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A potentially critical *Hpa* II site of the X chromosome-linked *PGK1* gene is unmethylated prior to the onset of meiosis of human oogenic cells

(development/X chromosome inactivation/DNA methylation/polymerase chain reaction/phosphoglycerate kinase)

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ABSTRACT **_Hpa_ II site H8 is in the CpG-rich 5′ untranslated region of the human X chromosome-linked gene for phosphoglycerate kinase 1 (*PGK1*). It is the only *Hpa* II site in the CpG "island" whose methylation pattern is perfectly correlated with transcriptional silence of this gene. We measured DNA methylation at site H8 in fetal oogonia and oocytes and found, using a quantitative assay based on the polymerase chain reaction, that purified germ cells isolated by micromanipulation were unmethylated in 47-day to 110-day fetuses, whereas ovaries depleted of germ cells and non-ovary tissues were methylated. We conclude that site H8 is unmethylated in germ cells prior to the onset of meiosis and reactivation of the X chromosome.**

Female primordial germ cells of mammals resemble somatic cells in having both an active and an inactive X chromosome. Evidence for inactivity of one X chromosome of oogonia has been found both cytologically and by studies on glucose-6-phosphate dehydrogenase heterodimer formation (1, 2). After migration of the oogonia to the genital ridge at, or shortly before, the time of entry into meiosis, reactivation of the previously inactive X chromosome occurs (1–6).

DNA methylation has been correlated with X chromosome inactivation in many studies (reviewed in refs. 7–9) and it is of interest to determine whether a loss of methylation is concomitant with X chromosome reactivation during oogenesis. Data on DNA methylation of oogenic cells are sparse. Several years ago evidence was obtained for undermethylation of some repeated sequences in female germ-line DNA (10, 11), and more recently, Driscoll and Migeon (12) obtained evidence for lack of methylation of several X-linked and autosomal genes in oogenic and spermatogenic cell fractions.

As the latter study was done on tissue fractions rather than preparations free of somatic cells, we thought it was important to measure DNA methylation of highly purified germ cells. As such cells are isolatable only in small quantities, we used a polymerase chain reaction (PCR)-based assay previously used to measure DNA methylation at a CCGG site in individual mouse embryos (13).

The site we chose to assay was *Hpa* II site H8 in the 5′ untranslated coding region of the human X-linked *PGK1* gene, 20 base pairs (bp) downstream of the major transcription start site (14). The 5′ region of the *PGK1* gene is now the most highly characterized X-linked promoter region, and the relationship between methylation, transcription, and transcription factors has been studied both in normal human lymphocytes and in cultured cell lines (15–19). Recently, ligation-mediated genomic sequencing has been used to establish that the promoter region of the *PGK1* gene on the inactive X chromosome is methylated at 60 of 61 CpG sites, whereas the active promoter has no methylated sites (19). Prior to these studies, methylation-sensitive restriction enzymes had been used to investigate methylation at the 8 *Hpa* II sites in the *PGK1* 5′ region in lymphocytes (15) and in cell lines before and after treatment with the potent methylation inhibitor 5-azacytidine (20). Clones were selected for reactivation of the X-linked gene for hypoxanthine (guanine) phosphoribosyltransferase; then the nonselected *PGK1* gene was studied. Only the methylation status of *Hpa* II site H8 was found to correlate perfectly with transcriptional silence (20). More recently, a ligation-mediated genomic sequencing study confirmed that all 5-azacytidine-treated clones that express PGK1 are demethylated at H8 whereas clones that do not express PGK1 are methylated at this site (17, 19). These studies suggested that H8 may be a critical site for maintaining transcriptional silence in human somatic cells; other studies have shown a similar correlation of DNA methylation and X inactivation for the analogous site in mice (13, 21). We show here that site H8 is completely unmethylated in germ cells well before reactivation of the X chromosome.

MATERIALS AND METHODS

Sources of Cells. All cells were obtained from human fetuses ranging in gestational age from 47 days to 112 days. Fetuses were collected by T. H. Shepard, Head of the Central Laboratory for Human Embryology at the University of Washington. The method of collection and the criteria for determining fetal ages have been described (22). The various kinds of cells analyzed were obtained as described below.

Pure germ cells (oogonia and oocytes) were dispersed by rupturing a gonad in Whittingham's medium (23). To obtain cells free of non-germ cells and debris, germ cells—which are recognizable by their relatively large size, spherical shape, and refractile characteristics—were transferred with a manually manipulated braking pipet (24) through a series of washes in chilled serum-free medium (23). Through observation under a dissecting microscope, one can detect the depletion of undesired material at each successive wash, and the washes were continued until no further contamination was seen. The pure cells were collected in tubes in an ice bath until sufficient numbers for further processing were obtained.

