Developmental and hormonal regulation of type II DNA topoisomerase in rat testis

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ABSTRACT

Type II DNA topoisomerase (topo II) is required for diverse biological functions including DNA replication, maintenance of genome stability, chromosome segregation and chromosome condensation. While the identity of topo II in rodent testis has been established, the regulation of topo II expression during the development of the postnatal testis and gametogenesis is unclear. Here, we report that rat testis topo II is developmentally and hormonally regulated. Topo IIα mRNA levels peaked prior to the onset of puberty, declined sharply thereafter and stabilized in adult testis. In contrast, the topo II enzyme content was lower in prepubertal testis but increased after the onset of puberty. Topo II was expressed in a cell-specific manner within germ cells, being detected only in pachytene spermatocytes. While testosterone markedly increased topo IIα mRNA levels in prepubertal testis, continued treatment failed to enhance topo IIα mRNA above postpubertal control levels. The extent of topo II activity remained steady regardless of the testosterone-induced increase in topo IIα mRNA levels. Inhibition of testosterone function in postpubertal animals by ethanedimethane sulphonate (EDS) and flutamide resulted in a significant decrease in topo IIα gene expression and topo II activity. The administration of exogenous testosterone (T) to EDS- and flutamide-treated rats restored topo IIα mRNA levels and topo II activity similar to the levels seen in the testis of age-matched control animals. Histochemical analyses of testes indicated that the effect of T on spermatogenesis was separable from its effect on topo IIα expression. Our results reveal that testosterone acts as a positive regulator of topo IIα gene expression and is required for the maintenance of topo IIα expression during the development of the postnatal testis and spermatogenesis.

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INTRODUCTION

Type II DNA topoisomerases (topo II) are a class of ubiquitous nuclear enzymes that perform essential roles in various processes related to DNA metabolism. Detailed biochemical, genetic and cytological studies have indicated that topo II plays a key role in DNA replication, chromosome segregation, maintenance of genomic stability, and chromosome condensation (reviewed in Wang 1996, Hsieh 1992, Watt & Hickson 1994). The biochemical functions include the removal of DNA supercoils generated during replication, recombination, transcription, and resolution of catenated intertwined duplex molecules to help disjunction of chromosomes. The canonical mechanism utilized by this dyadic enzyme involves the following steps: generation of a transient double-strand break, ATP-dependent passage of a double-stranded DNA segment through the break. After transport, the double-stranded break is resealed to form an intact duplex molecule (reviewed in Wang 1996, Watt & Hickson 1994, Berger & Wang 1996).

It has been demonstrated that the catalytic activity of topo II is essential for chromosome condensation and chromosome segregation during the metaphase–anaphase transition, implying that proper disjunction of 'sister' chromatids might involve the decatenation of topologically interlocked DNA molecules (Uemura *et al*. 1987, Holm *et al*. 1989, Shamu & Murray 1992, Clark *et al*. 1993). Likewise, cytogenetic analyses of *Saccharomyces*

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cerevisiae top2 mutants have demonstrated that topo II is essential for the segregation of homologs at meiosis I (Rose *et al*. 1990, Spell & Holm 1994).

Mammalian spermatogenesis is a complex and coordinated developmental process that normally culminates in the production of viable male gametes (Meistrich 1993). Notable features of this process include ordered progression through mitotic and meiotic cell divisions and chromatin reorganization in germ cells, which are influenced by a large number of hormones and growth factors (de Kretser & Kerr 1988, Meistrich 1993, Ward 1994). Therefore, it provides an instructive model for studying the regulated expression of topo IIα during the development of the postnatal testis and spermatogenesis. To investigate the relationship between topo II and the growth and differentiation of mammalian germ-line tissue, we have used the rat testis as a model. In this report, we present evidence to show that topo II expression is subject to regulation by developmental and hormonal signals during postnatal testicular development. Our results also suggest that the topo IIα gene is one of the targets of testosterone action *in vivo*.

MATERIALS AND METHODS

Animals

Male rats (*Rattus norvegicus*, Wistar strain) of an appropriate age were obtained from the Central Animal Facility of the Indian Institute of Science, Bangalore. Animals were killed by mild chloroform anesthesia followed by cervical dislocation. The testes were dissected out, rinsed in ice-cold saline, and frozen immediately in liquid nitrogen. Frozen testes were stored at − 70 °C.

Northern blot hybridization

Total RNA was extracted from testes by using the acid guanidinium isothiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). Northern blot analysis was performed as described (Sambrook *et al*. 1989). Briefly, RNA was resolved on a 1% agarose formaldehyde denaturing gel. Denatured RNA was transferred to Hybond-N membrane (Amersham–Pharmacia Biotech, Quarry Bay, Hong Kong) by capillary action. RNA was cross-linked to the membrane by UV irradiation and hybridized to an α-^{32}P-labeled topo IIα-specific probe. Topo IIα-specific cDNA was isolated by PCR amplification of a rat testis cDNA library (Stratagene, La Jolla, CA, USA) using *Taq* DNA polymerase (Amersham–Pharmacia Biotech). The primers were 5′-ATGATTATGACAGATCAGG AC-3′ and 5′-CTAACTGGGCAACTTTCACCT-3′, designed from the reported rat topo IIα gene sequence (GenBank accession number Z19552; Park *et al*. 1993). The PCR generated a 633 bp DNA fragment corresponding to a portion of topo IIα cDNA. The fragment was labeled using the random priming method (Megaprime labeling kit; Amersham–Pharmacia Biotech) and [α-^{32}P]dATP (specific activity 4000 Ci/mmol). The blot was hybridized with the labeled probe, washed, exposed to a film at –70 °C, and visualized as described (Church & Gilbert 1984, Sambrook *et al*. 1989). The probe for 18S rRNA was derived from the EcoRI fragment of the human 18S rRNA gene (GenBank accession number X03205; McCallum & Maden 1985).

Preparation of nuclear protein extracts

One gram of frozen testes was minced and homogenized at 4 °C in 5 ml homogenization buffer (10 mM Tris–HCl (pH 7·5), 5 mM $MgCl_2$, 25 mM KCl, 0·34 M sucrose, 0·1 mM phenylmethylsulfonyl fluoride (PMSF) in a Potter–Elvejhem homogenizer (Thomas Scientific, Swedesboro, NJ, USA). The homogenate was centrifuged at 5000 ***g*** for 10 min at 4 °C. The pellet was washed with homogenization buffer, and nuclei were resuspended in 5 ml lysis buffer (5 mM potassium phosphate buffer, pH 7·5, 100 mM NaCl, 10 mM 2-mercaptoethanol, 0·1 mM PMSF). The suspension was sonicated using a Branson (Danbury, CT, USA) sonicator with a microprobe for 15 s with the power output set at 5. The suspension was centrifuged at 5000 ***g*** for 10 min. The supernatant was removed and stored at –70 °C.

Decatenation assay

The decatenation reaction was performed in a buffer consisting of 40 mM Tris–HCl (pH 7·5), 100 mM KCl, 10 mM $MgCl_2$, 30 μg/ml BSA, 0·5 mM dithiothreitol, 150 ng kinetoplast DNA (kDNA) (TopoGEN, Columbus, OH, USA) with samples of nuclear protein extracts at 37 °C for 30 min. The reaction was terminated by the addition of SDS and EDTA to a final concentration of 1% and 10 mM respectively. Reaction products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. One unit of topo IIα activity was defined as the amount of enzyme required for the decatenation of 150 ng kDNA at 37 °C in 30 min. The specific activity was estimated from initial velocities well within the linear range of protein concentrations and expressed as units/mg

protein. The protein concentrations were determined by the dye binding method (Bradford 1976).

