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DNA methylation and methylase levels in normal and malignant mouse hepatic tissues

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Abstract

The status of DNA methylation, as measured by the 5-methylcytosine content of nuclear DNA, was examined in normal livers and in chemically induced or spontaneous primary hepatocellular carcinoma (PHC) arising in three strains of mice. The DNA from spontaneous tumors of genetic origin in C3H mice and also from acetylaminofluorene, chlordane, or 3′-methyl-4-dimethylaminoazobenzene-induced tumors in C57Bl and B6C3 mice was undermethylated compared to the levels in background and normal liver samples. The DNA methylase activities from normal liver, background liver, and PHC were assayed in C3H mice to determine whether the observed genomic undermethylation is related to a dysfunction of this enzyme and were compared to the rates of DNA synthesis in these tissues. Since DNA methylase levels from tumor nuclei were elevated compared to background, it is concluded that the undermethylation found in the tumor genomes of this system is not due to inactivation nor a significant deficiency of the activity of this enzyme relative to the demand in tumors for methylation of *de novo* synthesized DNA.

Introduction

The hypothesis that an alteration of DNA is a crucial step in the carcinogenic process is basic to the rationale for the use of mutagenicity assays as a means of identifying carcinogenic agents (1–3). This concept is supported by a strong correlation between mutagenic potential in bacterial tests (4–6) and other assays of DNA damage (7,8) with carcinogenicity *in vivo*. Further evidence has been derived from the susceptibility to malignant alterations of cells of patients with hereditary DNA repair-deficiency syndromes (9,10). However, this hypothesis does not adequately explain the induction of cancer by nonmutagenic agents (11,12) or hormones (13), nor does it offer an explantion for the escape of the neoplastic cell from normal regulatory mechanisms.

Barrett and Ts'o compared the rate of somatic mutation (as gene activation) in Syrian hamster embryo cells to the rate of development of anchorage-independent growth in soft agar and found that the latter, which is highly correlated with tumorigenicity, is 100-fold less frequent (14). They concluded that neoplastic transformation is more complex than a single gene mutation step. These results and other analyses of genetic and environmental factors in cancer suggest that carcinogenesis involves a multi-hit process affecting several genes or regulatory sites (15,16). However, it might also signify that transforming hit(s) correspond to 100-fold less frequent events, such as the inactivation of minor nucleotide(s) in noncoding or regulatory region(s) of the genome.

Although the function of postreplication modification of a small proportion of cytosines of nuclear DNA by methylation in eukaryotes (0.1%–8%) (17,18) has not been ascertained, recent findings have implicated undermethylation of DNA with the potential for gene expression (for review see 19,20). Ethionine, a hepatocarcinogen (21) that is not mutagenic in the Ames assay (5,6), has been reported to induce alpha fetoprotein (AFP)* synthesis in the liver (22) and globin synthesis in Friend erythroleukemia cells (23), while producing an undermethylation of these genomes (23–25). The basis for its action may be related to the formation of an inactive analog of S-adenosylmethionine (SAM) (25). Analogs of cytidine in which the C-5 position cannot be methylated have been shown to induce altered states of differentiation of 10T1/2 cells and a concomitant, transient undermethylation of the genome (26,27). In addition, analysis of the status of methylation of CCGG sequences detected by cleavage with the isoschizomer pair of restriction enzymes Msp I/Hpa II (28,29) has demonstrated a correlation with the undermethylation of certain of these sequences vicinal and internal to the genes being expressed (30–32). A similar undermethylation requirement has been noted for the expression of integrated viral genes in transformed cells (33–35).

Previous information on endogenous DNA methylation in malignancy has been scant and often contradictory. DNA methylation and methylase activity had been reported to increase in leukemia (36,37), whereas recently, the opposite finding has been reported (38). We have reported an undermethylation in the putative premalignant lesions in the rat liver during administration of N-acetylaminofluorene (AAF) as well as the primary

