Journal of Cell Science 113, 1635-1647 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JCS1014

During both interphase and mitosis, DNA topoisomerase II interacts with DNA as well as RNA through the protein's C-terminal domain

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Accepted 26 February; published on WWW 6 April 2000

SUMMARY

DNA topoisomerase II (topo II) is thought to be a nuclear enzyme; during interphase most was insoluble and could be recovered in the pellet after centrifugation of cell homogenates at 10,000 *g* (P-10). Upon entry into mitosis, the majority of topo II did not associate with condensed chromosomes but was apparently solubilized and redistributed throughout the cell. Although two non-chromosomal subfractions of mitotic topo II were defined by centrifugation at 130,000 *g*, the vast majority (≥90%) was recovered in the pellet (P-130). In vivo nucleic acid interactions with topo II were monitored by a recently developed approach of UV-photo-crosslinking, immunoprecipitation and ^{32}P-labeling. P-10 (interphase) topo II was largely associated with DNA. P-130 (mitotic non-chromosomal) topo II was primarily associated with RNA. These nucleic acid interactions with both interphase and mitotic topo II occurred through the catalytically inert and as yet, poorly understood C-terminal domain of the protein. P-10 topo II was highly active enzymatically. Activity, measured by the ability of topo II to decatenate kDNA minicircles, was reduced by treatment with phosphatase. In contrast, P-130 topo II was relatively inactive but activity could be *increased* by phosphatase treatment. In vivo, P-130 topo II was more heavily phosphorylated than P-10 topo II; in both, only the C-terminal domain of topo II was detectably modified. Our observations suggest that cell cycle-dependent changes in the distribution, nucleic acid interactions and enzymatic activity of topo II are regulated, at least in part, by phosphorylation/dephosphorylation.

Key words: Karyoskeleton, Nucleus, Mitosis, Topoisomerase, Lamin, *Drosophila*

INTRODUCTION

In vitro, DNA topoisomerase II (topo II) is an enzyme that mediates breakage and religation of double-stranded DNA, thereby catalyzing movement of one duplex past another (see e.g. Roca and Wang, 1994; Berger et al., 1996; Roca et al., 1996; for reviews, see Wang, 1985; Watt and Hickson, 1994; Berger and Wang, 1996). Complete reaction requires ATP hydrolysis. Eukaryotic topo II contains three domains (for reviews, see Watt and Hickson, 1994; Berger and Wang, 1996). Two are highly conserved (respectively homologous to the bacterial DNA gyrase subunits, GyrA and GyrB), have well-understood functions in vitro and are necessary in vivo. The third domain, located at the C terminus, while always present and highly charged, is poorly conserved, dispensable in vitro and of uncertain function in vivo.

In vivo, topo II is essential at mitosis (DiNardo et al., 1984; Holm et al., 1985; Uemura et al., 1987; Shamu and Murray, 1992; Buchenau et al., 1993; Rattner et al., 1996; Iwai et al., 1997) where participation in chromatin condensation (Wood and Earnshaw, 1990; Adachi et al., 1991; Garinther and Schultz, 1997) and in formation of the mitotic chromosome scaffold (Earnshaw and Heck, 1985; Earnshaw et al., 1985; Gasser et al., 1986) were implicated. It is similarly important in meiosis (Moens and Earnshaw, 1989; Rose et al., 1990; Klein et al., 1992; Rose and Holm, 1993). The precise role of topo II in maintaining mitotic chromosome structure remains uncertain (Hirano and Mitchison, 1993).

A structural role for topo II in organizing interphase nuclei was also suggested (Berrios et al., 1985; Fisher, 1989; Whalen et al., 1991; Meller et al., 1995). Interactions with both DNA and RNA have been reported (Meller and Fisher, 1995). During meiosis as well as mitosis in *Drosophila*, a substantial portion of topo II was non-nuclear and was located throughout the cell (Berrios et al., 1985; Whalen et al., 1991; Swedlow et al., 1993; see also Chaly et al., 1996; Meyer et al., 1997). Non-nuclear topo II was primarily in an RNase-sensitive, DNase-resistant 67 S particle (Meller et al., 1994).

We proposed a model whereby a proteinaceous extrachromosomal nucleoskeleton (karyoskeleton) organizes nuclei so that access to macromolecular information stores, i.e. DNA and RNA, is optimal (Rzepecki et al., 1998). Because of

abundance, activity with duplex DNA and potential for interaction with RNA, topo II seems ideally suited to be a key karyoskeletal component. To evaluate the hypothesis that topo II is a karyoskeletal protein as well as an enzyme, it was compared with lamin. Lamin, probably the best-characterized karyoskeletal protein, is a Type V intermediate filament protein that forms the nuclear lamina. The lamina surrounds and contributes to the organization of the nuclear contents. After meiosis during oogenesis (Smith and Fisher, 1989), in vinblastine-treated mitotic *Drosophila* tissue culture cells (Smith and Fisher, 1989) and after nuclear disassembly in vitro (Maus et al., 1995), we showed that a soluble isoform of lamin, termed lamin Dm_{mit}, accumulated. Accumulation correlated directly with lamin-phosphate rearrangement; i.e. both kinase and phosphatase activities are presumably involved. Recent mapping by mass spectroscopy suggests that phosphorylation of lamin S^{45} is at least partly responsible for meiotic disassembly of the lamina in vivo (Schneider et al., 1999).

As with lamin, *Drosophila* K_c tissue culture cells, blocked in mitosis by vinblastine, accumulate 'soluble' topo II. Using a recently developed strategy of photo-crosslinking/immunoprecipitation/^{32}P-labeling (Rzepecki et al., 1998), and highly specific anti-topo II antibodies, we identified novel interactions between topo II and nucleic acids. During both interphase and mitosis, these occur through the poorly understood C-terminal domain of the protein, and as with interphase lamin, involve both DNA and RNA. The majority of interphase topo II is associated with DNA. In contrast the vast majority of 'soluble' mitotic topo II associates with RNA. Relative to interphase topo II, the mitotic protein is less active in vitro and is somewhat hyperphosphorylated. Phosphorylation during both interphase and mitosis is confined to serine residues as well as to the C-terminal portion of the molecule. Evidence suggests that in vivo, phosphorylation and/or dephosphorylation is involved in cell cycle-dependent changes in topo II behavior.

MATERIALS AND METHODS

Antibodies

Domain-specific rabbit anti-*Drosophila* topo II antibodies directed against polypeptides encompassing amino acids 1-31, 1-202 and 534-950 were prepared and affinity purified (Meller et al., 1995). Rabbit anti-topo II antibodies directed against most of the cloned polypeptide (amino acids 32-1,030) were also affinity purified (Whalen et al., 1991) as were rabbit antibodies directed against full-length *Drosophila* lamin Dm_0 and derivatives (Fisher and Smith, 1988; see also Smith and Fisher, 1989). For both lamin and topo II, we used cloned, bacterially expressed proteins as affinity ligands immobilized on glutaraldehyde-activated glass beads (Boehringer, Indianapolis, IN). Affinity purified, alkaline phosphatase-conjugated goat anti-rabbit IgG was from Kirkegaard and Perry (Gaithersburg, MD).

Methods

Drosophila melanogaster (Oregon R, P2 strain) were maintained and embryos collected according to the method of Allis et al. (1977). SDS-PAGE was according to the method of Laemmli (1970) as modified (Fisher et al., 1982) on polyacrylamide minigels. Proteins were transferred electrophoretically from gels to nitrocellulose (Harlow and Lane, 1988) and resulting immunoblots were processed and probed with antibodies. Reactivity was visualized colorimetrically (McGadey, 1970) with calf alkaline phosphatase-conjugated goat anti-IgG antibodies (Blake et al., 1984; Smith and Fisher, 1984) and a one-solution alkaline phosphatase substrate (Kirkegaard and Perry).

Nucleases and nuclease digestion

DNase I was from Sigma (St Louis, MO) and was RNase free grade (catalog number D7291). RNase A was from Worthington (Freehold, NJ), was RASE grade (catalog number LS005679) and was determined empirically to be free of detectable DNase activity (M. McConnell and P. A. Fisher, unpublished; also see Meller et al., 1994; Meller and Fisher, 1995; Rzepecki et al., 1998). Micrococcal nuclease of the highest grade commercially available (catalog number 107 921) was from Boehringer. Mung bean nuclease was from New England Biolabs (Beverly, MA). RNase H was from BRL (Bethesda, MD). Generally, nuclease digestions were carried out exactly as described previously (Rzepecki et al., 1998). To study sensitivity of ^{32}P-labeled nucleic acid photo-crosslinked to topo II, nuclease digestions were performed for 30 minutes at 37°C with final concentrations of 66 μg/ml of DNase I, 60 μg/ml of RNase A and 45 μg/ml of micrococcal nuclease. All nuclease digestions were performed in a solution containing 20 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% (v/v) Triton X-100 and 0.02% (w/v) SDS (Buffer IPA) supplemented with 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$.

Tissue culture

K_c cells were the generous gift of Dr John Watson (UCSF) and were maintained according to Berrios et al. (1991). To enrich for mitotic cells, vinblastine was added to 2 μg/ml, culturing continued overnight, 2 μg/ml fresh vinblastine added (final vinblastine concentration of 4 μg/ml) and culturing continued for 6-12 hours for a total of 18-24 hours in the presence of vinblastine (Hanson and Hearst, 1973; see also Smith and Fisher, 1989). For routine 5-bromo-2-deoxyuridine (BrdU) incorporation, quantified with an anti-BrdU monoclonal antibody as previously (Rzepecki et al., 1998), 200 ml of exponentially growing cells ($1.2\text{-}1.8\times10^6$ cells/ml) were made 20 μM BrdU and maintained in culture for an additional 26-27 hours (Rzepecki et al., 1998). 5-Bromo-2-deoxycytidine (BrdC) incorporation was achieved similarly.