Impure germ cells were obtained by pressing a gonad under a siliconized coverslip in a drop of Whittingham's medium. The cells released from the ovary, which were preferentially (but not exclusively) germ cells, were picked up with a finely drawn Pasteur pipette (25).

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Ovarian remains consisted of material remaining as coherent tissue after the ovary had been ruptured to release oocytes. These remains contained primarily non-oocyte material, but some oocytes undoubtedly remained.

Non-ovary tissue consisted of peri-ovarian materials adhering to the ovary.

Estimation of Cell Purity. The purity of a sample of oogonia and early oocytes was determined by cytochemical detection of alkaline phosphatase activity (Sigma procedure no. 86). The assay was performed on an aliquot of pooled cells for each experiment. The presence of alkaline phosphatase as detected by this assay is a distinct marker of germ cells (26, 27).

Purification and Quantitative *Hpa* II–PCR Analysis of DNA. Pooled cells were pelleted in a Brinkman 3200 microcentrifuge. The supernatant was decanted and the cell pellet was frozen at −20°C until analysis. DNA was purified by a guanidinium hydrochloride-based method (13, 28). In some experiments an alternative purification method involving "salting out" of the DNA was used (29). Lysis buffer (75 mM NaCl/25 mM EDTA, pH 8/1% SDS with freshly dissolved proteinase K at 200 μg/ml) was added to the cells (1 ml per ≈1000 cells). After overnight incubation at 37°C, prewarmed saturated (≈6 M) NaCl was added to a final concentration of ≈1.5 M (250 μl/ml). After vigorous shaking, and centrifugation at 10,000 × *g* for 10 min at room temperature, the supernatant was extracted with 1 volume of chloroform. The DNA was reduced in size by 10 passages through a 26-gauge needle. Glycogen (20 μg) and M13 phage DNA (1 μg) were then added as carriers, and the DNA was precipitated by addition of 1 volume of 2-propanol. After washing with 70% (vol/vol) ethanol, the DNA was resuspended in 1 mM Tris·HCl, pH 8/0.1 mM EDTA. The DNA was digested with *Bam*HI to further reduce its size, and then half of the material was cut with *Hpa* II. After digestion, a 2-μl aliquot of each sample was subjected to gel electrophoresis to verify completeness of digestion of the M13 DNA in the reaction mixture. Quantitative PCR was then performed (13, 30) with primers PGK434(+) (5′-GCGGTGTTCCGCATTCTGCAAG-3′) and PGK616(−) (5′-GGGCAAACGTGCAGAATTACCT-3′), which bracket *Hpa* II site H8 of the human *PGK1* gene (14, 15). PCR was carried out for 37 cycles in PCR buffer (Cetus) at a pH of 8.5. Each cycle consisted of 1 min at 95°C, 2 min at 60°C, and 2 min at 72°C, except during the first 2 cycles, when the temperature was kept at 95°C for 4 min. The standard, a 142-bp fragment added to the PCR mixture for coamplification with the genomic template, was constructed by use of a "deletion primer" (5′-GCGGTGTTCCGCATTCTGCAAGAATCACCGACCTCTCTCC-3′) together with PGK616(−), as described (30).

Gel electrophoresis, transfer to a nylon filter, hybridization, and washing were as described (31). The radioactivity of each band on the filter was determined by an Ambis radioisotope scanning system II (Automated Microbiology Systems, San Diego). The ^{32}P-labeled probe used was PGK-552(−) (5′-CAGCTTGTTAGAAAGCGACATTTTGG-3′).

Sex Determination. The sex of the fetuses was confirmed by PCR assay for the *ZFY* gene as described (32), except that the downstream primer consisted of the unique sequence 5′-CACAGAATTTACACTTGTGCAT-3′ (33).

RESULTS

Germ cells were taken from human fetuses ranging in age from 1.5 to almost 4 months (47- to 112-day fetuses). In 2-month fetuses, close to 90% of the germ cells are oogonia, whereas in 4-month fetuses, only 40% of the normal germ cells are oogonia, and the remaining 60% are oocytes in various stages of premeiotic prophase, ranging from preleptotene to diplotene (34).

