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Methylation of DNA in mouse early embryos, teratocarcinoma cells and adult tissues of mouse and rabbit

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ABSTRACT

The distribution and amount of 5-methylcytosine (5-MeCyt) in DNA was measured for early embryos of mouse strain CF1 (2 to 4 cell stage to blastocyst) and mouse teratocarcinoma cells. In each case, the pattern of methylation was examined by use of the restriction enzymes Hha I and Hpa II, which cut DNA at the sites 5'GCGC and 5'CCGG respectively, when the cytosines at these sites are not methylated. Mouse embryo DNA was found to have the same level of methylation as adult mouse tissues, and no changes in methylation were seen during differentiation of the teratocarcinoma cells. The ratio of 5-MeCyt/Cyt in DNA was measured by high performance liquid chromatography for the differentiating teratocarcinoma cells and for several adult mouse and rabbit tissues. The variation between tissues or between teratocarcinoma cells at different stages of differentiation was less than 10 percent. These results are discussed in view of proposals that 5-MeCyt plays a role in differentiation.

INTRODUCTION

The DNA of higher organisms contains 5-methylcytosine (5-MeCyt), most often in the sequence CpG (1). 5-MeCyt is formed by the enzymatic methylation of cytosine after its incorporation into DNA (2,3,4). Its function is not known, but the ubiquity and amount of 5-MeCyt (~ 3% of cytosine in mammals) suggests that it must play some important role. No restriction enzymes have been reported in eukaryotes, so a function different from that of the simple modification-restriction systems of bacteria seems likely. The work reported here was stimulated by the possibility that 5-MeCyt plays a role in cellular differentiation by means of its distribution in clonally inheritable, stable patterns on the DNA (5,6,7,8).

There are two primary reasons for considering the involvement of 5-MeCyt in differentiation. First, the conversion of cytosine to 5-MeCyt introduces a methyl group into the major groove of the DNA helix, and it has been clearly established that the binding of DNA to proteins such as the lac repressor, restriction methylases, histones, and hormone receptors, can be affected

profoundly by changes in the major groove (9,10,11,12). For example, the 5-methyl group of thymidine is critically important for lac repressor binding to the lac operator (13). Therefore, as pointed out earlier (6), the conversion of cytosine to 5-MeCyt definitely should affect protein-DNA interactions in chromatin and thus could affect gene regulation and differentiation. Second, in bacteria, DNA usually is methylated in both strands in symmetrical sites and some methylases have a very strong preference for methylating half-methylated (one strand methylated) sites. Unmethylated sites (neither strand methylated) are methylated very poorly (14). It has been pointed out by Riggs (6) and Holliday and Pugh (8) that this property could provide a mechanism for the stable clonal inheritance of a differentiated state.

With these concepts in mind, we feel that several experimental results support the idea that 5-MeCyt may be involved in differentiation. Bird and Southern (15) have shown that the two DNA strands of Xenopus ribosomal DNA are symmetrically methylated at most (but not all) 5'CCGG and 5'GCGC sites, and that the methylation pattern (presence or absence of 5-MeCyt at a certain site) is maintained through DNA replication, i.e., is inheritable. Waalwijk and Flavell (16) and McGhee and Ginder (17) have obtained evidence for tissue specific differences in the methylation of specific sites in globin DNA of rabbit and chicken. Earlier experiments have been interpreted as showing small tissue specific differences in the overall level of methylation (18,19).

We reasoned that if a clonally inheritable pattern of methylation is established during differentiation, then in the fertilized egg or early embryo, the DNA might be unmethylated (or at least undermethylated) so that the new patterns could be set down on a clean slate. An early mammalian embryo contains only picogram amounts of DNA, so the measurement of 5-MeCyt in embryos seems formidably difficult. Recently, however, the use of restriction enzymes to probe for methylation (15,20,21,22) has provided a method sensitive enough to analyze early embryos. We report here the results of such experiments on mouse embryos, as well as direct measurement by high performance liquid chromatography of the 5-MeCyt content of the DNA of teratocarcinoma cells and adult tissues. For the latter two cases, 5-MeCyt also was measured directly by high performance liquid chromatography (HPLC). We find that the overall level of DNA methylation characteristic of differentiated tissues already is established in the early embryo. Our results effectively eliminate one model for differentiation, but are consistent with an alternative model which we present in the Discussion.

EXPERIMENTAL PROCEDURES

Animals and DNA.

CF1 mice, 1 to 2 months old, were obtained from Charles River Breeding Lab., Inc. Male rabbits, 8 weeks old, were obtained from Mission Lab Supply. Calf thymus DNA came from Sigma. DNA was purified by the Marmur procedure (23). To assure high molecular weight of DNA, tissues were disrupted in liquid nitrogen, and sterile technique was used through the purification (24).

Embryo DNA.

