieved by inactivation of one X chromosome (14, 28). Early in embryogenesis one of the X chromosomes becomes heterochromatic, late replicating, and genetically inert over most of its length. Once established, X inactivation in somatic cells is clonally inherited and extremely stable. However, in germ cells reactivation occurs just prior to meiosis (13, 22), and it has been reported that reactivation of the inactive X chromosome frequently occurs in human trophectoderm derivatives (30). The ontogeny of X inactivation is very likely a multistep process whose initiation is little understood at the molecular level. However, there is considerable evidence for the hypothesis that DNA methylation is involved in the later stages of X inactivation. These include studies of 5-azacytidine (5AC)-induced reactivation of the genes for hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), phosphoglycerate kinase (*Pgk*), glucose-6-phosphate dehydrogenase (*G6pd*), and α-galactosidase (17, 24, 31), transfection experiments with *Hprt* (24, 25, 48), and methylation-sensitive restriction enzyme analyses which have shown differential DNA methylation between active and inactive *Hprt*, *Pgk*, and *G6pd* genes (19, 26, 32, 37, 47, 51, 52, 56).

Clusters of CpG dinucleotides associated with the *Hprt*, *Pgk*, and *G6pd* genes are hypomethylated in active genes and methylated in inactive ones. The same regions in the *Hprt* and *G6pd* genes contain nuclease-hypersensitive sites which also differentiate active and inactive alleles (54). Nuclease-sensitive sites have also been described for the 3′ and 5′ regions of the *Pgk* gene on the active X chromosome (38, 39). The relative influence of particular methylation sites in determining nuclease sensitivity and gene expression could not be evaluated in these studies. In this report, we describe a variety of 5AC-treated cell hybrids which varied in their expression of human phosphoglycerate kinase (PGK) activity. The cell lines differed at the 5′ CpG cluster of *Pgk* in their *Hpa*II site methylation pattern and chromatin sensitivity to nuclease digestion. The data suggest that demethylation of specific sites in this region is critical for expression. Furthermore, we discovered a distinctive pattern of increased chromatin sensitivity to *Msp*I at demethylated sites in the 5′ region upon reactivation of *Pgk*.

MATERIALS AND METHODS

Cell culture. Standard growth conditions for cultured cells have been described (38). The 6-thioguanine-resistant human-hamster hybrid cell line X8/6T2 was characterized previously (10, 11). X8/6T2 cells originally contained two inactive human X chromosomes, but at least 95% of the cells used here had a single X chromosome (11). The rodent-cell parent was the Chinese hamster ovary cell line CHOYH21, which is deficient in hypoxanthine-guanine phosphoribosyltransferase and glucose-6-phosphate dehydrogenase (40). The human-cell parent was a 5X fibroblast cell line. X8/6T2 cells were grown in standard medium: RPMI 1640 containing

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10% (vol/vol) fetal calf serum and 40 μg of gentamicin sulfate (Garamycin; Elkins-Sinn, Inc.) per ml.

Reactivation experiments were initially performed as described by Mohandas et al. (31). This method results in a high frequency of mixed-population "clones" (11) and was therefore modified slightly. About 5×10^4 cells were plated into 25-cm^2 tissue culture flasks and treated the next day with standard medium containing 4 μM 5AC. The 5AC medium was replaced with standard medium after 24 h. The cells were grown for 2 days in standard medium and then cloned by limiting dilution with HAT medium (10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine in standard medium), using 79-cm^2 tissue culture dishes (100 cells per dish) or 96-well tissue culture plates (100 cells per plate). Clones were isolated after about 2 weeks and expanded for PGK extraction and for storage in liquid N_2. The frequency of *Hprt* reactivation was estimated after plating 10^3 5AC-treated cells into 79-cm^2 tissue culture dishes and growing the cells in standard versus HAT medium. Cells in HAT medium were allowed to grow longer than those in standard medium in order to reach equivalent colony sizes. Colonies were fixed and stained with 0.5% crystal violet in 20% ethanol.

X8/HAT cells are a population of HAT-resistant derivatives of X8/6T2 obtained by treating cells with 5AC and selecting in HAT medium (13). Y162/11C is a human-hamster hybrid cell line containing an active human X chromosome (M. Rocchi, unpublished data). X8/HAT, Y162/11C, and the HAT-resistant clones described in Results were routinely grown in HAT medium. Lymphoblastoid cell lines were derived from either Epstein-Barr virus-transformed male or 5X female lymphocytes (39) and routinely grown in standard medium containing 15% fetal calf serum.

Electrophoresis of PGK. Human and hamster forms of PGK were distinguished by cellulose polyacetate strip electrophoresis by the procedure described by Reddy et al. (35). For each cell line, cells from a 25-cm^2 flask with confluent growth were harvested, washed in phosphate-buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4], 137 mM NaCl, 2.7 mM KCl), and frozen as a cell pellet. The pellet was resuspended in 20 to 50 μl of PBS, and PGK activity was further extracted by two cycles of freeze-thawing. Samples were usually diluted 10-fold in PBS containing 0.1 mg of bovine serum albumin per ml and 0.05% bromophenol blue before application to the cellulose acetate strips. Electrophoresis was performed for 2 h at 150 V.