Cell fractionation

Cell fractionation was performed according to the method of Meller et al. (1994) with minor modifications. All steps were performed at 4°C unless indicated otherwise. Typically 200-ml of exponentially growing K_c cells ($1.4\text{-}1.8\times10^6$ cells/ml) were harvested by centrifugation and washed once with 10 mM KPO_4, pH 7.5, 140 mM NaCl (PBS). Cells were broken by Dounce homogenization with a tight pestle in 5-ml of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 250 mM sucrose, 5 mM $MgCl_2$, 0.1 mM EDTA and 1% Triton X-100 supplemented just before use with proteinase inhibitors 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), 1 mM L-1-tosyl-L-lysyl-chloromethyl ketone (TLCK), 1 μg/ml leupeptin, 1 μg/ml antipain and 1 μg/ml pepstatin A. PMSF, TPCK, TLCK, leupeptin, antipain and pepstatin A were from Sigma. Homogenates were filtered through four layers of Nytex (120-μm nylon mesh, Tetko Inc., Elmsford, NY) and centrifuged for 10 minutes at 10,000 ***g***. Resultant pellet and supernatant fractions were designated P-10 and S-10, respectively. The S-10 was further fractionated by centrifugation at 130,000 ***g*** for 60 minutes. The resultant pellet fraction was named the P-130. The same procedure was used for fractionation of *Drosophila* embryos except that 1% Triton X-100 was omitted from the homogenization buffer. For immunoprecipitation and labeling, SDS and DTT were added to all fractions to final concentrations of 5% and 20 mM, respectively. They were then boiled for 10 minutes and stored at –20°C.

In vivo photo-crosslinking

In vivo photo-crosslinking was according to Rzepecki et al. (1998).

Briefly, a fully broken-in hand-held lamp (UVGL-58, UVP Inc., San Gabriel, CA, >10-hours-old) emitting 366-nm light was used. Since lamps vary greatly with age in their absolute light output, exact conditions were established empirically and re-evaluated periodically. All quantities refer to 200-ml of cell culture starting material. Before photo-crosslinking, cells were harvested by centrifugation at 4°C for 5 minutes at 900 ***g***, resuspended in chilled PBS and washed by recentrifugation and resuspension three times. Finally, the cell pellet was resuspended in 8-ml of PBS and transferred to a plastic Petri dish (10-cm in diameter). The Petri dish was placed on ice and while uncovered, illuminated for 30 minutes with the hand-held lamp suspended such that the bottom surface of the lamp was 3-cm above the top of the cell suspension.

Immunoprecipitation and ^{32}P-end-labeling of coimmunoprecipitated nucleic acid

After in vivo photocrosslinking, cells were lysed, in some instances fractionated and ultimately denatured by addition of SDS and DTT; after addition of SDS and DTT, samples were boiled for 10 minutes. Typically, about 6×10^8 cells were harvested, resuspended in 1.5-ml PBS, lysed and either denatured immediately or fractionated and then denatured by addition of a solution containing 10% SDS and 40 mM DTT. Whole-cell lysates and/or subcellular fractions prepared in this way were stored at –20°C until use. Samples, each derived from about 1.5×10^7 cells, were thawed by boiling for 10 minutes, cooled to room temperature and trichloroacetic acid (TCA) was added to a final concentration of 10%. Samples were then incubated for 10 minutes at room temperature and precipitated proteins were collected by centrifugation, also at room temperature, for 10 minutes at 10,000 ***g***. The supernatant was discarded, 5 μl of 1.5 M Tris-HCl, pH 8.8, was added to the protein pellet and the pellet was dissolved in 200-μl of solution containing 20 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Triton X-100 and 0.02% SDS (Buffer IPA). Immediately before use, Buffer IPA was supplemented such that the final concentration was 2.5 mM $CaCl_2$, 0.5 mM PMSF, 1 mM TPCK, 1 mM TLCK, 1 μg/ml leupeptin and 1 μg/ml pepstatin A. Unless indicated otherwise, 5 μl of micrococcal nuclease (680 μg/ml; Boehringer) was then added and samples were incubated for 60 minutes at 37°C.

Immunoprecipitation was performed according to Rzepecki et al. (1998; see also Smith et al., 1987). Specifically, Protein A-Sepharose (Pharmacia, Piscataway, NJ) was hydrated in Buffer IPA supplemented with 5 mM EDTA (final concentration) (Buffer IPB), washed by suspension and brief centrifugation and finally resuspended in Buffer IPB. To 50-μl packed Protein A-Sepharose beads was added 2-4 μg of affinity purified IgG. The total volume was brought to 350-μl with Buffer IPB and the Protein A-Sepharose-IgG mixture was incubated with gentle agitation for 90 minutes at 37°C. Unbound IgG was removed by washing first with Buffer IPB (once) and then with Buffer IPA (twice). Micrococcal nuclease-treated K_c-cell lysates and/or subcellular fractions were then added to the washed Protein A-Sepharose-IgG beads and the mixture was incubated for 90 minutes at 37°C. The beads with IgG and antigen bound (immunoprecipitate) were recovered by centrifugation, and washed three times with 350-μl each of Buffer IPB followed by three identical washes with Buffer IPA.

After the final wash, the immunoprecipitate was resuspended in 80-μl of 1.5× concentrated T4 polynucleotide kinase buffer (New England Biolabs). To this resuspended immunoprecipitate was added 1-2-μl of [γ-^{32}P]ATP (4500 Ci/mmol; 10 μCi/μl; ICN Pharmaceuticals, Costa Mesa, CA) and 5 units of T4 polynucleotide kinase (New England Biolabs). ^{32}P-labeling with T4 polynucleotide kinase was for 30 minutes at 37°C. After this incubation, samples were washed three times, each with 350-μl of Buffer IPB, finally resuspended in 45-μl of 2.5× concentrated standard SDS-PAGE loading buffer, subjected to electrophoresis on an SDS-7% polyacrylamide gel and electrophoretically transferred to nitrocellulose for autoradiography and/or phosphorImager analysis, and immunoblot analysis. Because nucleic acid covalently crosslinked to topo II was ^{32}P-end-labeled, and the secondary antibody used for immunoblot analysis was conjugated to alkaline phosphatase, autoradiography (and/or phosphorImager analysis) was always performed before immunoblot analysis.

Quantification by scanning densitometry

Immunoblots and autoradiograms were quantified using an LKB Ultrascan XL laser densitometer (LKB Instruments Inc., Gaithersburg, MD). Quantitative data are expressed in arbitrary units. Amounts of topo II protein were estimated by examination of the intensity of the topo II protein bands seen after either Coomassie blue staining of polyacrylamide gels or Ponceau S staining of nitrocellulose blots.

Partial digestion of immunopurified topo II with trypsin

Topo II immunopurified from P-10 and P-130 (~0.4-μg each) was subjected to precipitation with TCA, the precipitated protein washed with ethanol and dissolved in 100-μl of 50 mM ammonium bicarbonate pH 8.3. Then, 10-ng of TPCK-treated trypsin (Sigma) was added to each sample and they were incubated on ice. Aliquots were withdrawn at the times indicated, boiled immediately for 5 minutes with standard SDS-PAGE loading buffer, and subjected to SDS-7% PAGE. Proteins were transferred to nitrocellulose blots; blots were subjected to autoradiography and/or phosphorImager analysis followed by immunoblot analysis with affinity purified domain-specific anti-*Drosophila* topo II antibodies.

Treatment of *Drosophila* topo II with potato acid phosphatase

Topo II immunopurified from P-10 and P-130 was treated with potato acid phosphatase (Sigma) according to the method of Shiozaki and Yanagida (1992). The enzyme was assayed before and after phosphatase treatment using the standard kDNA decatenation assay (see below for details). Treatments were also performed with calf alkaline phosphatase (Sigma) used as directed by the supplier.

Assay of topo II activity

For topo II activity assays, the P-10 and P-130 fractions (both non-denatured) were extracted for 10 minutes on ice with buffer containing 50 mM Tris-HCl, pH 8, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 10% glycerol, supplemented immediately before extraction to 0.5 mM PMSF, 1 mM TPCK, 1 mM TLCK, 1 μg/ml leupeptin, 1 μg/ml antipain and 1 μg/ml pepstatin A. The samples were then centrifuged at 4°C for 30 minutes at 100,000 ***g***. Resulting supernatants were collected and dialyzed against 25 mM Tris-HCl, pH 8, 50 mM NaCl, 7.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.5 mM PMSF, 1 mM TPCK, 1 mM TLCK, 1 μg/ml leupeptin, 1 μg/ml antipain and 1 μg/ml pepstatin A. Samples were frozen in liquid nitrogen and stored at –70°C until use. Aliquots were thawed for use only once and characterized both by activity assay and by immunoblot analysis with anti-topo II antibodies.