Mouse teratocarcinoma cells were supplied by Gail R. Martin. Teratocarcinoma DNA was purified as previously described (25). ^{3}H-labelled mouse embryos were collected and labelled as previously described (26), with minor modifications. CF1 female mice, 8 to 14 weeks old, were superovulated (27) with 10 units of pregnant mare's serum (Gestyl, Organon), and treated 46 hours later with 15 units of human chorionic gonadotrophin (HCG) (A.P.L., Ayerst). Then females were placed with CF1 fertile males for up to 13 hours. Mated females were sacrificed at 46 to 48 hours, or 69 to 70 hours after administration of HCG to obtain embryos at the two- to four-cell and morula stage respectively. Cells were rinsed in BMOC-3 (Gibco) and placed in the same medium with ^{3}H-thymidine (New England Nuclear, spa, 20 Ci/mmole) at a final concentration of 2 µCi/ml. Cells labelled during blastocyst stage were obtained by sacrificing mated females at 64 hours after HCG administration and culturing them in vitro in BMOC-3 medium for 28 hours before adding ^{3}H-thymidine.

After labelling, cells were rinsed three times in sterile phosphate buffered saline (PBS). They were resuspended in 50 µl of PBS, to which was then added 500 µl of lysing solution (Tris-HCl, 10 mM, pH 8.0; EDTA, 9 mM; sodium dodecyl sulfate, 1%). Carrier mouse liver DNA (10 to 30 µg) was added, followed by addition of RNAse A (heat treated, 20 µg/ml final concentration) for one hour at 37°C. After treatment with proteinase K at 37°C for 1.5 hours, sodium perchlorate was added to 1 M, and the mixture extracted twice with chloroform;isoamyl alcohol (24:1). Two volumes of 95% ethanol were added, and the tubes stored at -20°C overnight. The precipitate was resuspended in Tris-HCl, 5 mM, pH 7.4; EDTA, 0.05 mM, and dialyzed against the same buffer. The final recovery was 30 to 50% of initial acid precipitable counts.

High Performance Liquid Chromatography.

The measurement of 5-MeCyt content of DNA by high performance liquid chromatography was done as previously described (25). Approximately 30 µg of purified DNA was hydrolyzed to bases with 100 µl of 88% formic acid at

180°C for 20 minutes. The hydrolysate was evaporated to dryness, dissolved in 0.1 M HCl, and 5 to 10 µg applied to a Partisil SCX K218 column (Reeve Angel) run at a pressure of 550 p.s.i. The bases were isocratically eluted in ammonium phosphate or formate buffer, pH 2.4, ranging in molarity from 0.01 to 0.045, depending on the age of the column. Bases were detected by their absorbance at 280 nM; areas under the peaks were compared by weight after correction for extinction coefficients.

Restriction Enzyme Analysis.

Eco RI was a gift from Dr. P. Greene. Hha I, Hpa II, Alu, and Hae III were from Bethesda Research Laboratories (BRL). Msp I was from New England Biolabs (NEBL). Taq I was from Worthington. Incubation conditions for digestion were those suggested by the suppliers. Reactions with Eco RI were in 100 mM Tris HCl (pH 7.2), 5 mM $MgCl_2$, 50 mM NaCl, 100 µg/ml autoclaved gelatin. Reactions were terminated as previously described (22). Reaction products were subjected to electrophoresis on 1 to 3% agarose gels. The gels were stained in ethidium bromide and photographed on negatives of Polaroid PN-55 film, then traced by using a Loyce-Loebl microdensitometer. The size observed (weight average length) was taken to be the axis bisecting the area under the peak in each tracing. For fluorography, agarose gels were soaked in methanol + PPO, dried, and exposed to X-ray film (Kodak X-OMAT R film, preflashed) as described (28). Films were traced by use of a microdensitometer as outlined above for negatives.

RESULTS

5-MeCyt Content of DNA of Adult Tissues.

High performance liquid chromatography (HPLC) is a sensitive technique that allows the detection of as little as 10 ng of 5-MeCyt in small amounts of unlabelled DNA and circumvents artifacts that sometimes result from radioactive labelling (25). The DNA is hydrolyzed to free bases, which are separated on an ion exchange HPLC column. Figure 1 shows a typical elution profile. The mole percent of cytosine converted to 5-MeCyt is obtained from the relative peak areas of the two bases in the profile. The G to C ratio serves as an internal control.