***Msp*I digestion of nuclei.** Nuclei were isolated as previously described (38). For cell hybrids, six to nine 177-cm^2 dishes with nearly confluent growth were washed four times with PBS and sequentially harvested in PBS by scraping with a rubber spatula. The final suspension was brought to 50 ml in a 50-ml conical centrifuge tube and centrifuged at $450 \times g$ for 5 min at 4°C. Lymphoblastoid cells (~10^8) were harvested by centrifugation in 50-ml conical tubes, combined, and washed three times in PBS. The washed cell pellets were suspended with a disposable plastic pipette in 5 ml of ice-cold 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl–5 mM $MgCl_2$ (RSB) containing 0.5% Nonidet P-40. The suspension was brought to 50 ml with the same buffer and centrifuged for 2 min at $450 \times g$. The nuclei were washed two more times with RSB plus Nonidet P-40 and then suspended in 1 ml of RSB. DNA content was determined by A_{260} of samples placed in 0.1% sodium dodecyl sulfate (SDS). Nuclei were adjusted to 11.6 A_{260} units per ml with RSB, and 1 mM dithiothreitol was added. *Msp*I reactions were carried out in 1.5-ml plastic centrifuge tubes at 37°C for 20 min. The reactions were terminated by adding 10 mM EDTA, 0.1% SDS, and 0.5 mg of proteinase K per ml. Proteinase K digestion was carried out for 60 to 90 min at 37°C. Samples were stored frozen at −20°C until phenol extraction.

DNA analysis. Hybrid cell DNA was isolated from two 177-cm^2 dishes by washing nearly confluent monolayers four times with PBS and lysing the cells in 40 mM Tris hydrochloride (pH 7.5)–0.1 M NaCl–20 mM EDTA–0.5% SDS. The lysate was incubated with 0.5 mg of proteinase K per ml for 60 to 90 min at 37°C and stored at −20°C. DNA from nuclear and whole-cell lysates was purified as described previously (38). DNA was quantitated by fluorescent dye binding (23).

Restriction enzyme digestion conditions were usually those recommended by the manufacturer, with the addition of 0.1 mg of bovine serum albumin per ml, 0.1 mg of RNase A per ml, 1 mM dithiothreitol, and 1 mM spermidine. Restriction enzymes were added at 3 to 4 U/μg of DNA, and digestions were carried out at 37°C for 6 to 16 h. For *Eco*RI-*Bgl*II double digests, the reactions were carried out simultaneously. In *Hpa*II-*Eco*RI-*Bgl*II or *Msp*I-*Eco*RI-*Bgl*II triple digests, the *Hpa*II or *Msp*I reaction was carried out first overnight. *Eco*RI reaction buffer, *Eco*RI, and *Bgl*II were then added, and the reaction was continued for 6 h. Reactions were terminated by the addition of one-fifth volume of concentrated electrophoresis sample buffer (100 mM EDTA [pH 7.5], 0.5% SDS, 50% glycerol, 0.5% bromophenol blue).

Agarose gel electrophoresis and Southern blotting methods were essentially as described previously (38). Prehybridization buffer contained 50% formamide, 75 mM sodium citrate-HCl (pH 7.0), 0.75 M NaCl, 50 μg of salmon testes DNA per ml, 2.5 times concentrated Denhardt solution, and 0.05% SDS. Prehybridization was performed at 42°C for 6 to 16 h. Hybridization was carried out in the same buffer containing 10% dextran sulfate for 24 to 72 h at 42°C.

Probe BI 2.20 is a pUC18 subclone derived in our laboratory by M. A. Goldman from λEMBL3-PGKI (37), a *Pgk* genomic clone supplied by J. Singer-Sam, Molecular Biology Section, Beckman Research Institute of the City of Hope, Duarte, Calif.

RESULTS

Reactivation of the *Pgk* gene. The HAT-sensitive human-hamster cell line X8/6T2 was treated with 5AC and then selected in HAT medium to obtain cell lines in which the previously inactive human *Hprt* gene had become active. Clones derived from this procedure were tested for reactivation of the *Pgk* locus. The frequency of HAT-resistant clones relative to unselected 5AC-treated clones was about 25% (three experiments). Clones were expanded and analyzed by cellulose acetate electrophoresis for human PGK activity after at least 30 generations.