Germ-cell separation

Germ-cell separation was performed by centrifugal elutriation as described by Meistrich *et al*. (1981), with minor modifications. Twenty grams of testes from 5-week-old animals were rinsed briefly in ice-cold Dulbecco's phosphate-buffered saline (DPBS). Testes were detunicated and minced using a scalpel blade in a minimum volume of DPBS. The mince was transferred to 100 ml Dulbecco's modified Eagle's medium (DMEM). Collagenase (0·4 mg/ml) and DNase (0·01 μg/ml) were added and the mixture was incubated in a water-bath, with continuous shaking (170 r.p.m.), at 32 °C for 90 min. The suspension was filtered through a steel mesh and centrifuged at 1000 ***g*** for 10 min to sediment the cells. Cells were washed once in DPBS and resuspended in 50 ml DPBS (containing 0·2% BSA and 0·1% glucose). Approximately 1×10^9 cells were loaded into a JE6B elutriator rotor with the standard elutriator chamber (Beckman Coulter International, Nyon, Switzerland). The extent of enrichment of pachytene spermatocyte and round spermatid populations was assessed by flow cytometry as described by Suresh *et al*. (1992).

Immunoblotting

Proteins were separated by SDS-PAGE and were transferred to a nitrocellulose membrane as described elsewhere (Laemmli 1970, Galande & Muniyappa 1996). The membrane was blocked at 4 °C for 12 h with blocking buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl (pH 7·5), 0·5% BSA, 0·05% Triton X-100) and probed with testis anti-topo IIα antibodies (Galande & Muniyappa 1996). Following washing with PBS containing 0·1% Nonidet P40, the blot was incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA, USA) at 4 °C for 1 h. The blot was washed extensively with buffer, and topo IIα was visualized using chemiluminescence as described by Schneppenheim & Rautenberg (1987).

Treatment of prepubertal animals with testosterone

Twenty-six-day-old male rats were kept with a ratio of 12 h light:12 h darkness and had unrestricted access to food and water. Animals were injected daily for 6 days with testosterone (T) at a dose of 50 mg/kg body weight intra-peritoneally in propylene glycol, with the day of the first injection designated as day 0. Vehicle-treated animals served as controls. Animals were killed on day 3 (three injections) or day 6 (six injections) as described above. Testes were dissected out, rinsed in ice-cold 0·85% NaCl, frozen in liquid nitrogen and stored at − 70 °C. Northern blot hybridization (Sambrook *et al*. 1989) and kDNA decatenation assays (Galande & Muniyappa 1996) were performed as described.

Histochemical analysis

To examine the morphological changes in seminiferous tubules, a portion of testes rinsed in ice-cold 0·85% saline was fixed in Bouin's fixative (70% picric acid, 25% formaldehyde and 5% methanol) for 24 h (Luna 1968). Following washing with water, tissues were dehydrated in graded ethanol from 70% to 95%. Dehydrated tissue was dipped sequentially in series of ethanol:chloroform solutions from 75:25 to 0:100. The tissue was embedded in molten paraffin (Paraplast; Sigma–Aldrich, St Louis, MO, USA) for 24 h. Sections of paraffin-embedded tissue were cut to 5 μm, mounted on poly-L-lysine-coated glass slides, deparaffinized, and rehydrated. Sections were stained with Mayer's hematoxylin for visualization of cells. Stained sections were dehydrated and mounted in diphenyl xanthene. The slides were visualized using a BX60 microscope (Olympus Optical Company, Tokyo, Japan). Photomicrographs were generated by capturing images with an Olympus PM-30 automatic photomicrographic system.

Treatment of adult animals with ethane dimethane sulphonate and testosterone

Fifty-two-day-old male rats weighing about 125 g at the beginning of the experiments were used in this study. In the first experiment, rats were administered, intraperitoneally, 75 mg/kg ethanedimethane sulphonate (EDS) in of 25% DMSO (dissolved at a concentration of 75 mg/ml) (batch E) or with 25% DMSO alone (batch C); the latter served as controls. The day of injection was considered day 0. After injection, the rats were killed on day 2 (C2/E2), 4 (C4/E4) and 6 (C6/E6) by cervical dislocation, and the testes were quickly removed. In the second experiment, EDS-treated animals were administered, intraperitoneally, T (50 mg/kg) on days 3, 4 and 5 (batch ET). The rats treated with EDS followed by 1–3 doses of T were killed on days 4 (ET1), 5 (ET2) and 6 (ET3), and the testes were quickly removed. Three-day EDS treatment was chosen since this gives complete destruction of

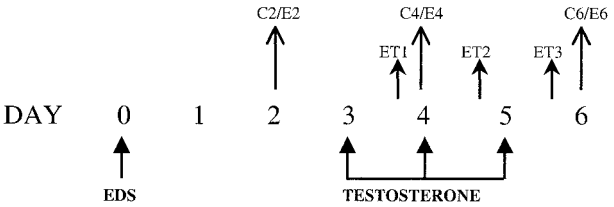

FIGURE 1. Schematic illustration of the treatment of adult males with EDS and T (see text for details).

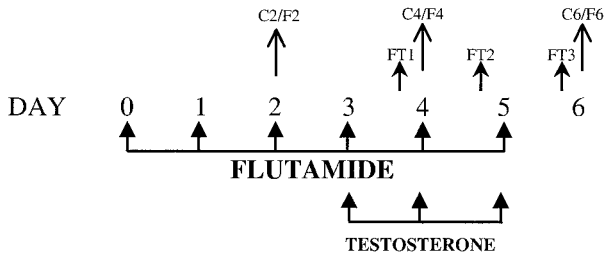

FIGURE 2. Schematic illustration of the treatment of adult males with flutamide and T (see text for details).

Leydig cells. In all of the experiments, each group contained an average of six rats. To assess the influence of EDS and T on the postpubertal testis, tissues were processed and analyzed for changes in the levels of topo IIα-specific mRNA, topo IIα activity and morphological features of seminiferous tubules as described above. The treatment regimen is schematically represented in Fig. 1.

Treatment of adult animals with flutamide and testosterone

Fifty-two-day-old male rats weighing about 125 g at the beginning of the experiments were used in this study. In the first experiment, rats were administered daily, intraperitoneally, flutamide (50 mg/kg; Sigma–Aldrich) in propylene glycol (batch F). The day of the first injection was considered day 0. Vehicle-treated rats served as controls (batch C). Rats were killed on days 2 (C2/F2), 4 (C4/F4) and 6 (C6/F6). The testes were removed, washed and stored as described above. In the second experiment, T (50 mg/kg) was administered intraperitoneally to flutamide-treated rats (on days 3, 4 and 5 (batch FT). Rats were killed on days 4 (FT1), 5 (FT2) and 6 (FT3). The testes were removed, washed and stored as described above. The influence of flutamide on the adult testis was evaluated by analyzing tissues for changes in topo IIα-specific mRNA levels, topo IIα activity and morphological features of seminiferous tubules as described above. The treatment regimen is schematically represented in Fig. 2.

