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5-Azacytidine-induced reactivation of the human X chromosome-linked *PGK1* gene is associated with a large region of cytosine demethylation in the 5′ CpG island

(X chromosome inactivation/hamster–human cell hybrid/*de novo* methylation)

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ABSTRACT **Hamster–human cell hybrids containing an inactive human X chromosome were treated with 5-azacytidine and derived clones were examined for phosphoglycerate kinase activity and cytosine methylation in the human *PGK1* (X chromosome-linked phosphoglycerate kinase) gene. Comparisons between expressing and nonexpressing clones indicated that demethylation of several methylation-sensitive restriction sites outside of the 5′ CpG island were unnecessary for expression. High-resolution polyacrylamide gel analysis of 25 *Hpa* II, *Hha* I, and *Tha* I sites revealed that all clones expressing *PGK1* were unmethylated in a large region of the CpG island that includes the transcription start site and 400 base pairs upstream. Many nonexpressing clones had discontinuous patterns of demethylation. Remethylation was often observed in subclones of nonexpressing hybrids. These data suggest that a specific zone of methylation-free DNA within the *PGK1* promoter is required for transcription. In addition, the presence of neighboring methylcytosines appears to decrease the heritable stability of unmethylated CpGs in this region.**

The sex difference in dosage of mammalian X chromosomes is compensated by random inactivation of all but one X chromosome in female somatic cells. The stable maintenance of transcriptional repression for several X chromosome-linked genes involves cytosine methylation (1). Islands of CpG-rich DNA are found in association with the promoter regions of a wide variety of mammalian genes and are generally methylation-free (2, 3). Several examples of this type of gene are found on the active X chromosome (1). A gene on the inactive X chromosome that escapes inactivation has also been found to have an unmethylated CpG island (4). Methylated islands have been discovered primarily on the inactive X chromosome in association with genes that undergo X chromosome inactivation (1).

These islands appear to be important in promoter activity (3) but the molecular mechanisms involved in controlling expression are not known. There are several reports that site-specific methylation, demethylation, or both may be involved in regulation (5). However, regions of methylation or demethylation may be necessary for control as in the promoter region of a human γ-globin gene (6). We have found (7) that reactivation of the X chromosome-linked *PGK1* gene with 5-azacytidine was correlated with demethylation of two *Hpa* II sites near the transcription start site in a hamster × human hybrid cell line harboring an inactive human X chromosome. However, the methylation status of all *Hpa* II sites in the 5′ CpG island was not determined in that study which used indirect end-labeling of probes and Southern blots for analysis.

In this paper, we employ the technique of PAGE to analyze small fragments of the CpG island obtained by digestion with one of three methylation-sensitive restriction enzymes (*Hpa* II, *Hha* I, or *Tha* I). The methylation patterns of expressing and nonexpressing hybrid cells indicate that a region of methylation-free DNA is required for gene expression that includes most of the known 5′ CpG island. In addition, subclone methylation patterns of several hybrids indicate that *de novo* methylation occurs frequently in partially methylated regions of the CpG island.

MATERIALS AND METHODS

Probes. Probes BI2.20 and RB0.8 are subclones of λEMBL3-PGKI, a *PGK1* genomic clone supplied by J. Singer-Sam (Molecular Biology Section, Beckman Research Institute of the City of Hope, Duarte, CA) (7, 8).

Phosphoglycerate Kinase (PGK) Electrophoresis. Human and hamster forms of PGK were distinguished by cellulose polyacetate strip electrophoresis as described (7).

Cell Culture. Standard growth conditions for cultured cells have been described (7, 9). X8-6T2 is a 6-thioguanine-resistant human–hamster hybrid cell line that contains a human inactive X chromosome (7, 10, 11). The hypoxanthine/aminopterin/thymidine (HAT)-resistant hybrids used in this study were described or similarly derived from 5-azacytidine-treated X8-6T2 cells (7). Clones and subclones were isolated from 96-well tissue culture plates and expanded for PGK and DNA extraction concurrently to control for possible changes in the cell population with continued growth. Y162-11CS3 is a subclone of Y162-11C, a human–hamster hybrid cell line containing an active human X chromosome (7). Lymphoblastoid cell lines were derived from either Epstein–Barr virus-transformed male or 5X female lymphocytes.

Y162-11CS3 and the HAT-resistant clones derived from X8-6T2 cells were routinely grown in HAT medium.

DNA Analysis. Hybrid cell DNA was isolated as described (7) except that incubation with proteinase K was at 55°C. DNA from whole-cell lysates was purified using an automated apparatus (Applied Biosystems) programmed for two extractions with 70% (vol/vol) phenol. DNA from donated blood lymphocytes was purified similarly after fractionation from erythrocytes (12). DNA was quantitated by fluorescent dye binding (13). RB0.8 and BI2.20 probes were purified from plasmids by restriction digestion and agarose electrophoresis. The probes were radiolabeled using the random hexanucleotide priming method (14).