The activity patterns of X8/6T2 and various HAT-resistant clones are shown in Fig. 1. Y162/11C is a human-hamster hybrid cell line which served as a control for full human PGK activity because it is similar to X8/6T2 except that it contains an active rather than an inactive X chromosome. The human PGK band of Y162/11C was about as strong as the hamster PGK band. Several HAT-resistant clones also showed strong human PGK activity, while a few had weaker activity (e.g., 11B, V-3D, and V-6C). The X8/HAT mass culture of HAT-resistant clones, which had been grown for a number of generations after 5AC treatment, appeared to have no human PGK. The limit of detection for human PGK activity was about 5% of the hamster PGK activity. The frequency of

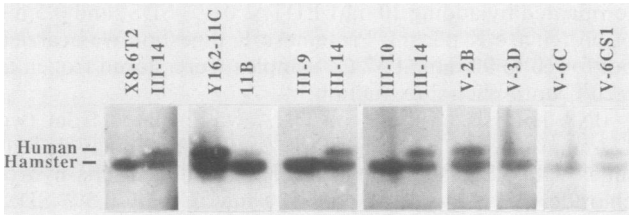

FIG. 1. Cellulose polyacetate electrophoresis showing PGK activity of human-hamster somatic cell hybrids. X8/6T2 is the untreated parental hybrid, containing an inactive human X chromosome. Y162/11C is a hybrid containing an active X chromosome. The other cell lines were derived from X8/6T2 after 5AC treatment.

human PGK-positive clones detected among HAT-resistant clones is shown in Table 1 for each of five experiments. In the 68 clones analyzed, the overall PGK reactivation frequency was 21%. Several PGK-positive and PGK-negative clones were analyzed further for DNA methylation status at the 5′ end of the gene.

Methylation of 5′ *Hpa*II sites in HPRT$^+$ "reactivants." Sequence data for many regions of the human *Pgk* gene are known, and several restriction enzyme sites have been mapped (29, 37, 44; M. Goldman, unpublished data). The localization of selected restriction sites in the 5′ region of the gene are illustrated in Fig. 2. Also shown are the locations of putative promoter elements (containing the Sp1 transcription initiation factor binding sequence GGGCGG [15]), the major transcription start site, the coding portion of exon 1, and the probe, BI 2.20, which was used to examine the methylation pattern and nuclease sensitivity of the region.

The C+G-rich region 5′ to exon 1 contains eight CCGG sites which could potentially be recognized by the methylation-sensitive restriction enzyme *Hpa*II. The DNA of various cell lines was tested for *Hpa*II sensitivity in this region by using the ^{32}P-labeled intronic probe BI 2.20. Genomic southern blots of *Eco*RI-*Bgl*II-*Hpa*II triple-digested DNA from these cells are shown in Fig. 3. Single bands were present in X8/6T2 and X8/HAT (both PGK negative) which comigrated with *Eco*RI-*Bgl*II-digested DNA, indicating that either all *Hpa*II sites were methylated or possibly just sites 2 to 8 given the proximity of site 1 to the *Eco*RI site. None of the predominant bands observed after *Hpa*II-*Bgl*II digestion of X8/6T2 DNA corresponded to cleavage at sites near the *Eco*RI site (data not shown). Therefore, all eight sites were methylated in the parental cell line, as was previously shown for the inactive X chromosome in normal females (37). One half of the PGK-negative hybrids appeared to be totally

TABLE 1. Reactivation of human PGK in X8/6T2 cells following 5AC treatment and HAT selection

Expt	No. of PGK-positive clones[a]	Total no. of clones	% PGK-positive clones
1	2	12	17
2	3	19	16
3	1	10	10
4	5	13	38
5	3	14	21

[a] Any detectable human PGK activity after cellulose acetate chromatography.

methylated, as in the parental cell line. *Hpa*II-derived bands, representing demethylation at sites 3 to 6, were present in the other seven PGK-negative cell lines (11BS7, 15A, 5AC D, 5AC M, III-8, III-9, and V-1B).

It is known from a previous study (19) that all eight CCGG sites in this region are unmethylated in normal male DNA. This is consistent with the finding that Y162/11C was demethylated at site 8 (Fig. 3). *Hpa*II-digested Y162/11C DNA comigrated with DNA extensively digested with *Msp*I, an isoschizomer of *Hpa*II which is generally insensitive to methylation at the internal cytosine (data not shown). DNA from male lymphoblastoid cells was also sensitive to *Hpa*II and *Msp*I at site 8. As in the active X chromosome cell lines, most of the PGK-positive *Hprt* reactivants were demethylated at site 8. However, an exception was V-2B, which was methylated at site 8 and demethylated at site 7. Although the methylation status of sites which are 5′ to the most 3′ demethylated site in a particular clone cannot be determined from this type of analysis, site 7 was found to be demethylated in PGK-positive hybrids (Y162/11C, 19A, III-4, III-14, and V-3D) by partial *Hpa*II digestion (data not shown). It should be noted that *Msp*I is inactive toward the sequence GGC^{m}CGG (3, 20), which occurs at sites 6 and 8. This specificity was observed in *Msp*I digestions of purified DNA from X8/6T2, III-9, and V-2B, which all gave bands corresponding to site 7 rather than site 8 cleavage (Fig. 3 and data not shown). Therefore, based on nuclease sensitivity experiments (discussed below), it can be concluded that site 6 was also demethylated in all the PGK-positive hybrids examined.

