 Nucleic Acids Research, 1999, Vol. 27, No. 15 **3229–3235**

Confluence-induced alterations in CpG island methylation in cultured normal human fibroblasts

Russell O. Pieper*, Kathryn A. Lester and Christie P. Fanton

Department of Neurological Surgery and the UCSF Cancer Center, Room N261, 2340 Sutter Street, University of California–San Francisco, San Francisco, CA 94115-0128, USA

Received January 29, 1999; Revised and Accepted May 21, 1999

ABSTRACT

Growth constraint of bacterial and human cells has been shown to trigger genetic mutation. We questioned whether growth constraint might also trigger epigenetic mutation in the form of CpG island methylation. Logarithmically growing normal human fibroblasts (NHF) displayed little (0–15%) CpG methylation in select regions of three CpG islands [estrogen receptor (ER), E-cadherin (ECAD) and *O*^{6}-methylguanine-DNA methyltransferase (MGMT)] examined. NHF grown to and left at confluence for 2–21 days showed little (<10%) CpG methylation in the ER and ECAD CpG islands. These confluent, growth-arrested cells, however, displayed extensive (~50%) methylation of the MGMT CpG island. CpG methylation in the MGMT CpG island was not associated with cellular senescence. The methylation was, however, heritable, but not permanent, as the level of CpG methylation in the MGMT CpG island of cells 4 population doublings following replating after confluence were no different from those in confluent cultures, but returned to levels noted in logarithmically growing cells by 10 population doublings following replating. These results suggest that growth constraint can trigger transient epigenetic change even in normal non-senescent human cells.

INTRODUCTION

Genomic stability is an important hallmark of normal human cells (1). Recent studies, however, have suggested that the processes that maintain genomic stability are regulated and, more specifically, down-regulated when cells are grown at high density. A significant increase in frameshift mutations was noted in mismatch repair-deficient human cells maintained at high density (2), while in bacteria, maintenance at stationary phase resulted in the down-regulation of the function of the mismatch repair protein mutL (3). In both cases prolonged confluence resulted in genomic instability and the ability of cells to undergo rapid genetic change in the face of selective pressure.

In addition to triggering genetic change, high density growth may also trigger epigenetic change. The factors involved in epigenetic control are numerous and poorly defined, although DNA cytosine methylation has been suggested to play a key role. These suggestions have been strengthened by recent studies demonstrating that the aberrant methylation of CpG dinucleotides in CpG-rich regions of the genome critical for gene expression (CpG islands) recruits methylated DNA-binding proteins which in turn interact with histone deacetylases. These histone deacetylases modify acetylation of histones in a manner that changes chromatin structure and favors loss of gene expression (4,5). In this manner alterations in CpG island methylation have the potential to heritably influence gene expression. Epigenetic change in the form of aberrant CpG methylation has been noted in the estrogen receptor (ER) CpG island in normal human cells as they approach senescence (6–8). Aberrant CpG island methylation is also common in tumor and transformed cells in which random aberrant methylation is believed to contribute to gene silencing and the selection of those cells with favorable patterns of gene expression (9). Unlike transformed or senescent cells, however, normal, non-senescent human cells are thought to retain epigenetic controls and to rigorously maintain CpG islands in an unmethylated state (10). The fidelity of these control mechanisms under conditions that relax controls on genetic stability, however, has not been examined.

To address the possibility that the processes that control epigenetic stability in the form of CpG island methylation can, like those processes controlling genetic stability, be altered or down-regulated, non-senescent normal human fibroblasts (NHF) were subjected to conditions that restrained and arrested growth without inducing senescence. Patterns of CpG methylation were then examined in three CpG island promoters known to be unmethylated in NHF but susceptible to methylation in senescent and/or transformed human fibroblasts.

MATERIALS AND METHODS

Cell culture

NHF (WI38) and SV40-transformed human fibroblasts (WI38VA4) were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's medium (DME-H16) supplemented with 20% calf serum (UCSF Cell Culture Facility). Cells were grown in a 95% air/5% CO_2 environment. For each independent experiment a new vial of cells from the supplier was thawed and used within 4 population doublings of thawing. In these

*To whom correspondence should be addressed. Tel: +1 415 502 7132; Fax: +1 415 502 3179; Email: rpieper@cc.ucsf.edu

studies population doublings were considered to be proportional to the extent of cell dilution upon passage, i.e. cells that were 70% confluent and split 1:4 were considered to have undergone 2 population doublings upon return to 70% confluence. Logarithmically growing cultures were maintained at <70% confluence by passage (1:4) every ~3–4 days. Cultures were considered to be confluent when the open area on the plate was <5% of the total area, when cells were in layered contact with one another and when the cells assumed a smaller, aligned phenotype. Following attainment of confluence, cells were left for 2–21 days during which time the morphology of the cells and the cell number remained unchanged. For confluent cultures, the medium was changed only on those cultures held at confluence for 21 days and then only once at 14 days post-confluence.

