

Review

5-Methylcytosine depletion during tumour development: An extension of the miscoding concept

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Summary We propose a general model for neoplastic development which postulates that the loss of methyl groups from 5-methylcytosines (5-mC) involved in the control of gene expression may initiate neoplastic transformation and give rise to the aberrant phenotype of the transformed cell. Interference with normal patterns of methylation can be envisioned to occur by a number of mechanisms: as a result of carcinogen-induced G:C→A:T transition leading to a loss of potentially methylatable cytosines; by mutations or chromosome rearrangement which disrupt the integrity of active DNA methylase genes; by separating methylated repressor regions of the genome from the genes they control; by direct interference with DNA methylation, as proposed for ethionine and 5-azacytidine; by spontaneous deamination of 5-mC to thymine, leading to accumulation of 5-mC:G→T:A transitions; by virus-induced perturbations in host cell methylation patterns; and by activation of DNA demethylases.

Perhaps the most ubiquitous aspect of tumours and the neoplastic cells of which they are composed is their loss of the ability to control cellular functions in a normal way. During the development of the malignant state, the neoplastic cell becomes increasingly refractory to both the internal and external stimulæ which integrate its normal counterparts into functional tissues and organ systems. This lack of integration is associated with alterations in the expression of a host of gene products, including, for example, the ectopic biosynthesis of hormones (Rees, 1975; Imura, 1980), various nuclear modifications (Rovera, 1975; Sarma *et al.*, 1975), changes in enzyme and isozyme patterns (Weinhouse, 1970, 1980, Weinhouse *et al.*, 1972; Ibsen, 1977; Ghosh *et al.*, 1978; Foti *et al.*, 1977; Greengard & Herzfeld, 1977; Shapira *et al.*, 1963), appearance of foetal antigens (Gold, 1971) and cell surface modifications (Robin & Nicholson, 1975; Mora, 1974). It is thus clear that a powerful interference with the normal machinery of gene expression accompanies the transformed state. Such aberrant gene expression may yield to the transformed cell the required release from physiological control that defines neoplasia. Altered metabolism resulting from the appearance of isozymic forms may be the key both to such loss of host control and to the unbridled growth that are

characteristic of rapidly growing tumours (Weinhouse, 1974).

As yet, altered gene expression has not been linked with the various processes known to produce tumours. Presented here is a model which proposes a basis for such a link, and which additionally provides a high degree of relevance to available data. The model is based upon a possible relationship between normal physiological methylation of DNA and chemically-induced alkylation of DNA, *viz.*, that carcinogen-induced alkylation leads to disappearance of sites of enzymatic methylation of DNA. Such hypomethylation would cause the expression of genes otherwise repressed, for example, those for isozymes, foetal antigens, ectopic hormones, etc.

Enzymatic methylation in gene expression

There is increasing evidence that the enzymatic methylation of DNA at the 5-position of cytosine exerts some influence over the expression of eukaryotic genes, as suggested by Holliday & Pugh (1975), Riggs (1975) and Scarano (1971). McGhee & Ginder (1979) have shown that in adult reticulocytes and erythrocytes which are expressing or have expressed the adult β -globin gene, CCGG sites near the ends of the gene sequence are unmethylated, whereas in oviduct, brain and embryonic red blood cells, which do not express this gene, such sites are at least partially methylated. Christman *et al.* (1977) found that DNA isolated from Friend erythroleukaemic cells induced to produce globin mRNA is

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hypomethylated when compared to DNA from uninduced cells. Mandel & Chambon (1979) demonstrated the existence of a class of methylation sites (m^{var}) within and around the conalbumin, ovomucoid, and β -globin genes, the variable methylation of which correlated with transcriptional activity. When a gene region is in an active chromatin conformation, as measured by DNase I sensitivity (Kuo *et al.*, 1979), there is extensive hypomethylation at many of these m^{var} sites. Sites of residual methylation near transcribed genes, as well as undermethylated sites near genes not being transcribed, were absent in DNase I-sensitive active chromatin. Based on these results, these authors suggest that in all tissues there is a certain possibility that an m^{var} site is present in the unmethylated state; thus only undermethylation at a substantial number of m^{var} sites of a given genomic region would be significantly correlated with the active state of chromatin. This and other information (for review see Doerfler, 1981) indicate that methylation at specific cytosine residues in DNA at specific times may be closely associated with the regulation of gene expression. Furthermore, the demonstration of specific unmethylated CG doublets in mammalian DNA by Browne *et al.* (1978) and the stringent conservation of the pattern of methylation throughout vertebrate evolution (Browne & Burdon, 1977) imply rigorous control of the DNA methylation system.

