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Chicken spermatogenesis is accompanied by a genomic-wide loss of DNA methylation

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Renaturation kinetics of DNA obtained from chicken testis cell nuclei separated by sedimentation at unit gravity showed that the undermethylation, previously observed in meiotic and postmeiotic cells, is not a peculiarity of repetitive sequences, but is also a feature of unique sequences. The large proportion of slowly renaturing, intermediately renaturing and rapidly renaturing DNAs contain 27, 32 and 31% less methylcytosines in meiotic and postmeiotic cells than the corresponding fractions of premeiotic cells. DNA methyltransferase activity is lower in meiotic and postmeiotic cells containing undermethylated DNA than in immature testis, enriched in spermatogonia, with higher levels of DNA methylation.

DNA methylation; Hypomethylation; Spermatogenesis; DNA methyltransferase; Enzyme activity

1. INTRODUCTION

In a previous study, we reported that the DNA of meiotic and postmeiotic chicken testis cells appears partially undermethylated, containing approx. 30% less methylcytosines than DNA obtained from somatic cells, such as liver cells or erythrocytes, and also 30% less than a fraction enriched in diploid premeiotic nuclei [1].

In order to determine whether genomic-wide hypomethylation is induced during spermatogenesis or if undermethylation is restricted to certain DNA sequences, we have here analyzed by HPLC the m^5C content of slowly renaturing, intermediately renaturing and rapidly renaturing DNA sequences obtained from chicken testis cell nuclei at successive stages of spermatogenesis. We have also assayed the DNA methyltransferase activity to determine a possible correlation between this enzymatic activity and the observed levels of DNA methylation.

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Abbreviations: HPLC, high-performance liquid chromatography; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]-methionine

2. MATERIALS AND METHODS

2.1. *Separation of chicken testis cell nuclei by sedimentation at unit gravity*

Sexually immature (6-week-old) and sexually mature (25-50-week-old) Hubbard White Mountain chickens were used in the experiments. Cell nuclei obtained from chicken testis were purified in citric acid and separated by sedimentation at unit gravity [2].

2.2. *DNA isolation and reassociation kinetics*

DNA was isolated as in [3]. The renaturation kinetics was investigated as described by Britten et al. [4]. DNA was fractionated according to its renaturation kinetics and each component was dialyzed against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.3 M NaCl and ethanol-precipitated.

2.3. *Determination of methylcytosine content of DNA by reversed-phase HPLC*

DNA samples were quantitatively hydrolyzed with DNase I, nuclease P_1 and bacterial alkaline phosphatase [5] and the resulting deoxyribonucleosides were separated by reversed-phase HPLC as described [1].

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2.4. *Assay of DNA methyltransferase activity*

Chicken testis was minced finely with scissors and homogenized in a medium containing 2 M sucrose, 10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF, by means of 15 strokes with a Teflon/glass homogenizer. Homogenates were filtered through miracloth and centrifuged at $52000 \times g$ for 60 min at 4°C. Nuclei were extracted with 0.35 M NaCl, 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10% glycerol as described by Simon et al. [6]. RNA was eliminated from the extract by DEAE-cellulose chromatography [7], the extract being dialyzed against 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM DTT, 1 mM PMSF and 10% glycerol. DNA methyltransferase activity was assayed in 200 μl of 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% glycerol, 2–5 μg hemimethylated *M. luteus* DNA and 10 μM [^{3}H]AdoMet (5 Ci/mmol). After incubation for 1 h at 37°C, reactions were stopped and processed as in [8].

3. RESULTS

3.1. *Loss of DNA methylation upon cell differentiation*

The profile of the reassociation kinetics obtained with total chicken DNA is shown in fig.1. The curve represents a composite of two repetitive components and a single-copy component. The relative amounts of the different kinetic classes show the existence of a very high proportion (87%) of slowly renaturing unique sequences (reassociation rate constant = 0.001); sequences in the intermediately renaturing class (reassociation rate constant = 0.226) account for a total of 7.8% of the DNA, while sequences in the very fast group (reassociation rate constant = 55.5) and foldback DNA account for 5.2% of chicken DNA. The renaturation profile obtained is in agreement with previous reports [9,10].

DNA obtained from chicken testis cell nuclei separated by sedimentation at unit gravity and fractionated according to its renaturation kinetics showed that in meiotic and postmeiotic cells, the large proportion of slowly renaturing, intermediately renaturing and rapidly renaturing DNAs contain 27, 32 and 31% less methylcytosines than the corresponding fractions of premeiotic cells (fig.2).

3.2. *DNA methyltransferase activity*

DNA methyltransferase activity, assayed in nuclear extracts, decreases in mature testis enriched in meiotic and postmeiotic cells (2.49 pmol $CH_3 \cdot mg^{-1}$ DNA $\cdot h^{-1} \cdot \mu g^{-1}$ protein), containing undermethylated DNA, relative to immature testis,