Topo II activity was assayed by measuring release by decatenation of minicircles from kinetoplast DNA (kDNA) prepared exactly as previously described (Marini et al., 1980; Luke and Bogenhagen, 1989). Assays were performed at 37°C in 40-μl containing 25 mM Tris-HCl, pH 8, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM ATP, 0.6 μg kDNA, 0.5 mM PMSF, 1 mM TPCK, 1 mM TLCK, 1 μg/ml leupeptin, 1 μg/ml antipain and 1 μg/ml pepstatin A. Reactions were stopped by addition of SDS and EDTA (to 0.5% and 10 mM, respectively), followed by addition of 10-μg of proteinase K and incubation for 45 minutes at 56°C. Samples were then subjected to electrophoresis on 1% agarose gels. Aliquots of each sample taken after incubation with kDNA but before addition of proteinase K, were also subjected to immunoblot analysis with affinity purified anti-*Drosophila* topo II antibodies. Exhaustive treatment of both P-10 and

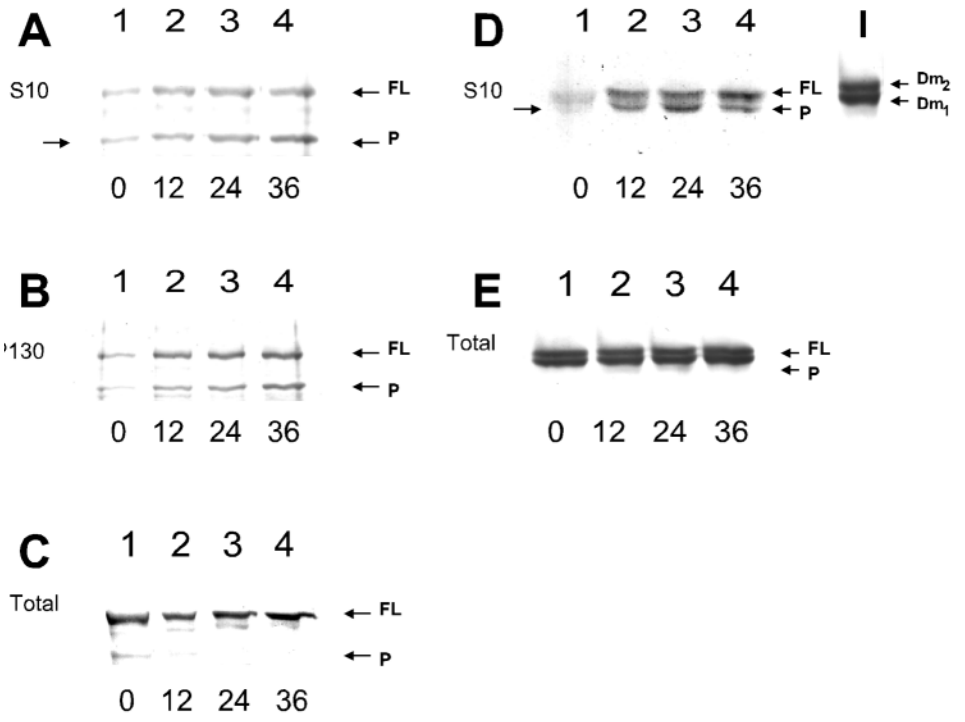

Fig. 1. Growth of K_c cells in vinblastine leads to accumulation of topo II in the S-10 and P-130 fractions. Immunoblot analyses of topo II and lamin levels were performed after fractionation of *Drosophila* K_c cells grown for various times in vinblastine. K_c cells were grown in suspension culture as described (Materials and Methods). Vinblastine was added, cell growth was continued and at the times after vinblastine addition as indicated in hours below each lane, 50-ml aliquots were taken; cells were harvested and fractionated into P-10 and S-10 as described in Materials and Methods. The S-10 was further fractionated by centrifugation (130,000 ***g*** for 60 minutes) into P-130. Material derived from equal amounts (2.5×10^5) of cells was loaded in each lane. To facilitate presentation, lanes in each panel are also numbered consecutively above. (A,B,C) Immunoblots of S-10, P-130 and total K_c cell extracts, respectively and as indicated, probed with affinity purified anti-topo II IgG. (D and E) Immunoblots of S-10 and total K_c cell extracts, respectively, and as indicated, probed with affinity purified anti-*Drosophila* lamin Dm_0-derivatives IgG. (D, lane I) *Drosophila* K_c cell nuclear extract subjected to electrophoresis on the same gel with the other samples shown in D to demonstrate the SDS-PAGE migration of interphase lamins Dm_1 and Dm_2. Arrows to the right of A, B and C designate the migration positions of full-length topo II (FL) and a major proteolytic breakdown product of topo II (P). The unlabeled arrow in A to the left of lane 1 designates this major proteolytic breakdown product. Arrows to the right of D and E designate the migration positions of full-length lamin Dm_{mit} (FL) and a major proteolytic breakdown product of lamin Dm_{mit} (P). The unlabeled arrow in D to the left of lane 1 designates this major proteolytic breakdown product.

P-130 fractions with RNase A was without any effect on topo II activity (not shown).

In vivo phosphate labeling and immunopurification of topo II

Exponentially growing K_c cells ($1.2\text{-}1.8\times10^6$ cells/ml, 200-ml) were collected by centrifugation (5 minutes, 900 ***g***, 23°C) and suspended in 100-ml of DM22 'low phosphate' medium (Sigma). Then 5-10 mCi of $[^{32}P]H_3PO_4$ (9,104 Ci/mmol, ICN Pharmaceuticals, Costa Mesa, CA) was added followed after 2 hours by addition of vinblastine (to 2 μg/ml). Cells were kept for 20-24-hours at room temperature with additional vinblastine added (4 μg/ml final concentration) after 12-hours incubation. Cells were collected and fractionated as described above into P-10 and P-130. Both pellets were dissolved in a 1:1 mixture of PBS:10% SDS, 40 mM DTT solution, boiled for 10 minutes and stored at –20°C. Samples were thawed by boiling for 10 minutes, allowed to cool to room temperature and proteins precipitated with TCA for 10 minutes at room temperature. Precipitated proteins were collected by centrifugation (10,000 ***g***, 10 minutes) at room temperature and pellets dissolved in 50 mM Tris-HCl, pH 8, 50 mM NaCl, 0.1% Triton X-100, 0.02% SDS and 5 mM $MgCl_2$, supplemented just before use with 0.5 mM PMSF, 1 mM TPCK, 1 mM TLCK, 1 μg/ml leupeptin, 1 μg/ml antipain and 1 μg/ml pepstatin A. DNase I (40 μg/ml) and RNase A (90 μg/ml) were then added and samples were incubated for 60 minutes at 37°C. EDTA and NaCl were then added to final concentrations of 5 mM and 500 mM, respectively, and samples were mixed with Protein A-Sepharose beads (1-3-ml) to which affinity purified anti-*Drosophila* topo II IgG was bound and glutaraldehyde coupled (Lin and Fisher, 1990). Incubation was continued for 90 minutes at 4°C with gentle rotation after which topo II-anti-topo II beads were poured into a column. The column was washed with 5-8 volumes of 50 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 and 0.02% SDS supplemented just before use with 0.5 mM PMSF, 1 mM TPCK, 1 mM TLCK, 1 μg/ml leupeptin, 1 μg/ml antipain and 1 μg/ml pepstatin A. Topo II protein was eluted from the column with 1.5-bed-volumes of 2.5 M NaSCN, 0.1% Triton X-100, 0.02% SDS, concentrated by precipitation with TCA and dissolved in standard SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE followed by either Coomassie blue staining and autoradiography (or phosphorImager analysis; Molecular Dynamics 445 SI) or immunoblot and autoradiography (or phosphorImager analysis). Because phosphorylation was being investigated and the secondary antibody used for immunoblot analysis was conjugated to alkaline phosphatase, autoradiography (and/or phosphorImager analysis) was always performed before immunoblot analysis.

Phosphoamino acid determination

K_c cells were labeled in vivo with ^{32}P and fractionated into P-10 and P-130 followed by immunopurification of topo II from each. Topo II was then subjected to SDS-7% PAGE. Gels were stained with Coomassie blue, dried and subjected to autoradiography and/or phosphorImager analysis. Topo II bands were excised from the gel, gel slices were rehydrated for 5 minutes at room temperature in 50 mM ammonium bicarbonate (pH 7.6) and homogenized. SDS and DTT were added (to 0.1% and 20 mM final concentrations, respectively) and samples were boiled for 5 minutes followed by rotation at room temperature overnight (~12-hours). Samples were then centrifuged (3 minutes, 5,000 ***g***), supernatants were collected, residual gel pellets were boiled with a fresh solution of SDS and DTT and extracted further for 3-hours at room temperature followed by another centrifugation. Both supernatants were pooled, clarified by brief centrifugation and proteins collected by precipitation with TCA (final concentration of 20%) for 90 minutes on ice followed by centrifugation for 10 minutes at 12,000 ***g*** (4°C). Pellets were washed with ice-cold ethanol (100%) and allowed to air dry. Dried samples were dissolved in 6 M HCl, incubated at 110°C for 90 minutes and dried under vacuum. Samples

