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Active gene sequences are undermethylated

(DNA methylation/deoxyribonuclease I)

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ABSTRACT **The degree of methylation of active regions of the chromosome has been investigated by several techniques. DNase I (deoxyribonuclease I, EC 3.1.21.1) was used to introduce nicks in the active regions of the nucleus and thereby specifically label these areas. By using the methylation-specific restriction enzymes *Hpa* II and *Hha* I it could be shown that active genes are more sensitive to these probes than are other parts of the genome. In order to measure the amount of methylation at all CpG residues, DNA was nick-translated in the presence of [α-^{32}P]dGTP as the sole nucleotide source and the methylated cytosine was detected by the standard nearest-neighbor analysis. Using this assay, we found that about 70% of all CpG sequences in animal cell DNA are methylated. In active nuclear regions that are sensitive to DNase I, only 30–40% of the CpG residues are methylated. This method was also employed to study the gene sequences that are complementary to cellular RNA. By this criterion expressed gene sequences are only 20–30% methylated. These data suggest that undermethylation is a general phenomenon in all actively transcribed genes.**

The factors that control gene expression in eukaryotes have not yet been well elucidated, and even in the case of specific individual genes one cannot pinpoint the exact chromosomal changes that accompany differentiation. There seems to be, however, two levels of modification that may be correlated with gene activity.

At the level of the chromosome it is clear that many specific active genes are in a special chromatin conformation that makes them more available to several enzymatic probes and especially to DNase I (deoxyribonuclease I, EC 3.1.21.1) (1–3). In tissues that do not express these functions the gene is not in this unique conformation. This sensitivity to DNase I is a general phenomenon that seems to be true for all active genes regardless of their extent of expression (4, 5) and is correlated with the presence of the high mobility group proteins HMG 14 and HMG 17 on active nucleosomes (6, 7).

In addition to chromosomal alterations there is strong evidence that modification at the level of DNA may be correlated with changes in gene expression. By using methylation-sensitive restriction enzymes to probe specific sequences it has been shown in several systems that the methylation pattern of a particular gene is tissue specific, with a general correlation between gene expression and undermethylation (8–10).

This paper deals with the general relationship between gene activity and methylation. We find that gene sequences that are in a DNase I-sensitive conformation and transcribed to RNA are relatively undermethylated at all CpG residues, including those located at specific restriction sites.

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METHODS

Nucleic Acids. Nuclei were prepared from chicken erythrocytes, mouse L cells, or calf thymus by homogenization using 0.5% Nonidet P-40 in a polyamine-containing buffer (7). DNA was obtained from isolated nuclei by using proteinase K digestion, extractions with phenol and chloroform, and RNase treatment as described (11).

RNA was extracted from mouse liver (2–3 g) by mixing in a blender with 50 ml of 10 mM Tris·HCl, pH 9/1 mM EDTA/0.04 M NaCl/1% $NaDodSO_4$/0.1 mg/ml of heparin per ml and 50 ml of saturated phenol containing 0.08% 8-hydroxyquinoline and 10% cresol for three bursts of 10 sec. The homogenate was mixed for 20 min at room temperature before separating the phases by centrifugation. After two additional phenol extractions the RNA was precipitated with 2 vol of ethanol and excess DNA was removed by LiCl precipitation (12). Poly(A)-containing RNA was obtained by passing 15 mg of the total RNA over a 5-ml oligo(dT) column equilibrated with 10 mM Tris·HCl, pH 7.9/0.3 M NaCl/0.1% $NaDodSO_4$ (13). The poly(A)-containing RNA (A_{260}/A_{280} = 2) was eluted with 10 mM Tris·HCl, pH 7.9/0.1% $NaDodSO_4$. Approximately 2% of the total RNA was found to contain poly(A). This RNA appeared relatively undegraded as determined by RNA gel electrophoresis (14).

