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DIFFERENTIAL REGULATION OF DNA METHYLATION IN RAT TESTIS AND ITS REGULATION BY GONADOTROPIC HORMONES

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Summary—Eukaryotic DNA methylation occurs exclusively at the 5′-position of cytosine and has been implicated in the regulation of gene expression. Using high-performance liquid chromatography, the methylation of testis DNA during its development, in different cell populations and during regulation by gonadotropic hormones, were studied. The 5-mC content of testis DNA increased significantly from days 30 to days 150, while in 2-yr-old testis 5-mC content decreased significantly. Among various populations of testicular cells, pachytene spermatocyte DNA contained a significantly high amount of 5-mC when compared to spermatogonia, spermatids and mature sperm DNA. However, the 5-mC content of elongated spermadids was significantly less when compared to the above four fractions. Administration of follicle stimulating hormone to immature rats caused hypomethylation of seminiferous tubular DNA while luteinizing hormone caused similar effects in Leydig cells. These results indicate that in testis, DNA methylation is differentially regulated during development and is controlled by gonadotropic hormones.

INTRODUCTION

In mammals, approximately 3–5% of all cytosine residues are modified enzymatically in a post replication process to give 5-methylcytosine (5-mC) [1]. The pattern of cytosine methylation is species as well as tissue specific [2–4] and 90% or more of 5-mC is symmetrically distributed in GC doublets [5]. Patterns of cytosine methylation are also somatically heritable [6, 7]. There is now considerable evidence implicating DNA cytosine methylation as a regulatory signal for vertebrate gene control [1, 8–11] and also in genome inprinting during development [12, 13]. In general, methylation of specific cytosines appears to inactivate the genes, while demethylation of such sites permits transcription [14–16]. DNA methylation has also been shown to be involved in different processes such as X-chromosome inactivation [17], recombination during gametogenesis [18], differentiation [19], count down of the number of cell divisions [9], nucleosome structure and packaging [20] and condensation of DNA in metaphase chromosomes [21].

Recently it was shown that the seminal vesicle secretory protein gene is hypomethylated in seminal vesicles of adult rats [22]. It was also shown that after estrogen stimulation the vitellogenin gene becomes demethylated at its 5′-end in the liver and oviducts of chicks [23, 24]. However, other hormones like hydrocortisone and testosterone did not cause demethylation of genes in the respective hormone-dependent tissues [25, 26]. There are also reports indicating age related changes in 5-mC content of liver and brain in rats [4] and thymus and heart in cows [27].

In view of these varying effects on the hormonal regulation of DNA methylation, it would be of interest to study the effect of various hormones on different organs and factors regulating methylation of DNA. The present work reports on the 5-mC content of rat testis DNA during development and in different testicular cell populations. The effects of gonadotropic hormones on the regulations of DNA methylation in immature rat testis was also studied using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Animals and chemicals

Wistar strain male rats which were bred in our animal house were used in this study. Ovine follicle stimulating hormone (FSH) amd luteinizing hormone (LH) were provided by National Hormone and Pituitary Program, Bethesda, U.S.A. Standard DNA bases cytosine (C), adenine (A), thymine (T), guanine (G) 5-methylcytosine (5-mC), collagenase, Tris and sucrose were obtained from Sigma Chemical Co., U.S.A. HPLC grade methanol, acetonitrile and tetrahydrofuran were obtained from Spectrochem. Ltd, India. All other chemicals were of analytical grade and were procured locally.

Separation of spermatogenic populations

The spermatogenic populations were separated as previously described [28] with minor modifications.

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Briefly, testes from adult rats (160–180 g) were decapsulated and dispersed in buffer A (10 mM phosphate buffer, pH 7.5/150 mM NaCl/0.1% glucose) and sedimented at 800 *g* for 15 min. The sediment was suspended in buffer A and filtered through two layers of cheese cloth. Collagenase (200 μg/ml) was added to the filtrate and incubated at 34°C for 20 min. After incubation, the solution was sedimented at 800 *g* for 10 min. The pellet was washed with buffer B (10 mM Tris–HCl, pH 7.5/5 mM $MgCl_2$/1 mM $CaCl_2$/150 mM NaCl/0.1% glucose/0.5% bovine serum albumin) and centrifuged at 800 *g* for 10 min. The pellet was again washed twice with buffer B and finally suspended in 30 ml of buffer B, homogenized gently with glass homogenizer 7–8 times and filtered through two layers of cheese cloth. The filtrate was loaded into an elutriator rotor (Beckman Instruments, Palo Alto) with a standard elutriation chamber: fractions were collected and centrifuged at 10,000 *g* for 10 min at 4°C. Pure fractions which have 80–85% homogenity were selected and DNA was isolated. Isolation of spermatogonia from testes of 9–10-day-old rats was carried out as per the above procedure, all operations being carried out at 4°C.

