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I am submitting herewith a thesis written by Rajashekar K. Reddy entitled "Analysis of the Expression of Topoisomerase I and IIα during Spermatogenesis in Mice." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

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We have read this thesis
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Analysis of the Expression of Topoisomerase I and IIα during Spermatogenesis in Mice

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Rajashekar K. Reddy

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DEDICATION

To my parents Smt. Gawramma and (Late) Sri. K. Pedda Reddy, and to my beloved aunt (Late) Smt. Saradamma

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ABSTRACT

Topoisomerases are nuclear enzymes functioning to remove torsional stress in DNA induced by replication, transcription, condensation, and recombination. Because of the potential importance of topoisomerases for various aspects of meiosis, I studied the expression of transcript and enzyme activity of topoisomerases I (topo I) and topoisomerase IIα (topo II) during testicular development and spermatogenesis in the mouse. Analysis by Northern blots was made of total RNA extracted from testes of different developmental ages and of total RNA extracted from spermatogenic cell fractions enriched by sedimentation at unit gravity. Comparative quantitation of transcript level in different samples was made by densitometric analysis of autoradiograms. During development, topo I transcript was expressed at quite similar and increased levels in the testes from mice aged 8, 16, and 21 days, and decreased by day 30 and thereafter . The level of topo II transcript was highest in those testes where meiotic cells are abundantly represented (day 16 and 21). Interestingly, topo I transcripts were high in the testes of XX*Sxr* (germ-cell deficient) mice, while levels of topo II were negligible in these testes, suggesting that most of the topo II activity in the whole testes derives from germ cells. From highly enriched cell populations, topo I transcript was high in type A and B spermatogonia, leptotene-zygotene spermatocytes, and pachytene spermatocytes, and then decreased dramatically in round spermatids. Enzyme activity, measured by a DNA relaxation assay, differed from transcript levels in also being high in round spermatids, thus suggesting enzyme stability. In the highly enriched spermatogenic cell populations, topo II transcript increased during spermatogenesis to high levels in pachytene spermatocytes and in round spermatids. These results demonstrate developmental regulation of both topo I and II and could imply meiotic function for these enzymes.

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CHAPTER I

Introduction

Topoisomerases are nuclear enzymes implicated in the relaxation of torsional stress in DNA, which usually follows transcription, replication, condensation and decondensation. Type I topoisomerases (topo I) accomplish this by single strand nicking and resealing whereas type II topoisomerases (topo II) accomplish this by causing transient double strand breaks (reviewed by Wang, 1985).

The molecular mechanism of topo I activity was elucidated by the use of anti-topo I drugs such as camptothecin, which is thought to block the religation reaction (Porter and Champoux 1989). In eukaryotes, topo I is thought to create a single strand break, and, while still covalently linked to the 3' hydroxyl end of the nicked DNA strand by a O^4-phosphotyrosine bond, allow the other strand to unwind or wind once before resealing the break (reviewed by Wang, 1985). The mouse topo I protein shows 96% amino acid sequence homology to the human counterpart and 42% to yeast (Koiwai et al., 1993).

Evidence for specific roles for topo I was obtained from both cytological and genetic analyses in yeast. A topo I mutation had shown that the enzyme was not essential for viability (Thrash et al., 1985). By Southern blotting the authors confirmed that the wild type gene had been disrupted. The authors analyzed several of these null mutations by sporulating the diploid cells and assaying the tetrads for viability. Many viable tetrads were found. In contrast to yeast, analysis of Drosophila topo I mutants had shown that the enzyme is essential for viability (Lee et al., 1993). Insertional mutation of the X-linked topo I gene resulted in no male or homozygous female progeny and the addition of an ectopic topo I gene copy into heterozygous mutant strains resulted in viable flies of both sexes. The authors also analyzed topo I transcript expression (by Northern hybridization)

and protein expression (immunoblots) during embryonic development in wild type flies. The highest levels of both transcript and protein were found during the earlier stages ie., 6 - 12 hour post fertilization, which corresponded to earlier results showing maximal DNA synthesis occurring during this time period. The authors compare their results to top I mutants in yeast which show that the enzyme was not essential. However yeast mutants do show a reduced growth rate (Thrash et al., 1985). They hypothesized that in a multicellular organism the absence of topo I could lead to a disruption in the coordination of DNA replication which could ultimately result in cell death and lethality.

Mutant analysis provides evidence for a role of topo I in transcription. Di Mauro et al. (1993) used the yeast mutants for topo I to show that the rates of transcription of certain genes were affected. They focused on a plasmid borne alcohol dehydrogenase II (*ADH2*) gene which is regulated at the level of initiation of transcription. The transcription of *ADH2* had earlier been shown to be directly affected by the topology of DNA, and to be activated 100-fold on the substitution of ethanol for glucose in the growth media. They observed increased transcription in the top1 mutant strain when compared to the wild type. They hypothesized that this could be due to the accumulation of negative supercoiling at the promoter region of the mutant in the absence of topo I relaxing activity, which could lead to an increase in accessibility of this gene to the transcriptional machinery, causing increased transcription. Roca and Mezquita (1989) showed a close correlation between topo I enzyme activity and transcriptional activity levels (as estimated by the uptake of ^{3}H uridine) in successive stages during rooster spermatogenesis. From this, and earlier evidence linking topo I activity to the nucleolus, the authors suggested that topo I could be linked to the transcriptional events catalyzed by RNA polymerase I and II. In polytene chromosomes of *Drosophila melanogaster,* topo I antibodies were shown to localize to transcriptionally active loci, including the nucleolus (Fleischmann et al. 1984). Puffs revealed prominent staining as did also heat shock loci after, but not before, the heat shock

induction.

Existing knowledge on the molecular mechanism of DNA cleavage by topo II comes from the use of anti-tumor drugs which block topo II function by trapping the enzyme in "clevable complexes". Rowe et al. (1986) had proposed a model for the action of topo II. From experiments dealing with the treatment of cultured cells with an anti-tumor drug, 4'-(9-acridinylamino)methane-sulfon-*m*-anisidide (*m*-AMSA), they showed that the enzyme is covalently linked to the 5'-ends of the broken DNA strands via a O^4-tyrosyl phosphoester bond. They suggested that topo II at first binds to the DNA substrate as a homodimer. It is then proposed to form a reversible cleavable complex after causing a double strand break staggered by four bases, while the complex itself is held together by noncovalent protein-protein interactions. The strands are religated after the relaxation process. In this manner topo II is able to release knots and tangles in DNA that cannot be resolved by the topo I enzyme. Topo II is functionally conserved across species. For instance the mouse topo II has been found to complement a yeast topo II mutation (Adachi et al., 1992).

The type II topoisomerases also have a role associated with transcription and replication, and as well, may play a significant role in the releasing of tangles and knots which could arise during replication, condensation and decondensation of DNA. They do this by causing transient double strand breaks in the DNA (reviewed by Wang, 1985). DiNardo et al. (1984) showed that yeast top2, temperature sensitive (ts) mutants were able to undergo a single round of DNA synthesis and then were arrested completely. By phase-contrast microscopy observation of budding, they showed that the mutant yeast were defective in the segregation of daughter chromosomes at the termination of DNA replication. The authors concluded that in the absence of topo II, the disjunction of sister chromatids could not occur properly, which blocked the nuclear division.

Analysis of ts topo II mutants in yeast had shown that the enzyme was essential for

viability during the mitotic cell division (Holm et al., 1985). Synchronized cell cultures were shifted to the restrictive temperature at various time intervals and the cells observed for viability and morphology. The inviability was apparent at the time of the mitotic cell division, and this could be rescued by the addition of agents which block mitosis. The authors hypothesized that topo II could be required for the normal segregation of sister chromatids during mitosis.

To date there is no direct evidence showing that topoisomerases are responsible for meiotic recombination events. Results from experiments *in vitro* have shown topo I to facilitate the topological linkage of homologous DNA molecules with the aid of RecA (Cunningham et al., 1981; Kmiec et al., 1983). RecA is known to facilitate pairing of homologous regions of DNA, which led the authors to the conclusions that during recombination events, duplex DNA molecules may pair proir to any strand breakage by enzymes such as topoisomerase I. Interestingly, topoisomerases have also been shown to suppress recombination events in the rDNA regions of yeast (Christman et al. 1988). The authors showed that in double mutants for topo I and II there was an increased level of recombination in the rDNA regions. This increase was observed to be evident only in the rDNA regions of the genome. The authors hypothesized that recombination in regions containing repeat sequences could be in some way deleterious to growth and is suppressed by the combined action of topo I and II. The two experimental results discussed above show contradictary funtions for topo I and/or II in recombination events, but topoisomerase activity could be influenced by other unknown factors in the rDNA regions. However, these results do indicate that these enzymes in some way play a role in recombination. Topo II could play a major role in the resolution of DNA following recombination events. Rose and Holm (1993) showed a meiosis-specific arrest of topo II mutant yeast cells. They suggest that the arrest, which occurs during the pachytene stage could result from the cells trying to ensure that there are no tangles, especially between nonhomologous

chromosomes. By fluorescence and electron microscopy they made observations on the formation of the synaptonemal complex (SC), a tripartite proteinaceous structure seen during the early stages of the meiotic cell division. The SC appeared to form normally but the cells were delayed in their exit from the pachytene stage. They concluded that topo II could play a role in the untangling of interlocks during the formation of the SC. Recombination between homologous chromosomes at meiosis may be facilitated by the SC. The SC is thought to be responsible for the pairing of the homologous chromosomes and the formation of chiasmata (Moens et al., 1992). Topo II antibodies have been shown to localize along the axial core of the SC in yeast (Klein et al., 1988). In rooster pachytene and diplotene spermatocytes immunogold labelled anti-topo II antibodies localized to specific regions of the tri-partite structure along the entire length of the SC (Moens and Earnshaw, 1989). The antibodies were shown to congregate increasingly during pachytene and were associated with the SC cores until the chromosomes separated at diplotene. The authors did not determine the role for topo II in the SC, but speculated that the enzyme could be involved in chromosome condensation through the resolution of interlocks in DNA and the generation of double-strand breaks which could serve as possible points for the initiation of meiotic recombination.

