An RNase-sensitive Particle Containing *Drosophila melanogaster* DNA Topoisomerase II

Victoria H. Meller, Maeve McConnell, and Paul A. Fisher

Department of Pharmacological Sciences, University Medical Center, State University of New York at Stony Brook, Stony Brook, New York 11794-8651

Abstract. Most DNA topoisomerase II (topo II) in cell-free extracts of 0–2-h old *Drosophila* embryos appears to be nonnuclear and remains in the supernatant after low-speed centrifugation (10,000 *g*). Virtually all of this apparently soluble topo II is particulate with a sedimentation coefficient of 67 S. Similar topo II–containing particles were detected in *Drosophila* K_c tissue culture cells, 16–19-h old embryos and extracts of progesterone-matured oocytes from *Xenopus*. *Drosophila* topo II–containing particles were insensitive to EDTA, Triton X-100 and DNase I, but could be disrupted by incubation with 0.3 M NaCl or RNase A. After either disruptive treatment, topo II sedimented at 9 S. topo II–containing particles were also sensitive to micrococcal nuclease. Results of chemical cross-linking corroborated those obtained by centrifugation. Immunoblot analyses demonstrated that topo II–containing particles lacked significant amounts of lamin, nuclear pore complex protein gp210, proliferating cell nuclear antigen, RNA polymerase II subunits, histones, coilin, and nucleolin. Northern blot analyses demonstrated that topo II–containing particles lacked U RNA. Thus, current data support the notion that nonnuclear *Drosophila* topo II–containing particles are composed largely of topo II and an unknown RNA molecule(s).

DNA topoisomerase II (topo II)[1] is a nuclear protein essential at mitosis (DiNardo et al., 1984; Holm et al., 1985; Uemura et al., 1987; Shamu and Murray, 1992) where it is required for separation of sister chromosomes. In meiosis I, it is also necessary for segregation of recombined homologous chromosomes (Rose et al., 1990); it has been histologically localized to the synaptonemal complex (Klein et al., 1992). In vitro, topo II can catalyze 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). It is essential for chromatin condensation at the onset of mitosis (Uemura et al., 1987) and in cell free systems (Wood and Earnshaw, 1990; Adachi et al., 1991). topo II was also 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; it was proposed to have structural as well as enzymatic roles (Mirkovitch et al., 1987; Fisher, 1989), but the role of topo II in maintaining chromatin structure is controversial (Hirano and Mitchison, 1993). In situ, topo II appears to be part of a substantially extrachromosomal network, at least in certain cells (Meller et al., 1994).

The interactions of topo II with DNA are biochemically complex. When selecting binding sites, topo II is thought to recognize features of DNA conformation and topology, including crossovers (Zechiedrich and Osheroff, 1990) and bends (Howard et al., 1991). It can also bind two DNA strands concurrently by a sequential process of binding and capture (Roca and Wang, 1992). topo II preferentially binds to and aggregates scaffold attachment region (SAR)-containing DNA; this preference apparently leads to cooperative binding by topo II of non-SAR containing flanking DNA (Adachi et al., 1989). SAR-binding by topo II is presumably sequence-specific. SARs are thought to be AT-rich regions which contain topo II cleavage sites (Gasser and Laemmli, 1986; Izaurralde et al., 1988; Lee et al., 1989). Although DNA and topo II interactions are well documented, to the best of our knowledge, interactions between topo II and RNA have not been reported.

Drosophila melanogaster apparently has only one topo II gene, encoding a 166-kD polypeptide (Nolan et al., 1986; see also Jenkins et al., 1992). Enzymatically active topo II is thought to be a homodimer (Shelton et al., 1983). During oogenesis, *Drosophila* oocytes are provisioned with large

1. *Abbreviations used in this paper*: DMF, dimethylformamide; DSP, dithiobis(succinimidylpropionate); RNasin, placental RNase inhibitor; SAR, scaffold attachment region; topo II, DNA topoisomerase II.

stores of topo II, as well as other nuclear proteins (Smith and Fisher, 1989; Whalen et al., 1991; see also King, 1970; Mahowald and Kambysellis, 1980). Results of cell fractionation experiments performed with 0–3-h old embryos revealed that during early development most topo II was apparently nonnuclear and soluble (Whalen et al., 1991). In this article, we demonstrate that this pool of topo II is present almost exclusively in NaCl-sensitive, RNase A-sensitive 67-S particles. This pool of topo II was detectable in situ. Similar particles were detected in extracts from older (16–19-h old) embryos and *Drosophila* K$_c$ tissue culture cells; a 75-S topo II–containing particle was detected in extracts of progesterone-matured oocytes from *Xenopus*.

Materials and Methods

Embryo Collection

Mass cultures of *Drosophila melanogaster* were maintained and embryos collected as described (Allis et al., 1977). Dechorionated embryos were stored frozen at −70°C.

Antibodies

Anti-*Drosophila* topo II antisera were raised against bacterially expressed PAGE purified polypeptides corresponding to amino acids 1-202, 32-518, and 534-950 of the authentic protein (Meller et al., 1994). Each fragment was used to immunize a different rabbit; two rabbits were immunized with the fragment containing amino acids 534-950. Anti-*Drosophila* topo II antibodies were affinity purified from each antiserum using an immobilized fragment containing amino acids 32- ⩾ 1030 (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 concentration of 31 ng/ml. Rabbit anti-*Drosophila* lamin antibodies were described previously (Smith and Fisher, 1989), as were monoclonal anti-*Drosophila* gp210 antibodies (Filson et al., 1985). Affinity purified anti-*Drosophila* RNA polymerase II subunits antibodies (Weeks et al., 1982) were the generous gifts of A. Greenleaf and were as previously described (Fisher et al., 1989). Anti-*Drosophila* PCNA antiserum was also previously described (Ng et al., 1990). Monoclonal anti-histone antibody mAb052 was from Chemicon International (Temecula, CA). Antiserum against *Xenopus laevis* oocyte topo II (Luke and Bogenhagen, 1989) was the generous gift of M. Luke and D. Bogenhagen. It was used at a dilution of 1:10,000 for probing immunoblots. Antiserum (autoimmune) against human coilin (Andrade et al., 1993) was the generous gift of Dr. E. Chan and was used at a final concentration of 1:500. Antiserum against *Xenopus* nucleolin (Heine et al., 1993) was the generous gift of P. DiMario and was used at a final concentration of 1:500. Alkaline phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Kirkegaard and Perry (Gaithersburg, MD).