Analysis of CpG island methylation

Genomic DNA was isolated from logarithmically growing or confluent fibroblast cultures as previously described (11). The DNA was sheared by passage through a 22 gauge needle and was quantitated and resuspended to ~1 μg/μl. The DNA was modified with sodium bisulfite as previously described (12,13). Select regions of the ER, E-cadherin (ECAD) and O^6-methylguanine-DNA methyltransferase (MGMT) CpG islands were amplified from bisulfite-modified DNA using two rounds of PCR with nested primers specific to the bisulfite-modified sequence of the island of interest. The primers used for amplification of the ER CpG island fragment were: ER primer 1 (nt 267–296), 5'-ATGGTTTTATTGTATTAGATTTAAGGGAAT-3', and ER primer 2 (nt 611–651), 5'-TATAACICTAAACTCITTCT-CCAAATAATA-3', for the primary amplification; ER primer 3 (nt 355–382), 5'-AGTGTATTTGGATAGTAGTAAG-3', and ER primer 4 (nt 587–610), 5'-CTAACCITAAAACTA-CAAAAAAAA-3', for the secondary amplification. Primers for amplification of the ECAD CpG island were: ECAD primer 1 (nt –307 to –282), 5'-TAAAAGAATTTAGT-TAAGTGTAAAAG-3', and ECAD primer 2 (nt 38–69), 5'-AACTAAAACIAACTAAAATCTAAACTAACTTC-3', for the primary amplification; ECAD primer 3 (nt –275 to –250), 5'-TTTGATTTTAGGTTTTAGTGAGTTAT-3', and ECAD primer 4 (nt 11–35), 5'-CAAACTCACAAATACTTTA-CAATTC-3', for the secondary amplification. The primers used for amplification of the MGMT CpG island were MGMT primer 1 (nt 442–465), 5'-TAGGGTTTTTGTTGGTTTGGG-GGT-3', and MGMT primer 2 (nt 1109–1133), 5'-AAACTAC-CCAAACACTCACCAAATC-3', for the primary amplification; MGMT primer 1 and MGMT primer 3 (nt 667–690), 5'-CC-TAAAACTCTATACCTTAATTTA-3', for the secondary amplification. All amplifications were carried out as previously described (13) except that the $MgCl_2$ concentration used was 1.5 mM. Both rounds of amplification used 35 cycles. For the second round of amplification, conditions used were shown to produce linear amplification of the product. Following the second round of amplification the resultant PCR products were electrophoresed in a 1.5% agarose gel, excised, purified (Wizard PCRPreps System; Promega) and ligated to a plasmid vector (pGEM-Easy-T; Promega) following the manufacturer's recommendation. Following ligation, competent bacteria (*Escherichia coli* JM109) were transformed with the ligated DNA and grown overnight on LB/agar/ampicillin (100 μg/ml) plates. Bacterial colonies (3 to 20) selected from each plate were grown overnight in 15 ml LB medium, after which recombinant plasmid was recovered (Wizard Plus Miniprep System; Promega). PCR-amplified CpG island inserts contained in the recombinant plasmid DNA were sequenced (TaqTrack Sequencing System; Promega) using a T7 or SP6 promoter primer. The methylation status of each individual CpG dinucleotide was determined by comparing the sequence of the recombinant DNA to the published sequence of the ER, ECAD or MGMT CpG islands (14–17). The percentage of clones methylated at any given CpG site was determined by dividing the number of methylated CpGs at a given site by the number of clones analyzed. The percentage of CpGs methylated for a given clone was determined by dividing the number of methylated CpG sites by the total number of CpGs analyzed in a given clone. Statistical significance was determined using Student's *t*-test. In all molecules examined, >95% of C residues not preceding G residues were chemically converted by the bisulfite treatment.

Bisulfite sequencing results were confirmed by Southern blot analysis using methylation-sensitive restriction enzymes. DNA (20 μg) from logarithmically growing fibroblasts or from fibroblasts held at confluence for 2, 14 or 21 days was cleaved with *Bam*HI and *Sst*I (10 U/μg DNA, 37°C). Following phenol/chloroform extraction and ethanol precipitation, the DNA was incubated with the methylation-sensitive restriction enzyme *Ava*I (5 U/μg DNA, 12 h, 37°C). The DNA was electrophoresed in a 1.1% agarose gel, transferred by capillary action to a nylon membrane and hybridized to a uniformly ^{32}P-labeled probe complementary to nucleotides 1–424 of the 1150 bp *Bam*HI–*Sst*I fragment of the MGMT CpG island. Following washing of the membrane (18), hybridized probe was detected by phosphorimager analysis.