Several cell lines analyzed gave multiple bands after *Hpa*II-*Bgl*II-*Eco*RI digestion. Such patterns may have resulted from mixed cell populations during the cloning procedure or an instability of methylation inheritance. Cells were recloned to test whether errors were made in the cloning procedure. Three bands were observed for the weakly PGK-positive cell line 11B. Of five subclones exam-

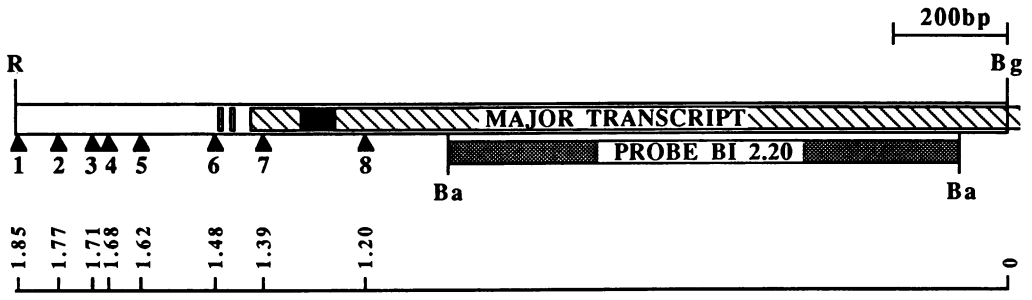

FIG. 2. Map of 5′ region of human *Pgk*, showing restriction enzyme sites, putative promoter elements, the major transcription start site, the protein coding portion of exon 1, and the genomic probe location. Ba, *Bam*HI; Bg, *Bgl*II; R, *Eco*RI; sites numbered 1 to 8 are *Hpa*II-*Msp*I sites. bp, Base pairs; kb, kilobases. Symbols: ▲, *Msp*I-*Hpa*II sites; ▯, GGGGCGG repeat; ■, coding region.