SDS-PAGE, Immunoblotting, and Immunocytochemistry

Proteins were separated by SDS-PAGE according to Laemmli (1970) and transferred electrophoretically to nitrocellulose (Towbin et al., 1979). Blots were incubated briefly with PBS containing 0.5% Tween-20 (PBST) and probed for 12 h with primary antibody in PBST. Immunoreactive bands were visualized colorimetrically after a 2-h incubation with alkaline phosphatase–conjugated goat anti–rabbit IgG (1:5,000) or goat anti–mouse IgG (1:1,000). Immunocytochemical detection of topo II in unfractionated *Drosophila* embryos was performed using the VECTASTAIN ABC Elite Kit (Vector Labs., Burlingame, CA) as specified by the vendor.

Cell Fractionation

All steps were performed at 4°C unless indicated otherwise. Dechorionated *Drosophila* embryos were broken by Dounce homogenization with a tight (A) pestle in 10 embryo volumes of 250 mM sucrose, 50 mM NaHPO$_4$ pH 7.5, 50 mM NaCl, 5 mM MgCl$_2$, 1 mM EDTA and 0.1 mM PMSF (homogenization buffer). For chemical cross-linking studies, 50 mM Hepes

pH 8.5, replaced NaHPO$_4$. Homogenates were filtered through three layers of 120 μm-mesh Nytex (Tetko Inc., Elmsford, NY) and centrifuged for 10 min at 10,000 g. The lipid layers were removed and discarded, the supernatant withdrawn, and pellets were washed twice by resuspending in homogenization buffer and centrifuging as above. The supernatant and pellet fractions were immediately aliquoted, frozen in liquid nitrogen, and stored at −70°C. The 10,000 g supernatant was further fractionated by centrifugation at 130,000 g for 30 min in a Beckman air-driven ultracentrifuge. The resultant pellet was termed the 130,000 g particulate fraction.

Stage VI *X. laevis* oocytes were progesterone treated, and only oocytes that had undergone germinal vesicle breakdown were processed further. Concentrated extract from 1,000 such oocytes was obtained by adding 400 μl of buffer containing 40 mM β-glycerophosphate pH 7.2, 15 mM MgCl$_2$, 20 mM EGTA, 1 mM DTT and 10 μg/ml each of leupeptin, pepstatin and chymostatin, and centrifuging for 15 min at 12,000 g. The clear supernatant just below the lipid layer was removed, aliquoted, and stored at −70°C.

Salt Solubility and Chemical Cross-linking

Protein solubility was determined by suspending the washed 10,000 g nuclear pellet or the 130,000 g particulate fraction in 250 mM sucrose, 20 mM Hepes pH 8.5, 4 mM EDTA, and NaCl concentrations ranging from 50 mM to 1 M. Suspended material was incubated for 15 min on ice, and then subjected to centrifugation at 12,000 g for 10 min (10,000 g nuclear pellet) or at 130,000 g for 30 min (130,000 g particulate fraction). Chemical cross-linking was performed using material prepared identically, but without the final centrifugation. The cross-linking agent, dithiobis(succinimidylpropionate) (DSP) (Pierce Chemical Co., Rockford, IL) was dissolved in dimethylformamide (DMF) immediately before use to achieve the indicated amount of DSP at a final DMF concentration of 3.85%. After a 5-min incubation at room temperature, reactions were terminated by addition of SDS to a final concentration of 6% followed by a 5-min incubation at 100°C. Samples were then mixed with SDS-PAGE loading buffer without reducing agent, subjected to electrophoresis on 5% polyacrylamide gels (Laemmli, 1970), and subjected to immunoblot analysis as detailed above.

Sucrose Velocity Gradient Centrifugation

Linear 3.75 ml 10–30% sucrose gradients were formed over 0.1 ml of 80% sucrose in 11 × 60 mm Beckman polyallomer ultracentrifuge tubes. For fractionation of *Drosophila* extracts, all solutions contained in addition to sucrose, 20 mM NaHPO$_4$ pH 7.5, 5 mM MgCl$_2$, 1 mM EDTA, and 50 or 300 mM NaCl, as indicated in the figure legends. When samples were treated with Triton X-100, 0.1% Triton X-100 was included in gradients. Samples of 125 μl were loaded and subjected to centrifugation in a Beckman SW60 rotor at 4°C as indicated in the figure legends. Fractions of 150 μl each were collected from the bottoms of tubes. S-values were calculated based on the sedimentation of intact ribosomes, EDTA-dissociated ribosomal subunits, and linear duplex DNA restriction fragments (Studier, 1965). DNA and ribosomal RNA were detected by agarose gel electrophoresis and ethidium bromide staining. Extracts of progesterone-matured *Xenopus* oocytes were fractionated identically, with the exception that gradient solutions contained the same buffer used in preparation of the extract. Gradients were loaded with 200 μl of extract, and fraction collection and analysis were performed as described above. Nondenaturing agarose gel electrophoresis was performed according to standard protocols (Sambrook et al., 1989).

Purification of Enzymatically Active topo II and Assay of topo II Activity

Purification of enzymatically active topo II was essentially according to Shelton et al. (1983). Purified topo II was the generous gift of N. Osheroff. Enzymatic activity of topo II was detected after nondenaturing agarose gel electrophoresis using an assay based on decatenation of kinetoplast DNA (Marini et al., 1980; Luke and Bogenhagen, 1989).

