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Dynamic Interrelationships between DNA Replication, Methylation, and Repair

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Although DNA methylation is required for mammalian development, the presence of 5-methylcytosine can render the nuclear genome unstable, leading both to heritable point mutations and to somatic events that culminate in cancers. This instability probably arises through several distinct mechanisms, some of which are only beginning to come to light. DNA-methylation patterns are remarkably plastic during development, showing clear differences in sperm and oocyte, followed by genomewide demethylation before a wave of de novo methylation establishes the adult pattern (Razin and Shemer 1995). In general, CpG-rich regions of DNA, termed "CpG islands," remain unmethylated throughout this time, with the exception of those regions associated with either imprinted genes or the promoters of genes located on the inactive X chromosome in females (Bird 1986). The biochemical mechanisms responsible for these dynamic changes are almost completely unknown, but methylation patterns are the same in different individuals (Achten et al. 1991), and they show strong exclusion of methylation from CpG islands. In contrast, the patterns are almost always altered in human cancers, with inappropriate CpG-island methylation being common (Jones and Gonzalgo 1997). Because genomic instability is a hallmark of human cancer, recent work in the field of DNA methylation has focused on the potential both for this process to create point mutations and chromosomal aberrations and for alterations in the DNA-repair machinery to change DNA-methylation patterns.

CpG Sites as Mutational Hotspots in Somatic Cells

CpG sites are widely recognized as hotspots for mutations in the germ line, where they contribute to ~30% of all point mutations (Cooper and Youssoufian 1988). In addition, CpG sites in the coding regions of tumor-suppressor genes are strong hotspots for acquired somatic mutations leading to cancer (Greenblatt et al. 1994). The CpG sites in the p53 coding region are methylated in all human tissues studied (Magewu and Jones 1994) and contribute to as many as 50% of all inactivating mutations in colon cancer and to as many as 25% of all inactivating mutations in cancers in general (Greenblatt et al. 1994). One hotspot, located at codon 248, is frequently mutated in both colon cancer and lung cancer. Here, however, the mutation is not the familiar C→T transition but, rather, a G→T transversion possibly due to preferential binding of benzo[a]pyrene diepoxide to the target guanine. In a fascinating recent study, Denissenko et al. (1996) showed that the presence of 5-methylcytosine at the CpG site actually increased the formation of the adduct at guanine, thus providing another biochemical pathway leading to somatic mutation.

The mechanism responsible for the increased mutability of 5-methylcytosine relative to cytosine has been known for some time. The pioneering work of Coulondre et al. (1978) established the base as a hotspot in *Escherichia coli,* and they proposed that this hotspot is due to difficulties inherent in the repair of T:G mismatches resulting from cytosine deamination in DNA (Coulondre et al. 1978). Figure 1 shows that there are three major variables involved in the fixation of these mutations: the rate of deamination, the rate of repair, and the rate of cell division. Because of the remarkable prevalence of C→T transition mutations in p53 in human cancers, we and others have investigated whether any of these variables is altered in cancer cells. The rate of deamination is probably constant, since (1) it is caused by a simple chemical reaction and (2) the rate is more than sufficient to account for all the mutations observed in double-stranded DNA (Shen et al. 1994). Alterations in the presumed major repair pathway catalyzed by thymine DNA glycolyase (TDG) do not seem to be common, since (*a*) we found no evidence for decreased enzymatic activity in colon tumors and (*b*) mutations in TDG have not been found (Schmutte et al. 1996). The

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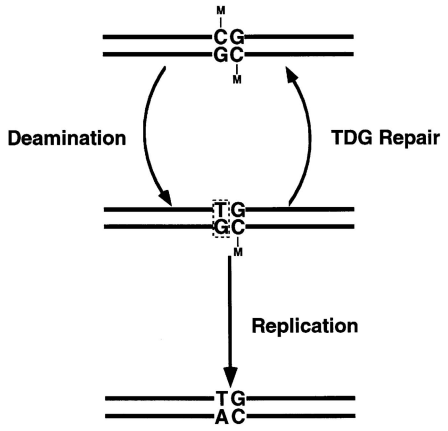

Figure 1 Pathways for the formation of signature C→T mutations at methylated CpG sites in mammalian cells. 5-Methylcytosine hydrolytically deaminates at a constant rate to form T:G mispairs (*boxed*). These mispairs are presumably repaired by the TDG enzyme (Schmutte et al. 1996). Unrepaired mispairs are fixed as C→T transitions if cell division occurs before repair is complete. Since the deamination rate is constant and alterations in the TDG-repair pathway have not been found, variations in the rate of cell division are most likely primarily responsible for mutation fixation.

prevalence of mutations is probably therefore a reflection of an increased rate of division (Lieb and Rehmat 1997). Indeed, CpG mutations are more common in those cancers where cell division is elicited by factors such as hormonal stimulation or repair of tissue damage after chronic stimulation (Greenblatt et al. 1994). The mutational pressure of 5-methylcytosine in the germ line is therefore also apparent in somatic cells. Once inactivating mutations occur in key genes such as p53, other alterations may follow, including substantial changes in methylation patterns and the silencing of other regulatory genes.

