Letter

Fidelity of the Methylation Pattern and Its Variation in the Genome

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The methylated or unmethylated status of a CpG site is copied faithfully from parental DNA to daughter DNA, and functions as a cellular memory. However, no information is available for the fidelity of methylation pattern in unmethylated CpG islands (CGIs) or its variation in the genome. Here, we determined the methylation status of each CpG site on each DNA molecule obtained from clonal populations of normal human mammary epithelial cells. Methylation pattern error rates (MPERs) were calculated based upon the deviation from the methylation patterns that should be obtained if the cells had 100% fidelity in replicating the methylation pattern. Unmethylated CGIs in the promoter regions of five genes showed MPERs of 0.018–0.032 errors/site/21.6 generations, and the fidelity of methylation pattern was calculated as 99.85%–99.92%/site/generation. In contrast, unmethylated CGIs outside the promoter regions showed MPERs more than twice as high ($P < 0.01$). Methylated regions, including a CGI in the *MAGE-A3* promoter and DMR of the *H19* gene, showed much lower MPERs than unmethylated CGIs. These showed that errors in methylation pattern were mainly due to de novo methylations in unmethylated regions. The differential MPERs even among unmethylated CGIs indicated that a promoter-specific protection mechanism(s) from de novo methylation was present.

[Supplemental material is available online at www.genome.org.]

CpG methylation is known to serve as cellular memory, and is involved in various biological processes, such as tissue-specific gene expression, genomic imprinting, and X chromosome inactivation (Jones and Takai 2001; Bird 2002; Futscher et al. 2002; Strichman-Almashanu et al. 2002). These important functions of methylations are based upon the fact that the methylated or unmethylated status of a CpG site is faithfully inherited. The methylated status of a CpG site is inherited upon DNA replication by the function of maintenance methylase, represented by DNA methyltransferase 1, which is located at replication forks and methylates hemimethylated CpG sites into fully methylated CpG sites (Leonhardt et al. 1992; Araujo et al. 1998; Hsu et al. 1999). The unmethylated status of a CpG site is inherited by not being methylated upon DNA replication or any other occasions. Unmethylated CpG sites generally cluster to form a CpG island (CGI), and most CGIs are kept unmethylated (Gardiner-Garden and Frommer 1987; Bird 2002). Methylations of CGIs in promoter regions are known to cause transcriptional silencing of their downstream genes by changing chromatin structures and blocking transcription initiation (Bird 2002; Richards and Elgin 2002). There are limited numbers of CGIs that are normally methylated (normally methylated CpG islands; NM-CGIs) (De Smet et al. 1999; Futscher et al. 2002). CpG sites outside CGIs, especially those in repetitive sequences, are also normally methylated (Bird 2002).

To keep the methylation pattern, maintenance of both methylated and unmethylated statuses of CpG sites during DNA replication is necessary. However, the fidelity of the methylation pattern has been analyzed only for the mainte-

nance of the methylated status (Wigler et al. 1981; Otto and Walbot 1990; Pfeifer et al. 1990). The fidelity in maintaining the methylated status of an exogenously introduced DNA was shown to be 94% per generation per site by Southern blot analysis (Wigler et al. 1981). The fidelity in maintaining the methylated status of a CGI in the 5′ region of the *PGK1* gene, which was derived from the inactive X chromosome, was estimated to be 98.8%–99.9% per site per generation by the ligation-mediated PCR method after chemical cleavage of DNA (Pfeifer et al. 1990).