Restriction enzyme digestion conditions for agarose gels were as described (7). Reactions were terminated by the

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Abbreviation: PGK, phosphoglycerate kinase.

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addition of 0.1 vol of 10× electrophoretic sample buffer [0.89 M Tris borate/0.45 M EDTA, pH 8.3/30% (wt/vol) Ficoll/0.25% xylene cyanole FF/0.25% bromophenol blue]. For PAGE, digested samples (13 μg of DNA) were concentrated by ethanol precipitation in the presence of 7.5 μg of sonicated salmon testes DNA and 1.9 M ammonium acetate. Washed pellets were dried and dissolved in 20 μl 1× sample buffer. Electrophoresis was performed on 7% acrylamide gels (0.75 mm thick) in 89 mM Tris borate, pH 8.3/25 mM EDTA at 150 V until the bromophenol blue dye front reached the bottom of the gel.

Agarose gels were Southern blotted onto nylon membranes (Bio-Rad Zeta-Probe) under alkaline conditions essentially as described (15). Native DNA fragments were electrophoretically transferred from polyacrylamide gels to nylon membranes in 20 mM Tris acetate, pH 7.4/0.5 mM EDTA at 10°C using a Bio-Rad Trans-Blot cell. DNA was denatured and fixed onto membranes by treatment with 0.4 M NaOH (15). Hybridization conditions were similar to those described (15). Filters were washed successively for 15-min periods in the following buffers: 2× SSC/0.1% SDS at 23°C; 2× SSC/0.1% SDS at 65°C; 0.5× SSC/0.1% SDS at 65°C; and 0.1× SSC/0.1% SDS at 65°C. (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.)

RESULTS

Agarose Gel Analysis of *PGK1* Methylation Patterns. A restriction map for the 5′ region of the human X chromosome-linked *PGK1* gene is shown in Fig. 1. The map was derived from published sequence data (16) and our restriction analysis of plasmid and genomic DNA (ref. 7, this paper, and unpublished results). The position and methylation status of several of these sites in human–hamster hybrid cell lines were determined from restriction fragments separated on agarose gels. Examples of such analyses are given in Fig. 2 where DNA was digested with *Hha* I and *Pst* I or *Hpa* II and *Pst* I and probed sequentially with BI2.20 and RB0.8. A major fraction of normal female lymphocytes were unmethylated on the inactive X chromosome at *Hha* I sites 4 and 5 as indicated by the 0.87/0.89-kilobase-pair (kb) doublet not present in male lymphocytes (BI2.20 and RB0.8 probes). *Hha* I site 7 was also unmethylated in some of the cells, but the fragments between *Hha* I site 7 and the *Pst* I site and between *Hha* I site 8 and the *Pst* I site were not well resolved in this gel. The presence of a faint 3.7-kb *Pst* I–*Pst* I fragment (5′ *Pst* I site not shown in Fig. 1) indicated that some cells were methylated at all the *Hha* I sites in the promoter region (best seen with RB0.8 probe). The inactive X chromosome of X8-6T2 was also methylated at *Hha* I sites 1–8 (data not shown).

Hpa II site analysis revealed that X8-6T2 was methylated at all eight *Hpa* II sites and active X chromosome-containing DNA (male or female lymphocytes) was unmethylated at *Hpa* II site 8, consistent with previous results (7, 17). A few PGK-negative hybrids were also demethylated at site 8 (VII-2D1). The RB0.8 probe identified two small bands (0.19 and 0.14 kb) in human lymphocytes corresponding to fragments cleaved at sites 5, 6, 7, and 8. The active X chromosome gene of normal lymphocytes is unmethylated at sites

