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Methylation of unique sequence DNA during spermatogenesis in mice

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ABSTRACT

In order to study whether changes in methylation of unique sequence DNA were related to meiosis, DNA was purified from F9 embryonal carcinoma (a "primordial germ cell" equivalent), germ cells from immature testes (containing germ cells up to early spermatocytes), sperm, and appropriate somatic tissues. Restriction was performed with the isoschizomers Msp I and Hpa II, and Eco RI as a control. Electrophoresis and Southern transfers were followed by hybridization to a mouse major β-globin clone (f7), a mouse pancreatic amylase clone (pMPa21), a type I, histocompatibility-2 clone (pH-2D-4), and a spermatid cDNA clone (pPM 459). The variably methylated sites were all hypomethylated in the embryonal carcinoma DNA and hypermethylated in DNA from immature testes and sperm, irrespective of the transcription state of the gene. The pattern in control tissues generally conformed to an inverse correlation of methylation with transcription. These results suggest that hypermethylation of sperm DNA persists from hypermethylation of these sequences early in testicular development, independent of gene expression.

INTRODUCTION

The widespread occurrence of m^5C has led to considerable speculation regarding its function. A primary focus of current research stems from the hypothesis that DNA methylation plays a role in gene regulation and cell differentiation (1, 2). Evidence showing a correlation between hypomethylation of specific sites, in the vicinity of a certain gene, and gene expression has come from studies on a variety of species and loci including: chicken--β-globin (3), α-globins (4), ovalbumin, ovotransferrin, and ovomucoid (5); rabbit--β-globin (6) and embryonic β-like globins (7); mouse--cell line immunoglobulin J chain (8) and heavy chain (9); and human--γ, δ, β-globins (10). Viral studies, in which free virions are generally found to be hypomethylated, whereas inactive, chromosomally-integrated or episomal proviruses are hypermethylated, support this correlation as well (11). These studies include Sv40 (12), adenoviruses (13), herpes viruses (14), and retroviruses (15).

Mammalian sperm DNA was reported to have a lower m^5C content than somatic DNA from the same species (16). This finding led to the hypothesis that the maintenance methylase is absent or inactive in germ cells during DNA replication immediately prior to meiosis resulting in hemimethylation of sperm DNA (17). Evidence supporting hemimethylation of repetitive elements in sperm DNA came from experiments involving denaturation and renaturation of sperm DNA to generate new hypomethylated sites (18), although the conclusions have been disputed (19). Previous reports indicate that variably methylated sites of two differentially regulated genes, the rabbit β-globin (6) and chicken ovalbumin (5) genes, are hypermethylated in sperm DNA while two genes for housekeeping functions, adenine phosphoribosyltransferase and dihydrofolate reductase, were not methylated differently in somatic tissue and sperm (20).

We wished to determine when during the course of sperm differentiation methylation patterns for differentiated genes are established, with special interest in meiosis as a possible critical event. We also wanted to know if sperm differentiation supports a methylation (1) or demethylation model of cell differentiation (21). Our approach was to digest genomic DNA from 1) germ cells from immature testis, 2) sperm, 3) F9 embryonal carcinoma, and 4) somatic control tissues with the restriction endonuclease isoschizomers, Msp I and Hpa II, separate the fragments by electrophoresis, and use the Southern blotting technique (22) to hybridize with specific gene probes. Since the majority of m^5C residues are found in 5'-CG-3' dinucleotide sequences (23, 24), the isoschizomers Msp I and Hpa II are ideally suited for studies of methylation. Both have the recognition sequence 5'-CCGG-3', however, Hpa II will not cleave DNA if the internal 3' cytosine is methylated (25), and Msp I will not cleave DNA if the external 5' cytosine is methylated (26).

F9 is a "nullipotent" embryonal carcinoma cell line derived from a testicular tumor that has long been maintained in culture (27). Although called "nullipotent", it has a low percentage of differentiation to primitive yolk sac-like cells (28) which can be greatly increased by treatment with trans-retinoic acid (29). Nonetheless, when grown in vivo, only small clusters of endoderm-like cells are found in addition to the embryonal carcinoma cells (28) and no H-2 antigen, normally found on differentiated embryonal carcinoma cell lines (30), can be detected (31). Even after treatment with retinoic acid, H-2 mRNA remains ≤1% the amount found in lymphoid cells (F. Benham, personal communication).