Nuclease Digestion

Bovine pancreatic RNase A was from Worthington Biochemical Corp. (Freehold, NJ); bovine pancreatic DNase I was from Sigma Chem. Co. (St. Louis, MO). RNase A and DNase I digestions were performed at 37°C in solutions containing 250 mM sucrose, 10 mM Hepes pH 8.5, 10 mM MgCl$_2$, 1 mM EDTA and 50 mM NaCl. Micrococcal nuclease digestions were performed at 25°C, in the same solution supplemented with either 2 mM CaCl$_2$ or 10 mM EGTA as indicated in the figure legends.

Inhibition of RNase A Activity by Placental RNase Inhibitor and Effects on Particle Stability

1 μg of RNase A was mixed with 30 μl of digestion buffer containing 5 mM DTT and either 10 μl of placental RNase inhibitor (RNasin) buffer (20 mM Hepes-KOH pH 7.6, 50 mM CKl, 8 mM DTT and 50% glycerol) or 10 μl RNasin (Promega Corp., Madison, WI) containing 400 U of activity. Glycerol was removed by 2 cycles of filtration through an Ultrafree-MC filtration unit with a 5-kD exclusion limit (Millipore, Bedford, MA) and addition of digestion buffer containing 5 mM DTT to the original volume. 10 μl aliquots were used to determine the effect of RNase A and RNasin on particle stability.

Detection of U RNA

Detection of U RNA was by dot-blot hybridization according to standard procedures (Sambrook et al., 1989). DNA clones corresponding to *Drosophila* U RNAs were the generous gift of P. Bingham; ^{32}P-labeled RNA transcripts of these clones were synthesized using T7 RNA polymerase as described (Spikes and Bingham, 1992).

Results

A Particulate Form of Drosophila topo II

Cell fractionation in combination with immunoblot analysis was used to investigate the apparently nonnuclear topo II found in early (0–2-h old) embryos. This pool was detectable immunocytochemically (not shown). The behavior of topo II was compared with that of gp210, an integral membrane protein thought to be a component of the nuclear pore complex (Wozniak et al., 1989; Greber et al., 1990; Berrios et al., 1994) and lamin, an intermediate filament protein which forms the nuclear lamina (see e.g., Smith and Fisher, 1989). Similar studies were performed with 16–19-h old embryos.

In early embryos (Fig. 1, lanes 1–4) all three nuclear proteins were more abundant in the supernatant after fractionation of homogenates at 10,000 g (Fig. 1 compare lanes 1 and 2). When this 10,000 g supernatant was subjected to centrifugation at 130,000 g for 30 min, all detectable gp210 and topo II was recovered in the pellet fraction; in contrast, most of the lamin present in the 10,000 g supernatant fraction of early embryos did not pellet during the 130,000 g centrifugation (Fig. 1 compare lanes 3 and 4).

With older embryos (16–19-h old) (Fig. 1, lanes 5–8), the
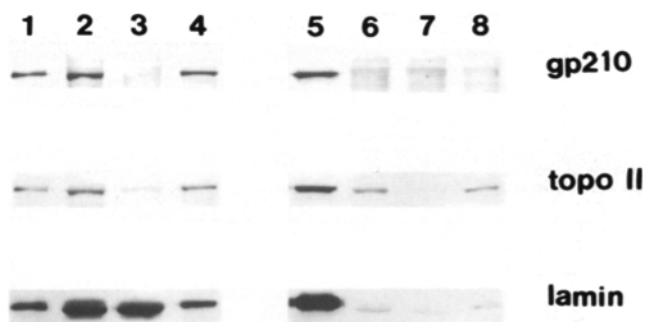
Figure 1. Immunoblot analysis of gp210, topo II, and lamin after fractionation of *Drosophila* embryos. (Lanes 1–4) 0–2-h old embryos; (lanes 5–8) 16–19-h old embryos. Lanes 1 and 5, washed nuclei; (lanes 2 and 6) 10,000 g supernatant. The 10,000 g supernatant fractions were centrifuged at 130,000 g to yield lanes 3 and 7, 130,000 g supernatant; (lanes 4 and 8) 130,000 g pellet. Material loaded in each lane was derived from 35 embryos.

majority of all three proteins was recovered in the 10,000 g pellet fraction (Fig. 1, lane 5). Consistent with previous results (Whalen et al., 1991), we detected a small fraction of topo II in the postnuclear supernatant of older embryos (Fig. 1, lane 6). This pool of topo II could be recovered entirely in the 130,000 g pellet fraction (Fig. 1, lane 8).

Initial ultracentrifugation experiments compared the 10,000 g supernatant fraction and its derivative, the 130,000 g pellet fraction. The mobility of topo II from these two sources on sucrose gradients was found to be indistinguishable (not shown), and, since the 130,000 g pellet fraction allowed significant enrichment for topo II, it was used in all subsequent experiments. topo II, lamin, and gp210 derived from the 130,000 g pellet fraction of 0–2-h old embryos (see Fig. 1, lane 4) were analyzed. Sucrose velocity gradient profiles demonstrated that at 50 mM NaCl (Fig. 2, *topo II a*), topo II migrated as a particle with an S value of 67 (± 5; n = 7). Decatenation activity was readily detected in topo II–containing fractions of the gradient, indicating that this material was enzymatically active (not shown). After treatment with 300 mM NaCl, all topo II was recovered near the top of the gradient with a calculated sedimentation coefficient of 9 S (Fig. 2, *topo II b*). This value remained constant at salt concentrations up to 1 M (not shown).

In contrast, treatment of topo II–containing particles with 1% Triton X-100 did not affect their migration through the gradient (Fig. 2, *topo II c*). This indicated that membrane or vesicle integrity was not required for the stability of the 67-S particle. Similarly, treatment of topo II–containing particles with EDTA was without effect on their sedimentation (not shown). This treatment dissociates ribosomes into subunits, and markedly alters their mobility (Fig. 3).

