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Differential Maintenance and De Novo Methylating Activity by Three DNA Methyltransferases in Aging and Immortalized Fibroblasts

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Abstract Genomic methylation, which influences many cellular processes such as gene expression and chromatin organization, generally declines with cellular senescence although some genes undergo paradoxical hypermethylation during cellular aging and immortalization. To explore potential mechanisms for this process, we analyzed the methylating activity of three DNA methyltransferases (Dnmts) in aging and immortalized WI-38 fibroblasts. Overall maintenance methylating activity by the Dnmts greatly decreased during cellular senescence. In immortalized WI-38 cells, maintenance methylating activity was similar to that of normal young cells. Combined de novo methylation activity of the Dnmts initially decreased but later increased as WI-38 cells aged and was strikingly elevated in immortalized cells. To further elucidate the mechanisms for changes in DNA methylation in aging and immortalized cells, the individual Dnmts were separated and individually assessed for maintenance and de novo methylating activity. We resolved three Dnmt fractions, one of which was the major maintenance methyltransferase, Dnmt1, which declined steadily in activity with cellular senescence and immortalization. However, a more basic Dnmt, which has significant de novo methylating activity, increased markedly in activity in aging and immortalized cells. We have identified this methyltransferase as Dnmt3b which has an important role in neoplastic transformation but its role in cellular senescence and immortalization has not previously been reported. An acidic Dnmt we isolated also had increased de novo methylating activity in senescent and immortalized WI-38 cells. These studies indicate that reduced genome-wide methylation in aging cells may be attributed to attenuated Dnmt1 activity but that regional or gene-localized hypermethylation in aging and immortalized cells may be linked to increased de novo methylation by Dnmts other than the maintenance methyltransferase. J. Cell. Biochem. 84: 324–334, 2002.

Key words: DNA methyltransferases; cellular senescence; immortalization; aging; methylation

Methylation of the 5′-position of cytosine is the most prevalent covalent modification of the genome in mammals. Cytosine methylation within gene regulatory regions correlates inversely with transcriptional activity [Yeivin and Razin, 1993] and is generally regarded to be an epigenetic determinant of gene expression. Methylation of cytosine has also been associated with several other cellular processes, including chromatin structuring [Adams, 1990; Kass et al., 1997], development [Lei et al., 1996], somatic X-chromosome inactivation [Shemer et al., 1996], carcinogenesis [Warnecke and Bestor, 2000], and aging [Wilson and Jones, 1983; Cooney, 1993; Tollefsbol and Andrews, 1993]. The enzyme DNA-methyltransferase (Dnmt; EC 2.1.1.37) catalyzes the transfer of a methyl moiety from *S*-adenosyl-L-methionine (SAM) to cytosines principally in the CpG dinucleotide [Adams et al., 1979; Ramsahoye et al., 2000]. Several Dnmts have been identified in somatic tissues of vertebrates. Dnmt1 is the most abundant methyltransferase in mammalian cells [Yoder et al., 1997]. This enzyme preferentially

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methylates hemimethylated DNA and is believed to be responsible for maintaining methylation patterns established in development [Lei et al., 1996]. Dnmt1b, another mammalian DNA methyltransferase, also methylates cytosines in CpG dinucleotides although its activity is approximately 2–5% of the level of Dnmt1 and likely exists as a minor DNA-methyltransferase isoform in human cells [Bonfils et al., 2000]. Dnmt2 is a human protein that displays strong sequence homology to DNA (cytosine-5)-methyltransferases of eukaryotes as well as prokaryotes [Dong et al., 2001]. No methyl transfer activity has yet been observed for Dnmt2 in vitro although it can bind DNA in a sequence-specific manner [Dong et al., 2001]. The recently identified DNA methyltransferases, Dnmt3a and Dnmt3b, appear to have primarily de novo methylation activities, that is, they are capable of methylating previously unmethylated DNA [Okano et al., 1998]. The DNA-methyltransferases 1, 3a, and 3b are encoded by three independent genes [Xie et al., 1999]. Additional diversity of the Dnmts has been shown to result from alternative translation initiation in the case of the murine Dnmt1 [Gaudet et al., 1998] or alternative splicing of the Dnmt1 mRNA in rat [Deng and Szyf, 1998] and human cells [Hsu et al., 1999]. Moreover, several novel alternatively spliced forms of Dnmt3b, which have altered enzymatic activity, can be expressed in a tissue-specific manner and in tumor tissue [Robertson et al., 1999].

