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In situ localization of DNA topoisomerase II, a major polypeptide component of the *Drosophila* nuclear matrix fraction*

(nuclear lamina/nuclear pore complex/DNA replication/transcription)

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ABSTRACT **DNA topoisomerase II has been immunochemically identified on protein blots as a major polypeptide component of the *Drosophila* nuclear matrix–pore complex–lamina fraction. Indirect immunofluorescence analyses of larval cryosections have confirmed the nuclear localization of topoisomerase II *in situ*. Although apparently excluded from the nucleolus, the topoisomerase protein is otherwise distributed throughout the interior of interphase nuclei. Similar immunocytochemical studies performed with permeabilized whole giant cells from third-instar larval salivary glands have shown topoisomerase II to be largely restricted to the polytene chromosomes. Upon nuclear disassembly during mitosis, the topoisomerase polypeptide appears to redistribute diffusely throughout the cell. Faint immunofluorescent staining of mitotic chromosomes is also observed.**

The *Drosophila melanogaster* embryo nuclear matrix–pore complex–lamina (NMPCL) fraction is operationally defined as that material which remains insoluble (pellets at 10,000 × *g*) during sequential treatments with RNase, DNase, 2% Triton X-100, and 1 M NaCl (1). By phase-contrast microscopy, NMPCL components resemble unfractionated nuclei. However, they are almost entirely devoid of DNA, RNA, phospholipid, and histones. By transmission electron microscopy, these nuclear remnants appear as ovoid or spherical structures surrounded by a limiting layer of fibrous material presumably derived from the nuclear lamina. They also contain an internal meshwork termed the nuclear matrix. Nucleolar remnants can be readily identified within the matrix, while higher-magnification examination of the NMPCL periphery reveals the presence of nuclear pore complexes apparently attached to the lamina. The *Drosophila* NMPCL is morphologically indistinguishable from fractions similarly derived from a variety of vertebrate nuclei (2–5). It is also similar to the rat liver nuclear pore complex–lamina fraction with regard to elements of the nuclear periphery (6–8).

When analyzed by one-dimensional $NaDodSO_4$/PAGE, the *Drosophila* NMPCL is heterogeneous (1). Two major polypeptides, migrating as a closely spaced doublet in the 74- to 76-kDa region of the gel, have been shown to be antigenically related and to be specifically localized to the nuclear envelope *in situ* (9). These polypeptides are apparently homologous to the three vertebrate lamins, A, B, and C (10, 11). A third major NMPCL band, migrating in the 170- to 190-kDa region of the $NaDodSO_4$/polyacrylamide gel, has been shown to be composed of two immunochemically distinct polypeptides of nearly identical molecular weight (12). One, identified as an ATPase/dATPase by direct UV photoaffinity labeling (13), has not yet been localized *in situ*. The other, a glycoprotein (1, 12), has been shown to be a specific component of the *Drosophila* nuclear envelope (14) and appears to be homologous to the 190-kDa rat liver nuclear pore complex glycoprotein (15). To date, no quantitatively major NMPCL polypeptide has been definitively localized to the nuclear interior *in situ*.

Recent work in our laboratory on NMPCL ATPases (12, 13) has led to the consideration of DNA topoisomerase II. Type II DNA topoisomerases catalyze the relaxation of superhelical turns in topologically constrained DNA by a double-strand breakage and rejoining mechanism that is dependent on the hydrolysis of ATP (16–18). DNA topoisomerase II has recently been purified to apparent homogeneity from *Drosophila melanogaster* embryos (19, 20). It has been shown to be a homodimer of a 166-kDa (19) to 170-kDa (20) subunit, and the catalytic activity has been studied in detail (19–23). Using an antiserum prepared against the near-homogeneous enzyme (19), we have been able to quantitate the distribution of the topoisomerase polypeptide during the preparation of the *Drosophila* embryo NMPCL. We have also been able to examine the localization of this enzyme *in situ* by indirect immunofluorescence. The results of these studies provide information on the polypeptide composition of the internal nuclear matrix and suggest a structural perspective from which to consider the biologic role of type II DNA topoisomerases.