Normally unmethylated regions might show different fidelities from normally methylated regions. Even among the unmethylated CGIs, the fidelities of their methylation pattern have been suggested to be different according to their location against a gene promoter. Methylation of CGIs in promoter regions almost always leads to transcriptional silencing while that of CGIs outside promoter regions does not (Gonzalgo et al. 1998; Jones 1999). Considering the cellular expense in maintaining methylation pattern, a cell could sacrifice the fidelity of methylation pattern for CGIs outside promoter regions. In addition, by recent genomic scanning techniques for methylation changes (Ushijima et al. 1997; Toyota et al. 1999; Costello et al. 2000; Jones and Baylin 2002), aberrant methylations of CGIs in cancers are observed in a nonrandom manner (Toyota et al. 1999; Costello et al. 2000; Kaneda et al. 2002a; Kaneda et al. 2002b). It is indicated that CGIs outside promoter regions were more frequently methylated than those in promoter regions (Nguyen et al. 2001; Takai et al. 2001; Kaneda et al. 2002a; Asada et al. 2003).

Here, we analyzed the methylation status of each CpG site on each DNA molecule by the bisulfite sequencing technique (Clark et al. 1994) in six clonal populations of normal human mammary epithelial cells (HMECs), for CGIs in the promoter regions, CGIs outside the promoter regions, and

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CpG sites outside CGIs. By analyzing the deviation from the most common two patterns, MPERs, which reflected the fidelity in replicating both methylated and unmethylated statuses, were measured.

RESULTS

Preparation of HMECs

A single HMEC in its log phase was plated, and expanded to 1.4×10^6 to 1.5×10^6 cells (Fig. 1). Plating efficiency during the two transfers of plates was 67 ± 0.9(mean $\pm$ SE)%. Based on these values, the number of cells that should have been produced at the time of harvest was calculated as 3.2×10^6 ($1.4 \times 10^6/0.67/0.67$). This value predicted that each cell harvested underwent 21.6 generations from the initial single cell. Doubling time was 48 h.

Gene Selection and Their Expression Levels

Methylation statuses were determined by bisulfite sequencing for CGIs in the promoter regions of the *E-cadherin*, *p41-Arc*, *SIM2*, *3-OST-2*, and *Cyclophilin A* genes; CGIs in the downstream exon/introns of the *E-cadherin*, *p41-Arc*, and *SIM2* genes; CpG sites outside CGIs of the *E-cadherin* and *p41-Arc* genes; a NM-CGI of the *MAGE-A3* gene; and differentially methylated region (DMR) of the *H19* gene (Fig. 2A). The former five genes were selected because they had CGIs in the downstream exon/introns that met a strict criterion of CGIs, regions of DNA of >500 bp with a G+C $\geq$ 55%, and observed CpG/expected CpG of 0.65 (Takai and Jones 2002). The *MAGE-A3* gene and the DMR of the *H19* gene were selected as a representative NM-CGI and a region critically involved in genomic imprinting, respectively. By quantitative RT-PCR analysis, their expression levels were shown to range from almost none (*SIM2* and *MAGE-A3*) to very high (*E-cadherin*), with *p41-Arc*, *3-OST-2* and *Cyclophilin A* being intermediate (Fig. 2B).
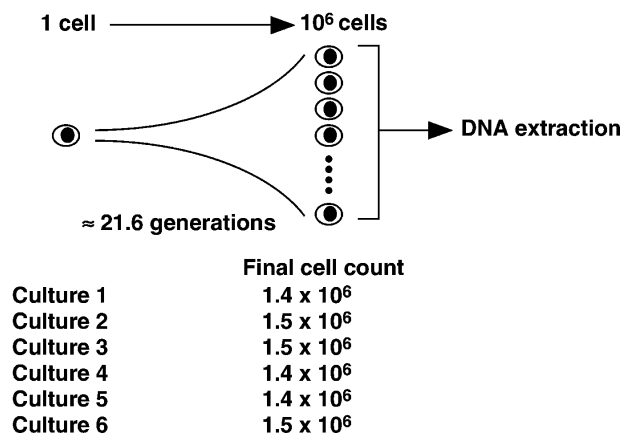

	Final cell count
Culture 1	1.4×10^6
Culture 2	1.5×10^6
Culture 3	1.5×10^6
Culture 4	1.4×10^6
Culture 5	1.4×10^6
Culture 6	1.5×10^6

Figure 1 Strategy of cell culture. A single HMEC was inoculated in a well by limiting dilution, and the cell was expanded up to approximately 10^6 cells. Based on the plating efficiencies during the two transfers and the actual final cell count, the number of cells that should have been produced at the time of harvest and the number of generations observed were calculated. DNA was extracted from the final cells, and used for bisulfite sequencing. Six independent cultures were performed.

