Zygote **20** (November), pp. 339–345. © Cambridge University Press 2011
doi:10.1017/S0967199411000293 First Published Online 18 May 2011

Analysis of DNA looped domains organization during *Triturus cristatus* spermatogenesis

L. Burlibaşa[1]
University of Bucharest, Genetics Department, Bucharest, Romania

Date submitted: 01.10.10. Date accepted: 07.03.11

Summary

Chromatin from eukaryotes is organized in DNA loops with sequential attachments to a nucleoskeleton named nuclear matrix. This organization plays major roles in replication, transcription, recombination, DNA repair, chromosome condensation and segregation. During spermatogenesis, chromatin undergoes several dynamic transitions, which are often associated with important changes not only in its physical conformation but even in its compositions and structure. To understand the periodical change in the functional organization of chromatin during spermatogenesis, the higher order organization of chromatin in different testicular cell types (pachytene spermatocytes, round spermatids) and the epididymal sperm of *Triturus cristatus* have been investigated. The expansion and the contraction of nucleoid DNA were measured with a fluorescence microscope following exposure of nucleoids to increasing concentrations of ethidium bromide (EtBr) (2.5–200 μg/ml) as an intercalating dye to induce DNA-positive supercoils. Nucleoids from all studied cell types exhibited a biphasic change (condensed–relaxed–condensed) in size as a consequence of exposure to increasing concentrations of EtBr, indicating that they contained negatively supercoiled DNA. At higher EtBr concentrations, maximum positive supercoiling occurred in pachytene DNA loops. Our data suggest that pachytene DNA is the most open chromatin conformation in terms of EtBr intercalation.

Keywords: Chromatin, DNA loops, Nucleoids, Spermatogenesis

Introduction

Many eukaryotic genes are organized into functional domains that coordinate regulation during development (Dillon & Grosveld, 1993; Wykes & Krawetz, 1999). The ability of cells to regulate the genes included within such chromatin domains is key to the establishment of tissue-specific gene expression and cell differentiation (Bonifer *et al.*, 1997). The way in which the genome is packaged in interphase nuclei and metaphase chromosomes is of fundamental importance for understanding the mechanics of nuclear structure and basic cell function. DNA packaging may also play a significant role in developmental regulation of gene expression. For example, changes in DNase I sensitivity that can extent tens of kilobases beyond the dimensions of the involved gene itself accompany changes in gene expression and are believed to reflect some alterations in chromatin organization (Gasser & Laemmli, 1987; Gerdes *et al.*, 1994).

The investigation of specific sequences and their relationship to putative loop domain has relied upon indirect approaches involving extraction of isolated nuclei, DNA digestion, and subsequent fractionation by electrophoresis to characterize the DNA that binds the residual nuclear matrix (Gerdes *et al.*, 1994). These sequences thought to localize to the base of chromatin loops are frequently termed matrix-associated region (MARs) or scaffold-associated region (SARs).

Our current understanding of DNA organization in sperm nuclei is very weak in comparison with that for somatic cell nuclei. During spermatogenesis, DNA is condensed into a compact, transcriptionally inert mass. The mechanisms that govern DNA condensation appear to be highly divergent because the chromatin protein complements in sperm that originate from different species are variable (Bloch,

[1]All correspondence to: Liliana Burlibaşa. University of Bucharest, Genetics Department, No 1–3 Aleea Portocalilor, Bucharest, Romania. Tel:/Fax: +4 0213181565. e-mail: liliana_burlibasa@yahoo.com.au

1969). For example, sperm from *Carassius auratus* contains somatic histones and the DNA is organized into nucleosomes (Muñoz-Guerra *et al.*, 1982). Sperm from *Rana* contains histones with one or more sperm-specific histone variants (Kasinsky *et al.*, 1985). The *Arbacia* (Spadafora *et al.*, 1976) and *Holothuria* sperm (Cornudella & Rocha, 1979, reviewed by Risley *et al.*, 1986) contain nucleosomes, but the repeat lengths are greater than that typical of somatic cells.