Genetic experiments have demonstrated that DNA methylation plays an important role in normal development [Li et al., 1992; Okano et al., 1999]. Dnmt1$^{-/-}$ and Dnmt3b$^{-/-}$ mutations in mice result in embryonic lethality while mice with mutation Dnmt1,3a$^{-/-}$ die at approximately 4 weeks of age. Mutations of human Dnmt3b are found in ICF (immunodeficiency, centromeric instability, facial abnormalities) syndrome, a developmental defect characterized by hypomethylation of pericentromeric repeats [Hansen et al., 1999; Xu et al., 1999]. DNA methylation is also implicated in tumorigenesis [Jones and Laird, 1999; Warnecke and Bestor, 2000]. Abnormal methylation patterns are observed in malignant cells and may contribute to tumorigenesis by improper silencing of tumor suppressor or growth-regulatory genes [Baylin et al., 1998]. Elevated levels of Dnmt1 mRNA and DNA methyltransferase activity have been observed in many cancer cells in vitro [Kautiainen and Jones, 1986; el-Diery et al., 1991] and in tumors [el-Diery et al., 1991; Belinsky et al., 1996]. Increased de novo methylation actually begins in the normal tissue as an age-related event and progresses to regional or gene-specific hypermethylation in generally hypomethylated cancer cells [Ahuja et al., 1998]. Thus, aging is a major contributing factor to regional or gene-specific hypermethylation in cancer; however, the potential role of de novo methylation in the processes of aging, replicative senescence, and immortalization is not well understood.

A steady decline in DNA methylation during cellular senescence [Wilson and Jones, 1983; Fairweather et al., 1987; Tollefsbol and Andrews, 1993] and aging of organisms [Singhal et al., 1987; Hornsby et al., 1992] appear to accompany a general decrease in Dnmt activity [Vertino et al., 1994; Young and Smith, 2001]. However, the changes in de novo and maintenance methylation or the methylating activity of the individual Dnmts have not yet been assessed in cellular senescence. Overall Dnmt activity may be elevated in cells transformed with SV40 T antigen [Rouleau et al., 1995] although other studies have indicated that the Dnmt1 activity stabilizes in immortalized cells [Vertino et al., 1994]. Consistent with a potential role for DNA methylation in cellular aging, immortalization and neoplastic transformation, Young and Smith [2001] have recently reported that inhibition of DNA methyltransferase activity in cultured cells induces the expression of p21, a cyclin-dependent kinase inhibitor which plays a major role in the entrance of cells into senescence. This link between Dnmt activity and cell cycle growth regulators suggests that Dnmt activity could serve as a mitotic division counting mechanism essential for cellular senescence [Young and Smith, 2001].

In this study, we have analyzed the relative de novo and maintenance methylation capacity of aging and immortalized WI-38 fibroblasts. We have also identified three major Dnmts in these cells which vary widely in their de novo and maintenance methylation activity. These Dnmts have striking differences in expression in aging and immortalized cells which may serve as an underlying basis for the major changes in overall DNA methylation and Dnmt activity that accompany cellular aging and immortalization.

MATERIALS AND METHODS

Cell Culture

WI-38 human fetal lung fibroblasts (AG06814) and SV40-immortalized WI-38 fibroblasts (AG07217) were obtained from the National Institute of Aging (Aging Cell Culture Repository; Coriell Institute for Medical Research, Camden, NJ). Cells were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in the University of Alabama at Birmingham Cell Senescence Culture Facility. Cell cultures were expanded through sequential subculturing using trypsin-EDTA (Gibco) to achieve a higher population doubling (PD) level [Pang and Chen, 1993]. Cells at PD less than 30 were considered young cells, cells at PD 30–50 were considered middle aged, and cells at more than 50 PD were considered presenescent or senescent [Sitte et al., 2000]. Cells grown to about 90% confluence were used for all the experiments. Figure 1 depicts the morphological features of living cultured WI-38 fibroblasts at different PDs as well as immortalized SV40 WI-38 fibroblasts using an Intel Digital Camera focused through the photo-port of an Olympus CK2 inverted phase contrast microscope (1,200×). To assess the presence of cellular senescence, β-galactosidase staining of cells was performed [Dimri et al., 1995]. Cells were fixed with glutaraldehyde for 30 min at room temperature, washed with phosphate buffered saline, and stained for 3 h at 37°C with β-galactosidase (Stratagene) in pH 4.0 or pH 6.0 citrate buffer as previously described [Dimri et al., 1995].