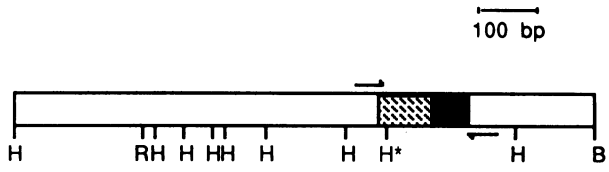

FIG. 1. The 5′ region of the human *PGK1* gene. Hatched region, 5′ untranslated region; solid region, translated portion of first exon; horizontal arrows, primers used for PCR (see *Materials and Methods*); H, *Hpa* II sites; R, *Eco*RI site; B, *Bam*HI site. Asterisk denotes *Hpa* II site H8, which is 21 bp downstream of the major transcription start site.

In early experiments, germ cells were isolated from genital ridges or ovaries by gentle pressing; in later samples, ≈200 germ cells per embryo were individually selected and rinsed (see *Materials and Methods*). In addition to germ cells, non-ovary tissue and ovary remains also were collected for DNA extraction and assay.

We used a sensitive assay developed to measure DNA methylation in a small number of cells (13). The assay is based on digestion of DNA with a methylation-sensitive restriction enzyme prior to PCR; only DNA methylated at a given restriction site will remain intact and serve as a PCR template when primers flanking the site are used (ref. 21 and Fig. 1). During the course of purification, M13 DNA is added both as a carrier and to verify completeness of digestion by *Hpa* II. After digestion, a known amount of an *in vitro* synthesized internal standard is added. The internal standard is amplified together with the sample DNA by the same set of primers; however, it produces a smaller PCR product. After each PCR, the amount of DNA initially present is determined by comparison of the signal due to the amplified sample with the signal due to the coamplified internal standard. The degree of methylation is then calculated as the ratio of the amount of amplifiable DNA remaining after *Hpa* II digestion to the amount of DNA present prior to *Hpa* II digestion.

For each sample, we measured the extent of methylation of *PGK1 Hpa* II site H8, which is located 21 bp downstream of the major transcription initiation site (14) (see Fig. 1). To confirm the sex of each fetus, samples were also assayed by PCR for the presence of the male-specific *ZFY* gene.

Fig. 2*A* shows an example of the results obtained using male fetus DNA. The expected PCR product (band I) was obtained only when there has been no prior *Hpa* II digestion (compare + and − lanes), demonstrating that this DNA was sensitive to *Hpa* II digestion and therefore unmethylated. The control PCR amplified product (band II), derived from the exogenously added internal standard, was present in both lanes as expected. A similar result was seen for the female germ-cell sample (Fig. 2*B*, two right lanes). The non-germ-

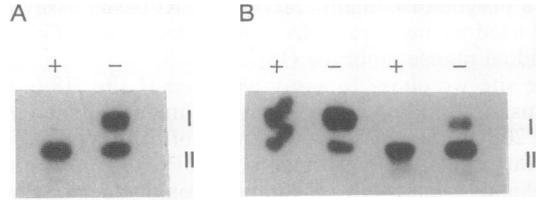

FIG. 2. Methylation of *Hpa* II site H8 in germ cell vs. somatic tissue. DNA was prepared from human fetuses and assayed for DNA methylation by PCR with (+) or without (−) prior *Hpa* II digestion. Band I corresponds to the expected position of the 183-bp PCR product; band II is the 142-bp coamplified product of the exogenous template (see *Materials and Methods*). (*A*) Male gonad DNA. (*B*) DNA from non-germ cells (two left lanes) and pure germ cells (two right lanes) of a 55-day female fetus.

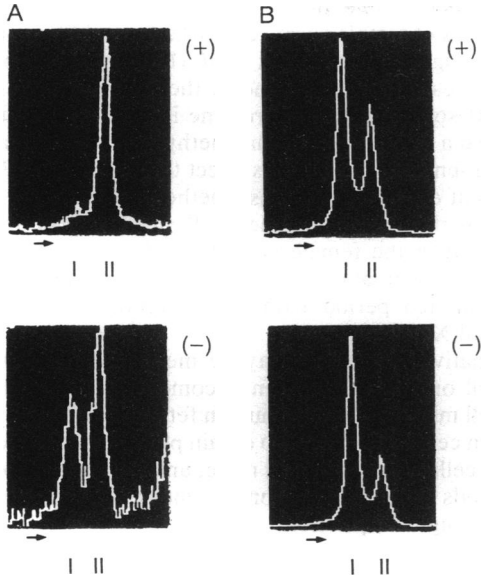

FIG. 3. Percent methylation of germ-cell vs. non-ovary DNA. Purified human fetal DNA that was uncut (−) or *Hpa* II-digested (+) was amplified by PCR in the presence of a second artificial template (see *Materials and Methods*). After gel electrophoresis, transfer, and hybridization to a ^{32}P-labeled oligonucleotide probe, each lane of the filters was scanned for radioactivity. Arrows show the direction of electrophoresis. Bands I and II are as indicated in the legend to Fig. 2. (*A*) Pure germ cell sample, 56-day fetus. (*B*) Non-ovary sample, 55-day fetus.

cell sample, however, gave an amplified product (band I) with and without prior *Hpa* II digestion (Fig. 2*B*, two left lanes).

