Developmental Regulation of *Drosophila* DNA Topoisomerase II

Anne M. Whalen, Maeve McConnell, and Paul A. Fisher

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

Abstract. Affinity-purified polyclonal antibodies were used to quantitate steady-state levels of DNA topoisomerase II (topo II) throughout *Drosophila* development. Although wide fluctuations were recorded at different stages, these fluctuations were paralleled by changes in levels of the nuclear lamin, a nuclear structural protein used as an internal standard. The exception to this was adult males where lamin levels were significantly elevated relative to topo II. Northern blot analyses of topo II and lamin mRNA, performed in conjunction with immunoblot analyses of protein revealed fluctuations in levels of the two different messages that paralleled changes in each other and in their respective translation products. Biochemical and immunochemical analyses were complemented by indirect immunofluorescence and immunoperoxidase experiments performed in situ. topo II was found distributed throughout nuclei in most but not all cell types examined. These results for *Drosophila* topo II are apparently at odds with those obtained by others working in vertebrate systems (see, for example, Heck, M. M. S. and W. C. Earnshaw. 1986. *J. Cell Biol.* 103:2569–2581; Heck, M. M. S., W. N. Hittelman, and W. C. Earnshaw. 1988. *Proc. Natl. Acad. Sci. USA.* 85:1086–1090) and suggest that in *Drosophila*, topo II may not be a useful marker for the proliferative state.

DNA topoisomerase II (topo II)[1] is an enzyme that in vitro, catalyzes the ATP-dependent double-stranded breakage and rejoining of duplex DNA (for review, see Wang, 1985). In vivo, the function or functions of topo II are not fully understood (for a review, see Sternglanz, 1989). Genetic studies in both *Saccharomyces cerevisiae* (DiNardo et al., 1984; Holm et al., 1985) and *Schizosaccharomyces pombe* (Uemura and Yanagida, 1984) demonstrated that topo II was essential; cells lacking enzymatically active topo II arrested in mitosis and subsequently died. It was suggested that this might be due to the inability of cells deficient in topo II to resolve multiply intertwined DNA catenanes resulting from termination of replication (see also, Sundin and Varshavsky, 1980; Sundin and Varshavsky, 1981). Other results indicate that topo II may be important in transcription (Brill et al., 1987; Brill and Sternglanz, 1988).

It also has been suggested that topo II plays a structural (karyoskeletal) role in organizing both mitotic chromosomes and interphase nuclei (for a review, see Fisher, 1988). topo II is a major component of mitotic chromosomes and mitotic chromosome scaffold fractions (Earnshaw et al., 1985; Gasser et al., 1986) as well as interphase-derived karyoskeletal protein-enriched fractions from *Drosophila* (Berrios et al., 1985; McConnell et al., 1987) and yeast (Berrios and Fisher, 1988; Cardenas et al., 1990). Moreover, specific DNA-scaffold attachment sites mapped in several systems have been found to be enriched in the consensus cleavage sequence defined by Sander and Hsieh (1985) for *Drosophila* topo II (for reviews, see Mirkovitch et al., 1987; Fisher, 1989).

Recently, the finding that nondividing vertebrate cells, i.e., those in phase G_0 of the cell cycle, apparently lack topo II (Heck and Earnshaw, 1986; Heck et al., 1988) raised concerns about the generality of topo II's role in organizing the interphase nucleus. Results of a developmental survey performed in *Drosophila* (Fairman and Brutlag, 1988) were interpreted as being consistent with the hypothesis that topo II was either absent or substantially reduced in nondividing cells of this organism as well. However, our initial impressions based on indirect immunofluorescence analyses (Berrios et al., 1985; McConnell et al., 1987) were contrary to the notion that *Drosophila* topo II was absent from any particular cell or cell type in third instar larvae, a stage at which many of the cells would be expected to be nondividing. Moreover, the developmental fluctuations in topo II levels reported by Fairman and Brutlag (1988) were reminiscent of those we had observed for *Drosophila* lamins (unpublished data). In the case of lamins, these fluctuations seemed to reflect inversely, fluctuations in the levels of nonnuclear proteins (e.g., muscle proteins such as actin and myosin) and correlated directly with the relative reduction in the numbers of nuclei as reflected by the decreasing DNA/protein ratio (Robertson, 1978).

To investigate further the expression of *Drosophila* topo II

1. *Abbreviations used in this paper*: PCNA, proliferating cell nuclear antigen; topo II, DNA topoisomerase II.

during development, we set about to systematically correlate levels of topo II mRNA and protein with levels of lamin mRNA and protein, respectively. Indirect immunofluorescence and immunoperoxidase analyses were used to study the distribution of these two proteins in situ. From our results, we conclude that with the exception of adult males, both topo II and lamin are present at relatively constant levels throughout development; topo II is found in nuclei of most but not all cell types examined.