It has been suggested that 5-methylcytosine (5-mC) may provide signals for regulatory protein binding (Yuan & Meselson, 1970). Indeed, it is well established that the *Escherichia coli* K restriction and modification enzyme has a strong affinity for an unmethylated site but no detectable affinity for a methylated site. Sista *et al.* (1979) have shown that the 5-methyl group of thymine is the only important functional group recognized by the lac repressor at position 13 of the lac operator. Replacing thymine with cytosine resulted in a 7–8 fold decrease in lac repressor binding, whereas 5-mC bound repressor at least as tightly as the wild type sequence. Thus methylation is already known to affect the binding of sequence-specific proteins to prokaryotic DNA. It seems a reasonable working hypothesis that the insertion of a hydrophobic methyl group into the major groove of DNA, as occurs with the methylation of cytosine, might affect protein binding in eukaryotic systems as well.

Chemical alkylation of DNA

Evidence has accumulated that non-enzymatic alkylation of DNA may also be biologically relevant. The site of alkylation of a nucleic acid, *in*

vivo or *in vitro*, is greatly dependent upon the type of alkylating agent (Jensen, 1978; Singer *et al.*, 1978). In general, alkylating agents with low mutagenicity or carcinogenicity, such as dimethyl sulfate, are weak electrophiles and tend to attack primarily ring nitrogens. Agents that produce more reactive electrophilic species, such as N-nitroso and related compounds (Magee, 1977a, 1978), show an increasing ability to alkylate oxygen, reacting with all ring oxygens as well as phosphodiester (Jensen & Reed, 1978; Singer, 1977, 1979). These compounds are very potent carcinogens, and many show an intriguing organ specificity (Druckrey, 1967).

Although N-nitroso and related compounds produce lesions at a variety of sites on DNA, alkylation at the O⁶-position of guanine is thought to be responsible for the subsequent induction of tumours by these agents (Magee, 1976, 1979; Pegg & Nicoll, 1976; Lawley, 1980; Frei *et al.*, 1978). The persistence of O⁶-alkylguanine in DNA of various rat and mouse tissues (Goth & Rajewsky, 1974; Kleihues & Margison, 1974; Margison *et al.*, 1976) correlates with the organ specificity of these compounds, especially when the rate of cell replication of the examined tissue is considered. The persistence of other lesions, such as those produced at the 7-position of guanine, the major site of alkylation, fails to predict which organs will serve as specific targets for tumorigenesis.

Goth & Rajewsky (1974) compared the persistence of O⁶-ethylguanine in brain and liver of 10 day old rats treated with a single dose of N-ethyl-N-nitrosourea under conditions known to be selectively tumorigenic for brain but not liver. Molar fractions of O⁶-ethylguanine, 7-ethylguanine, and 3-ethylguanine at one hour after injection were very similar in each tissue. The rate of removal of O⁶-ethylguanine over a 240 h period, however, was much slower in brain than in liver. Nicoll *et al.* (1975) have shown that after a single large dose of dimethylnitrosamine, O⁶-methylguanine is much longer lived in the kidney where tumours develop than in the liver, where they do not; and Kleihues & Margison (1974) found that in rats treated with methylnitrosourea, a potent neurogenic carcinogen which occasionally also produces tumours in the kidney but never in the liver, O⁶-methylguanine is removed from DNA least rapidly in the brain, most rapidly in the liver, and at an intermediate rate in the kidney. The resistance of the liver and the susceptibility of the brain and kidney to tumorigenesis induced by a single dose of either ethylnitrosourea, dimethylnitrosamine or methylnitrosourea therefore appears to be related to the ability of these tissues to remove O⁶-alkylguanine lesions from their respective DNAs.