Nick-Translation. Nuclei were washed and resuspended in 50 mM Tris·HCl, pH 7.9/5 mM $MgCl_2$/10 mM 2-mercaptoethanol at a concentration of 1 mg/ml and incubated with 0.05–0.2 μg of DNase I per ml at 37°C for 7 min. Polymerization was carried out by the addition of bovine serum albumin at 50 μg/ml, 4 μM dATP, dTTP, dCTP, and dGTP, with one of these nucleotides labeled with ^{32}P at the α position (New England Nuclear, specific activity 300–500 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and 10 units of DNA polymerase I (New England BioLabs) per ml at 15°C for 10–15 min. The labeled DNA was purified by phenol and chloroform extractions and the unincorporated nucleotides were removed by Sephadex G-50 column chromatography. The specific activity of the DNA obtained by this method was generally about 16,000 cpm/μg. Naked DNA was nick-translated to a specific activity of 6×10^6 cpm/μg in the same manner using, however, 0.5 μg of DNase I per ml. When necessary, nick-translated DNA was fractionated by sucrose gradient centrifugation (15) and only DNA larger than 30 kilobases was used for restriction enzyme analysis. It should be noted that the degree of replacement of the nucleotides in nuclear DNA is extremely small even if one takes into consideration that the DNase-sensitive regions of chromatin may represent 10–20% of the genome. Labeled naked DNA that underwent 500 times as much nucleotide replacement as nuclear DNA did not lose a significant number of methyl groups, as shown by the fact that *Hpa* II digested total and radioactive

DNA to the same extent (Table 1). Because *Hpa* II will not cut a hemimethylated C-C-G-G site (unpublished results), the replacement of one strand of the DNA with nonmethylated nucleotides will not affect the digestion pattern.

Agarose Gel Electrophoresis. Electrophoreses on gels of 0.6% agarose were run at 20 V for 20 hr at room temperature. Ethidium bromide-stained gels were photographed under short-wave ultraviolet light with Polaroid type 665 positive–negative film. Gels were then dried and autoradiographed. The film negatives and the autoradiographs were scanned with a Gilford spectrophotometer in the visible light range (600 nm). The scans were digitized by computer and the number-average molecular weights were calculated by reference to marker fragments.

Nearest-Neighbor Analysis. In order to determine the degree of methylation at the dinucleotide sequence CpG, DNA or nuclei were nick-translated in the usual manner, using [α-^{32}P]dGTP as the sole nucleotide source. The purified labeled DNA was digested to deoxynucleoside 3'-monophosphates in 100 mM Tris·HCl, pH 8.5/10 mM $CaCl_2$ containing micrococcal nuclease (Sigma) at 140 μg/ml and spleen phosphodiesterase (Worthington) at 7 units/ml for 4 hr at 37°C. A sample of this digest was applied directly to thin-layer cellulose chromatography sheets (Eastman Kodak) and chromatographed in two dimensions (16). The radioactive spots were quantitated by scintillation counting. In addition to the five expected nucleoside monophosphates we frequently obtained several additional spots, including one that migrates close to Ap. Although we have not identified these spots, they do not seem to interfere with the nearest-neighbor analysis. In all cases the relative amounts of ApG, TpG, GpG, and CpG were close to the values reported in the literature (17).

DNA·RNA Hybridization. Liver [α-^{32}P]dGTP-labeled DNA (5 μg) was denatured for 10 min at 100 °C and incubated with poly(A)-containing liver RNA (200 μg) in 100 μl of 10 mM Tris·HCl, pH 7.8/0.4 M NaCl/0.1% $NaDodSO_4$ for 50 hr at 70°C (R_0t, the product of RNA concentration in moles of nucleotide per liter and incubation time in sec = 1000). After this incubation the hybridization mixture was diluted into 3 ml of 10 mM Tris·HCl pH 7.9/0.3 M NaCl/0.1% $NaDodSO_4$ and passed over an oligo(dT)-cellulose column. Poly(A)-containing hybrids were then analyzed by the nearest-neighbor technology described above. Using these conditions, we obtained 7–10% hybridization of the DNA probe as determined by S1 nuclease digestion and by hydroxyapatite column chromatography (18). DNA incubated in the absence of added RNA hybridized approximately 1%.

RESULTS

In order to assay the state of methylation in a large population of active genes we have taken advantage of a modification of the method of nick-translation for labeling the active portions of the genome. In this technique DNase I in low concentrations is incubated with isolated *nuclei*, and the resulting nicks are used as primer for DNA polymerase in the presence of radioactive nucleotides. Because DNase I preferentially attacks active regions, these genes are specifically labeled (5). DNA labeled in this fashion can then be used as a substrate for studying the nature of active chromatin and in particular its methylation pattern.