Materials and Methods

Antibodies

Affinity-purified goat anti-rabbit IgG was from Cappel Laboratories (Malvern, PA). Rhodamine-conjugated affinity-purified donkey anti-rabbit IgG was from Jackson Immuno Research Laboratories Inc. (West Grove, PA). Biotinylated goat anti-rabbit IgG was from Vector Laboratories, Inc. (Burlingame, CA). Anti-*Drosophila* lamin antibodies (see, for example, Smith and Fisher, 1989), anti-*Drosophila* muscle myosin heavy chain antibodies (Berrios and Fisher, 1986), and anti-*Drosophila* proliferating cell nuclear antigen (PCNA) antibodies (Ng et al., 1990) were affinity purified essentially according to Fisher and Smith (1988). Anti-*Drosophila* topo II antiserum was prepared previously (McConnell et al., 1987) and anti-topo II antibodies were affinity purified using a fragment of *Drosophila* topo II that had been expressed in *Escherichia coli*, purified, and coupled to glutaraldehyde-activated glass beads (Boehringer Mannheim, Diagnostics, Inc., Indianapolis, IN) as detailed in Results.

Methods

Much of the methodology has been detailed previously. *Drosophila melanogaster* (Oregon R, P2 strain) were maintained in mass culture and embryos harvested according to Allis et al. (1977). Protein determination was according to Schaffner and Weissman (1973). SDS-PAGE was on 7% polyacrylamide gels according to Laemmli (1970). Proteins were transferred passively from SDS-gels to sheets of nitrocellulose (Fisher et al., 1982) and resulting immunoblots were processed, probed with primary antibodies, and bands of reactivity visualized colorimetrically with calf alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibodies (Blake et al., 1984; Smith and Fisher, 1984). Calf alkaline phosphatase was conjugated to affinity-purified goat anti-rabbit IgG according to Avrameas (1969); colorimetric detection was according to McGadey (1970). Additional details are provided in the figure legends.

Northern Blot Analyses

RNA was prepared from *Drosophila* embryos, larvae, pupae, and adults essentially according to O'Connell et al. (1987) using a combined method involving guanidinium thiocyanate extraction (Chirgwin et al., 1979) and CsCl purification (Glišin et al., 1974). Organisms were quick-frozen in liquid N_2, pulverized with a mortar and pestle, and then extracted with 4 M guanidinium thiocyanate, 1 M 2-mercaptoethanol, 50 mM sodium acetate pH 6, and 5 mM Na_2EDTA. Crude extracts were clarified by centrifugation for 10 min at 1,000 *g*; ~2 ml of clarified extract was layered over a 4-ml cushion of 5.7 M CsCl, 0.1 M Na_2EDTA, and the extract was centrifuged at 15°C for 24 h at 35,000 rpm in an SW41 rotor. The clear RNA pellet was dissolved in 10 mM Tris-HCl pH 7.5, 5 mM Na_2EDTA, and 1% SDS, extracted once with an equal volume of chloroform:1-butanol (4:1), ethanol precipitated, and the precipitate resolubilized in diethyl pyrocarbonate (DEPC) treated H_2O.

Hybridization of radiolabeled DNA probes to immobilized RNA was performed essentially according to Thomas (1980). RNA was denatured in formaldehyde and subjected to electrophoresis on horizontal 0.8% agarose slab gels containing 6% formaldehyde exactly as previously described in detail (Gruenbaum et al., 1988). After electrophoresis, gels were washed for 1 h in 10× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate pH 7.0) to remove formaldehyde and separated RNA species were transferred passively to Nytran (Schleicher & Scheull, Inc., Keene, NH) for 20 h using 10× SSC. After transfer, the blot was washed briefly in 10× SSC and the RNA was permanently immobilized by UV cross-linking. Blots were probed and washed exactly as previously described (Gruenbaum et al., 1988) using ^{32}P-labeled DNA probes generated by the random priming method of Feinberg and Vogelstein (1983). Generally, the probes were labeled to a specific activity of $\sim 5 \times 10^8$ cpm/μg of DNA.