Analysis of senescence

Degree of senescence of fibroblast cultures was assessed by measuring the percentage of cells expressing senescence-associated β-galactosidase activity (19). Logarithmically growing or confluent cells were left attached to culture plates and were washed twice with cold phosphate-buffered saline (PBS). The cells were then fixed in 2% formaldehyde/0.2% glutaraldehyde (3–5 min, room temperature), after which the cells were washed twice with PBS and incubated (37°C, 16 h) in staining solution (40 mM citric acid/sodium acetate buffer, pH 6, 5 mM potassium ferrocyanide, 5 mm potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM X-gal in dimethylformamide). After incubation the cells were washed twice with PBS, twice with methanol and air dried. The percentage of senescence-associated β-galactosidase-positive cells in each culture was assessed by counting the number of blue cells in a 10× microscope field and dividing this number by the number of blue cells plus the number of clear cells in the same field. The percentage of senescent cells per plate was the average of determinations from four fields per plate.

Analysis of cell proliferation

Extent of growth arrest was assessed by monitoring radiolabeled thymidine incorporation into the acid-insoluble fraction of fibroblasts. Logarithmically growing or confluent cultures were incubated with [methyl-^{3}H]thymidine (2 Ci/mmol, 5 μCi/ml culture medium; Amersham) for 20 h under standard culture conditions. Cells were then trypsinized, washed once in cold

Table 1. Methylation of CpG islands in logarithmically growing NHF or SV40-transformed human fibroblasts

CpG island	CpGs methylated in NHF (%)	CpGs methylated in transformed fibroblasts (%)
ER	14.1 ± 4.6 (*n* = 42)	70 ± 8.4 (*n* = 42)
ECAD	0 (*n* = 40)	100 (*n* = 40)
MGMT	15.9 ± 2.9 (*n* = 220)	86 ± 4.8 (*n* = 44)

Values are means ± SE.

PBS and resuspended in cold PBS. Cell number was determined using a hemocytometer, after which 10^5 cells from each group were filtered onto a pre-wetted GFC filter disc. The cells were washed with 5 ml cold PBS and lysed by the addition of 5 ml cold 10% trichloroacetic acid. The acid-insoluble fraction on the filter was washed with 5 ml cold PBS, after which the filter was dried, added to 10 ml scintillation fluid and subjected to scintillation counting. All experiments were performed in triplicate.

RESULTS

CpG methylation in select CpG islands of logarithmically growing NHF

Normal human lung fibroblasts (WI38) were grown to ~60% confluence after which DNA was isolated and subjected to sodium bisulfite sequencing. Extent of CpG methylation was analyzed in the ER CpG island at nucleotides 382–462 (the entire island spans nucleotides 293–670) (15), in the ECAD CpG island at nucleotides –250 to –130 (the entire island spans nucleotides (–250 to +155) (15) and in the MGMT CpG island at nucleotides 503–570 (the entire island spans nucleotides 503–1415) (16,17). These regions were all previously shown to be susceptible to CpG methylation (7,13). The results of this analysis are shown in Figure 1 and Table 1. There was very little methylation of CpG dinucleotides in any of the CpG island regions examined, regardless of whether this data was analyzed on a site by site basis (Fig. 1) or on an overall basis (Table 1). The regions analyzed were, however, susceptible to methylation as they were all extensively methylated (≥70%) in an SV40-transformed fibroblast cell line (Table 1).

CpG methylation in CpG islands of confluent NHF

DNA from cells that were left at confluence for 2, 14 or 21 days was subjected to sodium bisulfite sequencing, as was DNA from NHF that were allowed to proliferate at lower density (<70% confluence) for 2–21 days. Extent of CpG methylation was analyzed in regions of the ER, ECAD and MGMT CpG islands. The results of this analysis are shown in Figure 1 and Table 2. The DNA from cells left at confluence for 21 days contained very few methylated CpG sites in the region of the ER CpG island analyzed, with the extent of CpG methylation being no greater and perhaps less than that noted in logarithmically growing fibroblasts (Fig. 1 and Table 2). Similar results were noted in the analysis of the ECAD CpG island. Again, the extent of CpG methylation of the ECAD CpG island region examined in the DNA from cells left at confluence for 21 days was comparable to that noted in the DNA from logarithmically growing cells. In contrast, in the MGMT CpG island, the DNA from cells left at confluence for 21 days contained a higher percentage of methylated CpGs than the DNA from logarithmically growing cells. This same ~3-fold increase in methylation was noted whether the cells were left at confluence for 14 or 21 days or as short as 2 days (Table 2). The methylation noted appeared to be randomly distributed across the region analyzed, with the pattern of methylation differing in each molecule analyzed. The extent of methylation noted in confluent fibroblast cultures using bisulfite sequencing was also consistent with that noted by restriction enzyme-based Southern blot analysis of the same DNA. As shown in Figure 2, a ^{32}P-labeled 5'-end probe hybridized to an 1150 bp fragment of *Bam*HI–*Sst*I-digested DNA from logarithmically growing cells (lane 1) or from cells held at confluence for 2, 14 or 21 days (lanes 3, 5 and 7). When the DNA from logarithmically growing cells was additionally incubated with the methylation-sensitive restriction enzyme *Ava*I, the probe hybridized to a 530 bp fragment (lane 2), suggesting that the *Ava*I site nearest the 3'-end of the probe was completely unmethylated in this DNA. In *Bam*HI–*Sst*I–*Ava*I-digested DNA from cells held at confluence for 2, 14 or 21 days, however, the probe hybridized not only to a 530 bp fragment, but also to fragments generated by CpG methylation of *Ava*I sites at 530, 600 and 900 bp from the 3'-end of the probe (lanes 3, 5 and 7). These results are consistent with those derived from bisulfite sequencing and suggest that DNA from cells held at confluence contains methylated CpG dinucleotides at select restriction enzyme recognition sequences and throughout the island as a whole.