RESULTS AND DISCUSSION

Previous work has demonstrated that vertebrates possess two genetically and biochemically distinct isoforms of type II DNA topoisomerase: topo IIα and topo IIβ (Tsai-Pflugfelder *et al*. 1988, Drake *et al*. 1989, Tan *et al*. 1992). Topo IIα is expressed preferentially in actively proliferating tissues, whereas expression of topo IIβ is restricted to quiescent somatic tissues (Juenke & Holden 1993, Park *et al*. 1995), thereby implicating a relationship between growth regulatory signaling pathways and expression of topo II isoforms. These cell-specific differences in the expression of isoforms of topo II suggest that they play vital and non-redundant roles in cell growth and differentiation (Meyer *et al*. 1997, Grue *et al*. 1998). Previously, using biochemical and immunological methods, we and others have shown that the isoform expressed in rat testis is topo IIα (Juenke & Holden 1993, Park *et al*. 1995, Galande & Muniyappa 1996). Comparison of the topo IIα promoters from humans and animal models, including mice, hamsters and rats, has revealed a high degree of phylogenetic conservation. Furthermore, these studies have indicated the existence of both positive and negative *cis*-acting regulatory regions involved in the control of topo IIα expression (Hochhauser *et al*. 1992, Park *et al*. 1995, Ng *et al*. 1995). However, little is known about the external stimuli that govern the tissue-specific expression of different isoforms of topo II. Mammalian spermatogenesis provides an instructive model for studying the functional and developmental regulation of topo II. To further our understanding of this tissue-specific phenotype, we have studied the pattern of topo IIα expression during the development of the normal, functional postnatal testis and in the maintenance of spermatogenesis.

Expression of topo IIα mRNA is developmentally regulated

To determine when, during the development of the postnatal testis, topo IIα is expressed, we performed Northern blot hybridization of samples of total RNA extracts from the testes of rats ranging from 2 weeks to 10 weeks of age. A 6·2 kb topo IIα-specific transcript was detectable between 2 and 4 weeks of age, but the levels were relatively low (Fig. 3A), consistent with age-related development of the testis. The levels of topo IIα mRNA were highest at 5 weeks of age, indicating the possibility of a role for topo II in the establishment of puberty. Subsequently, the levels of

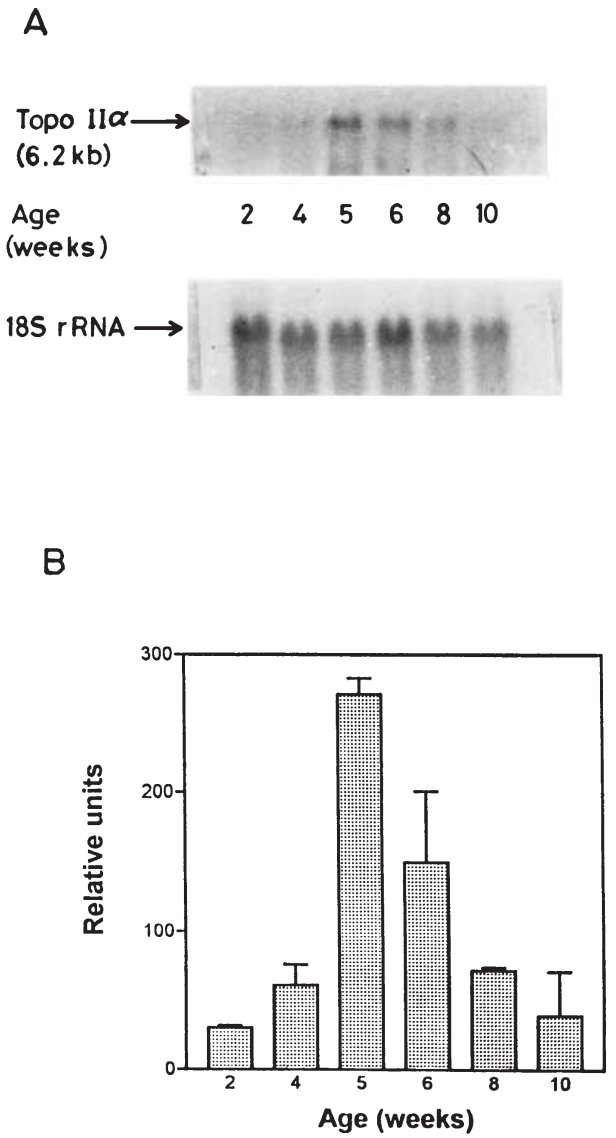

FIGURE 3. Expression of the topo IIα gene in the postnatal testis is developmentally regulated. (A) Northern blot analysis of topo IIα gene expression. Twenty micrograms of total RNA extract from testes of the indicated age groups were analyzed with the labeled topo IIα-specific probe as described in the Materials and Methods. Equal loading of RNA samples in each lane was verified by hybridizing the blot with a ^{32}P-labeled 18S rRNA-specific probe. The positions of topo IIα and 18S rRNA are indicated. The size of the topo IIα mRNA species is indicated in kb. (B) Relative levels of topo IIα mRNA from panel (A) as quantified by scanning the autoradiogram using an LKB Ultroscan laser densitometer; mRNA levels are expressed as relative densitometric units. Relative units of topo IIα mRNA were estimated using the following formula: (relative area under peak) topo IIα /(relative area under peak) 18S rRNA × 100. Results represent means ± S.D. of three independent experiments.

topo IIα mRNA fell progressively (3·5-fold) and were sustained in the adult testis (10 weeks old) at levels comparable to those seen in the prepubertal testis (Fig. 3B).

Regulation of topo II activity

To assess the possibility that translation of topo IIα mRNA to a functional protein might be physiologically regulated during the development of the postnatal testis, we used topo II-catalyzed ATP-dependent decatenation of kDNA to monitor its activity in nuclear protein extracts. Decatenation of kDNA is a hallmark of topo II and is especially useful in assaying topo II activity in cell-free extracts (Galande & Muniyappa 1996). In contrast to the mRNA levels, topo II activity (as indicated by the data shown in Fig. 4A) was relatively small in prepubertal extracts, increased significantly with the onset of puberty, then decreased slightly and remained stable in the adult testis. The increase in topo II activity might arise from an actual increase in the accumulation of topo II protein in the adult testis. We addressed this possibility by monitoring levels of topo II in nuclear protein extracts by using Western blot analysis with polyclonal antibodies raised against testis topo IIα (Galande & Muniyappa 1996). In nuclear protein extracts of testis at different stages of development, these antibodies recognized two prominent polypeptides with apparent molecular masses of 160–170 kDa and 125–140 kDa (Fig. 4B). Although the levels of topo II activity increased with the onset of puberty and were maintined in the adult testis, the levels of these polypeptides declined in the postpubertal testis. The doublet of bands is a hallmark of topo II in eukaryotes: the larger form (160–170 kDa) corresponds to the full-length protein, whereas smaller (125–140 kDa) forms are generated by proteolysis of the native form (Liu 1989, Osheroff *et al*. 1991 and references therein). To investigate the possibility that changes in topo II activity could arise from variations in other testicular proteins, we performed a complementary immunoblot assay for two extracellular signal-regulated MAP kinases, ERK1/ERK2, (Tamemoto *et al*. 1992) as above. Apparently, the levels of ERK1/ERK2 were unaltered (Fig. 4C), suggesting that changes in topo II activity are not completely attributable to variations in other testicular proteins. Furthermore, this observation excludes the possibility that differences in the amount of topo II detected are due to differences in the amount of protein loaded on the gel. These results demonstrate that the maintenance of topo II activity in the postpubertal testis is probably due to