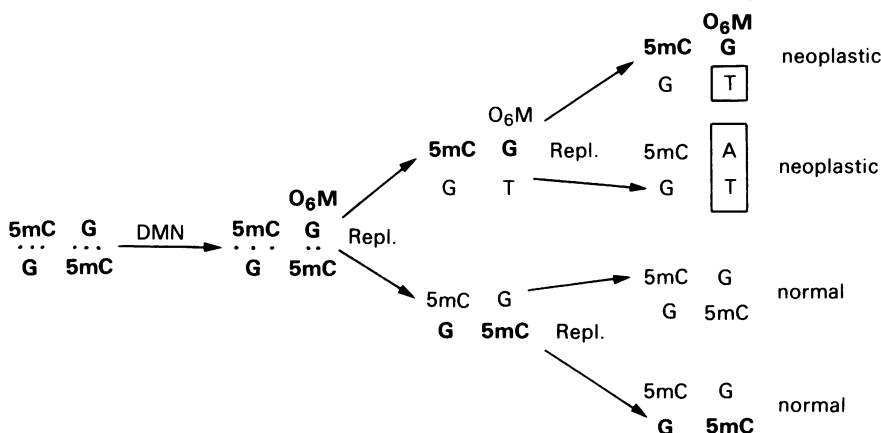


Figure 1 Diagrammatic representation of guanine alkylation-induced loss of methylatable cytosines following 2 or 3 rounds of replication. Original strands are shown in bold type, new strands in light type.

Do the biological effects of enzymatic and chemical alkylation intersect?

Based on the suggestion of Loveless (1969) that O⁶-alkylation of guanine could lead to mispairing during DNA replication, Gerchman & Ludlum (1973) have shown in their *in vitro* transcription system that O⁶-methylguanine mispairs with thymine in place of cytosine. Assuming that a similar mispairing event can occur *in vivo*, we suggest that the critical result of G:C→O⁶-MeG:C→O⁶-MeG:T→A:T transitions during DNA replication in N-nitrosamine-induced carcinogenesis is the loss of cytosine residues which may otherwise be potential sites of enzymatic methylation at the C-5 position (Figure 1). Such depletion of sites of enzymatic methylation may interfere with the otherwise rigidly fixed pattern of gene expression characteristic of fully differentiated cells. The aberrant gene expression and consequent unorchestrated attempt at differentiation that may result in a hypomethylated cell might then give rise, in both morphological and biological terms, to the transformed state. Transition mutations or other events (see below) leading to DNA hypomethylation are thus seen as capable of initiating the neoplastic phenotype by setting in motion programmes of transcription that disrupt the state of cellular differentiation.

Evidence in support of the model

Bryngelsson & Pero (1980) have presented evidence that the adenosine to guanine ratio of 1.272 in normal rat DNAs is significantly lower than that of 1.342 in tumours induced by a variety of agents, including 7,12-dimethylbenz(a)-anthracene, 3-

methylcholanthrene, 3,4-benzo(a)pyrene, and 1,2-dimethylhydrazine. Their data indicate that ~1% of rat tumour DNA or one in every 50 base pairs has an altered purine or pyrimidine residue compared to normal rat DNAs. Kasten *et al.* (1982) found that, following DNA damage induced in human diploid fibroblasts by ultraviolet irradiation, N-methyl-N-nitrosourea, or N-acetoxy-2-acetylaminofluorene, repaired patches of DNA remained permanently undermethylated. According to Lapeyre & Becker (1979), rats treated with acetylaminofluorene, DNA from resulting premalignant hepatic nodules was undermethylated by 20%. Primary hepatocellular carcinomas found in these animals were undermethylated by 45% and diethylnitrosamine-induced hepatocellular carcinomas were undermethylated by 32.5%. Further evidence in favour of the model comes from work with the amino acid analogue and hepatic carcinogen, L-ethionine. Ethionine is only one carbon atom larger than the essential amino acid methionine, is metabolized via the same pathways as methionine (Stekol, 1963), and yet gives rise to numerous hepatocarcinomas when included in the diet (Farber, 1963). Although significant transfer of the ethyl group of ethionine to RNA occurs (Farber & Magee, 1960; Craddock, 1969), very little reaction with DNA is observed (Swann *et al.*, 1971; Ortwert & Novelli, 1969). Significantly, Farber (1973) has pointed out that ethionine may be the exception to the rule that hepatocarcinogens damage DNA in a way detectable on alkaline sucrose gradients. It is impossible to rule out a role for direct ethylation of DNA in the mechanism of action of ethionine-induced carcinogenesis. However, levels of ethylation (Swann *et al.*, 1971; Ortwert & Novelli,

1969; Grilli *et al.*, 1974), and of repair of induced DNA single strand breaks (Craddock & Henderson, 1978; Farber, 1973), approach insignificance when compared to levels induced by other hepatocarcinogens. Furthermore, only 7-ethylguanine (Swann *et al.*, 1971) and not O⁶-ethylguanine or other miscoding and presumably oncogenic lesions, have been observed after ethionine treatment. This also contrasts with what is observed after treatment with other alkylating hepatocarcinogens, where O⁶-alkylguanine occurs to a very significant extent (Magee *et al.*, 1976; Margison *et al.*, 1976; Nicoll *et al.*, 1977). We would like to offer, within the context of the model we are developing here, a possible mechanism of action for ethionine carcinogenesis which is an alternative to the direct DNA ethylation concept.