Table I summarizes the results obtained for DNA from several tissues of adult mouse and rabbit. No significant differences are seen between tissues of either mouse or rabbit DNA or between DNA of male and female mice. The significance of the low value for mouse heart is questionable, because the

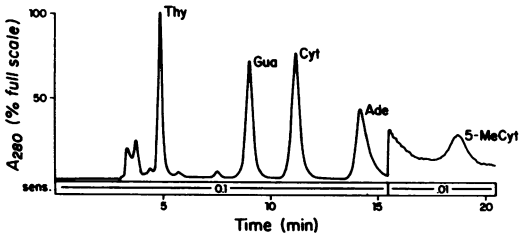

Figure 1. HPLC elution profile of bases from mouse spleen DNA. DNA (10 µg) was hydrolyzed in formic acid, applied to a Partisil SCX column, and eluted in 0.01 M NH_4COOH, pH 2.4, at a pressure of 500 p.s.i. The change in sensitivity of the ultraviolet monitor is indicated, either 0.1 or 0.01 full scale.

5-MeCyt content of rabbit heart DNA is the same as that from other rabbit tissues. We also find no significant difference when we compare chicken blood with chicken liver DNA or sea urchin sperm with pluteus DNA (results not shown).

Restriction of Adult Tissue DNA.

We and others have shown that restriction enzymes sensitive to the presence of 5-MeCyt can be used to probe methylation at specific sequences (15,16,20,21,22,29). The method can be used to estimate the percentage of

Table 1. 5-Methylcytosine Content of Adult Tissue DNA.

Source of DNA			Mole % $\frac{\text{5-MeCyt}}{\text{Cyt + 5-MeCyt}}$	Number of Determinations
Animal	Sex	Tissue		
Mouse	Male	Spleen	3.34 ± 0.08	3
Mouse	Male	Kidney	3.30 ± 0.04	3
Mouse	Female	Kidney	3.13 ± 0.14	4
Mouse	Female	Spleen	3.07 ± 0.22	4
Mouse	Female	Liver	2.74 ± 0.39	4
Mouse	Female	Brain	3.12 ± 0.16	4
Mouse	Female	Heart	2.39 ± 0.03	2
Rabbit	Male	Liver	2.61 ± 0.44	11
Rabbit	Male	Kidney	3.06 ± 0.20	10
Rabbit	Male	Heart	3.01 ± 0.28	7

DNA was purified and 5-MeCyt content analyzed by HPLC as described in Experimental Procedures. Significance figures are standard deviations except for mouse heart DNA, where the range is shown.

restriction sites methylated, and it has been demonstrated that the site 5'CCGG is highly methylated in mouse and rabbit DNA (16,20,22). Our aim here was to use restriction enzymes to test whether or not there was tissue specificity in the percentage methylation of CpG-containing sites.

We isolated DNA of high molecular weight, treated it with the restriction enzymes listed in Table 2, and electrophoresed the fragments on agarose gels. An example, showing the size distribution of mouse DNA treated with the enzymes Hpa II and Msp I, is shown in Figure 2. Quantitative data were obtained by the following procedures, which have proved to be reproducible to better than ten percent (22). Densitometer tracings of the ethidium bromide stained gels were made and, by cutting and weighing, we found the axis bisecting the peak of each tracing. The average length of DNA was obtained by comparison with standards (22).

Table 2 shows that, although several restriction enzymes cut mouse and rabbit DNA as expected, Hha I and Hpa II do not. The DNA fragments are 2 to

Table 2. Size of Adult Rabbit and Mouse DNA after Restriction.

Restriction enzyme	Site Recognized	Size Observed (Kbp)	Size predicted (Kbp)
Rabbit DNA			
Eco RI	G↓A A* T T C	4.1 ± 0.3	4.1
Hha I	G C G↓C	15.9 ± 1.1	3.4
Hpa II	C↓C* G G	6.8 ± 0.4	2.8
Hae III	G G↓C* C	0.7 ± 0.1	0.8
Msp I	C C G G	2.6 ± 0.1	2.8
Mouse DNA			
Hha I	G C G↓C	>18.0	5.1
Hpa II	C↓C* G G	17.8 ± 0.3	3.8
Msp I	C C G G	4.5 ± 0.5	3.8

DNA was treated with the enzymes listed, subjected to electrophoresis and the size determined as outlined in Experimental Procedures. Values for "size observed" for rabbit DNAs are averaged from DNA of liver, kidney and heart. "Size observed" for mouse DNAs are averaged from DNA of liver, spleen, kidney and brain. Differences between tissues were not significant (22). The size predicted (weight average length) was calculated based on the estimated size of 50 Kb for uncut DNA, and on the number of restriction sites expected from base composition and nearest neighbor analysis as described (22,34,43). The recognition sites for each restriction enzyme are taken from a summary by Roberts (44). Arrows indicate the site of cleavage. Asterisks show those bases, which, when methylated, prevent cutting by restriction enzymes (20,44).