In sperm of most other species, histones are replaced either partially or totally by sperm-specific proteins (SNBPs) like protamines or protamine-like proteins, which are more basic than histones and which vary in sequence between species (Bloch, 1969). The structure of chromatin in sperm that contains protamines (P) or protamine-like proteins (PL) is unclear as evidence has been presented to support both the presence (Sobhon *et al.*, 1982, reviewed by Risley *et al.*, 1986) and the absence (Balhorn, 1982; reviewed by Risley *et al.*, 1986) of nucleosome-like subunits in such sperm.

Chromatin organization that results from the interactions between the PL-P type proteins with DNA is quite different. Although PL and P proteins usually coexist with a small amount of histone in the sperm nucleus (Avramova *et al.*, 1984; Ausio, 1986; Gatewood *et al.*, 1987), the structure of the nucleoprotein complexes arising from the interaction of these proteins with DNA lacks the nucleosomal organization of the somatic chromatin type, as can be evidenced by X-ray diffraction (Ausio & Subirana, 1982; Ausio & Suau, 1983). The overall negative superhelicity of DNA is lost, most likely as a result of the topoisomerase II activity associated with the histone displacement/replacement by these PL or P proteins. Thus, the nucleohistone–nucleoprotamine (protamine-like) transition leads to a complete reorganization of chromatin, while possibly maintaining the specific three-dimensional organization of DNA and its DNA loop domain structure (Ausio, 1995). The detailed molecular structures of the nucleoprotein (P, PL) complexes are still unknown.

Little information is known about the dynamics of chromatin looped domains or the transitions in DNA topology that occur during the replacement of histones by diverse SNBPs. In somatic cells, the DNA in looped domains is folded around nucleosomes in left-handed toroidal supercoils and the later are constrained by histones, thus minimizing torsional tension (Risley *et al.*, 1986).

Our previous investigation showed that in *Triturus cristatus*, SNPBs are represented by PL-I proteins (Burlibaşa & Gavrilă, 2005). The information about topological organization of PL-I-containing chromatin is still scarce.

In present study, starting from Narayan & Raman investigations on topological organization of chromatin during mouse spermatogenesis (2004), the higher order chromatin organization in *Triturus cristatus* spermatogenesis has been analysed.

Materials and methods

Animals

Male newts (*Triturus cristatus*) were obtained from Bucharest Botanical Garden Pool, University of Bucharest, Romania. Animals were sacrificed in accordance with stipulations of European Council law 86/609/CEE/24.11.2004), regarding the protection of animals used for experimental and scientific aims.

Isolation of sperm and spermatogenic cells

Testes were dissected out and used immediately. A cell suspension from adult testes was prepared by stirring minced testes in cold OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, gentamicin 100 μg/ml, pH 7.8) for 15 min. Preparation of nucleoids was carried out according to Risley *et al.* (1986) with some minor modifications. Briefly, the cells were pelleted at 2500 r.p.m. for 10 min at 4°C in a Sigma 2K16C rotor and resuspended in cold NKETS buffer (10 mM NaCl, 40 mM KCl, 2 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM spermine, 0.25 mM spermidine, 15 mM Tris–HCl pH 7.4, 1 mM phenylmethanesulfonyl fluoride (PMSF)) then Triton X-100 was added to a final concentration of 0.5%. The suspension was vortexed and then mixed rapidly with 1 volume of 80% glycerol in NKETS buffer (−20°C). Nuclei were pelleted by centrifugation at 5000 rpm for 20 min at 0°C in the same rotor, then resuspended in 40% glycerol in NKETS buffer and maintained at −20°C prior to nucleoid preparation. This procedure stops protease and nuclease activities in order to avoid DNA supercoiling modifications in nucleoids. The nuclei were pelleted and resuspended in high salt buffer (2.0 M NaCl, 0.2 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5) in NKETS containing 2.5–200 μg/ml ethidium bromide (EtBr). After a few minutes of incubation on ice, a drop of nucleoids suspension was placed on a slide, and then covered with a coverslip. The slides were examined in an Olympus fluorescence microscope. Nucleoid diameter and halo radii from pachytene spermatocytes, round spermatids and sperm were measured before photography using an ocular micrometer.