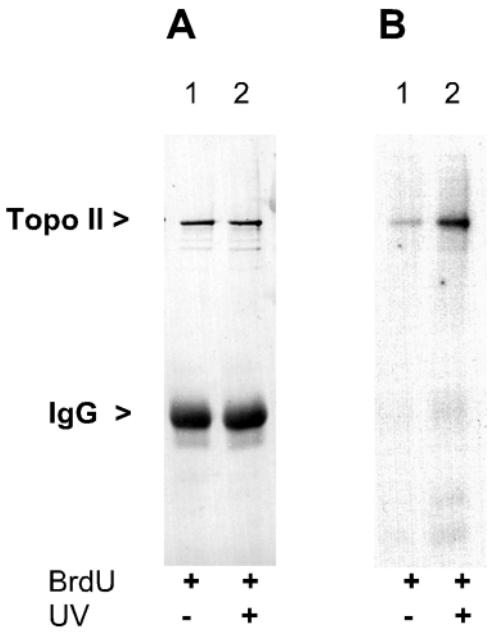

Fig. 2. Stimulation of ^{32}P-labeling of immunoprecipitated topo II by in vivo exposure to 366-nm light. All procedures were as described in Materials and Methods. *Drosophila* K_c cells were grown in suspension culture, maintained in 20 μM BrdU and where indicated, subjected to standard UV-crosslinking with 366-nm light. Samples were then subjected to immunoprecipitation and ^{32}P-labeling. Both immunoblot analysis (A) and autoradiography (B) were performed. SDS-PAGE mobilities of proteins covalently crosslinked to nucleic acids are similar to those of uncrosslinked proteins because samples are extensively treated with micrococcal nuclease before electrophoresis. Results were quantified by scanning densitometry.

were then dissolved in 400-μl of water and dried under vacuum. Samples were then dissolved in 300-μl of acetic acid:pyridine:water (100:10:1890, pH 3.5) solution, centrifuged for 5 minutes at 3,000 ***g*** and supernatant transferred into a fresh tube and dried. Samples were finally dissolved in 10-μl of acetic acid:pyridine:water (100:10:1890, pH 3.5) solution and unlabeled phosphoamino acid standards were added. Samples were loaded onto a cellulose thin layer plate (20× 20 cm; Sigma) and subjected to electrophoresis for 45 minutes at 1300 V in an HTLT-7000 thin layer electrophoresis apparatus (CBS Scientific Co., Del Mar, CA). After electrophoresis, plates were air dried, phosphoamino acid standards visualized with ninhydrin and plates subjected to autoradiography and/or phosphorImager analysis.

RESULTS

A non-nuclear and predominantly particulate fraction of topo II accumulates in *Drosophila* K_c cells blocked in mitosis by vinblastine

It was shown previously that early (pre-cellularization) *Drosophila* embryos contained non-nuclear (soluble) topo II as the major form defined by centrifugation at 10,000 ***g*** (Whalen et al., 1991; Meller et al., 1994). This non-nuclear (S-10) form was also found in K_c cells where it represented only a small fraction of the total topo II (Meller et al., 1994). In older embryos and in exponentially growing K_c cells, the predominant form of topo II was found in the pellet fraction after centrifugation at 10,000 ***g*** (P-10). This form was presumably derived from interphase nuclei. For both embryos and tissue culture cells, we speculated that S-10 topo II resulted from cell cycle-regulated nuclear disassembly during M-phase (Whalen et al., 1991; Meller et al., 1994). This hypothesis was tested using the drug, vinblastine to block *Drosophila* K_c tissue culture cells in mitosis (Hanson and Hearst, 1973; see also Smith and Fisher, 1989).

Results of immunoblot analysis with anti-topo II antibodies are shown in the left-hand panels of Fig. 1 (Fig. 1A,B,C). Parallel blots probed with anti-lamin Dm_0-derivatives antibodies are shown in the right-hand panels (Fig. 1D and E). Time points after the addition of vinblastine are indicated (in hours) below each panel. The S-10 contained detectable amounts of topo II (Fig. 1A, lane 1) as well as lamin Dm_{mit} (Smith and Fisher, 1989; Fig. 1D, lane 1) before incubation with vinblastine. Proteolytic breakdown products of both were also seen (Fig. 1A and D, see unlabeled arrows to the left of lanes 1). The SDS-PAGE mobilities of major species are indicated to the right of each panel. Growth of cells in vinblastine correlated with a substantial accumulation of topo II (Fig. 1A, lanes 2-4) and lamin Dm_{mit} (Fig. 1D, lanes 2-4) in the S-10 fraction. Densitometric quantification demonstrated that, after 24-hours cell growth in vinblastine (compared with no vinblastine added), there was a 450±110% (*n*=3) increase in the amount of S-10 topo II and an 860±160% (*n*=3) increase in the amount of lamin Dm_{mit}. During this same time period, the total levels of topo II (Fig. 1C) and lamins (Fig. 1E) did not change appreciably. These results also confirm that similar amounts of material were loaded in each lane. The mitotic index of the K_c cell population after 24-hours growth in vinblastine was less than 50% (see Smith and Fisher, 1989). Hence, both interphase and mitotic cells could be conveniently harvested from the same culture.

We previously reported that most or all S-10 topo II derived from early *Drosophila* embryos was present in an RNA-containing, salt-sensitive 67 S particle that could be recovered from a pellet fraction after centrifugation of the S-10 at 130,000 ***g*** (Meller et al., 1994). This fraction was designated the P-130. This was in contrast with S-10-lamin which remained largely soluble after similar centrifugation. From current experiments, it can be seen (Fig. 1B) that the amount of topo II recovered in the P-130 paralleled accumulation of topo II in the S-10 during vinblastine treatment. Densitometric quantification revealed that after 24-hours in vinblastine, there was a 510±80% (*n*=3) increase in the amount of P-130 topo II and that ≥90% of the S-10 topo II was recovered in the P-130 fraction.

Based on results similar to those shown in Fig. 1, we estimate that during mitosis, about 60% of the total cellular topo II is solubilized (found in the S-10) and distributed throughout the cell. This was independent of cell growth in vinblastine (i.e. similar estimates were made without and after 24-hours growth in vinblastine) and is consistent with previous observations made, where no vinblastine was present, in both embryos (Swedlow et al., 1993) and larvae (Berrios et al., 1985).

Interaction of topo II with nucleic acids involves both DNA and RNA

To study the interaction of *Drosophila* topo II with nucleic acids, we applied the in vivo photo-crosslinking/

immunoprecipitation/^{32}P-labeling strategy previously reported for lamin (Rzepecki et al., 1998). As before, 366 nm light-dependent labeling was substantially enhanced both by incorporation of BrdU and/or BrdC into K_c cell DNA (not shown) and by exposure of cells grown in BrdU to 366 nm light (Fig. 2). To quantify results, immunoblot analyses were performed with affinity purified antibodies directed against amino acids 32-1,030 of *Drosophila* topo II (Fig. 2A). Quantification was based on the intensity of the radiolabel (Fig. 2B) relative to the intensity of the immunoblot signal (Fig. 2A), both emanating from the full-length topo II band, and was determined by scanning densitometry. Densitometric quantification after several experiments including the example shown, revealed that exposure to 366-nm light increased topo II labeling 9.3-fold (± 0.8-fold; *n*=3).

After photocrosslinking/immunoprecipitation/^{32}P-labeling, the nuclease sensitivity of the radiolabel bound to topo II was studied. Treament of P-10 topo II with DNase I resulted in removal of about 70% of the covalently bound ^{32}P (±11%; *n*=2); treament with RNase A resulted in removal of about 35% of the covalently bound ^{32}P (±6%; *n*=2) (Fig. 3A and B). ^{32}P-label was almost completely sensitive to treatment with micrococcal nuclease (Fig. 3E and F; 94% removed ±4%; *n*=2), but was insensitive to either mung bean nuclease (not shown) or RNase H (not shown). Sensitivity of ^{32}P-label to nucleases was qualitatively similar when cells were exposed to UV but not to either BrdU or BrdC (not shown).

In contrast with above results (Fig. 3A and B), treatment of P-130 topo II with DNase I resulted in removal of only about 20% of the covalently bound ^{32}P (*n*=2); treatment with RNase A resulted in removal of about 85% of the covalently bound ^{32}P (*n*=2) (Fig. 3C and D). As observed for P-10 topo II, ^{32}P-label bound to P-130 topo II was almost completely sensitive to treatment with micrococcal nuclease (not shown) but was insensitive to either mung bean nuclease (not shown) or RNase H (not shown). Sensitivity of ^{32}P-label to nucleases was qualitatively similar when cells were exposed to UV but not to BrdU, BrdC or vinblastine (not shown). However, substantially reduced labeling precluded detailed quantitative analyses.

Interaction of topo II with nucleic acid occurs largely through the C-terminal domain of the protein

Limited digestion with trypsin was used to map grossly the site on the topo II molecule to which nucleic acid could be photo-crosslinked in vivo. Accordingly, P-10 topo II was immunoprecipitated with antibodies highly specific for the middle of the protein (amino acids 534-950), ^{32}P-labeled and partially digested with trypsin as indicated. After digestion,