Smith & Salmon (1965) demonstrated that ethionine administration leads to the accumulation of S-adenosylethionine (SAE); and Cox & Irving (1977) found that SAE, as a metabolic analogue of S-adenosylmethionine (SAM), competitively inhibits DNA methylase *in vivo*, resulting in methyl-deficient DNA. Such inhibition of methylation by SAE is reversible upon replenishment of SAM pools, and the toxic and carcinogenic effects of ethionine can be reversed by the simultaneous administration of methionine (Farber & Ichinose, 1958; Brada *et al.*, 1976). Methionine may thus overcome the carcinogenic effects of ethionine by maintaining the ratio of SAM to SAE at a sufficiently high level to allow normal methylation of cytosine.

Since tRNA is ethylated by ethionine *in vivo*, it has been suggested that tRNA is the target for ethionine carcinogenesis (Borek & Kerr, 1971; Srinivasan & Borek, 1964). There are very little data to support this suggestion however, since there is increasing evidence that perturbation of DNA, and not RNA or other cellular macromolecules, initiates carcinogenesis (see below). Significantly, ethylated bases observed in tRNA of ethionine-treated animals have their methylated analogues in normal tRNA (Craddock *et al.*, 1968; Dunn, 1959, 1963; Smith & Dunn, 1959), suggesting that tRNA methylases operate with a relaxed specificity for alkyl group donor and can utilize either SAE or SAM. On the other hand, since 5-ethylcytosine is not found in DNA from ethionine-treated animals (Craddock, 1971), DNA methylases apparently possess a strict requirement for SAM. Because DNA methylases cannot utilize SAE, methylation of specific cytosines would be blocked in its presence. As this block continues throughout subsequent replications in the presence of concentrations of SAE capable of inhibiting DNA transmethylation reactions, the degree of cytosine hypomethylation would increase. The consequence

would be an impaired gene regulation, leading to incomplete and abnormal differentiation. Since the replicative index of liver is very low, the model suggests that ethionine would be carcinogenic only at high concentration capable of inhibiting DNA methylase over extended periods. Such are the requirements for the induction of hepatic tumours by ethionine (Farber, 1963).

Other compounds that interfere with DNA methylation might also be expected to lead to changes in cellular differentiation and transformation. Whereas ethionine may inhibit DNA methylation by reducing cellular pools of SAM, 5-azacytidine (5-AzaCyt), an analogue of cytidine in which carbon 5 has been replaced by nitrogen, profoundly impairs methylation by masking the methyl acceptor site. Jones & Taylor (1980) have shown that 5-AzaCyt induces marked changes in differentiation of cultured mouse embryo cells. In testing other analogues substituted at the 5-position and elsewhere, these authors determined that changes in gene expression induced by such compounds correlated with their ability to inhibit DNA methylation.

Since certain DNA methylases ("maintenance methylases") may require hemimethylated double-stranded DNA as substrate (Pollack *et al.*, 1980; Bird, 1978), replications subsequent to a single treatment with 5-AzaCyt could produce fully unmethylated sequences, resulting in the permanent loss of 5-mC signal recognition sites for maintenance methylase activity. Constantinides *et al.* (1978) do, in fact, report that changes in the differentiated state induced in cell cultures by 5-AzaCyt occur by 8 to 11 cell divisions after treatment. At that time, the initial 5% substitution of 5-AzaCyt for cytosine bases in DNA have been reduced to a vanishingly small number. The 65% inhibition of DNA methylation observed by these authors in 5-AzaCyt-treated cultures would imply the erasing of previously established patterns of methylation by loss of a site of post-transcriptional modification. These observations suggest that 5-AzaCyt might be carcinogenic in tissues capable of incorporating it into their DNA. Likely targets might include rapidly replicating tissues such as the liver after partial hepatectomy, and the gastrointestinal tract. This suggestion may have some clinical relevance in view of the use of 5-AzaCyt as a chemotherapeutic agent (Armitage & Burns, 1977; Saiki *et al.*, 1978; Vogler *et al.*, 1976; Von Hoff *et al.*, 1976).