Establishment of How to Measure MPERs

The CGI in the promoter region of the *E-cadherin* gene (Fig. 3A), the non-CGI region of the *p41-Arc* gene (Fig. 3F), the CGI in the promoter region of the *MAGE-A3* gene (Fig. 3K), and the DMR of the *H19* gene (Fig. 3L) were found to contain two major populations of clones. The two major populations were considered to represent the methylation pattern of the two alleles in the original single cell. The methylation patterns of the two major populations were different from each other in the six cultures, which indicated that the HMECs before cloning had diverse patterns of methylation, but the patterns were relatively conserved during the culture from a single cell to approximately 10^6 cells. Therefore, we measured the number of errors in the methylation pattern based upon the culture from a single cell to approximately 10^6 cells. An MPER of a region in a culture was calculated from the number of errors in methylation pattern as described in Methods, and an average MPER of the region was calculated from the six MPERs obtained for the six cultures.
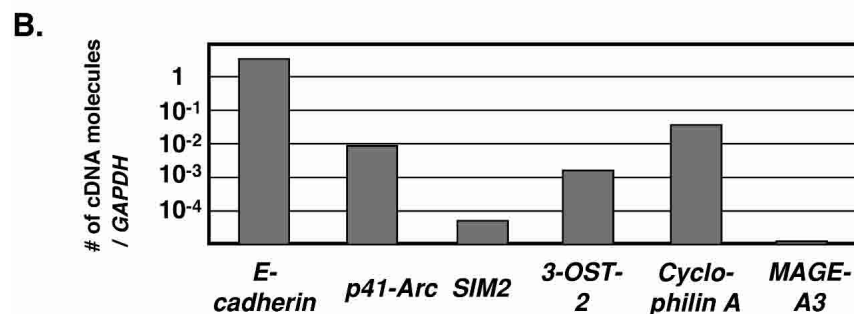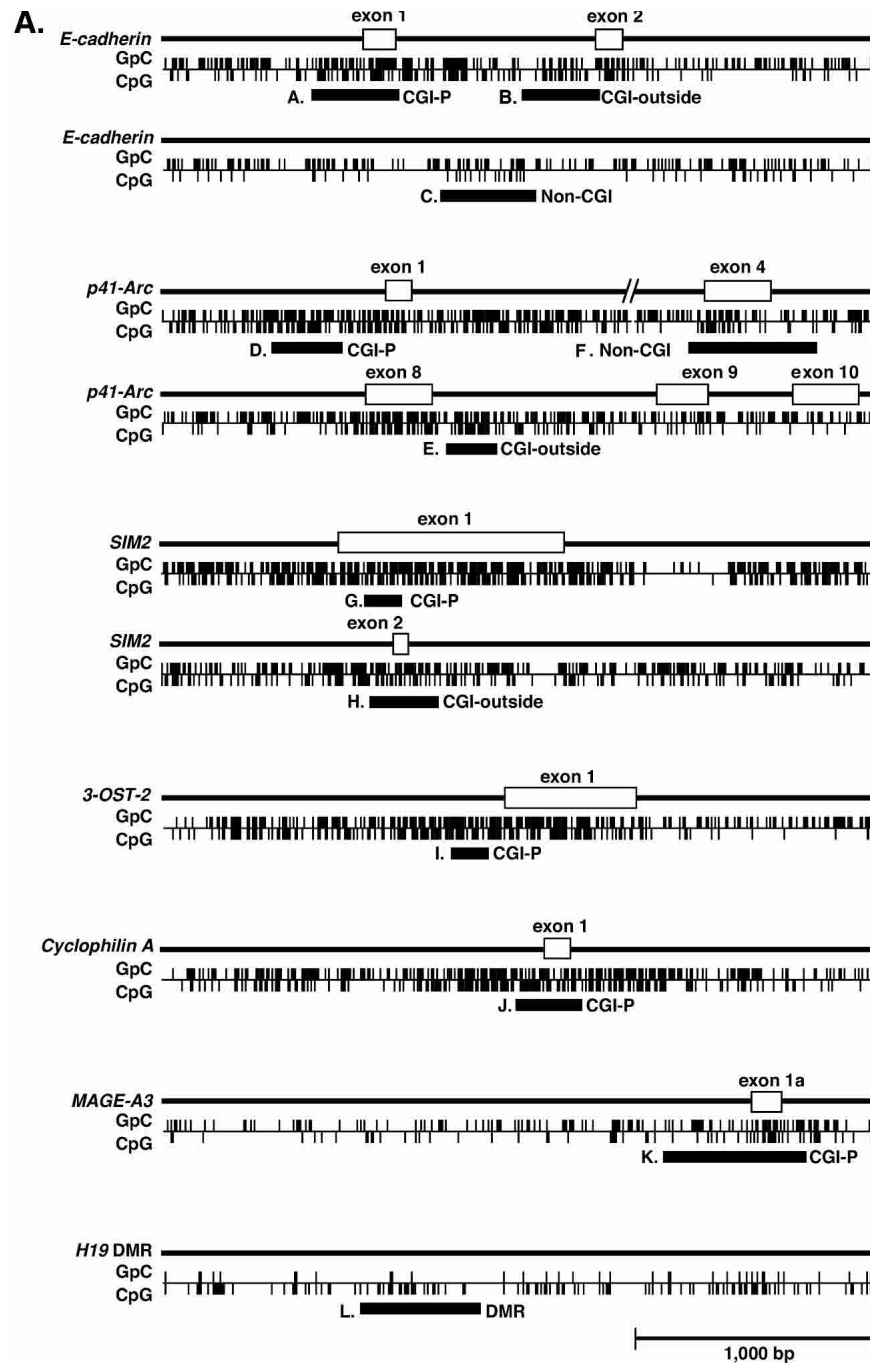
To examine the effect of an arbitrary selection of the "original methylation pattern" in ambiguous cases, a permutation test was performed for the CGI in the *E-cadherin* promoter region of HMEC10. One of the clones #5–#14 (Fig. 3A) was hypothesized as one of the original methylation pattern, and the number of errors in the methylation pattern was calculated. The numbers ranged from 18–22, and these values were expected to result in the average MPER ranging from 0.022–0.023. Similar permutation tests were performed for the CGI in exon 2 of the *E-cadherin* gene of HMEC12 and HMEC15. The numbers of errors in methylation pattern ranged from 13–16 for HMEC12 and from 12–15 for HMEC15, and these values were expected to result in the average MPER ranging from 0.050–0.058. These showed that arbitrary selection of the original methylation pattern in ambiguous cases does not seriously affect the resultant average MPER.