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*Abbreviations: 5-MeCyt, 5-methylcytosine; PHC, primary hepatocellular carcinoma(s); AAF, N-acetylaminofluorene; CRD, chlordane; MeDAB, 3′methyl-4-dimethylaminoazobenzene; THC, transplantable hepatocellular carcinoma(s); NMU, N-methyl-N-nitrosourea; MMTV, mouse mammary tumor virus; PMSF, phenylmethylsulfonylfluoride; DTT, dithiothreitol; TCA, trichloroacetic acid; DEN, diethylnitrosamine; AFP, alpha fetoprotein; SAM, S-adenosylmethionine; h.p.l.c., high pressure liquid chromatography; HF, hydrofluoric acid.

hepatocellular carcinomas (PHC) derived from these lesions and their transplantable hepatocellular carcinomas (THC) (39). Boehm and Drahovsky (40) have demonstrated a similar undermethylation of the genome of Raji cells after treatment with N-methyl-N-nitrosourea (NMU) that was found to persist even after cessation of treatment. A rationale for these findings is provided by the observation that DNA containing AAF adducts (and by supposition other carcinogen adducts to DNA bases) is not as efficient a DNA template for *in vitro* methylation with semi-purified DNA methylase as is an unmodified DNA template (41). In the cases where DNA methylation has been studied during viral oncogenesis, cells that are transformed by adenovirus, polyoma, or Rous sarcoma virus have been reported to have an elevated 5-methylcytosine (5-MeCyt) content in their genomes (42–44). In contrast, in cells transformed by mouse mammary tumor virus (MMTV), there is evidence the virus causes a generalized undermethylation of the genome (35).

In order to examine the possibility that altered methylation of nuclear DNA is a functional aberration of malignancy, we investigated the level of DNA methylation in tumors from three different strains of mice, two of which exhibit a strong tendency toward spontaneous carcinogenesis of the liver and one that does not.

Materials and Methods

Carcinogens

AAF was obtained from Tridom Chemicals (Hauppauge, NY). Chlordane (octachloro-4,7 methanotetrahydroindane) (CRD) was a gift from Velsicol Chemical Co. (Chicago, IL), and contained 90% CRD with ~ 10% heptachlor. 3′-Methyl-4-dimethylaminoazobenzene (MeDAB) was purchased from K & K Chemical Co. (Plainview, NY).

Each of these compounds was mixed with a semi-synthetic diet (#101, BioServ, Frenchtown, NJ) at 0.03% for AAF, 0.03% for MeDAB, and 0.005% for CRD, and fed in a pelleted form. A continuous dietary regimen of the above formulation was fed to the mice *ad libitum* beginning at 5 weeks of age for a period of 1 year. Control mice were maintained similarly, but on a pelleted diet, # 101.

Animals

Male C3H/HeN, male C57B1/6N, and the male F1 progeny from the cross of female C57B1/6H x male C3H/HeN, i.e., B6C3, were obtained from the Department of Mammalian Genetics of the National Institutes of Health. These mice were bred at the Charles River Farms (North Wilmington, MA), and were negative for MMTV.

DNA methylation

Nuclear DNA was isolated from the liver and PHC nuclei and analyzed for the methylated base 5-MeCyt as described in detail previously (39,45). Protein contaminants were removed from the DNA by Proteinase K digestions, repeated phenol-Sevag ($CHCl_3$:isoamyl alcohol, 24:1 v/v) deproteinization steps, and, to remove all traces of RNA, by digestion with a mixture of RNase A and T1 followed by base hydrolysis with 0.5M NaOH at 60°C for 30 min. The purified DNA was hydrolyzed to bases with concentrated hydrofluoric acid (HF) for 1.5 h at 80°C. This procedure has been found to produce less degradative losses of 5-MeCyt to thymine by deamination than procedures employing 88% formic acid hydrolysis at 170°C for 30 min or 1 N perchloric acid hydrolysis at 100°C for 30 min (45). Bases were analyzed by high pressure liquid chromatography (h.p.l.c.) on a Partisil 10-SCX column (0.46 x 25 cm, Whatman) eluted isocratically with 0.07 M ammonium formate, pH 3.2, at room temperature. Bases were identified relative to the elution of authentic compounds and their quantity determined from the peak areas divided by the extinction coefficient of the base at pH 3.2 and 280 nm. The steady state content of 5-MeCyt, reported as the quantity of the methylated base, 5-MeCyt, to total cytosines x 100 was used as a measure of genomic DNA methylation levels. The mean of triplicate determinations for each individual sample was entered for the calculation of the mean and standard deviation (σ) of the sample populations (N). Statistical significance of the results was examined by unpaired t-test and an analysis of variance (F-test).