Several restriction enzymes have been found useful for detecting the presence of m^5C at specific sequences. Thus *Hpa* II (C-C-G-G), *Hha* I (G-C-C-G-C), and other enzymes are unable to cut at their recognition site if the appropriate cytosine residue is methylated (8, 19). When considering a large population of DNA sequences this means that any fraction of the DNA that is relatively undermethylated will be more susceptible to digestion by *Hpa* II or any of these other enzymes. In general the number-average size of the DNA fragments resulting from such a digestion will be directly proportional to the degree of methylation.

The amount of methylation at C-C-G-G sequences of active genes can thus be determined by comparing the digestion pattern of total DNA with that for active region DNA. To this end nuclei were nick-translated and the DNA was purified and subjected to *Hpa* II digestion and size analysis. In order to ensure an accurate measurement of the digestion products it was advantageous to start with a homogenous large size class of DNA. Whereas in many cases the nick-translated DNA was found to be large, in other cases the labeled DNA was fractionated by sucrose gradient centrifugation and only DNA larger than 30 kilobases was used for digestion analysis.

After restriction enzyme digestion and gel electrophoresis the size distribution of total and active DNA was determined by scanning the photographic negative of ethidium bromide-stained gels and the autoradiographic film. The number-average molecular weight of each population was determined by computer analysis of the size distribution scans by reference to molecular weight markers (Fig. 1). Total chicken erythrocyte DNA has an unmethylated *Hpa* II site approximately every 7000 base pairs. In striking contrast to this, labeled active region DNA has an unmethylated *Hpa* II site, on the average, every 3500 nucleotides.

In order to convincingly demonstrate that this decrease is indeed due to undermethylation at these sites, one must show that the sequence C-C-G-G is equally represented in active and total DNA. To this end, labeled DNA was digested with the enzyme *Msp* I, an isoschizomer of *Hpa* II that recognizes the same site (C-C-G-G) but will cut the DNA even if the internal C is methylated. As shown in Table 1, this site appears with equal frequency in both active and total DNA, indicating that the observed differences in the digestion pattern of *Hpa* II must be due to a real undermethylation of C-C-G-G sequences in the region of active genes. In order to rule out the possibility of an artifact caused by the use of the enzyme *Hpa* II, or due to the

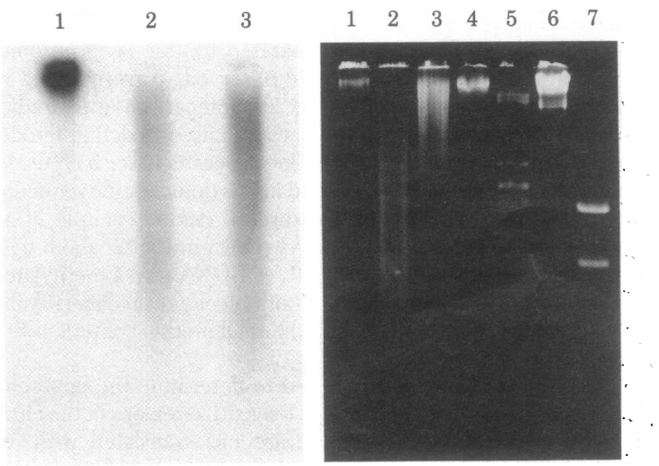

FIG. 1. *Hpa* II digestion of nick-translated DNA. Nick-translated DNA was fractionated according to size, digested by the enzymes *Msp* I and *Hpa* II, and analyzed by agarose gel electrophoresis (0.6%). (*Left*) Autoradiograph of agarose gel. Undigested (lane 1), *Msp* I-digested (lane 2), and *Hpa* II-digested (lane 3) nick-translated nuclear DNA. (*Right*) Ethidium bromide-stained gel containing undigested (lane 1), *Msp* I-digested (lane 2) and *Hpa* II-digested (lane 3) nick-translated naked DNA. Markers include λ DNA (lane 4) and λ DNA digested by *Eco*RI (lane 5) or *Kpn* I (lane 6) and pTk plasmid DNA digested by *Eco*RI (lane 7).

Table 1. Size analysis of *Hpa* II and *Msp* I digests of nick-translated DNA.