The majority of gp210 and a portion of lamin was detected at the bottom of gradients containing 50 mM NaCl. Unlike topo II, mobility was not affected by 300 mM NaCl. Also unlike topo II, treatment with Triton X-100 caused a dramatic shift in sedimentation of gp210, and to a lesser degree lamin (Fig. 2, *gp210 c; lamin c*).

Xenopus laevis Particulate topo II

Extracts prepared from progesterone-matured oocytes of *Xenopus* were subjected to sucrose velocity gradient analysis. The profile presented in Fig. 4 revealed that *Xenopus* topo II–containing particles migrated at ~75 S, slightly larger than those found in *Drosophila* embryos.

Nuclease Sensitivity of Drosophila topo II–containing Particles

The nuclease sensitivities of *Drosophila* topo II–containing particles were initially determined by sucrose velocity gradient centrifugation of enzyme-digested material. DNase I and RNase A digestions were performed on 130,000 g particulate fractions from 0–2-h old embryos (See Fig. 1, lane 4), and material was analyzed using sucrose gradients containing 50 mM NaCl. topo II gradient profiles are presented in Fig. 5 panel A. Parallel gradients were loaded with material incubated for 30 min at 37°C in 125 μl of digestion buffer containing either 15 μg DNase I, 19 μg RNase, A, or no enzyme (control). Incubation at 37°C caused the formation of some rapidly sedimenting material (compare Fig. 2 *topo II a* with Fig. 5 *A*, control); however, the majority of topo
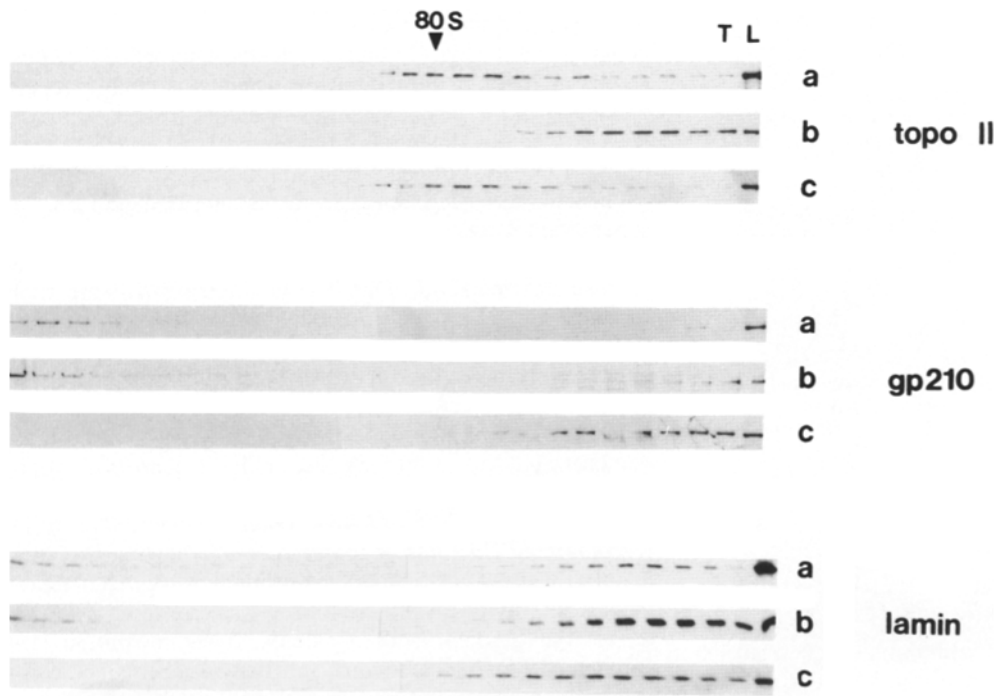
Figure 2. Sucrose velocity gradient analysis of topo II, gp210, and lamin from the 130,000 g particulate fraction of 0–2-h old *Drosophila* embryos. The particulate fraction from 2,250 embryos was separated on 10–30% sucrose gradients by centrifugation at 35,000 rpm for 1 h in the presence of 50 mM NaCl (*a*), 300 mM NaCl (*b*), or 50 mM NaCl plus 1% Triton-X 100 (*c*). 12 µl of each fraction was subjected to immunoblot analysis. *L*, load; *T*, top of gradient; *B*, bottom of gradient. Downpointing arrowhead indicates sedimentation position of 80-S monosomes.

II remained in the 67-S form. Digestion with high levels of DNase I had no effect on the mobility of topo II in the gradient (Fig. 5 *A*, DNase). In contrast, RNase A digestion completely converted topo II both from the 67-S particle and from the more rapidly sedimenting material to a form which sedimented at 9 S (Fig. 5 *A*, RNase).

The sensitivity of topo II–containing particles to nucleases was also demonstrable by digestion followed by centrifugation at 130,000 g for 30 min in an air driven ultracentrifuge (Fig. 5 *B*). This assay proved much simpler than sucrose velocity gradient centrifugation, and was used to characterize further the nuclease sensitivities of topo II–containing particles. RNase A caused detectable solubilization of topo II from particulate preparations at levels as low as 2.5 µg per ml (Fig. 6 *A*). topo II–containing particles were also sensitive to micrococcal nuclease, and this effect was dependent

on Ca^{2+}, a necessary cofactor for this enzyme (Fig. 6 *B*). RNase A solubilization of topo II from topo II–containing particles could be blocked by preincubation of RNase A with the specific inhibitor, RNasin (Fig. 7). The glycerol in which RNasin is supplied interfered with RNase A solubilization of topo II from the topo II–containing particle (not shown). RNase A was mixed with either RNasin storage buffer or RNasin, and glycerol removed by ultrafiltration as described (Materials and Methods).