MATERIALS AND METHODS

The sources of the materials and most of the methods have been described previously (1, 9, 14). Monoclonal antibody T-40 (24), directed against the major 74- and 76-kDa *Drosophila* NMPCL polypeptides, was the generous gift of Peter Symmons (Tübingen). Rabbit antiserum to 74- and 76-kDa NMPCL polypeptides was prepared against gel-purified antigen (1); rabbit antiserum to DNA topoisomerase II was prepared against a near-homogeneous preparation of the active enzyme (19). *Drosophila melanogaster* (Oregon R, P2 strain) were grown in mass culture and embryos were collected according to Allis *et al.* (25). The NMPCL fraction was prepared from *Drosophila* embryos as previously described (1). Antibodies were affinity purified by using antigens immobilized on nitrocellulose after blot transfer from $NaDodSO_4$/polyacrylamide gels (9). $NaDodSO_4$/PAGE was according to Laemmli (26) as modified (1, 12) on 7% (wt/vol) polyacrylamide gels. Nitrocellulose blot replicas of $NaDodSO_4$/polyacrylamide gels prepared as previously (1) were probed and developed also as described (9). Indirect immunofluorescence analysis of whole *Drosophila* cells and tissues and salivary gland polytene chromosomes was performed as previously (1), as was analysis of larval cryosections (9). Partial purification of the denatured DNA topoisomerase II polypeptide from the *Drosophila* NMPCL fraction was as recently described for the putative nuclear

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Abbreviation: NMPCL, nuclear matrix–pore complex–lamina.
*An abstract of this work has recently been published (42).
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pore complex glycoprotein (14). DNA topoisomerase II was purified to homogeneity in active form from nuclear extracts of *Drosophila melanogaster* embryos (19). For antibody-dependent activity neutralization studies, a typical assay (23) included 1 unit of topoisomerase and 0.6 μg of supercoiled pBR322 DNA in a final reaction volume of 20 μl containing 1 mM ATP, 10 mM Tris·HCl at pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and bovine serum albumin at 15 μg/ml. Incubations were for 6 min at 30°C. Samples were analyzed by electrophoresis on 1% (wt/vol) agarose gels run at 5 V/cm in 100 mM Tris borate, pH 8.3/2 mM EDTA. Gels were stained with ethidium bromide and photographed under UV illumination.

RESULTS

$NaDodSO_4$/PAGE and quantitative protein blot analyses of the subcellular fractions generated during the purification of nuclei indicated that in 6- to 18-hr-old embryos, approximately 50% of the 166-kDa topoisomerase II polypeptide was nuclear; the remaining 50% was apparently cytoplasmic. Blot analyses of the various fractions generated during the preparation of the *Drosophila* NMPCL from purified nuclei are shown in Fig. 1. The immunoreactive 166-kDa topoisomerase polypeptide, readily detectable in the nuclear fraction (Fig. 1*A*, lane N), was not affected by exhaustive digestion with DNase I and RNase A (lane DN). Its association with the insoluble subnuclear fraction was resistant to the nuclease treatment and to extraction with nonionic detergent (lane NS + TXS). This association was largely (approximately 75%) but not completely resistant to disruption with 1 M NaCl (lane SS-1 + SS-2). The putative topoisomerase subunit was somewhat more resistant to salt extraction than the 74- and 76-kDa NMPCL polypeptides and slightly less resistant than the putative pore complex glycoprotein (14). Histones, in contrast, were fully solubilized in 1 M NaCl after nuclease treatment (1). A Coomassie blue-stained $NaDodSO_4$/polyacrylamide gel electropherogram of the NMPCL fraction demonstrated that a quantitatively major polypeptide was coincident in $NaDodSO_4$/PAGE mobility with the putative topoisomerase subunit seen by blot analysis (compare Fig. 1 *A* and *B*, lanes NMPCL).