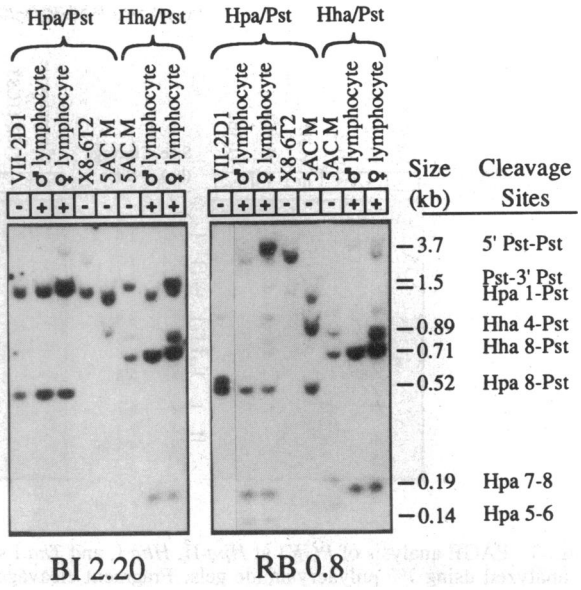

FIG. 2. Agarose gel analysis of *PGK1* at *Hpa* II and *Hha* I sites. X8-6T2 is the untreated parental cell line carrying the inactive human X chromosome; Y162-11C contains an active human X chromosome; ♂ and ♀ are male and female blood lymphocytes, respectively; the remaining cell lines were clones derived from X8-6T2 after 5-azacytidine treatment and HAT selection. The amount of human PGK detected in hybrids relative to hamster activity is indicated as follows: −, none; +, strong. DNA was digested with *Hpa* II and *Pst* I or *Hha* I and *Pst* I, electrophoresed on a 2% agarose gel, and probed sequentially with BI2.20 and RB0.8 (Fig. 1). The positions corresponding to *Pst* I–*Hha* I fragments (*Hha* I sites 1–8) shown in Fig. 1 were calculated relative to molecular weight standards (ϕX174 *Hae* III and λ*Hin*dIII fragments). Also shown are the positions and calculated sizes (kb) of observed *Pst* I–*Pst* I fragments.

2–8 (17). The fragments derived from cleavages at sites 2–5 were too small [<90 base pairs (bp)] to be analyzed on the 2% agarose gel. *Hpa* II–*Hpa* II fragments were present in many nonexpressing clones (VII-2D1), but none had both fragments from *Hpa* II site 5 to *Hpa* II site 6 and from site 7 to site 8, indicating that at least one of these sites was methylated. A number of the PGK-negative hybrids were found to have methylated and unmethylated CpGs in close proximity. For example, hybrid 5AC M was methylated at *Hpa* II site 7 and unmethylated 5 bp away at *Hha* I site 8.

Polyacrylamide Gel Analysis of *PGK1* Methylation Patterns. As shown above, the methylation status of several restriction sites in the promoter region was difficult to determine using agarose gel electrophoresis because potential fragments were too small. By using nondenaturing PAGE, fragments as small as 45 bp were detected (Fig. 3*B*). Examples of *Hpa* II–*Hpa* II fragment analysis using PAGE are shown in Fig. 3*A*. As expected, no such bands are observed for X8-6T2, but five bands corresponding to unmethylated *Hpa* II sites 2–8 are present in the Y162-11CS3 lane (sites 7–8 = 193 bp; sites 5–6 = 138 bp; sites 6–7 = 88 bp; sites 2–3 = 64 bp; sites 4–5 =

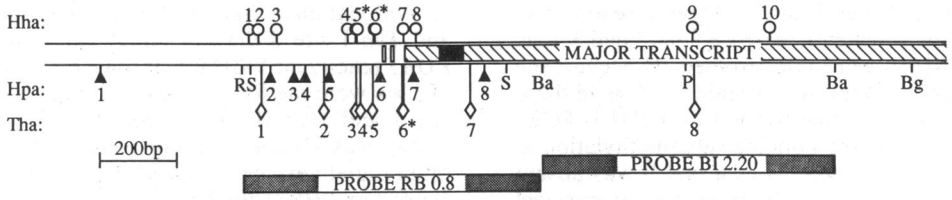

FIG. 1. Map of 5′ region of human *PGK1*. Shown are restriction enzyme sites, putative promoter elements (▮▮), a major transcription start site, a protein coding portion of exon 1 (■), and the genomic probe locations (RB 0.8 and BI 2.20). Ba, *Bam*HI; Bg, *Bgl* II; R, *Eco*RI; P, *Pst* I; S, *Sty* I. *Hpa* II/*Msp* I (▲), *Hha* I (○), and *Tha* I (◇) sites are numbered. Two overlapping sites are indicated by an asterisk.