Topoisomerases could also function post meiotically in the chromosome condensation process that occurs during the final stages of spermatogenesis leading to the tightly packed configuration apparent in the mature sperm head. Transient endogenous nicks in the DNA have been shown to be present during this period of chromatin condensation in the rat (McPherson and Longo, 1993). The authors showed topo II activity present in the nuclei of elongating spermatids and hypothesized that topo II might be involved in post meiotic chromatin condensation.

This study reports an investigation of topoisomerases during mouse spermatogenesis. Spermatogenesis in the mammal is a complex developmental process that

occurs in the testes. During this process primitive diploid cells undergo a series of morphological and cytological changes to finally produce haploid sperm. The temporal and stage-specific appearance of the various developmental stages have been well documented (reviewed by Russell et al., 1990; Bellvé, 1993). This system provides a valuable model for the analysis of topoisomerase gene expression in mitotic, meiotic and post meiotic stages of germ cells. A developmental profile of the transcripts or the enzyme activity of topo I and II can be compared with the known temporal DNA kinetics occurring in the various cell types, such as mitotic replication of DNA, meiotic recombination, transcription, chromosome condensation at meiotic metaphase and post-meiotic chromatin condensation for sperm head packaging. Mitotic replication occurs during the first few stages of spermatogenesis during which the primitive type A spermatogonia form the type A, then the intermediate, and subsequently the type B spermatogonia (Bellvé, 1993). Type B divide mitotically again to form the preleptotene spermatocytes which are presumably committed to complete the two meiotic divisions. The meiotic prophase stages are represented sequentially by the preleptotene, leptotene, zygotene, pachytene, diplotene, and diakinesis. Although there is no firm evidence when pairing of the homologous chromosomes and meiotic recombination occur, they are thought to begin at the leptotene stage and continue through the zygotene to the pachytene stage. Complete synapsis is apparent at the pachytene stage, during which there is also peak level of transcription activity (Roca and Mezquita 1989). The SC forms at the zygotene stage and is visible along the entire length of the paired chromosomes by the pachytene stage (reviewed by Alberts et al., 1989). The meiotic prophase takes about a week and is the longest of all the stages of spermatogenesis in the mouse. The spermatocytes then undergo two rapid meiotic divisions to produce the round spermatids. The post-meiotic differentiation, or spermiogenesis, includes differentiation of haploid spermatids to form the elongating spermatids, then the condensing spermatids and finally the mature sperm. A major

differentiative event of spermiogenesis is chromatin condensation, involving complex structural alterations and changes in nuclear proteins (reviewed by McPherson and Longo 1993).

The mouse has proved to be a valuable model for the study of spermatogenesis. From birth to maturity, the developmental stages of the testicular germ cells have been well documented (Bellvé et al., 1977). Mice at different ages can be used for developmental analysis of molecular events during spermatogenesis. The study of spermatogenesis has also been advanced by the ability to isolate and purify specific types of germ cells by the "STA-PUT" procedure (Bellvé et al., 1977). Stage-specific germ cells isolated by the STA-PUT protocol are highly enriched and can be used for *in vitro* manipulations. The study of meiotic events has been facilitated by the ability to culture pachytene spermatocytes for up to 48 hours, during which the cells maintain most of their morphological and cytological characteristics including pairing of homologous chromosomes (Handel et al., 1995). These cells can be used as a model for studying the meiotic metaphase chromosome condensation, since the cells can be induced to undergo the cell cycle transition from meiotic prophase to metaphase (Wiltshire et al., 1995).

Previous studies have already investigated some aspects of topoisomerase activity during spermatogenesis. Analysis of topo I and II expression during rooster spermatogenesis (Roca and Mezquita, 1989) did not include the specific developmental stages considered here, most probably due to the limitations imposed by the germ cell fractionation protocol that they used. McPherson and Longo (1993) studied topo II expression during post meiotic stages of spermatogenesis in the rat and related the enzyme activity levels during the successive spermiogenic stages to endogenous nicks present in the DNA. Topo II enzyme activity (by a decatenation assay) and protein levels (by immunoblots) were analyzed during *Xenopus laevis* spermatogenesis in the pachytene, round, elongating and mature sperm stages (Morse-Gaudio and Risley, 1994). These

authors also assayed DNA breaks induced by teniposide (a topo II-specific enzyme inhibitor) during the same stages, as well as in A and B type spermatogonia; these results were related to topo II protein levels. Their results showed the highest topo II levels in spermatogonia and spermatocytes, then lower in early-mid spermatids, still lower in elongate spermatids, and undetectable in sperm.

Our study aims to analyze and clarify the developmental expression of both topoisomerases I and II during male germ cell development in the mouse. Both transcripts and protein activity were studied in A/B type spermatogonia, leptotene/zygotene spermatocytes, pachytene spermatocytes, round spermatid stages (and in residual bodies) of spermatogenesis in the mouse. Comparison of topo I versus topo II levels could provide insights into the role of these enzymes during mitotic, meiotic and post meiotic stages of germ cell development. In addition, a study on the effects of okadaic acid (OA) on the transcriptional status of topoisomerase I and II was undertaken to determine if topoisomerases play a role in the OA-induced, rapid G2/M phase transition of cultured PS (Handel et al., 1995).

CHAPTER II

Materials and Methods

Materials and Animals

ICR mice were obtained from Harlan-Sprague and Dawley, Indianapolis, IN and maintained under standard conditions in the Walter's Animal Facility. Boxes with food and water (*ad libitum*) were changed twice weekly in accordance with PHS policy. The cages are maintained in light for 14 hours and dark 10 hours. Testes of XX Sex-reversed (XX*Sxr*) mice were obtained from frozen stocks in our laboratory. Topo I and IIα cDNA were a generous gift from Dr. Cho-Fat Hui (Institute of Molecular Biology, Academia Sinica, Nankang, Taipei, Taiwan Republic of China) and Dr. Stephen Kingsmore (Duke University Medical Center, Durham, NC).

Germ Cell Fractionation

Adult mice were used to obtain pachytene spermatocytes (PS), round spermatids (RS) and residual bodies (RB); eight-day old mice were used for A/B type spermatogonia (AB), and eighteen-day old mice were used for leptotene/zygotene spermatocytes (LZ). Mice were sacrificed by cervical dislocation, testes were excised, and detunicated, and the tubules dispersed (except for adult testes) and washed once in ice cold EKRB media (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 1.3 mM $CaCl_2$, 11 mM glucose, 1X Sigma essential amino acids, 1X Sigma nonessential amino acids). Prior to use, 5% CO_2 was bubbled through the EKRB for 20 minutes, and the EKRB was then filter sterilized. For solutions containing 0.5%, 2% and 4% BSA, the BSA was gently poured onto appropriate quantities of EKRB and allowed to dissolve overnight at 4^{0}C. Detunicated testes were subjected to enzymatic digestion (Bellvé, 1977 ; Romrell, 1976) with 0.5% collagenase (Sigma) for 20 minutes in a water bath at 33^{0}C, the cells were then washed with fresh ice cold EKRB three times by decanting the supernatant

containing most of the interstitial cells. Trypsin at 0.5% with DNase (1ug/ml) (both from Sigma) were added to the suspension of free seminiferous tubules and similarly digested for 13 minutes. During the enzymatic digestion the flasks were shaken at about 225 rpm in an atmosphere of 5% CO_2. The resulting cell suspension was then washed with 0.5% BSA in EKRB with DNAse (0.5ug/ml) to remove trypsin and filtered through 80μm nylon mesh. A single cell suspension (25ml) with a maximum cell count of 1X 10^9 cells was made up using fresh EKRB. The suspension was then sedimented at unit gravity through 2%-4% BSA density gradient in a STA-PUT sedimentation chamber (John's Scientific, Ontario, Canada) for 2.5 hours and fractions of approximately 10ml each (5ml for AB cells) were collected. The cells in each fraction were pelleted, resuspended, and scored for purity using Normarski optics. Peak fractions were pooled and scored again.

Criteria for morphological assessment of STA-PUT fractionated cells

Morphological assessment was done based on cell characteristics reviewed by Bellvé (1993). All cell types are classified based on the following general characteristics: a) shape and size, b) granularity of the nucleus, and c) presence or absence of distinguishing marks.

A and B type spermatogonia were obtained as a combined suspension. Type A spermatogonia were recognized by the presence of one or two flattened outer edges which results from the attachment of these cells to the basement membrane of the seminiferous tubules, and a nucleus occupying less than half the cell size present towards one side. Type B spermatogonia were more rounded with larger nuclei and a thicker peripheral region formed jointly by the nuclear and cell membrane. Contaminating cells present with the AB fraction were Sertoli and early spermatocytes. Sertoli cells were identified by their size (much larger than AB) and a distinctive nucleus which appears as a tri-globular structure. Early spermatocytes appeared generally much smoother, larger in size than the

AB spermatogonia and smaller than the Sertoli cells.

The leptotene and zygotene spermatocytes (LZ) had a spherical and a highly granular appearance. The cells were smaller in size compared to the contaminating Sertoli and pachytene spermatocytes. The nuclei appear granular, and occupy almost the entire cell, form a denser peripheral region since it is appears almost attached to the inner side of cell membrane.

The pachytene spermatocytes (PS) cells from adult mice were identified by their uniform appearance, smooth and rounded outer edges. A large nucleus appears as a distinct concentric circle with a rim of cytoplasm around it. The PS cells were the largest of the germ cells. Contaminating cells present in this fraction were mainly symplasts of three or more round spermatids.

The round spermatids (RS) were smaller in size than PS. The shape of RS ranged from circular to slightly oblong (elongating spermatids). Most of the cells had a distinct "pimple" at the central region of the nucleus. Contaminating cells in this fraction included PS and Sertoli cells.

The residual bodies (RB) were identified by their smaller size (less than 50% of RS), granular appearance with shapes ranging from round to irregular, and the absence of nuclei. Contaminating cells in this fraction were a number of sperm heads.