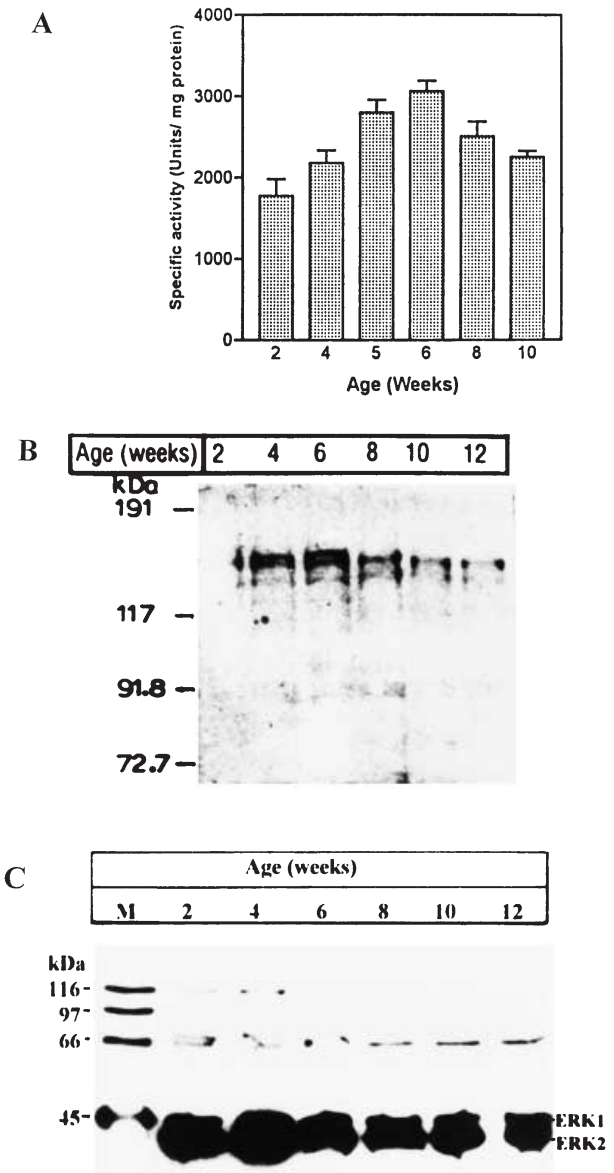

FIGURE 4. Regulation of topo II activity during the development of the postnatal testis. (A) Nuclear protein extracts were prepared from postnatal testis at the indicated stages of development. Topo II activity was assayed as described in the Materials and Methods. Data represent means ± S.D. of three independent experiments. (B) Nuclear protein extracts (50 μg protein) prepared from postnatal testis at the indicated stages of development were separated on SDS-PAGE (7·5%), transferred onto a nitrocellulose filter and incubated with anti-topo IIα antibodies as described (Galande & Muniyappa 1996). (C) Nuclear protein extracts (50 μg) were separated on SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-ERK1/ERK2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described for (A).

post-translational modification. It has been established that cells use protein phosphorylation as a mechanism to regulate the activity of a vast array of proteins and enzymes in a number of pathways (Hunter 1987). Accordingly, one possible source of enhancement of topo II activity may be through phosphorylation. Previous studies have shown that both isoforms of topo II exist as phosphoproteins *in vivo* (Kimura *et al*. 1994, 1996*b*) and that the phosphorylation state of topo IIα is regulated in a cell-cycle-dependent manner (Kimura *et al*. 1994, Wells & Hickson 1995, Ishida *et al*. 1996). It has been established, however, that phosphorylation has no effect on the catalytic activity of topo IIα (Kimura *et al*. 1996*a*, Redwood *et al*. 1998, Shapiro *et al*. 1999). We are unable to specify precisely the molecular event(s) responsible for the maintenance of topo IIα activity in the adult testis.

Topo IIα protein is synthesized in a cell-type-specific manner

The mammalian testis contains a heterogeneous cell population, the composition of which varies during the maturation and development of the testis (Knorr *et al*. 1970, Meistrich 1993). To investigate whether the expression of topo IIα is germ-cell type-specific, we isolated testicular germ cells from 5-week-old testes by centrifugal elutriation (Meistrich *et al*. 1981). The germ-cell pool of the 5-week-old testis is predominantly composed of pachytene spermatocytes and round spermatids (Knorr *et al*. 1970, Malkov *et al*. 1998). Western blot analysis of cell-free extracts, using topo II-specific antibodies, demonstrated that topo II was detectable only in pachytene spermatocytes, but not in round spermatids (Fig. 5), suggesting that significant topo II expression is restricted to pachytene spermatocytes. Previously, Longo and colleagues reported the presence of topo II-related peptides of apparent molecular mass 130 kDa (McPherson & Longo 1993) in fractions enriched for round spermatids of the rat testis.

During differentiation of the postnatal testis, pachytene spermatocytes appear first in the 3-week-old testis. Cytological analyses have revealed that their relative proportion increases gradually, reaching maximum levels in the 6-week-old testis; the proportion then begins to decline (Knorr *et al*. 1970, Malkov *et al*. 1998). Analysis of the topo IIα mRNA expression pattern (Fig. 3) discloses that the apparent variation in transcript level, in comparison to the relative abundance of pachytene spermatocytes, is restricted to a distinct stage during development. Significantly, the increase in topo

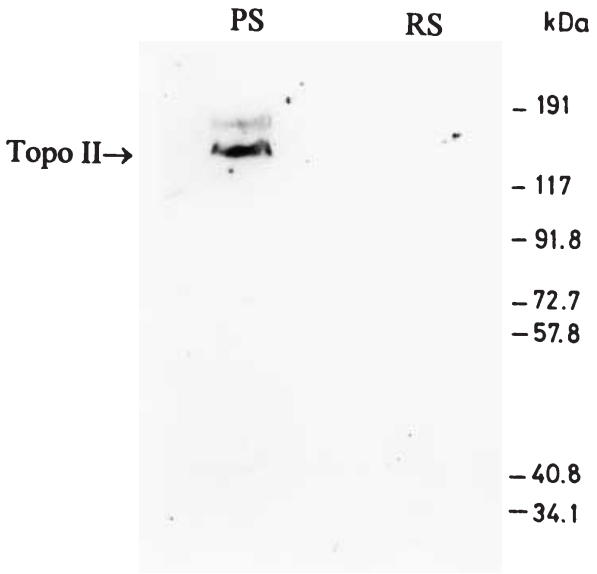

FIGURE 5. Expression of topo IIα is cell-type-specific. Cell-free extracts were prepared from 2×10^6 pachytene spermatocytes (PS) and round spermatids (RS) by lysis in 1% SDS. The entire sample was analyzed by SDS-PAGE (7·5%), transferred to a nitrocellulose membrane and then incubated with anti-topo IIα antibodies. Protein bands were visualized as described in the Materials and Methods. Molecular mass markers are given on the right.

II protein expression (Fig. 4B) corresponds to the emergence of pachytene spermatocytes in the seminiferous tublules (Knorr *et al*. 1970). The differential pattern of mRNA expression, topo II protein content and enzyme activity implies that topo IIα expression is influenced by multiple signaling factors during the development of the postnatal testis.