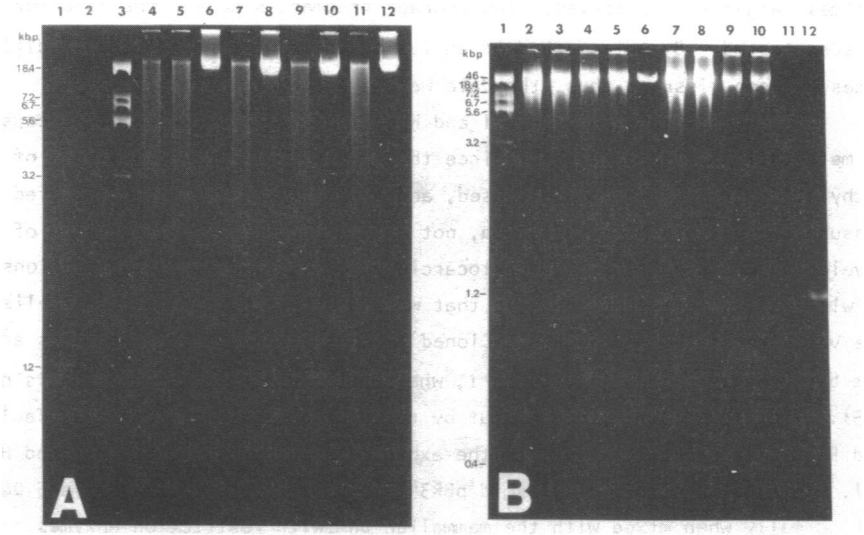

Figure 2. Restriction analysis of adult mouse DNA. (A) Agarose gel electrophoresis of DNA after digestion with restriction endonuclease Msp I. 1.5 µg of DNA from each tissue was digested with Msp I, applied to a 1.4% agarose gel, and electrophoresed as described in Experimental Procedures. Sizes shown are those of λplac 5 cut with Eco RI (41) and of pBR345 cut with Eco RI (42).

Lane 1, linear pBr345, 1 µg
2, pBR345 + Msp I, 2 U
3, λplac5 + Eco RI
4, mouse brain DNA + Msp I, 6 U
5, mouse brain DNA + Msp I, 4 U
6, mouse brain DNA, uncut
7, mouse spleen DNA + Msp I, 4 U
8, mouse spleen DNA, uncut
9, mouse kidney DNA + Msp I, 4 U
10, mouse kidney DNA, uncut
11, mouse liver DNA + Msp I, 4 U
12, mouse liver DNA, uncut

(B) Agarose gel electrophoresis of DNA after digestion with restriction endonuclease Hpa II. Two µg of DNA from each tissue were digested with Hpa II together with plasmid pBR345 as an internal control. The mixtures were applied to a 1.8% agarose gel and electrophoresed as described in Experimental Procedures.

Lane 1, λplac5 + Eco RI
2, mouse kidney DNA + Hpa II, 6 U
3, mouse kidney DNA + Hpa II, 3 U
4, mouse liver DNA + Hpa II, 6 U
5, mouse liver DNA + Hpa II, 3 U
6, λplac5 DNA
7, mouse spleen DNA + Hpa II, 6 U
8, mouse spleen DNA + Hpa II, 3 U
9, mouse brain DNA + Hpa II, 6 U
10, mouse brain DNA + Hpa II, 3 U
11, pBR345 + Hpa II
12, linear pBR345

4 times larger than expected. The average fragment size was the same for all tissues tested. By this method of analysis, there were no detectable differences between tissues; thus, the data were averaged for Table 2.

The lack of cutting by Hha I and Hpa II has been interpreted as caused by methylation of these sites. Since this restriction enzyme analysis of methylation is not yet commonly used, and since it was the method we used to measure the pattern of methylation, not only of adult tissue, but also of developing mouse embryos and teratocarcinoma cells (see following sections), we will review here some controls that we and others have done to establish the validity of the techniques. Cloned Xenopus ribosomal DNA sequences are Cut by the enzymes Hpa II and Hha I, whereas the natural uncloned DNA is not (15). The DNA we purify can be cut by restriction enzymes, since, as Table 2 and Figure 2a show, it is cut to the expected size by Msp I, Eco RI, and Hae III. Internal controls of plasmid pBR345 or bacteriophage lambda plac5 DNA are cut normally when mixed with the mammalian DNA with restriction enzymes. Additional purification of the DNA does not affect cutting by Hpa II or Hha I.

Msp I recognizes the same sequence as Hpa II and yet it cuts mammalian DNA normally. Thus, Msp I provides the important control showing that 5'CCGG sequences are present in mammalian DNA at the expected frequency. Cutting by Hpa II is prevented by the presence of 5-MeCyt (30). Moreover, Cedar, et al., (31) have shown recently that 5-MeCyt is at the 5' terminus of most DNA fragments produced by Msp I. Although such definite data are not available for Hha I, the most likely interpretation of the restriction data is that the sequence 5'GCGC is highly methylated.

From the data summarized in Table 2, we calculate that 70% to 80% of the Hha I sites (5'GCGC) are methylated in all tissues tested of mouse and rabbit. For the Hpa II site (5'CCGG), 75% to 85% is methylated in mouse tissues, whereas only 45% to 55% is methylated in rabbit tissues.

