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DNA topoisomerase II activity in nonreplicating, transcriptionally inactive, chicken late spermatids

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To study a possible differential involvement of type I and type II DNA topoisomerases in the functional and structural changes that chromatin undergoes during spermatogenesis, we have determined both enzymatic activities in chicken testis cell nuclei at successive stages of differentiation. Whereas DNA topoisomerase I varies in parallel with transcriptional activity, DNA topoisomerase II was present in both replicating, transcriptionally active chicken testis cells and nonreplicating, transcriptionally inactive late spermatids. The presence of DNA topoisomerase II activity in late spermatids and, in addition, the relative increment of drug-induced topo-II-mediated DNA cleavage detected in these cells, suggest that DNA topoisomerase II might modulate the topology of DNA during the marked changes that chromatin structure undergoes in the nucleohistone-nucleoprotamine transition at the end of the spermiogenesis and could be involved in the final organization of DNA within the nucleus of the male gamete.

Key words: DNA topoisomerase I/DNA topoisomerase II/spermatogenesis/nucleohistone-nucleoprotamine transition

Introduction

The topology of eukaryotic DNA is modulated by two classes of DNA topoisomerases, type I and type II (for reviews, see Cozzarelli, 1980; Gellert, 1981; Liu, 1983; Vosberg, 1985; Wang, 1985). Type I topoisomerases transiently cut one of the two DNA strands, whereas type II topoisomerases transiently break both strands. Cycles of breakage and rejoining of DNA catalysed by DNA topoisomerases are essential for the organization of DNA within the cell nucleus in respect of structure and function. DNA topoisomerase I is preferentially associated with transcriptionally active chromatin (Fleischmann *et al.*, 1984; Muller *et al.*, 1985; Gilmour *et al.*, 1986; Gilmour and Elgin,1987; Culotta and Sollner-Webb, 1988; Ness *et al.*, 1988) and could be involved in the elongation step during transcription (Zhang *et al.*, 1988). DNA topoisomerase II is preferentially associated with nontranscribed spacer segments close to the 5′ and 3′ boundaries of genes (Rowe *et al.*, 1986) and appears to be a major structural element of interphase nuclei and mitotic chromosomes (Berrios *et al.*, 1985; Heller *et al.*, 1986; Earnshaw *et al.*, 1985; Gasser *et al.*, 1986). DNA topoisomerase II is required for condensation and separation of mitotic chromosomes (Uemura *et al.*, 1987).

Spermatogenesis offers an excellent model to investigate the relationship between changes in DNA topoisomerase activities and the structural and functional transitions that chromatin undergoes during the differentiation of the germinal cell line. The most dramatic changes in chromatin structure observed in eukaryotes take place during the nucleohistone-nucleoprotamine transition when, in a highly decondensed hyperacetylated chromatin, nucleosomes are disassembled and the typical structure of interphase nuclei is replaced by a new form of organization of DNA in the nuclei of spermatozoa (Mezquita, 1985; Oliva and Mezquita, 1986; Oliva *et al.*, 1987). This structural transition, taking place in late spermatids, constitutes an ideal system to investigate the function of DNA topoisomerases in the topological changes of DNA occurring in spermatids in the absence of replication, recombination and transcription. We report here the DNA topoisomerase I activity and the DNA topoisomerase II activity at successive stages of chicken spermatogenesis. Our results indicate that while DNA topoisomerase I activity correlates with nuclear transcriptional activity, DNA topoisomerase II is present in both meiotic and premeiotic cells, active in replication and transcription, and also in nonreplicating transcriptionally inactive spermatids. The presence of topoisomerase II activity in terminally differentiated late spermatids suggests that this enzymic activity may be involved in topological changes of DNA, not unequivocally related to replication, recombination or transcription; these changes could be responsible for the final organization of DNA in the nucleus of the male gamete.

Results

DNA topoisomerase activities, type I and type II, at successive stages of chicken spermatogenesis

Chicken testis cell nuclei at successive stages of spermatogenesis were separated by sedimentation at unit gravity. Five different fractions previously characterized (I, diploid premeiotic and meiotic nuclei; II, tetraploid primary spermatocytes; III, round spermatids; IV, elongated spermatids; and V, spermatozoa from the vas deferens) were used to assay DNA topoisomerase I and DNA topoisomerase II activities. Each fraction of nuclei was extracted with 2 M KCl in the presence of protease inhibitors, and topoisomerase I activity was determined by relaxation of supercoiled pBR322 DNA. The assay was performed at protein concentrations within the linear range of activities in a medium without ATP and Mg^{2+}. Type I DNA topoisomerase activity was maximal in tetraploid primary spermatocytes, decreases in round spermatids and further decreases in elongated spermatids and mature spermatozoa (Figures 1 and 2).