Restriction enzyme	Average molecular size, base pairs		
	Total DNA	Labeled DNA	Labeled nuclei
Hpa II	7000	6600	3500
Msp I	1900	2000	2100

DNAs obtained from nick-translated naked DNA or nick-translated nuclei were digested with *Msp* I or *Hpa* II and fractionated by 0.6% agarose gel electrophoresis (Fig. 1). The distribution of total DNA and labeled DNA was determined by scanning of the ethidium bromide-stained gels and the autoradiographs. Number-average molecular weights were calculated by computer analysis. The average distance between restriction enzyme sites (base pairs) displayed in the table was derived from the molecular weight and consideration of the measured size of the undigested DNA, which was usually 30,000–50,000 nucleotide pairs. The data shown in the table were in each case an average from a minimum of 10 separate experiments.

replacement of nucleotides in the active regions, naked DNA was randomly labeled by nick translation in the usual manner and subjected to *Hpa* II digestion. As shown in Table 1, the labeled DNA is cut by this enzyme in the same manner as total DNA. These quantitative data suggest that about 70% of the C-C-G-G sequences in total DNA are methylated, whereas in active regions, only 43% of these sites are methylated. Experiments using the enzyme *Hha* I (G-C-G-C) gave results similar to those obtained for *Hpa* II (data not shown), suggesting that this phenomenon may apply to other cellular CpG residues.

Although the experiments using restriction enzymes are fairly convincing, it should be born in mind that the methylated CpG sequences detected in that assay represent only a small portion of the methylatable sites in animal DNA. In order to analyze the total methylation picture of DNA we have developed a method that is capable of detecting every methylated cytosine in the dinucleotide CpG. In this technique DNA is randomly nick-translated by using DNase I and DNA polymerase I in the presence of the single nucleotide [α-^{32}P]dGTP. When this labeled DNA is then digested by micrococcal nuclease and spleen phosphodiesterase the ^{32}P label is transferred to the nearest neighbor of G on its 5′ side (20). When the resulting mononucleotides are separated by thin-layer chromatography, five separate spots are observed, corresponding to Gp, Tp, Ap, Cp, and m^{5}Cp (Fig. 2). Comparison of the radioactivity present in Cp and m^{5}Cp can be used to define the degree of methylation at CpG residues. Because over 95% of the cellular methyl moieties are found in this dinucleotide sequence (21), this assay essentially measures a random sample of all methyl groups in animal DNA. As shown in Fig. 2, about 65% of the CpG sequences of mouse L cell DNA is in a methylated form. This is in agreement with both biochemical data (16) and restriction enzyme data (22) on the total methyl content of this DNA.

This method may also be used to determine the degree of methylation in the active DNase I-sensitive regions of the chromosome. When isolated *nuclei* are nick-translated with [α-^{32}P]dGTP in the same manner as total naked DNA, the resulting label will be incorporated preferentially in the active gene regions due to the specificity of the nicking agent, DNase I. As can be seen in Fig. 2, the DNA labeled in this manner has a strikingly low percentage of methylated cytosine. This result was obtained with various degrees of DNase I nicking, but was slightly less pronounced with high concentrations of DNase I. This phenomenon is absolutely specific to active regions of the chromosome. In a control experiment nuclei were treated with 0.4 M NaCl in order to elute the high mobility group proteins

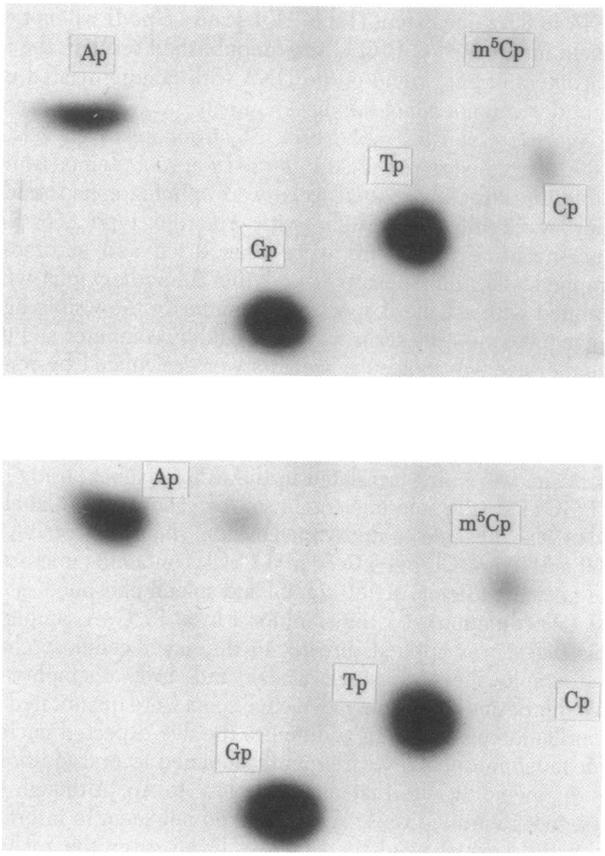

FIG. 2. Nearest-neighbor analysis of nick-translated DNA. Chicken erythrocyte nuclei (*Upper*) or naked DNA (*Lower*) were nick-translated using [α-^{32}P]dGTP, and the DNA was purified and enzymatically digested to nucleoside 3′-monophosphates, which were separated by two-dimensional thin-layer chromatography.