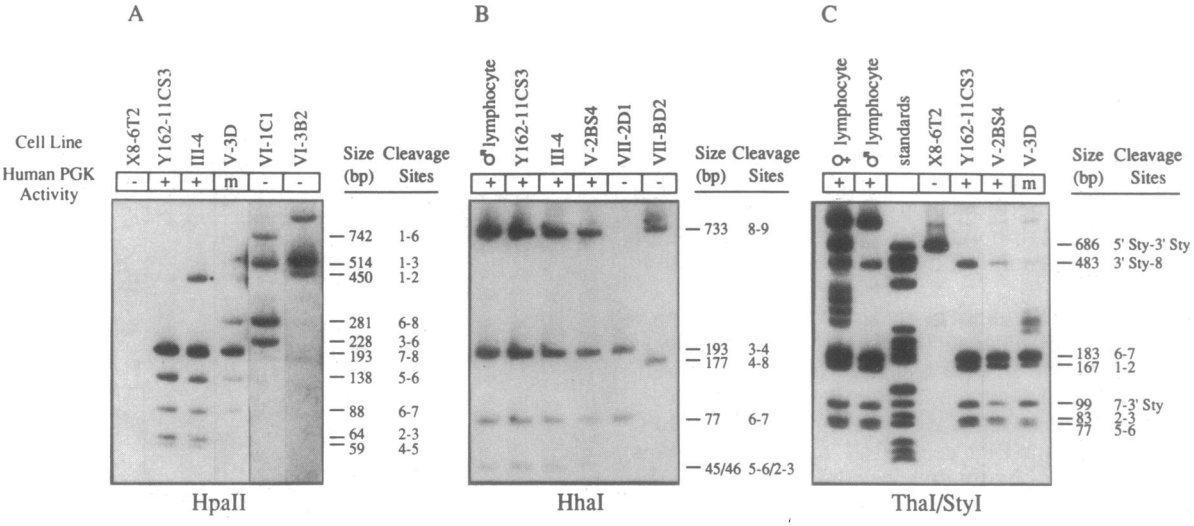

FIG. 3 PAGE analysis of *PGK1* at *Hpa* II, *Hha* I, and *Tha* I sites. DNA from hybrid cells and human peripheral lymphocytes was digested and analyzed using 7% polyacrylamide gels. Fragment cleavage sites (as depicted in Fig. 1) and lengths (bp) were obtained primarily from sequence data and positioned according to standards (pBR322 *Hae* III fragments). The amount of human PGK detected in hybrids relative to hamster activity is indicated as follows: −, none; m, moderate; +, strong. (*A*) DNA was digested with *Hpa* II. (*B*) DNA was digested with *Hha* I. (*C*) DNA was digested sequentially with *Tha* I and then *Sty* I.

59 bp). Male and female lymphocytes were identical to Y162-11CS3 in this region of the gel (not shown).

III-4 is a PGK-positive hybrid that is similar to Y162-11CS3 except it has an additional band at 450 bp corresponding to a fragment between *Hpa* II sites 1 and 2. This band was also observed in *Msp* I digestions of lymphocyte and hybrid DNAs (data not shown), indicating the absence of a *Hpa* II site 6 bp from the 5′ *Eco*RI site (16). Recent genomic sequencing data indicate that the sequence at this site in HeLa cells is CCAGG and not CCGG (18). The moderately PGK-positive V-3D appears to differ from III-4 by the presence of a 280-bp band and much weaker 64-bp and 59-bp bands. This pattern and that obtained after *Eco*RI/*Hpa* II digestion (not shown) indicate that a large fraction of cells in the V-3D population is methylated at sites 2, 4, and 5. The absence of a 514-bp band (sites 1–3) means that site 1 is methylated as well.

VI-1C1 and VI-3B2 are examples of PGK-negative hybrids that had *Hpa* II–*Hpa* II fragments. The most 3′ unmethylated *Hpa* II site for both of these hybrids was site 8, as determined by agarose gel analysis (not shown). The observed fragment lengths, therefore, indicate that VI-1C1 is predominantly methylated at all but sites 1, 3, 6, and 8. The weaker band at 740 bp indicates that some of the DNA is also methylated at site 3. Similarly, all but sites 1, 2, and 8 are methylated in VI-3B2, with some partial methylation at site 2 indicated by the presence of a 1-kb band.

Hha I–*Hha* I fragments were also examined with PAGE as shown in Fig. 3*B*. The following observed bands indicate that male lymphocytes, Y162-11CS3, and *PGK1* reactivants were unmethylated at *Hha* I sites 3–9: sites 8–9 = 770 bp; sites 3–4 = 193 bp; sites 6–7 = 77 bp; sites 2–3 and/or 5–6 = 45/46 bp. The sizes of fragments between *Hha* I sites 1 and 2 = 28 bp, sites 4 and 5 = 19 bp, and sites 7 and 8 = 32 bp were too small to be detected and the fragments between sites 2 and 3 = 46 bp and 5 and 6 = 45 bp were not resolved. However, the absence of a fragment between sites 4 and 6 = 66 bp in these DNAs means that site 5 is unmethylated. VII-2D1 is PGK-negative that had bands corresponding to demethylation at sites 3–7. The fragment between sites 8 and 9 was absent because of a methylated site 9 as determined by agarose gel analysis (not shown). VII-BD2, another nonexpressing hybrid, had a fragment between sites 8 and 9 and a 177-bp band that corresponds to a fragment between sites 4 and 8. The absence of fragments between sites 3 and 4 = 193 bp, sites 2 and 4 = 239 bp, or sites 1 and 4 = 267 bp indicates that sites 1–3 are methylated.