Quantitation of Immunoblots and Northern Blots

Bands of immunoblot reactivity were quantitated by excising regions of interest from the nitrocellulose and incubating them in DMSO. This results both in dissolution of the nitrocellulose and in solubilization of the purple phosphatase reaction product present on the immunoblot. The resulting DMSO-phosphatase reaction product solution was subjected to spectrophotometric analysis at 540 nm. We previously demonstrated immunoblot reactivity to be linearly dependent on antigen concentration (Smith and Fisher, 1984), and in a series of control experiments performed with purified *Drosophila* lamin, showed that the DMSO solubilization method of immunoblot quantitation gave identical results to quantitation of lamin protein in solution (Fisher, P., unpublished data). This method of immunoblot quantitation, as opposed to conventional scanning densitometry, was necessary due to the substantial variation in absolute amounts of immunoreactivity as well as in band morphology in the various lanes being analyzed (see Fig. 2).

Northern blot reactivity was quantitated by integrative scanning densitometry of autoradiograms. This was performed using an ULTROSCAN XL laser densitometer (LKB Instruments Inc., Gaithersburg, MD). Additional details are provided in the legend to Fig. 5.

Immunocytochemistry

Indirect immunofluorescence microscopy using third instar larval squash preparations and cryosections was performed essentially as previously described (Fisher et al., 1982; Smith and Fisher, 1984) and as follows. Muscle cell squash preparations were from adult fly thoraces. All whole tissue squashes were prepared in buffer A as originally described by Burgoyne et al. (1971; see also Paddy et al., 1990) supplemented with 0.1% Triton X-100 and 3.7% formaldehyde. Adult fly heads were dissected manually, prepared for cryosectioning, and cryosections cut as previously described for whole third instar larvae (Smith and Fisher, 1984). Because of intense autofluorescence, specific antibody binding to adult head cryosections was detected using a biotinylated secondary antibody followed by incubation with avidin-complexed biotinylated horseradish peroxidase and appropriate developing reagents as specified in the VECTASTAIN ABC Elite kit (Vector Laboratories, Inc.).

Preparation of Whole Organismal Extracts and Cell Fractionation

Whole embryo extracts were prepared by Dounce homogenization of developmentally staged embryos directly into 10 vol of boiling 10% SDS, 20 mM DTT. Boiling after homogenization was continued for ~3 min. Whole organismal extracts from larvae, pupae, and adults were prepared by first freezing in liquid N_2 followed by grinding to a fine powder under liquid N_2 with a mortar and pestle. The liquid N_2 was allowed to evaporate and ~10 vol of boiling 10% SDS, 20 mM DTT was added. Boiling was continued for about 3 min immediately thereafter. After cooling, boiled extracts were clarified by centrifugation and either used immediately or stored at −20°C.

For cell fractionation experiments, developmentally staged embryos were Dounce homogenized into standard extraction buffer (Fisher et al., 1982; McConnell et al., 1987) and homogenates subjected to centrifugation for 10 min at 10,000 *g*. Pellet fractions were resuspended in extraction buffer. Supernatants, resuspended pellets, and aliquots of the unfractionated homogenates were denatured by addition of an equal volume of 20% SDS, 40 mM DTT and boiling for ~3 min immediately thereafter.

Results

Specificity of Anti-topo II Antibodies

To determine reliably the levels of nuclear proteins in crude extracts by immunoblot analysis as well as to study their in situ localization by indirect immunofluorescence and/or immunoperoxidase staining, highly specific antibodies are required. A strategy which proved effective for obtaining suit-

