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Nuclear distribution of *Drosophila* DNA topoisomerase II is sensitive to both RNase and DNase

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SUMMARY

The nuclear distribution of *Drosophila* DNA topoisomerase II was determined by immunoblot analysis after nuclease digestion and cell fractionation. About 60% of DNA topoisomerase II could be removed from nuclei by RNase A, about 70% by DNase I, and about 90% by incubation with both enzymes together or with micrococcal nuclease. Nuclease treatment of nuclei did not affect the distribution of lamins Dm_1 and Dm_2 or other nuclear proteins similarly. Nuclease-mediated solubilization of DNA topoisomerase II from *Drosophila* nuclei was also dependent on NaCl concentration. Solubilization was not efficient below 100 mM NaCl. Sucrose velocity gradient ultracentrifugation demonstrated that DNA topoisomerase II solubilized from nuclei by either RNase A or DNase I migrated at about 9 S, as expected for the homodimer. Results of chemical crosslinking supported this observation. We conclude that DNA topoisomerase II has both RNA- and DNA-dependent anchorages in *Drosophila* embryo nuclei.

Key words: DNA topoisomerase II, karyoskeleton, nuclei, nuclear structure, *Drosophila*

INTRODUCTION

In vivo, DNA topoisomerase II (topo II) is a nuclear protein required at mitosis (DiNardo et al., 1984; Holm et al., 1985; Uemura et al., 1987; Shamu and Murray, 1992) and in meiosis I (Rose et al., 1990), where it was localized to the synaptonemal complex (Moens and Earnshaw, 1989; Klein et al., 1992). The requirement for topo II in mitotic chromosome condensation was demonstrated by reconstitution in cell-free systems (Wood and Earnshaw, 1990; Adachi et al., 1991). Moreover, topo II was reported to be a major component of mitotic (Earnshaw and Heck, 1985; Earnshaw et al., 1985; Gasser et al., 1986) and interphase (Berrios et al., 1985) chromosome scaffolds. Its precise role in maintaining chromatin structure is controversial (Hirano and Mitchison, 1993).

In vitro, topo II catalyzes ATP-dependent double-stranded DNA breakage and religation, thus allowing both passage of duplex DNA strands and changes in superhelicity of constrained DNA loops (Liu et al., 1980; Wang, 1985). The interactions of purified topo II with DNA are complex but relatively well studied (see e.g. Zechiedrich and Osheroff, 1990; Howard et al., 1991). In selecting binding sites, topo II can simultaneously interact with two duplex DNA strands by a sequential process of binding and capture (Roca and Wang, 1992). This, combined with the apparent localization of topo II in scaffold fractions, led to the hypothesis that stably bound topo II could act to anchor the bases of chromatin loops, thereby driving chromatin condensation (see Mirkovitch et al., 1987). In conjunction, topo II was proposed to have both structural and enzymatic roles (Mirkovitch et al., 1987; Fisher, 1989).

Recently, we demonstrated that virtually all topo II found in *Drosophila* early embryos (0- to 2-hour-old) was in a 67 S ribonucleoprotein particle (Meller et al., 1994; see also Whalen et al., 1991). This particle is presumably non-nuclear and could be disrupted completely by incubation with 0.3 M NaCl alone or RNase A in the presence of 50 mM NaCl. In contrast, it remained stable in the presence of EDTA, Triton X-100 or DNase I. These results suggested that topo II could interact directly with RNA as well as with DNA and prompted reinvestigation of topo II anchorage in nuclei. Results of current studies suggest that in *Drosophila* embryo nuclei, topo II interacts with both DNA and RNA. The interaction with RNA is different from that which occurs in the early embryo non-nuclear particle. Nuclear topo II remains associated with nuclei treated with nucleases in the absence of NaCl. This suggests that topo II is involved in protein-protein interactions that maintain nuclear localization and that these interactions are disrupted by 100 mM NaCl.

MATERIALS AND METHODS

Antibodies

Further details of antibody preparation will be reported elsewhere.