Typical cell purities obtained after scoring over a hundred cells were about 85% for AB, 77% for LZ, 90% for PS, 91% for RS and 85% for RB.

Germ cell culture and OA treatment

Germ cell culture was done as described by Handel et al. (1995). The PS fraction from adult mice was washed once with EKRB (0.5% BSA) and then with fresh, sterile filtered MEM-α media [1.02% MEM-α powder, 0.22% $NaHCO_3$, 0.59% HEPES, Sodium Lactate 0.175%, 0.059% penicillin, 0.01% streptomycin, pH to 7.2 (all from

Sigma)]. Cells were diluted with MEM-α media to a density of 2.5 X 10^6/ml and cultured overnight at 0.62ml per culture well in 4-well Nunclon dishes in a gaseous environment of 5% CO_2. OA (ICN Biomed) was added at 5μm concentration to each of the culture wells except controls. Cells were harvested at hourly intervals thereafter and pelleted for RNA isolation. Cell viability, as determined by trypan blue exclusion, was >95% before culture, 82% after overnight culture, and 76% four hours after OA treatment.

RNA Isolation and Northern Hybridization

RNA was isolated by the guanidium-isothiocyanate method of Cathala et al. (1983). All reagents were prepared in diethylpyrocarbonate (depc)-treated water and glassware baked overnight at 400°C was used. Testes frozen in liquid nitrogen were pulverized with a mortar and pestle, then homogenized in lysis buffer (5M guanidium isothiocyanate, 10mM EDTA and, 50mM Tris-HCl, pH 7.5) with 20 passes through a 20-gauge needle. RNA was precipitated overnight at 4°C in 7 volumes of 4M LiCl and pelleted by centrifugation (Beckman, JS-13 rotor) at 10K for 90 minutes at 4°C. The pellet was resuspended in solubilization buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA, and 0.1% SDS) and treated with proteinase K (Sigma) at 40μg/ml for 45 minutes at 37°C. The solution was then extracted twice with phenol:chloroform (1:1) and twice with chloroform. RNA was precipitated with NaAc at 0.2M, pH 7.0 and two volumes of cold ethanol overnight at -20°C and then pelleted at 10K for 20 minutes. The pellet was dried under vacuum and resuspended in depc-treated water. Quantitation was done with a Beckman DU650 spectrophotometer. All samples showed OD_{260} to OD_{280} ratios ranging from 1.9973 to 2.1349, which were indications of good purity of the samples. RNA was stored at -80°C until required.

Twelve μg of total RNA per sample was denatured at 65°C for 15 minutes in 50%

deionized formamide (Ambion, Austin, TX), 1X MOPS buffer, 6.5% formaldehyde(pH 6.0). Following the addition of 2 µl of loading dye (7.5% Ficoll 400, 0.125% BPB, 12.5mM EDTA, pH 8.0, and 50% sterile glycerol) the samples were loaded onto a denaturing 1% agarose gel (1X MOPS buffer, 2.2M formaldehyde). The samples were electrophoresed at 5V/cm until the dye front was about 8.5 cms from the wells. After several washes with distilled water (35 minutes) the gel was stained with ethidium bromide at 0.5µg/ml for 20 minutes and destained for 20 minutes. The gel was photographed to visualize RNA and the 28S and 18S bands in particular. It was then soaked in 20X SSC buffer (Sambrook et al., 1989) , and blotted onto nylon membrane (Hybond, Amersham) overnight. RNA was UV crosslinked to the nylon blot at 1280 µjoules/cm^2 in a Stratalinker (Stratagene, LaJolla, CA) . Blots were stored at room temperature till use.

Maxipreps (Quiagen, Chatsworth, CA) of each of the cDNAs were digested with the appropriate restriction enzyme and the insert separated by gel electrophoresis. The insert was then purified from the agarose gel by the Gene Clean method (BIO101, Inc.,) and quantitated by comparing with HindIII cut lambda DNA and ethidium bromide staining. 10ng of insert was labelled with radioactive ^{32}P dCTP (NEN Products, Boston, MA) by the random priming method described elsewhere (Feinberg et al., 1983). Probes were purified on Sephadex G-50 columns made using 5ml glass pipettes and added to the blots at appropriate cpm {topo I, topo II and hypoxanthine phosphoribosyl transferase (Hprt) at 2 X 10^6/ml and ß-actin at 0.75 X 10^6/ml of hybridization solution}.

Prehybridization [50% formamide, 5X SSC, 50µM NaPO4, pH 7.4, 0.25mg/ml sonicated fish sperm DNA (Boehringer Mannheim), and 5X Denhardt's solution] was at 42°C for 2-4hrs in heat sealable polythene pouches (Kapak Corporation, Minneapolis, MN). After removal of the pre-hybridization solution, the hybridization mix containing one

part 50% Dextran Sulfate, three parts fresh prehybridization solution and denatured probe solution was added to the same pouches which were heat sealed again. Hybridization was at a temperature of 42°C for 16-20 hours with shaking. Following hybridization the blots were washed twice in 1X SSC, 0.1% SDS at 42°C for 15 minutes with shaking, and once with 0.1XSSC at 60°C for 10 minutes (twice for a total of 30 minutes for ß-actin). Blots were wrapped in plastic and exposed to x-ray film (Sterling X-100) at -80°C for appropriate periods of time. Stripping of blots (using boiling 0.1% SDS) was done as recommended by the Amersham-Hybond catalogue. Efficiency of stripping was checked by autoradiography by exposing the blots to film at -80°C for over 4 days. On satisfactory stripping the blots were UV crosslinked and stored in a moist condition at 4°C or used immediately for reprobing.

Densitometric scanning of the autoradiographs was done on an Imaging Densitometer (Biorad, GS 670) and quantitation on Molecular Analyst program (Macintosh, Ver 1.1.1).

Preparation of Nuclear Extracts and Topoisomerase Enzyme Assays

Nuclei were obtained by a protocol modified from Wu (1989). All procedures were done on ice. All solutions used for preparation and storage of nuclear extract were made fresh and contained 1mM PMSF, 0.1mM leupeptin, 100U/ml aprotinin, 10mM 2-mercaptoethanol, and 0.5mM DTT, which were added just prior to use. The PS fraction from the STA-PUT was resuspended in ice cold Nuclei Buffer 1 (NB1) (0.3M sucrose, 60mM KCl, 15mM NaCl, 5mM $MgCl_2$, 0.1mM EDTA, 15mM Tris-HCl, pH7.5) and homogenized in a Dounce homogenizer by working the plunger 12-14 times. The resulting suspension (at 1 ml per column) was then spun through a column of 5-6 ml NB2 buffer cushion (same as NB1 but with 1.2M sucrose) at 8K for 20 minutes (Beckman, JS13 rotor). The supernatant was aspirated carefully and as completely as possible. The

white pellet of nuclei was used to prepare nuclear extract as described elsewhere (Roca et al. 1989). About 10^8 nuclei were resuspended in 200ul of solution A (1M Tris-HCl, pH 7.5 and 0.5M EDTA). To this suspension one ml of solution B (1M Tris-HCl, pH 7.5, 2.4M KCl) was added slowly. The nuclei were homogenized in a sonicator (Laboratory Supplies, Hicksville, NY, Model G112SPIT) at 600V, 80KC for about one minute . The DNA was precipitated for 30 minutes on ice following the addition of 600ul of solution C (1M Tris-HCl, pH 7.5, 18% PEG8000, 2.4M KCl). The solution was centrifuged at 15000g (11K in a Beckman JS13 rotor) for 25 minutes to pellet the precipitated DNA. The resulting supernatant containing the nuclear extract was mixed with either 15% or 30% of glycerol, aliquoted and stored at -80^{0}C. Nuclear extracts were quantitated by the Bradford assay (Biorad dye reagent) using BSA protein standards (Sigma).

The topo I relaxation assay was performed on serial dilutions of the nuclear extracts from PS and RS by a protocol modified from Hildebrandt et al. (1990). Twenty µl of reaction mix containing 2µl of a 10X reaction buffer (0.5M Tris-HCl, pH 7.5, 0.5M KCl, 0.1M $MgCl_2$, 1mM EDTA, 5mM DTT, and 0.3mg/ml BSA), and 0.25µg supercoiled pHOT1 DNA (TopoGen, Columbus, OH) was incubated at 37^{0}C for 20 minutes. A control reaction containing dilutions of purified human topoisomerase I (TopoGen) served as the standard for assessing the level of activity of the enzyme in the nuclear extracts. The reaction was immediately cooled on ice and stopped with 2 µl of a stop buffer (5% SDS, 0.1M EDTA, 60% glycerol, and 0.25mg/ml BPB). Samples were electrophoresed (5V/cm for 1.25hrs) on a 1% agarose gel and the bands visualized by ethidium bromide staining (2µg/ml) for 20 minutes and destaining for 20 minutes. The gel was photographed using a positive/negative polaroid film (polaroid#55). Band intensities are compared using an imaging densitometer as before.

A decatenation assay for topo II was performed according to a protocol modified from Morse-Gaudio and Risley (1994). Dilutions of the nuclear extract were added to 100ng of kinetoplast DNA (TopoGen) in a 20µl of a reaction mixture containing 4ul of a 5X buffer (0.25M Tris-HCl, pH 7.5, 0.5M KCl, 35mM $MgCl_2$, and 15mM DTT), and 10mM ATP. Serial dilutions of pure Drosophila topo II (USB, Cleveland, OH) served as a control for comparison. Following incubation at 37°C for 80 minutes, the reaction was stopped as in the topo I assay and electrophoresed similarly.

CHAPTER III

Results

Transcript levels of topo I and II α in testes and germ cells

Total cellular RNA was isolated from testes of mice aged 8, 16, 21, 30, and (adult) 55 days. The photograph of the ethidium bromide stained gel (Fig.1) demonstrates intact RNA by the appearance of the 18S and 28S bands. After transfer to a nylon membrane the blot was cut in half along the center of the molecular weight marker (Boehringer Mannheim) lane. One half of the blot was first probed with a control probe (ß-actin), and results indicated both uniform loading of samples and good RNA quality (results not shown). The second set was then sequentially probed and stripped with topo I, topo II, Hprt, and ß-actin. The autoradiographs for these hybridizations are shown in Figs 2A-2D and the corresponding densitometric scans are in Figs 3A-3D.