Restriction of Mouse Embryo DNA

Although adult tissues apparently are methylated to similar extents, we were interested in the possibility that early embryonic DNA might be completely unmethylated. We tested this hypothesis by the following experiment. Female mice of strain CF1 were treated to cause superovulation (26, 27). Embryos at the 2 to 4 cell stage, morula, and blastula were removed from the uteri, and cultured in vitro for 24 hours in the presence of ^{3}H-thymidine (26). The embryonic cells were lysed, mixed with carrier DNA from mouse liver, and the DNA was purified as described in Materials and Methods. The DNA was cut with

restriction enzymes, electrophoresed on agarose gels, stained with ethidium bromide to visualize adult DNA, and photographed. Then the gels were subjected to fluorography to visualize the embryonic DNA.

Figures 3 and 4 show that the size distribution appears exactly the same for the adult DNA and for the labelled embryo DNA. A comparison of lanes 1 and 3 of Figure 3a shows that the adult liver DNA was slightly reduced in

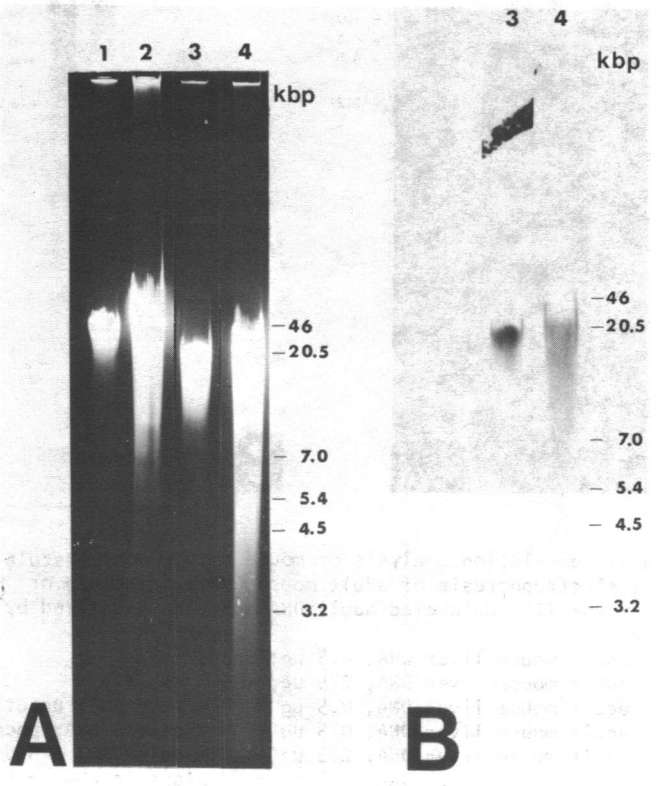

Figure 3. Hpa II restriction analysis of two- to four-cell mouse embryonic DNA. (A) Agarose gel electrophoresis of adult mouse DNA + ^{3}H-labeled two- to four-cell stage embryo DNA treated with Hpa II. Unlabeled adult DNA only is visualized by ethidium bromide staining.

Lane 1, adult mouse liver DNA, 0.75 µg; uncut.
2, adult mouse liver DNA, 2 µg; + Hpa II, 4.5 U
3, adult mouse liver DNA, 0.75 µg + ^{3}H-labeled two- to four-cell stage embryo DNA; uncut.
4, adult mouse liver DNA 3 µg + ^{3}H-labeled two- to four-cell stage embryo DNA; + Hpa II, 4.5 U.

(B) Embryonic DNA visualized by fluorography. The fluorogram is of lanes 3 and 4 of gel shown in 3A. Sizes shown are those of ^{3}H-labeled DNA treated with Eco RI (41), electrophoresed on the same gel.