MATERIALS AND METHODS

Isolation of DNAs

Immature testis, sperm, and somatic tissue DNAs were obtained from C57BL/6J mice. Germ cells (spermatogonia and spermatocytes with about 10% somatic cell contamination) were prepared from testes of 18 and 19 day old mice by enzymatic digestion (32). Liver tissue was obtained from adult mice that had been starved for 2 days. Newborn livers were obtained from C57BL/6J mice on day 1 following birth. F9 DNA was obtained from tumors resulting from subcutaneous injection of F9 cells in 129/Sv male mice. Sperm DNA was purified as described by Shiurba and Nandi (33). Somatic and F9 DNA was purified by the proteinase K method (34). DNA purifications were carried out either immediately after tissue dissection or subsequently following freezing in liquid nitrogen and storage at -70°F.

Enzyme Digestions and Southern Analyses

The restriction enzymes Eco RI, Msp I, and Hpa II were obtained from Bethesda Research Laboratories and PL Biochemicals. Enzyme digestions varied from 4-24 hrs, and 2-4 times the amount of enzyme (8 to 96-fold the time x units recommended for digesting λ DNA). Horizontal agarose gel electrophoresis was carried out at room temperature in continuously circulating 36 mM Tris, 30 mM NaH_2PO_4, 1 mM Na_2EDTA buffer or in 0.04 M Tris-acetate, 0.002 M EDTA. The gels were 1% agarose, 5-6 mm thick, and 200 mm x 245 mm. The apparatus was run at a constant voltage of 1.6 volt-cm for 16-18 hrs. DNA was transferred to nitrocellulose or Gene Screen according to the procedure of Southern (22). Hybridization to the filters was carried out at 68°C according to the procedure of Jefferys and Flavell (35) or at 42°C in 40% formamide (Gene Screen Literature).

Unique Sequence Probes

The specific gene probes used were a mouse β-major globin clone, f7 (36), the gift of Dr. Carolyn Jahn; a mouse pancreatic amylase clone, pMPa21 (37), the gift of Dr. Peter Wellauer; a mouse type I, histocompatibility-2 clone, pH-2D-4 (38), the gift of Dr. Gabriel Gachelin; and a mouse spermatid cDNA clone, which is post-meiotically expressed (39). Nick translations of the probes were done with the NEK-004C kit of New England Nuclear. Kodak XAR-5 film was used for autoradiography. Eco RI digested λ DNA was run in parallel lanes to yield DNA fragments of known molecular weight in order to determine the size of the bands on autoradiograms. The filters were cut into strips for hybridization but any one figure represents strips hybridized with the

same probe and exposed for the same length of time. Reproducible findings from multiple gels are discussed.

RESULTS

β-Major Globin Methylation Patterns

The methylation pattern around the mouse β-major globin gene in F9 tumor DNA (Fig. 1) indicated that the methylation sites were unmethylated. Fragments of 6.5, 5.4, and 3.8 Kb were present in both Msp I and Hpa II digestions. Eco RI digestion revealed fragments of 12.5 and 5.4 Kb. Germ cells from immature testis and sperm DNA showed patterns (Fig. 1) which indicated that the methylation sites were hypermethylated and which were nearly identical. Msp I digestions revealed fragments of 5.2, 4.6, and 3.6 Kb, while a distinct band was missing in Hpa II digestions and a high molecular weight smear was present. Other Hpa II digests of the same DNA preparations give distinct bands for the other probes indicating that incomplete digestion was not the reason for absent bands corresponding to the Msp I bands. Eco RI digestions indicate a fragment of 6.5 Kb in both immature testis and sperm which may correspond to the 7.0 Kb fragment reported for Eco RI digestion of BALB/c liver DNA hybridized with f7 by Jahn et al. (36). DNA from newborn liver, which is about one-half hematopoietic tissue (Fig. 1), showed a pattern of hypomethylation of the CCGG sites. Msp I and Hpa II digestions showed fragments of 5.2, 4.6 and 3.6 Kb as was seen only with Msp I in immature testis and sperm. A Hpa II fragment of 17.0 Kb suggests that at least one site was partially methylated, perhaps hypermethylated in the nonerythropoietic tissue. Fragments of less than 3.4 Kb which had not been resolved in the other DNAs probed with f7, were seen as well. F9 and the hematopoietic tissue (newborn liver) both revealed unmethylated sites whereas immature testis and sperm, where β-globin is not expressed, show highly methylated sites. The noncorrespondence of Eco RI size fragments between F9 and the other DNAs could be indicative of a DNA rearrangement in the F9 tumor or a restriction enzyme polymorphism between C57BL/6J and the 129/Sv strain in which the tumor originated. The newborn liver DNA also gave a different Eco RI pattern but the bands were very light.