Briefly, topo II fragments corresponding to amino acids 1-202, 32-518, and 534-950 of the authentic protein were expressed in *Escherichia coli* and purified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Each fragment was used to immunize a different rabbit; two rabbits were immunized with a fragment containing amino acids 534-950. Antibodies were affinity-purified from each antiserum using a fragment of *Drosophila* topo II (amino acids 32-≥1030) expressed in *E. coli* and coupled to glutaraldehyde-activated glass beads (Whalen et al., 1991). Four affinity-purified antibody fractions (anti-32-202, anti-32-518 and two different anti-534-950 antibodies) were mixed and used to probe blots at a final IgG concentration of 31 ng/ml (see Meller et al., 1994). Rabbit anti-*Drosophila* lamin Dm_0 antibodies were affinity-purified (Fisher and Smith, 1988) and characterized as previously described (Smith and Fisher, 1989). Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Kirkegaard and Perry (Gaithersburg, MD).

SDS-PAGE and immunoblot analysis

SDS-PAGE was performed on either 5% or 7% polyacrylamide gels essentially as described (Laemmli, 1970) and as modified (Fisher et al., 1982). For analysis of chemically crosslinked topo II on agarose/polyacrylamide composite gels, the separating gel contained 375 mM Tris, 150 mM boric acid, 0.75 mM EDTA, 3% polyacrylamide and 0.5% agarose. A 3% polyacrylamide stacking gel was formed with 125 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.1% SDS and Phenol Red tracking dye. The upper reservoir buffer was identical to that described by Laemmli (1970), and was used at 10× concentration in the lower reservoir. Electrophoresis was performed using a constant current at 15 mA.

For immunoblot analysis, proteins were transferred electrophoretically to nitrocellulose after SDS-PAGE (Towbin et al., 1979). After probing with primary and alkaline phosphatase-conjugated secondary antibodies (Smith and Fisher, 1984; McConnell et al., 1987; Meller et al., 1994), bands of reactivity were detected colorimetrically according to McGadey (1970).

Embryo collection and preparation of nuclei

Embryos were collected from mass *Drosophila* cultures and dechorionated essentially as described (Allis et al., 1977). Dechorionated embryos were stored at −70°C. Nuclei were purified from 0- to 12-hour-old *Drosophila* embryos essentially as previously described (Fisher et al., 1989). Briefly, they were subjected to Dounce homogenization (2 strokes with an A pestle) in 5 embryo vols of solution containing 1.3 M sucrose, 10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (Buffer A). The homogenizer was rinsed with an additional 2 embryo vols of Buffer A, and the homogenate was filtered through 4 layers of 120 μm Nytex mesh (Tetko Inc., Elmsford, NY). The filtered homogenate was layered on an equal vol. of Buffer A and centrifuged for 10 minutes at 8,000 rpm in a Sorvall HB-4 swinging bucket rotor. The supernatant (containing non-nuclear topo II) was carefully removed and purified nuclei (in the pellet) were suspended in 1 embryo vol. of solution containing 250 mM sucrose, 10 mM HEPES, pH 8.5, 10 mM $MgCl_2$ and 1 mM EDTA (Buffer B).

Treatment of nuclei with nucleases

Treatments with bovine pancreatic RNase A (Worthington Biochemical Corp., Freehold, NJ), bovine pancreatic DNase I (Sigma Chemical Co., St Louis, MO) or micrococcal nuclease (Sigma Chemical Co., St Louis, MO) were performed in Buffer B supplemented in the case of micrococcal nuclease with either 2 mM Ca^{2+} or 10 mM EGTA as indicated in the figures. RNase A, and DNase I digestions were performed for 30 minutes at 37°C. Micrococcal nuclease digestions were performed for 30 minutes at 25°C. Unless specified otherwise, digestions were performed with purified nuclei derived from 450 embryos in a total volume of 50 μl. The RNase A used was free of detectable DNase activity as determined using a ^{32}P-end-labeled synthetic template-primer as substrate (Ng et al., 1991).

Chemical crosslinking

Crosslinking studies were performed using the reagent, dithiobis(succinimidylpropionate) (DSP) (Pierce, Rockford, IL). DSP was dissolved in dimethylformamide (DMF) immediately before use and added to samples to achieve the indicated amount of DSP and a final DMF concentration of 3.85%. After a 5 minute incubation at room temperature, the reaction was terminated by addition of SDS to a final concentration of 6% and boiling for 5 minutes. Samples were then mixed with SDS-PAGE loading buffer without reducing agent, and subjected to standard electrophoresis followed by immunoblot analysis.