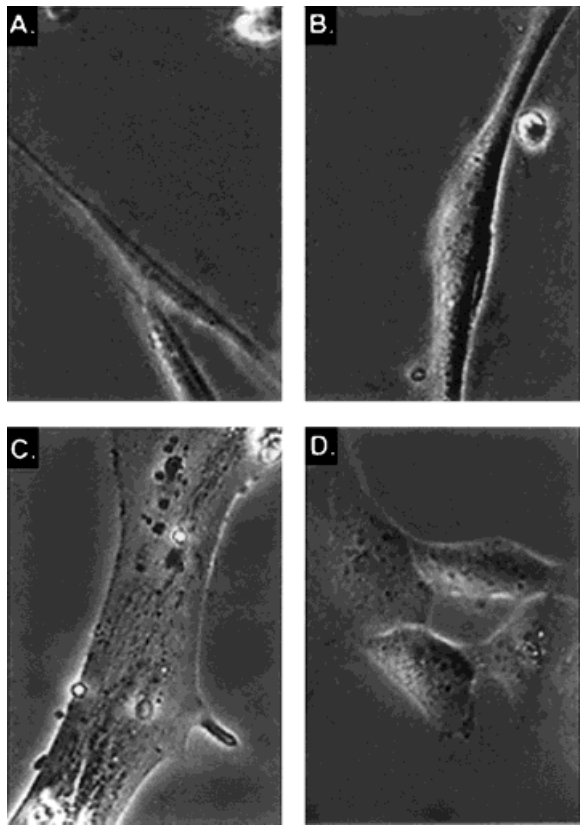

Fig. 1. Phase-contrast light microscopic digital images showing the morphological features of normal aging and immortalized cells. Aging and SV40-immortalized WI-38 fibroblasts are depicted at 1,200× as described in Materials and Methods. Panels **A**, PD 18; **B**, PD 40; **C**, PD 59; **D**, SV40-immortalized.

Oligonucleotide DNA Substrates

Oligonucleotides were synthesized as reported previously [Tollefsbol and Hutchison, 1995, 1997] using standard procedures and adding 5-methylcytosine (Glen Research) as the phosphoramidite where indicated. The sequence of the 60-mer oligonucleotide template was 5′-CATGGCCTAAGCAGGACTGAATGAGCAAG CTTCCGGAGAATTCTGCAGGACTGCAGAT GC-3′ containing one centrally located CpG dinucleotide (underlined) in a HpaII/MspI recognition sequence (CCGG). For synthesis of the nonmethylated template used for de novo methylation analysis, the complementary sequences were mixed at 500 ng/μl each, heated to 75°C for 10 min in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and annealed by slow cooling to room temperature [Tollefsbol and Hutchison, 1995, 1997]. For synthesis of the hemimethylated template to assess maintenance methylation, a 5-methylcytosine replaced the cytosine of the one centralized CpG dinucleotide, and this oligonucleotide was annealed with the nonmethylated complementary oligonucleotide to create a hemimethylated CpG in the central region of the oligonucleotide. The methylated state of the template was verified with the use of HpaII and MspI restriction digests. The double-stranded 60-mer oligonucleotides (nonmethylated and hemimethylated) were gel-purified and the concentration was determined in triplicate by absorbance at A_{280} on a Spectronic Genesys 5 spectrophotometer. All double-stranded oligonucleotides were analyzed for annealing efficiency on 3% (w/v) agarose gels and used only if the annealing process was complete.

Purification of Nuclear Extracts

Typically 6×10^7 cells were resuspended in 15 ml of lysis buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% sucrose, 10 μg/ml TPCK, 10 μg/ml TLCK, 5 μg/ml leupeptin, 7 μg/ml phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol) supplemented with 250 mM NaCl. The cell suspension was sonicated on ice for 30 s using a model W-225R (Ultrasonic) sonicator in pulse mode with 50% duty cycle. The extract was centrifuged at 45,000*g* in a JA18 rotor for 45 min at 4°C. The supernatant was collected and an equal volume of buffer A without NaCl was added to bring the final concentration of NaCl to 125 mM. This mixture was centrifuged for a further 30 min at 11,000*g* to remove the precipitate and used to assay the incorporation of [^{3}H]-methyl groups into DNA substrate in Dnmt assays.

Fractionation of the Dnmts

To fractionate the Dnmts, the nuclear proteins were precipitated by the addition of solid ammonium sulfate to 80% saturation [Lopatina et al., 1998]. The precipitates were collected by centrifugation at 105,000*g* for 1 h and dialyzed against buffer A without NaCl. The resulting proteins were processed on a Rotofor Cell Column apparatus (Bio-Rad) as described by the manufacturer. Twenty 2.5 ml fractions of each protein preparation applied were obtained as a result of the isoelectric focusing in the presence of ampholytes (Bio-Rad) ranging from pH 3 to 10 [Lopatina et al., 1998]. All procedures were carried out at 4°C in the presence of numerous protease inhibitors (10 μg/ml TPCK, 10 μg/ml TLCK, 5 μg/ml leupeptin, 7 μg/ml phenylmethylsulfonyl fluoride).

Protein Analysis

Protein concentration was determined using the Bio-Rad Coomassie assay kit. Standard curves were established using γ-globulin.

Dnmt Assay

The reaction mix (30 μl) contained 5 μM ^{3}H-*S*-adenosyl-L-methionine (15 Ci/mmol, NEN), 1 μg DNA substrate and nuclear extract in buffer A or protein from every fraction after isoelectric focusing. Incubation was for 2 h at 37°C. After completion of the assay, reaction mixtures were treated as described previously (Tollefsbol and Hutchison, 1998). DNA samples were applied to Whatman GF/C filters and radioactivity was quantified by liquid scintillation counting. Enzyme activity was expressed as cpm/μg of protein. The 60-mer oligonucleotide templates, either containing a centrally located de novo methylatable CpG to assess de novo methylation activity or a hemimethylated CpG to measure maintenance DNA-methyltransferase activity, were used as substrates in the Dnmt assays.