Type II DNA topoisomerase activity was determined by the P4 unknotting assay in the presence of ATP and Mg^{2+}, using protein concentrations within the linear range of activities. Topoisomerase II activity is present in meiotic,

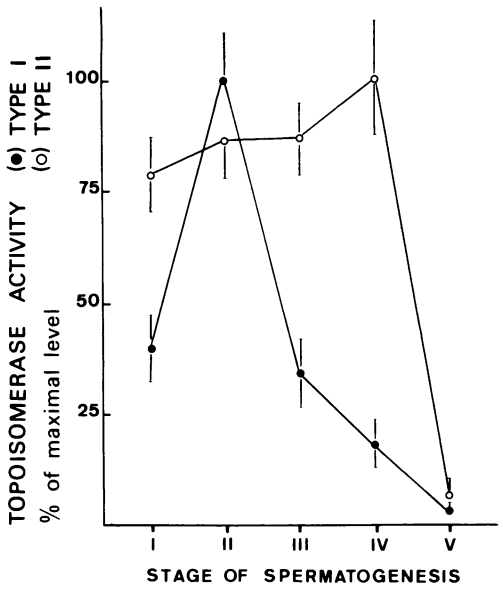

Fig. 1. DNA topoisomerase I and DNA topoisomerase II activities at successive stages of chicken spermatogenesis. Chicken testis cell nuclei at different stages of spermatogenesis were separated by sedimentation at unit gravity, extracted with 2 M KCl in the presence of protease inhibitors, and the topoisomerase activities present in the extracts were determined by relaxation of supercoiled pBR322 in the absence of ATP and magnesium (type I) and unknotting of P4 DNA in the presence of ATP and magnesium (type II). The 100% maximum level corresponds to 280 × 10^3 U/mg DNA for topoisomerase I and to 950 × 10^3 U/mg DNA for topoisomerase II. I, nuclei from diploid meiotic and gonial cells; II, nuclei from tetraploid primary spermatocytes; III, nuclei from round and elongating spermatids; IV, nuclei from elongated spermatids and testicular spermatozoa; V, nuclei from mature spermatozoa obtained from the vas deferens.

premeiotic and round spermatid nuclei (fractions I–III), and persists elevated in nonreplicating transcriptionally inactive late spermatids undergoing the nucleohistone–nucleoprotamine transition (fraction IV) only decreasing in mature spermatozoa (fraction V) (Figures 1 and 2).

DNA topoisomerase I activity parallels nuclear transcriptional activity at successive stages of chicken spermatogenesis

The comparison between DNA topoisomerase I activity at successive stages of spermatogenesis and the nuclear transcriptional activity detected in the same fractions (Mezquita and Teng, 1977) is shown in Figure 3. Both activities reach the highest level in tetraploid primary spermatocytes and decrease markedly in nonreplicating transcriptionally inactive late spermatids and in mature spermatozoa.

DNA topoisomerase I activity was lower in nontranscribing erythrocytes than in liver and mature testis (Table I and Figure 4).

Presence of DNA topoisomerase II activity in nonreplicating transcriptionally inactive late spermatids

The DNA topoisomerase II activity persists in chicken late spermatids when both replication and transcription have ceased (Figure 5). DNA topoisomerase II activity was not detected in the nuclei of nonreplicating, transcriptionally inactive chicken erythrocytes, and was several times higher in the nuclei of mature testis cells than in the nuclei of chicken liver cells (Table I and Figure 4). The presence of