Testosterone positively regulates topo IIα mRNA expression but not enzyme activity

The onset of puberty in the male rat is preceded, and accompanied, by a continuous rise in circulating plasma levels of T, implicating an important role for T in the growth and differentiation of the testis and in the maintenance of spermatogenesis (Knorr *et al*. 1970). The increase in topo IIα mRNA levels coincident with a rise in plasma T levels (Knorr *et al*. 1970) implies that T might play a regulatory role in topo IIα expression. To explore this possibility, we tested T-induced topo IIα mRNA expression by using Northern blot hybridization and kDNA decatenation assays in 4-week-old males. As shown in Fig. 6, T treatment for 3 days enhanced topo IIα mRNA levels by twofold relative

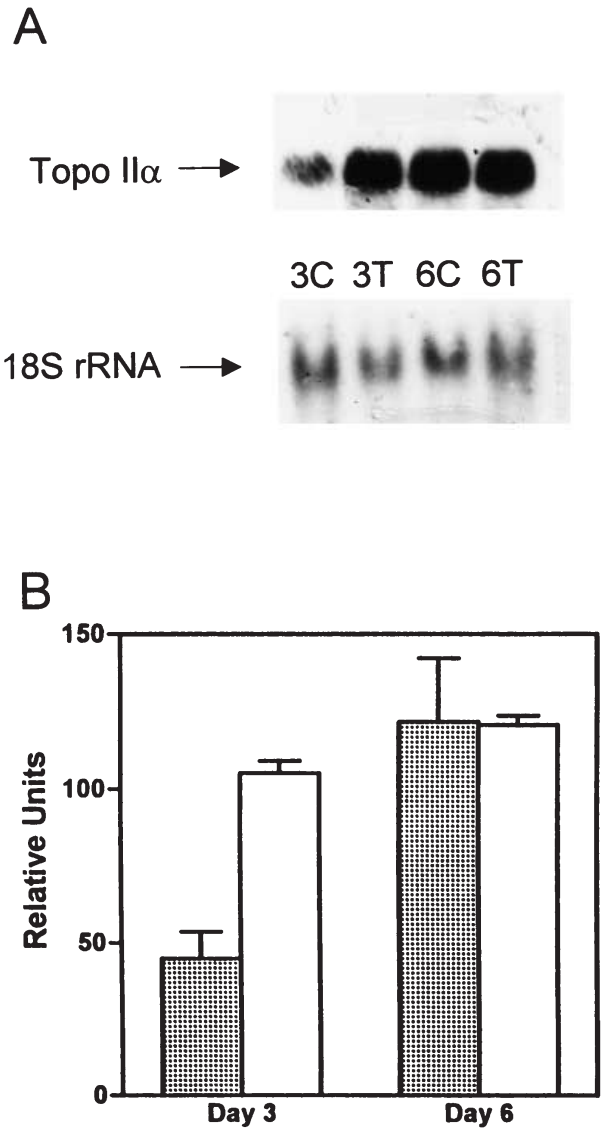

FIGURE 6. Upregulation of topo IIα mRNA, in prepubertal animals, by T. (A) Northern blot analysis of T-induced topo IIα gene expression. Twenty micrograms of total RNA extracts from testes of control and T-treated males were analyzed as described in the Materials and Methods. Equal loading of RNA samples in each lane was assessed by hybridizing the blot with a ^{32}P-labeled 18S rRNA-specific probe. The positions of topo IIα and 18S RNA are indicated. (B) Relative levels of topo IIα mRNA from (A), as quantified by scanning the autoradiogram using an LKB Ultroscan laser densitometer. Stippled and open bars correspond to control (3C and 6C) and testosterone-treated (3T and 6T) samples respectively. The mRNA levels are expressed as relative densitometric units calculated as described in the legend to Fig. 3B.

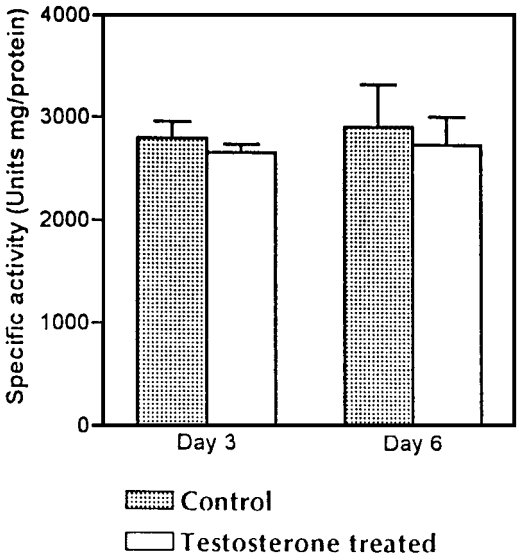

FIGURE 7. Testosterone fails to cause stimulation of topo II activity in the testis. Samples of nuclear protein extracts from T-treated and control males were assayed for topo IIα activity using the decatenation of kDNA as described in the Materials and Methods. Results represent means ± S.D. of three independent experiments.

to the levels seen in untreated controls. Significantly, continued T treatment for 6 days failed to enhance topo IIα gene expression relative to the controls, indicating that the system might already be saturated with the endogenous T. In contrast to its effect on mRNA levels, T treatment had no measurable effect on the levels of topo II activity (Fig. 7). The kinetics of expression of topo IIα in T-treated males correlates with its expression during the development of the postnatal testis.

Next, we examined whether T was necessary for topo IIα gene expression and enzymatic activity in the postpubertal testis; two approaches were used. In the first approach, T synthesis was abolished by ablation of Leydig cells with EDS. A single i.p. injection of 75 mg/kg EDS to male rats is sufficient to abrogate plasma T levels within 24 h and to cause Leydig cell apoptosis within 3 days (Morris & McCluckie 1979, Jackson *et al*. 1986, Morris *et al*. 1997). The cytotoxic effect of EDS is reversible, and normal testicular morphology is restored within 7 weeks (Morris & McCluckie 1979, Jackson *et al*. 1986). In the second approach, the endogenous T was suppressed by the non-steroidal competitive inhibitor flutamide. In both of these experiments, exogenous T was administered to the rats simultaneously to assess the reversibility of the EDS or flutamide treatments.