Topo I transcript levels are very high during early development where the representation in testes of cells in division is maximal (Figs. 2A and 3A). The level of topo I transcript decreases drastically after day 21 with the appearance of a higher proportion of haploid spermatids. Topo I transcript was also highly expressed in the germ cell deficient testes of the XX*Sxr* mice (Fig. 2A, lane 1). Topo IIα transcript levels (Fig 2B and 3B) indicate a negligible amount of transcript in the XX*Sxr* mice (lane 1). The level of topo IIα transcript increases from day 8 mice, peaks at day 21 and then decreases to the levels found in adult testes. Transcript levels for Hprt were almost similar to that observed in earlier experiments on Balb/c mice (Shannon and Handel, 1993); however they had shown a three fold increase in Hprt expression from day 8 to day 16, which was not observed here. This difference was re-examined by analysis of Hprt transcript in testes aged 8, 10, 12, 14 and 16 days (results not shown). No significant increase in Hprt transcript level was observed between day 8 through 16.

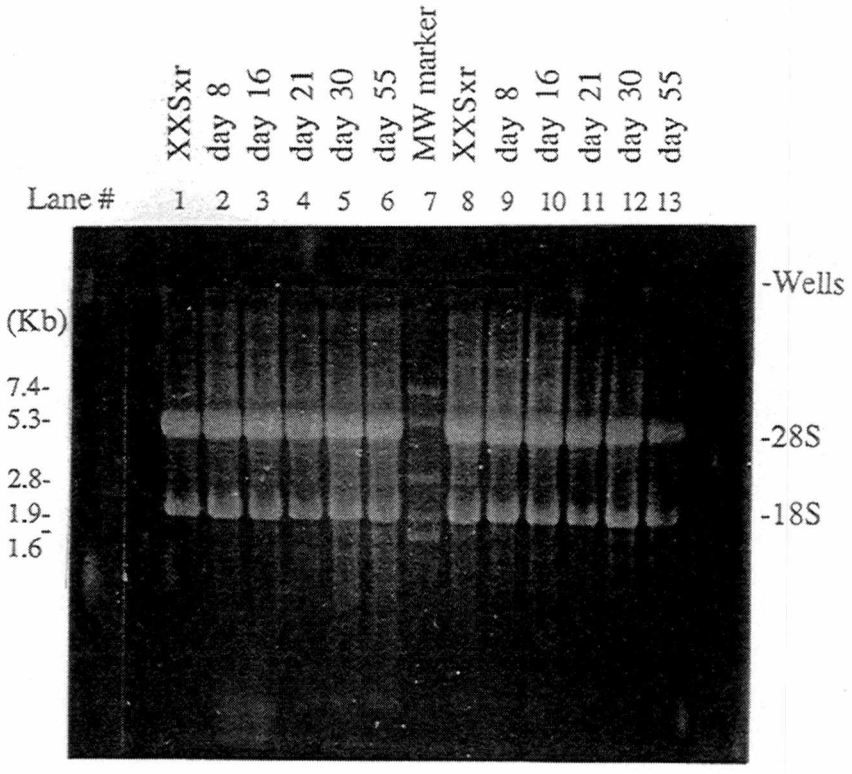
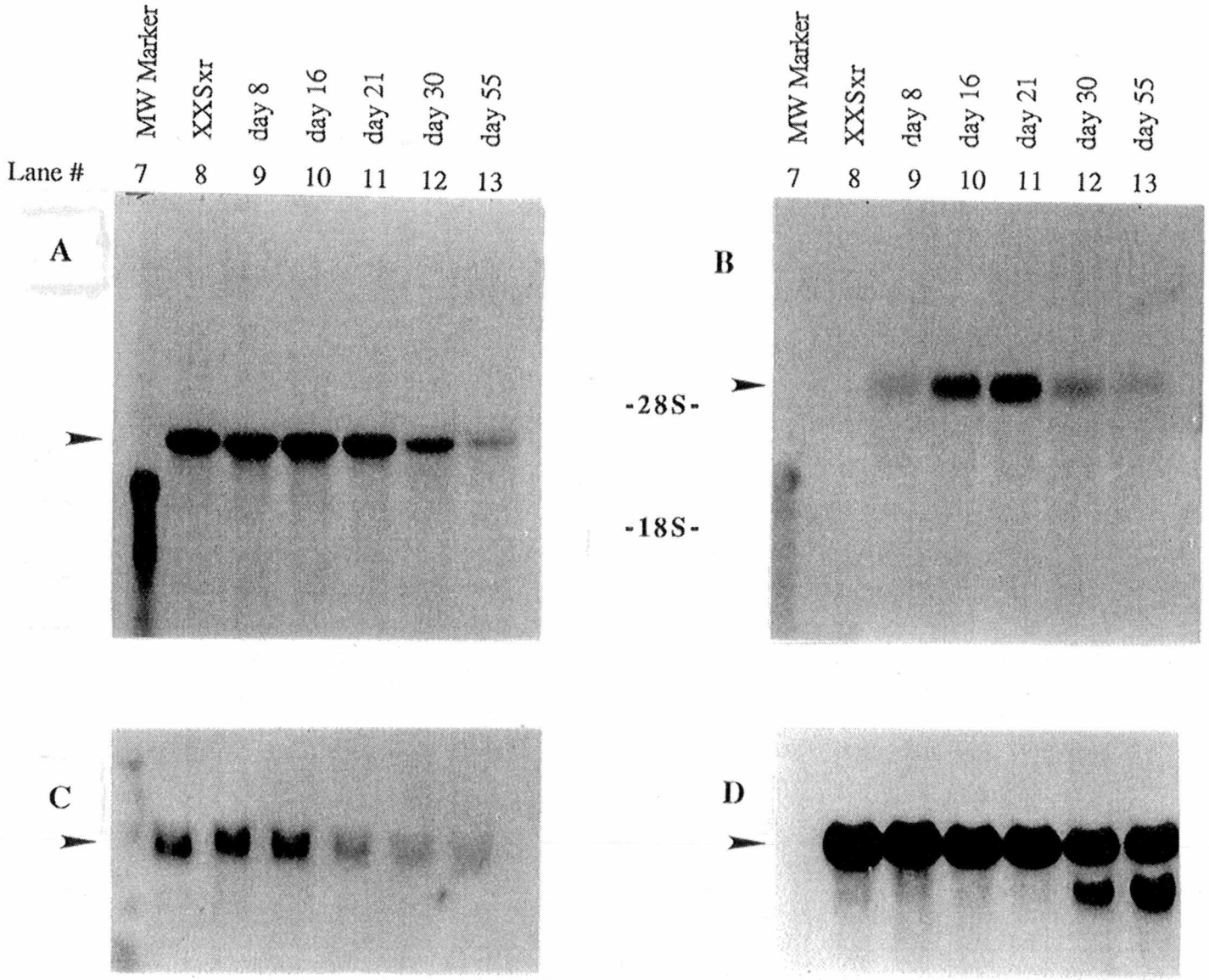


Fig. 1: Ethidium-bromide stained gel of total RNA(12µg) from prepubertal to adult mice testes. Electrophoresis was under denaturing conditions as described in Materials and Methods. The samples are XXSex reversed (XX*Sxr*) in lanes 1 & 8, day 8 (lanes 2 & 9), day 16 (lanes 3 & 10), day 21 (lanes 4 & 11), day 30 (lanes 5 & 12), and day 55 (lanes 6 & 13). Lane 7 contains 15µg of RNA molecular weight marker (Boehringer Mannheim). Positions of the wells, the 28S and 18S RNA bands, and the molecular weight markers are shown.

Fig. 2: Northern analysis of total RNA from testes. The blot from gel in Fig. 1 was sequentially probed with topo I (Fig. A), topo II (B), Hprt (C), and ß-actin (D). Band positions are indicated by thickened arrows. Autoradiograms were exposed to blots at-80^{0}C for 14, 10, 12, and 7 hours respectively. The lower molecular weight band seen in the case of ß-actin (Fig. 2D, lanes 12 and 13) is due to alternative splicing of the gene in RS.


MW Marker
XXSxr
day 8
day 16
day 21
day 30
day 55
Lane #
7
8
9
10
11
12
13
A
B
-28S-
-18S-
C
D