Table 2. Methylation of CpG islands in logarithmically growing or confluent NHF

CpG island	Growth	CpGs methylated (%)
ER	Logarithmic	14.1 ± 4.6
	Confluent (21 days)	5.5 ± 2.8 (*n* = 266)
ECAD	Logarithmic	0
	Confluent (21 days)	5.1 ± 3.3 (*n* = 60)
MGMT	Logarithmic	15.9 ± 2.9
	Confluent (2 days)	51.6 ± 3.6 (*n* = 99)[a]
	Confluent (14 days)	43.2 ± 6.8 (*n* = 44)[a]
	Confluent (21 days)	43.6 ± 5.9 (*n* = 55)[a]

Values are means ± SE.
[a]Significantly different from log cells, $P < 0.05$.

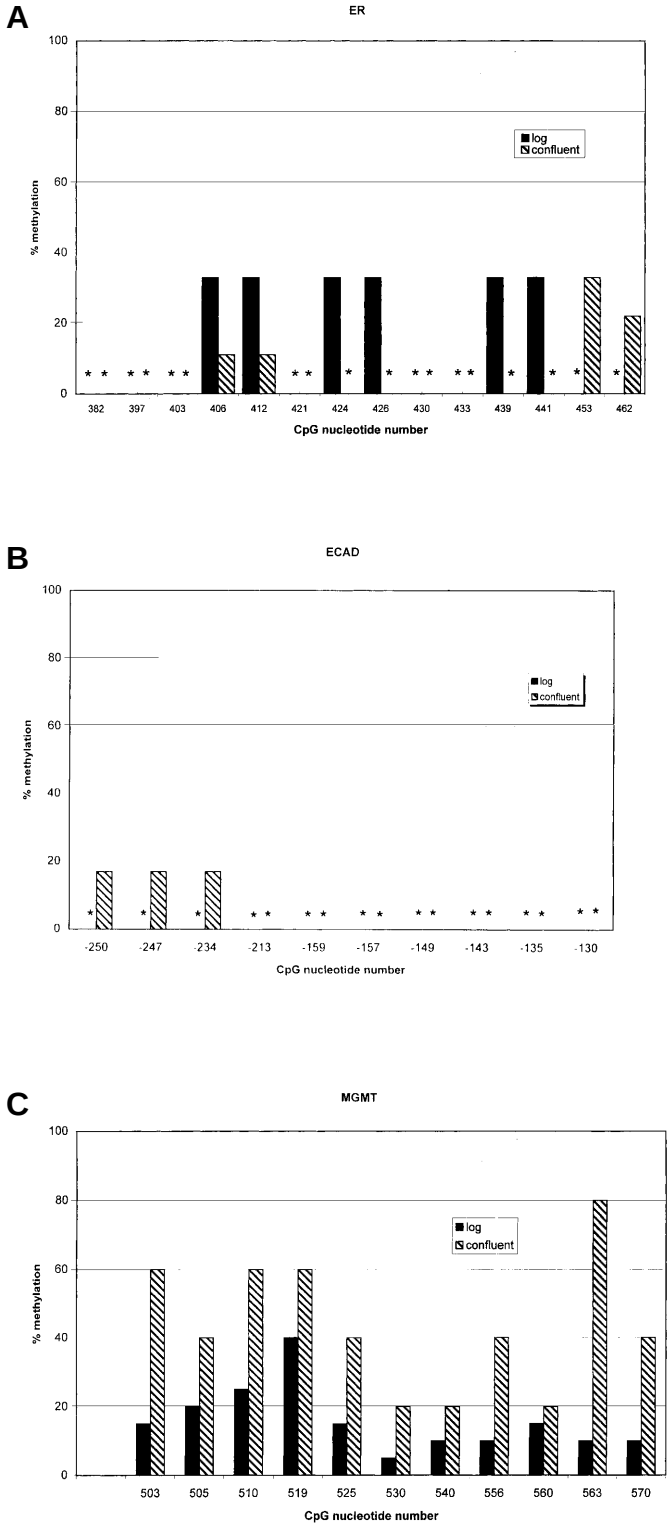

Figure 1. Extent of CpG dinucleotide cytosine methylation in three CpG islands of logarithmically growing (solid bars) or 21 day confluent (dashed bars) NHF. Select regions of the ER (**A**), ECAD (**B**) and MGMT (**C**) CpG islands were PCR amplified using bisulfite-modified genomic DNA from logarithmically growing or confluent NHF as template. PCR products were cloned and sequenced. *, value of 0. n = 3 (log) and 19 (confluent) for ER, 4 (log) and 6 (confluent) for ECAD and 22 (log) and 5 (confluent) for MGMT.