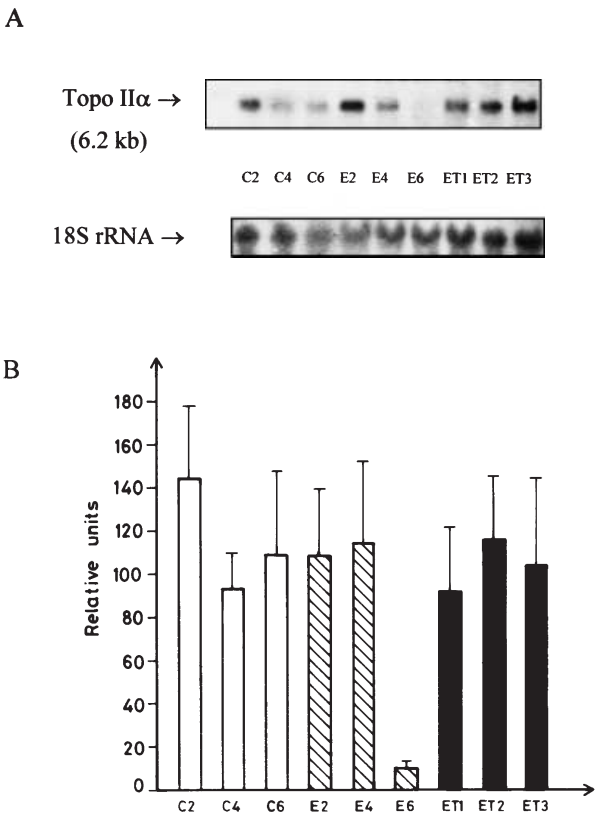

FIGURE 8. Effect of EDS treatment on topo IIα mRNA levels in the adult rat testis. (A) Northern blot analysis. Twenty micrograms of total RNA from the testes of vehicle-treated, EDS-treated, and EDS-treated–T-supplemented animals were separated on a 1·2% agarose-formaldehyde gel, transferred to nylon membrane and then hybridized with a ^{32}P-labeled topo IIα-specific probe, as described in the Materials and Methods. Topo IIα mRNA levels were normalized for the amount and integrity of RNA by hybridization of the same membrane with the constitutively expressed 18S rRNA. The size of topo IIα RNA is indicated in kb. (B) Histogram of the autoradiogram shown in (A). The intensities of bands in the autoradiogram were quantified using an LKB Ultroscan laser densitometer. The mRNA levels are plotted as relative units against the duration of treatment with the indicated chemical compound. Relative units were determined as described in the legend to Fig. 3B.

Altered expression of topo IIα mRNA and enzymatic activity in EDS-treated adult rat testis

Total RNA was isolated from the testes of vehicle-treated, EDS-treated, and EDS-treated–T-supplemented rats on days 2, 4 and 6 after treatment and then examined by Northern blot analysis. This analysis detected a single mRNA transcript

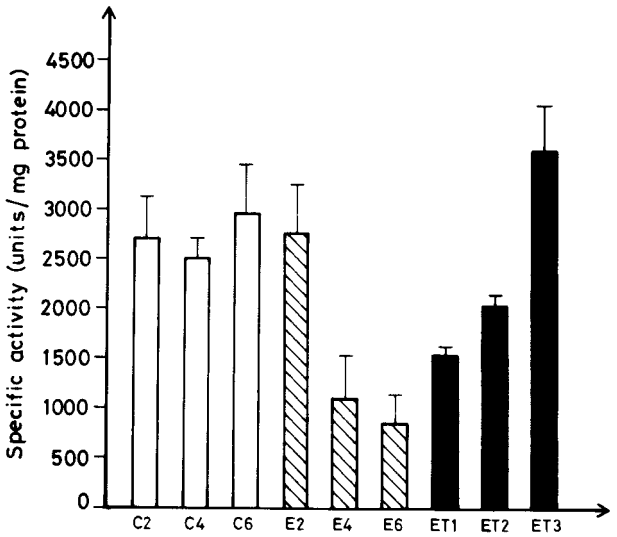

FIGURE 9. Effect of EDS on topo IIα activity. Nuclear protein extracts were prepared from testes of vehicle-treated, EDS-treated, and EDS-treated and T-supplemented rats, and topo II activity was assayed using a kDNA decatenation assay as described in the Materials and Methods. Data represent mean ± S.D. values from three independent experiments.

corresponding to a size of 6.2 kb (Fig. 8A). The autoradiogram was quantified by scanning in an Ultroscan laser densitometer (LKB–Pharmacia, Peapack, NJ, USA). Topo IIα expression was normalized for the amount and integrity of RNA, using constitutively expressed 18S rRNA. The intensities of bands, relative to 18S rRNA levels, were plotted as relative units against the duration of treatment with the indicated chemical compound. Topo IIα mRNA levels in the vehicle-treated testis was found to be higher at day 2, but lower on days 4 and 6 (Fig. 8B). The pattern of expression observed is consistent with the levels of topo IIα mRNA with decreasing developmental age (Fig. 3). In contrast, expression of topo IIα-specific mRNA decreased steadily from high levels at day 4, and was nearly abolished by day 6 in the EDS-treated testis. The levels of topo IIα mRNA in EDS-treated rats on day 6 declined by 10-fold, relative to the age-matched control animals (Fig. 8B). Interestingly, the level of topo IIα transcript was similar in the testes of EDS-treated–T-supplemented rats compared with the vehicle-treated control animals. The mechanism by which T enhances topo IIα transcript levels in the EDS-treated testis is unclear. To examine topo II expression more specifically, we investigated changes in topo II activity in the testes of vehicle-treated, EDS-treated, and EDS-treated–T-supplemented rats. Nuclear extracts were prepared from testes, and topo II activity was measured using the kDNA decatenation assay. Our analysis revealed that topo II activity was maintained at steady-state levels in vehicle-treated rats, as previously noted for the period of normal development of the postnatal testis (Figs 9 and 4). By contrast, topo II activity in EDS-treated rats had diminished by twofold by day 4 and had reached steady-state levels by day 6 after treatment

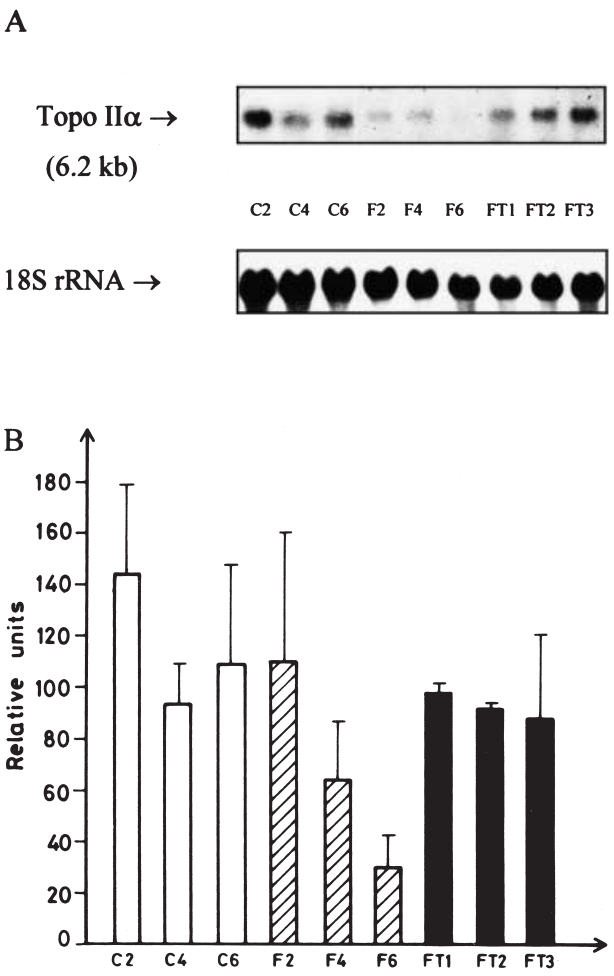

FIGURE 10. Effect of flutamide treatment on topo IIα gene expression. (A) Northern blot analysis. Twenty micrograms of total RNA from vehicle-treated, flutamide-treated, and flutamide-treated–T-supplemented testes after the indicated number of days of treatment were analyzed using a ^{32}P-labeled topo IIα-specific probe as described in the Materials and Methods. Equal loading of RNA samples was verified by hybridizing the blot with ^{32}P-labeled 18S rRNA probe. (B) Histogram of the autoradiogram shown in (A). Relative levels of topo IIα mRNA were quantified as described in the legend to Fig. 3B.