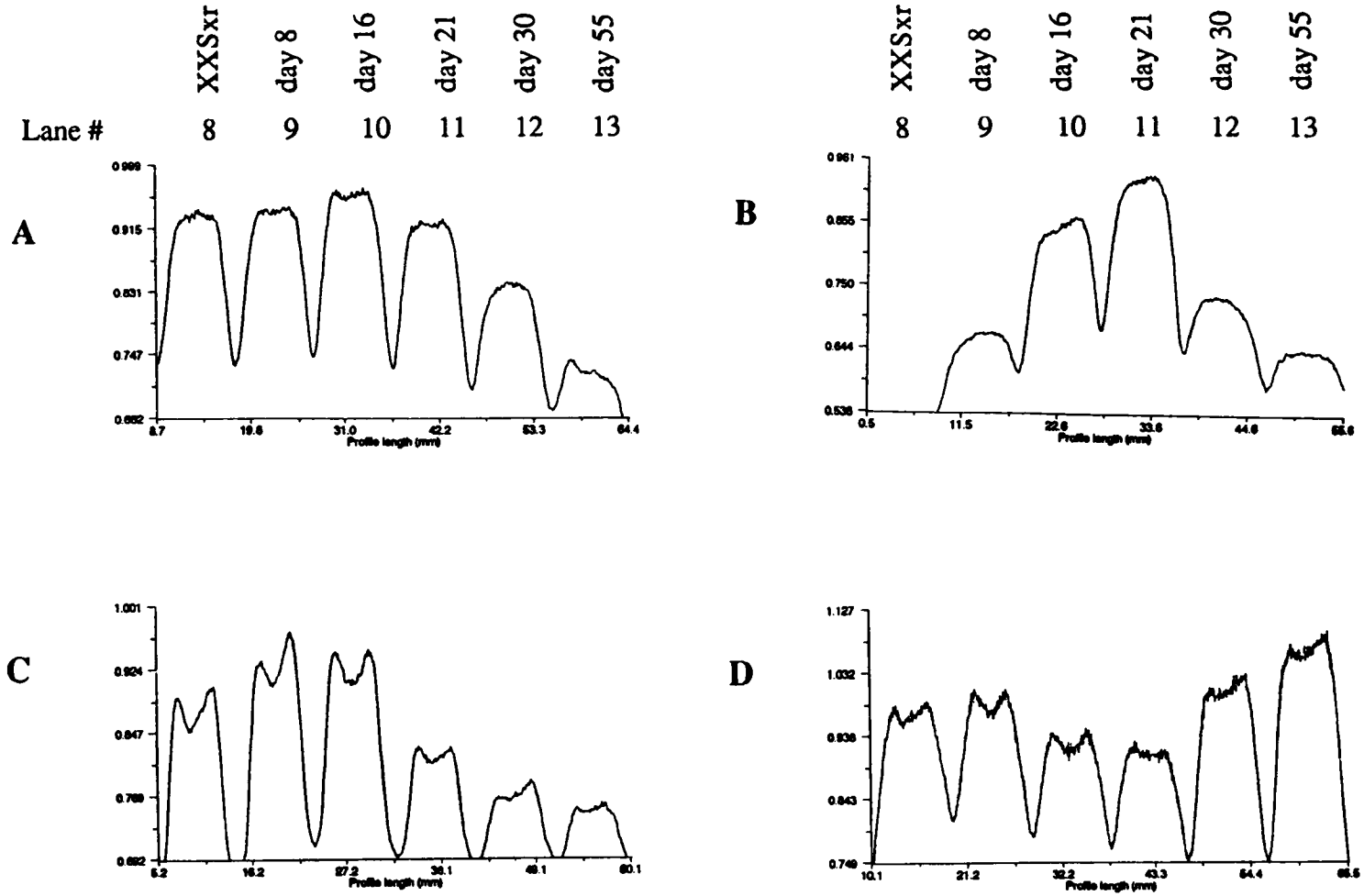


Fig. 3: Densitometric profiles for the corresponding autoradiographs in Fig.2.A) topo I, B) topo II, C) Hprt and D) ß-actin. Optical density readings are indicated on the Y-axis.

A Northern blot analysis of transcripts in specific cell types was done to extend the results of the above experiment. Fig 4 shows the ethidium bromide stained gel of the samples used (again in duplicate, except for the residual bodies on the second set). There is slight overloading of the RB(lane 7) but it did not affect the outcome of the experiment since most of the transcripts tested had negligible quantities expressed in the residual bodies. The nylon blot was cut between lanes 7 and 8 after the transfer to separate the two sets. The second set (minus RB) was used to check for uniformity of loading and RNA quality as before. The first set was probed with topo I, topo II, Hprt, and ß-actin. The autoradiographic results are shown in Figs.5A-D and the corresponding densitometric scans in Figs. 6A-D. Most of the topo I transcript expression (Figs. 5A and 6A) is from three specific cell types, ie. AB, LZ and PS (lanes 3, 4 and 5 respectively). There is very little expression of topo I transcript in the post meiotic RS (lane 6) and negligible quantity of transcript in the residual bodies (lane 7). The highest level of topo I transcript appears to be in the germ cell free testes of XX*Sxr* mice testes suggesting that somatic cells contribute much of the topo I transcript. In contrast topo II transcript levels (Figs. 5B, 6B) increase steadily from AB type spermatogonia and are maximum in PS. The RS also show a very high level of topo IIα expression. The levels in testes of XX*Sxr* mice was negligible (Fig.5B, lane1). The pattern or expression of the Hprt transcript (Fig. 5C) resembled that obtained in earlier experiments (Shannon and Handel, 1993). A high level of transcript is seen in the XX*Sxr* testes, AB and LZ, while RS show a low level of Hprt transcript and there is negligible transcript in the PS and RB lanes.

Topo I and II α transcript levels in OA-treated germ cells in culture

No significant differences were found in the levels of either topo I or topo IIα transcripts between PS cells cultured with and without 5μm OA (Figs. 7A and B). The cells treated with OA were analyzed at hourly intervals from 0 through 4 hours of OA

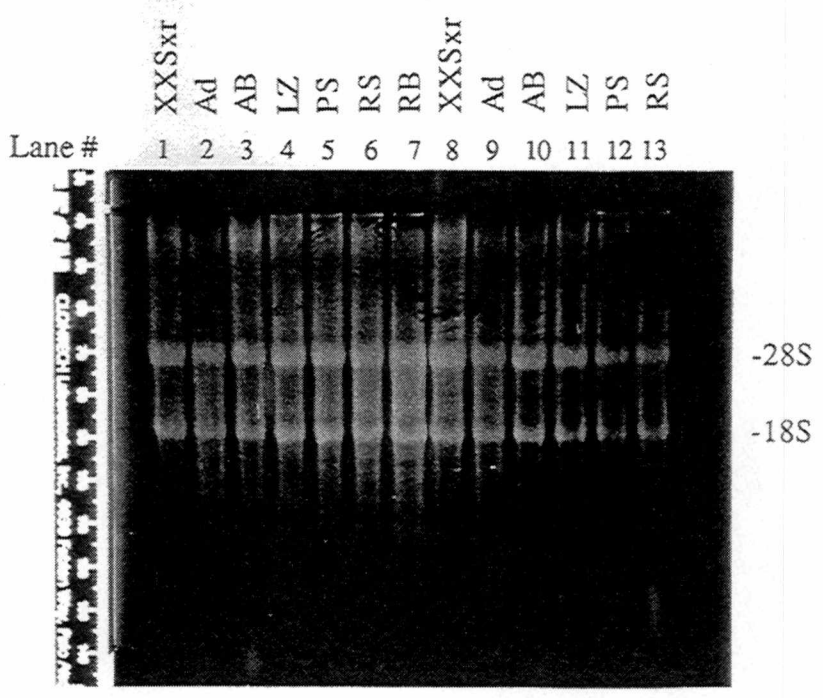
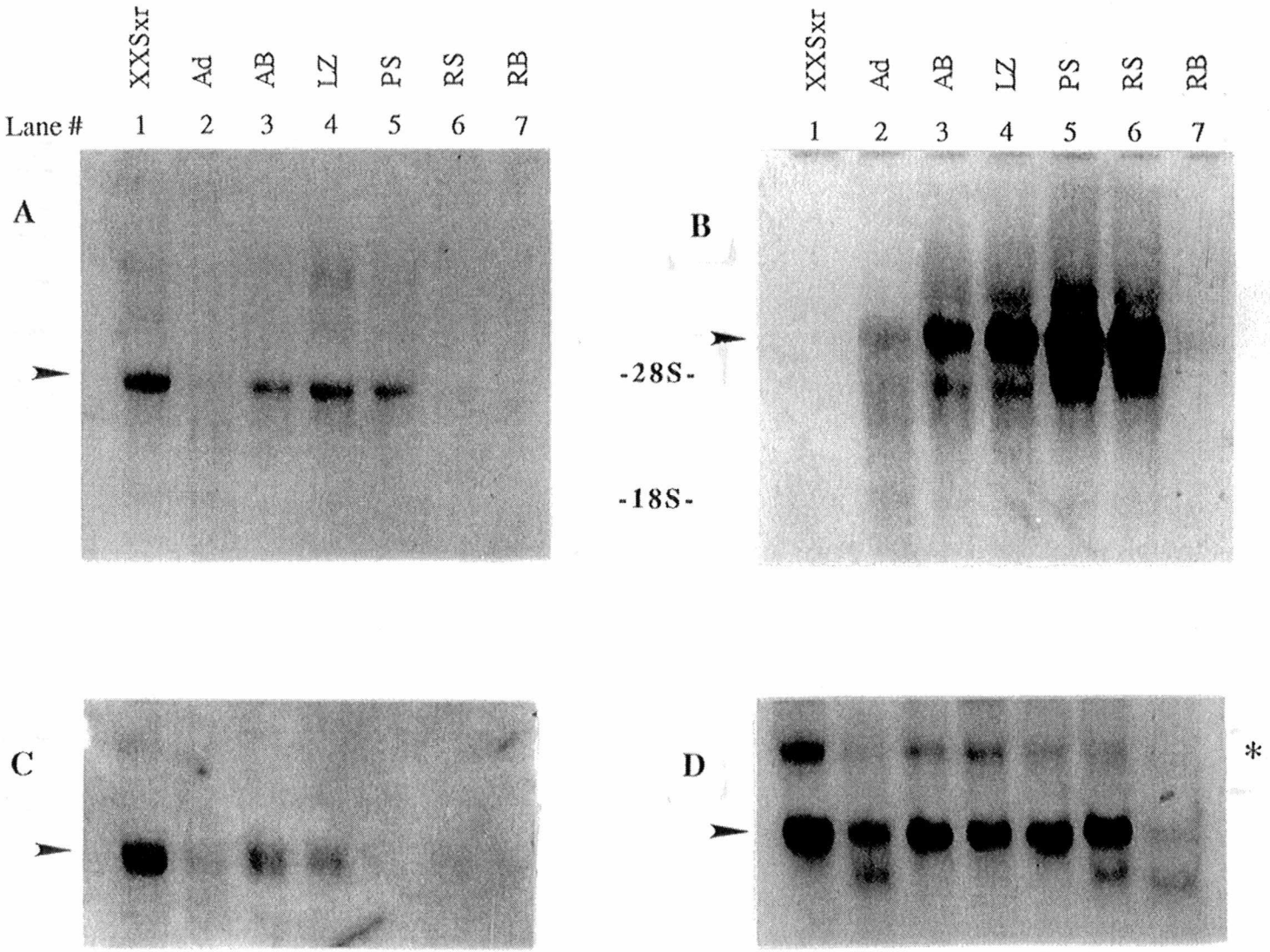


Fig. 4: Ethidium-bromide stained gel of total RNA (12 μg) from STA-PUT fractionated germ cells. The samples used are XX*Sxr* in lanes 1 & 8, Adult testes (Ad) in lanes 2 & 9, AB spermatogonia (AB) in lanes 3 & 10, leptotene/zygotene spermatocytes (LZ) in lanes 4 & 11, pachytene spermatocytes (PS) in lanes 5 & 12, round spermatids (RS) in lanes 6 & 13, and residual bodies (RB) in lane 7. Positions of the wells, 28S and 18S bands of rRNA are indicated. The scale to the left is in centimeters.