5-AzaCyt is mutagenic in *E. coli*, *Salmonella Typhimurium*, and V79 Chinese hamster cells (Fucik *et al.*, 1965; Marquardt & Marquardt, 1977), and limited evidence is available for its carcinogenic potential (Stoner *et al.*, 1973; NCI, 1978; IARC, 1981).

dependent on methionine, and that choline is effective for this purpose in so far as it provides the methyl group for methionine synthesis in this organ.

DNA repair, aging and viral carcinogenesis

We have discussed O⁶-alkylguanine as a significant lesion in tumour development because of its ability to eliminate methylation sites through G:C→A:T transitions. Because we are offering our model as a general description of the development of neoplasia, we wish to point out that any DNA adduct leading to the loss of methylation sites might be potentially tumorigenic. Conversely, genetic lesions which do not induce hypomethylation would not, according to the model, be carcinogenic.

Pfohl-Leskowitz *et al.* (1981) have shown that binding of 2-(acetyl-amino)-fluorene to C-8 of guanine inhibits DNA methylation irreversibly, thus possibly explaining the mechanism of carcinogenic action of this compound. In terms of carcinogen-DNA adducts leading to hypomethylation, one of the major predictions of our model is that alkylation of 5-mC moieties in DNA may be the most potently tumorigenic of all. Preliminary evidence from our laboratory suggests the formation of such lesions *in vitro*. Alkylation or other carcinogen-induced modification of 5-mC would significantly alter the character of the major groove of DNA. Any signal properties provided by 5-mC might consequently become impaired. Changes in the base pairing properties of 5-mC alkylation products are also possible. For example, alkylation at either O² or N⁴ might give 5-mC a base pairing bond configuration more characteristic of thymine than cytosine. In replicating tissues, the resulting alkyl 5-mC:G→T:A transitions would create the same disturbance in gene expression and subsequent development of neoplasia postulated for O⁶-alkylguanine-induced G:C→A:T transitions. Since 5-mC is not inserted into DNA *de novo* but occurs only by postreplicational methylation of cytosine, even the prompt repair of 5-mC alkylation products, if it occurs by a patch repair mechanism,

may leave DNA in a hypomethylated state. With such repair, inserted cytosines must be remethylated prior to replication in order to prevent the heritable loss of hemimethylated substrate for maintenance methylase activity. One might guess, therefore, that a transmethylase similar to that observed for O⁶-alkylguanine might be operative (discussed below). Repair effected by such a transmethylase would leave 5-mC in place, and hence be much less likely to upset the pattern of gene expression.

The model also offers an explanation for the observed increased susceptibility of older animals, including man, to spontaneous or chemically induced tumours (Ebbesen, 1974, 1977; Magee, 1978). Kudryashova & Vanyushin (1976*a, b*) and Vanyushin *et al.* (1980) have found that the DNA of old animals is hypomethylated when compared to that of young animals of the same sex and strain. That this may be due to spontaneous deamination of 5-mC to thymine is suggested by work with both prokaryotic and eukaryotic systems. Coulondre *et al.* (1978) have observed that spontaneous base substitution hotspots within the *lac I* gene of *E. coli* are due to deamination of 5-mC. Bird (1980) has discussed the relative scarcity of GC dinucleotides in vertebrate DNA along similar lines, providing evidence that 5-mC tends to mutate abnormally frequently to thymine. Although the deamination of adenosine to inosine and cytosine to uracil are also known to occur, the removal of these products can be effectively accomplished by the base excision repair system (Lindahl, 1979). However, since the deamination of 5-mC yields thymine, the excision of this product seems unlikely.

We suggest that spontaneous deamination of 5-mC to thymine, with the subsequent formation of 5-mC:G→T:A transitions (Figure 2), may explain the increased susceptibility to carcinogenesis that occurs with increasing age, since such transitions would accumulate over the lifetime of the animal. If relevant controlling regions become completely unmethylated as a result of such spontaneous deamination reactions, then the affected cell might