DNase I treatment of nucleoid DNA

The effects of DNase I on nucleoids structure were examined and measured as described before. DNase I

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Table 1 Nucleoid diameters (μm) at different ethidium bromide (EtBr) concentrations[a]

Cell types	Nuclear diameter control	EtBr concentrations (μg/ml) in salt buffer 1.5 M NaCl (Nucleoids $n \pm$ standard deviation)				
		2.5 μg/ml	10 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
Pachytene spermatocytes	8.2 (31 ± 1)	9.8 (33 ± 1)	13.8 (32 ± 1)	11.2 (30 ± 0.6)	10.8 (30 ± 1.2)	8.9 (34 ± 2.1)
Round spermatids	5.4 (22 ± 0.7)	5.5 (20 ± 0.5)	5.9 (27 ± 0.9)	5.8 (26 ± 2)	5.6 (20 ± 1.4)	5.4 (24 ± 0.8)
Epididymal sperm	2.1 (32 ± 0.3)	3.4 (30 ± 1.4)	3.4 (32 ± 0.5)	3.2 (31 ± 1.1)	3 (33 ± 1.8)	3 (35 ± 0.4)

[a]Nucleoids were measured at the maximum width region and an arithmetical mean was calculated.

Table 2 Halo radii (μm) at different ethidium bromide (EtBr) concentrations

Cell types	EtBr concentrations (μg/ml) in salt buffer 1.5M NaCl (Nucleoids $n \pm$ standard deviation)				
	2.5 μg/ml mean	10 μg/ml mean	50 μg/ml mean	100 μg/ml mean	200 μg/ml mean
Pachytene spermatocytes	1.1 (20 ± 2.1)	3.5 (10 ± 2.1)	2.8 (20 ± 0.5)	2.2 (30 ± 0.4)	1.5 (29 ± 1.3)
Round spermatids	– (19)	– (20)	– (16)	– (18)	– (14)
Epididymal sperm	1 (20 ± 1.2)	1.2 (20 ± 0.9)	1 (20 ± 0.4)	0.8 (23 ± 0.6)	0.4 (25 ± 0.5)

treatment of nucleoid DNA was carried out by adding 5 U/ml of DNase I to the nucleoid preparations and incubating for 5 and 30 min at 37°C. The reaction was stopped by chilling on ice and by adding 10 mM EDTA to a final concentration. All reagents were obtained from Sigma-Aldrich.

Results

Study of DNA topology in the nucleoids is one of the efficient tools providing information on chromatin organization in a cell.

Extraction of somatic cell nuclei with high salt concentrations removes constraints imposed by histones on nucleosomal supercoils resulting in the introduction of some torsional strain into the DNA loops or topological domains (Cook & Brazell, 1976; Risley *et al.*, 1986). At concentrations of EtBr sufficient to remove all negative supercoils (equivalence point), DNA became relaxed and extended giving the appearance of a 'halo'. Higher concentrations of EtBr introduced positive supercoils and torsional strain in topological DNA domains, resulting in a recontraction of the DNA loops (Tables 1 and 2).

As found for the somatic nuclei (Cook *et al.*, 1976; Nelson *et al.*, 1980; Robinson *et al.*, 1983; Pient & Coffey, 1984; VanderWaal *et al.*, 2002), DNA from sperm is in a particular association with the nuclear matrix (Balhorn, 1982; Risley *et al.*, 1986; Ward *et al.*, 1989; Ward & Coffey, 1991), but its higher organization differs in accordance with the proteins bound to it. The research on chromatin topology in spermatogenesis performed by Risley *et al.* (1996) showed that the histone-containing sperm nuclei of *Rana catesbiana* contain negatively supercoiled DNA; DNA from mature sperm from *Xenopus* and *Bufo fowleri* that contain protamines is not supercoiled.