Fig. 5: Northern hybridization of total RNA from germ cells. The blot from gel in Fig. 4 was probed with topo I (A), topo II (B), Hprt (C), and ß-actin (D). The higher MW band (*) in D is due to non specific binding of probe to the 18s band of ribosomal RNA. Alternatively spliced ß-actin band can be seen in lanes 2 and 6 in Fig. D. Autoradiographs were exposed for 17.5, 48.5, 126, and 9 hours respectively.


XXSxr
Ad
AB
LZ
PS
RS
RB
Lane #
1
2
3
4
5
6
7
A
B
-28S-
-18S-
C
D
*

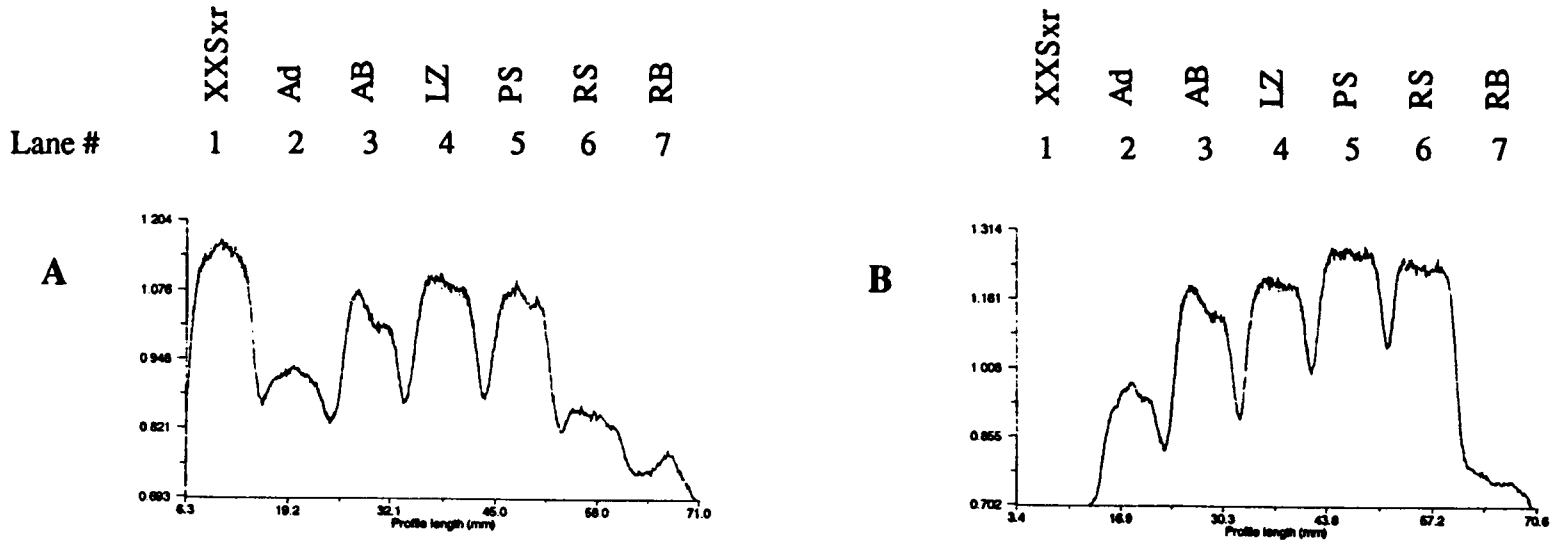


Fig. 6: Densitometric profiles of topo I (A) and topo II (B) in purified germ cells. Autoradiographic results shown in Fig. 5A and B were scanned as described in the Methods section.

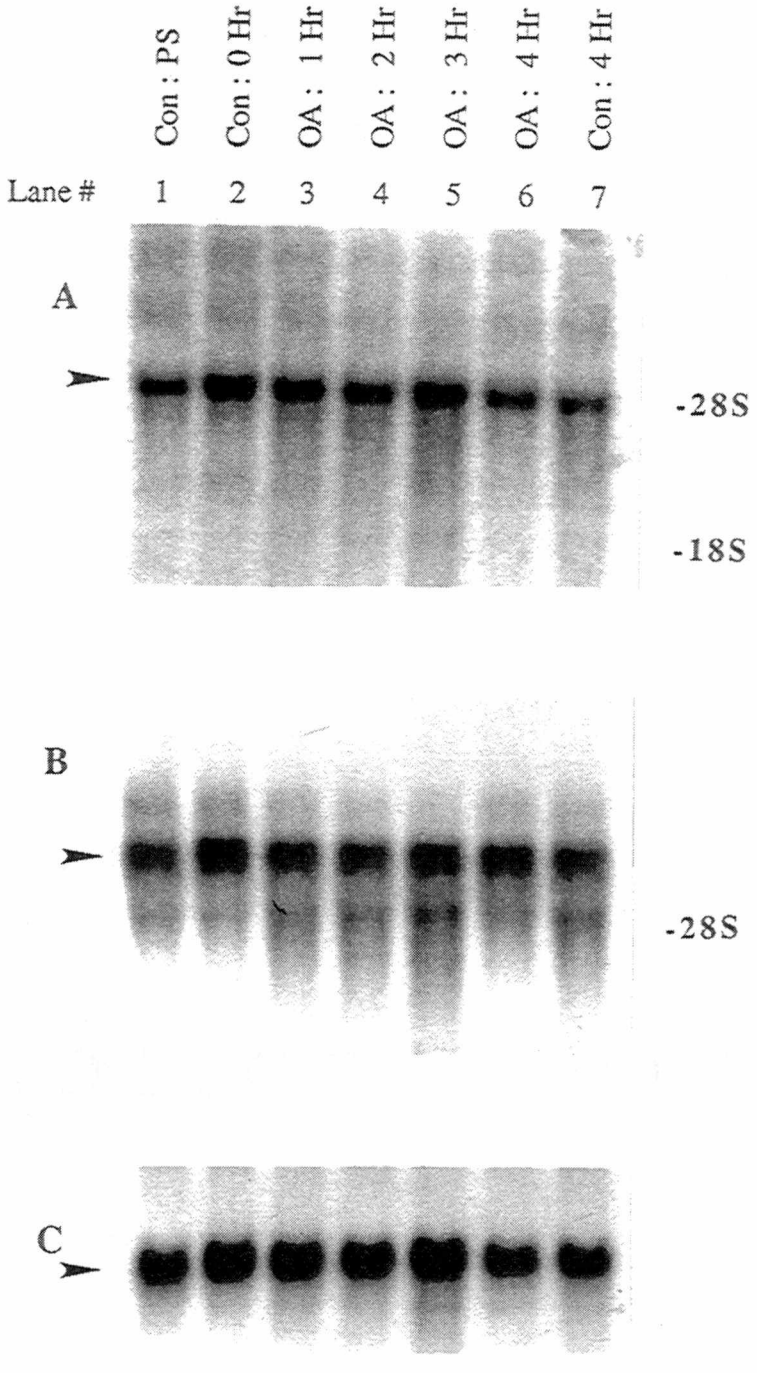


Fig. 7: Transcript levels for topo I (A) and topo II (B) expression in OA-treated PS in culture. Band positions (dark arrows) and 28S and 18S bands are marked. The blot was also probed with ß-actin cDNA probe (C). Control lanes (minus OA treatment) were lane 1 (before overnight culture), lane 2 (after overnight culture) and lane 7 (four hours later). Lanes 3-6 are OA treated samples collected at hourly intervals. Autoradiographs were exposed at -85^{0}C for 31, 11.5, and 7 hours respectively.

treatment, while control cells were analyzed at only two time points (0 and 4 hours). A very slight increase in transcript in topo I and II in lanes 3, 4 and 5 corresponds to an increase in the quantity of RNA in these samples as can be seen in the blot when probed with the ß-actin control (Fig. 7C).

Enzyme assays for topo I and IIα in nuclear extracts from PS and RS

Both topo I and IIα are known to relax supercoiled plasmid DNA. Topo IIα has an additional requirement for ATP and magnesium ions. Topo I activity is measured by the enzyme's ability to relax a supercoiled plasmid substrate in the absence of ATP and magnesium ions. Topo IIα activity, on the other hand, can be assayed by using knotted kinetoplast DNA as a substrate in the presence of ATP and magnesium ions. Nuclear extracts from PS and RS were prepared in the presence of protease inhibitors in 2.4M KCl. Serial dilutions of nuclear extracts standardized for protein content were used for the assays.

The topo I relaxation assay was performed with 200, 100, and 50ng of nuclear protein from both PS (Fig.8A, lanes 4,5,6 respectively) and RS (lanes 7,8,9 respectively) nuclear extracts. The supercoiled band in each lane was scanned by an imaging densitometer (Fig. 8B). There was no visible difference in topo I enzymatic activity between PS and RS. Serial dilutions of pure topo I (lanes 10-13) was used to establish a standard against which the samples could be compared for estimation of units of enzymatic activity. With increasing concentration of nuclear extract or the pure enzyme there was disappearance of the supercoiled band (marked 'S'). Pure enzyme quantity (in increasing units) on the X-axis was plotted against decreasing amount of supercoiled band (substrate disappearance) on the Y-axis (Fig.9). Estimates from the graph showed that roughly 1µg of nuclear extract in both samples had about 1.2 units of topo I activity.