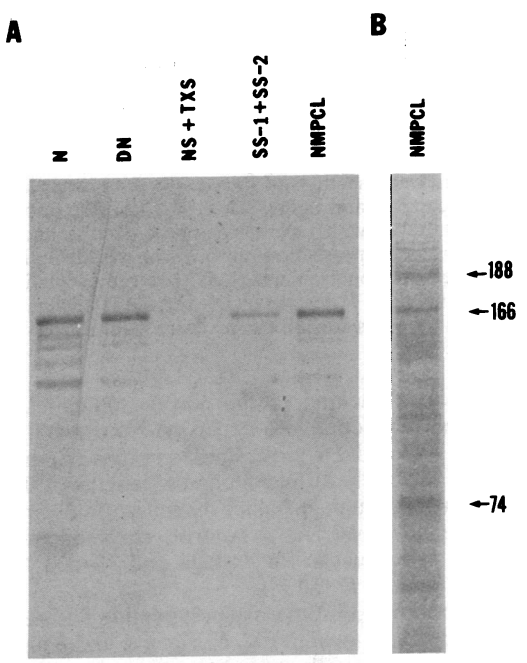

FIG. 1. Distribution of DNA topoisomerase II during subfractionation of purified *Drosophila* embryo nuclei. (*A*) Blot of fractions generated during the preparation of the NMPCL probed with rabbit anti-topoisomerase antiserum. Five units (1) of the respective fractions were loaded in each lane of the original gel. The serum was diluted 1:100 prior to probing the blot. Lane designations indicate the fractions loaded on the original gel: N, nuclei; DN, digested nuclei; NS, nuclease supernatant; TXS, Triton X-100 supernatant; SS-1, first salt wash supernatant; SS-2, second salt wash supernatant; NMPCL, nuclear matrix–pore complex–lamina fraction. (*B*) Coomassie blue-stained gel lane loaded with 20 units of the NMPCL fraction and electrophoresed in parallel with the segment used to generate the blot shown in *A*. Molecular masses are indicated in kDa.

The multiplicity of minor polypeptide species that were recognized by the anti-topoisomerase antiserum raised concerns regarding the identification of the major 166-kDa polypeptide as the topoisomerase subunit. Therefore, we first used the homogeneous 166-kDa topoisomerase polypeptide (19) to affinity purify anti-topoisomerase IgG from the unfractionated antiserum. This affinity-purified IgG was compared with the original antiserum in terms of reactivity with the NMPCL (Fig. 2). Serum (Fig. 2, lane S) and affinity-purified IgG (lane TOPO) gave similar patterns of reactivity. As an additional control, IgG was eluted from the 166-kDa polypeptide band shown in lane TOPO and used to probe a third blot (Fig. 2, lane NM). [This anti-topoisomerase IgG was therefore subjected to two cycles of affinity purification, first using the homogeneous 166-kDa topoisomerase subunit purified from the nuclear extract (19) as the affinity adsorbant and then using the immunoreactive 166-kDa NMPCL polypeptide.] Within the sensitivity limit of the analysis, the pattern of immunoreactivity seen with this twice-affinity-purified IgG was identical with that seen with the unfractionated serum (lane S).

The unfractionated anti-topoisomerase antiserum used for these studies was found to have neutralizing activity when added to a conventional, ATP-dependent, supercoiled DNA-relaxation assay of topoisomerase II. The anti-topoisomerase IgG, affinity purified with the NMPCL 166-kDa polypeptide used as the adsorbant, was also able to specifically neutralize DNA topoisomerase II activity (Fig. 3). Identical neutralization data were obtained when ATP-dependent decatenation of kinetoplast DNA was used as an alternative assay of topoisomerase II activity (data not shown).

Two additional steps of protein fractionation were performed to demonstrate that the major 166-kDa NMPCL

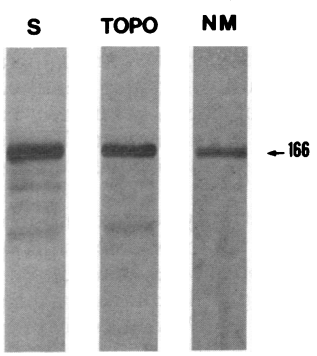

FIG. 2. Blot analysis of topoisomerase II polypeptides associated with the NMPCL fraction. Blots of the NMPCL fraction were prepared by electrophoresing 10 units in each lane of a gel. Lane S was probed with unfractionated antiserum diluted 1:1000. Lane TOPO was probed with IgG affinity purified from 20 μg of the homogeneous 166-kDa topoisomerase II polypeptide immobilized on nitrocellulose after $NaDodSO_4$/PAGE. Lane NM was probed with the IgG eluted from the 166-kDa band of a single nuclear matrix lane prepared and probed with affinity-purified anti-topoisomerase IgG in parallel with the one shown in lane TOPO.