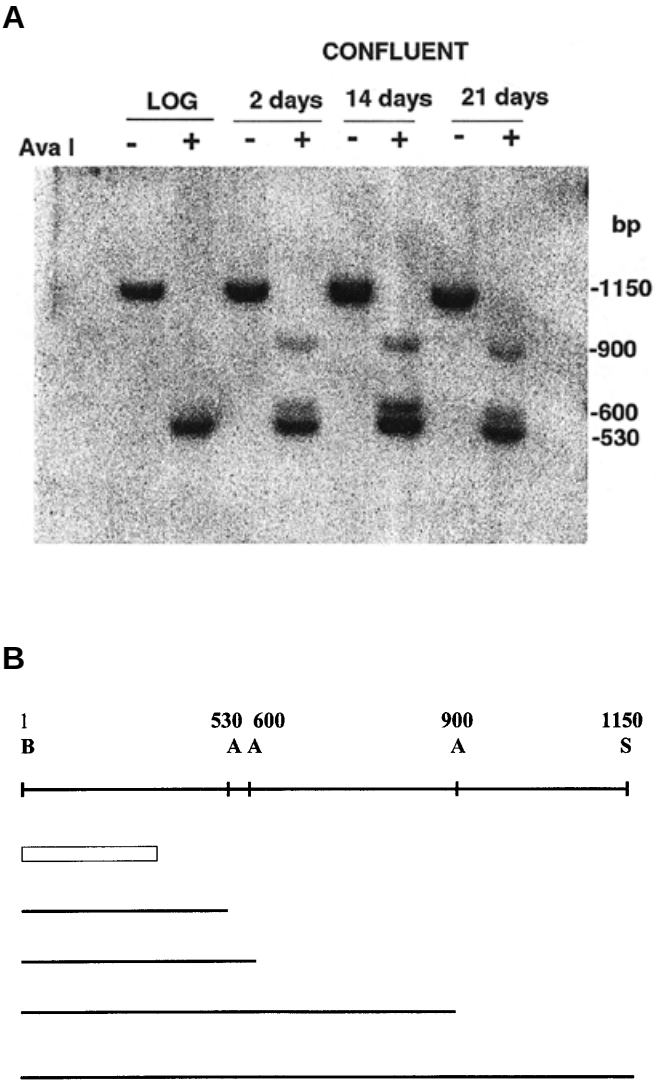

Figure 2. Southern blot analysis of methylation in the MGMT CpG island of logarithmically growing or confluent fibroblasts. (**A**) DNA from logarithmically growing fibroblasts or from fibroblasts held at confluence for 2, 14 or 21 days was cleaved with *Bam*HI and *Sst*I. Following precipitation the DNA was incubated with the methylation-sensitive restriction enzyme *Ava*I. The DNA was electrophoresed, transferred to a nylon membrane and hybridized to a ^{32}P-labeled probe complementary to nucleotides 1–424 of the 1150 bp *Bam*HI–*Sst*I fragment of the MGMT CpG island. Following washing of the membrane, hybridized probe was detected by phosphorimager analysis. (**B**) Location of *Ava*I (A) recognition sequences in the *Bam*HI (B)–*Sst*I (S) fragment of the MGMT CpG island. Hatched box, probe; solid bars, potential DNA fragments recognized by the probe. Numbering is consistent with that in Figure 1C.

Lack of association of CpG methylation with senescence

As aberrant methylation of CpG islands has been associated with senescence, the possibility that the aberrant methylation noted in the MGMT CpG island was senescence associated was examined. Parallel cultures to those used for methylation analysis were analyzed for DNA synthesis, senescence (as indicated by expression of senescence-associated β-galactosidase activity) and for ability to grow following replating at

subconfluence. Logarithmically growing NHF readily incorporated [^{3}H]thymidine into their acid-insoluble fraction while cells left at confluence for 2–21 days incorporated [^{3}H]-thymidine at levels that were 500-fold lower (Table 3). These growth-arrested, confluent cells were also fixed and incubated with X-gal at pH 6.0 in order to assess the percentage of cells which expressed senescence-associated β-galactosidase activity. The percentage of senescent cells was low in both logarithmically growing NHF (<1%) and in cells left at confluence for 2–21 days (~5–8%) (Fig. 3 and Table 4). Consistent with this data, confluent cells that were trypsinized and reseeded at ~20% confluence resumed doubling and within 12 h reassumed their subconfluent morphology. These results suggest that while >99% of the confluent cells were growth arrested, ~95% of the cells remained non-senescent. As ~50% of all copies of the MGMT CpG islands region examined contained aberrantly methylated CpGs, confluence-associated methylation did not appear to be a result of confluence-mediated senescence.