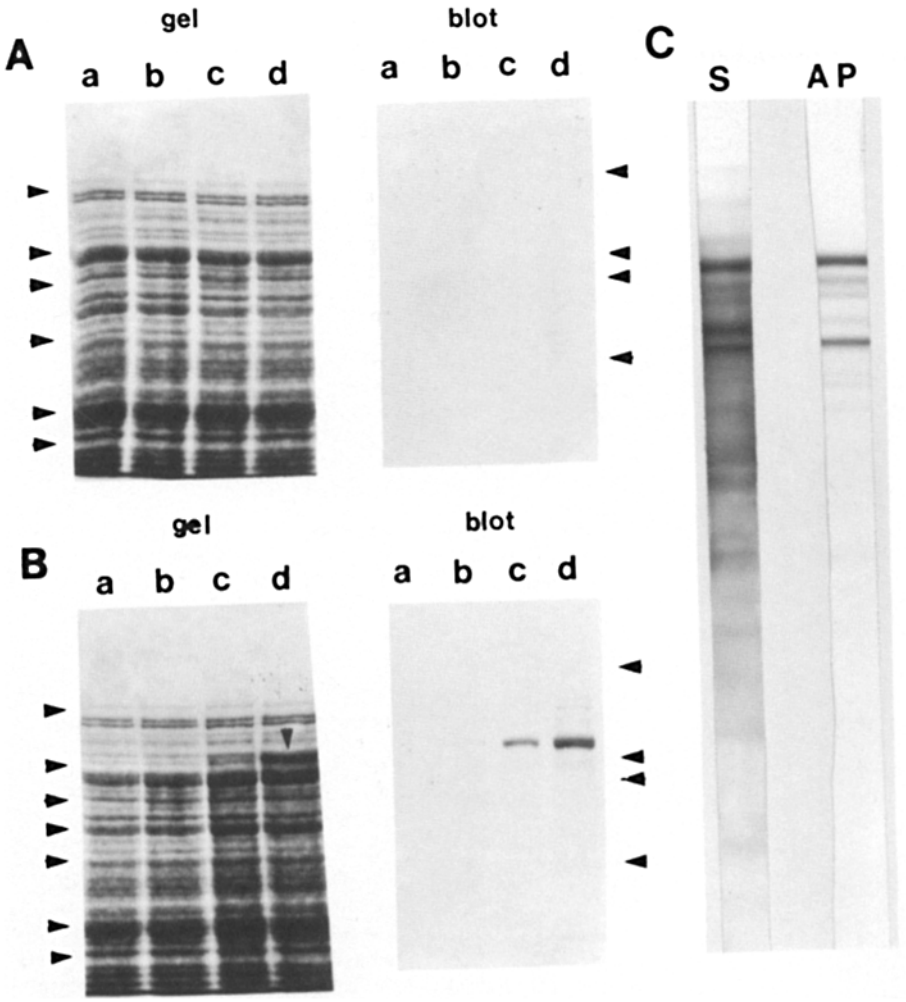

Figure 1. Bacterial expression of *Drosophila* topo II and affinity purification of anti-topo II antibodies. *E. coli* BL21(DE3) cells harboring bacteriophage T7 gene 1 coding for T7 RNA polymerase under control of the *lac*UV5 promoter (see Studier et al., 1990) were transformed with pET3a alone (*A*) or pET3DT2 (*B*), a pET3a derivative containing a cDNA insert encoding amino acids 32–1447 of *Drosophila* topo II. SDS-7% PAGE was on minigels (Hoefer Scientific Instruments, San Francisco, CA); gels as indicated, were stained with Coomassie blue; blots as indicated, of parallel gels were probed with anti-*Drosophila* topo II antiserum prepared against the native enzyme (see Berrios et al., 1985) diluted 1:1,000. For both gels and blots, *A* and *B*; lanes *a*, before induction; lanes *b*, 0.5 h after induction with 0.4 mM IPTG; lanes *c*, 1 h after induction; lanes *d*, 2 h after induction. Down-pointing arrowhead in *B* gel, lane *d* designates the new Coomassie blue-stainable protein band seen after induction only in cells harboring pET3DT2. The apparent mass of this polypeptide, ~120 kD, is smaller than expected based on the size of the topo II cDNA insert; this likely results either from premature termination of translation and/or proteolysis. Arrowheads to the left of gels in both *A* and *B* designate migration positions of (from *top* to *bottom*) α_2 macroglobulin (170 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), BSA (69 kD), ovalbumin (46 kD), and carbonic anhydrase (30 kD). Arrowheads to the right of blots in both *A* and *B* designate migration positions of (from *top* to *bottom*) myosin heavy chain (200 kD), β-galactosidase, phosphorylase b, and BSA. (*C*) Affinity purification of anti-*Drosophila* topo II antibodies. Nitrocellulose strips containing *Drosophila* nuclear proteins transferred after separation on a 20-cm-long SDS-7% polyacrylamide gel were probed either with unfractionated anti-*Drosophila* topo II antiserum diluted 1:1,000 (*S*), or with a comparable amount of anti-*Drosophila* topo II IgG purified by affinity chromatography on immobilized bacterially synthesized *Drosophila* topo II (*AP*). Minor bands below the main band on strip AP represent commonly encountered topo II proteolytic breakdown products (see, for example, Berrios et al., 1985).

able anti-lamin antibodies involved preparation of a rabbit antiserum by immunization with authentic lamin purified from *Drosophila* embryos followed by affinity purification using as the immobilized affinity ligand, clone-encoded antigen expressed in bacteria (see, for example, Gruenbaum et al., 1988; Fisher and Smith, 1988; Smith and Fisher, 1989).