Computer-Assisted Identification of Dnmts

Several studies have successfully used comparisons of computer-derived and actual isoelectric points for the identification and characterization of many different proteins [Hempe and Craver, 2000; Shimura et al., 2000; Xu et al., 2000]. For identification of Dnmts based on pI, we used the Isoelectric Program which is part of GCG SeqWeb v. 1.2 for use with the Wisconsin Package v. 10.1. The translated sequence of each human Dnmt, previously reported to the GenBank nucleotide database, was entered and a computer-derived pI was obtained. Although actual and computer-derived pI's can vary slightly depending upon the folding of a protein, the pI derived for Dnmt1, 7.67, matches well with the pI 7.2–7.4 Dnmt activity found in this study. No other Dnmt activity was found with a more similar pI. The Dnmt activity described in this study at pI 8.2–8.3 is consistent with Dnmt3b (pI = 8.37). None of the Dnmts recorded to date in the GenBank database matches the pI 4.2–4.5 Dnmt activity found previously [Lopatina et al., 1998] and in this study. We also used the Isoelectric Program to derive a pI for the previously reported cleavage products of Dnmt1 [Bestor, 1992] and neither the N-terminal or the C-terminal fragments yielded isoelectric points consistent with the acidic Dnmt found in this study, suggesting that the pI 4.2–4.5 may be a post-translationally modified Dnmt or the product of a gene which has not previously been described.

RESULTS

Culture of normal human diploid fibroblasts has long served as a model system for studying the cellular aging process. Human fibroblasts, like other nontransformed somatic cells, have a finite replicative capacity and exhibit characteristic morphological changes during cellular

senescence [Hayflick, 1965; Chen et al., 1995]. Figure 1 depicts the morphological features of the aging and immortalized WI-38 fibroblasts used in this study. The aging cells (Fig. 1C) are broader, flatter and more irregular in appearance compared to young cells (Fig. 1A) and contain more vacuoles in the cytoplasm. Multiple blebbing and protrusions were noted in the cell membranes of the senescent cells (Fig. 1C). The SV40-immortalized cells also have morphological features distinct from normal cells including a more cuboidal appearance as shown in Figure 1D. Thus, the cells used in this study displayed morphological changes consistent with aging or immortalization.

Previous studies have indicated that the Dnmts appear to undergo a generalized decrease in activity in aging cells [Vertino et al., 1994; Young and Smith, 2001], but the relative levels of maintenance versus de novo methylating activity of the Dnmts in aging and immortalized cells has not been fully elucidated. Table I depicts the maintenance and de novo methylating activity of aging WI-38 fibroblasts at incremental population doublings (young, PD 18; middle, PD 40; senescent, PD 59) as well as SV40-immortalized WI-38 fibroblasts. As early as 40 PDs, when the fibroblasts are considered to be middle aged, there is a decline not only in maintenance methylation, but also in de novo methylation as well (Table I). This decline in maintenance methylation by the Dnmts continues as the WI-38 cells reach senescence (PD 59). By contrast, however, de novo methylation actually increases as the cells progress from middle to old age. In addition, maintenance methylating activity by the Dnmts in SV40-immortalized fibroblasts is comparable to that of young normal cells while de novo methylating activity by the Dnmts is higher in SV40-immortalized fibroblasts as compared to young normal cells (Table I).

Figure 2 depicts the percent changes in maintenance and de novo methylating activity by the Dnmts in aging and immortalized WI-38 fibroblasts. Fibroblasts at middle age (PD 40) had much less than 50% of the maintenance methylation capacity of young cells and by senescence (PD 59), the maintenance methylating activity by the Dnmts was less than 25% of young cells whereas immortalized cells had close to 100% of the maintenance methylating activity of young cells, much higher than normal senescent fibroblasts (Fig. 2). The de novo methylating capacity of the Dnmts was completely different from those observed for maintenance methylation. Although de novo methylating activity by the Dnmts in middle aged WI-38 fibroblasts declined to about 50% that of young cells, a significant rise in de novo methylation to near levels of young cells occurred in the senescent fibroblasts (Fig. 2). Even more striking, however, was the finding of a greater than 70% increase in de novo methylating activity in SV40-immortalized fibroblasts as compared to young cells.

The ratio of de novo to maintenance methylation by the Dnmts in aging and immortalized WI-38 fibroblasts is depicted in Figure 3. In young normal cells, the capacity for de novo methylation by the Dnmts relative to maintenance methylation is quite small. However, this ratio increases by the time the cells have reached middle age and continues to rise dramatically in aged cells such that de novo methylation capacity of the senescent WI-38 fibroblasts at PD 59 is similar to the capacity for maintenance methylation in these cells (Fig. 3).