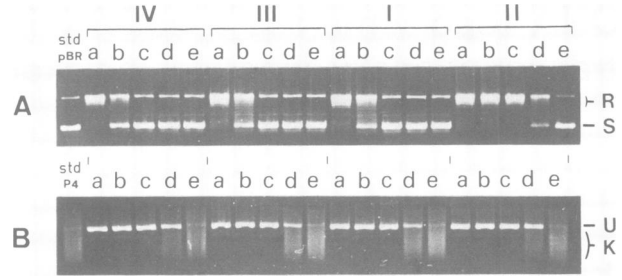

Fig. 2. Assays of type I and type II DNA topoisomerase activities in nuclear extracts of chicken testis cells by agarose gel electrophoresis. Serial dilutions of nuclear extracts (**a**–**e**) from chicken testis cells at different stages of spermatogenesis (I–IV) were assayed for relaxing activity of supercoiled pBR322 in the absence of ATP and magnesium (**A**) and for P4 DNA unknotting activity in the presence of ATP and magnesium (**B**). The amounts of protein (ng) in the nuclear extracts assayed were 160 (**a**), 80 (**b**), 40 (**c**), 20 (**d**) and 10 (**e**) for topoisomerase I and 90 (**a**), 30 (**b**), 10 (**c**), 3.3 (**d**) and 1.1 (**e**) for topoisomerase II. The positions of relaxed (R), supercoiled (S), unknotted (U) and knotted (K) DNA are indicated. Std is DNA incubated without nuclear extract. Stages of spermatogenesis (I–IV) as in legend to Figure 1.

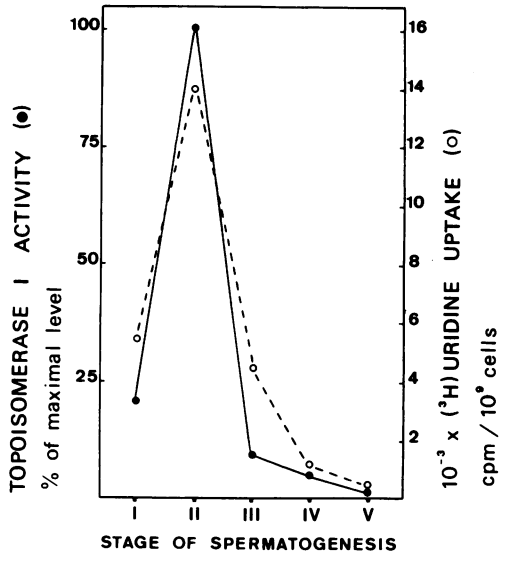

Fig. 3. Topoisomerase I activity and nuclear transcriptional activity at successive stages of chicken spermatogenesis. Topoisomerase I activities, corresponding to those plotted in Figure 1, are compared with the [^{3}H]uridine uptake at successive stages of chicken spermatogenesis (Mezquita and Teng, 1977). Stages of spermatogenesis (I–V) as in legend to Figure 1.

Table I. DNA topoisomerase activities in different chicken tissues

	Topoisomerase I (10^3 U/mg DNA)	Topoisomerase II (10^3 U/mg DNA)
Liver	320	200
Erythrocytes	53	0
Mature testes	107	844
Sperm	1	8

Nuclei and nuclear extracts were obtained as described in Materials and methods. One unit of topoisomerase I was defined as the amount of enzyme necessary to relax 50% of the 0.4 μg input supercoiled DNA in 30 min at 37°C. One unit of topoisomerase II was defined as the amount of enzyme necessary to unknot 50% of the 0.2 μg knotted DNA in 30 min at 37°C.

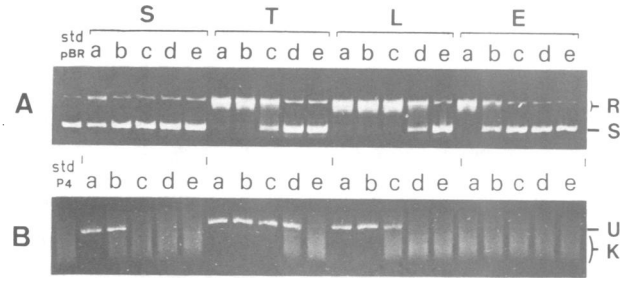

Fig. 4. Assays of type I and type II DNA topoisomerase activities in nuclear extracts from different chicken tissues by agarose gel electrophoresis. Serial dilutions of nuclear extracts from mature spermatozoa nuclei (S), mature testis cell nuclei (T), liver nuclei (L) and erythrocyte nuclei (E) were assayed for relaxing activity or supercoiled pBR322 in the absence of ATP and magnesium (**A**) and for P4 DNA unknotting activity in the presence of ATP and magnesium (**B**). The amounts of protein (ng) in the nuclear extracts assays were 160 (**a**), 80 (**b**), 40 (**c**), 20 (**d**) and 10 (**e**) for topoisomerase I and 90 (**a**), 30 (**b**), 10 (**c**), 3.3 (**d**) and 1.1 (**e**) for topoisomerase II. The positions of relaxed (R), supercoiled (S), unknotted (U) and knotted (K) DNA are indicated. Std is DNA incubated without nuclear extract.