Amylase Methylation Patterns

F9 tumor DNA (Fig. 2) revealed a pattern of unmethylated sites with identical fragments of 19.0, 11.0 and 9.5 Kb, and several less than 1.0 Kb, in both Msp I and Hpa II digestions. DNA of germ cells from immature testes and sperm (Fig. 2) showed nearly identical patterns for their major bands and

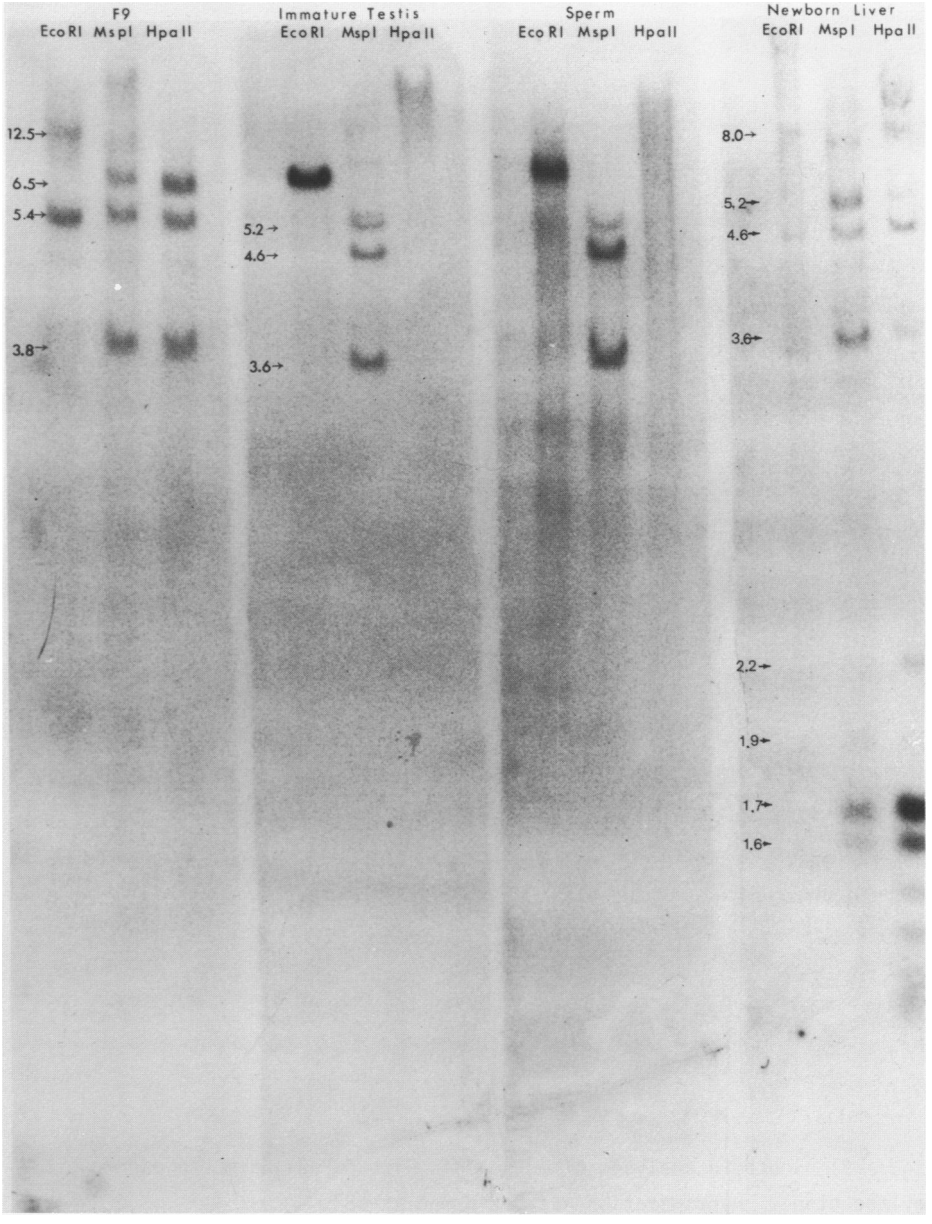

Figure 1: Southern analyses of mouse DNAs with f7, a mouse β-major globin clone. Hybridization was performed at 68°.

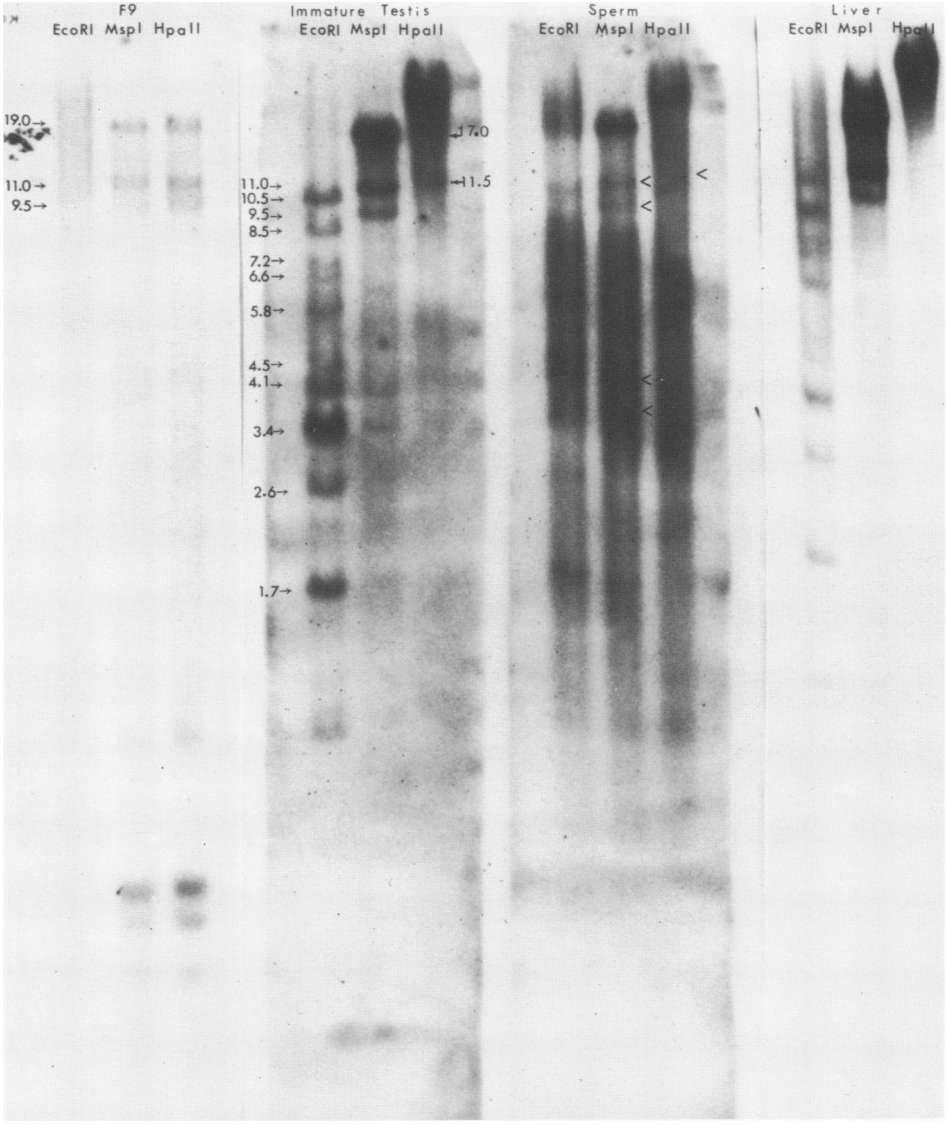

Figure 2: Southern analyses of mouse DNAs with pMPa21, a mouse pancreatic amylase clone. Hybridization was performed at 68°.