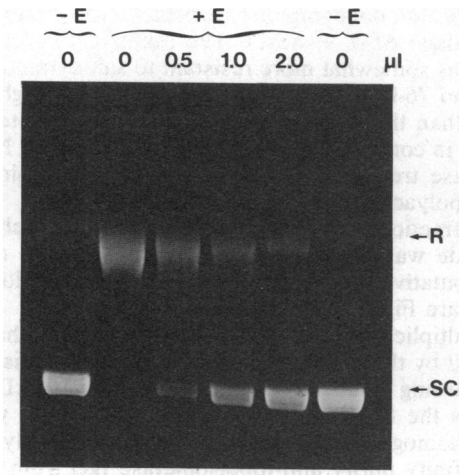

FIG. 3. IgG affinity purified from the 166-kDa NMPCL DNA topoisomerase II polypeptide neutralizes the enzymatic activity of purified DNA topoisomerase II. The NMPCL fraction (200 units) was electrophoresed on a preparative (no individual wells) NaDodSO$_4$/polyacrylamide gel and the proteins were transferred to nitrocellulose. The blot was probed with serum as in Fig. 2, lane S. IgG was eluted from the 166-kDa band, concentrated by ammonium sulfate precipitation, and dissolved in 100 mM NaCl/10 mM Tris·HCl, pH 7.9/in a final volume of 30 μl. A blank piece of nitrocellulose was "eluted" in parallel and the eluate was similarly processed to provide a control buffer. The affinity-purified IgG or a compensatory aliquot of the control buffer was added to a standard topoisomerase reaction mixture and preincubated with the enzyme (+E) for 3 min in the absence of substrates (ATP and supercoiled DNA). Amounts of IgG (μl) added are as indicated above each lane. −E, controls without topoisomerase. The migration positions of the supercoiled (SC) and relaxed (R) circular DNA molecules are indicated to the right.

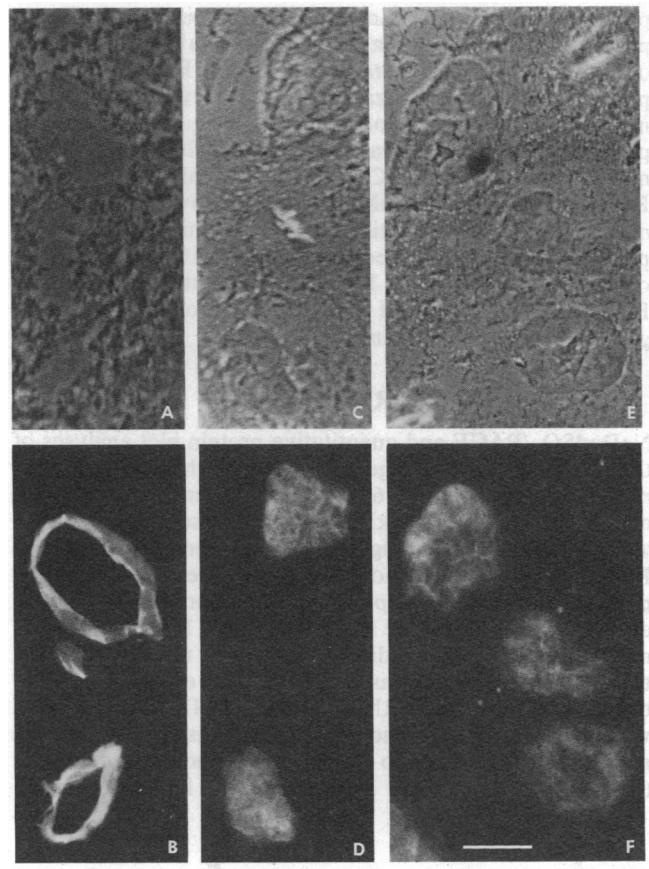

FIG. 4. Immunocytochemical localization of the DNA topoisomerase II polypeptide in *Drosophila* larval cryosections. Monoclonal antibody T-40 directed against the 74- and 76-kDa NMPCL nuclear envelope polypeptides, and immunoaffinity-purified anti-topoisomerase IgG, prepared as described in Fig. 3, were used to probe 6-μm-thick cryosections of *Drosophila* third instar larvae. (*A* and *B*) Phase-contrast and fluorescence micrographs, respectively, of the sections probed with T-40. (*C–F*) Phase-contrast and fluorescence micrographs of cryosections probed with a 1:10 dilution of the affinity-purified anti-topoisomerase IgG; two representative fields are shown. The bar in *F* designates 10 μm and is applicable to all panels. Exposure times for the fluorescence micrographs in *B*, *D*, and *F* were 45 sec.

