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Topoisomerase II expression and VM-26 induction of DNA breaks during spermatogenesis in *Xenopus laevis*

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SUMMARY

The relative content of topoisomerase II (topo II) and the induction of topo-II-mediated DNA damage and cellular abnormalities have been characterized in developing spermatogenic cells of *Xenopus laevis* to gain an insight into the roles of topo II during spermatogenesis. Decatenation assays identified topo II activity in nuclear extracts from spermatocytes and pre-elongate spermatids, but not in extracts from elongate spermatids or sperm. Extracts from early-mid spermatids contained 14% (per cell) of the decatenation activity found in spermatocyte extracts. Immunoblots of SDS extracts from whole cells and nuclei from both spermatocytes and pre-elongate spermatids, but not elongate spermatids or sperm, resolved a 180 kDa polypeptide that reacts with polyclonal antisera to *Xenopus* oocyte topo II, an antipeptide antibody (FHD29) to human topo IIα and β, and an antipeptide antibody to human topo IIα, suggesting homology between *Xenopus* spermatogenic cell topo II and mammalian topo IIα. Immunofluorescence microscopy of topo II in testis cryosections revealed the presence of topo II in nuclei of all spermatogenic stages, but not in sperm. The relative levels of topo II estimated from fluorescence intensity were highest in spermatogonia and spermatocytes, then early-mid spermatids, followed by elongate spermatids and somatic cells. Incubation of isolated spermatogenic cells with teniposide (VM-26), a topo II-targetted drug, resulted in a dose-dependent induction of DNA breaks in all spermatocytes and spermatid stages to nuclear elongation stages, as analyzed by alkaline single cell gel electrophoresis. Addition of 0.5-50 μM VM-26 to spermatogenic cell cultures for 27 hours resulted in stage-dependent abnormalities. Mid-late spermatid stages were relatively resistant to VM-26-induced damage. In contrast, meiotic division stages were arrested and spermatogonia B were killed by VM-26, and VM-26 induced abnormal chromosome condensation in pachytene spermatocytes. The results of these studies show that cellular levels of topo II are stage-dependent during spermatogenesis, that most spermatogenic stages are sensitive to topo II-mediated DNA damage, and that spermatogonia B, meiotic divisions and pachytene spermatocytes are particularly sensitive to induction of morphological abnormalities and cell death during acute exposure to topo II-targetted drugs.

Key words: topoisomerase II, spermatogenesis, DNA damage, *Xenopus laevis*

INTRODUCTION

During spermatogenesis, chromatin and chromosomes display structural and functional properties unique to the germline. In meiotic cells, chromosomes synapse, recombine, form chiasma, condense and segregate in two distinctive cell divisions. After meiosis, in late spermatid stages, histones and nucleosomes are usually replaced by protamines and DNA topology is altered (reviewed by Risley, 1990; Ward, 1993). Although many of the molecular mechanisms fundamental to meiotic and spermatid chromatin organization are not understood, several observations suggest that topoisomerase II (topo II) may play important roles in both the meiotic and spermatid stages.

topo II is an ATP-dependent enzyme that creates double-strand breaks in DNA to relax torsional stress, remove knots or catenate and decatenate separate DNA molecules (Wang, 1985; Liu, 1989). In somatic cells, the enzyme is found in all cycling cells with the highest levels present in the G_2-M phases of the cell cycle (Heck and Earnshaw, 1986; Heck et al., 1988). The high levels of topo II activity late in the cell cycle are consistent with proposed roles for topo II in chromosome condensation (Uemura et al., 1987; Newport and Spann, 1987; Wood and Earnshaw, 1990; Adachi et al., 1991; Hirano and Mitchison, 1993) and sister chromatid decatenation (Dinardo et al., 1984; Uemura et al., 1987; Shamu and Murray, 1992). A structural role for topo II has also been proposed, based on its presence in isolated nuclear matrices (Berrios et al., 1985) and mitotic chromosome scaffolds (Earnshaw et al., 1985; Gasser et al., 1986). There are two isoforms of topo II in mammals (Drake et al., 1987; Chung et al., 1989). Both isoforms have homology with the single topo II found in lower organisms such as yeast and *Drosophila* (Jenkins et al., 1992; Austin et al., 1993). topo IIα (170 kDa) is the cell cycle-

regulated topo II that is abundant in proliferating cells, while topo IIβ (180 kDa) predominates in quiescent cells (Woessner et al., 1991). Both isoforms have been localized to the nucleoplasm and nucleoli (Petrov et al., 1993), but some reports suggest that topo IIβ is primarily in the nucleolus (Zini et al., 1992).

topo II has been immunolocalized in spermatocytes in chickens (Moens and Earnshaw, 1989) and rats (McPherson and Longo, 1993), and in yeast meiocytes (Klein et al., 1992). The enzyme is found throughout chromatin, but concentrated in chromosome axes adjacent to synaptonemal complexes (Moens and Earnshaw, 1989; Klein et al., 1992). Roca and Mezquita (1989) have shown that topo II isolated from chicken spermatocytes is catalytically active. Catalytically active topo II has also been isolated from chicken (Roca and Mezquita, 1989) and rat (McPherson and Longo, 1993) spermatids. Isoform expression has not been well defined in spermatogenic cells; however, a 161 kDa polypeptide in rat spermatocytes and a 137 kDa polypeptide in rat spermatids reacts in western blots with an antibody to topo II (McPherson and Longo, 1993).

Understanding the roles of topo II during spermatogenesis could provide insights into the mechanisms of meiosis and spermatid development. In yeast meiotic cells, *top2* conditional mutants do not progress through the first meiotic division unless recombination is suppressed by a *rad50* mutation (Rose et al., 1990). topo II is also required for normal segregation during meiosis II, independent of recombination levels. Meiotic arrest in *top2* mutants occurs in late pachytene, presumably due to a meiotic regulatory checkpoint (Rose and Holm, 1993). topo II activity may be required to resolve interlocked nonhomologous chromosomes during pachytene (Rose and Holm, 1993) and may also play a role in chromosome condensation, homologue disjunction and sister chromatid segregation during the meiotic divisions. In spermatids, topo II may be important for maintaining the looped domain organization of chromatin and may also aid in the topological transitions that accompany the loss of nucleosomes during the replacement of histones by protamines (Risley et al., 1986; Roca and Mezquita, 1989).

Given the timing of topo II functions in meiosis, topo II may also be a target for drug-induced chromosome aberrations in the germline. In somatic cells, topo II interacts with diverse chemicals to produce DNA breaks, which lead to chromosome aberrations and cell death (reviewed by D'Arpa and Liu, 1989; Liu, 1989). In germ cells, topo II-drug interactions may result in chromosome damage, which is heritable and a direct cause of congenital abnormalities. Teniposide (VM-26), an epipodophyllotoxin that traps topo II in cleavable complexes (Chen et al., 1984), inhibits meiotic chromosome segregation in oocytes (Wright and Schatten, 1990) and induces DNA breaks in chicken spermatids (Roca and Mezquita, 1989).

Investigation of topo II functions in vertebrate spermatogenesis has been hindered by the lack of genetic approaches equivalent to those available in yeast and by difficulties in the isolation and culture of germ cells. We have been developing an in vitro system using cultured spermatogenic cells from *Xenopus laevis* to study topo II functions in vertebrate spermatogenesis. Many of the spermatocyte and spermatid stages in *Xenopus* continue to develop in cell (Risley, 1983) and organ cultures (Risley et al., 1987). Thus, specific spermatogenic stages are accessible to the application of experimental approaches to neutralize cellular topo II activity, including drug inhibition, immunoneutralization by microinjected antibodies (cf. Buchenau et al., 1993), and antisense oligonucleotide inhibition of topo II mRNA translation. Treated cells may be examined to identify topo II-dependent stages of development and topo II functions. In this report, the expression of topo II in *Xenopus laevis* spermatogenesis is characterized and compared to the stage-dependent induction of DNA damage and developmental abnormalities by teniposide. We show that topo II is present at stage-dependent levels in all stages to the late elongate spermatid stage. Teniposide induces DNA breaks in most spermatogenic stages, but teniposide-induced cell death and developmental arrest are stage-dependent. A preliminary account of this study has been reported (Risley et al., 1992).