A similar analysis of the region was done using the methylation-sensitive restriction enzyme *Tha* I (recognition sequence CGCG, sensitive to methylation at either cytosine). Examples of *Tha* I/*Sty* I double digests are shown in Fig. 3*C*. The DNA from cells expressing PGK had the expected fragments for unmethylated *Tha* I sites 1–7: between sites 6 and 7 = 188 bp; sites 1 and 2 = 167 bp; site 7 and a *Sty* I site = 99 bp; sites 2 and 3 = 83 bp; sites 5 and 6 = 77 bp; absence of a fragment between sites 3 and 5 = 47 bp. The fragment between a *Sty* I site and site 8 = 480 bp was also observed in these samples, although weak in V-3D. The *Sty* I–*Sty* I 686-bp band was prominent only in the female lymphocytes and X8-6T2, indicating that the inactive X alleles were methylated. However, the female lymphocytes also had a number of prominent bands in the 280- to 400-bp range in addition to the smaller fragments common to male lymphocytes. These *Sty* I–*Tha* I bands correspond to mosaic cleavages at single *Tha* I sites in the region of sites 3–6. V-3D was also mosaic in that bands were observed at 250 bp and 280 bp. The simplest interpretation of these data is that site 2 is methylated in some of the cells (*Tha* I sites 1–3 = 250 bp) and both sites 1 and 2 are methylated in another population (*Sty* I site–site 3 = 278 bp).

Summary of *PGK1* Methylation Patterns. The methylation patterns of 63 5-azacytidine-treated hybrid cell clones were analyzed by restriction mapping. A potential of 64 CpGs could be determined with this technique, including both strands. Some of the deduced patterns are represented in Fig. 4, which includes those of lymphocyte DNA and 28 hybrids. The PGK-positive cells were generally methylation-free at the restriction sites in the region bounded by *Tha* I sites 1 and 7. Methylation at *Hpa* II sites 1 and 8, *Hha* I sites 9 and 10, and *Tha* I site 8 did not affect expression. The moderate *PGK1* reactivant V-3D was mosaic in that a major population of cells were methylated in the 5′ region bounded by *Hha* I site 1 and *Hpa* II site 5. Demethylation in PGK-negative clones was always discontinuous, with unmethylated sites often found adjacent to methylated ones (for example, *Hpa* II site 7 and *Hha* I site 8 in several hybrids shown in Fig. 4*B*).

Occurrence of Remethylation in Subcloned Hybrids. The clones derived from 5-azacytidine-treated hybrids were often mosaic in their methylation patterns even though the cells