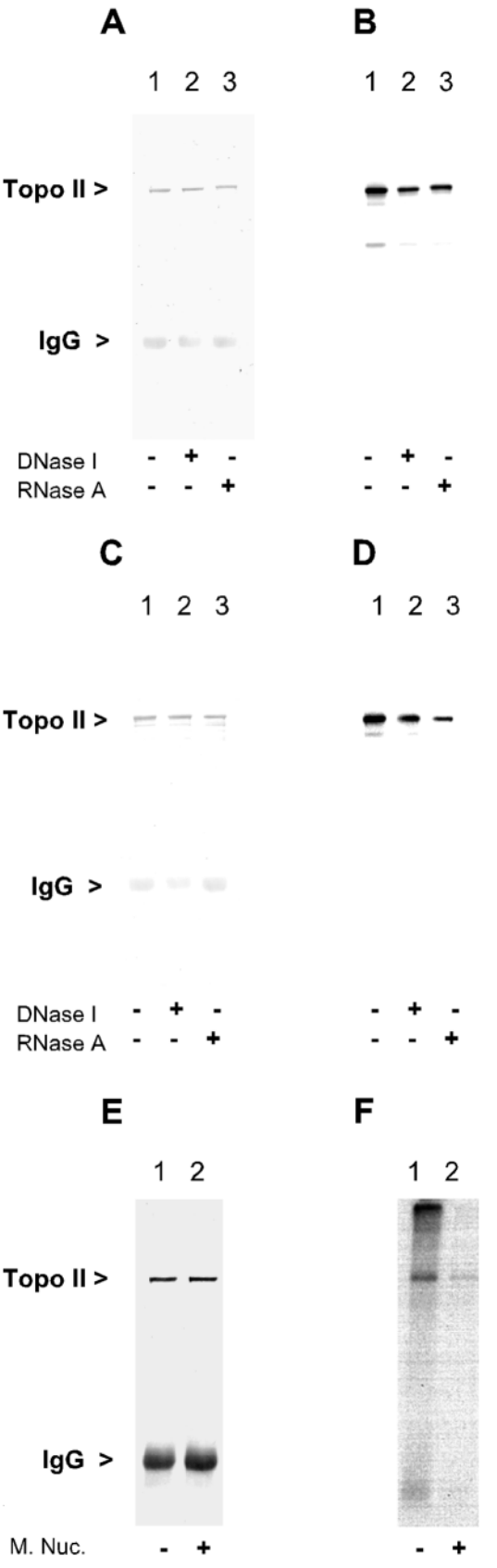

Fig. 3. Effect of nuclease treatment on the ^{32}P-labeled nucleic acid bound to topo II after standard in vivo photo-crosslinking, immunoprecipitation and labeling. All procedures were as described in Materials and Methods. P-10 (A,B,E,F) and P-130 (C and D) were analyzed. After labeling, samples were subjected to further nuclease treatment as indicated beneath each panel. To facilitate presentation, lanes in each panel are also numbered consecutively above. Immunoblot analyses (A,C,E) were with affinity purified anti-topo II antibodies. First, autoradiography of all blots shown was performed (B,D,F, respectively). The arrowheads labeled Topo II and IgG to the left of A, C and E designate the migration positions of the full-length topo II subunit and IgG heavy chain, respectively, and apply to all lanes in the corresponding autoradiograms (B,D,F).

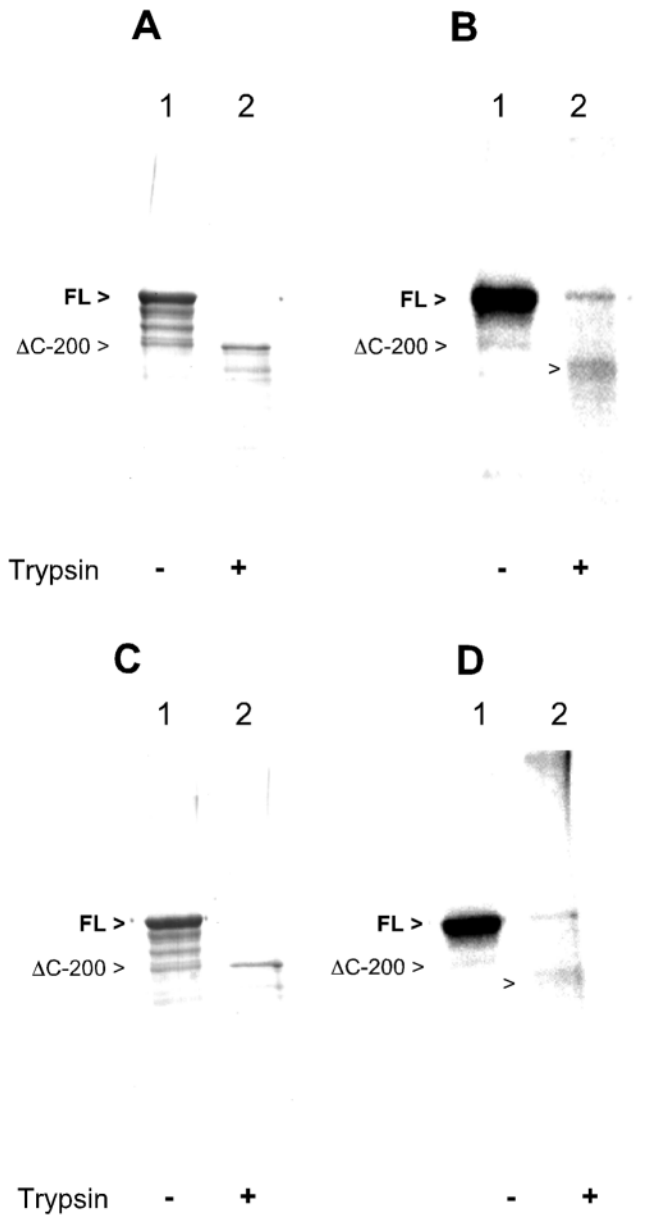

Fig. 4. Effect of trypsin treatment on ^{32}P-labeled, nucleic acid bound topo II after in vivo photo-crosslinking and immunoprecipitation; immunoblot analysis with antibodies against the N terminus of topo II (amino acids 1-31) and autoradiography. All procedures were as described in Materials and Methods. Trypsin digestion was for 8 minutes and as indicated below each lane and was performed to such an extent that afterwards, no full-length topo II was detectable by immunoblot analysis. P-10 (A and B) and P-130 (C and D) were analyzed. After labeling, samples were subjected to treatment with trypsin as indicated beneath each panel. To facilitate presentation, lanes in each panel are also numbered consecutively above. Immunoblot analyses (A and C) were performed with affinity purified anti-*Drosophila* topo II antibodies directed against N-terminal amino acids 1-31. First, the blots shown in A and C were subjected to autoradiography (B and D, respectively). The unlabeled arrows to the left of lanes 2 in B and D designate the major labeled tryptic product of *Drosophila* topo II that does not react with the anti-N-terminal IgG used to probe the blots shown in A and C, respectively. This species also migrates reproducibly more quickly than ΔC-200. The arrowheads labeled FL and ΔC-200 to the left of all panels designate the migration positions of the full-length topo II subunit and C-terminally truncated (trypsin proteolyzed) topo II subunit, respectively, and apply to all lanes.

samples were subjected to SDS-PAGE and immunoblot analysis with antibodies highly specific (Meller et al., 1995) for the N terminus of topo II (amino acids 1-31; Fig. 4A) as well as autoradiography (Fig. 4B). It was apparent from results of this experiment that of the immunoreactive polypetides, full length P-10 topo II, designated FL, was labeled. In contrast, the major immunoreactive species after trypsin treatment, designated ΔC-200 (Fig. 4A), was unlabeled (Fig. 4B). The nearby radiolabel, designated by the unlabeled arrow, migrates somewhat more quickly than ΔC-200 as indicated (Fig. 4B). We also think it noteworthy that since our ^{32}P-labeling approach is more sensitive than immunoblot analysis, a small amount of apparently full-length ^{32}P-labeled topo II can still be seen, even after trypsin treatment (Fig. 4B lane 2).

To determine which domain of P-130 topo II was associated with nucleic acids, a similar strategy (trypsin digestion after standard in vivo photo-crosslinking, immunoprecipitation and ^{32}P-labeling) was applied. This was followed by SDS-PAGE, immunoblot analysis using antibodies directed against the N terminus (amino acids 1-31) of topo II and autoradiography. As can be seen, full-length P-130 topo II, designated by the arrows labeled FL (Fig. 4C, lane 1) was also intensely labeled (Fig. 4D, lane 1). In contrast, after trypsin digestion such that no full-length topo II was detectable by immunoblot analysis, a major polypeptide about 20 kDa smaller than the full-length protein, designated by the arrows labeled ΔC-200, was seen using antibodies directed against the N terminus of topo II (Fig. 4C, lane 2). This species was unlabeled (Fig. 4D, lane 2). As above (Fig. 4A,B) and because ^{32}P-labeling is more sensitive than immunoblot analysis, a small amount of apparently full-length ^{32}P-labeled topo II can still be seen, even after trypsin treatment (Fig. 4D, lane 2). For both P-10 and P-130 topo II, results similar to those shown above (Fig. 4) were obtained with antibodies specific for amino acids 32-1,030 of *Drosophila* topo II (not shown).

Based on extensive tryptic proteolysis results of others (Lindsley and Wang, 1991; Dang et al., 1994; Lee and Hsieh, 1994), continued reactivity with a highly specific anti-N terminus of topo II antibody (see Meller et al., 1995) and estimated size reduction upon SDS-PAGE, we concluded that the polypeptide designated ΔC-200 in all panels (Fig. 4) was truncated by about 200 C-terminal amino acids. Since virtually no ^{32}P-labeling of ΔC-200 was observed, we also concluded that UV-photocrosslinking of both P-10 and P-130 topo II to nucleic acid involved predominantly, the C-terminal domain of the protein. Quantitatively minor interactions elsewhere (e.g. the active site) cannot be excluded.