DNA methylase

For the determination of total DNA methylase activity, purified nuclei were sedimented once in 1.0 M sucrose, 1mM $MgCl_2$ 0.5 mM phenylmethylsulfonylfluoride (PMSF) at 4800 x g for 15 min and extracted with 3.7 volumes of 0.8 M KCl, 0.5% Triton X-100, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 7.4) essentially as described by Simon *et al.* (46). After being stirred for 30 min on ice, the KCl concentration of the solution was lowered to 0.3 M by addition of 1.1 volumes of 10 mM Tris-HCl (pH 7.8), 0.5 mM DTT, and 0.5 mM PMSF. An extract containing greater than 90% of the enzyme activity was obtained by centrifugation at 20,000 x g for 15 min. The crude enzyme was subsequently dialyzed at 4°C with 20 mM Tris-HCl, (pH 7.4), 1 mM EDTA, 0.5 mM DTT, 0.05% Triton X-100, and 10% glycerol. Enzyme assays were performed in triplicate in a volume of 200 μl with identical amounts of methylase extract protein (130 μg) determined by the method of Bradford (47). The assay contained 10 μg of denatured *Micrococcus luteus* DNA, 1 μM [^{3}H methyl]SAM (9.0 Ci/mmol, ICN, final specific activity 19,800 d.p.m./pmol) in 0.02 M Tris-HCl pH 7.4, 1 mM EDTA, and 0.5 mM DTT. It was incubated for 1 h at 37°C. Reactions were stopped by the addition of 200 μl of 1 N NaOH followed by the addition of 40 μg carrier DNA, and by heating for 15 min at 60°C. After cooling to ambient temperature and neutralization with one-tenth volume of 5N H_3PO_4, the samples were deproteinized twice with phenol-Sevag (1:1 v/v) and the labeled DNA was precipitated with 10% trichloroacetic acid (TCA) and collected on GF/C

filter for scintillation counting. DNA methylase activity was expressed as net pmol CH_3/mg protein/h incorporated over zero time controls (where the reaction was stopped with base prior to the addition of enzyme).

DNA synthetic rates

One hour prior to sacrifice, 1.5 μCi/g body weight [^{3}H-methyl]thymidine (10 Ci/mmole, ICN) in sterile saline was injected i.p. The DNA in the methylase extract pellet was processed according to a modified Schmidt-Thannhauser procedure using perchloric acid (48). The specific activity of the 1 N perchloric acid DNA hydrolysate was determined by counting an aliquot in a perchloric acid-compatible liquid scintillation cocktail (Scintiverse, Fisher), and assaying the quantity of DNA therein by the method of Giles and Myers (49).

Results

Male C3H mice demonstrate a genetically determined predisposition toward the formation of spontaneous PHC, the incidence of which rises to ~ 52% at 14 months of age (50). Male C67B1/6N exhibit virtually no incidence of spontaneous PHC and generally require substantial exposure to known carcinogens to evoke malignancy (51). However, continous exposure, beginning at 5 weeks of age and continuing for 1 year, to the following regimens regularly induced PHC: 0.03% AAF, 0.03% MeDAB, and 0.005% CRD in #101. B6C3, the F1 progeny of these strains, demonstrate a lesser tendency toward spontaneous carcinogenesis (~ 20% – 30% that of C3H/HeN) and approximately the same susceptibility to AAF induction of PHC as C57B1/6N. In many instances, the gross appearance of chemically induced PHC in B6C3 mice was sufficiently distinctive to allow discrimination from spontaneous PHC. However, further confirmation by subsequent histologic examination of each tumor was utilized for positive identification of the tumor type. Tumors were excised from surrounding liver tissue (background liver) and, where necessary, gross necrosis and hemorrhage were trimmed away. Nuclear DNA was prepared from isolated nuclei and purified for the analysis of the minor base, 5-MeCyt by h.p.l.c. as described in **Methods.**

DNA methylation levels

Figure 1 shows histograms of the level of methylation of nuclear DNA in PHC, background, and normal liver from the three mouse strains. Ten spontaneous PHC from C3H mice were found to have a 5-MeCyt content of 3.75 ± 0.29%, N = 10, 5-MeCyt/total cytosines (mean ± S.D., N). The background tissue (grossly free of involvement) demonstrated a 5-MeCyt content of 4.26 ± 0.12%, N = 3. The 5-MeCyt content of livers of age-matched controls (>40 weeks) and juvenile mice (5 weeks) were 4.62 ± 0.28%, N = 4 and 4.62 ± 0.18%, N = 4, respectively. Using an unpaired t-test, the difference in methylation between PHC/normal liver was found to be statistically significant, i.e., $t = 2.39$, $p < 0.05$.