The effect of different concentrations of EtBr on nucleoid DNA of different testicular cell types from *Triturus cristatus*

Nucleoids were prepared in a range of saline buffers (1.5 M NaCl) that contained different concentrations of EtBr (2.5–200 μg/ml). The general shape of the nucleus without the nuclear envelope was preserved in various cell types, regardless of the superhelical configuration of DNA.

Almost all cell types encountered (pachytene spermatocytes, spermatids and testicular sperm) showed a biphasic change in their nucleus, with a halo whose size was dependent on the concentration of EtBr.

The maximum sized halo was observed at a concentration of 10 μg/ml EtBr for pachytene spermatocytes and round spermatids, after which the reduction of the halo took place proportionally with increasing concentration of EtBr. A complete reduction of the halo was not achieved even at the maximum concentration of 200 μg/ml EtBr.

Testicular sperm nucleoids were relaxed only at a concentration of 2.5 μg/ml and 10 μg/ml and gradually condensed at increased concentrations of EtBr.

At the concentration of 10 μg/ml EtBr the size of the nucleoid diameter increased differently in various types of cells. The pachytene nucleoid had the maximum length of the loop domains (an average of 3.5 μm). In the round spermatids, the formation

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Table 3 Nucleoid diameters following DNase I treatment

Cell types	Nucleoid diameter (Nucleoids $n \pm$ standard deviation)		
	Control[a]	5 U/ml DNase I 5 min	5 U/ml DNase I 30 min
Pachytene spermatocytes	13.8 (32 ± 0.6)	12.3 (30 ± 0.3)	10.9 (30 ± 0.7)
Round spermatids	5.9 (27 ± 0.7)	5.5 (20 ± 0.7)	5.2 (20 ± 0.5)
Epididymal sperm	3.4 (32 ± 1.2)	3.4 (30 ± 1.2)	2.9 (30 ± 0.9)

[a]Control, no DNase I treatment (ethidium bromide 10 μg/ml).

Table 4 Halo radii following DNase I treatment

Cell types	Halo radii (Nucleoids $n \pm$ standard deviation)		
	Control[a]	5 U/ml DNase I 5 min	5 U/ml DNase I 30 min
Pachytene spermatocytes	3.5 (30 ± 2.5)	2.7 (30 ± 0.5)	1.5 (30 ± 1.1)
Round spermatids	– (20)	– (20)	– (20)
Epididymal sperm	0.8 (30 ± 1.4)	0.8 (30 ± 0.3)	0.3 (30 ± 0.2)

[a]Control, no DNase I treatment (ethidium bromide 10 μg/ml).

of the halo was not notable, however we noticed a biphasic change (condensed–relaxed–condensed) in the nucleus radius. When the round spermatids were subjected to treatment with a concentration higher that 2.6 M NaCl for the preparation of nucleoids, it resulted in much enlarged nucleoids with an average of 9.8 μm in radius (for 24 measured nucleoids) and an average halo of 2.6 μm.

Increasing concentrations of EtBr caused the testicular sperm nucleoids to condense gradually. Significant changes in nucleoid size, however, were not detected at 2.5–10 μg/ml EtBr.

Sensitivity of the loop domains to DNase I in different spermatogenic cells

The sensitivity of chromatin to the action of DNase I depends on its functional organization, therefore the sensitivity of DNA loops to the concentration of 5 U/ml for 5 and 30 min has been investigated.

The loop domains of different cells showed different reactions to DNase I treatment. The maximum effect of DNase I was observed for pachytene nucleoids. Sperm nucleoids showed a minimal effect at 5 min treatment.

While the demembranated nuclei of the pachytene cells lost 10.8% of their radius and 22.8% of the halo radius, the demembranated nuclei of the spermatozoids stayed stable without DNA loss. After prolonged treatment with DNase I (5 UI/ml for 30 min), the decrease in the nucleoids and halo radii was comparable in all cell types (Tables 3 and 4).