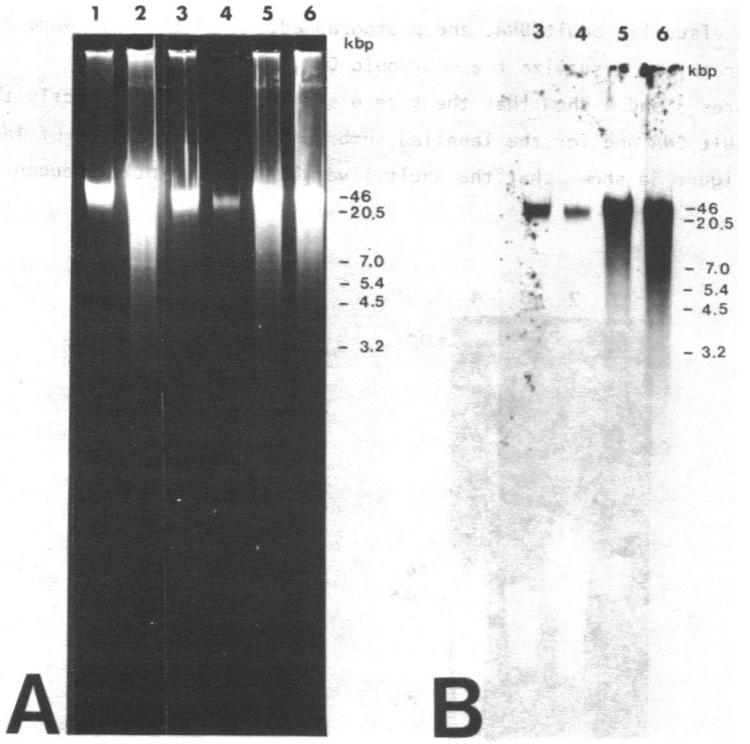

Figure 4. Hpa II restriction analysis of mouse morula and blastula DNA. (A) Agarose gel electrophoresis of adult mouse DNA + ^{3}H morula or ^{3}H blastula DNA treated with Hpa II. Unlabeled adult DNA only is visualized by ethidium bromide staining.

Lane 1, adult mouse liver DNA, 0.5 µg; uncut.
2, adult mouse liver DNA, 2.5 µg; + 5 U Hpa II.
3, adult mouse liver DNA, 0.5 µg + ^{3}H morula DNA; uncut.
4, adult mouse liver DNA, 0.5 µg + ^{3}H blastula DNA; uncut.
5, adult mouse liver DNA, 2.5 µg + ^{3}H morula DNA; + 5 U Hpa II.
6, adult mouse liver DNA, 2.5 µg + ^{3}H blastula DNA; + 5 U Hpa II.

(B) Embryonic DNA visualized by fluorography. The fluorogram is of lanes 3 to 6 of the gel shown in Fig. 4A. Sizes shown are as in Fig. 3.

size by its copurification with embryo DNA. However, the conclusion remains unambiguous because the percent protection from cutting by Hpa II of both the unlabelled and labelled DNA seen in Figure 4 is 70% and is very similar to that calculated for adult mouse DNA (Ref. 22 and previous section).

Similar results were obtained for ^{3}H-thymidine-labelled morula and blastula DNA cut with Hpa II (Figure 4). In addition, when these DNAs were

treated with Hha I and Msp I, they gave the same size distribution as adult DNA (results not shown). We conclude that there is little change (less than 10%, the limit of detection of our assay) in the overall level of methylation of the sites 5'CCGG and 5'GCGC between early embryos and adult tissues.

5-Methylcytosine Content and Restriction Pattern of DNA from Differentiating Teratocarcinoma Cells.

Direct measurement of the level of 5-MeCyt in mouse embryo DNA is impractical because of the difficulty of obtaining enough material. However, the finding that mouse teratocarcinoma cells can be made to differentiate in vitro (32,33) has enabled us to get enough DNA from differentiating cells to measure directly by high pressure liquid chromatography their ratio of 5-MeCyt/(5-MeCyt + Cyt). We also have treated the DNA with restriction enzymes sensitive to the presence of 5-MeCyt to measure indirectly any changes in methylation at the restriction sites.

The cells used were clonal lines from two mouse teratocarcinomas: LT, derived from an ovarian tumor, containing two X chromosomes; and PSA1, derived from an ectopic transfer of normal embryo, containing only one X chromosome, XO (33). These cells can be maintained in the undifferentiated state or they can be stimulated in vitro to form embryoid bodies, a process that closely resembles normal postimplantation development. Cells were harvested at 0, 4, 7, and 13 days after the start of in vitro embryoid body formation. During this time (at about 8 days), there is a twofold reduction in the activity of X-linked enzymes in LT cells, but not in PSA1 cells, suggesting that X inactivation, an event associated with the late blastocyst stage of embryonic differentiation, is occurring (33).

The DNA was purified and assayed for 5-MeCyt by HPLC. Table 3 shows

Table 3. 5-Methylcytosine Content of Differentiating Teratocarcinoma Cells.

Time of differentiation in culture	Mole % 5-MeCyt / Cyt + 5-MeCyt	
	Strain LT (XX)	Strain PSA 1 (XO)
0 days	2.73 ± 0.26	3.61 ± 0.10
4 days	2.56 ± 0.32	3.78 ± 0.36
7 days	2.91 ± 0.33	3.46 ± 0.44
13 days	3.32 ± 0.26	3.56 ± 0.26

DNA was purified and assayed for 5-MeCyt by HPLC as described in Experimental Procedures. Each value shown is an average of six determinations; significance figures are standard deviations.

that the ratio of 5-MeCyt/(Cyt + 5-MeCyt) does not vary significantly during differentiation of PSA1 strain. A small but statistically significant increase in methylation ($p < 0.01$) is seen 13 days after the start of differentiation of strain LT. The average 5-MeCyt content we observed, 2.9 ± 0.4% for strain LT and 3.6 ± 0.3% for PSA1, is comparable to what we and others have found for adult mouse tissue (22,34).