The Methylation Machinery

DNA methylation, the covalent addition of a methyl group to the C-5 position of cytosine in the context of the CpG dinucleotide, is mediated by the DNA methyltransferase enzyme. This large enzyme (~200 kD) displays a marked preference for hemimethylated DNA, DNA that carries a methyl-cytosine on one strand (Bestor and Verdine 1994). Hence, it had been proposed that this enzyme's primary function is to maintain the methylation patterns laid down during development, and it is often referred to as the "maintenance" methlytransferase. This methyltransferase will copy the preexisting pattern of methylation, from the original parental strand to the newly synthesized daughter strand, after each round of DNA synthesis. The mechanism by which this copying is performed is rather unusual. The target cytosine is extruded from the double helix into the active-site cleft in the enzyme (Klimasauskas et al. 1994). There, the now extrahelical cytosine is attacked by a conserved active-site cysteine (Bestor and Verdine 1994), followed by transfer of a methyl group from the cofactor S-adenosyl-L-methionine (SAM) to the 5 position (Smith et al. 1992).

Is all of the DNA methylation at CpG accomplished by a single enzyme? To date, only the maintenance methyltransferase has been cloned and characterized. However, this enzyme does not appear to be well suited for the task of de novo methylation, because of its preference for hemimethylated DNA, and a developmentally regulated de novo methyltransferase has been postulated. Proteolytic removal of the regulatory domain of the maintenance methyltransferase results in an increase in de novo methylation ability in vitro; such processing has not been observed in vivo, however (Bestor and Verdine 1994). Recently, compelling evidence for the existence of an independently encoded de novo methyltransferase has come to light. Mice bearing a null mutation of the known methyltransferase gene showed arrested development prior to the 8-somite stage but contained low but stable levels of cytosine methylation (Lei et al. 1996). Embryonic stem (ES) cells used for the creation of the knockout mice are viable, indicating that methylation may be essential only in differentiated cells (Lei et al. 1996). Retroviral infection of methyltransferase-null ES cells leads to de novo methylation of proviral DNA, as observed in normal ES cells, presumably a defense mechanism against invading parasitic sequences (Bestor and Coxon 1993; Lei et al. 1996). This de novo methyltransferase is then thought to be down-regulated during differentiation, once the desired methylation pattern has been established. This pattern is then propagated by the maintenance methyltransferase for the life of the organism. Validation of this idea, however, will await cloning and characterization of this de novo enzyme.

Methylation Changes in Cancer

As with the demethylation and de novo methylation observed during development, changes in methylation patterns during neoplasia have been recognized for some time (Counts and Goodman 1995). Initially it was shown that malignant cells have lower levels of methylation than do normal cells (Feinberg et al. 1988). This global hypomethylation accompanies a hypermethylation of CpG islands, DNA regions often associated with promoters of human genes that are normally protected from methylation (Bird 1986). The mechanism by which these regions remain unmethylated in the normal cell is not known, but it may be mediated by the binding of certain transcription factors. In malignant cells, these CpG-island regions become methylated and expression

of the associated gene is silenced (Counts and Goodman 1995). In the case of a tumor-suppressor gene, this may result in a growth advantage for the cell. DNA methylation–mediated transcriptional inhibition has thus been proposed as a mechanism that is alternative to mutation and deletion, in the removal of tumor suppressor–gene function (Jones 1996). Examples of such genes include the two cell-cycle regulators p16 Ink4a (Gonzalez-Zulueta et al. 1995) and p15 Ink4b (Herman et al. 1996), the von Hippel–Lindau gene VHL in some renal carcinomas (Herman et al. 1994), the retinoblastoma gene product Rb (Stirzaker et al. 1997), BRCA1 (Dobrovic and Simpfendorfer 1997), the angiogenesis inhibitor thrombospondin (Ahuja et al. 1997), and the metastasis-suppressor gene E-cadherin (Graff et al. 1995).