The efficiency of bisulfite conversion was examined by analyzing DNA with no methylation in the CGIs in the promoter region and exon 2 of the *E-cadherin* gene. In the CGI in the promoter region, none of the 600 cytosines at CpG sites (30 CpG sites per clone, 20 clones analyzed) remained unconverted, showing that unconversion rate was almost 0 in this region under our experimental condition. In the CGI in exon 2, one of 483 cytosines at CpG sites (23 CpG sites per clone, 21 clones analyzed) remained unconverted, showing that the unconversion rate was 0.0021. These values showed that the MPERs in CGIs in the promoter regions are 10-fold more than the unconversion rates.

MPERs and Fidelities of Methylation Pattern in the Genome

The average MPERs obtained for each region are summarized in Table 1. Unmethylated CGIs in the promoter regions showed MPERs between 0.018 and 0.032 errors/site/21.6 generations. In contrast, CGIs outside promoter regions showed significantly higher MPERs, ranging from 0.037 to 0.091 ($P < 0.01$ or 0.005). MPERs in the CGIs outside the promoter regions were more than twice as high as those in the promoter regions of the same genes.

NM-CGI of the *MAGE-A3* gene and methylated alleles of the DMR of the *H19* gene showed MPERs of 0.002 and 0.007, respectively. Any genomic regions that were normally methylated, whether or not they were in CGIs, showed significantly lower MPERs than those unmethylated. This was par-

ticularly clear when the MPER of the allele methylated at DMR of the *H19* gene was compared with that of the other unmethylated allele.

DISCUSSION

It was first demonstrated here that the fidelity of replicating methylation patterns of CGIs in the promoter regions is significantly higher than that of CGIs outside the promoter regions. It was also demonstrated here that methylated genomic regions show much higher fidelity than unmethylated genomic regions. These showed that maintenance methylation of hemimethylated CpG sites into fully methylated CpG sites at DNA replication was highly reliable, while unmethylated CpG sites tended to be methylated by de novo methylation. It is well-known that exogenous DNA is exposed to a de novo methylation pressure (Doerfler et al. 2001; Bird 2002), and a similar methylation pressure seems to be working on the endogenous DNA. To maintain the unmethylated status of CGIs, protection mechanisms from the de novo methylation pressure seem to be necessary. Since the MPERs were significantly lower in CGIs in the promoter regions than in CGIs outside the promoter regions, the presence of a protection mechanism(s) specific to the promoter regions, in addition to a mechanism(s) common to all CGIs, was indicated. Although the details of the mechanisms are still unknown, binding of transcriptional factors, such as Sp1, has been indicated as a promoter-specific mechanism (Han et al. 2001).

The differential fidelities in replicating methylation patterns of CGIs in the promoter regions and those outside indicated that aberrant methylation of CGIs would occur at different rates depending upon their locations. This will be important when tumors are analyzed for the CGI methylator