The levels of DNA methylation in PHC induced in

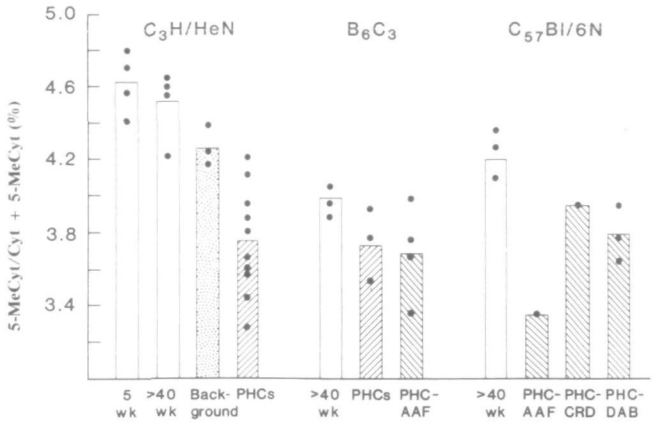

Fig. 1. Nuclear DNA methylation in PHCs, background liver, and normal (nontumor-bearing) liver in three mouse strains. Each point (●) is the mean of triplicate determinations of each nuclear DNA sample for 5-MeCyt determined by h.p.l.c. reported as the percentage of 5-MeCyt/total cytosines. (□), normal, i.e., nontumor-bearing liver at 5 and >40 weeks of age; (▨), background, i.e., grossly uninvolved liver from PHC-bearing mice; (▨), spontaneous PHC; and (▨), AAF-, CRD-, or MeDAB-induced PHC.

C57B1/6N mice following exposure to AAF, CRD, and MeDAB were 3.35, N = 1; 3.95 N = 1; and 3.79 ± 0.13%, N = 3; respectively, compared to a level of 4.20 ± 0.09%, N = 3, in the control livers from age-matched mice. These results, which suggest that a similarity exists in the undermethylation in both spontaneous and chemically induced tumors, were examined in a B6C3 hybrid strain in which both tumor forms occur. In B6C3, the 5-MeCyt content in spontaneous PHC from unexposed mice was 3.75 ± 0.21%, N = 3, while those tumors that bore the histologic characteristics of the AAF-induced type of tumor from animals exposed to carcinogen was 3.69 ± 0.23%, N = 4. Compared to the level of methylation in the DNA of normal livers of age-matched controls, i.e., 3.99 ± 0.23%, N = 3, these tumors demonstrate an undermethylation of their genomes. In the latter groups, the number of sample determinations was insufficient to assess statistical significance.

DNA methylase and DNA synthesis

This led us to determine whether an inactivation or a deficiency in the DNA methylase (methyltransferase) in the spontaneous tumors could account for the undermethylation of the tumor genomes. The experiment was designed not only to measure the rate of DNA synthesis and compare it to the levels of DNA methylation, but also to assess if a relative deficiency of the methylase by dilution in the form of rapid cell division takes place in the tumors. Thus, 1 h before sacrifice, each tumor-bearing animal received an i.p. injection of [^{3}H]thymidine at a dose of 1.5 μCi/g body weight. Paired samples of background and tumor tissue were prepared from five animals. DNA methylase was solubilized from isolated nuclei (see **Methods**), the specific activity of the DNA recovered in the pellet resulting from high-speed centrifugation of a nuclear

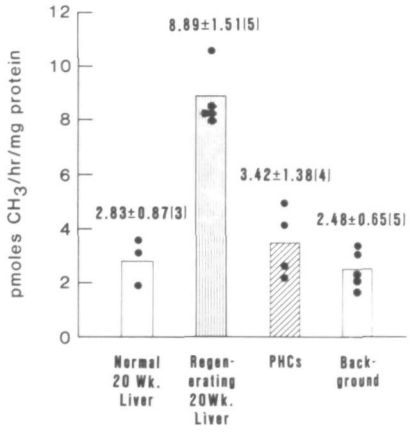

Fig. 2. DNA methyltransferase activity in normal 48 h regenerating and background livers compared with PHCs in C3H mice. Points (●) represent the mean of triplicate determinations from single PHCs and their background in five mice. (□), normal, 20 week liver; (▥), regenerating liver, (▤), background liver; and (▨), spontaneous PHC.