Isolation of sonicate-resistant spermatid (SRS) nuclei from rat testis

The SRS nuclei (elongated spermatids, steps 13–19) from rat testis were isolated according to Platz *et al.*[29] with minor modifications. Briefly, testes from adult rats (150–180 *g*) were decapsulated and homogenized in 6–8 vol of buffer C (10 mM Tris–HCl, pH 7.4/0.1 mM phenyl methyl sulfonyl fluoride (PMSF)/ 0.34 M sucrose/0.1% Triton X-100). The homogenate was filtered through two layers of cheese cloth and centrifuged at 1000 *g* for 10 min. The crude nuclear pellet was suspended in 3–5 vol of buffer D (10 mM Tris–HCl, pH 7.4/0.1 mM PMSF) and subjected to sonication for 14–16 bursts each of 15 s with 45 s intervals. The sonicate was centrifuged at 1000 *g* for 10 min. The pellet was resuspended in 3–5 vol of buffer D and uniform suspension was layered on a 10 ml cushion of buffer D containing 1.5 M sucrose and centrifuged at 1000 *g* for 30 min. The final pellet was found to contain SRS nuclei (late spermatids, steps 13–19) of greater than 95% purity on examination under a light microscope. All operations were carried out at 4°C.

For isolation of sperm, the epididymides from adult rats (150–180 *g*) were removed and immediately minced with scissors in 4–5 vol of buffer E (50 mM Tris–HCl, pH 7.5/0.15 M NaCl), stirred at 25°C for 15 min and filtered through two layers of cheese cloth. The filtrate was centrifuged at 1500 *g* for 10 min and the pellet was suspended in distilled water for 30 min so as to lyse the erythrocytes and the lysate was centrifuged at 1500 *g* for 10 min at 4°C. The pellet was suspended in buffer E and sperm were observed under a light microscope.

For isolation of Leydig cells and seminiferous tubules, the decapsulated testes from 6–8 immature animals (aged 21–22 days) were pooled and incubated in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.1% collagenase, 1% bovine serum albumin and 10 mM glucose at 37°C for 30 min. Leydig cells and seminiferous tubules were separated as described [30].

Isolation of DNA

High molecular weight DNA was isolated according to Maniatis *et al.*[31] with some modifications. Briefly, tissues were homogenized in 10 vol of homogenizing buffer (10 mM Tris–HCl, Ph 7.8/0.15 M NaCl/0.05 M EDTA), SDS was added to make final concentration to 0.5% and the solution was incubated at 60°C for 15 min. The solution was extracted with equal volume of distilled phenol saturated with homogenizing buffer and once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and several times with chloroform/isoamylalcohol (24:1, v/v) until clear interface was obtained. DNA was precipitated with chilled ethanol, spooled on to a glass rod or centrifuged, washed with 80% ethanol, dried and dissolved in sterile 10 mM Tris–HCl (pH 8.0), 1 mM EDTA buffer. DNA was then treated with preboiled RNase (100 μg/ml) for 2 h at 37°C and with autodigested pronase (50 μg/ml) for 2 h at 37°C. The phenol and chloroform extraction steps were repeated and DNA was isolated as described above and finally it was dissolved in sterile buffer containing 10 mM Tris–HCl, 1 mM EDTA (pH 8.0) and stored at 4°C. For isolation of DNA from spermatogenic populations, the cells were suspended in homogenizing buffer containing 0.5% SDS and proteinase K (100 μg/ml) and lysate was incubated at 37°C for 4 h and phenol and chloroform extraction steps were repeated as described above and DNA was isolated.