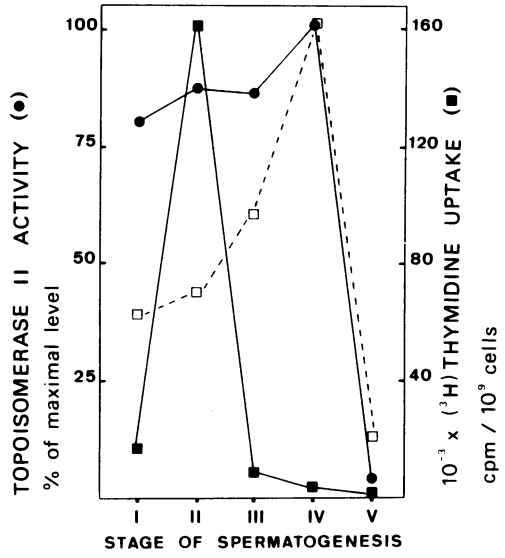

Fig. 5. Topoisomerase II activity, [^{3}H]thymidine incorporation and DNA binding sites at successive stages of chicken spermatogenesis. Topoisomerase II activities, corresponding to those plotted in Figure 1, are compared with the [^{3}H]thymidine incorporation and DNA binding sites for [^{3}H]actinomycin D (−□−) at successive stages of chicken spermatogenesis (Mezquita and Teng, 1977). Stages of spermatogenesis (I−V) as in legend to Figure 1.

high levels of DNA topoisomerase II activity in late spermatids in contrast with its absence in nonreplicating transcriptionally inactive terminally differentiated chicken erythrocyte nuclei, could be related to the unique properties of the spermatidal chromatin during the nucleohistone−nucleoprotamine transition. We have previously shown that chicken late spermatids possess chromatin with hyperacetylated histones, high capacity for binding of actinomycin D and an initiation pattern of RNA synthesis *in vitro* with characteristics of transcriptionally active chromatin (increased number of RNA polymerase binding sites, increased rate of propagation of growing RNA chains) (Mezquita and Teng, 1977) (Figure 5).

Teniposide-induced topo-II-mediated DNA cleavage is maximal in nonreplicating transcriptionally inactive late spermatids undergoing the nucleohistone−nucleoprotamine transition

To investigate if the pool of salt-soluble DNA topoisomerase II activity present in late spermatids is active on DNA *in vivo*, we have treated chicken testis cells at successive stages of spermatogenesis, with the DNA topoisomerase II inhibitor, teniposide. This drug stabilizes the DNA−topo II linkage and, after SDS treatment, the number of DNA strand breaks detected measures the topoisomerase II molecules acting on DNA *in vivo* (Ross *et al.*, 1984). Three different fractions of chicken testis cells were separated by centrifugal elutriation: meiotic and premeiotic cells (I and II); round spermatids (III); and elongated spermatids (IV). An additional fraction consisting of mature spermatozoa (V) was obtained from the vas deferens. Each fraction was incubated for 30 min with teniposide and cells were lysed in SDS and treated with proteinase K. The strand breaks induced on DNA were quantified by comparing the size distribution of DNA from treated and control cells after electrophoresis in an alkaline 0.4% agarose gel. The maximum cleavage was detected in nonreplicating transcriptionally inactive late spermatids undergoing the nucleohistone−nucleoprotamine transition (Figure 6). DNA strand breaks were not induced in chicken erythrocyte nuclei where salt-soluble DNA topoisomerase II activity was not detectable (Table I and Figure 4).

Discussion

During spermatogenesis of most higher eukaryotes two types of structural changes of chromatin occur, those related to genetic activities (replication, transcription and recombination), taking place in spermatogonia, spermatocytes and early spermatids, and those involved in the nucleohistone−nucleoprotamine transition occurring in late spermatids at the end of spermiogenesis (Mezquita, 1985). We have chosen chicken spermatogenesis as a model system to investigate a putative differential role of DNA topoisomerase I and DNA topoisomerase II in both types of structural changes of chromatin.

In a fraction enriched in tetraploid primary spermatocyte nuclei, active in DNA replication, recombination and transcription, both enzymatic activities, topo I and topo II, were detected. DNA topoisomerase activities have been implicated in replication (Liu *et al.*, 1980; Duguet *et al.*, 1983; Taudou *et al.*, 1984; Muller *et al.*, 1985; Sullivan *et al.*, 1985; Heck and Earnshaw, 1986; Nelson *et al.*, 1986; Brill *et al.*, 1987; Chow and Ross, 1987; Heck *et al.*, 1988) segregation of daughter DNA molecules after S phase (DiNardo *et al.*, 1984; Uemura and Yanagida, 1986), recombination (Kikuchi and Nash, 1979) and transcription (Fleischmann *et al.*, 1984; Muller *et al.*, 1985; Gilmour *et al.*, 1986; Glikin and Blangy, 1986; Gilmour and Elgin, 1987; Culotta and Sollner-Webb, 1988; Ness *et al.*, 1988; Zhang *et al.*, 1988). Particularly in meiotic nuclei the interlocking of synaptonemal complexes, frequently observed during zygotene, requires for their resolution breaking and rejoining of the DNA molecules of one or both sister chromatids, a function played by DNA topoisomerases (Wettstein *et al.*, 1984). A clear correlation between DNA topoisomerase I activity and nuclear transcription is observed