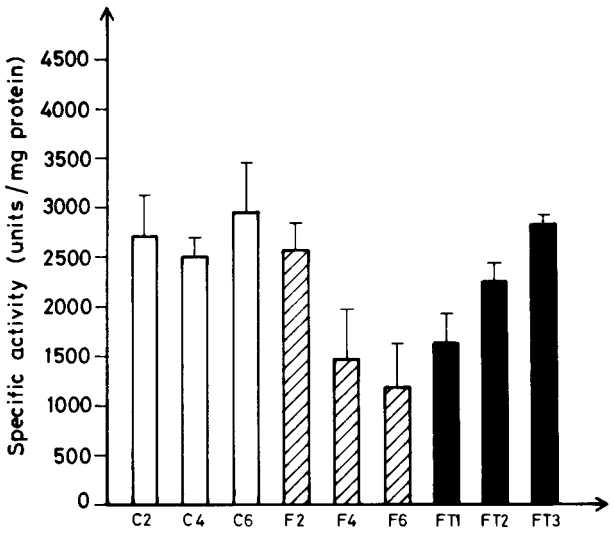

FIGURE 11. Effect of flutamide treatment on topo II activity. Nuclear protein extracts were prepared from testes of vehicle-treated, flutamide-treated, and flutamide-treated–T-supplemented animals, and topo II activity was assayed using a kDNA decatenation assay as described in the Materials and Methods. Data represent mean ± S.D. values from three independent experiments.

(hatched bars, Fig. 9). This pattern does not correlate with the decrease in topo IIα mRNA levels in EDS-treated animals (Fig. 8B). Significantly, in contrast to the topo IIα mRNA levels (Fig. 8A), the full restoration of topo II activity to control levels in EDS-treated–T-supplemented rats required continued administration of T over a period of 3 days (filled bars, Fig. 9).

Effect of flutamide on topo IIα mRNA expression and enzymatic activity

The experiments described above suggest that T is required for both stimulation and maintenance of topo IIα expression in the testis. However, one caveat of these studies is that EDS causes complete destruction of Leydig cells (Jackson *et al*. 1986, Morris *et al*. 1997), the site of synthesis of a number of factors involved in the growth of different cell types in the developing testis (Sharpe *et al*. 1988). To confirm the importance of T in topo IIα gene

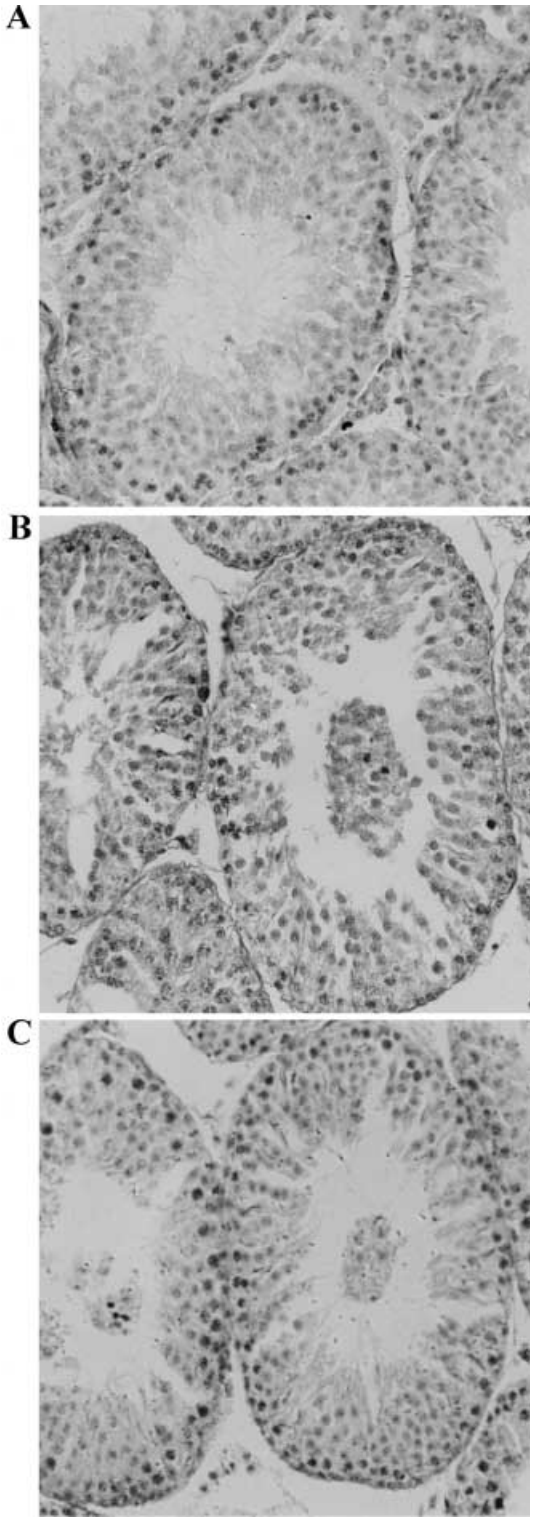

FIGURE 12. Histochemistry of vehicle-treated (A), EDS-treated (B), and EDS-treated–T-supplemented (C) adult rat testis. Testis cross-sections were stained with hematoxylin and visualized (× 20) as described in the Materials and Methods. Note the presence of round spermatids in the lumen and the absence of Leydig cells in (B) and (C). The sections are representative of testis samples from three independent experiments.

expression, an independent approach involving perturbing the T signaling pathway was employed. The action of T was blocked with the use of flutamide. Total RNA was isolated from testes of vehicle-treated, flutamide-treated, and flutamide-treated–T-supplemented rats on days 2, 4 and 6 after treatment. Samples were then examined by Northern blot analysis. The levels of topo IIα mRNA displayed a progressive decline in the testes of flutamide-treated rats, relative to control animals, during the course of treatment (Fig. 10A). We next investigated the ability of T to influence topo IIα expression in the presence of flutamide. The level of topo IIα mRNA was restored to levels similar to those seen in the testes of control animals. To reinforce these results, the autoradiogram was quantified by scanning in an LKB Ultroscan laser densitometer. Topo IIα expression was corrected for the amount and integrity of RNA used as described above. The intensities of bands, relative to 18S rRNA levels, were plotted as relative units against the duration of treatment with the indicated compound. As shown in Fig. 10B, topo IIα-specific mRNA was markedly reduced by day 2 in flutamide-treated animals (hatched versus open bars, Fig. 10B). The levels of topo IIα mRNA in flutamide-treated rats on day 6 were fivefold less relative to age-matched controls. The differences in the pattern and magnitude of variation of topo IIα mRNA with EDS and flutamide treatments are likely to be a reflection of the modes of action of the agents employed.