HPLC analysis of DNA bases

10–40 μg of purified tissue DNA was suspended in 100–200 μl of 90% formic acid and sealed in glass tubes. The DNA was hydrolysed at 175–180°C for 25 min. The tubes were cooled and the hydrolysate was evaporated for complete dryness under vacuum. The residue was dissolved in sterile distilled water before HPLC analysis. About 15–20 μl of sample was applied to a column (Hypersil-ODS, 250 × 4 mm, Knauer) via an injection port and bases were isocratically eluted with a mixture of buffer containing 20 mM KH_2PO_4, pH 4.0/20 mM hexanesulfonic acid/methanol/acetronitrile/ and tetrahydrofuran (39:55:3:1.5:1.5, v/v). The flow rate of the elution buffer was 0.8 ml/min at a pressure of 300 psi. All samples were run at 0.0025 sensitivity scale on the u.v. detector to ensure detection of 5-methylcytosine. The data were plotted and processed by a Shimadzu C-R3A chromatopac integrator. The DNA bases were identified by comparing with retention time of standards at 280 nm. The nanomolar amounts of all five DNA bases were calculated by comparing with the peak areas obtained by standard authentic bases.

To determine the precision and reproducibility of assay of 5-mC, standard mixtures of five DNA bases of different concentrations (10–300 ng) were analysed by HPLC. The percent relative standard deviation obtained from these analyses is less than 1% and the values are reproducible. The percentage of methylation was calculated as follows:

$$\% \text{ methylation} = \frac{\text{nmols 5-mC}}{\text{nmols 5-mC} + \text{nmols C}} \times 100$$

RESULTS

HPLC is a very sensitive technique that detects less than 1% of minor modified bases relative to the four common bases in DNA. We have adopted this technique for optimal separation of 5-mC from four bases to allow precise quantitation of the 5-mC/cytosine ratio. Figure 1 shows typical elution profile of standard and sample testis DNA bases. The percent methylation is obtained from the relative peak areas of the two bases cytosine and 5-mC in the profile. The guanine (G) and cytosine (C) ratio serves as an internal control. In our system it is possible to detect even 5 ng of 5-mC in the sample DNA. There was no degradation of 5-mC and C during formic acid hydrolysis at 175°C for 20 min. To check any degradation 5 μg of standard 5-mC or cytosine were hydrolysed in separate tubes and eluted on HPLC column in similar conditions to that of standard bases. Earlier it was also reported that formic acid hydrolysis does not break down 5-mC or cytosine [32, 33]. In this study the 5-mC content is slightly

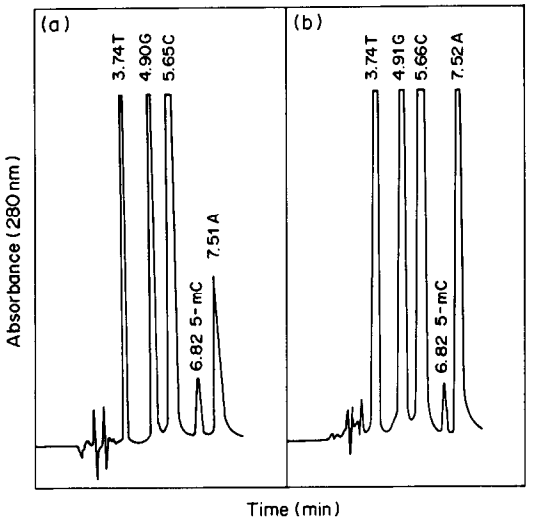

Fig. 1. HPLC elution profile of DNA bases. (a) Mixture of standard DNA bases containing 5-methylcytosine (5-mC, 20 ng), adenine (A, 200 ng), thymine (T, 200 ng), guanine (G, 200 ng) and cytosine (C, 200 ng) was injected to a HPLC column and eluted isocratically with elution buffer as described in "Materials and Methods". At the top of peaks numerals indicate retention time in minutes while capital letters designate the five bases T, G, C, 5-mC and A. (b) Isolated testis DNA was hydrolysed with formic acid and bases were separated.

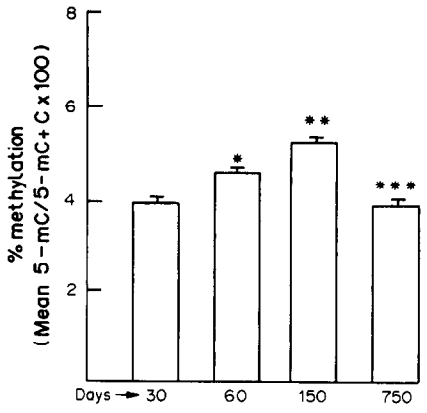

Fig. 2. 5-Methylcytosine content of rat testis DNA at various ages. Rat testes DNA from various age groups was prepared and 5-mC content was determined by HPLC analysis as described in "Materials and Methods". Each bar represented mean ± SEM of 3–4 observations. For each observation triplicate HPLC runs of acid digested DNA were performed and mean was taken. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to 30 day testis, $^{***}P < 0.0001$ as compared to 150 day testis. Significance is measured by the Student's t-test.

high compared to already reported data in other tissues in literature. The percentage methylation of tissues measured in this study such as ventral prostate, seminal vesicles and liver was 4.31, 3.55 and 4.41% respectively.