Topo IIα levels were determined by the decatenation assay using kinetoplast DNA

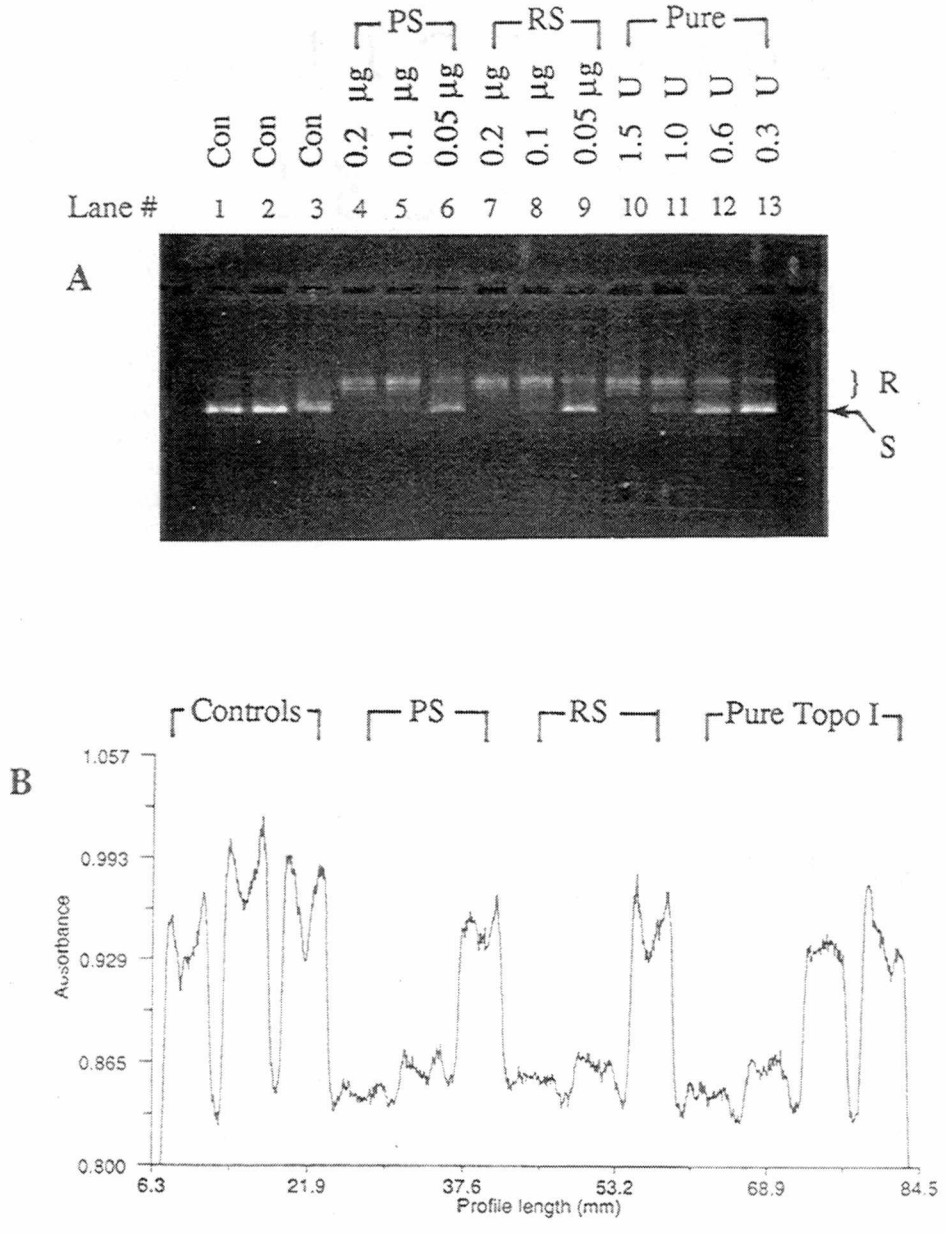


Fig. 8: **A)** Results of relaxation assay for topo I activity in nuclear extracts (NE) from PS and RS. Negative controls are in lanes 1 (minus NE, plus nuclear extract dilution buffer), 2 (topo I replaced by topo II), and 3 (minus NE). Dilutions of PS NE was in lanes 4-6, and RS NE in lanes 7-9. The protein concentrations in µg has been indicated. Positions of the supercoiled band (S) and relaxed bands (R) have been marked. Pure topo I was in lanes 10-13 (Units activity indicated). **B)** Densitometric profile of negative film of fig A. Only the lower (S) band was quantitated.

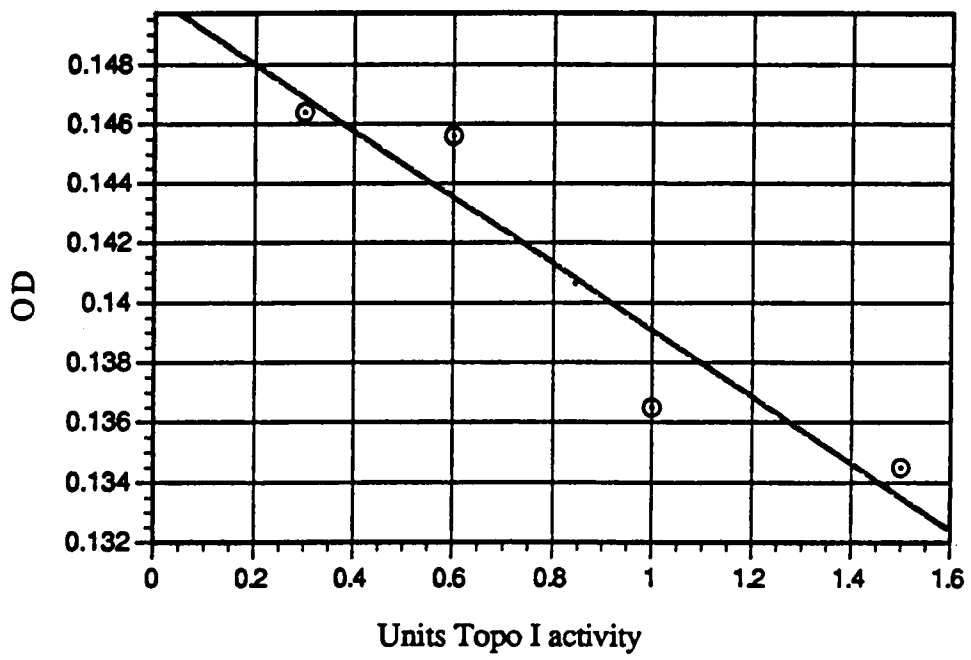


Fig. 9: Graphical representation (Delta Graph) of pure topo I profile from Fig. 8. Since only the lower band of supercoiled plasmid was scanned, increasing OD readings on the Y-axis represents increasing activity of the enzyme on the X- axis.

(kDNA). kDNA is a highly networked and catenated form of DNA derived from mitochondria of the protozoan *Crithidia fasciculata,* and consists of a complex of mini circles (Rauch et al., 1993) which can be released as monomers by the double strand nicking and resealing action of Topo IIα. Nuclear extract was assayed at concentrations ranging from 1ug - 200ng. Preliminary results revealed inhibition of Topo IIα activity with increasing concentration of nuclear extract and a total inhibition beyond 1ug. Further experiments are required, but have not yet been done, to determine the linear range of protein concentrations resulting in increasing amount of substrate converted. However, from ethidium bromide stained gels of the released monomers of kDNA, it was evident that the levels of Topo IIα activity shows no evident differences between PS and RS (Fig.10); a similar lack of difference between the two cell types was found for topo I activity.

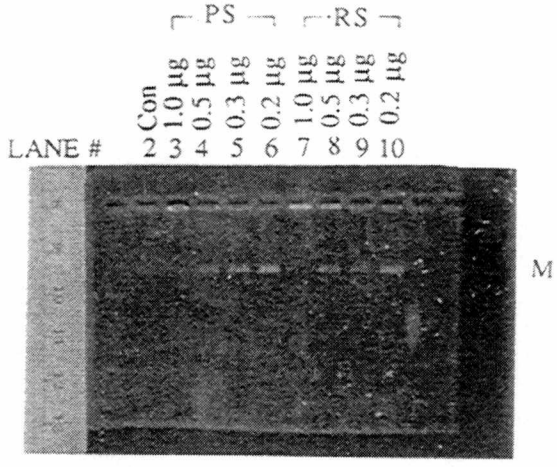


Fig. 10: Results of topo II decatenation assay. Nuclear protein extracts from PS (lanes 3-6) and RS (7-10) were incubated with 100ng of kDNA as mentioned in Materials and Methods. Lane 1 is a negative control minus NE. The knotted kDNA substrate (seen in the wells) is converted into monomers by the enzyme which is shown as a lower molecular weight band. The protein concentration in µg is indicated. Position of the wells and decatenated monomer (M) has been indicated. Increasing conversion of the substrate is seen as the NE concentration is decreased from 1.0 to 0.2µg.

CHAPTER IV

Discussion

The main objective of this study was to analyze the expression levels of transcripts for topo I and II in the different developmental stages of spermatogenesis in the mouse. Additionally an analysis of topo I and II enzyme activity in the pachytene and round spermatid stages was also undertaken. This analysis permits a correlation of the transcript/enzyme activity levels with known changes or existing conditions in DNA topology during mitotic, meiotic and post meiotic cell divisions. The mouse provides an ideal model for such an analysis because the temporal appearance of spermatogenic cells in the mouse testis has been well documented (Bellvé et al., 1977). Day 8 seminiferous tubules contain A and B type spermatogonia (27%) as well as the somatic Sertoli cells (73%). By day 16 the AB germ cells divide and differentiate to form the preleptotene to zygotene cells (17%) and PS (15%). The relative proportion of Sertoli cells thus gradually diminishes with age, and is about 37% by day 16. By day 21 the PS account for the largest percentage of the total germ cells in the seminiferous tubules (36%), and the RS begin to appear at this stage. In the adult testes (day 55 and above) the full complement of germ cells are present. These ages mark temporal points at which developmental analyses can be conducted. Additionally, the mouse is advantageous for the study of developmental gene expression because highly enriched stage-specific populations of germ cells can be obtained by the STA-PUT procedure. Results from analyses of these cell populations augment and extend results from analyses of whole testes at different ages.