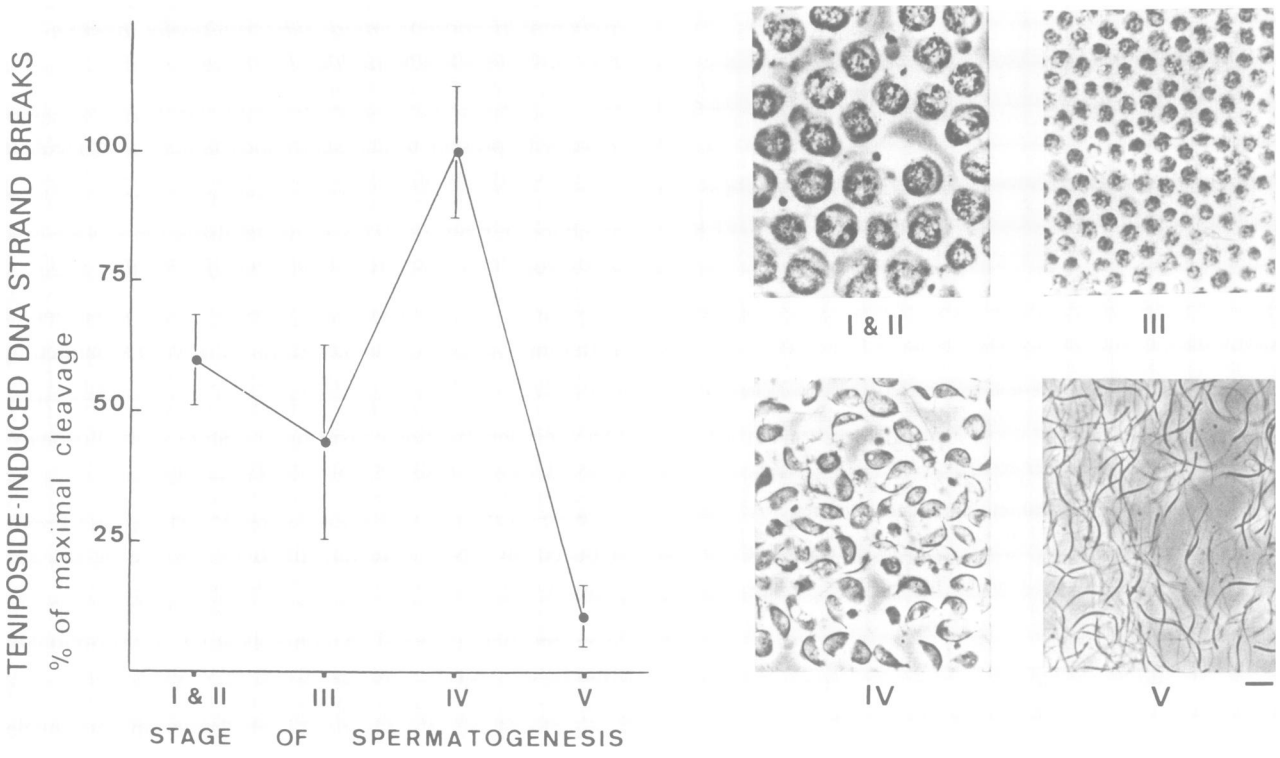

Fig. 6. Teniposide-induced topoisomerase-II-mediated DNA cleavage at successive stages of chicken spermatogenesis. **Left**. Chicken testis cells separated by centrifugal elutriation and spermatozoa obtained from the vas deferens were incubated for 30 min in the presence or absence of 45 μM VM26 (teniposide). After incubation cells were immediately lysed in SDS, treated with proteinase K and the strand breaks induced on DNA were quantified by comparing the size distribution of DNA from treated and control cells after electrophoresis in an alkaline 0.4% agarose gel. **Right**. Phase-contrast photomicrographs of chicken testis cells separated by centrifugal elutriation and spermatozoa obtained from the vas deferens. Cell fractions were observed through a Zeiss ST-143 phase-contrast microscope. **I** and **II**, meiotic and premeiotic cells; **III**, round spermatids; **IV**, elongated spermatids. An additional fraction, consisting of mature spermatozoa (**V**), was obtained from the vas deferens. Bar = 10 μm.