Figure 2 Structures and expressions of the genes analyzed. (*A*) Schematic representation of the genomic regions analyzed. Regions analyzed by bisulfite sequencing are shown by closed boxes, and designations A–L correspond to panels in Fig. 3. CGI-P: a CGI in the promoter regions; CGI-outside: a CGI outside the promoter regions; Non-CGI: CpG sites outside CGIs; and DMR: differentially methylated region. All panels are drawn to the same scale. (*B*) Expression levels of the seven genes in HMECs.
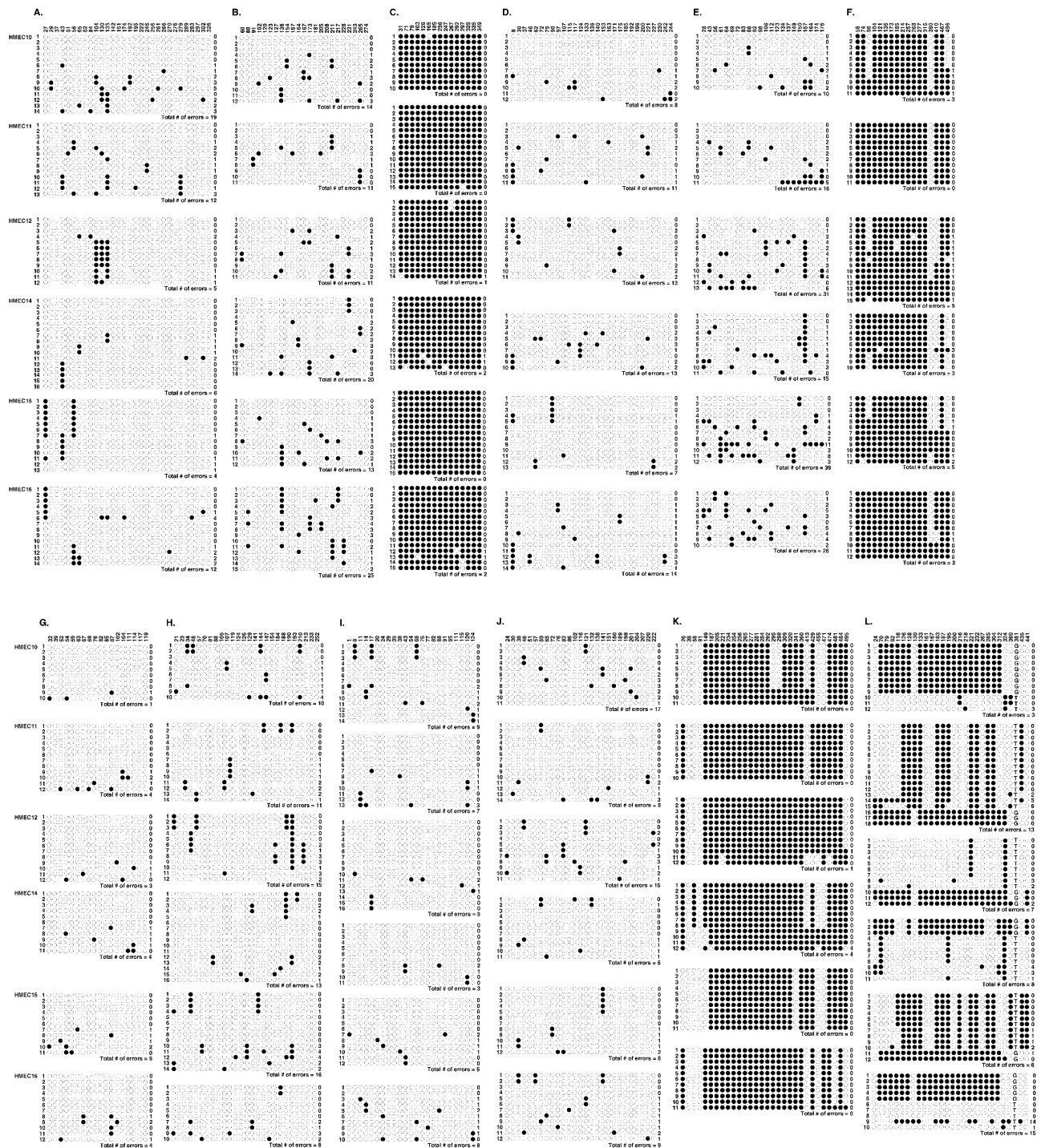
Figure 3 Distribution of unmethylated and methylated CpG sites shown by bisulfite sequencing. Unmethylated and methylated CpG sites are shown by open and closed circles, respectively. (*A*)–(*C*) A CGI in the promoter region, a CGI outside the promoter region and CpG sites in non-CGIs of the *E-cadherin* gene. (*D*)-(*F*) A CGI in the promoter region, a CGI outside the promoter region and CpG sites in non-CGIs of the *p41-Arc* gene. (*G*), (*H*) A CGI in the promoter region and a CGI outside the promoter region of the *SIM2* gene. (*I*) A CGI in the promoter region of the *3-OST-2* gene. (*J*) A CGI in the promoter region of the *Cyclophilin A* gene. (*K*) A CGI in the promoter region of the *MAGE-A3* gene, which is normally methylated. (*L*) A CGI in the differentially methylated region of the *H19* gene.

phenotype (CIMP), which are considered to be caused by molecular defects that allow accumulation of aberrant CGI methylations (Toyota et al. 1999). The differential fidelities shown here suggest that there are two types of CIMP, one due to a defect(s) in the protection mechanisms common to all CGIs and the other due to a defect(s) in the protection mechanisms specific to CGIs in the promoter regions. Actually, a correlation between the CIMP and the diffuse-type histology was clearly observed in gastric cancers when CGIs in the promoter regions were used for CIMP analysis (Kaneda et al. 2002b),