polypeptide seen by Coomassie blue staining after one-dimensional NaDodSO$_4$/PAGE was, in fact, the immunoreactive topoisomerase subunit. We have previously shown differential urea extraction to be an effective means of separating *Drosophila* NMPCL polypeptide components (14). Using this approach, we were able to demonstrate that the solubilization of the majority species of the 166-kDa polypeptide visualized by Coomassie blue staining paralleled the solubilization of the topoisomerase antigen as shown by blot analysis (data not shown). Further correlation of the major 166-kDa Coomassie blue-stainable NMPCL polypeptide with the immunoreactive topoisomerase subunit was obtained by using NaDodSO$_4$/hydroxylapatite chromatography (12, 14). A single major 166-kDa polypeptide, identified by Coomassie blue staining of a NaDodSO$_4$ gel of hydroxylapatite eluate fractions, was found to chromatograph with the immunoreactive topoisomerase II polypeptide identified on blots (data not shown).

NMPCL antigen–affinity-purified anti-topoisomerase IgG was used to localize the topoisomerase polypeptide *in situ*. Indirect immunofluorescence analyses of larval cryosections are shown in Fig. 4. In contrast to the peripheral localization of the 74-kDa and 76-kDa nuclear envelope polypeptides shown in Fig. 4 *A* and *B* (9, 14), the topoisomerase was detected virtually throughout the nuclear interior; nucleoli stained relatively less intensely (Fig. 4 *C–F*).

Our analysis of topoisomerase II localization was extended to the giant cells of third instar larval salivary glands. Cryosections of salivary gland cells showed diffuse intranuclear staining similar to that seen in Fig. 4 *D* and *F* (data not shown). However, whole cell squash preparations demonstrated that DNA topoisomerase II in the giant cell nuclei was largely restricted to the polytene chromosomes (Fig. 5 *A* and *B*). (This could not be appreciated in the cryosections due to the close packing of the chromosomes.) Chromosomes probed after complete extrusion from the fixed cell nuclei (Fig. 5 *C* and *D*) gave similar results. Staining appeared to be relatively diffuse along the lengths of the chromosome arms, although at higher magnification some banding could be observed. As a control, chromosomes were probed with IgG specific for 74-kDa and 76-kDa NMPCL polypeptides (Fig. 5 *E* and *F*).

Location of the topoisomerase polypeptide during mitosis was determined as shown in Fig. 6. Fig. 6 *A* and *B* shows the results obtained with antiserum directed against the 74- and 76-kDa nuclear envelope polypeptides. As has previously been reported for the rat liver lamins (10, 11, 22), the 74- and 76-kDa *Drosophila* NMPCL polypeptides become diffusely localized throughout mitotic cells. As shown in Fig. 6 *C* and *D*, similar results were obtained when mitotic cells were probed with the affinity-purified antibodies against topoisomerase. Faint staining of mitotic chromosomes could also be detected.

DISCUSSION

An antiserum prepared against purified *Drosophila* DNA topoisomerase II has been used to identify the topoisomerase polypeptide in subcellular fractions of *Drosophila melano-*