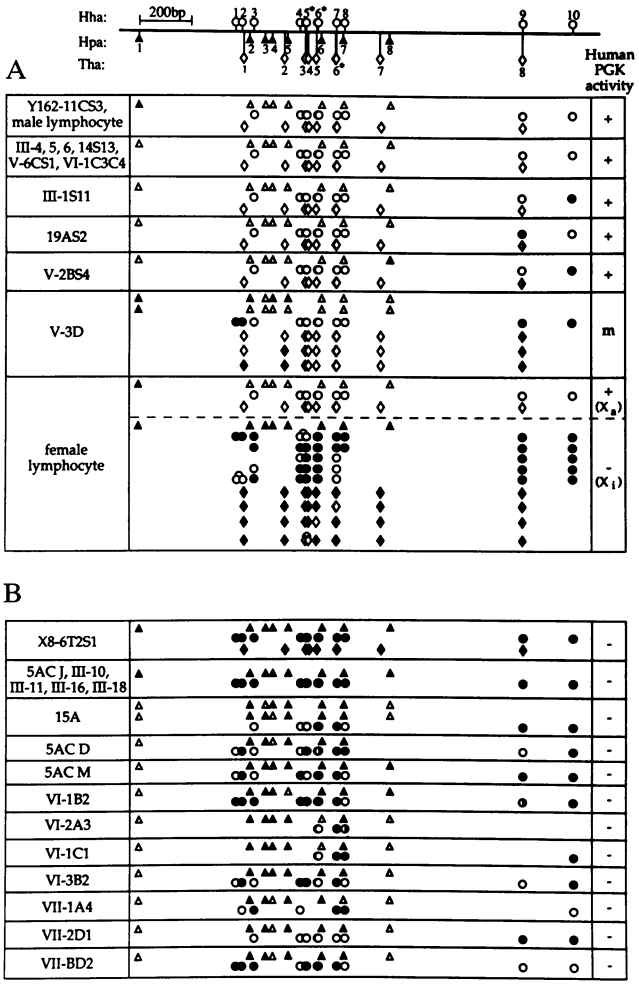

FIG. 4. Methylation patterns of PGK-positive and -negative cells. Data from agarose and polyacrylamide gel analyses are summarized. The positions of numbered *Hpa* II (▲), *Hha* I (○), and *Tha* I (◇) sites are shown for reference at the top of the figure. Two overlapping sites are indicated by an asterisk. In the body of the figure, methylated sites are depicted with solid symbols (▲, ●, ◆) and the symbols for unmethylated sites are open (△, ○, ◇). Partially methylated sites are shown as half-solid symbols (◭, ◑, ◈). Partial methylation is also illustrated by multiple lines in cases where cis methylation patterns could be deduced and designation by multiple half-solid symbols would be ambiguous. The most abundant populations are uppermost in these mosaic cases as estimated from band intensities (bands of a mosaic population were at least 10% the intensity of major populations). Linked symbols (e.g., ⧓) indicate unresolved sites, of which at least one site is unmethylated. Sites are not shown whose methylation status was not clear. (*A*) PGK-positive cells. (*B*) PGK-negative cells.

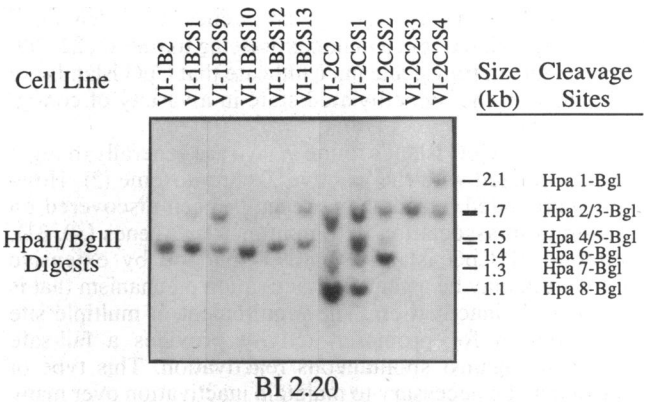

FIG. 5. Methylation mosaicism in hybrid subclones. PGK-negative hybrid cell lines and derivative subclones were analyzed using 1.5% agarose gel electrophoresis. DNA was digested with *Hpa* II and *Bgl* II. Positions expected for fragments cleaved at *Bgl* II and at each of *Hpa* II sites 1–8 are shown with the corresponding size (kb).

were cloned at a stage in which methylation inheritance should have stabilized (7, 11). Mosaicism was also observed in subclones of many of these cell lines. Fig. 5 shows two examples of PGK-negative cell lines that were subcloned and analyzed using agarose gel electrophoresis. VI-1B2 was unmethylated at *Hpa* II site 5 and not mosaic before subcloning. The five derived subclones all showed some cellular mosaicism at this site as evidenced by the variable appearance of a 1.7-kb band corresponding to an unmethylated site at or near site 2. VI-2C2 was mosaic before subcloning, with bands observed at sites 8 and 7 and near site 2. Three of four subclones were also mosaic. VI-2C2S4 had a new band corresponding to site 1 cleavage.

DISCUSSION

We have found (7) that reactivation of the *PGK1* gene correlated with demethylation of *Hpa* II sites 6 and 7. However, the methylation status of all sites in the 5′ region was difficult to determine in that study which employed agarose gel analysis and indirect end-labeling. We have shown here that the technique of PAGE is quite useful for detailed mapping of the closely spaced methylation-sensitive restriction sites. More than 60 5-azacytidine-treated hybrid clones have now been examined for their methylation patterns in the 5′ region.

The methylation maps of several PGK-expressing and -nonexpressing clones, which are summarized in Fig. 4, suggest that transcription is inhibited by methylation in a region that includes the transcription start site and extends 400 bp upstream. This region is unmethylated in strong PGK reactivants at all sites examined. PGK-negative hybrids were demethylated much less extensively, with no large areas of unmethylated restricted sites. Nuclease-hypersensitive sites in chromatin that are specific for expressing hybrids are known to span this same area of the gene (7) and are thus associated with demethylation. The entire region, therefore, appears to be important for transcriptional control of *PGK1*. This is supported by the observation that methylation in the 5′ end of V-3D was associated with diminished expression (Fig. 4*A*).

Although regional demethylation appears to be important in controlling transcription from the CpG-rich *PGK1* promoter, the possibility remains that only certain CpGs are required whose demethylation is unstable when neighboring sites remain methylated. Data arguing against this possibility are the PGK-negative clones found to have a variety of isolated unmethylated sites (Fig. 4). However, the methylation patterns observed in subclones of hybrids with discontinuous demethylation do suggest a tendency to remethylate under cloning conditions (Fig. 5). Possible explanations for this phenomenon include a direct involvement of the methyltransferase enzyme responsible for *de novo* methylation. The *de novo* activity of the mammalian methyltransferase enzyme is increased *in vitro* by premethylation at neighboring sites (19, 20). A methylation-specific binding factor that promotes the spread of methylation to neighboring sites might also be involved.