This study of topo I gene expression was the first of its kind. Determination of topo I transcript levels during the above ages indicated increasing and high levels of expression from day 8 to day 16. There is a slight decrease by day 21 and a drastic drop thereafter until maturity is reached. The transcript level from day 8-16 can be assumed to

reflect the high proportion of mitotic and early meiotic or prophase germ cells in these testes. A major contributor could also be topo I transcript in the Sertoli cells, which form a large fraction of the cell population at this stage and which divide until about day 10. A high level of topo I transcript in the testes of the germ-cell-deficient XX*Sxr* mice (containing mainly Sertoli cells) also supports this interpretation. The drop in topo I transcript levels after day 16 coincides temporally with the appearance of an increasing number of non-dividing post meiotic round spermatids. Thus it seems that in the adult mouse testes the topo I transcript detected is associated with the AB, LZ, PS and the Sertoli cells. This developmental analysis has a major shortcoming in that it is difficult to ascertain the exact level of transcript contributed by each of the cell types at any given age. These results can therefore be augmented by determination of topo I transcript levels in specific germ cell types isolated by the STA-PUT protocol. Results from this analysis (Fig. 5A, 6A) in fact did support the above assumptions. The highest level of topo I expression was seen mainly in three cell types ie., AB, LZ and PS. The PS and LZ fraction showed a slightly higher level that AB. In comparison to these three stages, there was negligible transcript in the haploid RS.

These results demonstrate a differential topo I transcript expression during spermatogenesis. As mentioned above there is negligible transcript for topo I in the post-meiotic RS when compared to the RS. However, the relaxation assay for topo I enzyme activity in nuclear extracts from PS and RS indicated an almost equal level of activity in both these cell types. Thus there must be carry over of the enzyme from the PS to the post-meiotic RS. Results for the enzymatic activity of topo I or topo II are more definitive than transcript analysis in designating a functional role for these enzymes in the stages considered. Estimation of transcript levels does not neccessarily define function but does suggest the possibility of a functional role. My results clearly indicate that topo I transcript is present at a high level during the mitotic (AB spermatogonia), meiotic (PS) and post

meiotic RS (only enzyme activity) stages of spermatogenesis and this may reflect requirement for the enzyme. Roca and Mezquita (1989) assayed topo I enzyme activity in successive stages of chicken spermatogenesis. They showed that topo I activity paralleled transcriptional activity as determined by the uptake of ^{3}H labeled uridine, estimated by liquid scintillation spectrometry. Their results indicated that topo I activity was low in Stage I (premeiotic and gonial cells) and stage III (haploid spermatids). In comparison topo I activity peaked sharply in stage II ("tetraploid cells"- a misnomer they used for diploid PS). Comparison of my results to theirs was possible for the two cell types in which I measured topo I enzyme activity (ie., PS and RS, which corresponds to their description of stages II and III respectively). For these two stages there was a contrast in results since the topo I enzyme activity in my results showed an almost equal level in both cell types. However it is evident that their nuclear isolation and germ-cell fractionation protocols were not efficient in obtaining enriched fractions of (more specifically) the earlier stages of spermatogenesis (pre-pachytene stages). No mention was made in their results on the purity of any of the fractions obtained by either protocol. The differences in results in the enzyme activity in PS and RS are unlikely to be methodological since we followed almost the same protocol for the extraction of nuclear extracts from these stages and the assay for enzyme activity. However, the differences could be attributed to species differences. In my results, I found topo I transcript levels fairly high in all the stages, AB (premeiotic), LZ and PS (diploid/4C) and RS (haploid).

Topo I has been implicated mainly in modifying DNA topology following stress resulting from the action of the transcriptional machinery. The movement of the polymerase creates a positive supercoiling effect ahead and a negative supercoiling behind it. Since there is active transcription of genes during all the four stages during which the transcript/activity levels are high (AB, LZ, PS and RS), these data are consistent with the idea that topo I could be necessary during these stages; however these results are temporal

correlation and do not define function. The fact that topo I transcript is also expressed at a high level in the somatic cells of the XX*Sxr* testes which are essentially non-dividing, also implies a generalized and house keeping nature of its function.

The developmental expression of topo II transcript during spermatogenesis is markedly different when compared to topo I expression. Topo II transcript was marginally expressed in day 8 testes, which contain mainly AB germ cells and Sertoli cells. There was a drastic increase (> three fold) by day 16 when preleptotene-zygotene cells are predominant. The highest expression was in day 21 where there was a large number of PS and RS. Following day 21 there was a sharp drop in relative levels of transcript, correlating with the appearance of an increasing number of elongating and fully differentiated sperm in the testes. The topo II transcript could therefore be produced mainly by the meiotic prophase (preleptotene to PS) or, the post meiotic RS. In order to more definitively ascertain the cell types contributing to the topo II levels, a Northern blot analysis of specific cell types was done as in the case of topo I. These results (Fig. 5B, 6B) clearly indicate increasing transcript levels as the germ cells progress from AB type to PS. RS have almost an equal (but slightly less) transcript than the PS. Interestingly, unlike topo I, there was a negligible level of topo II transcript in the XX*Sxr* testes. The somatic cells of the XX*Sxr* testes are nondividing but known to be transcriptionally very active. This does suggest a unique role for topo II in spermatogenesis, possibly related to mitosis or meiosis. My results for the decatenation assay of topo II activity in PS and RS are in the preliminary stage. The enzyme activity was detected at about 0.5µg of nuclear extract in a 20µl assay. There was increasing inhibition of the enzyme between 0.2 and 1.0µg of nuclear extract in the assay. The reason for this inhibition was later determined (by Cynthia Park in our laboratory) to be due to an excessively high salt concentration in the buffer used for dilution of the nuclear extracts. The concentration levels at which the nuclear extract should give a linear range of activity are yet to be worked out. However

from the results so far it was evident that the level of topo II activity in PS is almost equal to that of RS.

Heck et al. (1988) had shown the synthesis and degradation of topo II to be coupled to parallel condensation and decondensation of DNA during the mitotic cell cycle. A major role for the topo II in the AB spermatogonia could therefore be a similar one, i.e., assisting in chromosome condensation and decondensation during the mitotic cell division cycles. In my results there was a steady increase in topo II transcript during the earlier stages of spermatogenesis, with a peak during the pachytene stage. This is probably a reflection of a high level of requirement for the enzyme either during or just after this stage.

With respect to meiosis, topo II might play a role in the SC. Moens and Earnshaw (1989) have shown anti topo II antibodies to localize to specific regions of the SC, although the authors state that the functional significance of this association is not known. There is no firm evidence for a role of topoisomerases in establishing meiotic crossing over between homologous chromosomes.

The peak topo II transcript level in the PS stage of spermatogenesis could be a reflection of a high level of requirement of the enzyme either during or just after this stage. In their analysis of yeast topo II mutants during meiosis, Rose and Holm (1993) had shown that the meiosis specific arrest of the mutant strain was primarily at the pachytene stage. Topo II might play a role in the various meiotic events occurring during and immediately after the PS stage, such as pairing and resolution of homologous chromosomes, formation of chiasmata, metaphase condensation and segregation at the following anaphase. In their study of rooster spermatogenesis, Roca and Mezquita (1989) showed a high and increasing level of topo II activity during the first four stages (premeiotic to haploid). They found highest levels of activity in the stage IV (haploid) and linked this to the requirement of topo II during the nucleohistone-nucleoprotamine transition. My results for topo II enzyme activity in PS and RS were quite similar.

McPherson and Longo (1993) showed topo II activity levels in rat post-meiotic germ cells, consistent with my results for topo II enzyme activity. Topo II could therefore be an important player in helping the nucleoprotein-nucleohistone transition.

Experiments on the effects of okadaic acid treatment on cultured PS were conducted in order to identify a possible role for topoisomerase enzymes in the rapid G2/M phase transition artificially induced by OA. The hypothesis was that since topoisomerases are known to be involved in chromosome condensation, OA treatment of PS would lead to an increase in the transcript levels of these enzymes. No change in either topo I or II transcript levels was encountered in treated versus untreated cells. A possible reason for this could be that the topo transcripts are already "switched on" to the maximum during the pachytene stage, even before OA treatment, since it is speculated that these enzymes are needed for meiotic prophase events. The existing levels of topoisomerases in the cultured PS could therefore have been sufficient to play any necessary role in altering the DNA topology during the induced G2/M transition. Further experiments such as OA treated PS in culture with and without the addition of specific topoisomerase inhibitors would be quite interesting. An assay for either enzyme activity, or observation of morphological changes could provide an alternative source of answers whether either or both of the enzymes play an important role in the metaphase condensation of chromatin.

In conclusion, the dissimilarity in profiles between topo I and II transcript levels suggest differential function for these enzymes during the stages of spermatogenesis considered. Topo I transcript was found at very high and almost equal levels in germ cells known to be actively transcribing and dividing mitotically or meiotically. These data appear to be consistent with known functions for topo I in modification of DNA topology during transcription and/or replication. Topo I enzyme activity results indicate a carry over of enzyme from the diploid PS to the haploid RS following the two meiotic divisions since levels of transcript in RS are negligible. For unknown reasons the transcription of topo I

appears to be turned off following the PS stage of spermatogenesis, probably reflective of a low level of requirement of topo I during the final stages of spermiogenesis. Topo II transcript is present in increasing quantities as the germ cells progress from AB to PS and RS. These results suggest higher requirement for topo II in meiotically dividing than in mitotically dividing germ cells. Possible functions for topo II during these stages could include aiding in chromosome condensation at metaphase by resolution of interlocks, and unknown functions associated with the SC. Topo II levels were also high in the post meiotic haploid spermatids which is consistent with earlier results and hypotheses that topo II could play an important role in aiding the higher order compaction of chromatin seen in the mature sperm. Further experiments using *in situ* hybridization technique on cross-sectioned testes and purified germ cells could be done in order to assess more accurately the topo I and II transcript production at the specific stages considered.

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