MATERIALS AND METHODS

Animals

Xenopus laevis (South African clawed frogs) were puchased from NASCO (Ft. Atkinson, WI) and Xenopus I (Ann Arbor, MI), or obtained as a generous gift from Dr John Gerhart (Department of Molecular Biology, University of California at Berkeley, Berkeley, CA). Frogs were maintained in water purifed by reverse osmosis and charcoal filtering, and fed twice weekly with Purina Trout Chow. Frogs were killed by decapitation and pithed prior to dissection. Lewis rats were maintained on 12 hours light/12 hours dark cycles and fed ad libitum. Rats were killed by CO_2 asphyxiation prior to dissection.

Testis dissociation and cell separations

Xenopus testes were dissociated with collagenase and filtered through Nitex screens to remove undissociated cells, as described previously (Risley, 1983; Li et al., 1989). Released cells were then separated by centrifugation at 850 ***g*** for 15 minutes in 28% Percoll (Sigma Chem. Co., St Louis, MO) or 30% Percoll if elongate spermatids were to be isolated (Li et al., 1989). The pellets, consisting primarily of sperm, erythrocytes and late elongate spermatids, were further fractionated by centrifugation (4°C) in 30% to 50% Metrizamide gradients, as described (Risley and Eckhardt, 1979). Sperm were also isolated by centrifugation in Metrizamide step gradients consisting of 3 ml 30% Metrizamide, 3 ml 35% Metrizamide and sperm and 3 ml 40% Metrizamide. After centrifugation at 7000 rpm for 15 minutes in an HB4 rotor, sperm (98-99%) were collected from the 35/40% interphase and the pellet.

Cells in the 28% or 30% Percoll supernatants, consisting largely of spermatogonia (5-10%), spermatocytes and spermatids (80-90%), and Sertoli cells (3-5%), were further separated by unit gravity sedimentation in 900 ml, 5% to 12% Percoll gradients formed in a CELSEP chamber (DuPont, Wilmington, DE), as described by Li et al. (1989). Spermatocyte-enriched populations obtained by unit gravity sedimentation consisted mostly of primary spermatocytes (90%), with spermatids (3.2%) and spermatogonia plus secondary spermatocytes (2.6%), as the principal contaminants (n=8). Mixed spermatid populations (round through early elongate stages) obtained from the CELSEP were either used directly or further purified by centrifugation through 10 ml linear gradients of 17% to 28% Percoll (prepared in 70% Hanks' balanced salt solution) formed over 1 ml cushions of 40% Percoll. Gradients were centrifuged (4°C) at 5000 ***g*** for 15 minutes in a SW40.1 rotor. Gradients were collected (0.5 ml fractions) from the top with a Haake Buchler Auto Densiflow IIC, and examined microscopically. Fractions were pooled to collect populations enriched for acrosome vesicle stages (92% round+acrosome vesicle, 5% elongate, 2% sperm; n=5) or early elongate spermatid stages (75% elongate, 19% round+acrosome vesicle, 4% sperm; n=6).

Nuclear isolation and topoisomerase II extraction

Crude nuclei were isolated from spermatogenic cells and testes by modifications of procedures used for *Xenopus* somatic cell nuclei (Richard and Bogenhagen, 1991). Cells or minced testes were Dounce homogenized in ice-cold 10 mM HEPES (pH 7.9), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100, 1 mM benzamidine-HCl, 0.2 mM PMSF, and 1 μg/ml aprotinin and leupeptin, and nuclei were pelleted by centrifugation at 1000 ***g*** for 10 minutes. Nuclei were also released from decapsulated rat testes by homogenization (Potter Elvehjem) in ice-cold PBS containing 0.1% Triton X-100, 1 mM benzamidine-HCl, 10 μg/ml soy bean trypsin inhibitor, 50 μg/ml leupeptin, and 1 mM PMSF, and pelleted as above. Supernatants (cytosol) were mixed with concentrated SDS-gel sample buffer (Laemmli, 1970). Whole cells and nuclear pellets (maximum 10^7 cells or nuclei/100 μl) were sonicated in SDS sample buffer (without boiling) to shear DNA. Most assays were quantified using extract volumes containing specific numbers of cells or nuclei, as determined from hemacytometer counts. Protein assays were also performed using the Bradford assay (Bradford, 1976).

Nuclei were extracted with 0.5 M NaCl to obtain crude preparations of topo II for catalytic assay. Nuclei (10^6 to 10^7/100 μl) were resuspended in ice-cold 20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM benzamidine-HCl, 0.2 mM PMSF, 1 μg/ml aprotinin and leupeptin, and an equal volume of 1 M NaCl in the same buffer was added. After 10 minutes on ice, nuclei were pelleted in a microfuge at 10,000 ***g*** for 10 minutes, and solubilized in SDS-gel sample buffer as described above. Samples of all extracts were stored frozen at –90°C.

Catalytic assays

topo II activities in nuclear extracts were evaluated using the kDNA decatenation assay (Marini et al., 1980), as described by Luke and Bogenhagen (1989). *Crithidia fasciculata* kDNA (TopoGEN, Columbus, OH) was added (140 ng) to assay buffer (50 mM Tris-HCl, pH 7.6, 100 mM KCl, 7 mM $MgCl_2$), followed by 3 mM DTT and 1 mM ATP just prior to use. Yeast tRNA (140 ng) was also added to serve as an internal control for gel loading and to compete for nucleic acid binding proteins (e.g. histone H1). Varied dilutions of nuclear extracts were added (1 μl/18 μl assay) and reactions incubated for 80 minutes at 35-37°C. The reactions were stopped by addition of 100 mM EDTA, pH 7.5, 60% glycerol, 2% SDS and samples were electrophoresed (1 hour, 6.2 V/cm) in 1% agarose gels using 40 mM Tris-HCl, sodium acetate, 1 mM EDTA (pH 8). Some samples were digested (45 minutes, 37°C) with proteinase K (50 μg/ml) prior to electrophoresis. The DNA was stained with ethidium bromide (0.5-2 μg/ml) and photographed with a Fotodyne transilluminator (Fotodyne, Inc., New Berlin, WI) using Polaroid type 55 P/N film. On occasion, teniposide (VM-26, Bristol Myers Squibb, Wallingford, CT) was added to the assay mixtures from 50 mM stocks prepared in DMSO. DMSO was also added to controls to maintain constant DMSO levels.

All assays were performed with varied dilutions of extracts to assure a linear correspondence between minicircle formation and extract input, as determined by densitometry. Densitometry was performed on photographic negatives using the transmission mode on the Bio-Rad model 620 Video Densitometer (Bio-Rad Laboratories, Hercules, CA) and minicircle density was determined with 1D Analyst II software (Bio-Rad). We also performed densitometry by digitizing (PC Vision Plus, Imaging Technology, Inc., Woburn, MA) reflected images of the negatives viewed with a Pulnix TM845 CCD camera (Pulnix America, Inc., Sunnyvale, CA). The digitized images of minicircle bands were analyzed using the vertical pixel averaging feature of the JAVA image analysis program (Jandel Scientific, San Rafael, CA). Linear regressions were performed to determine the linear range of decatenation rate relative to extract input in cell equivalents. Slopes were compared to obtain relative decatenation activities in extracts from different cell types isolated at the same time and assayed concurrently on the same gel. Values reported are the averages for extracts from three separate cell separations. Results from the two densitometric methods were in close agreement.