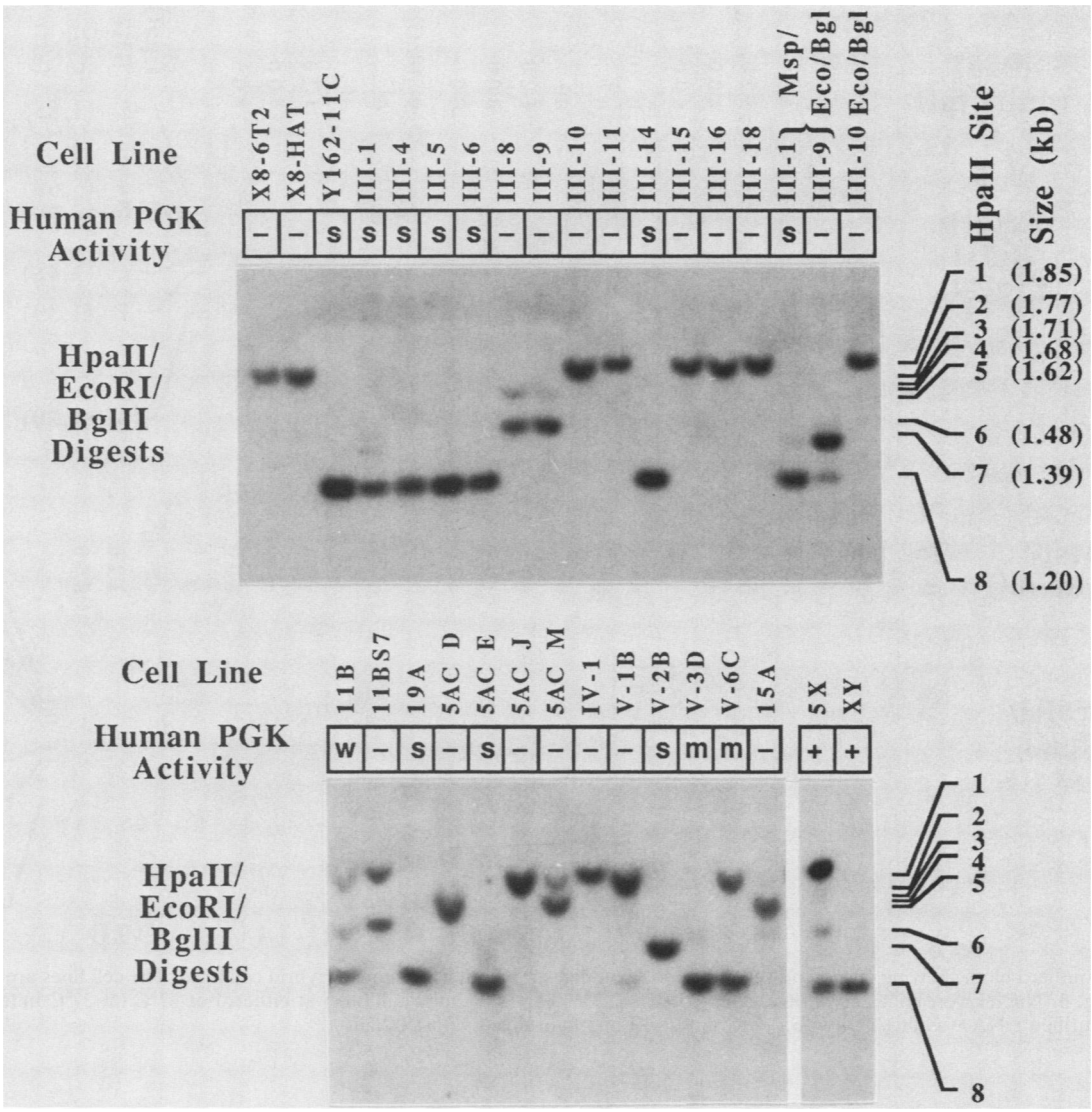

FIG. 3. Southern blots showing demethylation of CCGG sites in the 5′ region of 5AC-treated hybrid cell lines. X8/6T2 is the untreated parental cell line carrying the inactive human X chromosome; X8/HAT is an uncloned culture of 5AC-treated and HAT-selected hybrids derived from X8/6T2; Y162/11C contains an active human X chromosome; XY and 5X are male and 5X-female lymphoblastoid cell lines, respectively; the remaining cell lines are clones derived from X8/6T2 after 5AC treatment and HAT selection. DNA was digested with *Hpa*II, *Eco*RI, and *Bgl*II and hybridized with BI 2.20 (Fig. 2). The positions corresponding to each of the eight CCGG sites shown in Fig. 2 were calculated relative to molecular size standards and are shown on the right. The amount of human PGK detected in hybrids relative to hamster PGK activity is indicated as follows: —, none; w, weak; m, moderate; s, strong (the strong PGK activity of human cell lines is indicated by +).

ined, no PGK-positive reactivants were detected (data not shown). The PGK-negative subclone 11BS7 was found to have the two higher-molecular weight bands (Fig. 3). Another negative subclone, 11BS5, also had these two bands (data not shown). Therefore, the cell mosaicism in these instances probably arose after cloning. It is unlikely that these cells contain multiple *Pgk* sequences because in situ hybridization with a human X chromosome-specific centromeric probe (K. Dyer, T. Canfield, and S. Gartler, manuscript submitted) showed a single human X chromosome in 11BS7 (data not shown). V-6C was a moderately PGK-positive cell line which had two *Hpa*II-generated bands (Fig. 1 and 3). However, the V-6CS1 subclone showed a single band corresponding to site 8 and had strong PGK activity (data not shown and Fig. 1). These data suggest that V-6C heterogeneity was a result of improper cloning or unstable methylation inheritance in the early stages of clonal growth (36, 53, 55). The 5X lymphoblast cell line gave two major bands corresponding to full methylation and site 8 demethylation in the proportion expected for the presence of one active and four inactive genes. However, faint bands were also observed at site 6 and at one of sites 2 to 4 (Fig. 3).

***Msp*I sensitivity of nuclear DNA in the 5′ region of *Pgk*.** The 5′ CCGG sites were tested for nuclease accessibility in nuclear chromatin by using *Msp*I. Nuclei were treated with increasing concentrations of *Msp*I, and the DNA was purified and then digested with *Eco*RI and *Bgl*II. The different patterns of *Msp*I sensitivity that were observed are shown in Fig. 4. The cell lines containing a single active X chromosome (Y162/11C and male lymphoblast) were quite sensitive