Phosphorylation regulates the enzymatic activity of topo II

To explore further, differences among topo II found in P-10 and P-130, the in vitro enzymatic activity of each was characterized both without and with prior treatment with potato acid phosphatase (PAP). As described (Materials and Methods), decatenation of kinetoplast DNA, a highly specific assay, was used to evaluate topo II activity in relatively crude fractions. Incubation with topo II-containing extracts in the

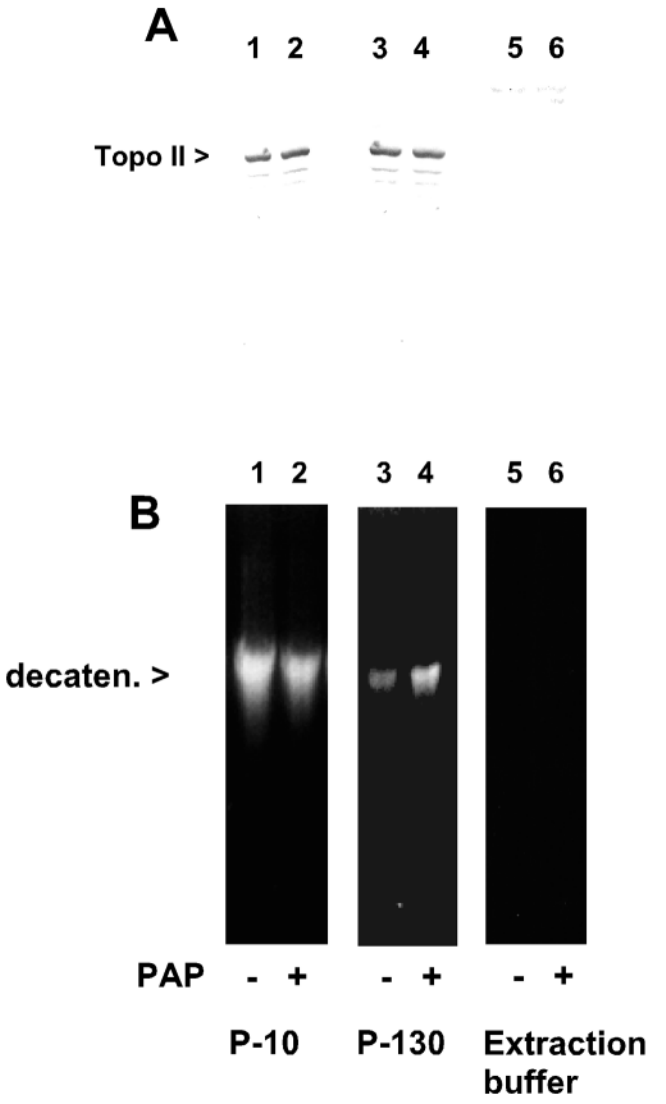

Fig. 5. Effect on enzymatic activity, of treatment of K_c cell topo II with potato acid phosphatase, as a function of subcellular fractionation. All procedures were as described in Materials and Methods. *Drosophila* K_c cells were grown, maintained for 24-hours in vinblastine, harvested and fractionated into P-10 and S-10; the S-10 was further fractionated into P-130. Topo II was extracted from the P-10 and P-130 and each was dialyzed separately. Afterward, a portion of each dialyzed fraction was treated with heat-inactivated potato acid phosphatase (PAP) and a second portion was treated with active PAP. After incubation with phosphatase, each was divided into two aliquots. One aliquot was subjected to standard immunoblot analysis to monitor both the absolute amount and integrity of the topo II subunit. The second aliquot of each was assayed for topo II activity. (A) Immunoblot analysis of each fraction after treatment either with heat-inactivated PAP (lanes 1, 3 and 5) or active PAP (lanes 2, 4 and 6). Analyzed were P-10 (lanes 1 and 2), P-130 (lanes 3 and 4) and extraction buffer only (lanes 5 and 6). The arrowhead labeled Topo II to the left of A designates the migration position of the full-length topo II subunit and applies to all lanes. (B) Topo II activity assay after either inactive (–) or active PAP-treatment (+), as indicated, of P-10 (lanes 1 and 2), P-130 (lanes 3 and 4) or extraction buffer only (lanes 5 and 6) were as indicated beneath each segment. Essential segments of nondenaturing agarose gels, stained with ethidium bromide, are shown. The arrowhead labeled decaten. to the left of B designates the migration position of DNA minicircles released from kinetoplast DNA by the enzymatic activity of topo II, and applies to all agarose gel segments.

presence of ATP results in the release by decatenation, of kinetoplast minicircles. Minicircles have a distinct mobility upon agarose gel electrophoresis. We found that release was ATP-dependent (not shown) and completely inhibited by 2 mM Na_3VO_4, a specific topo II inhibitor (not shown). In the absence of incubation with extract (topo II activity), no decatenation was seen (Fig. 5, lanes 5 and 6).

Immunoblot analysis was used to quantify amounts of topo II protein as well as subunit integrity after treatment either with heat-inactivated (Fig. 5A, odd numbered lanes) or active PAP (Fig. 5A, even numbered lanes). P-10 topo II was apparently less active after PAP treatment (Fig. 5B). In contrast, P-130 topo II was apparently activated, albeit by only about twofold, by treatment with PAP (Fig. 5B). Identical results were seen over a 20-fold range of kDNA concentration (half of that shown to 10 times higher than that shown). Identical results were also obtained when calf alkaline phosphatase was substituted for PAP (not shown).

In vivo phosphorylation of topo II

We investigated the phosphorylation of the forms of *Drosophila* topo II identified by cell fractionation. K_c cells were grown in the presence of vinblastine and [^{32}P]H_3PO_4, lysed and fractionated, all as described (Materials and Methods). ^{32}P-labeled topo II was immunopurified from each fraction. It was then subjected to SDS-PAGE and immunoblot analysis/autoradiography, also as described (Materials and Methods). Because the vinblastine-induced mitotic block was only partial, all topo II-containing fractions were purified from the same ^{32}P-labeled cells. Comparison of immunoblot results (Fig. 6A) with those from autoradiography (Fig. 6B) demonstrated that relative to P-10 topo II, P-130 topo II was more heavily phosphorylated by about 2-fold. Based on scanning densitometry, relative phosphorylation levels were 100±17% (n=4) for P-10 topo II and 197±40% (n=4) for P-130 topo II. Phosphoamino acid analyses revealed that, consistent with results of others (Ackerman et al., 1988), both P-10 and P-130 topo II contained exclusively phosphoserine (Fig. 6C).

Phosphorylation of topo II occurs predominantly in the C-terminal domain

We took advantage of the fact that minimal trypsin treatment results in removal only of the topo II C-terminal domain to determine the region associated with nucleic acid (see Fig. 4 above). We used trypsin similarly to define the domain of in vivo phosphorylation of K_c cell topo II. After vinblastine treatment and ^{32}P-labeling, cells were harvested, lysed, fractionated and fractions were subjected to immunopurification with affinity purified anti-*Drosophila* topo II IgG, all procedures performed as described (Materials and Methods). Immunopurified topo II was incubated with TPCK-treated trypsin on ice for the times indicated, subjected to SDS-PAGE, transfer to nitrocellulose and autoradiography. Highly similar results with both P-10 and P-130 topo II were obtained (Fig. 7). As digestion progressed, ^{32}P-label disappeared such that after 8 minutes, none was detectable (Fig. 7A and D). A blot prepared in parallel was probed with highly specific