during chicken spermatogenesis. The maximum level of DNA topoisomerase I activity and nuclear transcription was observed in a fraction enriched in tetraploid primary spermatocyte cell nuclei. During mammalian spermatogenesis the greatest rRNA synthesis occurs in tetraploid primary spermatocytes (Kierszenbaum and Tres, 1978). During chicken spermatogenesis the greatest RNA synthesis per nucleus also occurs in tetraploid primary spermatocytes (Mezquita and Teng, 1977). DNA topoisomerase I has been found heavily concentrated in the nucleolus of the cell (Muller *et al.*, 1985). Covalent topo I–DNA complexes have been isolated in association with rDNA sequences and a primary role in transcription of ribosomal genes has been postulated for topoisomerase I (Muller *et al.*, 1985). This enzymic activity has been found also associated with other active genes (Gilmour and Elgin, 1987). Heterogeneous RNA is synthesized in both spermatocytes and early spermatids but not in late spermatids or spermatozoa (Mezquita and Teng, 1977; Kierszenbaum and Tres, 1978). The distribution of DNA topoisomerase I activity during chicken spermatogenesis agrees with observations that suggest a role of this enzymic activity in transcriptional events catalysed by RNA polymerase I and II.

At the end of spermiogenesis DNA topoisomerase I markedly decreases in nonreplicating transcriptionally inactive late spermatids undergoing the nucleohistone–nucleoprotamine transition, while DNA topoisomerase II remains elevated in these cells. The drug-induced topo-II-mediated DNA cleavage is maximal in late spermatids in relation to other stages of spermatogenesis. This observation constitutes additional evidence for the involvement of DNA topoisomerase II in the topological changes of DNA occurring at the end of spermiogenesis. Although the physiological role played by DNA topoisomerase II during spermiogenesis is unknown at present, the persistence of this enzymic activity in nonreplicating, transcriptionally inactive late spermatids, may be related either to the ability of these cells to repair their genetic damage before the final condensation of chromatin occurs (Lähdetie *et al.*, 1983), or with two other putative functions: (i) modulation of DNA topology during the nucleohistone–nucleoprotamine transition; and (ii) reorganization of DNA in new looped domains in the sperm nucleus.

It has been suggested that, in late spermatids, changes in the topology of DNA within a looped domain may contribute to destabilization of nucleosomal structure throughout the domain, facilitating the displacement of histone by protamine and the clustered loss of nucleosomes (Risley *et al.*, 1986). High levels of DNA supercoiling induced by DNA gyrase have been suggested to lead to a partial disruption of the compact form of the nucleosome (Garner *et al.*, 1987). Although we have shown that the chicken protamine, galline, is highly efficient in the disassembly of hyperacetylated nucleosomes near physiological conditions (Oliva and Mezquita, 1986; Oliva *et al.*, 1987), the process may be facilitated *in vivo* by topoisomerase II activity. Further studies on the effect of topoisomerase II inhibitors *in vivo* and *in vitro* on the nucleohistone–nucleoprotamine transition will

shed new light on the physiological role of this enzymic activity in the process.

As a consequence of histone removal during spermiogenesis the superhelicity of DNA should increase. The final topological state of DNA in late spermatids may be modulated by the topoisomerase II activity present in these cells. Nucleoids from *Xenopus* sperm, where histone has been completely replaced by protamine, contain DNA predominantly in an unsupercoiled form (Risley *et al.*, 1986).