In a recent issue of *Science,* a report by Chuang et al. (1997) has shed new light on how methylation patterns are maintained and how they may become altered in cancer. It was shown that the DNA methyltransferase is targeted to newly replicated DNA by the replication-associated protein PCNA (proliferating cell nuclear antigen). PCNA is the polymerase-processivity factor for the δ and ϵ DNA polymerases, is homologous to the *E. coli* β subunit, and is required for DNA replication (Krishna et al. 1994). PCNA is also the target of the cell-cycle regulator $p21^{WAF1/CIP1}$ (Waga et al. 1994). The binding of p21 and DNA methyltransferase to PCNA appears to be mutually exclusive, since their levels have been shown to be inversely related in both normal and transformed cells (Chuang et al. 1997). In response to DNA damage, p53 is up-regulated, leading to increased synthesis of p21 (El-Deiry et al. 1993). p21 binding to PCNA then inhibits DNA synthesis and thus mediates the ability of p53 to arrest cell division (Waga et al. 1994). Targeting of the DNA methyltransferase to PCNA may then allow for the rapid remethylation of newly synthesized daughter strands before being packaged into chromatin. This may be important, since one of the principle chromatin-organizing proteins, histone HI, has been shown to inhibit DNA methylation (Carotti et al. 1996); thus this tight spatial and temporal linkage of DNA methylation and DNA synthesis may be essential.

In either a malignant cell or a premalignant cell, however, p53 function is very often lost (Vogelstein and Kinzler 1992), sometimes as a result of point mutations in CpG dinucleotides (Greenblatt et al. 1994; Magewu and Jones 1994). Thus, loss of p21 is also a common event in cancer. One might then propose that loss of p21 would perturb a balance between the p21 and DNA-methyltransferase interaction with PCNA, which would allow for an increase in methylation errors (fig. 2), as we have proposed elsewhere (Jones 1996). For example, DNA methyltransferase has an affinity for certain forms of damaged DNA (Smith et al. 1994). PCNA is required for both mismatch repair (Umar et al. 1996) and nucleotide-excision repair (Nichols and Sancar 1992). Loss of p21, which also associates with PCNA at sites of DNA repair, might then grant greater access of the DNA methyltransferase to these regions, resulting either in a methylation error or in de novo methylation at a CpG site that is not normally methylated. This type of random methylation error will then be propagated and may act as a nucleus for other such events, leading, over time, to the type of inappropriate de novo methylation seen in cancer cells and to the transcriptional silencing of key tumor-suppressor genes (fig. 2).

DNA Methylation and DNA Repair

Another interesting corollary to the relationship between DNA methylation and DNA repair comes from two recent papers. The first, by Kane et al. (1997), showed for the first time that the genes coding for mismatch-repair proteins may themselves be subject to methylation-mediated transcriptional silencing. The *hMLH1* promoter was found to be methylated in certain sporadic colon tumors and tumor cell lines that failed to express hMLH1—but in which no coding-sequence mutation was detected. Understanding of both the importance and the frequency of this mode of inactivation of the mismatch-repair pathway will have to await cloning and characterization of the promoter regions of other mismatch-repair genes. The second paper, by Lengauer et al. (1997), describes an interesting phenomenon, in which retroviral infection of mismatch repair–deficient cell lines led to de novo methylation of and transcriptional silencing of the proviral DNA whereas mismatch repair–proficient cell lines were competent to express the retrovirally encoded reporter gene. Another recent study has also noted methylation-pattern differences between mismatch repair–deficient and mismatch repair–proficient cell lines (Ahuja et al. 1997). In light of the results of Chuang et. al. (1997), these effects may result from the interaction of the DNA methyltransferase with PCNA. PCNA associates with the mismatch-repair proteins MLH1 and MSH2 in yeast, and PCNA is required for efficient mismatch repair. Furthermore, the presence of PCNA appears to be required at a step preceding DNA resynthesis, so PCNA may not simply be acting as a polymerase-accessory factor (Umar et al. 1996). Loss of p21 from sites of DNA repair, by inactivation of p53, may also perturb a balance between various interacting repair proteins, leading to aberrant DNA methylation. Increased cell division, due to the loss of the cell-cycle inhibitor p21, would then allow these mutations and methylation changes to become fixed in the genome, as indicated in figure 2.