Sucrose velocity gradient ultracentrifugation

Sucrose velocity gradient ultracentrifugation was performed as previously described (Meller et al., 1994). Briefly, linear 3.75 ml 10% to 30% sucrose gradients were formed on top of 0.1 ml of 80% sucrose in 11 mm × 60 mm Beckman polyallomer ultracentrifuge tubes. In addition to sucrose, all gradient solutions contained 20 mM $NaHPO_4$, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA and NaCl as indicated in the figure legends. Before analysis, nuclei from 4,500 embryos were washed briefly in Buffer B and suspended in 150 μl Buffer B supplemented either with 300 mM NaCl or with the indicated amounts of nucleases. After incubation, residual nuclear material was removed by centrifugation at 12,000 ***g*** for one minute; 125 μl of the supernatant was loaded directly onto each sucrose gradient. Sucrose gradients were subjected to ultracentrifugation at 50,000 rpm for 2 hours in an SW60 rotor at 4°C. Fractions of 150 μl each were collected from the bottom of each tube. S values were calculated based on the migration of ribosomal subunits (60 S and 40 S) and on the migration of double-stranded DNA restriction fragments (Studier, 1965) run in parallel gradients centrifuged identically.

Scanning densitometry of immunoblots

Immunoblots stained for topo II were quantified using an LKB Ultrascan XL laser densitometer (LKB Instruments Inc., Gaithersburg, MD). Quantitative data are expressed as the percentage of total topo II which could be recovered in the postnuclear supernatant.

RESULTS

Effect of NaCl concentration on the association of topo II with unfractionated *Drosophila* embryo nuclei

As was previously demonstrated for enzymatic activity (see e.g. Shelton et al., 1983), the association of topo II polypeptide with *Drosophila* embryo nuclei could be disrupted by NaCl alone (Fig. 1). This was demonstrable by first incubating nuclei with solutions of various NaCl concentrations, separating incubated material into a 12,000 ***g*** (nuclei-containing) pellet and a 12,000 ***g*** supernatant, and then subjecting both pellet and supernatant fractions to immunoblot analysis with anti-topo II antibodies. Note that when *Drosophila* nuclei were extracted with only 100 mM NaCl, the vast majority of topo II was recovered in the 12,000 ***g*** pellet fraction (Fig. 1).

Effect of NaCl concentration in combination with nucleases on the association of topo II with unfractionated *Drosophila* embryo nuclei

The effect of NaCl on the association of topo II with *Drosophila* embryo nuclei could be altered dramatically by the

addition of nucleases. Addition of either RNase A (Fig. 2A, compare P 100 + with S 100 +) or DNase I (Fig. 2B, compare P 100 + with S 100 +) resulted in recovery of the majority of topo II in the 12,000 ***g*** supernatant fraction. Below 75 mM NaCl, addition of nuclease had relatively little effect on the distribution of topo II (Fig. 2). In contrast with effects on topo II, RNase A had the opposite effect on lamins Dm_1 and Dm_2 (Fig. 2A); DNase I had little or no effect on lamins Dm_1 and Dm_2 (Fig. 2B). Simlarly, extraction of several other nuclear proteins was not affected by nuclease digestion (not shown).

Micrococcal nuclease is a Ca^{2+}-dependent enzyme that can degrade either DNA or RNA. At 100 mM NaCl, micrococcal nuclease treatment resulted in nearly complete removal of topo II from *Drosophila* embryo nuclei; removal of topo II was Ca^{2+}-dependent and did not occur in the presence of EGTA (Fig. 3A). Micrococcal nuclease had little or no effect on the distribution of lamins Dm_1 and Dm_2 (Fig. 3A). The effects of micrococcal nuclease on the distribution of topo II and lamins Dm_1 and Dm_2 (Fig. 3A) were similar to the effects of combining RNase A and DNase I (Fig. 3B).