To clarify the influence of flutamide on topo II activity during the course of treatment, kDNA decatenation assays were performed using nuclear extracts prepared from testes of vehicle-treated, as well as flutamide-treated, rats. The extent of total topo II activity was significantly reduced in the testicular extracts of flutamide-treated rats compared with vehicle-treated rats (Fig. 11). The administration of flutamide plus T resulted in gradual restoration of topo II activity. However, it was not until day 3 that the levels of topo IIα enzymatic activity were restored relative to the levels seen in the vehicle-treated rats. More importantly, the overall pattern of topo IIα expression is consistent with that observed with Leydig-cell ablation using EDS. Together, these

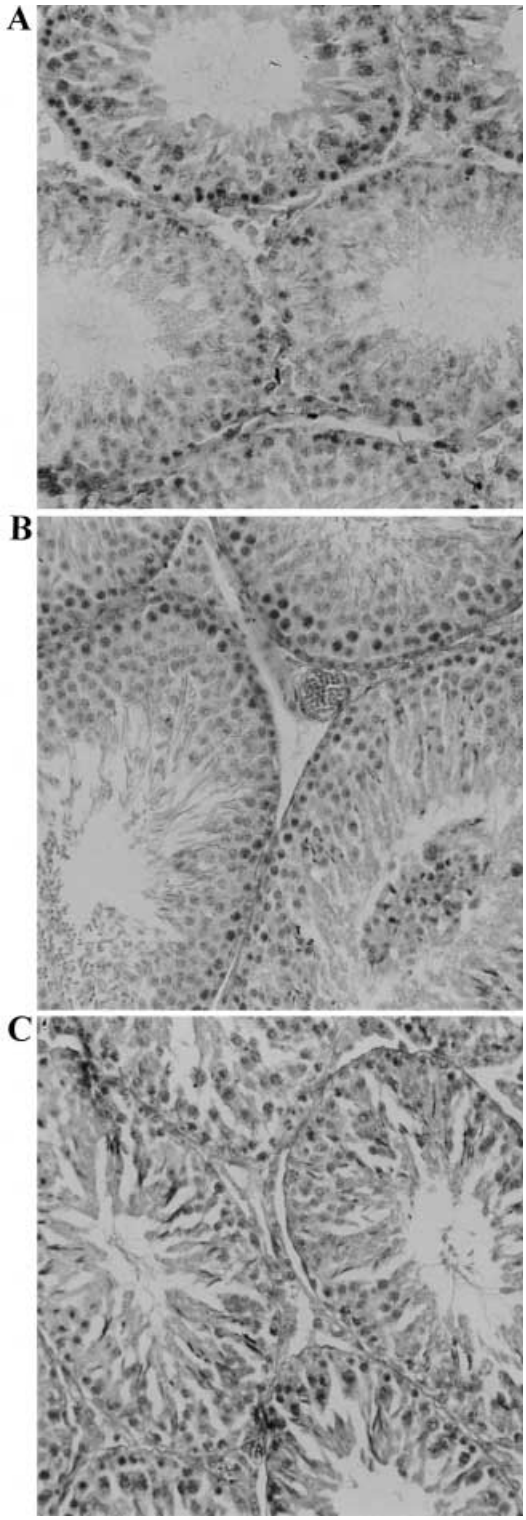

FIGURE 13. Histochemistry of vehicle-treated (A), flutamide-treated (B), and flutamide-treated–T-supplemented (C) adult rat testis. Testis cross-sections were stained with hematoxylin and visualized (× 20) as described in the Materials and Methods. Note the presence of round spermatids in the lumen, the presence of Leydig cells, and the aberrant morphology of Sertoli cells in (C). The sections are representative of testis samples from three independent experiments.

results indicate that T plays a key role in topo IIα gene expression in the testis of the adult rat.

Histochemical examination of testis from EDS- and flutamide-treated rats

The effects of EDS and flutamide on individual cell types during spermatogenesis were examined by staining 5 μm cross-sections of day 6 vehicle-treated testis, EDS-treated testis, and flutamide-treated testis with hematoxylin. As shown in Figs 12 and 13, treated and untreated animals exhibited qualitatively similar seminiferous epithelium. Treatment with EDS led to elimination of Leydig cells (Fig. 12B). In addition, EDS led to an increase in germ-cell sloughing into the lumen and enlarged lumen size relative to the age-matched control animals (compare Fig. 12A with Fig 12B). Progressive shedding of germ cells from the periphery of tubules into the tubule lumen is a hallmark of androgen deprivation (O'Donnell *et al*. 1996). Interestingly, the numbers of pachytene spermatocytes, and their gross morphology, were not significantly altered. Evaluation of sections from EDS-treated and T-supplemented rats showed that T failed to confer protection to the testis against the cytotoxic effects of EDS (Fig. 12C). Histochemical analysis also revealed that the overall qualitative changes, including the centrally located spermatocytes, spermatids and germ-cell sloughing into the lumen, were similar. Intriguingly, despite dramatic effects of EDS on the phenotype of the T-supplemented testis, topo IIα gene expression and enzymatic activity were unaffected. It has been previously demonstrated that EDS treatment leads to selective germ-cell death on day 6 and that perfusion-fixed testes do not show sloughing of germ cells into the lumen (Sharpe *et al*. 1990, Kerr *et al*. 1993). Control testis, which is treated in a manner identical to EDS-treated testis, demonstrates intact morphology (Fig. 12A). Additionally, androgen deprivation obtained using an independent agent (flutamide) results in similar morphology (see below). Therefore, it is unlikely that the observed effect is an artifact of handling or fixation. The exact mechanism of topo IIα gene expression in EDS-treated and T-supplemented rats remains to be determined.

Although the inhibitory effects of EDS on functions mediated by T are widely recognized, the molecular mechanisms of these effects are obscure. To corroborate the ablation of T with EDS, we also examined whether the testis phenotype is affected by flutamide. Chronic exposure of adult rats to flutamide impaired the normal morphology of seminiferous tubules (compare Fig. 13A with Fig. 13B). Significantly, flutamide treatment did not have any detectable effect on Leydig cells. However, a fraction of the tubules retained the normal morphology, suggesting that the effect of flutamide was less severe than the EDS treatment. Although exogenous T appeared to reduce germ-cell sloughing into the lumen, spermatogenesis was not quantitatively restored (Fig. 13C). The ability of flutamide to impair the normal morphology of the testis, and to repress topo IIα gene expression (Fig. 11), confirms the role of T in regulation of topo IIα gene expression. Additionally, the differential effect of T deficiency on topo IIα mRNA levels and enzymatic activity argues for multiple mechanisms of regulation of topo II expression in the adult testis.

CONCLUSIONS

In summary, we have shown that topo IIα is differentially expressed during the development of the postnatal testis, with an apparent lack of correlation with mRNA, protein and enzyme activity levels. Our results also demonstrate that T acts as a positive regulator of topo IIα expression in prepubertal animals. Furthermore, T is required for topo IIα expression even after the onset of puberty; this effect being separable from the requirement of T for maintenance of spermatogenesis. It has been demonstrated that deregulated expression of topo IIα can lead to apoptosis (McPherson & Goldenberg 1998). In this context, it is possible that the endogenous level of topo IIα is tightly controlled in the testis because an excessive supply of topo II may be lethal for differentiating germline cells. The observation that T is important for regulating topo IIα gene expression is significant because of the importance of T for germ-cell proliferation in the testis and for spermatogenesis. In the prepubertal rat, T is required for the development of male secondary sex characteristics and hormonal imprinting of the liver, prostate and hypothalamus. In the adult, T is essential for spermatogenesis, sperm maturation and sexual function (Sundaram & Kumar 1996). Intriguingly, germ cells lack androgen receptors (Grootegoed *et al*. 1977), and the promoter of the rat topo IIα gene is devoid of a canonical androgen response element (Park *et al*. 1995). In this scenario, further studies are required to obtain a full understanding of the regulation of topo IIα expression in the testis, and of its roles in the growth of various cell types in the developing testis and in the maintenance of spermatogenesis.

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