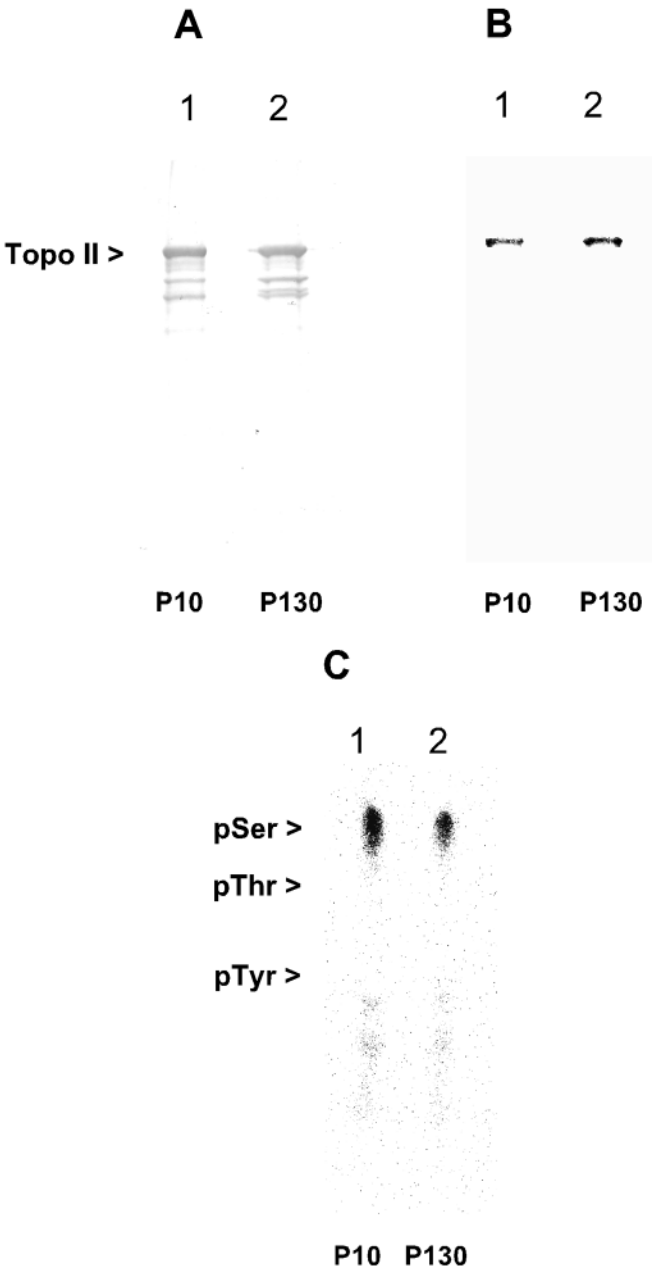

Fig. 6. In vivo phosphorylation of K_c cell topo II as a function of subcellular fractionation. All procedures were as described in Materials and Methods. *Drosophila* K_c cells were grown, maintained for 24-hours in vinblastine, harvested and fractionated into P-10 and S-10; the S-10 was further fractionated into P-130. During maintenance in vinblastine, proteins were ^{32}P-labeled by inclusion of $[^{32}P]H_3PO_4$ in the tissue culture medium. Topo II was immunoprecipitated from the P-10 and P-130, subjected to SDS-PAGE and electrophoretic transfer to nitrocellulose sheets. Resulting blots were subjected to autoradiography followed by immunoblot analysis with affinity purified anti-*Drosophila* topo II antibodies. Both immunoblot analysis (A) and an autoradiogram of the nitrocellulose (B) are shown. On both panels and as indicated beneath each lane, lanes 1 were loaded with P-10 topo II and lanes 2 were loaded with P-130 topo II. The arrowhead labeled Topo II to the left of A designates the migration position of the full-length topo II subunit and applies to both panels. (C) Phosphoamino acid analysis as a function of subcellular fractionation after in vivo phosphorylation of K_c cell topo II. Topo II was immunoprecipitated and subjected to standard radioactive phosphoamino acid analysis. The phosphoamino acid compositions of P-10 and P-130 topo II were analyzed as indicated. Arrowheads to the left of C labeled pSer, pThr and pTyr designate the migration positions of nonradioactive phosphoserine, phosphothreonine and phosphotyrosine standards, respectively.

affinity purified IgG directed against amino acids 32-1,030 of *Drosophila* topo II (Fig. 7B and E). The position of full-length (undigested) topo II is indicated by the arrows labeled FL to the left of all panels. By comparison of the autoradiogam with the immunoblot (Fig. 7, compare A with B and D with E), it can be seen that only full-length topo II was labeled intensely. Radiolabel was lost, coincident with disappearance of undigested topo II, even though a major immunoreactive species about 20-kDa smaller than full-length topo II was seen to increase in intensity relative to full-length topo II during tryptic proteolysis (Fig. 7; see the arrows labeled ΔC-200 to the left of all panels).

To confirm C-terminal degradation of *Drosophila* K_c cell topo II, the same nitrocellulose blots that were initially subjected to autoradiography (Fig. 7A and D) were subsequently probed with antibodies highly specific for topo II N-terminal amino acids 1-31 (Fig. 7C and F). As seen (compare Fig. 7B with C and E with F), reactivity of the major tryptic-digestion products of *Drosophila* topo II (indicated by the arrows labeled ΔC-200 to the left of all panels) was unchanged relative to reactivity with antibodies against topo II amino acids 32-1,030 thus confirming that the N-termini of these fragments were intact. Attempts to prepare antibodies directed against the C-terminal domain of *Drosophila* topo II in our laboratory were unsuccessful (V. H. Meller and P. A. Fisher, unpublished).

DISCUSSION

Like lamin, a majority of topo II is apparently solubilized upon entry of *Drosophila* K_c cells into mitosis (Fig. 1). Consistent findings were reported previously in early embryos (Whalen et al., 1991). During the final stage of *Drosophila* oogenesis, the oocyte arrests in metaphase of meiosis I. In apparent anticipation of the rapid nuclear division cycles characteristic of early embryogenesis, and in a process biochemically related to events in mitosis, polymeric and physiologically insoluble proteins such as lamin are phosphorylated, depolymerize, solublize and redistribute throughout the oocyte cytoplasm (Smith and Fisher, 1989). Current evidence, in conjunction with that reported previously, suggests that topo II behaves similarly.

UV-photo-crosslinking between protein and BrdU/BrdC-containing nucleic acid occurs only when the two principal molecules are within several Å of each other (see e.g. Simpson, 1979). Hence, our current results suggest that topo II is intimately associated with both DNA and RNA. Interactions of topo II with both DNA and RNA were proposed after extraction of topo II from isolated nuclei mediated by nucleases (Meller and Fisher, 1995). Except for the fact that both current and previous conclusions rely on nuclease specificity, two entirely different methods were employed. We

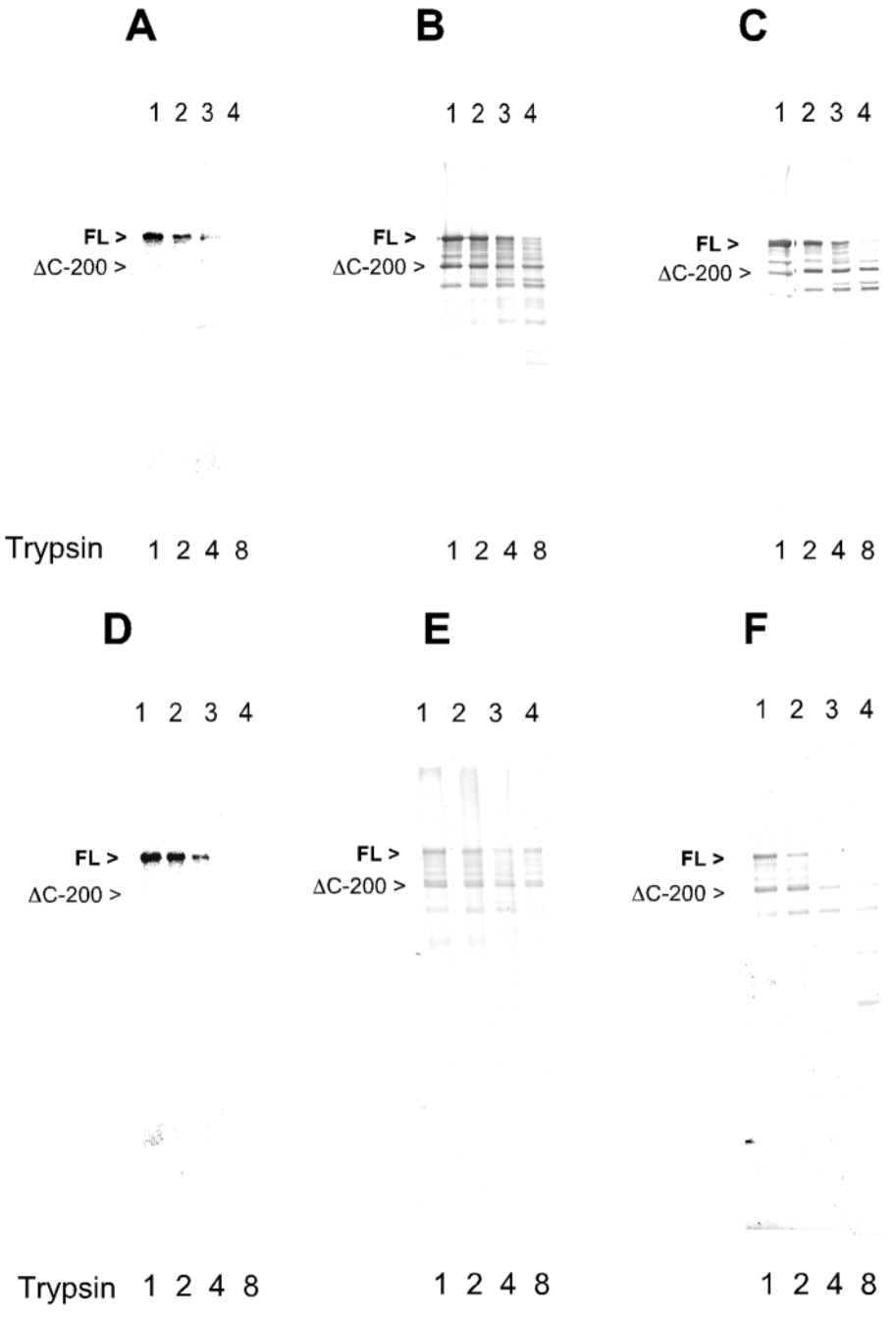

Fig. 7. Tryptic 'fingerprinting' of in vivo phosphorylation of P-10 and P-130 K_c cell topo II. All procedures were as described in Materials and Methods. *Drosophila* K_c cells were grown, maintained for 24-hours in vinblastine, harvested and fractionated into P-10 and S-10; the S-10 was further fractionated into P-130. During maintenance in vinblastine, proteins were ^{32}P-labeled by inclusion of [^{32}P]H_3PO_4 in the tissue culture medium. Topo II was immunoprecipitated from P-10 and P-130, subjected to SDS-PAGE and electrophoretic transfer to nitrocellulose sheets. Resulting blots were subjected to autoradiography followed by immunoblot analysis with affinity purified anti-*Drosophila* topo II antibodies. (A-C) P-10 topo II. (A) Autoradiogram; (B) immunoblot analysis of gel run in parallel to the one used for A and probed with rabbit antibodies directed against *Drosophila* topo II amino acids 32-1,030; (C) immunoblot analysis of blot shown in A and probed with rabbit antibodies directed against *Drosophila* topo II amino acids 1-31. (D-F) P-130 topo II. (D) autoradiogram; (E) immunoblot analysis of gel run in parallel to the one used for D and probed with rabbit antibodies directed against *Drosophila* topo II amino acids 32-1,030; (F) immunoblot analysis of blot shown in D and probed with rabbit antibodies directed against *Drosophila* topo II amino acids 1-31. Treatment with trypsin on ice was as indicated in minutes below each lane. To facilitate presentation, lanes in each panel are also numbered consecutively above. The arrowheads labeled FL and ΔC-200 to the left of all panels designate the migration positions of the full-length topo II subunit and C-terminally truncated (trypsin proteolyzed) topo II subunit, respectively.

have gone to considerable lengths to demonstrate that nucleases used were indeed, specific (M. McConnell and P. A. Fisher, unpublished). Additionally, they were used for this purpose several times previously (see Meller et al., 1994; Meller and Fisher, 1995; Rzepecki et al., 1998).