Densitometric analysis of effects of NaCl, individual nucleases and/or combinations of nucleases demonstrated that about 90% of the nuclear topo II could be solubilized by either 300 mM NaCl alone, DNase I plus RNase A or micrococcal nuclease in the presence of Ca^{2+} (Fig. 4A). Moreover, incubation with increasing concentrations of RNase A (Fig. 4B) or DNase I (Fig. 4C) revealed a maximal effect for each nuclease. Neither enzyme alone could solubilize more than about 60-70% of the nuclear topo II. In these experiments (Fig. 4), greater than 100% of the nuclear topo II was apparently recovered. We think it likely that this reflects a technical artifact of using scanning densitometry of immunoblots to quantify topo II. Experiments performed with nuclei prepared differently (Fisher et al., 1982) yielded identical results (not shown).

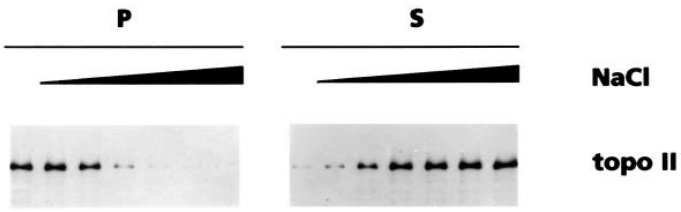

Fig. 1. Salt solubility of topo II associated with 0- to 12-hour-old embryo nuclei. Washed nuclei were subjected to a 15 minute extraction at 4°C in buffers, the NaCl concentration of which was increased from 50 mM to 350 mM in 50 mM increments. Pellet (P) and supernatant (S) fractions were separated by a 10 minute 12,000 ***g*** centrifugation. Material derived from 17 embryos was loaded in each lane, and gels were subjected to immunoblot analysis with anti-topo II antibodies.

The topo II solubilized from *Drosophila* embryo nuclei by either nuclease or NaCl is dimeric

To evaluate the quaternary structure of the topo II released from *Drosophila* embryo nuclei, sucrose velocity gradient ultracentrifugtation was used. After treatment with nucleases or NaCl as indicated, residual nuclear material was removed