HMG 14 and 17 and thus remove the DNase I sensitivity from the active nucleosomes (6, 7). When these nuclei were nicked with DNase I and subjected to nearest-neighbor analysis, the m^{5}C content was similar to that found in total DNA (Table 2). The undermethylation observed for the active regions of nuclei was not unique to mouse L cells. Calf thymus nuclei and chicken erythrocyte nuclei gave similar results (Table 2).

From these data it is clear that areas that are considered active by reason of their sensitivity to DNase I are clearly undermethylated at all CpG sequences. In order to validate this correlation in a more direct manner we constructed an experiment that would determine the degree of methylation of all DNA sequences that undergo transcription to RNA. To this end deproteinized mouse liver DNA was nick-translated with [α-^{32}P]dGTP and hybridized to total mouse liver poly(A)-RNA. RNA·DNA hybrids were isolated by oligo (dT) chromatography and the ^{32}P-labeled DNA in these hybrids was subjected to

Table 2. 5-Methylcytosine content of nick-translated DNA

Template	m^{5}CpG, % of total CpG
Chicken erythrocyte DNA	66
Chicken erythrocyte nuclei	30
Chicken erythrocyte nuclei + 0.4 M NaCl	64
Calf thymus DNA	69
Calf thymus nuclei	42

DNA or nuclei were nick-translated with [α-^{32}P]dGTP and subjected to nearest-neighbor analysis. The results are expressed as the percent of total CpG sequence that are methylated.

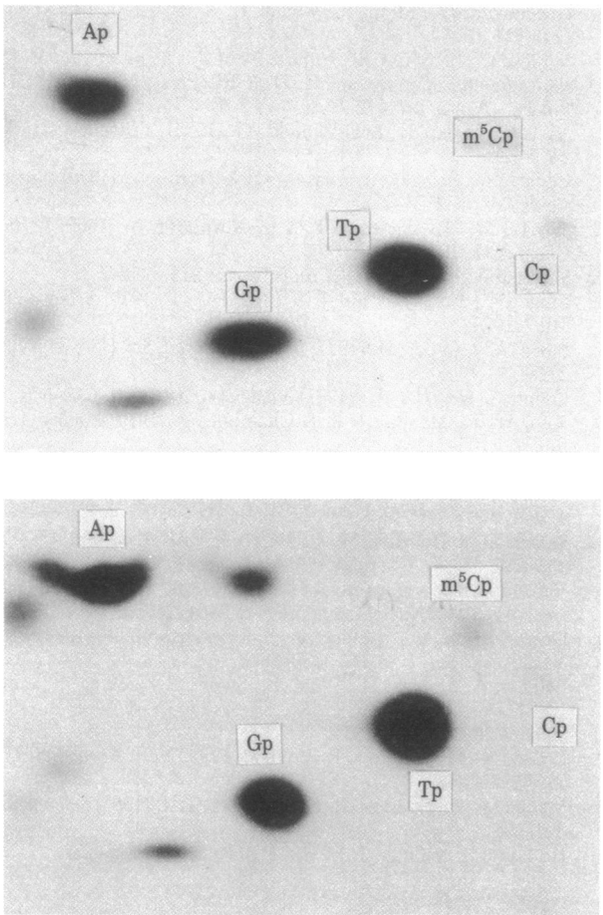

FIG. 3. Nearest-neighbor analysis of DNA complementary to poly-(A)-RNA. Mouse liver DNA was nick-translated with [α-^{32}P]dGTP and hybridized with total poly(A)-containing mouse liver RNA. After the reaction the DNA·RNA hybrids (*Upper*) were isolated and the DNA was subjected to the nearest-neighbor analysis as in Fig. 2. (*Lower*) Nearest-neighbor analysis of the DNA fraction that did not hybridize to RNA.

dized served as control. The results shown in Fig. 3 clearly demonstrate that the DNA sequences representing the actively expressed RNA are strikingly undermethylated. Whereas unhybridized DNA is 65% methylated at its CpG residues, the hybrid DNA was only 25% methylated at these sites. It should be noted that this hybridization was carried out to saturation. Methylation was analyzed after reaching 7–10% hybridization at a R_0t of 1000 M·sec. Thus these results are representative of most of the active gene population.

nearest-neighbor analysis. The DNA that remained unhybri-

DISCUSSION

The results of this paper clearly show that active genes are to a large extent undermethylated in comparison to the rest of the genome. Two different approaches were used to arrive at this conclusion.