Immunoblot assays

Extracts were centrifuged at 16,000 ***g*** for 20 minutes. Samples of the supernatant were electrophoresed on 0.75 mm 6% acrylamide-SDS gels (Laemmli, 1970). Pre-stained molecular mass standards were included in each gel (Sigma). Proteins were electrically transferred to immobilon P using the methods of Towbin et al. (1979) or they were electrically transferred (0.4 amp, 1 hour) to nitrocellulose in 50 mM 3-cyclohexylamino-1-propanesulfonate (CAPS) buffer (pH 11). Gels were stained with 0.1% Coomassie Blue R-250 after transfer to assess uniformity of protein transfer.

The blots were blocked by incubation for 1 hour in TBST (10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween-20) containing 2% bovine serum albumin (BSA) and were then incubated for 2 hours in blocking buffer containing primary antibodies. The antibodies used included a rabbit polyclonal antiserum directed to *Xenopus* oocyte topo II (Luke and Bogenhagen, 1989). This antibody was generously provided by Dr Daniel Bogenhagen (Department of Pharmacological Sciences, State University of New York, Stony Brook, NY) and was used at dilutions of 1:2500 to 1:50,000 with similar results. An affinity-purified antibody (FHD29) directed to a common oligopeptide in mammalian topo IIα (sequence 148-162) and topo IIβ (Webb et al., 1993) was obtained from Dr Fred Drake (Department of Cellular Biochemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, PA) and used at a dilution of 1:500. An affinity-purified, antipeptide antibody directed to a 16-residue sequence in the carboxyl terminus of human topo IIα was purchased from TopoGEN (Columbus, OH) and used at a dilution of 1:2500. After incubation in primary antibodies, blots were washed in TBST, and incubated in blocking buffer containing a 1:15,000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Blots were washed with TBST and developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT) using standard procedures (Harlow and Lane, 1988).

Relative levels of topo II in different extracts were determined by reflectance mode densitometry of the blots (not negatives), as described above. Each blot contained multiple dilutions of spermatocyte extracts to establish concurrent standard curves and the linear range of band density relative to extract input.

Immunofluorescence microscopy

Testes were excised and frozen in isopentane cooled with liquid nitrogen. Cryosections (6-8 μM) were prepared at –20°C, applied to gelatin-coated coverslips, air-dried for 5 minutes, and fixed by immersion in methanol (–20°C) for 10 minutes. Sections were blocked by incubation for 1 hour in PBS (0.7×) with 2% BSA and 0.05% Tween-20. Sections were then incubated overnight in blocking solution containing 1:50 to 1:250 dilutions of FHD29 (Webb et al., 1993). Controls consisted of FHD29 preincubated 1 hour with 10 μg/ml of its oligopeptide antigen prior to addition to the sections. After washing, sections were incubated for 2 hours in blocking solution containing a 1:500 dilution of goat anti-rabbit IgG conjugated to FITC (Sigma). The sections were stained with 0.5 μg/ml Hoechst 33258, mounted in glycerol mounting medium (Sigma) containing 0.2% *p*-phenylenediamine and examined with a Nikon Optiphot fluorescence microscope. *Xenopus* A6 cells (American Type Culture Collection, Rockville, MD) were also examined by immunofluorescence after growing on Matrigel-(Collaborative Research) coated coverslips overnight. The cells were fixed and processed as described above.

Single cell gel electrophoresis

Spermatogonia through early elongate spermatid stages were recovered from 30% Percoll supernatants following centrifugation of

collagenase-dissociated cells (see above). The cells were cultured at 1×10^6 to 4×10^6 cells/ml in XSCM containing 10% heat-inactivated fetal calf serum (Risley, 1983). Teniposide was added at 0, 0.5, 5 or 50 μM to cultures and DMSO was added to controls to 0.1% to maintain constant DMSO levels. All treatments were conducted in duplicate and two independent replicates were performed.

After 4 hours, cells were pelleted and resuspended in ice-cold PBS (0.7×) with or without the teniposide concentration they were exposed to in culture. The cells were then analyzed for DNA breaks by alkaline single-cell gel electrophoresis, using modifications of published procedures (Singh et al., 1988, 1989). Cells were mixed with an equal volume of 1.5% low-gelling agarose prepared in PBS (0.7×). Then, 50-60 μl (1000 cells/μl) were immediately applied to the frosted end of a glass slide previously coated with a thin layer of 0.5% agarose. Two slides were prepared from each culture replicate. The agarose was covererd with a plastic coverslip and gelled for 5 minutes at 4°C. After removing the coverslip, slides were immersed (10 ml/slide) in 1% sodium lauryl sarcosine, 100 mM EDTA, 10 mM Tris-HCl (pH 10) and incubated overnight at 4°C. The next day, slides were incubated 20-30 minutes in 2.5 M NaCl prepared in the same buffer lacking detergent. Slides were then transferred to alkaline running buffer (300 mM NaOH, 1 mM EDTA) for 20 minutes followed by alkaline electrophoresis in a horizontal gel unit for 10 minutes at 20 V. Slides were neutralized by incubation for 10 minutes in 0.4 M Tris-HCl (pH 7.6), stained with 50 μl of ethidium bromide (20-50 μg/ml), coverslipped and examined by fluorescence microscopy. Slides remained stable during storage for 2-3 days at 4°C in a humidified chamber.

DNA damage was evidenced by the occurrence of comet-like fluorescent structures resulting from migation of DNA fragments away from residual nucleoids. Residual nucleoids remained spherical in the absence of DNA breaks. Spermatogenic cell types were characterized as pre-spermatids or spermatids, on the basis of the relative size and shape (elongate spermatids have elongate nucleoids) of the nucleoids. DNA damage in the two cell categories was compared with VM-26 dose by counting the percentage of each nucleoid type (out of 100/slide) showing comet tails. This approach has been shown to be a simple and effective technique for the analysis of VP-16-induced DNA breaks in Chinese hamster cells (Olive et al., 1990).

Teniposide effects on cultured spermatogenic cells

Mixed spermatogenic cells from 28% Percoll supernatants were cultured in serum-supplemented XSCM and treated with VM-26, as described above. Some cultures were conducted in multiwell plates, while others were cultures of cells attached to concanavalin-A-coated chamber slides, prepared as described (Risley et al., 1990). Cultures were maintained for 27 hours and observed periodically using an inverted microscope. Differential cell counts were performed (500-1000 cells/duplicate) to determine the dose dependence of VM-26-induced morphological abnormalities in different cell types resolved with Nomarski DIC optics. Numbers of abnormal cells of each type were compared with numbers of normal appearing cells in concurrent solvent controls. Data presented are means of two independent replicates, with each replicate conducted in duplicate.

RESULTS

Relative topo II activities in different phases of spermatogenesis were compared in kinetoplast decatenation assays, which measure the release of minicircles from kinetoplast DNA networks (Fig. 1). Nuclear extracts from spermatocyte-enriched populations (Fig. 1A, lanes 1-4) and round-to-acrosome vesicle stage spermatids (lanes 5-7) contained decatenation activity that was ATP-dependent (see lane 1) and inhibited by VM-26 (Fig. 1B). Nuclear extracts from elongate spermatids (Fig. 1A, lanes 8-9) and sperm (Fig. 1A, lane 10) lacked measurable decatenation activity. The absence of decatenation activity in extracts from elongate spermatids and sperm was not due to the occurrence of stage-specific catalytic inhibitors or topo II proteolysis because co-extraction of nuclei from these cells with nuclei from spermatocytes did not inhibit spermatocyte decatenation activity (Fig. 1C). Densitometric analysis of minicircle formation relative to extract input showed that extracts from early-mid spermatids contained 14.4±0.4% (mean ± s.e.m.) (n=3) of the decatenation activity in spermatocyte extracts. Since spermatid DNA content is 25% of spermatocyte DNA content, extractable spermatid topo II activity per unit of DNA is about 57% of spermatocyte topo II activity.