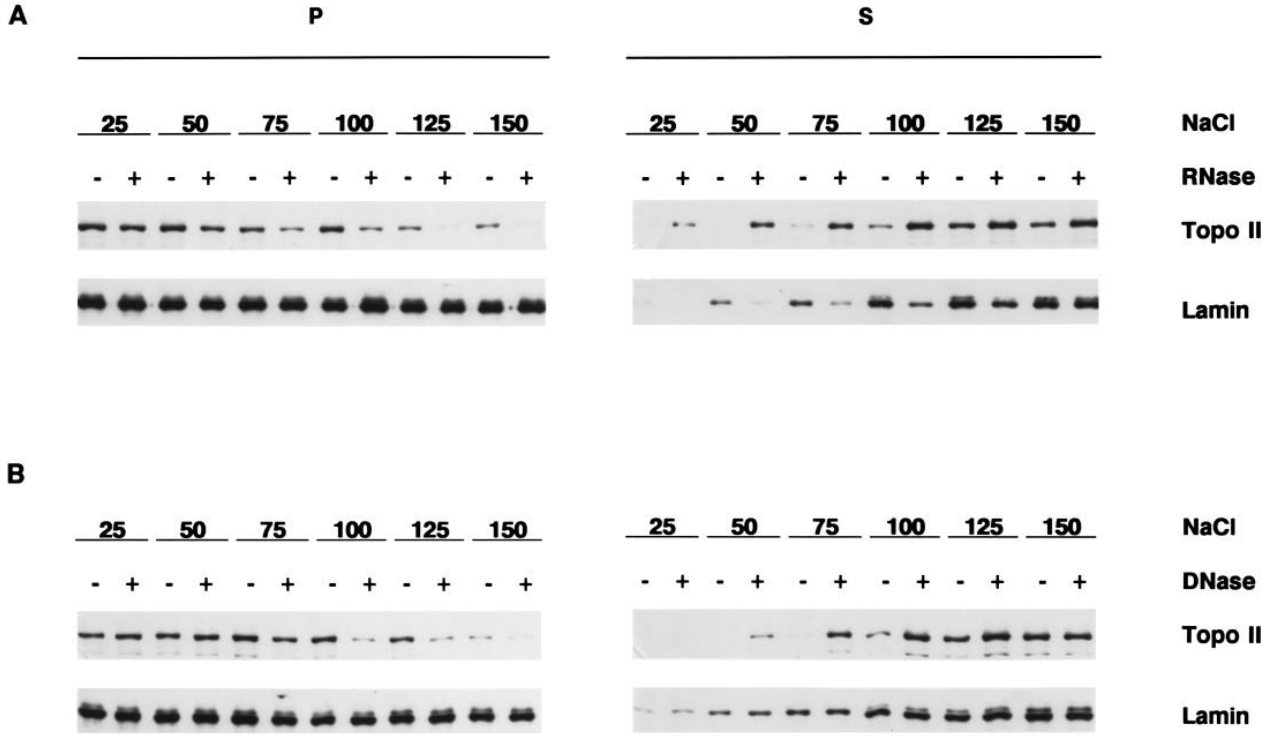

Fig. 2. NaCl dependence of topo II solubilization by RNase A and DNase I. Nuclei were digested with 40 μg/ml RNase A (A) or 25 μg/ml DNase I (B) at NaCl concentrations increasing from 25 mM to 150 mM as indicated. Pellet (P) and supernatant (S) fractions were subjected to immunoblot analysis for both topo II and lamins Dm_1 and Dm_2. Nuclear proteins from 27 embryos were loaded in each lane.

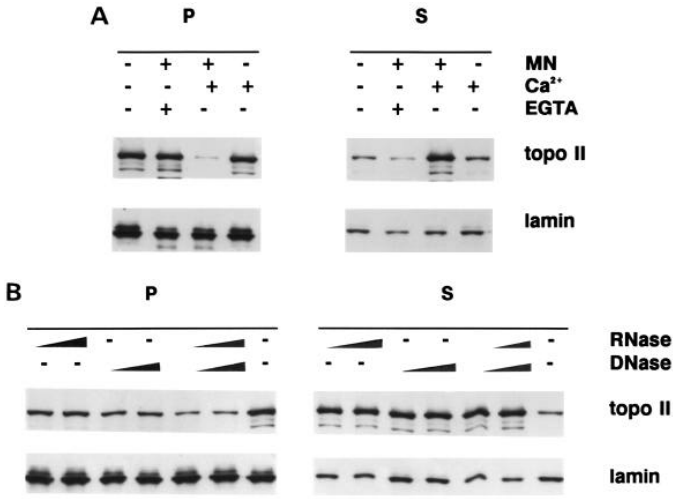

Fig. 3. Micrococcal nuclease, RNase A and DNase I solubilization of topo II from *Drosophila* nuclei. (A) Nuclei were digested for 30 minutes at 25°C with 33 μg/ml micrococcal nuclease (MN) and either 2 mM Ca^{2+} or 10 mM EGTA as indicated. Pellet (P) and supernatant (S) fractions were subjected to immunoblot analysis for both topo II and lamins Dm_1 and Dm_2. (B) RNase A digestion with 20 and 40 μg/ml enzyme, DNase I digestion with 12.5 and 25 μg/ml enzyme, and digestion using both enzymes simultaneously as indicated were performed for 30 minutes at 37°C. Pellet and supernatant fractions were analyzed as for A, nuclear proteins from 27 embryos were loaded in each lane.

by low speed centrifugation and the supernatant was loaded directly onto 10% to 30% sucrose gradients. Gradients were subjected to centrifugation for 2 hours at 50,000 × rpm, and topo II was identified by immunoblotting. In all cases, the topo II released from nuclei migrated at about 9 S (Fig. 5).

Similar results to those obtained by ultracentrifugation were observed after treatment with the homobifunctional chemical crosslinking reagent, DSP (Fig. 6). *Drosophila* embryo nuclei were treated with nucleases or NaCl as indicated, subjected to chemical crosslinking by brief incubation with DSP and residual nuclear material was removed by low speed centrifugation. After crosslinking, supernatant fractions were subjected to electrophophoresis on composite agarose/polyacrylamide gels run in the presence of SDS but without reducing agents, followed by standard immunoblot analysis. Results of these experiments demonstrated that in the absence of either extraction with NaCl or nuclease digestion, topo II was detected in a high molecular mass smear (Fig. 6, lane - - +). However, after digestion with RNase A, DNase I or a combination of DNase and RNase, or extraction with 300 mM NaCl alone as indicated, the soluble fraction contained a single major immunoreactive band with a calculated mass of about 310 kDa. This was in good accordance with the anticipated value for the topo II homodimer (332 kDa) and was identical to results seen when material was subjected to standard electrophoresis on 5% polyacrylamide gels (not shown). Moreover, the position of the putative topo II homodimer was identical to that seen when highly purified *Drosophila* topo II was subjected to DSP crosslinking under the same conditions (Meller et al., 1994).