Transient expression assays with methylated CpG-rich promoters indicate that only a few sites of methylation are necessary to inhibit expression of reporter genes (6, 20). Limited methylation in the CpG island of an integrated thymidine kinase gene also inhibits expression in cell culture (21). Our data examining the *PGK1* gene in its normal chromosomal location also suggest that complete demethylation of the 5′ CpG island is necessary for the full expression. CpG islands are apparently under special control with respect to methylation as they are generally unmethylated. CpG

islands associated with tissue-specific genes have been found to be unmethylated, even in nonexpressing tissues (2, 22–28). Experiments in transgenic mice indicate that CpG islands are maintained in the unmethylated state in a variety of configurations (29).

Methylated CpG islands found *in vivo* are generally thought to be associated with the inactive X chromosome (2). However, methylated islands have recently been discovered on autosomes in association with nonfunctional genes (30, 31). Inhibition of CpG island promoter function by extensive methylation may be a general inactivation mechanism that is utilized in X inactivation. The requirement of multiple site demethylation for promoter activity provides a fail-safe mechanism against spontaneous reactivation. This type of control may be necessary to maintain inactivation over many cell divisions, an extended cell lifetime, or both because it would tolerate multiple mutations due to demethylation (32) and methylcytosine deamination (3). Demethylation events might explain the significant populations of normal peripheral lymphocytes whose inactive *PGK1* promoter regions were unmethylated at certain *Hha* I and *Tha* I sites (Figs. 2, 3*C*, and 4). The inactive X chromosomes of a 5X lymphoblastoid cell line were also found to be unmethylated at various *Hha* I sites in the 5′ region (data not shown).

In contrast to the unmethylated *Hha* I and *Tha* I sites found in lymphocytes, *Hpa* II sites in the 5′ region were all methylated. Several PGK-negative hybrids also appeared to favor *Hha* I over *Hpa* II demethylation (Fig. 4*B*). VII-2D1 is an interesting example in that it was methylated at all but one *Hpa* II site in this region, yet unmethylated at all *Hha* I sites. The difference in *Hpa* II vs. *Hha* I methylation may reflect differential recognition of sequences containing these sites by a factor that influences methylation. However, more examples of this phenomenon are needed to make any generalizations.

Methylation mosaicism was always observed upon subcloning demethylated PGK-negative hybrids (Fig. 5). This was most likely the result of methylation because several subclones were more methylated than any observed population in the parental clone (Fig. 5). In addition, the frequency of spontaneous demethylation is probably low and there are many precedents for the occurrence of *de novo* methylation in culture (33, 34). Mosaic subclones of nonexpressing hybrids were often found whose partial methylation pattern was identical to the parent "clone." This observation indicates that a probability of methylation is somehow inherited. Similar patterns of methylation inheritance have also been described for other sequences (35, 36). The probability of methylation may be determined, in part, by the methylation pattern of neighboring CpG sites.

In vitro binding assays have identified several regions in the 5′ CpG island of *PGK1* that specifically interact with proteins present in cell extracts (37). Two binding activities generate DNase I-protected footprints centered about 360 bp and 130 bp upstream of the transcription start. Genomic footprint (38) comparisons between our hybrid cell lines that differ in methylation and *PGK1* expression may help to identify the necessary transcription control factors for the inactive and/or active genes.