Immunoblotting was conducted with a polyclonal antiserum to *Xenopus* oocyte topo II (Luke and Bogenhagen, 1989), a *Xenopus* homolog of mammalian topo IIα (Hirano and Mitchison, 1993). This antibody reacted with a 180 kDa polypeptide in SDS extracts of whole cells (Fig. 2A) and nuclei

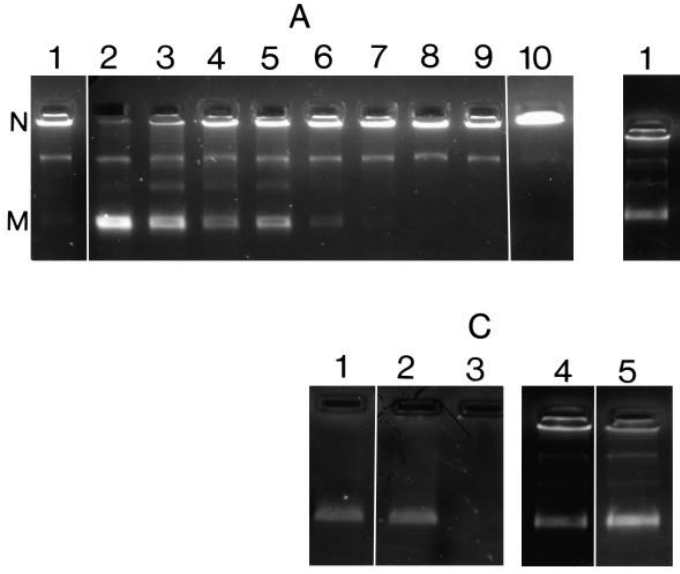

Fig. 1. Decatenation of kDaNA by 0.5 M NaCl nuclear extracts. (A) Lane 1, spermatocytes (1×10^4 nuclei) minus ATP; lanes 2-10, plus ATP; lanes 2-4, spermatocytes (1.5×10^4, 1×10^4, 7.5×10^3); lanes 5-7, round and acrosome vesicle spermatids (6×10^4, 4×10^4, 3×10^4); lanes 8-9, early elongate spermatids (1.2×10^5, 6×10^4); lane 10, sperm (3×10^5). N, kDaNA networks; M, minicircle monomers. (B) VM-26 inhibition of decatenation. Spermatocyte extracts (lanes 1-4, 1×10^4 nuclei; lanes 5-6, 4×10^3 nuclei) without VM-26 (lanes 1 and 5) or with 10 μM (lane 2), 50 μM (lane 3), 100 μM (lane 4), or 500 μM (lane 6) VM-26. (C) Decatenation of kDNA by extracts from mixed spermatocytes and elongate spermatids or sperm. Lane 1, spermatocytes (6×10^3); lane 2, spermatocytes (6×10^3) plus elongate spermatids (2.4×10^4); lane 3, elongate spermatids (2.4×10^4); lane 4, spermatocytes (1×10^4); lane 5, spermatocytes (1×10^4) and sperm (4×10^4).

(Fig. 2B) from *Xenopus* spermatogenic cells. The size of the immunoreactive polypeptide is consistent with the size of topo II in oocytes (Luke and Bogenhagen, 1989) and somatic cells of *Xenopus* (Hirano and Mitchison, 1993). Cytosol fractions lacked measurable levels of topo II (Fig. 2B, lane 4). topo II was relatively abundant in extracts from spermatocytes (Fig. 2A, lane 1; Fig. 2B, lane 2), but not detected in sperm extracts (Fig. 2A, lane 3) or elongate spermatid extracts (Fig. 2B, lane 3). Early-mid spermatid extracts contained 15.4±2% (n=3) of the topo II present in spermatocyte extracts (or 61% per unit DNA).

Immunoblotting was also employed to examine the distribution of topo II in nuclei after salt extraction (Fig. 3). Densitometric comparison of topo II levels in SDS and 0.5 M NaCl nuclear extracts demonstrated that salt extracts contained 74% (n=2) of the SDS-soluble topo II in spermatocyte nuclei (Fig. 3A) and 85% of the SDS-soluble topo II in early-mid spermatid nuclei (Fig. 3B). Most of the topo II that was not recovered from nuclei in salt extracts was present in the salt-insoluble nuclear fraction. Early elongate spermatid nuclei lacked measurable topo II in salt-soluble or insoluble fractions (Fig. 3B, lanes 6 and 7). Thus, the immunoblot assays and the decatenation assays are consistent and support the conclusion that the levels of topo II polypeptide and catalytic activity are relatively high in spermatocytes, decline by 39-43% (per unit of DNA) in early-mid spermatids, and then decline further to very low levels in elongate spermatids and sperm.

Immunoblots of SDS extracts were also probed with antisera to mammalian topo II to compare homology with that of *Xenopus* topo II and to investigate further the occurrence of germline-specific isoforms. An affinity-purified, antipeptide antibody (FHD29) directed to a highly conserved sequence in both topo IIα and topo IIβ (Webb et al., 1993) reacted with a 180 kDa polypeptide in extracts from spermatocytes and spermatids (Fig. 4A, lanes 1-4), but no reactivity was observed in extracts from elongate spermatids (lane 7). As expected, 170 kDa and 180 kDa isoforms of mammalian topo II were detected in rat testes extracts (lane 6). An affinity-purified, antipeptide antibody directed to the carboxy terminus of human topo IIα (TopoGEN) reacted only with the 170 kDa isoform present in rat testes extracts (Fig. 4B, lane 1) and the 180 kDa polypeptide in *Xenopus* spermatocytes (lanes 2-4). Evidence for germline-specific isoforms was not detected with any of the antibodies.

Immunofluorescence was used to investigate further topo II distribution in different germ cell stages. These experiments used the affinity-purified, antipeptide antibody FHD29 (Webb et al., 1993), since it reacts well with *Xenopus* spermatogenic cell topo II and shows little cross-reactivity to other proteins (Fig. 4A). Immunostaining of the *Xenopus* A6 cell line showed strong reactivity of this antibody with metaphase chromosomes (Fig. 5A). Reactivity was also detected in the nucleoplasm and nucleoli, but the fluorescence intensity showed intercellular heterogeneity, probably reflecting the cell cycle dependence of topo II levels. A similar observation was reported following immunostaining of A6 cells with the less specific polyclonal antiserum to *Xenopus* oocyte topo II (Fischer et al., 1993). Cryosections from *Xenopus* testes immunostained with FHD29 showed widespread reactivity of the antibody (at 1:50 and 1:100 dilutions) with spermatogenic cells and somatic cell nuclei (Fig. 5C). Immunoreactivity was eliminated if the

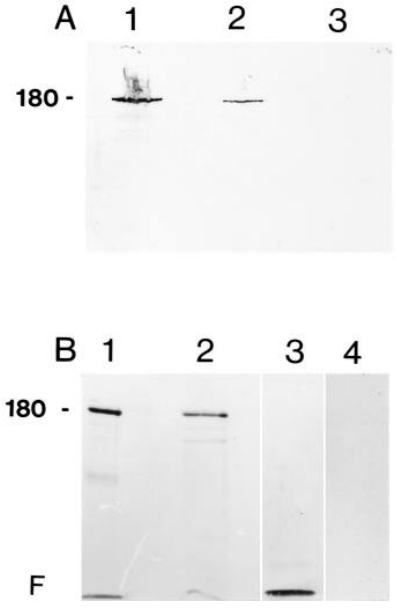

Fig. 2. Immunoblots of SDS extracts from whole cells (A) and nuclei (B). (A) Lane 1, spermatocytes (2.5×10^6 cells); lane 2, round-acrosome vesicle spermatids (1×10^7 cells); lane 3, sperm (1×10^7 cells). (B) Lane 1, spermatocytes (2.5×10^6 nuclei); lane 2, round-acrosome vesicle spermatids (1.5×10^6 nuclei); lane 3, elongate spermatids (5×10^6 nuclei); lane 4, spermatocyte cytosol (4×10^5 cells). Blots were developed with antisera to *Xenopus* oocyte topo II diluted 1:2500 (A) and 1:50,000 (B). F, electrophoretic front.