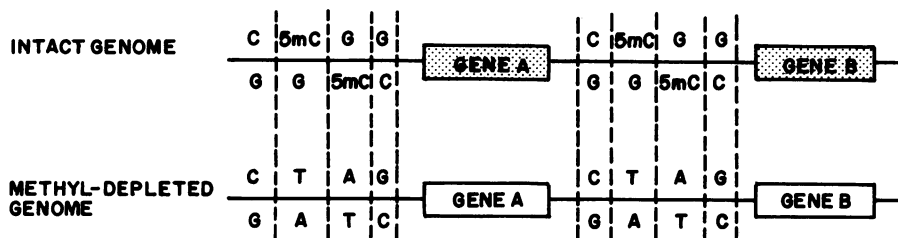


Figure 2 Diagrammatic representation of gene activation by multiple 5mC:G→T:A transition mutations. Shading indicates inactive genes.

give rise to a "spontaneous" neoplasm. But even the partial methyl-depletion of multiply methylated controlling regions would increase the probability that a carcinogenic insult to DNA would result in a neoplastic effect. Simply stated, if "old" DNA is undermethylated, fewer hypomethylating mutations would be required to put it over the regulatory threshold.

A recent statistical analysis of tumours occurring in patients over 50 years old suggested that the tumours studied, and their numbers, could be related to a decrease in the integrity of the genome (Dix *et al.*, 1980). The spontaneous deamination of 5-mC and the subsequent generation and collection of 5-mC:G→T:A transitions may, according to our model, contribute to the age-associated decline in genome integrity suggested by these authors. If true, then chemical carcinogens might be said to exert their tumorigenic effects by mimicking a natural phenomenon.

Since the induction of tumours by viruses is well established, we wish to point out that variable patterns of DNA methylation in virus-transformed cells have been reported (Rubery & Newton, 1973; Browne & Burdon, 1977; Greene *et al.*, 1975; DeWichter *et al.*, 1971; Berneman *et al.*, 1978). Groudine *et al.* (1981) recently showed that the 5-AzaCyt-induced hypomethylation of an endogenous retroviral gene of chick embryo erythrocytes results in the activation of this genome as judged by DNase sensitivity, transcription and synthesis of viral proteins. Other observations relating DNA hypomethylation to viral carcinogenesis have been made. For example, proviral mouse mammary tumour virus (MMTV) DNA sequences acquired from milk in animals at high risk for breast cancer are undermethylated compared to endogenous MMTV sequences that are associated with a much reduced risk (Breznik & Cohen, 1982). Since an inverse relationship has been demonstrated between proviral methylation and transcriptional activity, derepression of endogenous proviruses through demethylation of these sequences may represent the mechanism of MMTV-induced tumorigenesis (Breznik & Cohen, 1982). Analysis of the methylation pattern of the viral thymidine kinase (TK) gene in Herpes simplex virus (HSV)-transformed mouse cells showed that when the gene was being actively transcribed it was unmethylated, when inactive it was methylated, and when induced to activity by 5-AzaCyt it was again unmethylated. This finding was extended by Waechter & Baserga (1982) who found that when the cloned gene for HSV-TK was methylated with Eco RI methylase and microinjected into the nucleus of TK⁽⁻⁾ cells, methylation at particular sites markedly reduced or abolished the expression of the gene. These authors pointed out the possibility that different genes

might respond differently to methylation, since similar Eco RI methylation of the gene coding for Simian virus 40 T antigen had no effect upon its expression after microinjection. A causative relationship between DNA methylation and decreased gene expression in Herpes simplex TK genes was also observed by Christy & Scangos (1982). Diala & Hoffman (1982) observed hypomethylation of HeLa cell DNA and the absence of 5-mC in SV40 and adenovirus (Type 2) DNA. Desrosiers *et al.* (1979) observed that viral DNA in cells of *Herpesvirus saimiri* transformed non-producing lymphoid cell lines contains DNA methylated at cytosine positions which are unmethylated in virion DNA and in DNA of lymphoid cell lines that produce virus. In other studies an inverse correlation was observed between the levels of methylation of integrated adenovirus (Type 12) DNA sequences and viral gene expression in transformed hamster cells (Sutter & Doerfler, 1980). These results suggest that hypomethylation of DNA may provide a common transforming mechanism shared by chemical carcinogens and oncogenic viruses.

Genetic vs. epigenetic considerations

A purely epigenetic model of carcinogenesis, also concerned with the effects of hypomethylation upon cell populations, has been put forward (Holliday, 1979). This model proposes that damage to DNA results in the potentially reversible loss of methyl groups as a function of repair processes. This would occur either as a result of repair before DNA synthesis, and before appropriate methylases have remethylated newly inserted cytosines, or as a result of recombination following damage to DNA.