TABLE I. DNA Methyltransferase Activity in Aging and Immortalized WI-38 Fibroblasts

Cell type	Maintenance methylation (cpm/μg protein)[a]	De novo methylation (cpm/μg protein)[b]
Normal WI-38 (young), PD-18	266 ± 5.5	72 ± 7.3
Normal WI-38 (middle), PD-40	90.3 ± 5.7	40.5 ± 4.8
Normal WI-38 (senescent), PD-59	63 ± 7.3	56.2 ± 8.5
SV40-immortalized WI-38	260 ± 8.8	120 ± 0.6

[a]Maintenance methylation activity was assessed in duplicate using a 60-mer double-stranded oligonucleotide containing one centralized hemimethylated CpG. Cpm values represent the amount of radioactivity transferred by the enzyme from tritiated *S*-adenosyl-L-methionine to the double-stranded template in 2 h. Mean values ± SEM (standard error of the mean) are depicted.
[b]De novo methylation activity was assessed in duplicate as for maintenance methylation except using a 60-mer oligonucleotide containing one centralized CpG with no pre-existing methylation in the template. Values are mean ± SEM.

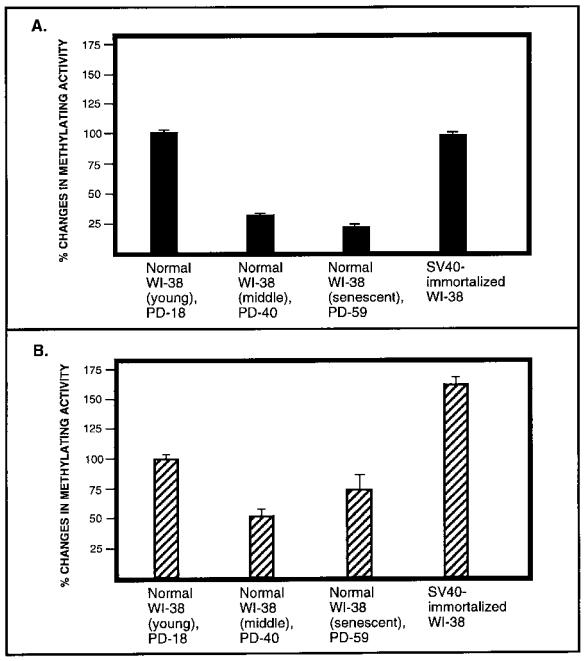

Fig. 2. Changes in maintenance and de novo methylating activity of aging and immortalized WI-38 fibroblasts. **Panel A**: The percent maintenance methylating activity (solid bars) of aging (PD 18, PD 40, PD 59) and immortalized (SV40-infected) WI-38 fibroblasts is depicted relative to the values of young (PD 18) cells shown at 100% methylating activity. **Panel B**: The percent de novo methylating activity (hatched bars) of aging (PD 18, PD 40, PD 59) and immortalized (SV40-infected) WI-38 fibroblasts is depicted relative to the values of young (PD 18) cells shown at 100% methylating activity. Values represent the mean of duplicate assessments and the standard errors of the mean (SEMs) are shown at the top of each bar.

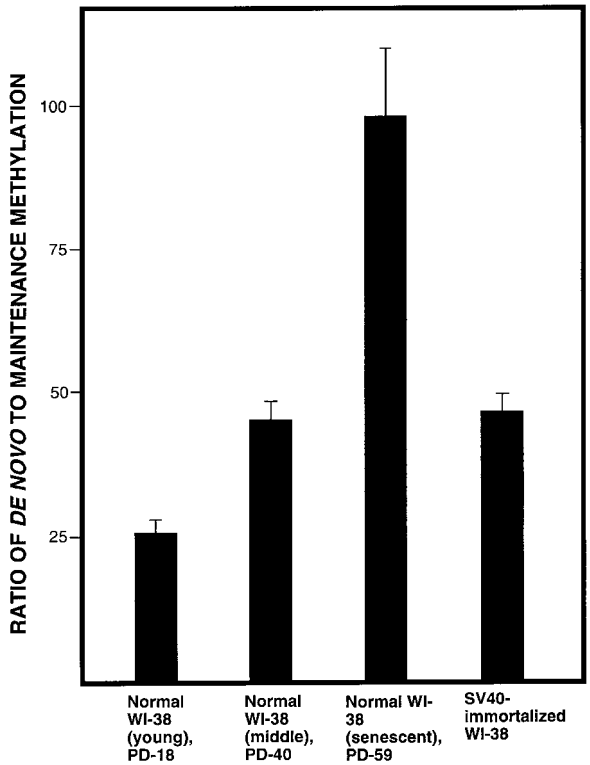

Fig. 3. Ratio of de novo to maintenance methylating activity of aging and immortalized WI-38 fibroblasts. The ratio of de novo to maintenance methylating activity of aging (PD 18, PD 40, PD 50) and immortalized (SV40-infected) cells are depicted. Values are the mean of duplicate measurements and the SEMs are depicted at the top of each bar.