Table 1. MPERs in Various Genomic Regions

Gene/location	G + C content (%)	CpG score	Methylation status	MPER (number of errors/site/21.6 generations)	Fidelity (%/site/generation)
E-cadherin					
CGI-P	64.6	0.70	U	0.022 ± 0.012	99.89
CGI-outside	66.7	0.66	U	0.053 ± 0.012***	99.75
Non-CGI	50.2	0.59	M	0.004 ± 0.005*	99.98
p41-Arc					
CGI-P	68.9	0.69	U	0.032 ± 0.010	99.85
CGI-outside	66.4	0.72	U	0.091 ± 0.039**	99.56
Non-CGI	65.2	0.31	M	0.017 ± 0.012*	99.92
SIM2					
CGI-P	65.7	0.76	U	0.018 ± 0.004	99.92
CGI-outside	64.8	0.70	U	0.037 ± 0.008***	99.83
3-OST-2					
CGI-P	66.1	0.80	U	0.021 ± 0.008	99.90
Cyclophilin A					
CGI-P	61.6	0.90	U	0.032 ± 0.017	99.85
MAGE-A3					
CGI-P	63.7	0.46	M	0.002 ± 0.004	99.99
H19-DMR					
both alleles	59.5	0.65	U/M	0.026 ± 0.016	99.87
unmethylated			U	0.043 ± 0.034	99.80
methylated			M	0.007 ± 0.011*	99.98

MPERs (number of errors/site/21.6 generations) were calculated from the observed number of errors in six clonal populations that underwent 21.6 generations. Fidelity (%/site/generation) was calculated by the equation $M = 1 - F^{21.6}$. MPERs in CGIs outside the promoter regions and non-CGI regions were compared with those in the promoter regions by the *t*-test, *P < 0.05, **P < 0.01, ***P < 0.005. CGI-P: CGI in the promoter region; CGI-outside: CGI outside the promoter regions; Non-CGI: CpG sites outside CGIs. G + C content and CpG score of the regions analyzed were calculated for the most suitable region larger than 500 bp using a program at the "CpG Island Searcher" web site (http://www.uscnorris.com/cpgislands/). Methylation statuses of these regions in physiological conditions were described as U: unmethylated, M: methylated.

while it was unclear when CGIs outside the promoter regions were used.

In order for an impaired fidelity in maintaining a methylation pattern to exert any biological effect, methylation statuses of multiple CpG sites in a CGI must be altered. A significant increase of MPERs would be necessary for this, and quantitative analysis of MPERs in cells with suspected increase of MPERs is necessary. DMR of the *H19* gene had a polymorphism at nt. 391 (nt. 8217; GenBank accession no. AF125183), and this served to distinguish the two alleles clearly. The G-allele was methylated in all of the six cultures, and the T-allele was unmethylated. The methylation patterns of the T-alleles were similar in HMEC11 and HMEC15, but were essentially variable among the six cultures. This indicated that, although the original cells in HMEC11 and HMEC15 might have had a common ancestral cell, methylation patterns in a tissue alter significantly during a human life span.

Future clarification of what protection mechanisms are involved and how they are impaired in various diseases will contribute to understanding of aging (Ahuja et al. 1998; Issa et al. 2001) and various pathological conditions.

METHODS

Cell Culture and DNA/RNA Extraction

HMECs were purchased from Clonetics, and cultured in MEBM (Clonetics). HMECs are known to have a stable normal diploid karyotype (Stampfer and Bartley 1988; Berthon et al. 1992). A single cell in its log phase was plated in a well of a 96-well plate, and inoculation of a single cell was confirmed by observing stochastic distribution of positive wells in the plate and a single colony in a positive well. Cells were transferred serially to a well of a 12-well plate and to a 10 cM dish. When the cells were subconfluent, they were collected, and high molecular weight genomic DNA was extracted by serial extraction with phenol/chloroform and ethanol precipitation. Culturing and DNA extraction were performed for six progenitor single cells. Plating efficiencies were measured by parallel plating of 300 cells and observing their viability. The number of cell generations observed was calculated from the plating efficiencies and the final cell count.