Discussion

These experiments have shown permanent changes in chromatin organization during spermatogenesis. These differences in nucleus size without nuclear envelope and in the halo are a measure of the different DNA topology in different cell types.

The biphasic changes of the nucleoid and halo sizes of testicular cells in response to increasing concentrations of EtBr show that, like many other cell types studied (Vogelstein *et al.*, 1980, Risley *et al.*, 1986, Narayan & Raman, 2004), DNA of newt germinal cells is organized in negatively supercoiled domains. A remarkable finding is the similar biphasic modulation of mature spermatozoids of epididymes but on a much smaller scale, which would lead to the assumption that the nucleosome organization is not completely lost in these highly differentiated cells.

One of the most interesting findings is the absence of a visible halo in the round spermatids at all concentrations of EtBr (1.5 M NaCl) and the appearance of the halo at a concentration of 2.6 M NaCl (Fig. 1*D*).

The absence of the halo indicates that, in the round spermatids, the organization of the chromatin domains and of the loops is considerably different from that of other germinal cell types, even of spermatozoids.

The presence of the typical biphasic changes of the demembranated nucleus and of the halo dimensions after the extraction of NaCl 2.6 M leads to the suggestion that the negative supercoil of DNA resists changes even at male germinal cell differentiation.

An unexpected outcome was the fact that, although spermatozoa are in the final phases of differentiation, and therefore have a greater degree of chromatid condensation than the round spermatids, they are less resistant to NaCl extraction.

The apparent lack of loop domains is the consequence of attaching an even greater number of regions to the nuclear matrix.

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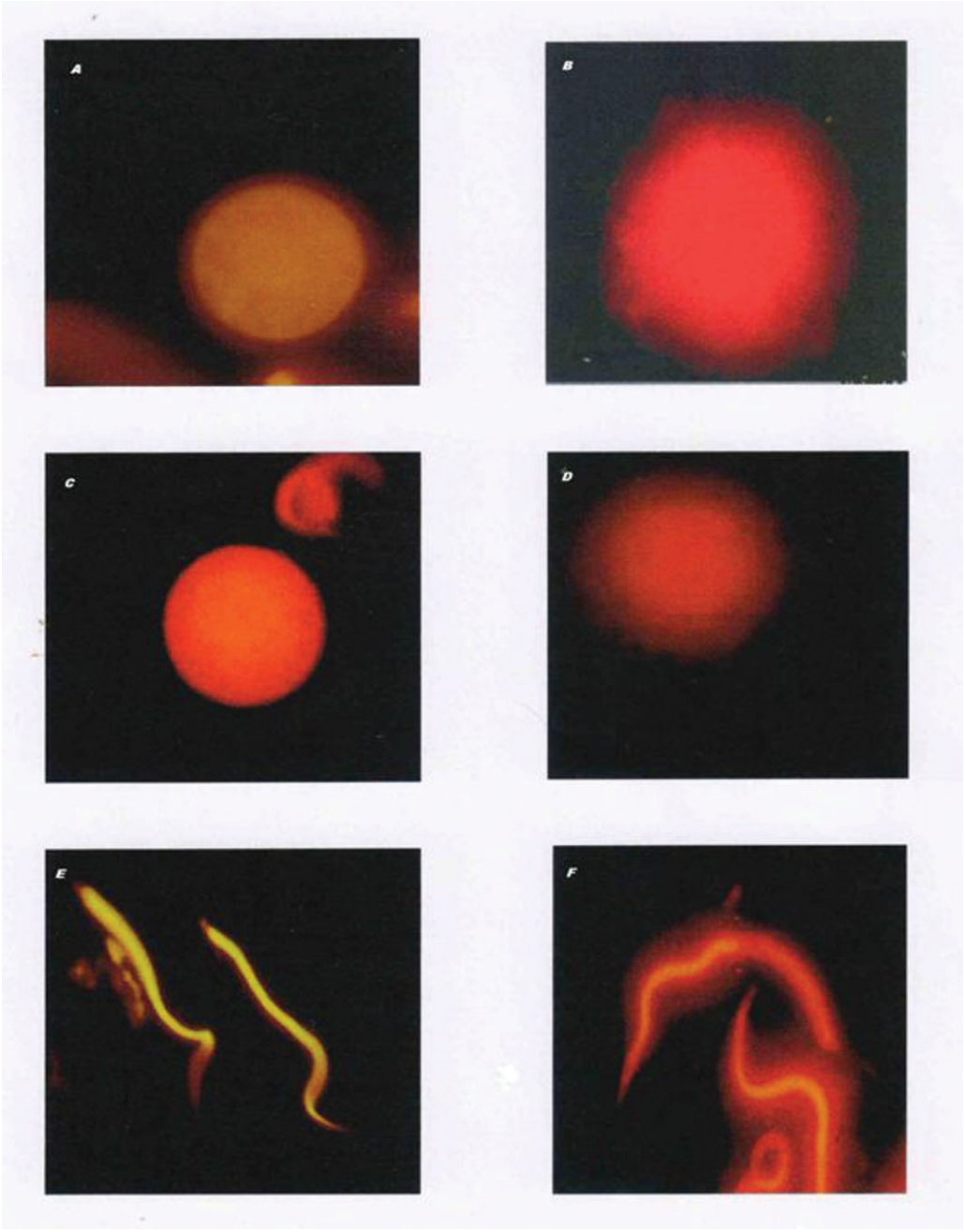