To probe for changes at specific sites, each DNA was treated with the restriction enzymes listed in Table 4, and the resulting size of the fragments determined from ethidium bromide-stained gels. No change occurred during differentiation of LT or PSA1 cells in the size of the fragments after treatment with any of the restriction enzymes tested. The values obtained at each stage for LT and PSA1 cells were averaged, and are shown in Table 4. These sizes are compared with those predicted from the frequency of occurrence of each restriction site.

As in the case of rabbit DNA, enzymes that have no CpG in their recognition sites (Eco R1, Hae III, and Alu) cut the DNA to the predicted size.

Table 4. Size of Teratocarcinoma DNA Fragments after Restriction.

Restriction Enzyme	Site Recognized	Size Observed (Kbp)		Size Predicted (Kbp)
		LT	PSA1	
Hha I	G C G↓C	16.6 ± 1.4 (4)	15.7 ± 0.6 (5)	5.0
Msp I	C C G G	3.0 ± 0.1 (4)	2.9 ± 0.1 (5)	3.5
Hpa II	C↓C* C G G	10.2 ± 1.1 (4)	10.9 ± 1.1 (5)	3.5
Eco R1	G↓A A* T T C	4.5 ± 0.3 (4)	4.6 ± 0.2 (5)	5.5
Hae III	G G↓C* C	0.85 ± 0.02 (4)	0.85 ± 0.02 (5)	0.76
Taq I	T↓C G A	2.9 (2)	2.9 ± 0.03 (3)	2.5
Alu	A G↓C T	0.62 ± 0.01 (4)	0.6 ± 0.02 (5)	0.43

DNA was purified from LT and PSA1 teratocarcinoma cells harvested 0, 4, 7, and 13 days after the start of differentiation. DNAs were treated with the enzymes listed, subjected to electrophoresis, and their size determined as outlined in Experimental Procedures. Values for "size observed" represent the averages for DNA from cells harvested 0, 4, 7, and 13 days after the start of embryoid body formation, since no difference was observed among these samples. The significance figures are standard deviations, and the numbers in parentheses are the number of determinations. The size predicted (weight average length) was calculated as described in the legend for Table 2, based on an estimated size of 30 Kb for uncut DNA. The recognition sites for each restriction enzyme are taken from a summary by Roberts (44). Arrows indicate the site of cleavage. Asterisks show those bases which, when methylated, prevent restriction enzyme cutting (30, 44).

However, the enzymes Hha I (5'GCGC) and Hpa II (5'CCGG) cut the DNA to a larger size than predicted, suggesting that the DNA of LT and PSA1 cells is approximately 70% methylated at both the Hha I and Hpa II sites. As before, the number of Hpa II sites present is as predicted, as seen from the size of the fragments cut by Msp I. The finding that Taq I cuts DNA to the predicted size implies that the site 5'TCGA is not methylated, or that, like Msp I, Taq I is insensitive to the presence of 5-MeCyt.

DISCUSSION

Adult Tissues.

The results obtained by HPLC clearly establish that in mouse and rabbit, the overall level of DNA methylation varies less than ten percent from tissue to tissue. Our data do not actually contradict that of earlier workers (18, 19) where differences between tissues were claimed. By labelling with ^{14}C-deoxyuridine, Kappler (18) found that embryonic chicken tissues contained from 3.6 to 4.2 mole percent 5-MeCyt/Cyt, with the average being 3.9 percent. Vanyushin (19) found significant differences between tissues of fish, but rat tissue 5-MeCyt content varied only 4 percent from the average.

The work of Waalwijk and Flavell (16) established that differences between tissues do occur when specific sites are observed. Recent results by McGhee and Ginder (17) confirm this observation. Our work, taken together with the earlier work, establishes that the specific changes must take place against a high background level that remains constant or nearly so. The restriction analysis results reported here establish that the overall percent methylation of the specific sites 5'CCGG (Hpa II) and 5'GCGC (Hha I) also does not vary more than ten percent. Seventy to eighty percent of the 5'GCGC (Hha I) sites are methylated in all mouse and rabbit tissue examined. The 5'CCGG (Hpa II) sites also show no tissue variation in percent methylation; however, it is important to note that there is significant species variation. Seventy-five to eighty-five percent of the 5'CCGG sites in the mouse are methylated, whereas only 45 to 55 percent of these same sites are methylated in the rabbit. This result suggests that constraints on the percent methylation of a specific site are loose enough to allow considerable variation between species. Many of the changeable sites must be in single copy and moderately repetitive sequences, since differences in satellite DNA alone cannot account for the differences we observe in fragment size. Each methylated 5'CCGG site contains one 5-MeCyt per strand (31), and if the same is

true for 5' GCGC these sites account for only about 12% of the total 5-MeCyt.