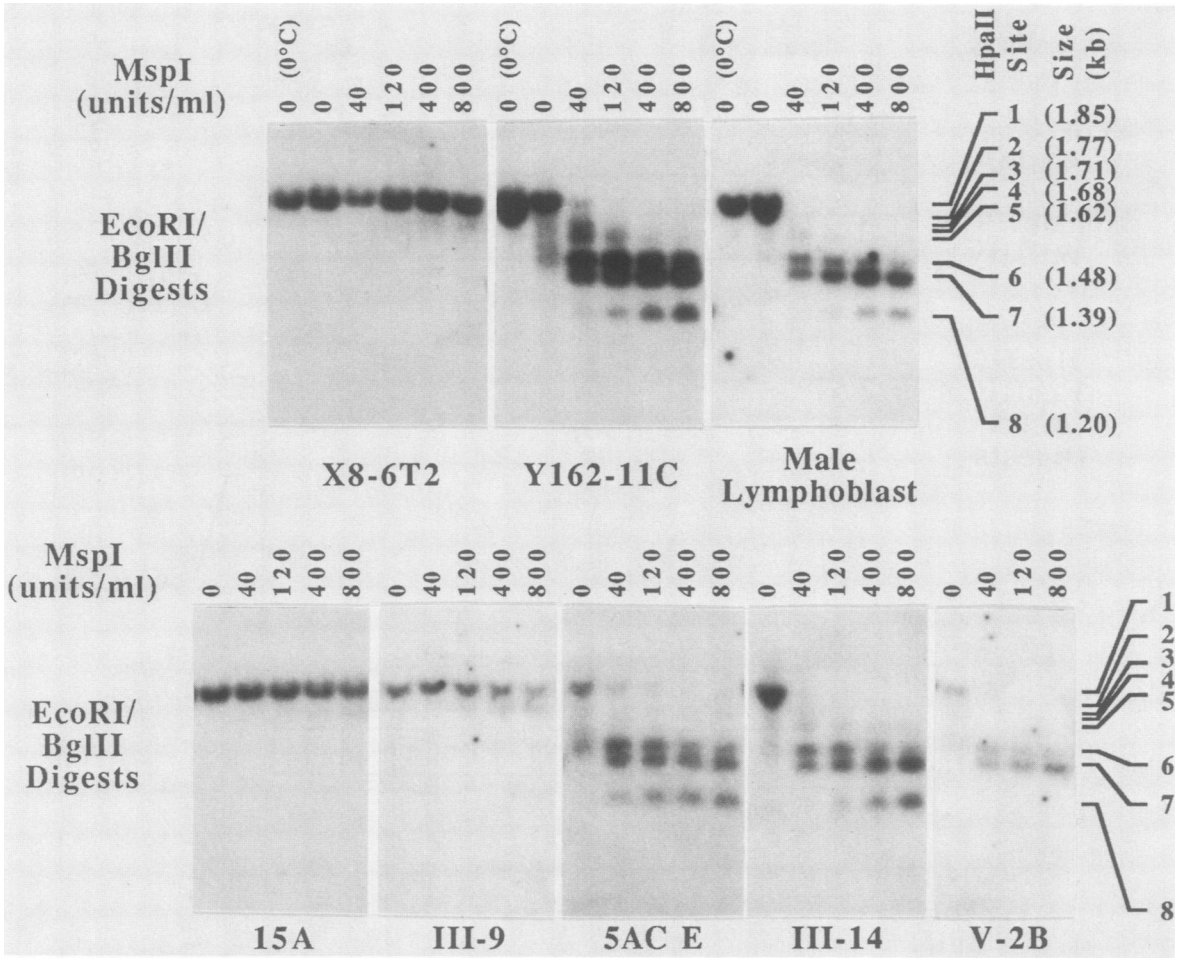

FIG. 4. Southern blots showing chromatin sensitivity to *Msp*I digestion in the 5′ region of hybrid cell lines. The cell lines are among those shown in Fig. 3. Nuclei were digested with various concentrations of *Msp*I (units/milliliter) at either 0 or 37°C (at 37°C unless otherwise indicated). Purified DNA was digested with *Eco*RI and *Bgl*II and hybridized with BI 2.20.

to *Msp*I digestion in that very little undigested DNA remained at the lowest concentration of enzyme used. The moderate-to-strong *Pgk* reactivants were also very sensitive to *Msp*I digestion. At the lowest *Msp*I concentration, the major bands observed in all PGK-positive clones indicate cleavage at sites 6 and 7. A weak doublet was also observed in the site 2 to site 5 region. A band corresponding to site 8 cleavage appeared at higher *Msp*I concentrations (except in V-2B, which was methylated at site 8). In contrast, X8/6T2 and most of the PGK-negative clones were insensitive to *Msp*I digestion, showing an undigested 1.9-kilobase band. The weaker positive cell lines, 11B and V-6C, contained both of these patterns, with the *Msp*I-undigested band predominating (data not shown). V-3D also showed moderate PGK activity (Fig. 1), yet its pattern of digestion resembled that of strong positive clones. III-9, a PGK-negative clone with site 6 demethylated, was somewhat sensitive at 400 and 800 U of *Msp*I per ml relative to the other PGK-negative cell lines. In *Msp*I digests of nuclei from PGK-positive cells, an endogenous nuclease activity resulted in limited cleavage at a number of sites extending from site 6 to near the 5′ *Eco*RI site (e.g., Y162/11C, 5AC E, and V-2B). This phenomenon was never observed in nuclei from PGK-negative cells. The *Msp*I sensitivity and methylation status of a number of PGK-negative and -positive cell lines are given in Table 2.