RNase A-sensitive Particles Containing topo II Are Present in Drosophila K_c Tissue Culture Cell Extracts

The 130,000 g postnuclear particulate fraction from *Drosophila* K_c tissue culture cells was analyzed on sucrose gra-
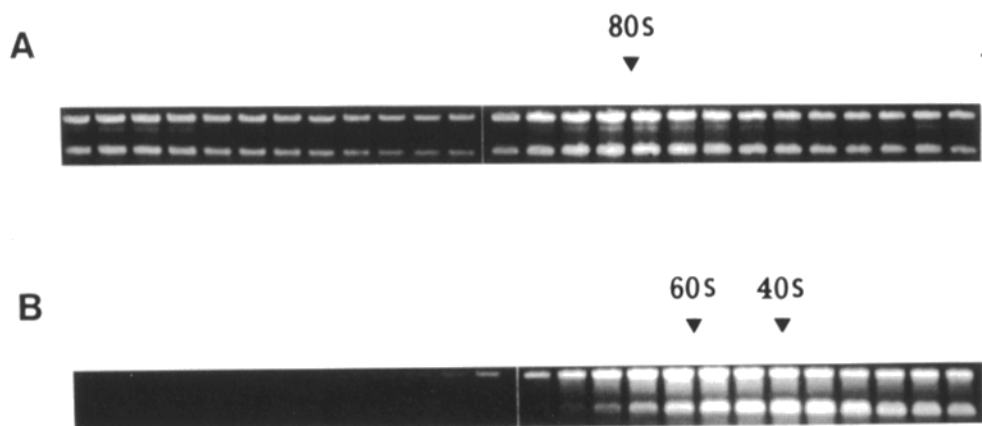

Figure 3. Sucrose velocity gradient analysis of nucleic acid from the 130,000 g particulate fraction of 0–2-h old *Drosophila* embryos. The particulate fraction from 2,250 embryos was separated on 10–30% sucrose gradients by centrifugation at 35,000 rpm for 1 h in the presence of either 5 mM $MgCl_2$ (*A*) or 5 mM EDTA (*B*). 12 µl of each fraction was subjected to phenol/chloroform extraction and electrophoresis on a nondenaturing 1.2% agarose gel; nucleic acid was detected by ethidium bromide staining. *T*, top of gradient. Downpointing arrowheads designate the sedimentation positions of 80-S monosomes, 60-S ribosomal subunits, and 40-S ribosomal subunits as indicated.

B   **80 S**   **T**
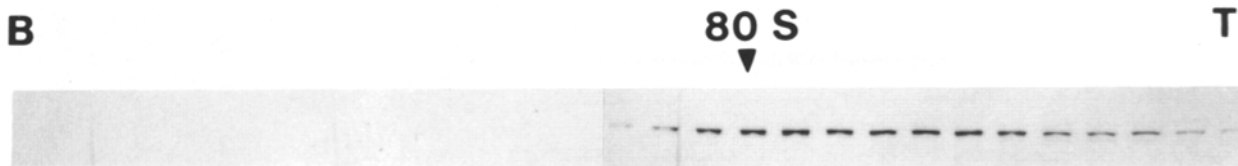
Figure 4. Sucrose velocity gradient analysis of *Xenopus* topo II. A low speed supernatant prepared from 500 progesterone-treated oocytes was fractionated on a 10–30% sucrose gradient at 35,000 rpm for 1 h. 7 μl of each fraction was subjected to immunoblot analysis using anti-*Xenopus* topo II antibodies. *T*, top of gradient; *B*, bottom of gradient. Downpointing arrowhead indicates sedimentation position of 80-S monosomes.

dients, exactly as described for embryo extracts. A topo II–containing particle of ∼68 S was identified (Fig. 8 *A*). Like topo II–containing particles from early embryos, those prepared from K_c cells were insensitive to DNase I (Fig. 8 *B*, lanes *5*), but could be at least partially disrupted by either RNase A (Fig. 8 *B*, lanes *4*) or micrococcal nuclease in the presence of 2 mM Ca^{2+} (Fig. 8 *B*, lanes *3*). topo II was not released by micrococcal nuclease in the absence of Ca^{2+}
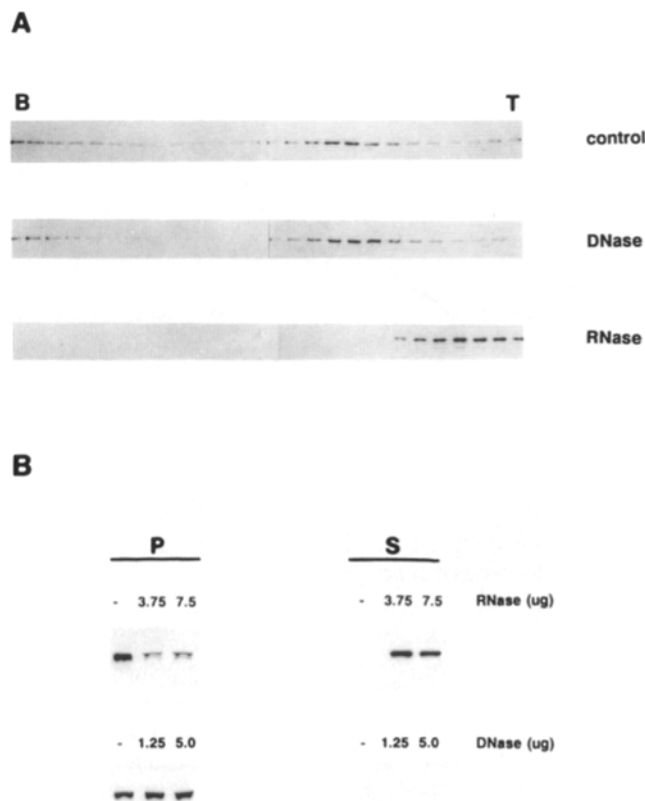
Figure 5. DNase I and RNase A treatment of *Drosophila* topo II–containing particles. (*A*) topo II profiles of gradients loaded with 130,000 *g* particulate fractions from 3,400 0–2-h old embryos, incubated for 30 min at 37°C in 125 μl digestion buffer (*control*), or with either 15 μg DNase I or 19 μg RNase A as indicated. Centrifugation, fractionation, and analysis were performed exactly as in Fig. 2. (*B*) RNase A and DNase I digestions were performed on particulate fractions from 360 embryos in a total volume of 50 μl. Pelletable material (*P*) was separated from supernatant (*S*) by centrifugation at 130,000 *g* for 30 min, and immunoblot analysis was performed. Each lane contains material derived from 65 embryos.