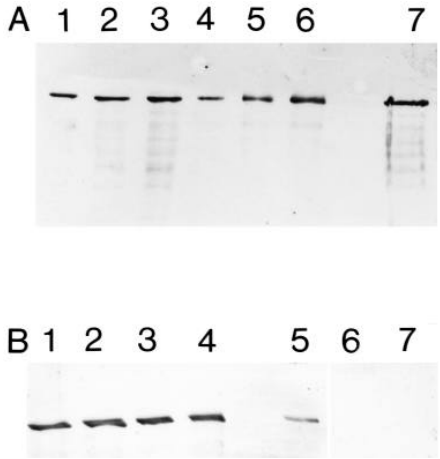

Fig. 3. Immunoblots of salt-extractable topo II from nuclei of spermatocytes (A) and spermatids (B). (A) SDS-soluble topo II (lanes 1-3) and 0.5 M NaCl-extracted topo II (lanes 4-6) from spermatocyte nuclei (lanes 1, 4, 2×10^5; lanes 2, 5, 3×10^5; lanes 3, 6, 4×10^5 nuclei); lane 7, 0.5 M NaCl-insoluble topo II (1×10^6 nuclei). (B) SDS-soluble topo II (lanes 1, 2) and 0.5 M NaCl-extracted topo II (lanes 3, 4) from 1.6×10^6 round-to-acrosome vesicle spermatid nuclei; lane 5, 0.5 M NaCl-insoluble topo II (5.2×10^6 nuclei). Lanes 6 and 7, 0.5 M NaCl-extracted (lane 6) and insoluble (lane 7) topo II from elongate spermatid nuclei (lane 6, 1.6×10^6 nuclei; lane 7, 3.5×10^6 nuclei). Blots were developed with antiserum to *Xenopus* oocyte topo II (1:50,000).

antibody was pre-incubated with its peptide antigen (Fig. 5D), showing the specificity of the reaction for the peptide sequence found in topo II.

Testis cryosections stained with FHD29 showed relatively strong fluorescence in spermatogonia and spermatocyte nuclei. Meiotic chromosomes were also fluorescent (Fig. 6A). Sperm nuclei were not immunoreactive, but nuclei in all spermatid stages through the nuclear elongation stage were immunostained (Fig. 6C). The reactivity of late elongate spermatids was a surprise, since immunoblotting and decatenation assays failed to resolve extractable topo II in late spermatids. The level of topo II in late spermatids appears relatively low, however, since immunostaining with higher dilutions (1:250) of FHD29 resulted in a loss of fluorescence in late spermatids but only reduced fluorescence in early-mid spermatids. Somatic cell nuclei in the interstitium also lacked fluorescence at FHD29 dilutions that immunostained most spermatogenic stages except elongate spermatids. Similar immunofluorescence results were obtained when the less-specific antiserum to *Xenopus* oocyte topo II was used (data not shown).

Single-cell gel electrophoresis was employed to determine if teniposide (VM-26), a topo II-targetted drug, induces DNA breaks in *Xenopus* spermatogenic cells. Mixtures of isolated spermatogonia through early elongate spermatid stages were incubated with 0-50 μM VM-26 for 4 hours, embedded in agarose, subjected to alkaline electrophoresis, and examined by fluorescence microscopy (Singh et al., 1988, 1989). DNA in controls was retained in spherical nucleoids that were proportional in size to the cell of origin (Fig. 7A). Thus, DNA from spermatocytes and spermatogonia (prespermatids) was in large nucleoids and DNA from haploid spermatids was present in small nucleoids. Only 5-8% of control nucleoids showed evidence (fluorescent comets) of DNA breaks. Interestingly, untreated *Xenopus* sperm nucleoids contained extensive DNA breaks after alkaline electrophoresis (not shown), as first shown for mouse and human sperm by Singh et al. (1989). Incubation in VM-26 for 4 hours resulted in the induction of DNA breaks and fluorescent comets in nucleoids (Fig. 7B,C).

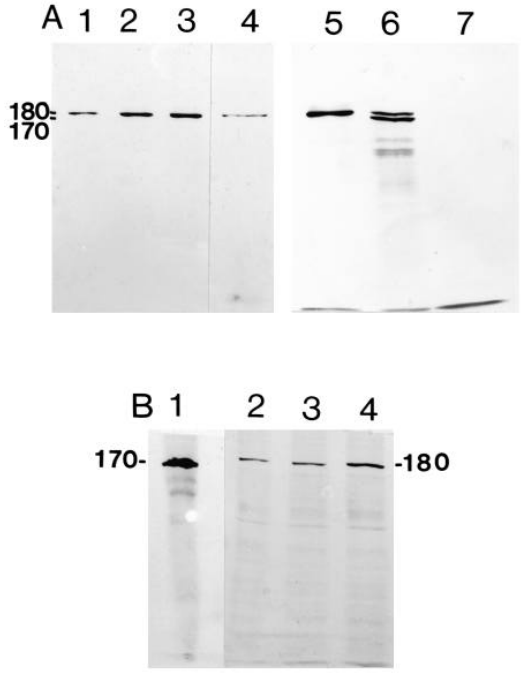

Fig. 4. Immunoblots of SDS extracts of nuclei developed with FHD29 (A) and anti-topo IIα (B). (A) Lanes 1-3, spermatocyte nuclei (1×10^5, 1.5×10^5, 2×10^5); lane 4, round-to-acrosome vesicle spermatid nuclei (1.5×10^6); lane 5, mixed (1:3) spermatocytes and spermatids (7.5×10^5 nuclei); lane 6, 50 μg of SDS-soluble rat testis nuclear protein; lane 7, elongate spermatid nuclei (5×10^6). Blot developed with 1:500 dilution of FHD29. (B) Lane 1, 50 μg rat testis nuclear protein; lanes 2-4, spermatocyte nuclei (2×10^5, 3×10^5, 4×10^5). Blot developed with 1:2500 dilution of anti-topo IIα (TopoGEN).

Counts of comets from prespermatid stages (Fig. 8A) and spermatids (Fig. 8B) showed that VM-26 induction of DNA damage was dose-dependent. At the highest dose (50 μM), 98% of prespermatid stages and 88% of spermatids had extensive DNA damage and much of the DNA migrated in the comet tail (see Fig. 7C). Low dose exposures (0.25-0.5 μM) resulted in a more heterogeneous response. Some nucleoids lacked broken DNA. Other nucleoids contained DNA breaks, but the break frequency was lower than that seen in nucleoids from high dose exposures, since most DNA did not migrate out of the nucleoids. Breaks induced by 0.25 and 0.5 μM VM-26 were repaired in prespermatids and spermatids after placing treated cells in fresh medium for 20 hours. Repair was only partial, however, in cells treated with 5 and 50 μM VM26. These results suggest that some fraction of the topo II in most spermatogenic stages is catalytically active and a target for drug-induced DNA damage.

To determine which spermatogenic stages may develop gross abnormalities due to acute exposure to topo-II-targetted drugs, isolated spermatogenic cells were cultured for 27 hours in medium with 0-50 μM VM-26 and differential cell counts were performed (Fig. 9). Morphological abnormalities were detected in relatively few mid-late (acrosome vesicle to elongate) spermatid stages, even at the highest VM-26 dose. Early (round) spermatids, however, were absent from cultures containing 5 and 50 μM VM-26, and were reduced to 44% of controls in cultures with 0.5 μM VM26. This may be due primarily to the inhibition of meiosis by VM26 and the resultant failure to replace round spermatids that develop to acrosome vesicle stages. Meiotic divisions were reduced to 14.4% of controls in 0.5 μM VM-26, 2.2% of controls in 5 μM VM-26, and were absent from cultures containing 50 μM VM-26. Periodic observations indicated that morphological abnormalities (condensed chromosome masses) were present in meiotic cells as early as 4 hours of culture.