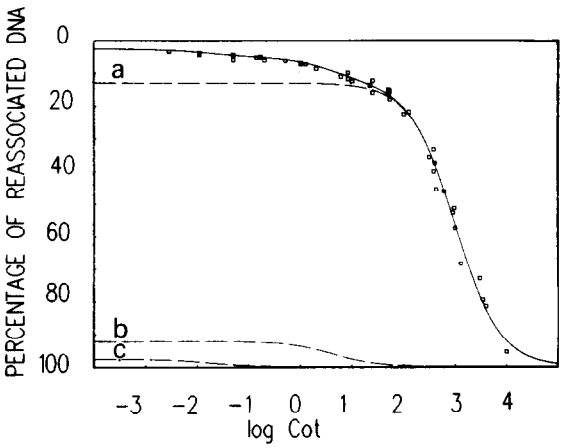

Fig.1. Reassociation kinetics of chicken DNA. Ordinate: percentage of reassociated DNA after incubation to various Cots. Cot is plotted on a log scale on the abscissa. A computer operating with a non-linear fitting procedure of the BMDP statistical software (University of California Press, Berkeley) has been used to match the renaturation data to the equation derived by Britten et al. [4]. Traces: (a) renaturation in whole DNA of fragments bearing only single-copy sequences (this component includes 87% of the DNA); (b) renaturation in whole DNA of fragments bearing intermediately repetitive sequences (this component includes 7.8% of the DNA); (c) renaturation in whole DNA of fragments bearing highly repetitive sequences (this component and the foldback DNA account for 5.4% of chicken DNA).

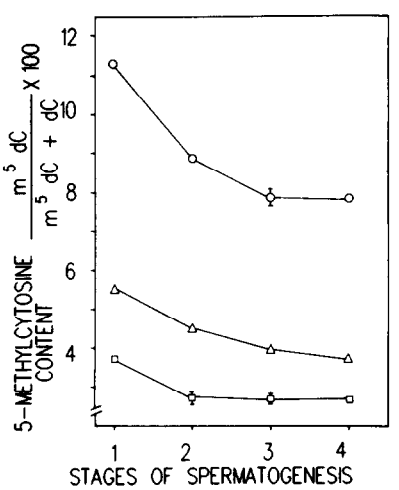

Fig.2. Mean methylcytosine levels in three different kinetic classes of chicken DNA obtained from chicken testis cell nuclei at successive stages of spermatogenesis. Methylcytosine content of DNA was determined by reversed-phase HPLC as indicated in section 2. Stages of spermatogenesis: diploid nuclei (1), tetraploid meiotic nuclei (2), haploid nuclei of round spermatids (3), haploid nuclei of elongated spermatids (4). Rapidly renaturing and foldback DNA (○—○), intermediately renaturing (△—△) and slowly renaturing DNA (□—□).

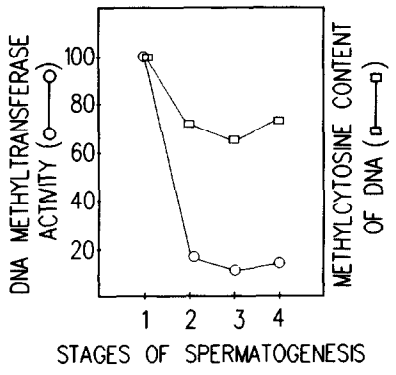

Fig.3. DNA methyltransferase activity and methylcytosine levels during chicken spermatogenesis. Chicken testis cells at different stages of spermatogenesis were obtained from immature testis enriched in spermatogonia (1) and by centrifugal elution [22]: meiotic and premeiotic cells (2), round spermatids (3) and elongated spermatids (4). The DNA methyltransferase activity (100%) corresponds to 14.31 pmol CH_3 incorporated $\cdot mg^{-1}$ DNA $\cdot h^{-1} \cdot \mu g^{-1}$ protein. Mean methylcytosine levels (100%) correspond to 4.52 ± 0.10 ($m^5dC/m^5dC + dC$) $\times$ 100.

enriched in spermatogonia (14.31 pmol $CH_3 \cdot mg^{-1}$ DNA $\cdot h^{-1} \cdot \mu g^{-1}$ protein), with higher levels of DNA methylation. The decrease in DNA methyltransferase activity was also observed on comparing immature testis enriched in spermatogonia with cell fractions enriched in meiotic and postmeiotic cells obtained by centrifugal elution (fig.3).

4. DISCUSSION

Previously it has been suggested that undermethylation of many sites in the satellite DNA can probably account for the lower level of methylation of sperm DNA [11]. Here we describe genomic-wide undermethylation occurring approximately in the same proportion (about 30% loss of m^5C) in both repetitive and unique DNA sequences. These results are consistent with and extend our previous studies on undermethylation of total DNA in meiotic and postmeiotic stages of chicken spermatogenesis [1]. Undermethylation of certain DNA sequences during spermatogenesis has been observed in different species [12–14]. Genomic-wide undermethylation occurs in mammalian extraembryonic tissues [15–17] and in cells induced to differentiate [18–20]. The physiological role of genome-wide undermethylation is unknown at present. Although the precise involvement, if any, of DNA methylation in the structural and functional changes undergone by chromatin during spermatogenesis or in the genomic stability of the male gamete cannot be deduced from the present data, the genomic-wide undermethylation observed during chicken spermatogenesis could be consistent with two proposed roles of hypomethylation: (i) Identification of DNA sequences that should be available to the machinery of transcription, recombination, repair or other genetic activities occurring during spermatogenesis or in preparation for embryonic development. (ii) Stabilization of the genome of the male gamete, avoiding, through undermethylation, the risk of spontaneous deamination of m^5C to thymine in critical DNA sequences. If a specific mismatch repair system [21] does not exist in the chicken male gamete, or if the DNA sequences are not accessible to the repair system, as a consequence of the compact structure of the nucleoprotamine complex, the sperm cell could be especially vulnerable to this mutagenic hazard at the internal temperature of the chicken (42°C), due to the propensity of m^5C residues to undergo thermal deamination to thymine residues. Many clusters of CpG have apparently been able to escape mutation through evolution because they are not methylated in the sperm cell [13].

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