(Fig. 8 *B*, lanes *2*). Results similar to those obtained with *Drosophila* K_c tissue culture cells were also obtained with extracts of 16–19-h old embryos (not shown).

Chemical Cross-linking Analysis of topo II Quaternary Structure

Drosophila topo II was extractable from non-heat stabilized nuclei by solutions of elevated NaCl concentration (McConnell et al., 1987). Similarly, the association of topo II with 67-S particles prepared from 0–2-h old embryos could be disrupted by incubation with 300 mM NaCl (see Fig. 2). Extraction of topo II from 0–12-h old embryo nuclei and the 130,000 *g* particulate fraction from 0–2-h old embryos exhibited similar dependence on NaCl, occurring at concentrations ≥200 mM (not shown). At relatively low NaCl concentrations (50 mM), nuclear topo II was present in an extended structure which, after treatment with the cross-linking reagent, DSP, was detectable as a diffuse, high molecular mass smear upon immunoblot analysis (Fig. 9 *A*). As NaCl concentration was increased to ≥200 mM, DSP cross-linking resulted in quantitative production of what was apparently the topo II dimer. These results were consistent with solubilization studies; both indicate a change from participation in an extended quaternary structure at low salt concentrations (<150 mM) to existence in a soluble form of greatly reduced size, most likely the 330-kD homodimer, at higher salt concentrations (≥200 mM).

Comparison of the topo II products resulting from chemical cross-linking of 130,000 *g* particulate fractions or nuclei revealed essentially similar patterns which depended on the level of NaCl present (Fig. 9 *B*). At 50 mM NaCl, most of the topo II from both nuclei and particles was retained in the stacking gel, especially at the highest DSP levels. In the presence of 300 mM NaCl, the primary product of cross-linking had a mobility appropriate for the topo II homodimer, and the majority of immunoreactive topo II entered the separating gel even at the highest DSP concentrations. The mobility of this dimer increased at higher levels of cross-linking reagent. Under the conditions used in these experiments, nuclear and particulate topo II both appeared to participate in comparably complex quaternary structures, each of which was subject to dissolution by 300 mM NaCl.

Immunoblot analysis revealed that RNase A digestion of topo II–containing particles produced a similar effect to treatment with 300 mM NaCl before cross-linking with DSP (Fig. 9 *C*). In the absence of RNase A or 300 mM NaCl, the products of topo II cross-linking did not enter the separating gel (lanes *1–3*, see also the *blotted stacking gel region* of Fig. 9 *B*). As the RNase A concentration was in-

A

P   S

RNase

B

-	+	-	+	+	+		-	+	-	+	+	+	**MN**
-	-	+	+	+	-		-	-	+	+	+	-	**Ca^{2+}**
-	-	-	-	+	+		-	-	-	-	+	+	**EGTA**
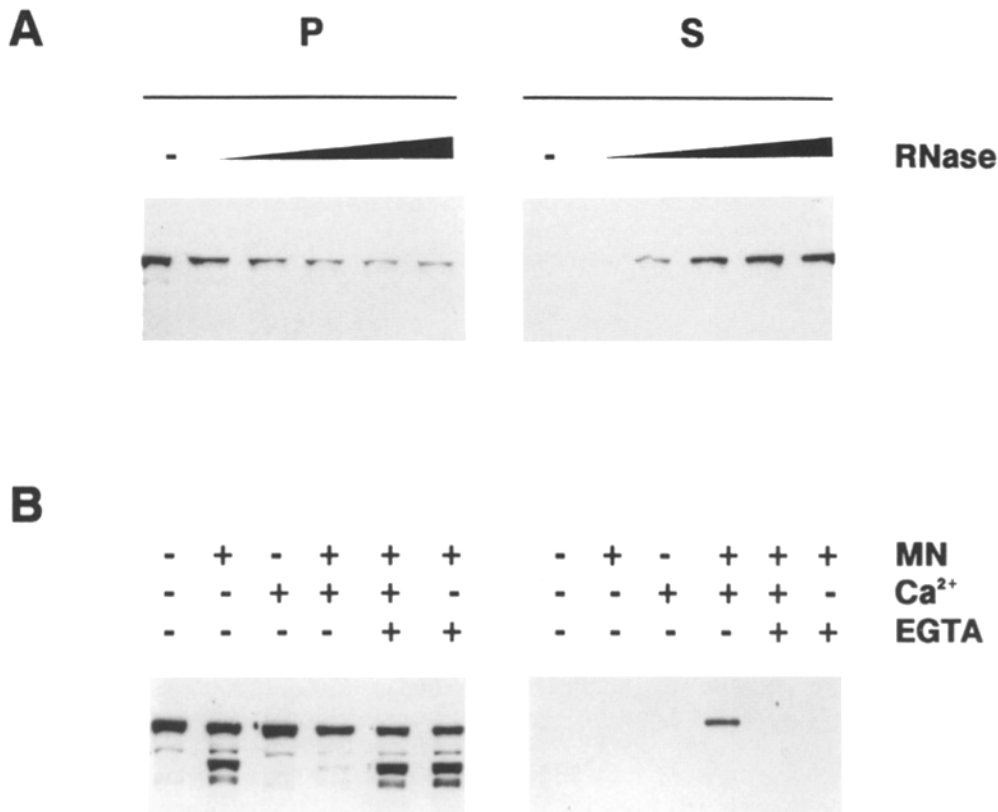
Figure 6. Nuclease sensitivity of topo II–containing particles. Pellet and supernatant fractions from each set of experiments are indicated on the top of A. (A) RNase A digestions using (*from left to right*) 0, 0.125, 0.25, 0.5, 1.0, and 2.0 μg of enzyme. (B) Pellets are to the left; supernatants are to the right. Ca^{2+} dependence of micrococcal nuclease (*MN*) release of topo II. Digestions contained 1.65 μg of nuclease, 2 mM Ca^{2+}, and/or 10 mM EGTA as indicated. Analysis is exactly as in Fig. 5 B.