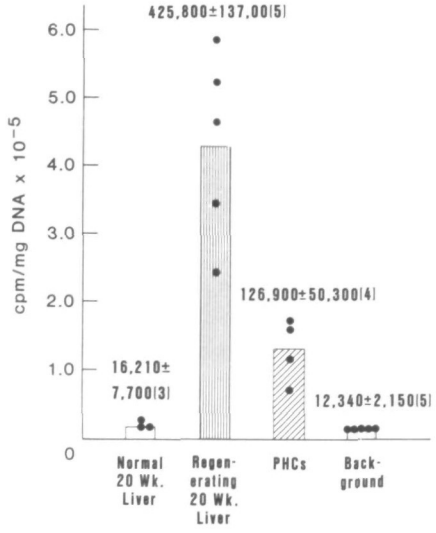

Fig. 3. DNA synthesis in normal, 48 h regenerating and background liver compared with PHC in C3H mice. Rates of nuclear DNA synthesis were determined as the specific activity of nuclear DNA 1 h following a single i.p. injection of [^{3}H]thymidine at a dosage of 1.5 μCi/g body weight. These DNAs were isolated from the pellet resulting from high-speed centrifugation of the nuclear extracts used to assay the DNA methyltransferase activity in Fig. 2.

extract was determined by a modified Schmidt-Thannhauser procedure. The methylase extracts were adjusted to equal protein concentrations and assayed with single-stranded *M. luteus* DNA and [^{3}H-methyl]SAM. As shown in Figure 2, the DNA methylase activity in PHC was higher (3.42 ± 0.65, N = 5). During the period of peak DNA synthesis in the liver (48 h after partial hepatectomy), the methylase level increased 3.15-fold to 8.89 ± 1.51, N = 5 pmol CH_3/h/mg protein from 2.84 ± 0.87, N = 3 in normal livers from 20 week old rats.

In Figure 3, the relative rate of DNA synthesis, determined from the specific activity of the nuclear DNA from each sample used to measure the DNA methylase, was 126,900 ± 50,300 c.p.m./mg DNA, N = 4 for PHC, or a 10.2-fold increase over the mean found in background liver tissues, i.e., 12,340 ± 2,150, N = 5. In the 39 h regenerating liver, the increase in DNA synthesis over normal liver is 26.2-fold, i.e., 425,800 ± 137,000, N = 5 to 16,210 ± 7,700, N = 3 c.p.m./mg DNA.

Using the ratio of the values in (PHC minus background) to (regenerating minus normal) x 100 as a normalized statistic to measure the relative activity in tumor versus the induced maximal values obtained in the regenerating liver, we found that the DNA synthesis in PHC as 27.9% of the maximum induced level whereas the increase in DNA methylase in PHC was only 16.6%. This leads to the conclusion that although DNA methylase activity is elevated in PHC, the induced level in PHC is 10% less than predicted from the rate of DNA synthesis in normal liver. However, since the maximum induction of methylase is ~ 3.2-fold to accomodate a 26.2-fold increase in DNA synthesis in regenerating liver, and the likelihood that the cell maintains a slight excess rather than a limiting amount of DNA methylase, *viz.*, the basal level in the quiescent normal liver, it seems unlikely that the quantity of DNA methylase in the PHC is limiting. Experiments in this laboratory (52) have also demonstrated an elevated DNA methylase activity in a transplantable rat hepatoma despite an undermethylation of the hepatoma DNA (39) that was found to accept more methyl groups than that from regenerating or normal liver. Measurements of the relative levels of normal and malignant tissue enzyme activity were invariant when assayed with three different diagnostic DNA templates including *M. luteus* DNA used in this study.