There are, however, observations which detract from this otherwise attractive hypothesis. Thus, while the persistence of O⁶-alkylguanine correlates well with the tumour susceptibility of various tissues and that of 7-alkylguanine does not, these lesions would be predicted by the purely epigenetic model to be equally tumorigenic if sequences containing them were excised, then repaired, but not remethylated prior to DNA replication. Yet methyl methanesulfonate (MMS), which produces extensive methylation at the N₇ position of guanine and only insignificant amounts of the O⁶-alkylated product, is not hepatocarcinogenic in the rat even when administered in the wave of DNA replication which follows partial hepatectomy (Craddock, 1975). Furthermore, evidence has accumulated which suggests that, while 7-alkylguanine is removed from DNA by a base excision process presumably susceptible to repair-associated DNA hypomethylation, O⁶-alkylguanine is repaired in a transmethylation reaction where base integrity

remains intact (Olson & Lindahl, 1980; Pegg *et al.*, 1982; Waldstein *et al.*, 1982; Renard *et al.*, 1981; Regan & Setlow, 1974). Thus the repair mechanism for O⁶-alkylguanine does not proceed by an excision system that would deplete 5-mC. Sequences containing methylated cytosines in the vicinity of such lesions remain intact. Since there are no gaps to fill, there is no reason to expect that O⁶-alkylguanine repair would lead to hypomethylation. The repair-associated hypomethylation model therefore suffers from the flaw that it predicts that 7-alkylguanine, but not O⁶-alkylguanine, is a critical oncogenic lesion, when exactly the opposite seems to be true. It would appear, then, that fixation of O⁶-alkylguanine lesions via G:C→A:T transition mutations is a more likely mechanism of carcinogenesis, at least for alkylating carcinogens, than that postulated in the purely epigenetic model.

The molecular basis underlying the relationship between mutagenesis and carcinogenesis has become increasingly substantial (see Magee, 1977b). Thus, most chemical carcinogens are also mutagens (Ames & McCann, 1976; McCann & Ames, 1977); tumours appear to be clonal in origin, suggesting the occurrence within a single cell of a permanent molecular alteration (Gould *et al.*, 1978); cells that are more sensitive to mutagenic lesions are also more sensitive to malignant transformation (Cleaver & Bootsma, 1975; Mortelman *et al.*, 1976; Takebe *et al.*, 1977); mutagenic metabolites produced by metabolic activation within tumour-susceptible organs have been identified as ultimate carcinogens (Felton & Nebert, 1975); and the direct perturbation of DNA has been shown to be sufficient to initiate neoplastic transformation (Barrett *et al.*, 1978). Yet Cairns (1981) has convincingly championed the argument that many cancers may be caused by non-mutational mechanisms (e.g., cancers arising in tissues next to implanted sheets of plastic, or induced by implantation of ovary or embryo cells into sites where constraints upon their cellular multiplication are removed). He has concluded that there is no obvious connection between the many ways that tumours can be induced. One strength of the hypomethylation model is that it provides such a common link between diverse carcinogenic stimulæ. Another strength is that it provides a basis for believing the difference between mutational and epigenetic mechanisms may be more apparent than real, with both mechanisms sharing considerable overlap. As an example, one may consider the loss of hemimethylated sites for DNA methylase that would occur if 5-mC were to be modified by an alkylating carcinogen, repaired but not remethylated before the next round of DNA synthesis. Similarly, interference with DNA methylase induced by ethionine could lead to

permanent alteration in the pattern of methylation of the genetic material. Are such changes in the expressible nature of the involved DNA to be considered mutational or not? They do, after all, disrupt the clonal inheritance of the genomic methylation pattern (Riggs, 1975; Holliday & Pugh, 1975). Yet, in the case of ethionine-induced hypomethylation, if a sequence-specific methylase capable of acting upon completely unmethylated sites could be induced, the methylation pattern could theoretically be fully restored. In this scenario, the boundaries between epigenetic and mutational mechanisms of carcinogenesis become obscure.

We might point out here that short-term bacterial mutagenesis assays may produce some false negative results when testing the relationship between carcinogenesis and mutagenesis because of differences in the methylated bases occurring in prokaryotes and eukaryotes, and their different functions. For example, a compound like ethionine which is not overtly genotoxic does not test positively in a *Salmonella typhimurium* revertant assay (McCann *et al.*, 1975), yet is clearly carcinogenic in eukaryotes. The number of other substances tested which are either mutagens or carcinogens but not both offer perhaps one of the most intriguing avenues for research into the basic mechanism of tumorigenesis.