DISCUSSION

The interphase chromosome scaffold or internal nuclear matrix is thought to function in genome replication (see e.g. Nakayasu and Berezney, 1989; Blow et al., 1989; Mills et al., 1989; Cox and Laskey, 1991; Manders et al., 1992; Hozak et al., 1993; Hassan and Cook, 1993; for reviews, see Berezney, 1991; Cook, 1991; Georgiev et al., 1991), transcription and transcript processing (see e.g. Spector, 1990; Li and Bingham, 1991; Xing and Lawrence, 1991; Huang and Spector, 1992; Jackson and Cook, 1993; Jackson et al., 1993; Carter et al., 1993; Xing et al., 1993; Jiménez-Garcia and Spector, 1993; Wansink et al., 1993; for a review, see Spector, 1993) and DNA methylation (Leonhardt et al., 1992). The proposition that during these

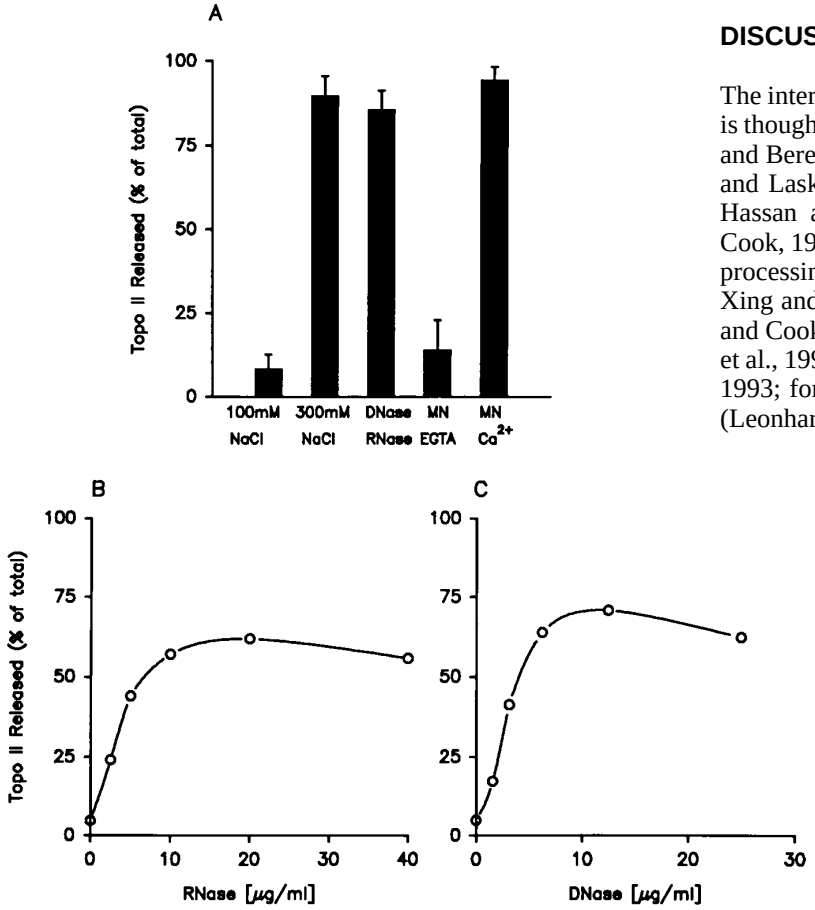

Fig. 4. Nuclease-mediated solubilization of topo II is dependent on nuclease specificity and concentration. (A) The fraction of nuclear topo II solubilized by nuclease treatments was quantified by scanning densitometry of immunoblots. Error bars represent one s.d.; 100 mM NaCl plus Buffer B (n=7); 300 mM NaCl plus Buffer B (n=4); RNase A (20 μg/ml) and DNase I (12.5 μg/ml, n=6); MN, 33 μg/ml micrococcal nuclease with 10 mM EGTA (n=4); or with 2 mM Ca^{2+} (n=4). (B) The fraction of total topo II released by RNase A digestion is plotted versus the concentration of enzyme used. Data are from a single experiment. (C) The fraction of total topo II released by DNase I digestion is plotted versus the concentration of enzyme used. The data are from a single experiment.