Discussion

In this study, nuclear DNA from spontaneous PHC and PHC induced by three different carcinogens were demonstrated to be undermethylated in three mouse strains. The results also indicated that the defect in DNA methylation in spontaneous C3H tumors is not due to inactivation of deficiency in DNA methylase activity. The resultant undermethylation may be due to other factors, such as: (1) aberrant metabolism or unavailability of the methyl-donor SAM; (2) altered chromatin configuration or accessibility, possibly due to a difference in tumor nonhistone chromosomal protein complement in C3H PHC (53) or, (3) a change in the specificity in DNA methyltransferase during carcinogenesis.

It is presently not possible to assess experimentally the functional effect of changes in endogenous DNA methylation on gene function or altered states of expression during carcinogenesis. However, several lines of evidence cited previously suggest that DNA methylation may play a role in the regulation and integration of gene

function at a higher level required for differentiation and development (54–57). Because of this possible function, an aberration in DNA methylation during carcinogenesis either by chemicals or by some genetic imbalance may play an etiologic role in the conversion of normal differentiated cells to neoplastic and fully malignant cells. Thus, the findings bearing on the change in methylation observed in spontaneous and chemically induced hepatomas reported here; in PHC and THC induced by AAF and diethylnitrosamine (DEN) (39); in Raji cells with NMU (40); and, in murine breasts carcinoma induced by MMTV (35) may be highly significant.