Sodium Bisulfite Modification and Sequencing

Sodium bisulfite modification was performed according to previous reports (Clark et al. 1994; Rein et al. 1997). Genomic DNA was restricted with *Bam*HI restriction enzyme (New England Biolabs), and 500 ng of the restricted DNA was denatured in 0.3 N NaOH. The denatured DNA was sulfonated in a solution of 3.1 M $NaHSO_3$ (pH 5.3) and 0.5 mM hydroquinone, which underwent 15 cycles of denaturation at 95°C for 30 sec and incubation at 50°C for 15 min. The sample was desalted with the Wizard DNA clean-up system (Promega), and desulfonated by treatment in 0.3 N NaOH at room temperature for 5 min. The DNA sample was ethanol-precipitated with ammonium acetate, and dissolved in 20 µL of TE buffer. For bisulfite sequencing, 1 µL of the DNA solution was used for PCR with the primers common for methylated and unmethylated DNA sequences (See Supplementary Table at www.genome.org). PCR products were cloned into pGEM-T Easy Vector (Promega), and 10–18 clones from each sample were cycle-sequenced using T7 and Sp6 primers with a BigDye

Terminator kit (PE Biosystems) and an ABI automated DNA sequencer (PE Biosystems).

To measure unconversion rates during the bisulfite modification, a completely unmethylated DNA was prepared by PCR of a 6,629-bp fragment, which covered CGIs in the promoter region and exon 1 of the *E-cadherin* gene, with primers shown in the Supplementary Table and LA-Taq (Takara). The PCR solution contained 1M betaine, and the PCR was performed for 30 cycles consisting of 10-sec denaturation at 94°C and 15-min annealing/extension at 72°C. The PCR product was purified, and added to the rat genomic DNA at an equimolar concentration. In the same manner with the samples, the rat genomic DNA with the PCR product was restricted with *Bam*HI, modified with bisulfite, and sequenced.

Quantitative Reverse-Transcription-PCR

cDNA was synthesized from 3 µg of DNase-treated total RNA in 20 µL with oligo $(dT)_{12-18}$ primer and SuperScript II reverse transcriptase (Life Technologies). One µL of the cDNA solution was amplified in a solution that contained SYBR Green PCR Core Reagents (Applied Biosystems) and 200 nM of primers. Real-time PCR analysis was performed using an iCycler iQ detection system (Bio-Rad Laboratories), with a PCR condition of 40 cycles of denaturation at 94°C for 30 sec, annealing at a specified temperature for 30 sec, and extension at 72°C for 30 sec. The sequences of the primers and annealing temperature are listed in the Supplementary Table. The absence of nonspecific amplification was confirmed by electrophoresing the PCR products in agarose gels. The number of cDNA molecules was quantified by comparing amplification of an unknown sample to those of standard samples that contained 10^1–10^7 copies of the gene. The amount of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) of each cDNA solution was also quantified, and the amount of a gene of interest was normalized to the amount of *GAPDH*.

Calculation of MPER and Fidelity, and Statistical Analysis

To calculate MPERs, clones sequenced for each region were classified by their methylation patterns. The most and second most prevalent patterns were determined. When the second most prevalent patterns were present in multiple, a pattern that would minimize the number of deviations in the remaining clones from the original two patterns was regarded as an original pattern. By counting the number of deviations from the original two patterns (numbers shown to the right of each clone in Fig. 3), the total number of methylation pattern errors in each clone was calculated (shown for each culture). To obtain a MPER of a culture, the total number of methylation errors was divided by the total number of CpG sites examined in the culture. Fidelity of methylation pattern (*F*: %/site/generation) was calculated from MPERs (*M*: error/site/21.6 generations) by an equation: $M = 1 - F^{21.6}$.

The MPERs of two regions were statistically compared using a *t*-test.

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Fidelity of the Methylation Pattern and Its Variation in the Genome

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