Although large differences between adult organ tissues of the same animal can be ruled out, it should be pointed out that organs are a mixture of cell types; thus small differences between different cell types could be easily obscured.

Embryos and Teratocarcinoma Cells.

Even though adult tissues are uniformly methylated, the possibility remained that egg DNA and/or early embryo DNA was unmethylated. We reasoned that if methylation of DNA did not occur during the first few rounds of DNA replication following fertilization, any earlier pattern of methylation would be eliminated and the embryonic DNA would be a clean slate upon which a new methylation pattern could be established during differentiation.

We have measured the methylation of embryonic DNA in two ways. The first involved the restriction enzymes Hpa II and Hha I and was sensitive enough to allow us to analyze, for the first time, DNA from early mouse embryos cultured in vitro. Our results clearly indicate that there are no major differences between embryo and adult DNA in the average percent methylation of 5'CCGG (Hpa II) and 5'GCGC (Hha I) sites. If a change occurs, it must be less than about ten percent.

The above results, which concern only two specific sites and only about 12% of the total methyl groups, were confirmed by a second type of experiment involving teratocarcinoma cell lines. The cell lines we used are thought to correspond to cells from early embryos prior to differentiation. In vitro differentiation to a variety of cell types and embryoid bodies can be induced by the removal of the feeder layer. A study of teratocarcinoma cells has the advantage that enough material can be obtained for direct HPLC analysis of total 5-MeCyt content. Prior to differentiation, the 5-MeCyt content of the two cell lines analyzed (strains LT and PSA1) is close to that of adult tissues and no change or only a slight change occurs during in vitro differentiation into embryoid bodies (Table 3). Restriction analysis experiments on the teratocarcinoma cells also show no change in methylation during differentiation for the sites 5'CCGG or 5'GCGC. Pollock, Swihart, and Taylor (35) recently established by HPLC analysis that, contrary to earlier reports (36,37), there is no change in 5-MeCyt level during sea urchin development.

A Demethylation Model.

The idea that the DNA of an early mammalian embryo would be unmethylated, thus allowing a methylation pattern to be set down on a clean slate during cellular differentiation is clearly untenable. The methylation model (Model

A) shown in Figure 5 is rendered unlikely by our results.

However, the idea that methylation is involved in differentiation remains attractive because, in addition to the logical arguments given in the introduction, several favorable experimental results have been obtained recently that are in agreement with earlier predictions (6,7,8). (1) Methylation is involved in the maternal inheritance of chloropoast DNA in Chlamydomonus (38); (2) Methylation is symmetrical (15,31); (3) The methylation pattern is inherited through DNA replication (15); (4) Maintenance type methylation enzymes (see Figure 5) with a preference for CpG and undermethylated DNA have been isolated (39,40); and (5) Waalwijk and Flavell (16) and McGhee and Ginder (17) have shown that there is tissue specific methylation at specific Hpa II sites (CCGG) within or near β-globin genes of rabbit and chicken.

Model B in Figure 5 is a demethylation model that we think is in keeping with all current experimental evidence and the emerging consensus that undermethylation is correlated with gene activity. According to this model, the DNA of an embryo is fully methylated, and all sites are saturated. During differentiation, certain key sites would be specifically demethylated by the specific binding of a protein to a given site, thereby stearically preventing the maintenance methylating enzyme from acting. The stem cells may remain methylated at the site, but after another round of DNA replication, the progeny cells would be unmethylated at the specific site. As we did earlier (6), we propose that in somatic cells, the maintenance enzyme cannot methylate *de novo*, i.e., it cannot methylate unmethylated sites. Thus, the change becomes permanent. After the initial specific demethylation, the continued presence

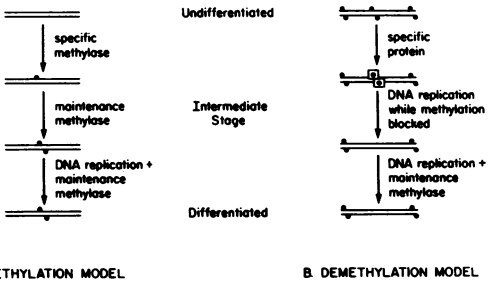

Figure 5. Models showing how the pattern of methylation of DNA might be stably altered to affect differentiation. The experiments described here rule out Model A, which assumes undermethylation early in development. Model B (see Discussion) assumes that specific demethylation events occur against a high background of noncritical methylation.

of the sequence specific protein would be unnecessary. The methylation system would not initiate specific changes, but would serve mainly to ensure stable clonal inheritance of the differentiated state. The main point we wish to make is that models based on demethylation retain *all* of the attractive features of earlier models based on methylation (6,8).

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