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References

1. Heidelberger,C. (1975), Chemical carcinogenesis, *Annu. Rev. Biochem.*, **44**, 79-121.
2. Irving,C.C. (1973), Interaction of chemical carcinogens with DNA, in Busch, H. (ed.), *Methods in Cancer Research*, Vol. 7, Academic Press, New York, pp. 189-244.
3. Barrett,C., Tsutsui,T., and Ts'o, P.O.P. (1978), Neoplastic transformation induced by a direct perturbation of DNA, *Nature* **274**, 229-232.
4. Ames,B.N., Durston,W.E., Yamasaki,E., and Lee,F.N. (1973), Carcinogens are mutagens: a sample test system combining liver homogenates for activation and bacteria for detection, *Proc. Natl. Acad. Sci. USA*, **70**, 2281-2285.
5. McCann,J., Choi,E., Yamashi,E., and Ames,B.N. (1975), Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay for 300 chemicals, *Proc. Natl. Acad. Sci. USA*, **72**, 5135-5139.
6. McCann,J., and Ames,B.N. (1976), Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Discussion, *Proc. Natl. Acad. Sci. USA*, **73**, 950-954.
7. Loeb,L.A., Springgate,C.F., and Battula,N. (1974), Errors in DNA replication as a basis of malignant changes, *Cancer Res.*, **14**, 2311-2321.
8. Sirover,M., and Loeb,L.A. (1976), Metal-induced infidelity during DNA synthesis, *Proc. Natl. Acad. Sci. USA*, **73**, 2331-2335.
9. Cleaver,J.E. (1968), Defective repair replication of DNA in xeroderma pigmentosum, *Nature*, **218**, 652-656.
10. Cleaver,J.E. (1969), Xeroderma pigmentosum: a human disease in which an initial stage of DNA repair is defective, *Proc. Natl. Acad. Sci. USA*, **63**, 428-435.
11. Reddy,J.K., Azarnoff,D.L., and Hignite,C.E. (1980), Hypolipidaemic hepatic peroxisome proliferators for a novel class of chemical carcinogens, *Nature*, **283**, 297-398.
12. Anti-thyroid and related substances (1974), in *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man*, IARC 7, 9-109.
13. Lingeman,C.H. (1979), Hormones and hormonomimetic compounds in the etiology of cancer, in Lingeman,C.H. (ed.), *Recent Results in Cancer Research: Carcinogenic Hormones*, Springer Verlag, New York, Vol. **66**, pp. 1-48.
14. Barrett,J.C., and Ts'o, P.O.P. (1978), Relationship between somatic mutation and neoplastic transformation, *Proc. Natl. Acad. Sci. USA*, **75**, 3297-3301.
15. Mole,R.H. (1964), Cancer production by chronic exposure to penetrating gamma irradiation, *Natl. Cancer Inst. Monograph*, **14**, 271-290.
16. Knudson,A.G. (1968), Mutation and cancer: statistical study of retinoblastoma, *Proc. Natl. Acad. Sci. USA*, **68**, 820-823.
17. Wyatt,G.R. (1951), The purine and pyrimidine composition of deoxypentose nucleic acids, *Biochem. J.*, **48**, 584-590.
18. Vanyushin,B.F., Tkacheva,S.G., and Belozersky,A.N. (1970), Rare bases in animal DNA, *Nature*, **225**, 948-949.
19. Razin,A., and Riggs,A.D. (1980), DNA methylation and gene function, *Science (Wash.)*, **210**, 604-610.
20. Drahovsky,D., and Boehm,T.L.J. (1980), Enzymatic DNA methylation in higher eukaryotes, *Int. J. Biochem.*, **12**, 523-528.
21. Farber,E. (1963), Ethionine carcinogenesis, *Adv. Cancer Res.*, **7**, 383-418.
22. Hancock,R.L., Forrester,E.L., Lorscheider,P.C., Lai,P.C.W., and Hay,D.M. (1976), Ethionine-induced activation of embryonic genes, in Fishman,W.H. and Sell,S. (eds.), *Oncodevelopmental Gene Expression*, Academic Press, New York, pp. 247-251.
23. Christman,J.K., Price,P., Pedrinan,I., and Acs,G. (1977), Correlation between hypomethylation of DNA and expression of globin genes in Friend erythroleukemia cells, *Eur. J. Biochem.*, **81**, 53-61.
24. Sneider,T.W., Teague,W.M., and Rogachevsky,L.M. (1975), S-adenosylmethionine: DNA-cytosine 5-methyltransferase from a Novikoff rat hepatoma cell line, *Nucleic Acids Res.*, **2**, 1685-1700.
25. Cox,R., and Irving,C.C. (1977), Inhibition of DNA methylation of S-adenosyl-ethionine with the production of methyl deficient DNA in regenerating rat liver, *Cancer Res.*, **37**, 322-325.
26. Taylor,S.M., and Jones,P.A. (1979), Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine, *Cell*, **17**, 771-780.
27. Jones,P.A., and Taylor,S.M. (1980), Cellular differentiation, cytidine analogs and DNA methylation, *Cell*, **20**, 85-93.
28. Waalwiji,C., and Flavell,R.A. (1978), DNA methylation at a CCGG sequence in the large intron of the rabbit ß-globin gene: tissue-specific variations, *Nucleic Acids Res.*, **5**, 4631-4641.
29. Singer,J., Robert-Ems,J., and Riggs,A.D. (1979), Methylation of mouse liver DNA studied by means of the restriction enzymes Msp I and Hpa II, *Science (Wash.)*, **203**, 1019-1021.
30. McGhee,J.D., and Ginder,G.D. (1979), Specific DNA methylation sites in the vicinity of the chicken ß-globin gene, *Nature*, **280**, 419-420.
31. Mandel,J.L., and Chambon,P. (1979), DNA methylation: organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes, *Nucleic Acids Res.*, **7**, 2081-2103.
32. Van der Ploeg,L.H.T., and Flavell,R.A. (1980), DNA methylation in the γδß globin locus in erythroid and nonerythroid tissues, *Cell*, **19**, 947-958.
33. Desrosiers,R.C., Mulder,C., and Fleckenstein,B. (1979), Methylation of *Herpesvirus saimiri* DNA in lymphoid tumor cell lines, *Proc. Natl. Acad. Sci. USA*, **76**, 3839-3843.
34. Sutler,D., and Doefler,W. (1980), Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression, *Proc. Natl. Acad. Sci. USA*, **77**, 253-256.
35. Cohen,J.C. (1980), Methylation of milk borne and genetically transmitted mouse mammary tumor virus proviral DNA, *Cell*, 19, 653-662.
36. Silber,R., Berman,E., Goldstein,B., Stein,H., Farnham,G., and Bertino,I.R. (1966), Methylation of nucleic acids in normal and leukemic leukocytes, *Biochim. Biophys. Acta*, **123**, 638-640.
37. Federov,N.A., Kuzmirhev,V.A., Kritskii,G.A., and Vinogradova,Y.E. (1977), Methylation of pyrimidine isopliths of newly synthesized DNA from human lymphocytes under normal conditions and in chronic lymphocytic leukemia, *Biokhimiya*, **42**, 791-793.
38. Burtseva,N.N., Azizeo,Y.N., Ithern,B.Z., and Vanyushin,B.F. (1980), Changes in the methylation of DNA of cattle lymphocytes during chronic lympholeukemia, *Biokhimiya*, **42**, 1331-1335.
39. Lapeyre,J.N., and Becker,F.F. (1979), 5-Methylcytosine content of nuclear DNA during chemical hepatocarcinogenesis and in carcinomas which result, *Biochem. Biophys. Res. Commun.*, **87**, 698-705.
40. Boehm,T.L.J., and Drahovsky,D. (1981), Hypomethylation of DNA in Raji cells after treatment with N-methyl-N-nitrosourea, *Carcinogenesis*, **2**, 39-42.
41. Salas,C.E., Pfohl-Leszkowicz,A., Lang,M.C., and Dirheimer,G. (1979), Effect of modification of N-acetoxy-N-2-