Direct measurements of methylation in active regions obtained by analyzing DNA sequences that specifically hybridize to cellular RNA revealed a sharp undermethylation in active regions. These data suggest that transcribed sequences have 30–50% as many methyl groups as nontranscribed DNA. Because extensive evidence points to the fact that active genes in the nucleus are preferentially digested by DNase I, we took advantage of this to show that these same sensitive areas are undermethylated at CpG sequences in general and at several restriction sites in particular. According to this criterion, active regions are also about 50% as methylated as total DNA. In addition to the experiments presented in this paper, other data from our laboratory suggest a correlation between DNase I sensitivity and undermethylation. The early digestion products of DNase I on isolated nuclei when analyzed by mass spectrometry (23) were found to be low in m^{5}C. Furthermore, by using *Hpa* II methylase to assay the number of unmethylated C-C-G-G sites (24) it was shown that these sites in nuclei are preferentially digested by DNase I. Finally, the *Hpa* II site near the 5′ end of the ovalbumin gene is both undermethylated and sensitive to DNase I in the oviduct but not in other tissues (25). There is also a close correlation between DNase I sensitivity and undermethylation in the chicken hemoglobin gene family (26).

A considerable amount of evidence now suggests a strong correlation between specific gene activity and undermethylation (8–10, 26–27). In adenovirus-transformed hamster cells the expressed portion of the viral genome was found to be undermethylated in comparison to untranscribed adenovirus genes (28, 29), and a similar effect was observed for the methylation pattern of herpes saimiri virus (30). Despite this strong correlation it should be noted that many sites within the region of active genes remained methylated in all tissues (9), and other sites were found to be unmethylated in every tissue. Thus there may be a considerable number of sites that are not modulated during changes in gene expression.

Some data suggest that undermethylation may be a requirement for gene expression. The pBR322 plasmid containing the herpes virus thymidine kinase (*tk*) gene that was artificially methylated at its C-C-G-G sites was found to efficiently transfer this gene activity to *tk*$^-$ L cells in a DNA-mediated gene transfer experiment (11). Whereas most of the pBR322 sequences inherited their methyl groups in a semiconservative manner after integration into the cellular genome, certain sites within the *tk* gene itself always remained unmethylated (unpublished data). In an analysis of herpes virus *tk* L cell transformants and their revertants, one *tk*$^-$ revertant was found to be highly methylated at several restriction sites within the region of the *tk* gene (31). Other evidence suggests that X chromosome inactivation may involve modification at the level of DNA. Inactive X chromosome DNA, containing the hypoxanthine phosphoribosyltransferase gene (*hprt*), for example, was unable to transform *hgprt*$^-$ cells in a DNA-mediated gene transfer experiment (32). Furthermore, this gene could be induced from an inactive chromosome using 5-azacytidine, a compound that is known to lead to DNA demethylation (33).

The data in this paper greatly expand our current concepts with regard to the relationship between methylation and gene function, because they suggest that many, if not all, active gene sequences are subject to this modulation and that this phenomenon is true, not only at specific restriction sites but at all CpG sequences. Although our evidence shows a 50% decrease in methylation at active sites, this number must represent a minimum, because in all of the techniques used in this paper the "active genes" may contain other nonrelevant sequences.

Assuming the methylation pattern of cellular DNA indeed varies during differentiation, what is the mechanism of this process? In this regard it is of interest that in cases in which certain gene sites were undermethylated in a tissue-specific manner, these sites were always fully methylated in germ-line DNA (8–10, 26, 27). This suggests that there is a carefully directed demethylation of specific sites during differentiation, probably by a mechanism that involves several rounds of replication in the absence of methylation (34). Once this change takes place the new methylation pattern would probably be inherited in a semiconservative manner (11). It is worthwhile to point out that

these data provide a strong correlation between DNA modification and function but do not prove that changes in methylation control gene expression.

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