revealed highly methylated sites. Msp I digestions resulted in fragments of 19.0, 11.0, 9.5, 4.1 and 3.4 Kb while Hpa II digestions revealed a fragment of 11.5 Kb and an indistinct area of hybridization greater than 19.0 Kb. Eco RI digestions resulted in fragments ranging from 10.5 Kb to small fragments

less than 3 Kb. DNA from adult liver, which expresses a very low level of amylase (37), revealed a pattern similar to sperm and immature testis, except that no 11.5 Kb fragment was distinguishable in Hpa II digestions (Fig. 2). Thus, F9 teratocarcinoma DNA is unmethylated about the amylase genes whereas germ cells from immature testis and sperm show highly methylated sites. Liver DNA was also highly methylated. Although liver expresses the Amy 1^A gene to a slight extent (10^{-2} of salivary glands), perhaps only a small number of liver cells are expressing the gene, while the majority are not.

Type I, Histocompatibility-2 Methylation Patterns

F9 tumor DNA again revealed unmethylated sites, as Msp I and Hpa II digestions indicated identical fragments ranging from 12.0 Kb to small fragments less than 3 Kb from the multiple copies of these genes (Fig. 3). Germ cells from immature testis and sperm DNA revealed nearly identical patterns in which the sites were highly methylated. Msp I digestions resulted in many fragments ranging from 10.5 Kb to small fragments less than 3 Kb. Hpa II digestions resulted in larger fragments ranging from 14.5 to 4.2 Kb. Eco RI digestions resulted in many fragments ranging from 16.0 Kb to small fragments less than 3 Kb. F9 cells do not express H-2 antigens (31, 40, 41) while there is some controversy as to whether type I antigens are expressed on sperm (42-44). Thus, the result with the H-2 probe does not fit a simple model correlating hypomethylation with gene expression. However, there are many copies of the type I genes potentially obscuring methylation patterns of the expressed copies.

Methylation Pattern of Spermatid cDNA Clone Which is Post-Meiotically Expressed

Msp I digestion of immature testes, sperm, and liver DNA revealed bands of 3.9, 6.2 and 6.7 Kb while Hpa II digestions of the same DNA samples only revealed a smear of hybridizing DNA centered at 15.5 Kb (Fig. 4). Thus, this gene, which is transcribed in spermatids (39) is as methylated in immature testes and sperm as it is in liver, a non-expressing tissue.

DISCUSSION

As stated in the Introduction, our two major objectives were to determine when during sperm differentiation sperm-specific methylation patterns are first observed and whether a methylation or demethylation model of differentiation might be supported. Our studies were limited to determining methylation of the internal cytidine in CCGG sequences (but not including GGCCGG sequences) and, thus, only address a limited sample of sites. Nonethe-