p   s

a b c   a b c
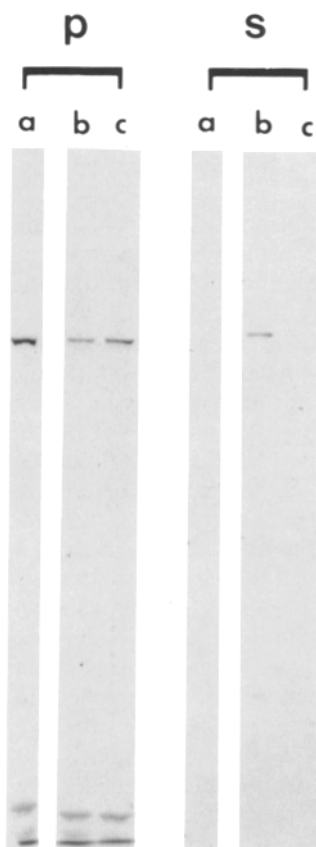
Figure 7. RNasin blocks RNase A-dependent solubilization of topo II from topo II–containing particles. RNase A was preincubated with either buffer alone or RNasin and dialyzed as described (Materials and Methods). Preincubated RNase A was used for particle digestion experiments exactly as described in the legend to Fig. 5 B. Pellet (*p*) and supernatant (*s*) fractions are as indicated. (Lanes *a*) No RNase A was added to incubations with topo II–containing particles; (lanes *b*) RNase A was preincubated with buffer alone; (lanes *c*) RNase A was preincubated with RNasin.

creased or after incubation with 300 mM NaCl, topo II mobility shifted to that expected for the cross-linked homodimer. When highly purified topo II was incubated with DSP only the topo II homodimer was seen (Fig. 9 D). As higher levels of DSP were used, the mobility of the cross-linked topo II dimer increased.

RNase-sensitive topo II-containing Particles from Early Embryos Lack U RNA as Well as Several Well-Characterized Nuclear Proteins

Dot-blot analysis was used to evaluate the possibility that topo II–containing particles from early *Drosophila* embryos contained U RNA. The 10,000 g supernatant from 0–2-h old *Drosophila* embryos was subjected to centrifugation at 130,000 g for 30 min; the resulting pellet fraction was resuspended, digested with DNase I, and then subjected to standard sucrose velocity gradient ultracentrifugation. Immunoblot analysis for topo II revealed a typical pattern of sedimentation with a peak at ~67 S (Fig. 10 A). Dot-blot analysis suggested that the vast majority of U RNA present in the 10,000 g supernatant from 0–2-h old *Drosophila* embryos remained in the supernatant fraction after 130,000 g centrifugation (not shown). This RNA was sensitive to digestion with micrococcal nuclease (see Fig. 10 B). That which was detectable in the 130,000 g pellet fraction remained largely at the top of the sucrose gradient run subsequently; U1 RNA did not cosediment with topo II (Fig. 10 C). Similar results were obtained with other U RNAs (not shown).
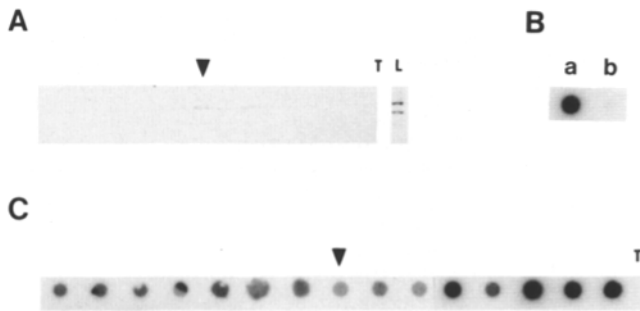
A

B

A

C

B

D

Figure 10. The 67-S *Drosophila* topo II–containing particle does not contain U1 RNA. The particulate fraction from 0–2-h old *Drosophila* embryos was separated on 10–30% sucrose gradients by centrifugation at 35,000 rpm for 1 h in the presence of 50 mM NaCl. Before centrifugation, samples were treated with DNase I as described in the legend to Fig. 5. *T*, top of gradient. *L*, load. (*A*) topo II profile from sucrose velocity gradient after immunoblot analysis; downpointing arrowhead indicates the peak fraction of topo II sedimentation. (*B*) Dot-blot hybridization showing U1 RNA from 4–5 embryos present in the 10,000 *g* supernatant fraction before (*a*) and after (*b*) treatment with 33 μg/ml micrococcal nuclease. (*C*) Dot-blot hybridization showing U1 RNA present in the same gradient fractions shown in *A*; downpointing arrowhead indicates the peak fraction of topo II sedimentation. Before analysis, gradient fractions were phenol/chloroform extracted according to Sambrook et al. (1989). *T*, top of gradient.