Pachytene was less sensitive to VM-26-induced damage than the meiotic divisions, but more sensitive than earlier prophase stages. VM-26 induced extreme chromosome condensation in pachytene cells in a dose-dependent manner. Affected cells may be developmentally arrested, since 98% of pachytene cells cultured with 5 μM VM-26 for 90 hours showed extreme chromosome condensation (not shown). Unlike pachytene cells, only 35% of earlier meiotic prophase cells showed abnormal chromosome condensation after 27 hours in 50 μM and longer exposures did not increase this value.

Spermatogonia B appeared to be sensitive to cell killing by VM-26, since these cells became highly pycnotic, resembling apoptotic cells (cf. Kerr and Harmon, 1991). At 5 μM VM-26,

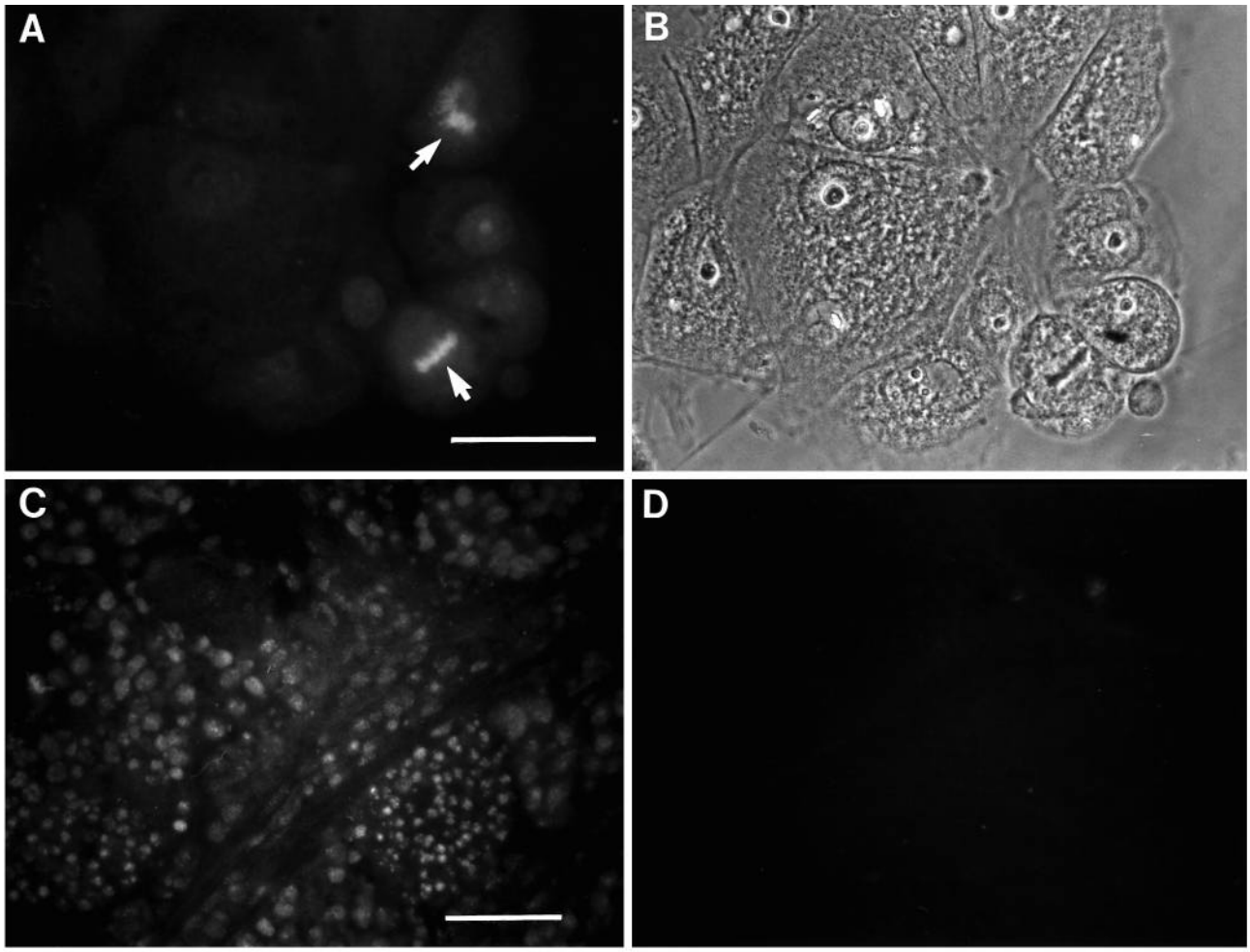

Fig. 5. Immunofluorescence microscopy of *Xenopus* A6 cells (A,B) and *Xenopus* testis cryosections (C,D) incubated with FHD29 (A,C) and FHD29 plus 10 μg/ml of its peptide antigen (D). Fluorescence (A,C,D) and phase-contrast (B) micrographs. Arrows indicate metaphase chromosomes. Bars, 50 μm.

only 15% of the spermatogonia B in controls were present after 27 hours. On the other hand, spermatogonia A were relatively resistant to VM-26, since 73% of spermatogonia A remained after 27 hours in 50 μM VM26. A condensed morphology was also not seen in spermatogonia A.

DISCUSSION

A 180 kDa topo II was detected in immunoblots of SDS extracts from *Xenopus* spermatogenic cell nuclei. The mass is similar to that of topo II in *Xenopus* oocytes (Luke and Bogenhagen, 1989) and somatic cells (Hirano and Mitchison, 1993). Fischer et al. (1993) recently reported the occurrence of a 170 kDa topo II in *Xenopus* A6 cells, but we have not detected this presumptive isoform. The 170 kDa polypeptide may be a degradation product of the 180 kDa topo II, since only one size class of topo II consisting of two isoforms has been detected in cDNA libraries from *Xenopus* (Hirano and Mitchison, 1993). The combined results obtained from immunoblotting with three different antisera to topo II suggest that the predominant topo II present in spermatogenic cells is homologous to mammalian topo IIα. The *Xenopus* oocyte topo II antiserum reacts strongly with spermatogenic cell topo II and with oocyte topo II (Luke and Bogenhagen, 1989), which is a *Xenopus* topo IIα homolog (Hirano and Mitchison, 1993). Also, we showed that a mammalian topo IIα-specific antibody (TopoGEN) reacted with spermatogenic cell topo II. We have also found that FHD22, an affinity-purified antibody to the sequence from amino acids 195-209 in topo IIα (Webb et al., 1993), reacts strongly with *Xenopus* spermatocyte 180 kDa topo II (data not shown). McPherson and Longo (1993) have shown that large pachytene spermatocytes in rats also have abundant topo IIα, and Moens and Earnshaw (1989) demonstrated an accumulation of a 170 kDa topo II on peripheral chromatin and on chromosome cores in chicken pachytene spermatocytes. These observations suggest that topo IIα is the predominant isoform in the male germline of diverse species.