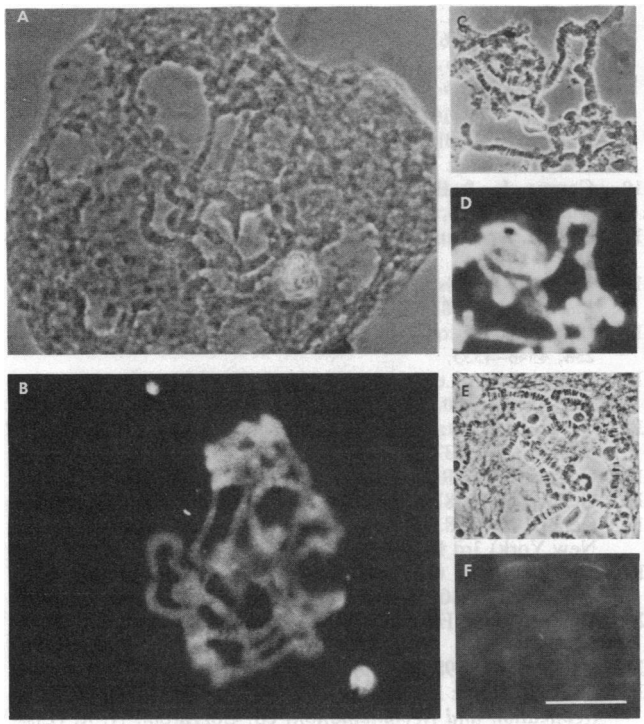

FIG. 5. Immunocytochemical localization of the DNA topoisomerase II polypeptide in *Drosophila* salivary gland giant cell polytene chromosomes. Salivary gland cells were squashed and probed with affinity-purified IgG fractions. (*A* and *B*) Phase-contrast and fluorescence micrographs, respectively, of a squashed cell probed with a 1:10 dilution of affinity-purified anti-topoisomerase IgG as in Fig. 4. (*C* and *D*) Micrographs of fully extruded chromosomes probed similarly. (*E* and *F*) Micrographs of chromosomes probed with affinity-purified IgG specific for 74- and 76-kDa NMPCL polypeptides. Exposure times for the fluorescence micrographs in *B* and *D* were 45 sec; the exposure in *F* was 4 min. The bar in *F* designates 50 μm and is applicable to all panels.

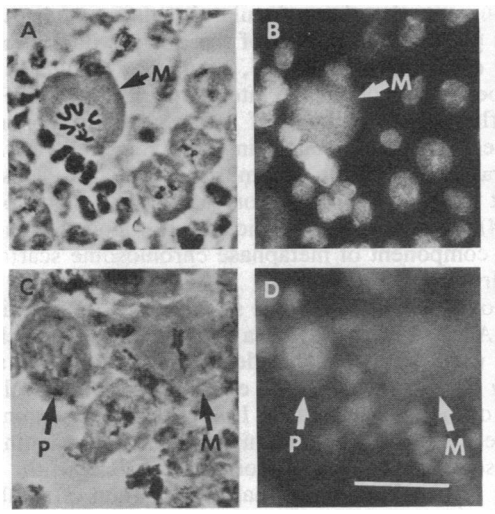

FIG. 6. Immunocytochemical localization of the DNA topoisomerase II polypeptide in mitotic cells from the *Drosophila* larval neural ganglion. Permeabilized neural ganglion cells were probed as in Fig. 5. (*A* and *B*) Orcein stain and fluorescence micrographs, respectively, of a field probed with unfractionated anti-74- and 76-kDa polypeptides antiserum diluted 1:200. The fluorescence micrograph in *B* was from a 30-sec exposure. The large cell designated M in *A* and *B* appears to be in metaphase; fluorescence staining is seen diffusely throughout this cell (*B*). (*C* and *D*) Orcein and fluorescence micrographs, respectively, of a field probed with affinity-purified anti-topoisomerase II IgG diluted 1:10 as in Fig. 5. The fluorescence micrograph in *D* was from a 90-sec exposure. The large cell designated M in *C* and *D* appears to be in metaphase; fluorescence staining is seen diffusely throughout this cell (*D*). Faint staining of mitotic chromosomes can also be observed. The cell designated P appears to be in mitotic prophase. Partially condensed chromosomes are seen in *C*; the nucleus is still identifiable by fluorescence staining but the cytoplasm also stains (*D*). The bar in *D* designates 25 μm and is applicable to all of the panels.

gaster embryos. Two pools of this protein have been found. A "cytoplasmic" fraction remains to be further characterized. The nuclear fraction has been studied in detail. Upon subfractionation of nuclei, the majority of the associated topoisomerase copurifies with the NMPCL. In this respect, it behaves similarly to the major *Drosophila* NMPCL proteins characterized previously. Further, topoisomerase II has been shown to be similar to these other polypeptides in terms of abundance in the NMPCL fraction. This conclusion has been substantiated through correlation of polypeptide identity with immunoreactivity, using both differential urea extraction and $NaDodSO_4$/hydroxylapatite chromatography. These results, in conjunction with the activity neutralization data shown in Fig. 3, establish DNA topoisomerase II as a major component of the *Drosophila* NMPCL fraction.¶

In contrast with other major NMPCL components studied to date, DNA topoisomerase II has been found to be diffusely localized throughout the nucleus. No major NMPCL polypeptide has previously been identified as a specific component of the nuclear interior. Internal nuclear matrix localization may also be the case in other species, since it has recently been reported that approximately 60% of the cellular topoisomerase II is associated with nuclear matrix fractions isolated from a variety of vertebrates (27).