The role for DNA methylation in causing somatic mu-

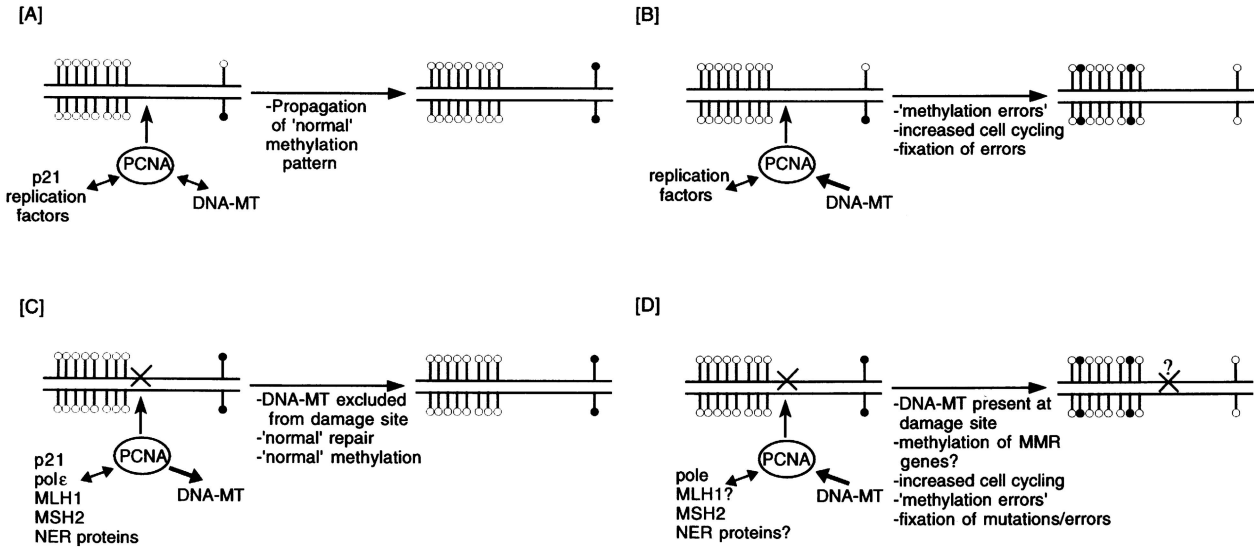

Figure 2 Pathways for the formation of methylation errors and mutations in cancer cells, and the central role of PCNA. In a normal cell (*A*), some interplay between p21 and the DNA methyltransferase (DNA-MT) allows for DNA replication and maintenance methylation (Chuang et al. 1997). Typically, cytosines in isolated CpG dinucleotides are methylated (*blackened lollipops*), but those in CpG islands are not methylated (*unblackened lollipops*). In a malignant cell (*B*), the loss of p21 perturbs the balance and allows for methylation errors to occur and for these to become fixed, potentially leading to inactivation of tumor-suppressor genes. In a normal cell (*C*), the DNA methyltransferase may be excluded from a site of damage (indicated by "×") by p21, allowing repair to be completed by mismatch repair (MLH1 and MSH2) or nucleotide excision repair (NER). In a malignant cell (*D*), the loss of p21 may allow the methyltransferase access to sites of DNA damage, causing methylation errors because of its affinity for abnormal or damaged DNA structures (Smith et al. 1994). Furthermore, we suggest that the repair pathways themselves may be affected by both the loss of p21 and the presence of the methyltransferase, which may increase the error rate (indicated by "?").

tations is firmly established. Recent evidence—such as the finding of (1) de novo methylation of a mismatch-repair gene in cancer and (2) linkage between the DNA methyltransferase and PCNA, a critical protein involved in both DNA replication and repair—even suggests that methylation and genomic instability may be linked. The role of DNA damage in the alteration of patterns of DNA methylation, due to the requirement for PCNA in both mismatch repair and nucleotide-excision repair and to the association of PCNA with the DNA methyltransferase, is an exciting new idea that will no doubt be a major focus of research in the field in coming years.

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References

Achten S, Behn-Krappa A, Jucker M, Sprengel J, Holker I, Schmitz B, Tesch H, et al (1991) Patterns of DNA methylation in selected human genes in different Hodgkin's lymphoma and leukemia cell lines and in normal lymphocytes. Cancer Res 51:3702–3709

Ahuja N, Mohan AL, Li Q, Stolker JM, Herman JG, Hamilton SR, Baylin SB, et al (1997) Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res 57:3370–3374

Bestor TH, Coxon A (1993) The pros and cons of cytosine methylation. Curr Biol 3:384–386

Bestor TH, Verdine GL (1994) DNA methyltransferases. Curr Opin Cell Biol 6:380–389

Bird A (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209–213

Carotti D, Funiciello S, Lavia P, Caiafa P, Strom R (1996) Different effects of histone H1 on de novo DNA methylation in vitro depend on both the DNA base composition and the DNA methyltransferase. Biochemistry 35:11660–11667