Filters were scanned for quantitation as shown in Fig. 3. As in Fig. 2, the peaks labeled I and II correspond to the amplified products of the cellular DNA being assayed and the added internal standard, respectively. DNA from 55-day pure germ cells showed a greatly reduced I/II ratio after *Hpa* II digestion [Fig. 3*A*, compare I/II ratio in the (+) scan vs. the (−) scan]. However, DNA from non-germ cells gave a different pattern (Fig. 3*B*), where the I/II ratio decreased after *Hpa* II digestion, to 57% of the ratio for undigested DNA. Notice that the absolute peak sizes, which may reflect tube-to-tube variation in amplification efficiency, do not affect the results, as comparison is always made to the coamplified internal standard (35).

The results of the assay of embryonic germ cells, ovary, and non-ovary material are summarized in Fig. 4. Significant methylation was found for germ cells isolated by traditional pressing methods, ranging from ≈20% to ≈80% purity as determined by alkaline phosphatase activity ("impure germ cells"). The methylation values for these samples ranged from <5% to >50%, confirming that this method results in preparations contaminated to varying degrees. Ovary remains and non-ovary tissue gave average values of 39 ± 6% and 43 ± 5% methylation, respectively (mean ± SEM, n = 4). These values are consistent with genomic sequencing and Southern blot analysis showing this site to be fully methylated on the inactive X chromosome in adult tissue and hybrid cells in culture (15, 19, 20).

Pure germ cells showed very low or no methylation even at the earliest stages assayed; non-germ-cell tissues retained an almost 50% methylation level, as expected from somatic cells with one of their two X chromosomes inactivated. Five of the six pure germ-cell samples, isolated from 47- to 110-day-old fetuses, had methylation levels of 0–3%. The slightly higher value found for the remaining 57-day sample was most likely due to an artifact, or perhaps just statistical fluctuation.

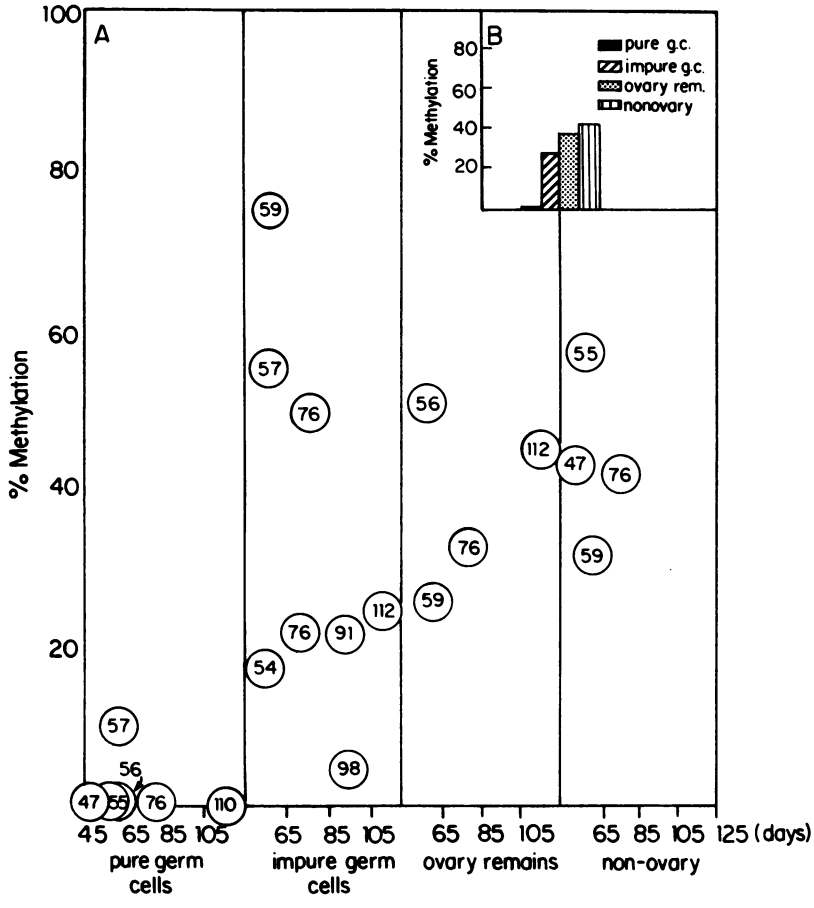

FIG. 4. Summary of results. Numbers in circles represent gestational age (in days) of individual fetuses assayed. *Inset* shows averages obtained for each tissue type. One aberrant value for 59-day impure germ cells (g.c.) was excluded from the average (see text). An attempt was made to obtain a control tissue [e.g., ovary remains (rem.) or non-ovary tissue] for each pure germ-cell sample. However, in two instances (57-day and 110-day specimens) such controls were not obtained.