The second proposed function for type II DNA topoisomerase is the reorganization of DNA in new looped domains in the sperm nucleus. It has been suggested that topoisomerase II plays an essential role in the higher order folding of DNA necessary for the compaction observed in the interphase nucleus and the metaphase chromosome. DNA topoisomerase II has been identified as one of the proteins involved in the organization of DNA in looped domains (Earnshaw *et al.*, 1985; Gasser and Laemmli, 1986; Gasser *et al.*, 1986). In a similar way DNA topoisomerase II may be required for the compaction of DNA in the sperm nucleus. The DNA helix is first folded as a fibre of nucleosomes, 100 Å in diameter, which accounts for 6- to 7-fold compaction of the linear molecule. At a second level the 100 Å fibre is folded into a 300 Å fibre, but another 10- to 15-fold compaction is still necessary to account for the organization of DNA in the interphase nucleus and another 110-fold to account for the size of the metaphase chromosome (Paulson, 1981). The DNA concentration in the chicken sperm nucleus, calculated from a nuclear DNA content of 1.1 pg (Mezquita and Teng, 1977) and a volume of 2 μm^3 (McIntosh and Porter, 1967) is ~ 0.55 pg/μm^3. This concentration is similar to that reported for mouse sperm and 4-fold higher than concentrations calculated for sea urchin sperm with nucleosomal structure, and for mitotic chromosomes (0.13–0.18 pg/μm^3) (Green and Poccia, 1985; Bennet *et al.*, 1983). The highest order of DNA folding could be achieved by topological constraining of the molecule into loops ranging in size from 30 to 100 kb (Laemmli *et al.*, 1978). During *Xenopus* spermiogenesis the size of the loops has been estimated to change from 105 kb (spermatids) to 25 kb (sperm) (Risley *et al.*, 1986).

Although the majority of DNA is organized in a highly compact form in the head of spermatozoa, the possibility that specific sequences of DNA become packed in a different form during spermiogenesis cannot be excluded. Evidence exists that demonstrates genomic imprinting in the male gamete (Surani *et al.*, 1986). Whereas most of the nonhistone proteins are lost during spermiogenesis, a small set of nonhistone proteins in conjunction with DNA topoisomerase II activity might be responsible for a putative selective organization of certain genes in the sperm nucleus.

Materials and methods

Separation of chicken testis cell nuclei and testis cells by sedimentation at unit gravity and by centrifugal elutriation

Hubbard White Mountain roosters (6–12 months old) were used in all the experiments. Cell nuclei obtained from testis, erythrocyte and liver were purified as previously described (Mezquita and Teng, 1977). Chicken testis cell nuclei were separated by sedimentation at unit gravity (Mezquita and Teng, 1977). Chicken testis cells were prepared and separated by centrifugal elutriation essentially as described in Meistrich (1977). Mature testes were decapsulated and minced finely with scissors. The minced tissue was gently suspended in 10 vol of minimum essential medium (Eagle) containing 0.1% (w/v) trypsin and 2 μg of DNase I/ml. The suspension was incubated at 31°C for 30 min with gentle stirring in a water bath. After incubation, the cell suspension was filtered through four layers of surgical gauze and centrifuged for 20 min at 1500 *g* in a JE-7.5 Beckman rotor. The sample was resuspended in 50 ml of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 0.02% (w/v) soybean trypsin inhibitor, 0.1% bovine serum albumin and 0.1% glucose. The cell suspension was diluted in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline to a final concentration of $25-30 \times 10^6$ ml. A cell suspension of 20 ml was loaded into a JE-6 Beckman elutriator rotor and separations were performed with speeds of 3000 r.p.m. and flow rates of 3–100 ml/min. Fractions of 125 ml were collected. The following cell types were obtained: testicular spermatozoa and residual bodies (3 ml/min flow rate); elongated spermatids (11 ml/min flow rate); round spermatids (20 ml/min flow rate); meiotic and premeiotic cells and multinucleate cells (37 ml/min flow rate). Mature spermatozoa were obtained from the vas deferens.

[^{3}H]Thymidine incorporation

Seminiferous tubules were labelled for 1 h at 37°C in Hanks' balanced salt solution in the presence of 30 μCi/ml of [^{3}H]methylthymidine (Amersham, sp. act. 40 Ci/mmol). The tissue was washed three times with the culture medium, and the nuclei were prepared and separated by the procedure described above. The nuclear fractions were washed with 5% trichloroacetic acid, ethanol, ethanol/ether and ether to remove free [^{3}H]thymidine. The residue was suspended in 0.2 M $HClO_4$ and portions of the solution were used for determination of DNA and radioactivity.

Preparation of nuclear extracts

Chicken testis cell nuclei ($\sim 10^8$) were suspended in 200 μl of 40 mM Tris–HCl pH 7.5, 4 mM EDTA. To the suspension, 1 ml of a solution containing 2.4 M KCl, 40 mM Tris–HCl, pH 7.5 was slowly added and after sonication (10 s at 60 W) the DNA was precipitated for 30 min by adding 600 μl of 18% polyethylene glycol (PEG 6000), 2 M KCl, 40 mM Tris, pH 7.5) and removed by centrifugation at 15 000 *g* for 25 min. The supernatant was mixed with an equal volume of glycerol and stored at −40°C. All the solutions used for preparation of nuclear extracts contained 1 mM PMSF, 0.1 mM leupeptin, 100 U/ml trasylol, 10 mM 2-mercaptoethanol and 0.5 mM dithiothreitol and were maintained at 4°C.