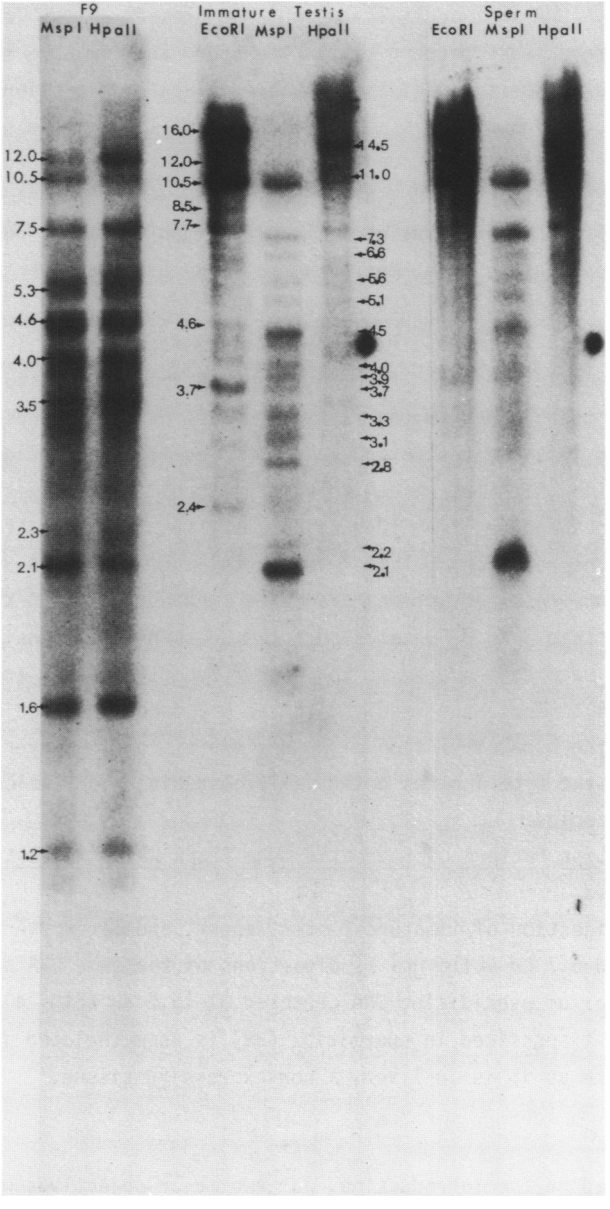

Figure 3: Southern analyses of mouse DNAs with pH-2D-4, a type I, histocompatibility-2 clone. Hybridization was performed at 68°.

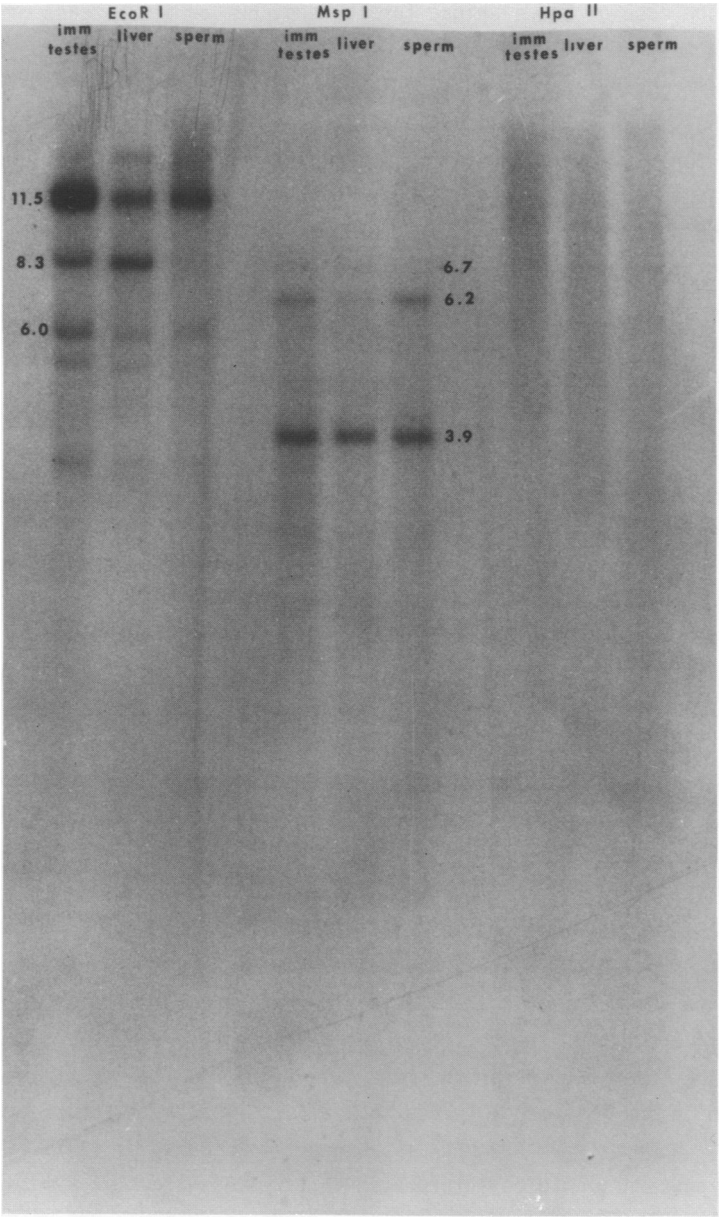

Figure 4: Southern analyses of mouse DNAs with pPM 459, a post-meiotically expressed, spermatid cDNA clone. Hybridization was performed in the presence of 40% formamide.