Since maintenance methylating activity by the Dnmts in SV40-immortalized fibroblasts is comparable to that of young cells, the marked rise in de novo methylating activity in these cells (Fig. 2) yields a ratio of de novo to maintenance methylation in the SV40-immortalized WI-38 fibroblasts comparable to that of middle aged normal WI-38 fibroblasts but still greater than that observed in young cells (Fig. 3).

To explore potential mechanisms for the generalized decline in maintenance methylation capacity in aging cells and the rise in immortalized cells as well as the dramatic increase in de novo methylation in senescent and immortalized fibroblasts, we fractionated the Dnmts by isoelectric focusing and assessed the maintenance and de novo methylating activity of each individual Dnmt in aging and immortalized WI-38 fibroblasts (Fig. 4). Three different methylating activities were resolved at pIs 7.2–7.4, 4.2–4.3, and 8.2–8.3. Based in part on propensity for hemimethylation, high overall activities, and closely corresponding actual and computer-derived pIs, we have identified the pI 7.2–7.4 activity as the maintenance methyltransferase (Dnmt1) which has much greater hemimethylation capacity than the pI 4.2–4.3 and pI 8.2–8.3 activities [Lopatina et al., 1998]. The pI 8.2–8.3 Dnmt has been previously observed in liver cancer cells where it has a propensity for de novo methylation [Lopatina et al., 1998]. The pI of this Dnmt, which has preferential de novo methylating activity, closely matches that of Dnmt3b [Robertson et al., 1999; Xie et al., 1999]. The identity of the pI 4.2–4.3 (acidic) Dnmt is currently unknown but consistently appears in all of the WI-38 cells we have examined. In normal young WI-38 cells, most of the maintenance and de novo methylating activity can be attributed to Dnmt1 (Fig. 4A). A minor

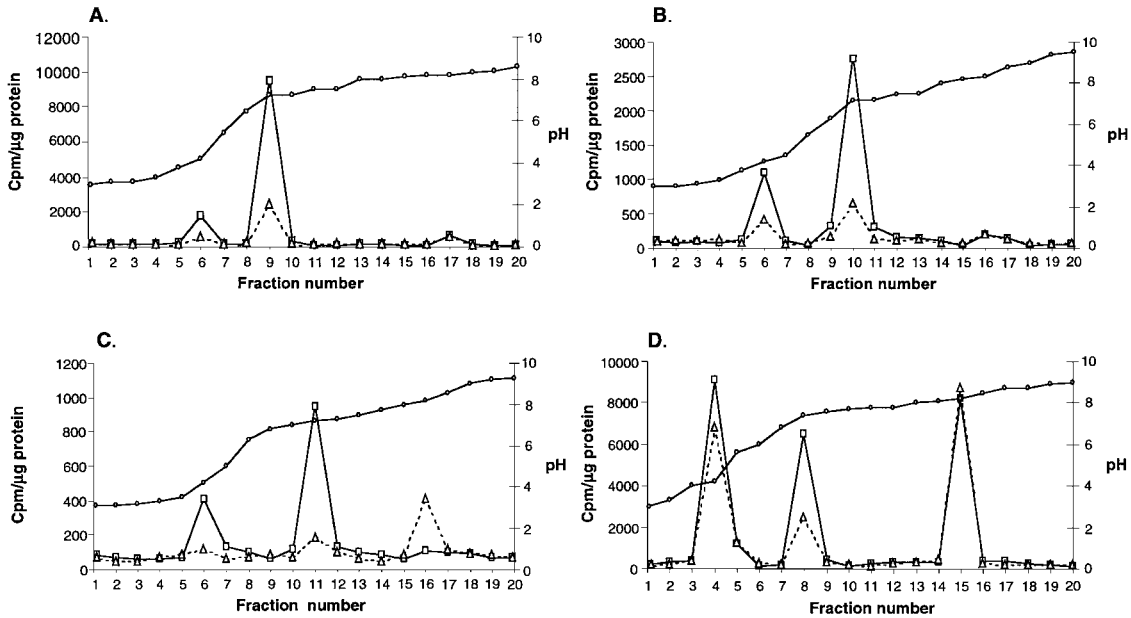

Fig. 4. Isoelectric focusing of the Dnmts from aging and immortalized fibroblsts. For each panel, maintenance methylation is depicted by squares, de novo methylation by triangles and dotted lines, and pH by circles. Panels: **A**, young WI-38 normal fibroblasts at PD 18; **B**, middle-aged WI-38 normal fibroblasts at PD 40; **C**, senescent normal fibroblasts at PD 59; **D**, SV40-immortalized fibroblasts.

amount of maintenance methylation activity in these young cells is contributed by the newly-identified acidic Dnmt, and only slight maintenance and de novo methylating activity by Dnmt3b was observed. By PD 40 when the WI-38 fibroblasts reach middle age, however, greater methylating activity by Dnmt3b and the acidic Dnmt increased though not to the level of Dnmt1. This trend continued as the cells approached senescence (Fig. 4A–C). A dramatic increase in both maintenance and de novo methylating activity by these Dnmts relative to Dnmt1 was observed in SV40-immortalized WI-38 fibroblasts (Fig. 4D).