DISCUSSION

We used 5AC-induced gene reactivation in human-hamster cell hybrids containing an inactive human X chromosome to analyze the effect of DNA methylation on the chromatin conformation and expression of the human *Pgk* gene. The high frequency of reactivation in this system allowed for the isolation of many PGK-positive clones by selecting only for *Hprt* reactivants. The methylation status of a number of CCGG sites in *Pgk* was determined for PGK-positive and -negative cell lines. No correlation was found between expression and methylation for a number of sites beginning about 4 kilobases downstream from exon 1 (*Hpa*II-*Eco*RI-digested DNA; data not shown). Methylation patterns in the body of other X-linked genes also appear to be unimportant for expression, because they are generally similar in active and inactive alleles present in the same tissue (8, 47, 54). However, we found that the methylation status of certain sites in the 5′ region of *Pgk* did appear to be important for gene expression.

As in the normal inactive X chromosome (37), the parental hybrid was methylated at all eight *Hpa*II sites in the 5′

TABLE 2. Data for cell lines tested for chromatin sensitivity to *Msp*I

Cell line	PGK activity[a]	CCGG site methylation[b]				*Msp*I sensitivity
		2–5	6	7	8	
X8/6T2	−	+	+	+	+	−
III-10	−	+	+	+	+	−
5AC D	−	−	+	+	+	−
15A	−	−	+	+	+	−
III-9	−	?	−	+	+	−/+[c]
11BS7[d]	−	+, ?	+, −	+	+	−
Male lymphoblast[e]	+++	−	−	−	−	+
Y162/11C	+++	?	−	−	−	+
11B[d]	+	+, ?	+, −	+, ?	+, −	+/−[f]
V-6C[d]	++	+, ?	+, −	+, ?	+, −	+/−[f]
V-3D	++	?	−	−	−	+
19A	+++	?	−	−	−	+
5AC E	+++	?	−	?	−	+
III-1	+++	?	−	?	−	+
III-4	+++	?	−	−	−	+
III-5	+++	?	−	?	−	+
III-6	+++	?	−	?	−	+
III-14	+++	?	−	−	−	+
V-2B	+++	?	−	−	+	+

[a] Human PGK activity relative to hamster PGK activity: −, none detected; +, weak; ++, moderate; +++, strong.
[b] −, Demethylation of any site in the site 2 to site 5 region; ?, unknown methylation status.
[c] Slight *Msp*I sensitivity was observed at 400 and 800 U/ml.
[d] Multiple *Hpa*II-*Eco*RI-*Bgl*II cleavage products were observed.
[e] Methylation status of sites 2 to 5 and 7 was inferred from published data for male DNA (21).
[f] Some *Msp*I-insensitive material was present at all *Msp*I concentrations.

region. All of the *Pgk* reactivants and some of the PGK-negative hybrids were demethylated in this region (Fig. 3 and Table 2). Demethylation at a *Hpa*II site in the region of sites 3 to 6 was not sufficient for *Pgk* expression (Fig. 3 and Table 2). By analogy with other housekeeping genes (12, 37), six base pairs downstream from site 6 are putative promoter elements which contain the consensus binding sequence for RNA polymerase II transcription factor Sp1 (15). Therefore, it is interesting that site 6 demethylation was not sufficient for reactivation (clones 11BS7, III-8, and III-9). However, all 11 PGK-positive hybrids were demethylated at site 6. One *Pgk* reactivant (V-2B) was also demethylated at site 7, yet was methylated at site 8. The other 10 PGK-positive hybrids were demethylated at site 8. Five of these positive hybrids were examined for methylation status by partial *Hpa*II digestion and were all found to be demethylated at site 7 in addition to sites 6 and 8.

These data suggest that reactivation of *Pgk* is best correlated with demethylation at both sites 6 and 7. Because demethylation of site 6 alone was not sufficient for reactivation, it appears that site 7 demethylation plays a critical role. In many instances, regulatory methylation domains appear to function as general controlling regions rather than containing specific control sites (7, 18, 33, 34). Activation of the *Pgk* gene may also require a minimal region of generally demethylated CpG's. However, demethylation in the region of *Hpa*II site 7 appears to be necessary for expression and may be a dominant site of regulatory control by methylation. Several other genes have been shown to be inhibited by site-specific methylation in transient expression assays of in vitro-methylated DNA (9, 42, 57). Some of the 24 other CpG's between sites 6 and 8 may also be important for *Pgk* expression. A few of these CpG sites could be analyzed with other methylation-sensitive restriction enzymes, but a complete understanding of the relationship between expression and methylation in this region will require analysis of these sites by genomic sequencing methods (41).

Chromatin hypersensitivity to endonuclease digestion is a characteristic property of regulatory sequences in eucaryotic genes (6, 50). The data presented in Fig. 4 and Table 2 show a strict correlation between *Msp*I sensitivity of the region 5′ to the *Pgk* gene and the expression of PGK activity. Site 6 and two sites near site 3 appear to be the most sensitive (Fig. 4, Y162/11C). A site 7 cleavage product was also evident at all *Msp*I concentrations. Site 8 cleavage began to occur at enzyme concentrations higher than those at which cleavage of the other sites began. In the cell line V-2B, site 8 was not sensitive to *Msp*I digestion because, even in purified DNA, the enzyme does not efficiently cleave the sequence GGC^{m}CGG. Examination of this cell line with other endonucleases which cleave near site 8 may provide evidence for a role for methylation in determining nuclease hypersensitivity.