Preparation of plasmid pBR322 and knotted P4 head DNA

Plasmid pBR322 was propagated in *Escherichia coli* strain HB 101 and supercoiled pBR322 DNA was prepared from a chloramphenicol-amplified culture by an alkaline SDS lysis procedure (Maniatis *et al.*, 1982). Knotted P4 head DNA was prepared as described by Liu and Davis (1981).

Topoisomerase assays

Topoisomerase assays were performed on serial dilutions of nuclear extracts (Duguet *et al.*, 1983; Taudou *et al.*, 1984). Both topoisomerase I and II were present in the same extract and separate determinations of each activity were performed by using specific assays. Topoisomerase I was measured by relaxation of supercoiled DNA (pBR322) in the absence of ATP and magnesium ions, and topoisomerase II activity was assayed measuring the unknotting activity using knotted P4 DNA as substrate in the presence of ATP and magnesium. For the topoisomerase I assay, 20 μl of the reaction mixture, consisting of 2 μl of nuclear extract dissolved in a medium containing 40 mM Tris pH 7.5, 100 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 30 μg/ml bovine serum albumin, 0.4 μg pBR322 DNA (form I) were incubated at 37°C for 30 min. Topoisomerase II was determined by assaying the ATP-dependent unknotting activity (Liu and Davis, 1981). Knotted P4 head DNA (0.2 μg) was incubated with the nuclear extract for 30 min at 37°C in 40 mM Tris–HCl pH 7.7, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1.0 mM ATP, 30 μg/ml bovine serum albumin (total volume 20 μl). Reactions were terminated by the addition of 5 μl of 5% SDS, 50 mM EDTA and 0.25 mg/ml bromophenol blue. The reaction products were analysed in agarose gel electrophoresis. Electrophoreses were run in a 90 mM Tris–borate buffer, pH 8.3, 2.5 mM EDTA for 2 h at 5 V/cm in 1% agarose gels for type I DNA topoisomerase assays or 1 h at 8 V/cm in 0.6% agarose gels for type II DNA topoisomerase assays. After electrophoresis the gels were stained with 2 μg/ml of ethidium bromide, destained with water, and photographed under UV light. Densitometric scans of the negatives were made with a Hoefer densitometer and the peak areas of supercoiled and relaxed DNA in the case of topoisomerase I and the circular unknotted DNA in the case of topoisomerase II were integrated and compared.

The possible interference of nuclease activities was tested by analysing the DNA in agarose gels containing 3 μg/ml chloroquine, a drug that changes the torsion of DNA, allowing a clear separation of nicked DNA from

covalently closed forms, or by two-dimensional electrophoresis.

One unit of topoisomerase I was defined as the amount of enzyme necessary to relax 50% of the 0.4 μg input supercoiled DNA in 30 min at 37°C. One unit of topoisomerase II was defined as the amount of enzyme necessary to unknot 50% of the 0.2 μg knotted DNA in 30 min at 37°C.

Teniposide-induced topoisomerase-II-mediated DNA cleavage

Chicken testis cells separated by centrifugal elutriation ($\sim 5 \times 10^6$) were incubated for 30 min at 37°C in 500 μl minimum essential medium in the presence or absence of 45 μM VM26 (teniposide). After incubation cells were immediately lysed in an equal volume of 20 mM Tris−HCl, pH 8.0, 40 mM EDTA, 2% SDS previously heated to 60°C. Proteinase K was then added to a final concentration of 200 μg/ml and the mixture was incubated overnight at 37°C. The cell lysate was extracted with phenol−chloroform and chloroform. The aqueous phase was recovered and NaOH (2 M) was added to a final concentration of 150 mM. After incubation for 3 h at 37°C., 25 μl of denatured DNA samples were mixed with 10 μl of 10% glycerol, 0.05% bromophenol blue and electrophoresed in a 0.4% agarose alkaline gel at 100 mA for 40 h. After electrophoresis the gel was treated with renaturing buffer containing 0.6 M NaCl, 1 M Tris, pH 7.4. The gel was stained for 20 min with ethidium bromide (0.3 μg/ml) in water. After an additional 60 min in the renaturing buffer pictures were taken under UV light using a Polaroid 665 positive−negative film. Densitometer scans of the negatives obtained with a Hoefer densitometer were used to evaluate the average number of breaks according to the equation described by Kohen *et al.* (1986).

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