It is a limitation of this technique that to date, qualitative rather than quantitative data have been obtained. Although it would certainly be of interest to measure the fraction of total topo II in intimate contact with nucleic acids, current experimental design precludes such interpretation. It is our impression that careful isotope dilution experiments (i.e. with unlabeled ATP) would make such estimates possible. Meanwhile, substantial new insights have been derived from qualitative results.

Eukaryotic topo II functions in vitro as a homodimer. All subunits sequenced contain three domains. One, located in the N-terminal 'third,' is homologous to the B subunit of bacterial DNA gyrase (the GyrB subunit) and is thought to contain the ATP binding/hydrolysis site. The second, represented by the middle 'third' of the protein and homologous to the A subunit of bacterial DNA gyrase (the GyrA subunit), contains the active site for DNA strand cleavage (centered around Y 785 of *Drosophila* topo II; see Lee and Hsieh, 1994), as well as the domain required for polypeptide dimerization. Both the N-terminal and middle domains of topo II are required, both in vitro and in vivo. In contrast, the C-terminal 'third' (~250 amino acids) is poorly understood. Although all eukaryotic topo II molecules contain such a domain, implying key biological function, the primary amino acid sequence is poorly conserved. The only common characteristic noted is an unusually high density of charged amino acids, both positive and negative.

One in vivo function clearly associated with the C-terminal domain of topo II is nuclear localization. With most eukaryotic

topo II molecules, the C-terminal nuclear localization signal (NLS) is both necessary and sufficient to direct topo II to the nucleus (Crenshaw and Hsieh, 1993a,b; Caron et al., 1994; Lee and Hsieh, 1994). NLS necessity accounts, at least in part, for the essentiality of the C-terminal domain of topo II in vivo. In *S. pombe*, the C-terminal NLS is one of two such signal sequences; the other is located near the N terminus of the protein (Shiozaki and Yanagida, 1992). Hence, neither the N terminus nor the C terminus is essential by itself. However, a construct lacking both termini, while retaining all in vitro activity, was nonfunctional in vivo.

Despite the necessity of nuclear localization, the NLS occupies only a very small portion of the C-terminal domain (<20 amino acids of ~250 total). What other function(s) of this domain provided the selection pressure required to maintain its existence, albeit in poorly conserved form, throughout eukaryotic evolution? Current results suggest that this domain may function to modulate topo II-nucleic acid interactions during both interphase and mitosis. The C-terminal domain of topo II was previously reported by several groups to be a major site of regulated phosphorylation (Cardeñas et al., 1992; Shiozaki and Yanagida, 1992; Kimura et al., 1996). C-Terminal phosphorylation was reported by others to regulate both topo II-DNA interactions and topo II-protein interactions (Dang et al., 1994; Vassetzky et al., 1994). To relate these several observations, we here suggest that a major function of regulated phosphorylation is to modulate topo II-nucleic acid interactions. Such modulation may be necessary during chromatin remodeling associated with intranuclear processes including replication, repair, gene expression and mitotsis.

Considerable literature exists on the phosphorylation of topo II (for a review, see Watt and Hickson, 1994). In vivo, it was demonstrated that topo II from a variety of eukaryotes was a phosphoprotein (see e.g. Ackerman et al., 1988; Cardeñas et al., 1992; Saijo et al., 1992; Shiozaki and Yanagida, 1992; Burden et al., 1993; Wells et al., 1994; Kimura et al., 1994, 1996). Analysis of topo II amino acid sequence reveals a number of potential targets for phosphorylation, both for casein kinase II and for protein kinase C. The targets are clustered in the poorly understood C-terminal domain. In vitro, the activity of topo II purified from interphase nuclei could be stimulated by phosphorylation with either purified casein kinase II or protein kinase C (2- to 3-fold for *Drosophila*; DeVore et al., 1992; Corbett et al., 1993; up to 20-fold for *Saccharomyces*, Cardeñas et al., 1993); it could be reduced by treatment with phosphatase (Cardeñas et al., 1993). During mitosis, it was shown that phosphorylation of *S. cerevisiae* topo II increased (Cardeñas et al., 1992).

Our data on the phosphorylation of K_c cell topo II are consistent with and extend previous findings. Like others, we showed that during interphase, topo II phosphorylation occurs at serines (Fig. 6). Moreover, phosphorylation was found to take place within the C-terminal domain (Fig. 7). Since previous activity studies focused on topo II purified from interphase cells, our observation that treatment of P-10 topo II with phosphatase leads to reduction in topo II activity (Fig. 5) is also consistent with earlier work. To summarize, phosphorylation stimulates interphase topo II; dephosphorylation inhibits it. Phosphorylation of P-130 topo II also occurs at serines (Fig. 6) and on the C-terminal domain of the protein (Fig. 7). However, in contrast to results obtained with P-10 topo II, phosphatase treatment of P-130 topo II leads to an *increase* in enzyme activity in vitro (Fig. 5). To the best of our knowledge, our studies of mitotic topo II coupled with those of meiotic topo II reported previously (Whalen et al., 1991) represent the first biochemical characterization of M-phase enzyme and suggest that it may have properties qualitatively different from those reported previously for interphase topo II.

The effects of phosphatase treatment on topo II activity, though consistent with the work of others, are minimal. Moreover, since our assays were performed in unfractionated extracts, they may in fact be indirect. We suggest that in vivo, the primary role of topo II phosphorylation is not to modulate enzyme activity but rather, to regulate interactions with nucleic acids. In this context, a model can be offered to explain the behavior of topo II through the cell cycle in higher organisms. During interphase, a major role of topo II is to organize the cell nucleus structurally. To do this, topo II must interact with both DNA and RNA. Such interactions are primarily non-covalent and are mediated through the protein's C-terminal domain, not through the topo II active site. As cells approach mitosis, one role of topo II is to promote chromosome condensation. This function is presumably accomplished in conjunction with disentanglement of newly replicated DNA. Roles for topo II in both chromosome condensation and mitotic separation of newly replicated DNA have been demonstrated in other systems (DiNardo et al., 1984; Holm et al., 1985; Uemura et al., 1987; Wood and Earnshaw, 1990; Adachi et al., 1991; Shamu and Murray, 1992; Buchenau et al., 1993; Garinther and Schultz, 1997).

When chromosomes condense in preparation for mitosis, far less DNA is available to interact with topo II. To allow chromosome condensation to occur therefore, most topo II-DNA binding must be eliminated. Some topo II is thought to participate in forming the mitotic chromosome scaffold (Earnshaw et al., 1985; Gasser et al., 1986; Taagepera et al., 1993). Accordingly, it is likely that scaffold-associated topo II retains its ability to interact with (bind) DNA. We have not studied mitotic scaffold-associated topo II specifically. That which can no longer bind DNA now binds preferentially to RNA. This is unlike lamin which during mitosis, appears to no longer bind any nucleic acid (Rzepecki et al., 1998). Since RNA is distributed throughout the cell, this switch in binding specificity is consistent with and/or promotes the observed redistribution of topo II throughout the M-phase cell. We further speculate that the alteration in the nucleic acid binding preference of the C-terminal (nucleic acid binding) domain is regulated by phosphorylation, also of that domain. Phosphorylation may be catalyzed by M-phase specific kinases (see e.g. Taagepera et al., 1993) widely thought to regulate the process of mitosis.

In this context, we were concerned that because of the relatively high mitotic index (≤50%) after vinblastine treatment of K_c cells, topo II associated with the mitotic scaffold might be present as a greater fraction of the total topo II and might therefore constitute a significant contaminant of the subcellular fractions analyzed. Based on known sedimentation properties of scaffolds, the P-10 fraction was the most likely to be contaminated. However, in all cases, conclusions regarding P-10 topo II, particularly in comparison to P-130 topo II, would be unaffected by possible P-10 contamination. Moreover,

results qualitatively identical to those shown were obtained when the P-10 fraction was derived from cells that were not exposed to vinblastine (Rzepecki and Fisher, unpublished). In such cells, the mitotic index was ≤5%.

From a methodologic perspective, it was formally possible that, despite dependence on exposure to 366-nm light (Fig. 2), some or all of the labeling of topo II in our procedure resulted not from association with nucleic acids as we suggest (this article; see also Rzepecki et al., 1998) but from direct phosphorylation of immunoprecipitated protein by some unknown protein kinase contaminating the commercial T4 polynucleotide kinase preparation. However, in none of the samples analyzed was significant labeling of IgG heavy chain observed. IgG heavy chain was present during ^{32}P-labeling and was much more abundant than topo II in immunoprecipitated material. These observations, coupled with the nuclease sensitivity of most or all of the labeled material seen, provide strong evidence that labeling depends on the interaction of topo II with nucleic acids. We were also concerned that interactions detected were dependent on either BrdU, BrdC or vinblastine. However, the fact that qualitatively similar results were obtained with embryos and/or K_c cells maintained in the complete absence of these chemicals, argues against any explicit effect.

These studies were supported by a grant from the Human Frontier Science Program Organization. It is a pleasure to acknowledge D. Mozzherin for assistance with figures, M. McConnell for expert technical assistance and C. Dingwall for critical reading of the manuscript.

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