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1. Grant, S. G. & Chapman, V. M. (1988) *Annu. Rev. Genet.* **22,** 199–233.
2. Bird, A. P. (1986) *Nature (London)* **321,** 209–213.
3. Gardiner-Garden, M. & Frommer, M. (1987) *J. Mol. Biol.* **196,** 261–282.
4. Goodfellow, P. J., Mondello, C., Darling, S. M., Pym, B., Little, P. & Goodfellow, P. N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5605–5609.
5. Doerfler, W. (1983) *Annu. Rev. Biochem.* **52,** 93–124.
6. Murray, E. J. & Grosveld, F. (1987) *EMBO J.* **6,** 2329–2335.
7. Hansen, R. S., Ellis, N. A. & Gartler, S. M. (1988) *Mol. Cell. Biol.* **8,** 4692–4699.
8. Riggs, A. D., Singer-Sam, J. & Keith, D. H. (1985) in *Biochemistry and Biology of DNA Methylation,* eds. Razin, A. & Cantoni, G. L. (Liss, New York), pp. 211–222.
9. Riley, D. E., Canfield, T. K. & Gartler, S. M. (1984) *Nucleic Acids Res.* **12,** 1829–1845.
10. Dracopoli, N. C., Rettig, W. J., Albino, A. P., Esposito, D., Archidiacono, N., Rocchi, M., Siniscalco, M. & Old, L. J. (1985) *Am. J. Hum. Genet.* **37,** 199–207.
11. Ellis, N., Keitges, E., Gartler, S. M. & Rocchi, M. (1987) *Somat. Cell Mol. Genet.* **13,** 191–204.
12. Poncz, M., Solowiejczyk, B., Harpel, B., Mory, Y., Schwartz, E. & Surrey, S. (1982) *Hemoglobin* **6,** 27–36.
13. Labarca, C. & Paigen, K. (1980) *Anal. Biochem.* **102,** 344–352.
14. Feinberg, A. P. & Vogelstein, B. (1984) *Anal. Biochem.* **137,** 266–267.
15. Reed, K. C. & Mann, D. A. (1985) *Nucleic Acids Res.* **13,** 7207–7221.
16. Singer-Sam, J., Keith, D. H., Tani, K., Simmer, R. L., Shively, L., Lindsay, S., Yoshida, A. & Riggs, A. D. (1984) *Gene* **32,** 409–417.
17. Keith, D. H., Singer-Sam, J. & Riggs, A. D. (1986) *Mol. Cell. Biol.* **6,** 4122–4125.
18. Pfeifer, G. P., Steigerwald, S. D., Mueller, P. R., Wold, B. & Riggs, A. D. (1989) *Science* **246,** 810–813.
19. Weissbach, A., Nalin, C. M., Ward, C. A. & Bolden, A. H. (1985) in *Biochemistry and Biology of DNA Methylation,* eds. Razin, A. & Cantoni, G. L. (Liss, New York), pp. 79–94.
20. Rachal, M. J., Yoo, H., Becker, F. F. & Lapeyre, J.-N. (1989) *Nucleic Acids Res.* **17,** 5135–5147.
21. Tasseron-de Jong, J., Aker, J., den Dulk, H., van de Putte, P. & Giphart-Gassler, M. (1989) *Biochim. Biophys. Acta* **1008,** 62–70.
22. Bird, A. P., Taggart, M. H., Nicholls, R. D. & Higgs, D. R. (1987) *EMBO J.* **6,** 999–1004.
23. Edwards, Y. H., Charlton, J. & Brownson, C. (1988) *Gene* **71,** 473–481.
24. Broad, P. M., Symes, A. J., Thakker, R. V. & Craig, R. K. (1989) *Nucleic Acids Res.* **17,** 6999–7011.
25. Clevers, H., Lonberg, N., Dunlap, S., Lacy, E. & Terhorst, C. (1989) *EMBO J.* **8,** 2527–2535.
26. Dvorak, M., Urbanek, P., Bartunek, P., Paces, V., Vlach, J., Pecenka, V., Arnold, L., Travnicek, M. & Riman, J. (1989) *Nucleic Acids Res.* **17,** 5651–5664.
27. Linton, J. P., Yen, J.-Y. J., Selby, E., Chen, Z., Chinsky, J. M., Liu, K., Kellems, R. E. & Crouse, G. F. (1989) *Mol. Cell. Biol.* **9,** 3058–3072.
28. Sakamoto, S., Ortaldo, J. R. & Young, H. A. (1989) *J. Biol. Chem.* **264,** 251–258.
29. Kolsto, A.-B., Kollias, G., Giguere, V., Isobe, K.-I., Prydz, H. & Grosveld, F. (1986) *Nucleic Acids Res.* **14,** 9667–9677.
30. Woodcock, D. M., Crowther, P. J., Diver, W. P., Graham, M., Bateman, C., Baker, D. J. & Smith, S. S. (1988) *Nucleic Acids Res.* **16,** 4465–4482.
31. Nur, I., Pascale, E. & Furano, A. V. (1988) *Nucleic Acids Res.* **16,** 9233–9251.
32. Mays-Hoopes, L. L. (1989) *Int. Rev. Cytol.* **114,** 181–220.
33. Holliday, R. (1987) *Science* **238,** 163–170.
34. Dobrovic, A., Gareau, J. L. P., Ouellette, G. & Bradley, W. E. C. (1988) *Somatic Cell Mol. Genet.* **14,** 55–68.
35. Schmookler-Reis, R. J. & Goldstein, S. (1982) *Nucleic Acids Res.* **14,** 4293–4304.
36. Wolf, S. F. & Migeon, B. R. (1982) *Nature (London)* **295,** 667–671.
37. Yang, T. P., Singer-Sam, J., Flores, J. C. & Riggs, A. D. (1988) *Somatic Cell Mol. Genet.* **14,** 461–472.
38. Mueller, P. R. & Wold, B. (1989) *Science* **246,** 780–786.