Figure 2 shows 5-mC content of rat testis DNA at various ages. The 5-mC content of rat testis DNA increased significantly from 30-day to 150-day-old rats. Afterwards the 5-mC content fell significantly in 2-yr-old testis when compared to 150-day-old rat testis. However, the 5-mC content of 2-yr-old testis DNA was similar to that of 30-day-old testis DNA. It was shown that in rat liver a significant increase in cytosine methylation has occurred between prenatal day 20 and 2 weeks postnatal, while the brain exhibited significant decrease in cytosine methylation between prenatal 15 day and 2 weeks postnatal followed by a significant increase by adulthood [4]. Other workers [34] showed 14–17% fall in the 5-mC content of rat brain and liver DNA between 1 and 12 months after birth. Age-related changes in DNA methylation content have also been reported for salmon, where 5-mC levels fell to half in various tissues during spawning [35]. In bovine, thymus and heart, DNA lose up to 27% of their 5-mC content during the 12 yr after birth [27]. The results presented in Fig. 2. indicate that during development of rat testis, the 5-mC content also changes with age.

Figure 3 shows the 5-mC content of different populations of rat testicular cells. The results indicate that the 5-mC content increased significantly from spermatogonia to pachytene nuclei and afterwards decreased significantly in elongated spermatids. The 5-mC content again increased in mature sperm when compared to that of elongated spermatids. However, there is no significant difference in the 5-mC content

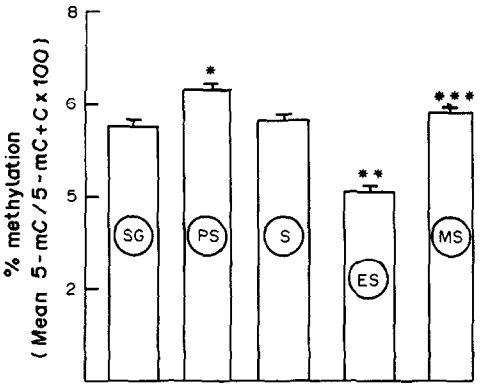

Fig. 3. 5-Methylcytosine content of rat testicular cells. Rat testicular cells populations spermatogonia (SG), pachytene spermatocytes (PS), spermatids (S), elongated spermatids (ES) and mature sperm (MS) were separated and DNA was prepared as described in "Materials and Methods". 5-mC content was estimated using HPLC. Values are mean ± SEM of 3–4 observations. $*P < 0.01$ compared to spermatogonia. $**P < 0.001$ compared to pachytene cells. $***P < 0.001$ compared to elongated spermatids.

of spermatogonia and sperm. These results show that during spermatogenesis the DNA methylation is differentially regulated in rat.

To study the effects of gonadotropic hormones on DNA methylation immature rats (aged 21–22 days) were used. These rats were treated with either FSH or LH and seminiferous tubules and Leydig cells were separated and 5-mC content was quantitated in these two fractions using HPLC. The 5-mC content of FSH-treated seminiferous tubular DNA decreased significantly over that of saline-treated controls (Fig. 4). LH has also caused significant reduction in the 5-mC content in Leydig cells.

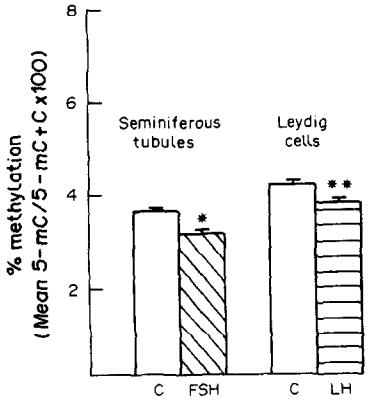

Fig. 4. Effects of gonadotropic hormones on 5-mC content of immature rat testis. Animals aged 21–22 days were injected subcutaneously with LH or FSH (50 μg/day) in saline at 0 h and animals were sacrificed 48 h after treatment. Seminiferous tubules and Leydig cells were separated after collagenase dispersion and 5-mC content was estimated by HPLC. Values represent mean ± SEM of four observations. $*P < 0.01$ as compared to saline treated control seminiferous tubules, $**P < 0.001$ as compared to saline treated control Leydig cells.