Chuang LS-H, Ian H-I , Koh T-W, Ng H-H, Xu G, Li BFL (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex is a target for p21Waf1. Science 277:1996–2000

Cooper DN, Youssoufian H (1988) The CpG dinucleotide and human genetic disease. Hum Genet 78:151–155

Coulondre C, Miller JH, Farabaugh PJ, Gilbert W (1978) Molecular basis of base substitution hotspots in Escherichia coli. Nature 274:775–780

Counts JL, Goodman JI (1995) Alterations in DNA methylation may play a variety of roles in carcinogenesis. Cell 83: 13–15

Denissenko MF, Pao A, Tang M, Pfeifer GP (1996) Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in p53. Science 274:430–432

Dobrovic A, Simpfendorfer D (1997) Methylation of the

BRCA1 gene in sporadic breast cancer. Cancer Res 57: 3347–3350

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trant JM, Lin D, et al (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75:817–825

Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 48:1159–1161

Gonzalez-Zulueta M, Bender CM, Yang AS, Nguyen T, Beart RW, van Tornout JM, Jones PA (1995) Methylation of the CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res 55:4531–4535

Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, et al (1995) E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 55:5195–5199

Greenblatt MS, Bennett WP, Hollstein M, Harris CC (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54: 4855–4878

Herman JG, Jen J, Merlo A, Baylin SB (1996) Hypermethylation associated inactivation indicates a tumor suppressor role for p15INK4B. Cancer Res 54:722–727

Herman JG, Latif F , Weng Y, Lerman MI, Zbar B, Liu S, Samid D, et al (1994) Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci USA 91:9700–9704

Jones PA (1996) DNA methylation errors and cancer. Cancer Res 56:2463–2467

Jones PA, Gonzalgo ML (1997) Altered DNA methylation and genome instability—a new pathway to cancer? Proc Natl Acad Sci USA 94:2103–2105

Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, Jessup JM, et al (1997) Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res 57:808–811

Klimasauskas S, Kumar S, Roberts RJ, Cheng X (1994) HhaI methyltransferase flips its target base out of the DNA helix. Cell 76:357–369

Krishna TSR, Kong X-P, Gary S, Burgers PM, Kuriyan J (1994) Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. Cell 79:1233–1243

Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122: 3195–3205

Lengauer C, Kinzler KW, Vogelstein B (1997) DNA methylation and genetic instability in colorectal cancer cells. Proc Natl Acad Sci USA 94:2545–2550

Lieb M, Rehmat S (1997) 5-Methylcytosine is not a mutagen hot spot in nondividing Escherichia coli. Proc Natl Acad Sci USA 94:940–945

Magewu AN, Jones PA (1994) Ubiquitous and tenacious methylation of the CpG site in codon 248 of the p53 gene may explain its frequent appearance as a mutational hot spot in human cancer. Mol Cell Biol 14:4225–4232

Nichols AF, Sancar A (1992) Purification of PCNA as a nucleotide excision repair protein. Nucleic Acids Res 20: 2441–2446

Razin A, Shemer R (1995) DNA methylation in early development. Hum Mol Genet 4:1751–1755

Schmutte C, Yang AS, Nguyen T, Beart RW, Jones PA (1996) Mechanisms for the involvement of DNA methylation in colon carcinogenesis. Cancer Res 56:2375–2381

Shen J-C, Rideout WM, Jones PA (1994) The rate of hydrolytic deamination of 5-methylcytosine in double-stranded DNA. Nucleic Acids Res 22:972–976

Smith SS, Kaplan BE, Sowers LC, Newman EM (1992) Mechanism of human methyl-directed DNA methyltransferase and the fidelity of cytosine methylation. Proc Natl Acad Sci USA 89:4744–4748

Smith SS, Laayoun A, Lingeman RG, Baker DJ, Riley J (1994) Hypermethylation of telomere-like foldbacks at codon 12 of the human c-Ha Ras gene and the trinucleotide repeat of the FMR-1 gene of fragile X. J Mol Biol 243:143–151

Stirzaker C, Millar DS, Paul CL, Warnecke PM, Harrison J, Vincent PC, Frommer M, et al (1997) Extensive DNA methylation spanning the Rb promoter in retinoblastoma tumors. Cancer Res 57:2229–2237

Umar A, Buermeyer AB, Simon JA, Thomas DC, Clark AB, Liskay RM, Kunkel TA (1996) Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell 87:65–73

Vogelstein B, Kinzler KW (1992) p53 Function and dysfunction. Cell 70:523–526

Waga S, Hannon GJ , Beach D, Stillman B (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369:574–578