Previous reports have suggested that topo II may be important in elongate spermatids to resolve torsional strain resulting from nucleosome displacement by protamines (Risley, 1986; Roca and Mezquita, 1989; McPherson and Longo, 1993). Assays of topo II unknotting activities extracted from chicken spermatids suggested that topo II activity rises

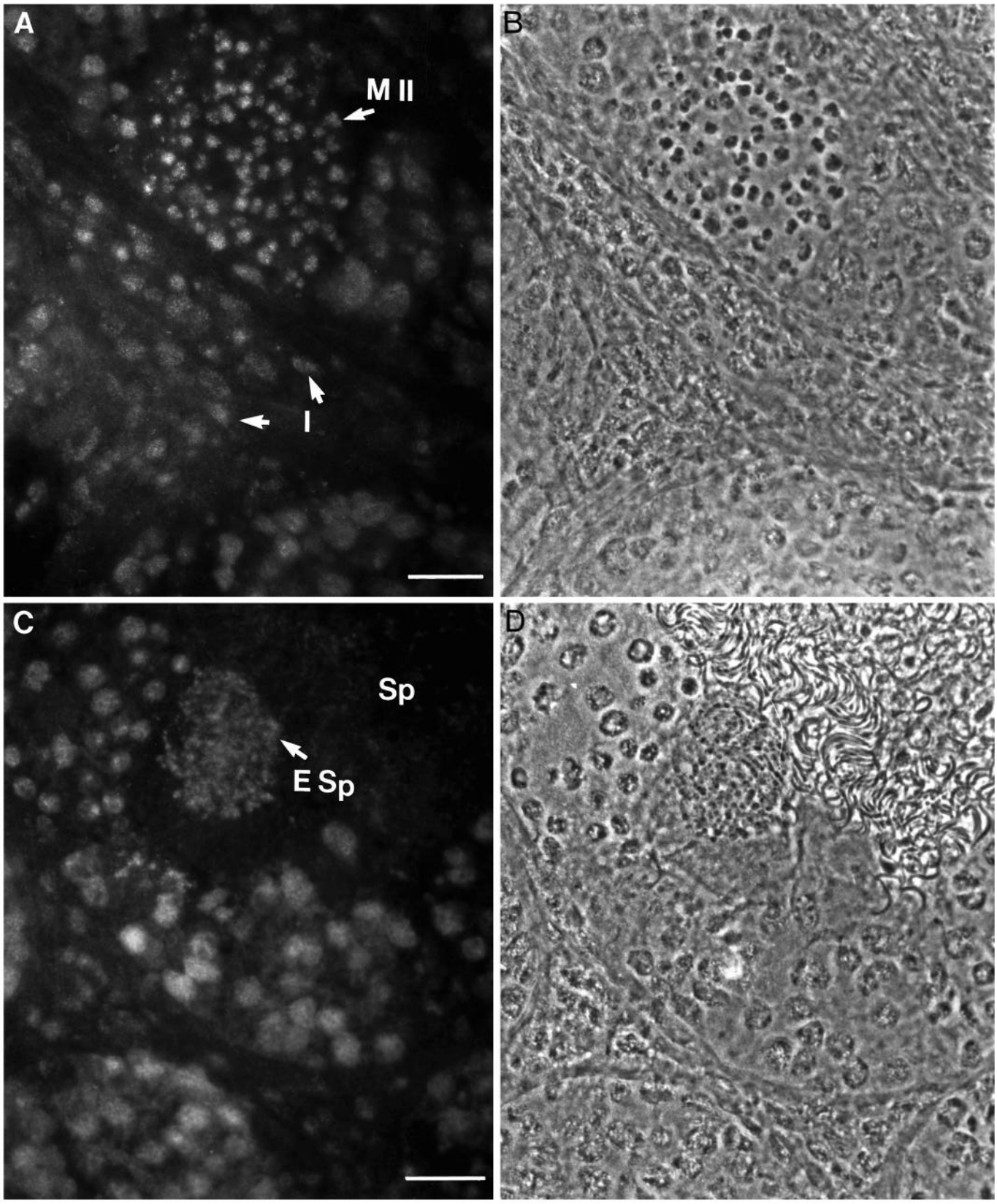

Fig. 6. Immunofluorescence microscopy of *Xenopus* testis cryosections incubated with FHD29. Fluorescence (A,C) and phase-contrast (B,D) micrographs. In (A) MII is meiotic metaphase II; arrows indicate interstitial cells (I). In (C) Sp are sperm; ESp are late elongate spermatids. Bars, 25 μm.

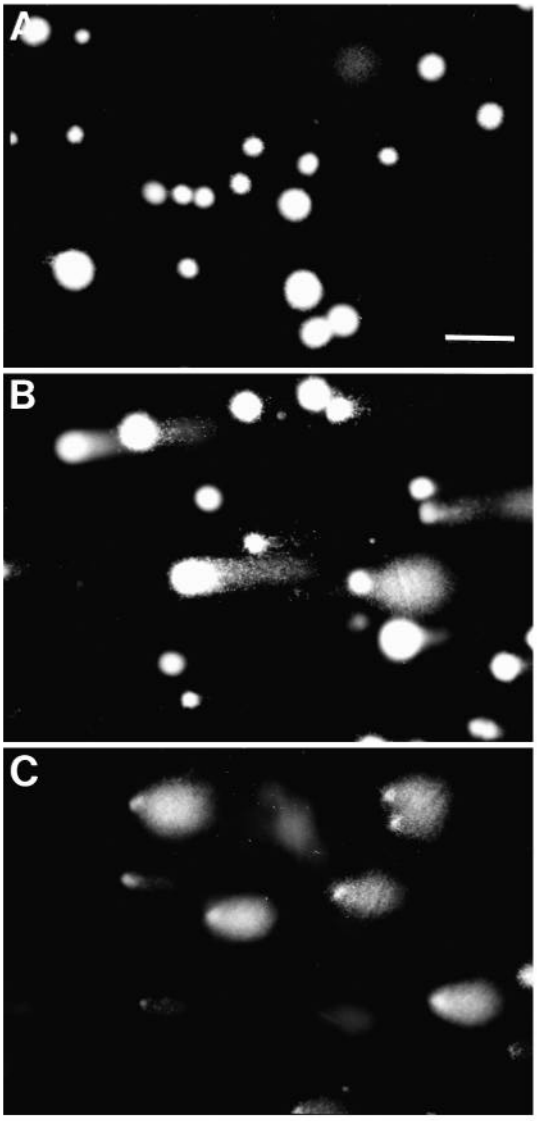

Fig. 7. Fluorescence micrographs of spermatogenic cell nucleoids incubated for 4 hours with 0 μM (A), 0.25 μM (B), or 50 μM (C) VM-26 and subjected to alkaline single-cell gel electrophoresis. Bar, 100 μm.

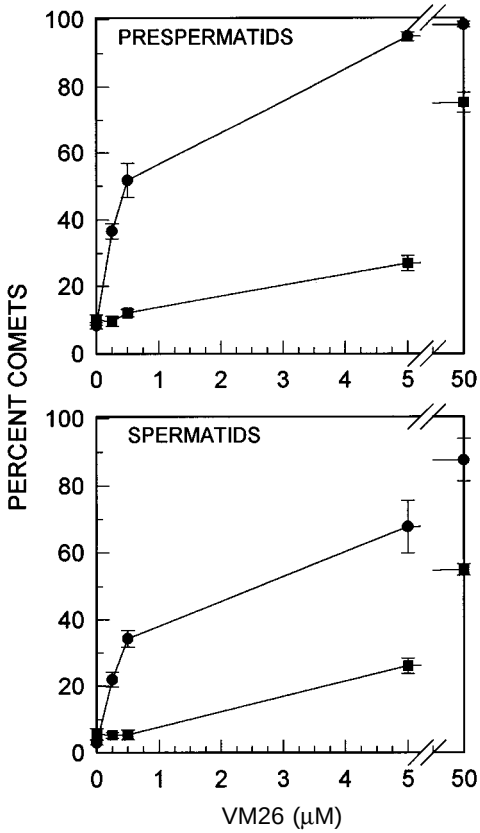

Fig. 8. Percentage of nucleoids showing DNA breaks (comets) after incubation for 4 hours in 0-50 μM VM-26. (●——●) 4 hours VM-26; (■——■)4 hours VM-26 and 20 hours in fresh medium. Data are means ± s.e.m. (*n*=2).

after meiosis to a peak level in elongate spermatids (Roca and Mezquita, 1989). In contrast, McPherson and Longo (1993) found only low levels of topo II polypeptides in round spermatids from rats and relatively low topo II activities in elongate spermatids. McPherson and Longo (1993) also described a germline-specific 137 kDa topo II in rat elongate spermatids. We have shown that the 180 kDa topo II and decatenation activity both decline after meiosis in *Xenopus* and continue to decline as spermatid maturation progresses. This pattern of topo II expression resembles that seen during erythropoiesis in chickens (Heck and Earnshaw, 1986). Immunofluorescence demonstrated the presence of topo II in elongate spermatids, but decatenation assays and immunoblot analyses of SDS extracts suggest that the level of topo II is less than 1% of that in spermatocytes. The combined results indicate that the relative topo II levels in *Xenopus* testes are highest in spermatogonia and spermatocytes, then lower in early-mid spermatids, followed by elongate spermatids and somatic cells. topo II was not detected in sperm by any method, consistent with results reported by Hirano and Mitchison (1993). Germline-specific topo II isoforms were also not detected.