DISCUSSION

The data presented in Fig. 2 shows age-related changes in the 5-mC content of rat testis during development. The 5-mC content increased significantly from day 30 to day 150 and afterwards it fell significantly in 2-yr-old testis. The decrease in 5-mC content of bovine and rat DNA with increased age of the animals was also reported earlier in thymus and heart [27, 34]. Recently it was reported that in mice, the liver content of 5-mC is high at 6 months of age and decreases significantly in 2-yr-old animals [36]. Demethylation of mouse intracisternal A particle genes [37] and major mouse long interspersed DNA [38] was also observed in aged animals. As the patches of DNA produced on repair of damaged regions of DNA tend to be undermethylated [39] and as demethylating events are reported to increase on aging [27], greater level of recombination may occur leading to heightened levels of chromosomal rearrangements. It has also been shown that damage to hemimethylated DNA, especially alkali-labile lesions and single-stranded breaks, strongly inhibits the activity of mouse DNA methyltransferase [40]. It is also possible that the lower levels of testis DNA methyltransferase in aged animals may be responsible for decreased level of DNA cytosine methylation [41]. However, undermethylation of DNA in older animals is found largely in the moderately and highly repeated sequences where it can have little effect on coding potential [25]. This might lead to an elevated level of genome rearrangement and altered DNA-protein interactions, finally leading to aging process.

The results presented in Fig. 3 indicate differential regulation of DNA cytosine methylation during rat spermatogenesis. Recently it was reported that *de novo* methylation occurs between the spermatogonial and primary spermatocyte stage of chick spermatogenesis [42]. Using antibodies to 5-mC it was also reported that the 5-mC content of human testicular cells increased significantly from early to mid-pachytene spermatocyte stage of meiosis [43]. After second maturation division the elongated spermatids show low levels of DNA methylation. The 5-mC content increased significantly from elongated spermatids to sperm. Increased level of methylation in sperm DNA as compared to somatic tissue DNA has been found in most vertebrates [10]. Our results indicate that the differential regulation of DNA methylation could play a role in protein-DNA (or protamine) interactions during rat spermatogenesis.

Luteinizing hormone and follicle stimulating hormone regulate the postnatal development of the growth and maturation of testis in males. Our results presented in Fig. 4 show that both follicle stimulating hormone and luteinizing hormone caused hypomethylation of DNA in their respective target cells, seminiferous tubules and Leydig cells. It was shown earlier that FSH stimulates general protein synthesis [44], RNA synthesis [45], ODC [46, 47] and poly

(A) polymerase [48] in seminiferous tubules while LH stimulates synthesis of testosterone in Leydig cells [49]. FSH also stimulates DNA and androgen binding protein synthesis in Sertoli cells [50]. Spermatogonial cell divisions are said to be under FSH control [50]. Recently LH was shown to stimulate DNA topoisomerase I activity in Leydig cells after 24–48 h treatment [51]. LH also caused an increase in the replicative DNA synthesis in Leydig cells at 48 h intervals [52]. Human chorionic gonadotropin caused a transient activation of C-myc oncogene in Leydig cells [53], while FSH caused C-fos gene expression in Sertoli cells [54]. In an earlier study we observed inhibition of DNA methyltransferase activity by FSH in immature rat testis, however LH did not cause any effect [41]. It is possible that due to the presence of low populations of Leydig cells (3%) in testis it was not possible to detect changes in the DNA methyltransferase levels in Leydig cells. The present study shows that both LH and FSH caused hypomethylation of DNA in their respective target cells. It is possible that the observed hypomethylation of DNA by gonadotropic hormones may facilitate the expression of genes for protein synthesis in the testis by affecting protein-DNA interactions. This differential regulation of DNA methylation during spermatogenesis might play a role in their transcriptional activation of genes. These possibilities can be tested by isolating genes specific for spermatogonial and postmeiotic stages and determining the methylation pattern and chromatin structure during various stages of rat spermatogenesis. The effects of gonadotropic hormones on specific genes of the testis needs further ir.vestigation.

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