less, in the case of the 4 unique (or low copy number in the case of H-2) sequence genes examined, hypermethylated patterns were observed in sperm DNAs and were already present in DNA from a mixture of spermatogonia and spermatocytes. The highly methylated patterns are reminiscent of findings for the rabbit β-globin (6) and chicken ovalbumin genes (5) in sperm DNA. Meiosis does not appear to be a critical event involved with the establishment of methylation patterns as the methylation pattern is already established in germ cells from immature testis.

Our inclusion of the "nullipotent" (see Introduction) embryonal carcinoma, F9, in this study was designed to determine the degree of methylation of an available analogue of a primordial germ cell. The methylation model states that pluripotent germ cells are originally unmethylated and methylation occurs, inactiving certain genes, during differentiation. The demethylation model states that the pluripotent germ cells are originally fully methylated, and demethylation occurs resulting in the activation of certain genes during differentiation. Our findings in F9 tumor DNA indicated that three unique sequence genes examined were unmethylated. A previous study of a related embryonal carcinoma cell line revealed that the repetitive fragments were methylated and the authors argued for a demethylation model of differentiation (21). If F9 cells are truly developmental equivalents of pluripotent germ cells, our findings of hypomethylation for all three genes examined, which later become highly methylated in sperm, support a methylation model of differentiation. We believe that our findings for mouse β-major globin, pancreatic amylase, and type I histocompatibility-2 genes should be representative of the differentiation process.

The correlation of undermethylation and gene expression had many exceptions in our study: sperm with the H-2 and pPM 459 probes, and all the results with the F9 tumor. It is possible that very few of the heterogeneous liver cells are expressing the Amy 1^{A} gene and therefore a hypermethylated pattern might be expected for the majority of liver cells. Similarly, H-2 expression in sperm is probably due to a minority of the multiple copies of this gene. However, pPM 459 mRNA is abundant in spermatids (39). The F9 tumor DNA patterns could be interpreted in terms of a lack of a methylase in these cells. The finding of unmethylated sites in F9 tumor DNA using the H-2 probe differs from the results found with this probe in cultured F9 cells (G. Gachelin, personal communication). Other teratocarcinoma cell lines which spontaneously differentiate generally express H-2 genes on the differentiated cells (45). However, the hypomethylation of the β-globin and amylase genes

agrees with other studies that find hypomethylation to be a necessary but not sufficient correlation with gene expression (10), since there is no evidence for β-globin or amylase expression in these lines. Studies of the albumin gene in rat hepatoma cell lines also revealed that some cell lines did not express the gene even though the gene was hypomethylated (46). Studies on cultured human cell lines have also shown variation in methylation without apparent differences in gene expression (45).

Although early reports (16) indicated a lower m^5C level in spermatozoal than in somatic DNA, HPLC determinations of m^5C in mouse tissues revealed 0.83 mol% in sperm compared to 0.84 mol% in heart and lungs with a range of 0.89 to 1.09 mol% in other tissues and cells (48); a similar slight degree of hypomethylation was found in human sperm (0.84 mol%); a lower degree of methylation (0.76 mol%) was found in human placental DNA (49). Human sperm DNA (and placental DNA) are comparatively less methylated in the most repetitive fractions, a finding confirmed for bovine spermatozoal satellite I DNA (50). Thus, hypermethylation of differentiated genes, hypomethylation of repetitive elements, and no change in the methylation of "housekeeping" genes may result in the 0.83 mol% m^5C seen in spermatozoal DNA. The apparent increase in number of m^5C sites detected with an antibody to m^5C as spermatocytes proceed from early to mid-pachytene (51) may reflect changes in accessibility of antibody to m^5C secondary to changing chromatin structure, and not reflect changes in gene regulation. Our data do not support a finding of decreased methylation of unique sequence DNA during spermatogenesis.

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