In *Xenopus*, protamine deposition begins in acrosome vesicle stage spermatids (Risley, 1977, 1983; Abe and Hiyoshi, 1991), but protamine accumulation occurs predominantly during spermatid nuclear elongation (Risley, 1977, 1983; Yokota et al.; 1991; Moriya and Katagiri, 1991). Single-cell gel electrophoresis of VM-26-treated *Xenopus* spermatids showed that topo II is catalytically active in intact cells engaged in protamine deposition, but the levels of topo II determined from blotting and decatenation assays appear to be inversely proportional to the nuclear levels of protamine. This suggests that topo II is displaced from nuclei during the transition from nucleohistone to nucleoprotamine. The loss of topo II in *Xenopus* spermatids is also temporally correlated with the rearrangement of chromatin from negatively supercoiled looped domains typical of somatic cell chromatin to the relaxed domains seen in *Xenopus* sperm (Risley et al., 1986) and hamster sperm (Ward et al., 1989). Displacement of topo II may be responsible for the alteration of domain structure in late spermatids, since topo II has been implicated in the attachment of looped chromatin domains to the nuclear matrix (Berrios et al., 1985) and chromosome scaffold (Earnshaw et al., 1985;

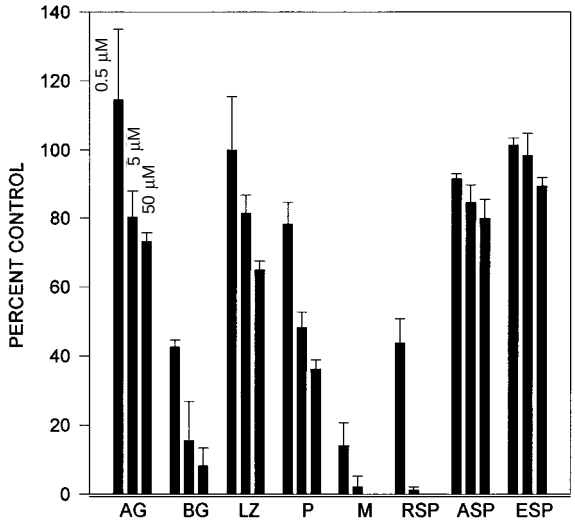

Fig. 9. Differential cell counts of spermatogenic cells after 27 hour culture with 0-50 µM VM-26. Data are percentage of normal cells relative to concurrent solvent controls (means ± s.d; *n*=2). AG, spermatogonia A; BG, spermatogonia B; LZ, leptotene-zygotene; P, pachytene; M, meiotic divisions; RSP, round spermatids; ASP, acrosome vesicle spermatids; ESP, early elongate spermatids.

Gasser et al., 1986). Since protamine deposition occurs in cultured *Xenopus* spermatids (Risley, 1983; Abe and Hiyoshi, 1991), this in vitro system may be useful for studying the possible relationship of topo II activity and displacement to protamine deposition and domain restructuring.

Despite evidence that topo II is a major constituent in the nuclear matrix, most (74-85%) topo II was extracted with low salt (0.5 M NaCl) from *Xenopus* spermatocyte and spermatid nuclei. Low salt extractions have also been effective in removing topo II from mammalian cell nuclei (Woessner et al., 1991) and from sperm chromosomes assembled in *Xenopus* egg extracts (Hirano and Mitchison, 1993). In contrast, Klein et al. (1992) have shown that topo II in yeast meiotic cells is resistant to extraction even at high salt concentrations. When we used 2 M NaCl to extract *Xenopus* spermatogenic cell nuclei, the yields of topo II were lower than those obtained with 0.5 M NaCl (M. Morse-Gaudio, unpublished). This suggests that high salt extraction may induce aggregation and precipitation of topo II in residual nuclei.

The occurrence of topo II in *Xenopus* spermatocytes is consistent with the report by Moens and Earnshaw (1989) showing topo II in leptotene through diplotene stages of chicken spermatocytes. Single-cell gel electrophoresis of *Xenopus* spermatocytes treated with VM-26 suggested that some portion of the topo II present in each meiotic prophase cell is engaged in catalytic cycles of DNA breakage and reunion. During 27 hours of culture in 0.5-50 µM VM-26, both meiotic divisions of *Xenopus* spermatocytes were inhibited. VM-26 also inhibits meiosis in clam oocytes (Wright and Schatten, 1990). Chromosomes condensed and aggregated to one side of the nuclear envelope in many pachytene spermatocytes cultured for 27 hours in 5 or 50 µM VM-26. In long-term cultures (4-6 days) containing 5 or 50 µM VM-26, >90% of pachytene spermatocytes assumed a pycnotic morphology and failed to develop (M. Risley, unpublished data). Since VM-26 induces apoptosis in several mammalian cell lines (Bertrand et al., 1991; Roy et al., 1992), it will be important to determine if the pycnotic morphology observed in VM-26-treated pachytene cells is a result of apoptosis.

It is difficult to separate the effects of VM-26-induced DNA breaks from the effects of topo II inhibition by VM-26. Nevertheless, the relative sensitivities of meiotic divisions and pachytene spermatocytes to VM-26-induced abnormalities are consistent with the stage-dependent effects of *top2* conditional mutants during yeast meiosis. Rose et al. (1990) have shown that topo II acts during both meiotic divisions in yeast meiocytes with normal levels of recombination. Rose and Holm (1993) have shown that normal topo II activities are required for progression from pachytene to later meiotic stages, suggesting the occurrence of a meiotic checkpoint at pachytene. Cultured *Xenopus* spermatocytes may provide experimental opportunities for manipulating topo II activities in meiotic cells of a higher eukaryote in order to explore the roles of topo II during meiosis. Comparisons of the effects of VM-26 with drugs that inhibit topo II without inducing DNA breaks may help resolve meiotic stages requiring topo II activity from stages sensitive to DNA damage (cf. Clarke et al., 1993).

The single-cell gel experiments showed that most spermatogenic stages in *Xenopus* accumulate DNA breaks during exposure to VM-26. VM-26 traps topo II in cleavable complexes that are rapidly reversed upon removal of VM-26, but the complexes may induce endonucleolytic cleavages that persist until DNA is repaired (Liu, 1989). The breaks observed in this study are likely to be the result of endonuclease activity, since they persist in spermatocytes and spermatids for relatively long periods (hours) after removal of the VM-26, although the DNA repair systems in spermatogenic cells are capable of reversing the breaks. The inducibility of topo II-mediated DNA damage at diverse spermatogenic stages suggests that this enzyme may be an important molecular target in both meiotic and postmeiotic stages for the induction of germline DNA damage and chromosome aberrations that are heritable. topo II activity is required during yeast mitosis to prevent nondisjunction and chromosome breaks (Holm et al., 1989). Furthermore, topo II inhibitors induce sister chromatid exchanges (Pommier et al., 1985) and quadriradial chromosomes, presumably by illegitimate recombination (Charron and Hancock, 1991). Cultured *Xenopus* spermatogenic cells may be useful for the study of topo II-mediated genomic aberrations during spermatogenesis.

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