Figure 1 Fluorescence images of ethidium bromide (EtBr)-stained newt (*Triturus cristatus*) germinal cells. (*A*) Pachytene nucleus. (*B*) Pachytene nucleoid following 1.5 M NaCl salt extraction. (*C*) Round spermatid nucleus. (*D*) Round spermatid nucleoid following 2.6 M NaCl salt extraction. (*E*) Epididymal sperm. (*F*) Nucleoids from epididymal sperm following 1.5 NaCl salt extraction. Magnification ×100.

The fact that DNase I digestion did not resulted into a complete collapse of the halo even after prolonged treatment (30 min), suggests that DNase I does not cleave randomly in DNA loops. The regions closer to the matrix could be less sensitive to cleavage than the remote ones.

These experiments have shown permanent changes in chromatin organization during spermatogenesis. The differences that affect the size of the nucleus without the nuclear envelope and the halo reflect the different DNA topology in various cell types.

The biphasic changes in the diameter of the nucleus without a nuclear envelope from testicular cells in response to increasing concentrations of EtBr show that DNA from newt germinal cells is organized into topological domains that are negatively supercoiled. We have also observed a similar biphasic modulation in mature spermatozoids in epididymis but on a much

https://doi.org/10.1017/S0967199411000293 Published online by Cambridge University Press

smaller scale, which would lead to the assumption that nucleosomal organization is not completely lost in these highly differentiated cells. The presence of typical biphasic changes in the nucleoids and the halo size after extraction with 2.6 M NaCl leads to the idea that negative supercoiling of DNA persists even at higher levels of male germinal cell differentiation. The apparent lack of looped domains is the consequence of attachment of an even greater number of regions to the nuclear matrix. It is appropriate to assay the sensitivity of histone-depleted and EtBr-relaxed chromatin, as DNase I sensitivity could be a function of the local displacement of histones to form a nucleosome-free region of chromatin (Narayan & Raman, 2004). DNase I does not cleave randomly on the loop: the region closest to the matrix may be less sensitive than the most distant region (Narayan & Raman, 2004).

Changes in DNA organization are of particular interest and are likely to be a result of important and finely regulated mechanisms that lead to the formation of the chromatin peculiar to sperm. The appearance of nucleosome-depleted regions during spermiogenesis presumably leaves a great number of unconstrained DNA supercoils in the spermatid's genome. The molecular mechanism at the origin of the nucleosome removal is not yet known yet but may involve a combination of destabilizing factors (Boissonneault, 2002).

Acknowledgements

This work was supported by CNCSIS Bucharest, grant IDEI 142/2007.

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