In a previous study all assays were performed at two concentrations of template, to assure that the assay could be interpreted quantitatively. For these germ-cell preparations, in most cases the sample size was so small that it was impossible to perform the assay at two different concentrations of template. Nevertheless, Wilcoxon rank sum tests revealed a statistically significant difference in degree of methylation between pure germ cells and the other samples ($P = 0.003$ when pure germ cells were compared with impure germ cells; $P = 0.002$ when pure germ cells were compared with ovary remains and non-ovary tissue). No statistically significant difference was seen between impure germ cells and ovary remains or non-ovary tissue ($P = 0.318$), as expected given the wide variation (20–80%) in the purity of the samples. However, a trend toward lower methylation was observed in these cells as well, with some samples showing values well below those seen for non-germ-cell material.

DISCUSSION

We have analyzed oogenic cells for methylation of *Hpa* II site H8, near the transcription start sites of the human *PGK1* gene. All evidence so far has correlated methylation at this site with inactivation of the *PGK1* gene (see Introduction). Additionally, the analogous site in the mouse X-linked *Pgk-1* gene, which shows a similar correlation (21), becomes methylated in the whole embryo at 5.5–6.5 days, when X inactivation is first established (13). This contrasts with the mouse gene for hypoxanthine (guanine) phosphoribosyltransferase, in which the methylation of several restriction sites in the first intron occurs only several days after X chromosome inactivation (36).

We find that site H8 is unmethylated in germ cells of human female fetuses, even as early as 47 days. At this time in development, the germ cells are beginning to migrate into the genital ridges and consist of ≈90% oogonia (34). As judged by glucose-6-phosphate dehydrogenase heterodimer formation, the inactive X chromosome does not undergo reactivation until after about 85 days, >1 month later than the time of assay of our earliest samples (1–3). Thus, this study shows that site H8 is unmethylated prior to oogenic reactivation of the X chromosome.

Driscoll and Migeon (12) have reported the only other study of human germ-cell methylation. Using Southern blotting and impure samples 8–21 weeks of age, they analyzed 57 *Hpa* II sites in six X-linked genes and one autosomal gene. Faint bands indicative of hypomethylation of a small percentage of the cell population were seen in both sexes of the youngest, 8-week, gonadal samples, and this was interpreted to mean that primordial, premeiotic germ cells are unmethylated in both sexes. In their study, H8 was not analyzed separately from seven other sites clustered in the 5′ promoter and CpG island of the *PGK1* gene. We confirm here that the interpretation of Driscoll and Migeon is probably correct and that the most critical region of this cluster is indeed hypomethylated prior to the onset of meiosis. These results are consistent with a locking function for DNA methylation, with the X-linked *PGK1* gene being unlocked prior to its reactivation. The absence of methylation of this critical site in oogonia is consistent with results reported for a transgene whose methylation in developing mouse has been examined (37). In that study hypomethylation of female gametogenic cells was followed by sex-specific methylation after birth (37).

It is not known whether *PGK1* site H8 is ever methylated in primordial germ cells. Previous work on mice (13) suggests that at the time of embryonic X inactivation in late blastula, the equivalent of *Hpa* II site H8 becomes methylated in the embryo as a whole, although we would not have been able to detect a small number of cells escaping such methylation. Thus, it is possible that site H8 is never methylated in progenitors of female germ cells, which would be consistent with the suggestion of Monk *et al.* (10) that the germ line is set aside early in development, thereby avoiding methylation. At some point the germ line is methylated, since we know that a number of sites are methylated in sperm and that even the female germ line is subject to methylation (37). The main point of interest to us is whether a critical site like H8 is ever methylated in oogenesis. If sites like H8 are never methylated in the female germ line, this would mean that random X inactivation can occur and be maintained for at least a limited period without differential methylation of individual X-linked genes.

Alternatively, the site may be methylated very early in germ-cell ontogeny and then become unmethylated during germ-cell migration. While human fetal material and primordial germ cells are difficult to obtain prior to the time of entry of germ cells into the genital ridge, analysis of cells obtained by methods such as flow cytometry may eventually be helpful in answering this question.

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