Immunoblots similar to those shown (see e.g., Fig. 10 *A*) were probed with anti-*Drosophila* PCNA antiserum (Ng et al., 1990), anti-*Drosophila* RNA polymerase II antibodies (Weeks et al., 1982; see also Fisher et al., 1989), monoclonal anti-histone antibody mAb052, autoimmune anti-human coilin antiserum (Andrade et al., 1993), and anti-*Xenopus* nucleolin antiserum (Heine et al., 1993). None of these proteins was detected in the particle (not shown).

Discussion

In early *Drosophila* embryos (0–2-h old), most of the topo II is extranuclear when localized immunocytochemically; it is recovered in the 10,000 *g* supernatant in the form of NaCl-sensitive, RNase A-sensitive 67-S particles. Identification of topo II–containing particles was previously reported (Noguchi et al., 1983; Jazwinski and Edelman, 1984). It is likely that the large pool of particulate topo II found in early *Drosophila* development results from maternally derived stores released upon germinal vesicle breakdown at the end of oogenesis. Similar topo II–containing particles were found in extracts of both K$_c$ tissue culture cells and older embryos, although particulate topo II comprised a much smaller fraction of the cellular total at these stages (see Fig. 1; see also Whalen et al., 1991). The current analysis of the distribution of topo II during embryogenesis corroborates an earlier, more extensive developmental study (Whalen et al., 1991), which showed that a relatively constant amount of topo II was found in the 10,000 *g* supernatant, while the amount associated with the 10,000 *g* pellet increased as embryogenesis proceeded. Current results indicate that virtually all of the topo II which is found in the 10,000 *g* supernatant throughout embryogenesis can subsequently be recovered in a 130,000 *g* pellet derived from the 10,000 *g* supernatant.

topo II was localized to both nuclear and extranuclear compartments of the *Drosophila* syncytial blastoderm (Buchenau et al., 1993; Swedlow et al., 1993), and cell cycle-dependent shuttling of topo II between nucleus and cytoplasm has also been documented (Swedlow et al., 1993). The localization of the 67-S topo II–containing particle is unknown, but the potential exists for both cytoplasmic and nuclear localization. The function of these large, topo II–containing particles is unclear, but their persistence throughout embryogenesis and presence in K$_c$ tissue culture cells suggests functions in addition to providing storage for topo II solely during early development. A similar (75 S) topo II–containing particle was identified in extracts of progesterone-matured *X. laevis* oocytes, suggesting that participation of topo II in extrachromosomal structures may be common.

Treatment of 67-S topo II–containing particles from early embryos with either 300 mM NaCl or RNase A leads to recovery of topo II in a 9-S form. Sedimentation values of 9.2 S (Shelton et al., 1983) and 10 S (Sander and Hsieh, 1983) have been reported for the purified *Drosophila* topo II homodimer. Chemical cross-linking of particulate topo II after treatment either with 300 mM NaCl or RNase A leads to detection of a single cross-linked product with a mobility appropriate for the homodimer. Together, these data suggest that the integrity of the particle is due solely to the organizing effect of RNA on topo II. In contrast to sensitivity to RNase A, *Drosophila* topo II–containing particles were insensitive to DNase I, and appeared to contain no high molecular mass DNA, as determined by agarose gel electrophoresis (Meller, V. H., unpublished observations). topo II from *S. pombe* was shown to bind cooperatively to SAR-containing DNA (Adachi et al., 1989); however, the absence of chromatin in the 67-S particle indicates that *Drosophila* topo II can participate in an extended, quaternary structure which does not require the organizing influences of DNA or chromatin.

Chemical cross-linking followed by immunoprecipitation with anti-topo II antibodies, reversal of cross-linking and SDS gel electrophoresis failed to yield evidence of any protein associated with topo II in either the nucleus or in particles (Meller, V. H., unpublished observations). This, coupled with the fact that RNA appears to provide the structural basis for particle integrity, suggests that topo II may not frequently be involved in protein–protein interactions, other than dimerization, in either the particle or in its proposed role as a chromosome scaffold protein.

Initial results from our laboratory have shown that a portion of nuclear topo II can be released by RNase A digestion (Meller, V. H., and P. A. Fisher, manuscript in preparation), suggesting that RNA-topo II interactions are widespread, occurring in the nucleus as well as in the 67-S particle. The interaction of a well-characterized DNA-binding protein such as topo II with RNA was unexpected, but not unprecedented. The transcription factor TFIIIA associates with both specific DNA sequences coding for 5 S rRNA, and with the 5-S rRNA itself to form a storage particle unique to oogenesis (Honda and Roeder, 1980; Pelham and Brown, 1980). The specificity of TFIIIA binding to DNA vs RNA has been attributed to different subsets of the 9 zinc fingers of the TFIIIA protein (Bogenhagen, 1993; Clemens et al., 1993). We currently have no understanding of the regions of topo II responsible for interaction with RNA, the nature of the RNA(s) involved in particle formation, or the possibility of

concurrent DNA and RNA binding. The answers to these questions will be essential to fully understanding topo II–nucleic acid interactions.

The association of other proteins with *Drosophila* topo II–containing particles is of immediate importance. Data shown in Fig. 2 indicate that neither lamin nor gp210 are associated with the particles. Similar immunoblot experiments were used to exclude significant participation of RNA polymerase II subunits, proliferating cell nuclear antigen, histones, coilin, or nucleolin. However, low-level participation of these proteins cannot be ruled out. If other proteins are present, we anticipate that they will be components of a functional complex whose activity requires topological manipulation of DNA, i.e., replication or transcription.

Also of immediate importance is identification of the RNA(s) associated with *Drosophila* topo II–containing particles. Initial attempts to do so were frustrated by the large amounts of ribosomal RNA present in fractions enriched for topo II–containing particles (see e.g., Fig. 3). In addition, use of U RNA-specific hybridization probes indicated that the particle lacked U RNA (see Fig. 10). At this time, it seems that complete particle purification (e.g., away from ribosomal contaminants) will be required to positively identify the RNA molecule(s) responsible for *Drosophila* topo II–containing particle integrity. Such purification is underway.

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