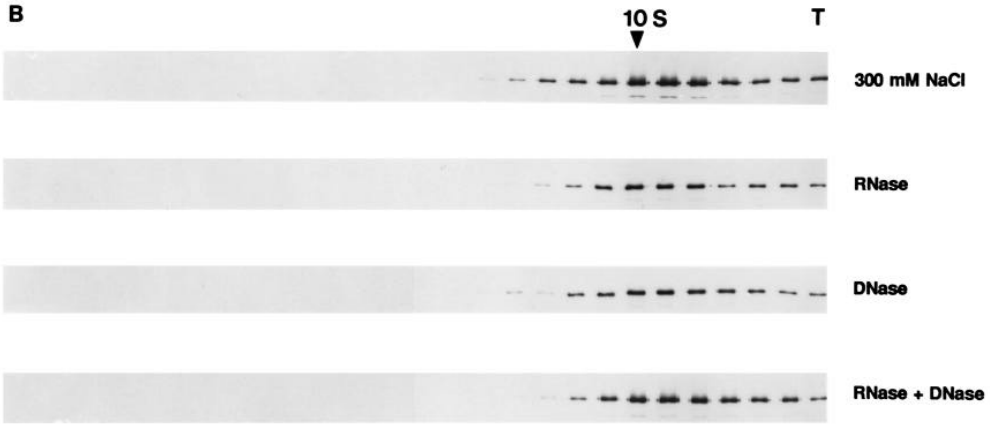

Fig. 5. Sucrose velocity gradient analysis of topo II solubilized from nuclei by nuclease digestion or NaCl extraction. Nuclei were digested for 30 minutes at 37°C with 40 μg/ml RNase A, 25 μg/ml DNase I, or both enzymes together at these concentrations, or in Buffer B supplemented with 300 mM NaCl but with no added nuclease. Material released from nuclei derived from 3,750 embryos was loaded directly on 10% to 30% sucrose gradients and subjected to centrifugation at 50,000 rpm for 2 hours. Aliquots (6 μl) of each fraction were subjected to immunoblot analysis. T, top of gradient; B, bottom of gradient. Arrow indicates the fraction with a calculated mobility of 10 S.

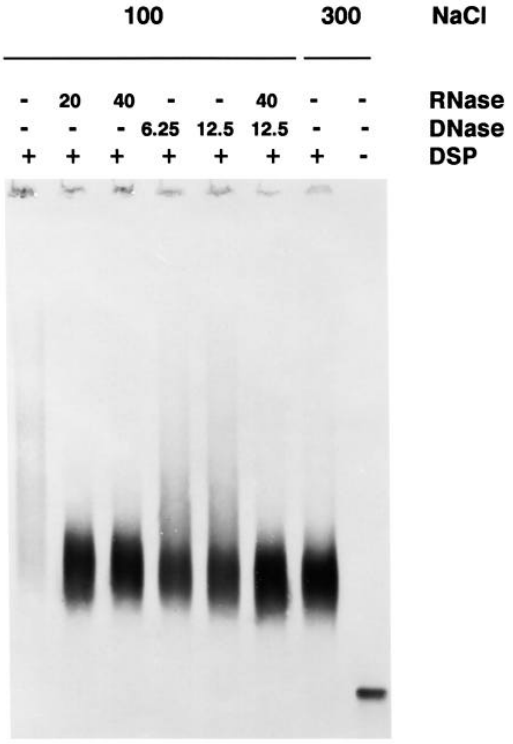

Fig. 6. Immunoblot analysis of chemically crosslinked topo II from nuclease-digested nuclei. Nuclei were incubated at 37°C for 30 minutes with 20 or 40 μg/ml RNase A, 6.25 or 12.5 μg/ml DNase I, both RNase A (40 μg/ml) and DNase I (12.5 μg/ml), or without nucleases but with 300 mM NaCl. Crosslinking was performed for 5 minutes at room temperature with 0.2 mg/ml DSP, and proteins from 68 embryos were loaded per lane.