Cellular oncogenes

While the model presented here implies widespread changes in gene expression as a result of chemically-induced hypomethylation of DNA, it is entirely possible that only one or a few of the newly activated genes are responsible for transformation. Much of the recent work on cellular oncogenes suggests that this may be the case (for a review see Weinberg, 1982). From our point of view, the cellular oncogene (or oncogenes) would be one or a few of many genes activated during carcinogen-induced hypomethylation. Certain retro-viruses incorporate into their DNA a cellular gene which is thus released from host control (Weinberg, 1981). This collapse of host regulatory function for the oncogene might involve actual demethylation of the host sequence incorporated into the virion (Breznik & Cogen, 1982) or physical separation of the oncogene from the methylated host DNA sequence responsible for its repression. Integration of the avian leukosis viral genome next to a specific cellular oncogene has been shown to occur, and to transcriptionally activate the gene (Hayward *et al.*, 1981).

Possible candidates for genes with oncogenic potential may include those genes for 5-mC demethylase or 5-mC deaminase. While the former

activity has been reported in murine erythro-leukemic cells (Gjerset & Martin, 1982), no evidence exists for the latter, although its function during early development remains an attractive theoretical possibility (Holliday & Pugh, 1975). Since the levels of hypomethylation following treatment with alkylating agent appear to be larger than would be expected based upon the number of pro-mutagenic O⁶-alkylguanine lesions induced, it is possible that low level hypomethylation occasionally releases the gene for a putative 5-mC demethylase from repression, initiating a cascade of newly expressed genes which release the cell from growth control.

Tumour promotion

An early and profound effect of tumour promoters is the stimulation of mitotic activity and cell proliferation (Diamond *et al.*, 1980). All that may be required of a promoter is that it induce a condition of chronic regenerative hyperplasia in the target tissue (Argyris, 1981). Since replication is required in order for hypomethylation-inducing lesions to become fixed in DNA, promoters may exert their effect primarily by increasing the rapidity of cell turnover. As can be seen in Figure

1, an initiating event such as the formation of O⁶-alkylguanine or spontaneous deamination of 5-mC to thymine will not be expressed until successive DNA replications lead to hypomethylation.

This would be consistent with the long latent periods observed between initiation and administration of promoter that are possible in some experimental systems. Once the genetic damage has been permanently fixed into the DNA in the form of lost sites of enzymatic methylation, many months or even years may elapse before that cell is induced to undergo proliferation, or does so spontaneously. When proliferation is resumed, however, the undermethylated genome may allow the expression of previously repressed genes, some of which may release the cell from growth control. We conclude our discussion by drawing attention to Table I. It is remarkable that in every system in which DNA hypomethylation is encountered, neoplasia follows. It is, of course, possible that this accumulation of circumstantial evidence may be misleading, and it is always difficult to separate cause from effect, especially in a subject as complex as neoplastic transformation. Nevertheless, the correlation between DNA hypomethylation and neoplastic transformation is intriguing and certainly deserving of serious investigation.

Table I Systems in which hypomethylation of DNA may initiate the transformed state

AGENT OR CONDITION AFFECTING DNA METHYLATION	AFFECTED SYSTEM	MECHANISM OF INDUCTION OF DNA HYPOMETHYLATION
Chemical alkylation (e.g., by N-nitroso-or related compounds)	DNA	G:C → A:T transition mutations which delete present or potential sites of enzymatic DNA methylation
Other carcinogen-induced modifications (e.g. Benzo (a) Pyrene, aflatoxin B1, AAF, DAB)	DNA	E.g., AAF insertion-denaturation of DNA and subsequent irreversible inhibition of DNA methylase (Pfohl-Leskowicz <i>et al.</i> , 1981)
Ethionine, hereditary tyrosinemia, choline/methionine deficiency	DNA methylase system	Reduction of SAM pool size and subsequent inhibition of DNA methylase
Pyridoxine deficiency	DNA methylase system	Cofactor depletion and subsequent inhibition of DNA methylase
5-azacytidine	DNA	Loss of methylatable cytosine residues in CpG sequences
Aging	DNA	5mC:G → T:A transition mutations due to spontaneous deamination
Viruses	Host DNA, DNA methylase system	a) differential methylation of host vs viral DNA by virus associated DNA methylase? b) insertion mutation?

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