Table 3. DNA synthesis in logarithmically growing or confluent NHF

Growth	[^{3}H]Thymidine incorporation/10^5 cells (d.p.m.)
Logarithmic	76 217 ± 1551 (n = 3)
Confluent (2 days)	145 ± 12.5 (n = 3)
Confluent (14 days)	160 ± 15.8 (n = 3)
Confluent (21 days)	138 ± 11.1 (n = 3)

Values are means ± SE.

Table 4. Percentage of senescence-associated β-galactosidase-positive cells in logarithmically growing or confluent NHF cultures

Growth	Senescence-associated β-Gal$^+$ cells (%)
Logarithmic	<1
Confluent (2 days)	5 ± 3 (n = 4)
Confluent (14 days)	6 ± 4 (n = 4)
Confluent (21 days)	8 ± 4 (n = 4)

Values are means ± SE.

Heritability of growth arrest-induced CpG island methylation

To determine if the epigenetic mutation induced by confluence could be inherited by progeny in the absence of growth arrest, cells which had been held at confluence for 21 days were reseeded at subconfluence and grown at subconfluence for 4 or 10 population doublings. DNA from these cells was then subjected to sodium bisulfite sequencing and the extent of CpG methylation in the MGMT CpG island was analyzed. The results of this analysis are shown in Table 5. The DNA from cells allowed 4 population doublings following confluence contained levels of CpG methylation in the MGMT CpG island that were not statistically different from those noted in the parental confluent cells, but which were significantly higher

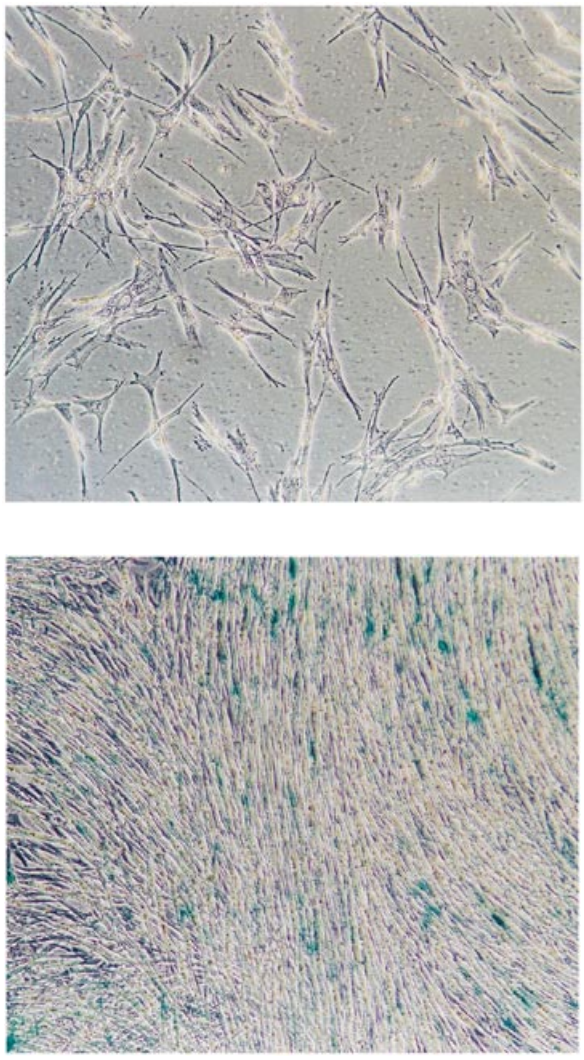

Figure 3. Expression of senescence-associated β-galactosidase activity in logarithmically growing or confluent NHF. NHF were maintained in logarithmic growth (**A**) or were grown to and left at confluence for 14 days (**B**). Cells were then fixed and incubated at acidic pH with X-gal.

(P < 0.05) than those noted in the DNA from logarithmically growing cells. The DNA from cells allowed 10 population doublings following confluence, however, contained very few methylated CpG sites in the region of the MGMT CpG island analyzed, with the extent of CpG methylation being no different (and perhaps less) than that noted in logarithmically growing fibroblasts.

DISCUSSION

The data presented in this manuscript show that confluence-induced growth constraint can trigger transient alterations in epigenetic stability in the form of altered CpG island methylation. These data suggest that even non-senescent NHF have the potential to alter CpG island methylation in a semi-heritable fashion in response to external stimuli. As such, confluence appears capable of triggering both genetic and epigenetic instability.

Table 5. Methylation of CpG islands in logarithmically growing NHF, confluent human fibroblasts or confluent human fibroblasts following replating and return to logarithmic growth

Growth	CpGs methylated (%)
Logarithmic	15.9 ± 2.9
Confluent (21 days)	43.6 ± 5.9 (n = 55)[a]
Confluent (21 days) + 4 doublings	44.0 ± 4.5 (n = 77)[a]
Confluent (21 days) + 10 doublings	6 ± 4 (n = 33)

Values are means ± SE.
[a]Statistically different from log cells, $P < 0.05$.