The distribution of the maintenance and de novo methylating activity by the three Dnmts we have observed in aging and immortalized WI-38 fibroblasts is depicted in Table II. Dnmt1

TABLE II. Distribution of DNA Methyltransferases in Aging and Immortalized Fibroblasts

		% Distribution[b]	
DNA methyltransferease[a]	Cell type	Maintenance methylation	De novo methylation
pI 7.2–7.4 (Dnmt1)	Young (PD-18)	100	100
	Middle (PD-40)	83.0	77.0
	Senescent (PD-59)	80.1	67.8
	Immortalized (SV40)	31.4	16.3
pI 4.2–4.3 (novel)	Young (PD-18)	100	100
	Middle (PD-40)	205.6	250.0
	Senescent (PD-59)	247.7	275.0
	Immortalized (SV40)	355.1	471.3
pI 8.2–8.3 (Dnmt3b)	Young (PD-18)	100	100
	Middle (PD-40)	203.1	222.2
	Senescent (PD-59)	140.0	317.5
	Immortalized (SV40)	1093	776.6

[a]DNA methyltransferases in various cell types were resolved into three fractions at pI 7.2–7.4, 4.2–4.3, and 8.2–8.3 (see Figure 4).
[b]Percent distribution of the DNA methyltransferases is depicted for levels of maintenance or de novo methylation (see Figure 4). The values for young (PD-18) cells are set at 100%.

(pI 7.2–7.4) decreased slightly more in de novo methylating activity relative to maintenance methylation in aging cells, and both methylating activities of Dnmt1 decreased with cellular senescence. More striking, however, was the marked reduction in Dnmt1 maintenance and de novo methylating activity in immortalized cells. The reduction in percent distribution of Dnmt1 in aging and immortalized cells is likely the result of the relatively marked increase in percent methylating activity by Dnmt3b and the acidic Dnmt. The acidic Dnmt accounted for a greater than twofold increase in maintenance and de novo methylating activity by the time the cells reached middle age (PD 40). A similar pattern was observed for Dnmt3b (Table II). This increase in percentage of maintenance and de novo methylating activity continued as the cells reached senescence (PD 59) and was markedly elevated in SV40-immortalized cells. In senescent cells, the percent distribution of maintenance methylation by Dnmt3b was about 40% higher than in the young cells but was a little less than that observed for middle aged cells. However, the percentage of overall de novo methylating activity by this Dnmt continued to rise as cells underwent senescence and was over sevenfold higher in immortalized cells than in young cells. The percentage of maintenance methylation in the SV40-immortalized fibroblasts by Dnmt3b was about 10-fold that of its activity in the young cells (Table II). Thus, there is a dramatic increase in the methylating activity of the Dnmt3b and novel acidic Dnmt in aging and immortalized cells relative to the methylating activity of Dnmt1, the major maintenance DNA methyltransferase.

DISCUSSION

The utility of extended-passage fibroblast cells in investigating the underlying mechanisms of cellular senescence and aging is generally accepted and has yielded important information about telomere shortening [von Zglinicki, 1998], stress resistance [Liu et al., 1989], turnover of total or individual proteins [Mellmann et al., 1972; Bradley et al., 1976], and the effects of proliferative senescence on gene expression [Saretzki et al., 1998]. Although it has been known for quite some time that genomic cytosine methylation decreases during cellular senescence and aging [Cooney, 1993; Tollefsbol and Andrews, 1993], the mechanisms responsible for the age-related decline in cytosine methylation have not yet been elucidated. The loss of cytosine methylation or aberrant de novo methylation have been proposed as alternative counting mechanisms for aging [Wilson and Jones, 1983; Tollefsbol and Andrews, 1993], and some studies have indicated that overall DNA methyltransferase (Dnmt) activity declines in aging cells and stabilizes or increases in immortalized cells [Vertino et al., 1994; Rouleau et al., 1995; Young and Smith, 2001]. These studies suggest that a decrease in Dnmt activity may be an underlying mechanism for the loss of cytosine methylation in aging cells resulting in inappropriate activation of oncogenic genes [Tollefsbol and Andrews, 1993] or aberrant expression of cell cycle regulating proteins such as p21 [Young and Smith, 2001]. In contrast to the generalized hypomethylation in aging cells, it has been proposed that some genes may undergo de novo methylation during cellular senescence leading to inactivation of key genes essential for cellular processes [Tollefsbol and Andrews, 1993] and subsequent studies have found evidence for hypermethylation of specific genes in aging cells [Issa et al., 1994; Gonzalez-Zulueta et al., 1995; Ahuja et al., 1998].