The characteristic *Msp*I digest pattern indicates that site 6 was more susceptible to attack than site 7, and site 8 was the least accessible of the three. The observation that sites 7 and 8 were not susceptible to endogenous nuclease digestion is further support for partial digestion due to a gradient of decreasing accessibility from site 6 to site 8 (Fig. 4). Therefore, the region of nuclease hypersensitivity appears to be related to promoter activity, because sites 7 and 8 are in transcribed regions of the gene (Fig. 2). Sites 7 and 8 may be more resistant to digestion because of either bound blocking factors or an unfavorable DNA conformation. Alternatively, cleavage at one site might render other sites less accessible.

The 5′ nuclease-sensitive region in the active *Pgk* gene extends at least from about *Msp*I site 3 to site 8, a length of 500 base pairs (Fig. 4). An endogenous nuclease activity that cleaved at a number of sites between the *Eco*RI site and *Msp*I site 6 was observed in most PGK-positive nuclei (Fig. 4). Therefore, the 5′ boundary of the sensitive region extends at least to the *Eco*RI site (650 base pairs from *Msp*I site 8). This minimal length of the *Pgk* nuclease-sensitive region is somewhat larger than those in other active genes, which are usually about 200 to 500 base pairs long (6, 50). The use of other restriction enzymes for nuclear digestion should help to map the boundaries more precisely.

Chromatin conformation and nuclease sensitivity of the 5′ region of *Pgk* may be controlled by factors which recognize specific demethylations at *Hpa*II sites 6 and 7 or a general demethylation up to and including site 7. It is interesting that a slight *Msp*I sensitivity at or near site 3 and at site 6 was observed in III-9, whose 3′-most demethylated site is site 6 (Fig. 4). This type of sensitivity was not observed for 5AC D which is unmethylated at site 3, 4, or 5. Detailed examination of the methylation patterns in the reactivant cell lines may reveal the boundaries of sequences which control the local chromatin conformation.

An important role for methylation in gene activity and chromatin conformation is supported by reports of tissue-specific methylation patterns in the 5′ nuclease-sensitive sites of several autosomal genes (4, 16, 49). A number of other studies (5–7) have more directly implicated changes in methylation pattern as responsible for altering chromatin structure and thus affecting gene expression. For example, the demethylation kinetics for CpG's in the avian vitellogenin gene correlate well with the appearance of two major DNase I-hypersensitive sites (41). Also, in vitro-methylated genes transfected into mouse L cells contained no DNase I-hypersensitive sites, while the same unmethylated genes

transfected into L cells did (21). Nuclease-hypersensitive sites in hypomethylated regions of active X-linked housekeeping genes have been previously described (38, 39, 46, 47, 54). However, conclusions about the importance of methylation at specific sites within the CpG clusters could not be determined because these sites were not resolved.

Although 5′ CpG clusters are associated with many widely expressed housekeeping genes and are not unique features of the X chromosome (1, 2, 12, 43, 45), it appears that they are important inactivation control elements on the differentiated X chromosome. DNA methylation may be a late event in the inactivation process, as suggested by a recent report (27) that sequences 5′ to the *Hprt* allele on the inactive X chromosome in female rodent embryos become methylated in most cells several days after inactivation is believed to occur. However, it remains to be determined whether control of *Pgk* or other genes on the X chromosome by methylation is secondary to an initial inactivation mechanism in vivo. Our data indicate that demethylation at the 5′ end of the *Pgk* gene, perhaps at specific sites, results in increased nuclease sensitivity of the region and is necessary for reactivation of the gene. It is possible that more primary methylation control sites for *Pgk* expression exist outside the DNA region examined, even at the locus of another gene which could induce secondary demethylation events (5, 6). The possible existence of another controlling locus in the experiments reported here is supported by the moderate PGK activity of the V-3D hybrid (Fig. 1) despite its similarity to strong reactivants in patterns of methylation and chromatin sensitivity (Fig. 3 and 4).

The inhibition of transcription by methylation of CpG islands in widely expressed genes appears to be unique to those present on the inactive X chromosome. X-chromosome inactivation therefore provides a useful model system to investigate the role of these islands and their associated proteins by comparative analysis of the active and inactive loci. An insufficient number of reactivants were analyzed in studies of other genes to allow any conclusions about the importance of particular sites within CpG clusters. The stability of 5AC-induced demethylation events and the high frequency of reactivation in the X8/6T2 cell line indicates that this system will be useful in the examination of other X-linked loci for transcriptional control by methylation.

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