In the present study, confluence-induced growth constraint triggered CpG methylation, but did not appear to do so in a global fashion. The three islands examined for methylation alteration were chosen based on their propensity for becoming methylated in tumor and transformed cells. Within these islands we examined those regions that had previously been shown to be methylated in tumor cells (13,19,20,21) and that were demonstrated in our hands to become methylated in virally transformed NHF. Given the high susceptibility of these CpG islands to aberrant methylation, confluence-induced CpG methylation in only one of the three CpG islands examined suggests that growth constraint-induced methylation may not be a global phenomenon, but rather may be targeted to select genes. Such targeting is consistent with the gene-selective CpG island methylation noted in aging colon mucosa (8) and also with the apparent wide range of susceptibility of various CpG islands to aberrant methylation. Alternatively, the results may simply be a consequence of which CpG island regions were examined. Aberrant methylation of the MGMT CpG island has been studied extensively by us and others (13,18,22) and the region analyzed in the present study has been shown to be highly susceptible to aberrant methylation. As similar regions have not been as well defined in the ER and ECAD CpG islands, it remains possible that the ER, ECAD and MGMT CpG islands all display some degree of confluence-induced methylation, but that such methylation is limited to the most methylation-sensitive regions of the islands, which to date have not been identified in the ER or ECAD CpG islands.

An additional puzzling aspect of the confluence-induced methylation noted in the MGMT CpG island is the observation that aberrant methylation appears to be heritable but not permanent. After confluent NHF were replated and allowed to grow for 4 population doublings, parental cells could be predicted to make up only 6% of the culture. The extent of methylation of the MGMT CpG island in these expanded cells, however, was not statistically different than that in the original confluent culture, suggesting that confluence-induced methylation was passed from parental to daughter cells. This inheritance did not, however, seem to be permanent, as by 10 population doublings following replating the NHF had reverted to their pre-confluent level of methylation. While it may be possible that those cells which contained aberrant methylation proliferated until population doubling 4 but did not proliferate or contribute to the culture between population doublings 4 and 10, it seems more likely that the methylation was inherited and then reversed following cessation of the conditions (confluence) that triggered the event. This pattern of mutation inheritance is quite different from the stably inherited genetic mutations noted in bacterial and human cells grown to confluence. It thus appears that confluence may induce both short-term epigenetic instability and longer term genetic instability.

The cause of CpG methylation in NHF grown to confluence can only begin to be understood from the present studies. It is possible that cells grown to and retained at confluence are nutrient deprived and severely stressed and that such cells may activate or deactivate various mechanisms controlling CpG island methylation. As previously noted, bacterial and human cells seem to down-regulate mechanisms controlling genetic stability under conditions of nutrient deprivation, the result being the generation of cells perhaps more capable of surviving such selective pressure. While starvation may have played a role in the confluence-mediated methylation noted in this study, the fact that cells left at confluence for 2 days showed the same extent of methylation as those left for 21 days suggests that the medium and/or growth conditions were unlikely to be the primary cause.

Senescence has also been associated with aberrant methylation in normal cells and as previous studies have shown that confluence can to some degree trigger senescence in NHF (19), we considered the possibility that confluence induced CpG island methylation by triggering senescence. This appeared not to be the case in the present study, however, as in confluent NHF the percentage of senescent cells (<10%) was much less than the percentage of copies of the MGMT CpG island that were aberrantly methylated (~50%). It may also be possible that confluence triggers a transcriptional down-regulation of MGMT and potentially other genes and that the response to such down-regulation and decreased promoter usage is the spread of methylation from methylated regions of the genome into the CpG island. This spread may not be sufficient to silence the gene and may in fact be controlled by mechanisms in the NHF that limit the spread of methylation. The spread of methylation may, however, precede and set the stage for gene silencing should the control mechanism fail, as might be true in SV40-infected fibroblasts. A return to normal growth conditions would result in gene re-expression and perhaps the gradual removal of aberrant methylation. These possibilities are consistent with the recently proposed idea that CpG island promoters remain unmethylated in normal cells as a result of very low levels of transcription initiating from these islands (23) and also with the finding that protein binding may specify sites of demethylation and/or protection from methylation (24). It is currently unknown, however, if growth of cells at high density decreases MGMT expression. Additionally, while the degree of MGMT CpG island methylation noted in confluent NHF may be consistent with gene silencing, the patterns and extent of methylation in individual gene copies is highly variable and is considerably different from the extensive methylation noted in the MGMT CpG island of SV40-transformed cells, in which MGMT is transcriptionally silent. Furthermore, if MGMT silencing occurs only in cells in the population undergoing the most dramatic changes in methylation, it is unclear if such changes in gene expression could be detected by conventional methodologies. Finally, the idea that CpG island methylation may be altered in response to changes in gene expression seems at odds with the observation that the CpG islands of

many tissue-specific genes remains unmethylated in non-expressing tissues. Perhaps the clearest link between confluence and induced CpG island methylation is growth arrest itself. NHF held at confluence for 2 days showed the same degree of growth arrest as cells held at confluence for 21 days, mirroring the similarity in aberrant MGMT CpG island methylation seen in the two groups. Additionally, the reversal of growth arrest also reversed confluence-induced aberrant methylation. As growth arrest entails many aspects of cell regulation, including cell–cell contact and the entire cell cycle regulatory machinery, it is difficult to predict which component might be altered by confluence-induced growth constraint. Untangling the links between confluence, growth arrest and aberrant methylation would be challenging but ultimately might be helpful in understanding how normal cells maintain epigenetic stability.