Many studies focusing on the underlying causes for changes in cytosine methylation of aging or immortalized cells have limited their investigation to overall maintenance Dnmt activity. However, it is now known that cytosine methylation is mediated by at least three different Dnmts that have different propensities for maintenance or de novo methylation [Lei et al., 1996; Gaudet et al., 1998; Lopatina et al., 1998; Okano et al., 1998; Bonfils et al., 2000]. In this study we have found striking differences in maintenance and de novo methylating activity by the Dnmts in aging and immortalized cells. Consistent with previous findings of a genome-wide decrease in methylation in aging cells, we have found that maintenance methylating activity by the Dnmts is decreased in aging cells which could lead to a gradual loss in methylated cytosines in these cells. However, we have observed a late increase in de novo methylating activity in these cells which, although much lower than maintenance methylation, could be a cause for the increased incidence of de novo methylation silencing of a subset of genes in aging cells. We have also observed that maintenance methylating activity in immortalized cells is greater than that of

senescent cells but comparable to that of young cells. Moreover, de novo methylating activity is markedly elevated in immortalized WI-38 fibroblasts relative to normal cells of any age that we have examined suggesting that aberrant de novo methylation could play a role not only in cellular senescence, but also in immortalization. Thus, although previous studies have indicated that overall Dnmt activity may be stabilized [Vertino et al., 1994] or elevated [Rouleau et al., 1995] in immortalized cells, our studies indicate that only maintenance methylation is stable in immortalized cells relative to young cells and that de novo methylating activity is markedly elevated in these cells.

Investigations assessing overall Dnmt activity [Vertino et al., 1994; Young and Smith, 2001; this study] as well as distinguishing relative maintenance and de novo methylating activity (as done in this study) in aging or immortalized cells are informative. However, discoveries of more than one Dnmt that can carry out cytosine methylation in mammalian cells [Lei et al., 1996; Gaudet et al., 1998; Okano et al., 1998; Bonfils et al., 2000] suggest the possibility that these Dnmts could undergo changes in expression or activity in aging or immortalized cells leading to the observed changes in overall Dnmt maintenance or de novo methylation. We have found dramatic differences in the activity of individual Dnmts in aging and immortalized cells. The amount of maintenance methylating activity carried out by Dnmt1, the major DNA methyltransferase of mammalian cells, is reduced in aging cells which is likely to be an underlying cause for the general genomic hypomethylation of aging cells. However, the amount of de novo methylating activity by Dnmt1 is also decreased in aging cells although regional hypermethylation of certain genes can occur in these cells. Our findings of a greater de novo methylating activity attributed to Dnmt3b and the acidic Dnmt in aging cells could provide an explanation for regional hypermethylation in these cells. Since the activity of these Dnmts is much less than Dnmt1 in normal aging cells, however, it is unlikely that their increase in maintenance methylating activity could counteract the decline in Dnmt1 maintenance methylating activity in aging cells. Thus, these studies indicate that a major factor in the decline in overall cytosine methylation of aging cells is a decrease in Dnmt1 and suggest that an increase in the distribution of minor Dnmts may lead to a regional hypermethylation of specific genes in aging cells.

Our studies comparing SV40-immortalized WI-38 fibroblasts with normal WI-38 fibroblasts have also revealed major differences in the activities of the individual Dnmts in these cells. The amount of de novo and maintenance methylation by Dnmt1 in immortalized cells is strikingly lower than in normal cells while Dnmt3b and the acidic Dnmt contribute relatively more than Dnmt1 to maintenance and de novo methylation in immortalized cells. These findings strongly suggest that, although the mechanisms for immortalization in these cells are not known, underlying changes in the distribution of the Dnmts could contribute significantly to aberrant methylation in immortalized cells and could play a role in altered gene expression in these cells.

The isoelectric points of the three different Dnmts we have observed in human cells closely resemble those previously described in liver tumor tissue of rats fed a methyl-deficient diet [Lopatina et al., 1998]. We have identified the pI 7.2–7.4 Dnmt as the major "maintenance methyltransferase" (Dnmt1) of mammalian cells. The pI 8.2–8.3 Dnmt was identified as Dnmt3b, but the identity of the pI 4.2–4.3 (acidic) Dnmt is unknown although this Dnmt is endogenous, was present in all of the cells we examined and no other Dnmt activity besides the three we have identified was detected. It is known that several different Dnmts are present in mammalian cells and that these Dnmts have minor methylating activity compared to Dnmt1 as well as different propensities for maintenance and de novo methylation [Robertson et al., 1999]. Further studies will be required to determine the exact identity of the minor acidic Dnmt described previously [Lopatina et al., 1998] and in this study. Phosphorylation as a posttranslational modification of Dnmt1 would be expected to yield a more acidic Dnmt with potentially different methylating properties. Alternatively, the acidic Dnmt may be the product of a gene which has not previously been described. It is likely that increased activity by the acidic Dnmt along with Dnmt3b in aging and immortalized cells could contribute to many of the changes in cytosine methylation in these cells. Additional studies of the gene expression of the Dnmts in aging and immortalized cells could contribute greatly to our understanding of changes in maintenance and de

novo methylation in cellular senescence and immortalization and the potential role of these processes as alternative cell counting mechanisms.

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