processes DNA strands are drawn past immobile, scaffold-bound enzyme complexes emphasizes the need for topological manipulation of DNA as a corequisite. Although topo II was identified as a major component of the interphase chromosome scaffold (nuclear matrix) fraction in *Drosophila* (Berrios et al., 1985), relatively little is known about the organization of topo II within the interphase nucleus, the structure of the interphase chromosome scaffold or the role of topo II in contributing to this putative structure. Two recent observations may be relevant to addressing this problem.

First, confocal immunocolocalization of topo II and histones demonstrated that *Drosophila* topo II was distributed in a pattern distinct from that of the chromosomes (Meller et al., 1995). Three-dimensional reconstruction of topo II localization data suggested that topo II was distributed throughout nuclei in a loosely defined network that was at least partly extra-chromosomal. Second, we recently identified a topo II-containing ribonucleoprotein particle in early (0- to 2-hour-old) *Drosophila* embryos (Meller et al., 1994), suggesting that topo II can form stable associations with RNA. RNase A digestion of this particle at 50 mM NaCl resulted in its complete disintegration, with consequent identification, of topo II in homodimeric form.

Current results suggest that topo II interacts with both RNA and DNA in unfractionated *Drosophila* nuclei. Accordingly, RNase A digestion can solubilize only a portion of nuclear topo II, and efficient release requires 100 mM NaCl. Nuclear topo II which resists RNase A extraction can be solubilized by DNase I, and efficient release also requires 100 mM NaCl. Thus, topo II could indeed play a structural role in the nucleus, interacting with both nucleic acids (evidenced by results of nuclease digestion) and proteins, including itself (evidenced by the NaCl requirement for solubilization). In contrast with topo II, distribution of the lamins Dm_1 and Dm_2 as well as several other nuclear proteins was largely unaffected by nuclease digestion. Like topo II, lamins are known to bind DNA (Ludérus et al., 1992, 1994). Based on these results, there are at least three possible models to account for topo II attachment in nuclei. The first is that topo II binds RNA and DNA concurrently. The second is that topo II can bind either RNA or DNA, but not both simultaneously. The third is that protein-protein interactions anchor topo II in the nucleus. None of these models are mutually exclusive.

The first possibility, concurrent binding of RNA and DNA, seems relatively unlikely, in that either RNase A alone or DNase I alone released large portions of nuclear topo II, and each enzyme appeared to release topo II as an unencumbered homodimer, as determined by sucrose gradient mobility and corroborated by chemical crosslinking. If DNase I digestion released an RNA-bound topo II or conversely, RNase A

digestion released a DNA-bound topo II, we would have expected this to be reflected upon sedimentation. Our value of about 9 S for topo II released from nuclei with either RNase alone or DNase alone agrees well with values of 9 S (Shelton et al., 1983) and 10 S (Sander and Hsieh, 1983) reported for the purified topo II homodimer.

In this context, the second possibility, that topo II can bind to either RNA or DNA, but not both concurrently, seems more likely. In addition, neither DNase I nor RNase A can efficiently release topo II from nuclei at very low ionic strength; rather, efficient release requires the presence of 100 mM NaCl. This observation lends credence to the third model, suggesting that protein-protein interactions play a role in anchoring topo II within nuclei. There is at present no molecular evidence, e.g. results of chemical crosslinking studies, to define these potential protein-protein interactions further. Moreover, it is possible that these interactions are artifactual, occurring only secondarily to nuclease-mediated degradation of nucleic acid or at very low ionic strength.

We also think it should be noted that our analyses pertain to the bulk of topo II, but would not necessarily have revealed associations involving minor fractions of this protein. Given the dynamic nature of chromatin and the abundance of topo II in the nucleus, an event which is transient or involves only a small fraction of topo II could be of substantial biological importance, yet might have gone unrecognized. Similarly, we used embryos for all experiments. It will be particularly interesting to determine how nonembryonic cells behave.

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