REFERENCES

1. Modrich,P. (1991) *Annu. Rev. Genet.*, **25**, 229–253.
2. Richards,B., Zhang,H., Phear,G. and Meuth,M. (1997) *Science*, **277**, 1523–1526.
3. Harris,R.S., Feng,G., Ross,K.J., Sidhu,R., Thulin,C., Longerich,S., Szigety,S.K., Winkler,M.E. and Rosenberg,S.M. (1997) *Genes Dev.*, **11**, 2426–2437.
4. Nan,X., Ng,H.H., Johnson,C.A., Laherty,C.D., Turner,B.M., Eisenman,R.N. and Bird,A. (1998) *Nature*, **393**, 386–389.
5. Jones,P.L., Veenstra,G.J., Wade,P.A., Vermaak,D., Kass,S.U., Landsberger,N., Strouboulis,J. and Wolffe,A.P. (1998) *Nature Genet.*, **19**, 187–191.
6. Issa,J.P., Ottaviano,Y.L., Celano,P., Hamilton,S.R., Davidson,N.E. and Baylin,S.B. (1994) *Nature Genet.*, **7**, 536–540.
7. Vertino,P.M., Issa,J.-P., Pereira-Smith,O. and Baylin,S.B. (1994) *Cell Growth Differ.*, **5**, 1395–1402.
8. Ahuja,N., Li,Q., Mohan,A.L., Baylin,S.B. and Issa,J.-P. (1998) *Cancer Res.*, **58**, 5489–5494.
9. Baylin,S.B., Herman,J.G., Graff,J.R., Vertino,P.M. and Issa,J.-P. (1998) *Adv. Cancer Res.*, **72**, 141–196.
10. Bird,A.P. (1987) *Trends Genet.*, **3**, 342–347.
11. Costello,J.F., Futscher,B.W., Kroes,R.A. and Pieper,R.O. (1994) *Mol. Cell. Biol.*, **14**, 6515–6521.
12. Clarke,S.J., Harrison,J., Paul,C.L. and Frommer,M. (1994) *Nucleic Acids Res.*, **222**, 2990–2997.
13. Watts,G.S., Pieper,R.O., Costello,J.F., Peng,Y.-M., Dalton,W.S. and Futscher,B.W. (1997) *Mol. Cell. Biol.*, **17**, 5612–5619.
14. Greene,S., Walter,P., Kumar,V., Krust,A., Bornert,J.-M., Argos,P. and Chambon,P. (1986) *Nature*, **320**, 134–139.
15. Bussemakers,M.J.G., Giroldi,L.A., van Bokhoven,A. and Schalken,J.A. (1994) *Biochem. Biophys. Res. Commun.*, **203**, 1284–1290.
16. Patel,S.A., Graunke,D.M. and Pieper,R.O. (1997) *Mol. Cell. Biol.*, **17**, 5813–5822.
17. Harris,L.C., Potter,P.M., Tano,K., Shiota,S., Mitra,S. and Brent,T.P. (1991) *Nucleic Acids Res.*, **19**, 1663–1667.
18. Pieper,R.O., Patel,S., Ting,S.A., Futscher,B.W. and Costello,J.F. (1996) *J. Biol. Chem.*, **271**, 13916–13924.
19. Dimiri,G.P., Lee,X., Basile,G., Acosta,M., Scott,G., Roskelley,C., Medrano,E.E., Linskens,M., Rubelj,I., Pereira-Smith,O., Peacocke,M. and Campisi,J. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 9363–9367
20. Lapidus,R.G., Nass,S.J., Butash,K.A., Parl,F.F., Weitzman,S.A., Graff,J.R., Herman,J.G. and Davidson,N.E. (1998) *Cancer Res.*, **58**, 2515–2519.
21. Graff,J.R., Herman,J.G., Lapidus,R.G., Chopra,H., Xu,R., Jarrard,D.F., Isaacs,W.B., Pitha,P.M., Davidson,N.E., and Baylin,S.B. (1995) *Cancer Res.*, **55**, 5195–5199.
22. Qian,X.C. and Brent,T.P. (1997) *Cancer Res.*, **57**, 3672–3677.
23. Macleod,D., Ali,R.R. and Bird,A. (1998) *Mol. Cell. Biol.*, **18**, 4433–4443.
24. Hsieh,C.-L. (1999) *Mol. Cell. Biol.*, **19**, 46–56.