In the giant cell nuclei of the *Drosophila* larval salivary gland, DNA topoisomerase II is apparently restricted to the polytene chromosomes. Thus, it seems that in this specialized cell, topoisomerase II is associated with a structure that is topologically coextensive with chromosomes. Although the distribution of topoisomerase II along the polytene chromosomes is not entirely homogeneous, it is sufficiently so as to preclude simple or immediate conclusions regarding the significance of the banding pattern seen. Using identical polytene chromosome preparations, we have been able to identify another NMPCL component of low abundance that is specifically localized only to a small group of individual bands (unpublished observation). It therefore seems unlikely that diffuse localization of DNA topoisomerase II is simply an artifact of poor chromosome preservation. The diffuse distribution of DNA topoisomerase II is in contrast with the recent observation that DNA topoisomerase I is associated primarily with transcriptionally active regions of *Drosophila* polytene chromosomes (28).

During mitosis, the topoisomerase polypeptide appears to be redistributed throughout the mitotic cell. In this respect, it is similar to the well-characterized nuclear envelope components from the *Drosophila* NMPCL as well as the rat liver lamins (10, 29) and nuclear pore complex glycoprotein (15).

¶The identification of topoisomerase II as a NMPCL component was unanticipated. Topoisomerase II has previously been regarded as a soluble enzyme and has been purified as such from *Drosophila* embryos (19, 20). However, our current observations are not inconsistent with this. First, it is likely that the cytoplasmic antigen identified in the present analysis is the source of at least some of the soluble topoisomerase activity purified by Sander and Hsieh (20) from whole embryo extracts. It may also be significant that the specific conditions used by Shelton *et al.* (19) to extract nuclear topoisomerase II are qualitatively different from those used to prepare the NMPCL fraction, and they result in nuclear lysis and release of chromatin. Preliminary results indicate that they also lead to destabilization of the NMPCL structure.

This suggests that the molecular dissolution and reassembly of the peripheral elements of the nucleus during mitosis in higher eukaryotes (10, 11, 15, 29) may be paralleled by similar processes involving the internal nuclear matrix. Faint immunofluorescent staining of metaphase chromosomes has also been observed, suggesting involvement of DNA topoisomerase II in mitotic chromosome structure. This is consistent with recent observations of Earnshaw and colleagues (43, 44) regarding the identification of topoisomerase II as a major component of metaphase chromosome scaffolds isolated from vertebrate cells.

In conclusion, it should be stressed that the identification of DNA topoisomerase II as a component of the nuclear matrix *in vitro* cannot be considered proof of such an association *in vivo*. Neither does it establish the biological significance of the nuclear matrix. It is possible, for example (the complete solubilization of histones notwithstanding), that the association of DNA topoisomerase II with the nuclear matrix results from artifactual precipitation of soluble protein during cell fractionation. However, even in view of these reservations, there are a number of implications to the observation that DNA topoisomerase II is a major nuclear matrix component *in vitro*. The duplex DNA binding site of the topoisomerase seems ideally suited for anchoring and regulating the topology of chromosome loops (30–33). Roles for type II topoisomerases in chromosomal replication, segregation, and transcription, events that have previously been associated with the nuclear matrix (see, e.g., refs. 3 and 34–37), have also been proposed (38–41). [With respect to transcription, it is perhaps significant that, unlike DNA topoisomerase I (28), DNA topoisomerase II does not appear to be specifically localized to regions of increased transcriptional activity on the polytene chromosomes.] Roles in chromosome condensation and decondensation during mitosis seem plausible. From a structural perspective, the use of topoisomerase II as a marker for the internal matrix, in conjunction with markers specific for the nuclear lamina and pore complexes, may now allow direct investigation of the molecular interactions among these three topological "subdomains" of isolated NMPCL structures. It is similarly reasonable to approach the biochemistry of nuclear assembly and disassembly with respect to DNA topoisomerase II as well as with respect to the lamins and the pore complex glycoprotein. It is likely that information forthcoming from such experiments will ultimately be important for a detailed biological understanding of the NMPCL fraction and, perhaps, of DNA topoisomerase II as well.

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