acetylaminofluorene on the level of DNA methylation, *Nature,* **278**, 71-72.
42. Gunthert,U., Schweiger,M., Stupp,M., and Doerfler,W. (1976), DNA methylation in adenovirus. Adenovirus-transformed cells and host cells, *Proc. Natl. Acad. Sci. USA,* **73**, 3923-3927.
43. Rubery,E.D., and Newton,A.A. (1973), DNA methylation in normal and tumour virus-transformed cells in tissue, *Biochim. Biophys. Acta,* **324**, 24-36.
44. Nass,M.M.K. (1973), Differential methylation of mitochondrial and nuclear DNA in cultured mouse, hamster and virus-transformed hamster cells, *in vivo* and *in vitro* methylation, *J. Mol. Biol.,* **80**, 155-175.
45. Lapeyre,J.N., and Becker,F.F. (1980), Analysis of highly repeated DNA sequences of rat with EcoRI endonuclease, *Biochim. Biophys. Acta,* **607**, 23-35.
46. Simon,D., Grunert,G., v.Acken,U., Doring,H.P., and Kroger,H. (1978), DNA-methylase from regenerating rat liver: purification and characterization, *Nucleic Acids Res.,* **5**, 2153-2167.
47. Bradford,M.M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dyé binding, *Anal. Biochem.,* **72**, 248-254.
48. Schmidt,G., and Thannhauser,S.J. (1945), A method for the determination of DNA, RNA and phosphoproteins in animal tissues, - *J. Biol. Chem.,* **161**, 83-89.
49. Giles,K.W., and Myers,A. (1965), Improved diphenylamine method for estimation of DNA, *Nature,* **206**, 93.
50. Becker,F.F., Stillman,D., and Sell,S. (1977), Serum α-fetoprotein in a mouse strain (C3H-A^{vy} fB) with spontaneous hepatocellular carcinomas, *Cancer Res.,* **37**, 870-872.
51. Becker,F.F., and Sell,S. (1979), α-Fetoprotein levels and hepatic alterations during chemical carcinogenesis in C57B4/6N, *Cancer Res.,* **39**, 3491-3494.
52. Walker,M.S., and Becker,F.F. (1981), Methylase activity of normal liver, regenerating liver, and a transplantable hepatocellular carcinoma, *Cancer Biochem. Biophys.* in press.
53. Rodriguez,L.V., and Becker,F.F. (1979), Analysis of chromosomal proteins of fractionated chromatin from rat liver and transplantable hepatocellular carcinomas, *Int. J. Cancer,* **24**, 490-497.
54. Scarano,E. (1971), The control of gene function in cell differentiation and in embryogenesis, *Adv. Cytopharmacol.,* **1**, 13-23.
55. Holliday,R., and Pugh,J.E. (1975), DNA modification mechanisms and gene activity during development, *Science (Wash.),* **187**, 226-232.
56. Riggs,A.D. (1975), X inactivation, differentiation, and DNA methylation, *Cytogenet. Cell Genet.,* **14**, 9-25.
57. Sager,R., and Kitchin,R. (1975), Selective silencing of eukaryotic DNA, *Science (Wash.),* **189**, 426-433.