To pursue a similar strategy for affinity purification of anti-topo II antibodies, a full-length *Drosophila* topo II cDNA clone (Nolan et al., 1986) was obtained from Dr. T.-s. Hsieh (Duke University, Durham, NC). In preliminary experiments, we demonstrated that the in vitro translation product synthesized in a wheat germ lysate after transcription of this clone could be specifically immunoprecipitated (not shown) by either of two anti-topo II antisera available in the laboratory (see McConnell et al., 1987) as well as by anti-topo II antibodies affinity purified using authentic *Drosophila* topo II as the affinity ligand (see Berrios et al., 1985). A 4.6-kb Bam HI fragment of the topo II cDNA, encoding all but a small portion of the NH_2 terminus of topo II (amino acids 32–1,447; Wyckoff et al., 1989) was ligated in frame into the T7 RNA polymerase-dependent expression vector, pET3a (Rosenberg et al., 1987; Studier et al., 1990) to generate the plasmid pET3DT2. An appropriate strain of bacteria was transformed either with this plasmid or with a control plasmid lacking the topo II cDNA insert. Transformed cultures were induced with isopropyl β-D-thiogalactoside (IPTG) and after various periods, cells were lysed, and proteins subjected to SDS-PAGE and immunoblot analysis with anti-topo II antiserum.

When cells were transformed with the control plasmid, there was no change in the Coomassie blue staining pattern during the time course of induction (Fig. 1 *A*, gel) nor were any immunoreactive polypeptides detectable on a parallel immunoblot probed with anti-topo II antiserum (Fig. 1 *A*, blot). In contrast, induction of cells transformed with pET3DT2 resulted in synthesis of a new polypeptide as determined by Coomassie blue staining (Fig. 1 *B*, gel); this polypeptide was highly reactive with anti-topo II antiserum (Fig. 1 *B*, blot). This bacterially synthesized polypeptide was purified from cell extracts under denaturing conditions (see

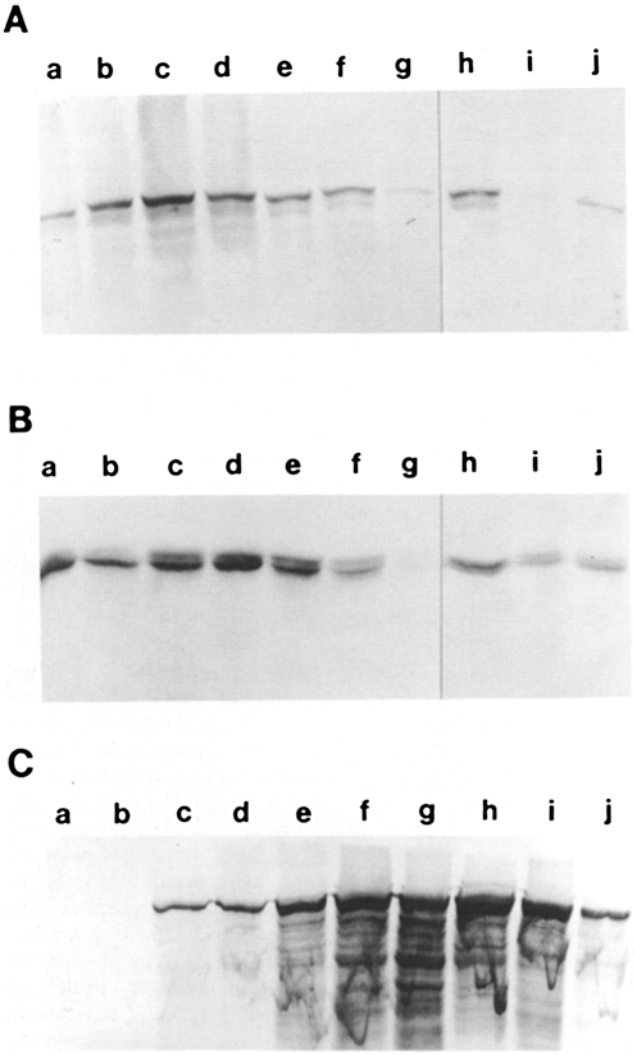

Figure 2. Steady-state levels of topo II, nuclear lamin, and muscle myosin heavy chain during *Drosophila* development. Extracts were prepared as described (Materials and Methods), and 500-μg aliquots of each subjected to electrophoresis on 20-cm-long SDS-7% polyacrylamide gels. After electrophoresis, separated proteins were transferred passively (Fisher et al., 1982) to nitrocellulose and resulting immunoblots were probed with specific antibodies. Only primary regions of reactivity are shown. The blot shown in *A* was probed with antiserum raised against native *Drosophila* topo II diluted 1:1,000; identical results were obtained using comparable amounts of affinity-purified anti-*Drosophila* topo II IgG (not shown). The blot shown in *B* was probed with affinity-purified anti-*Drosophila* lamin IgG. The blot shown in *C* was probed with affinity-purified anti-*Drosophila* muscle myosin heavy chain IgG. Samples loaded in individual lanes were the same in all three panels; lanes *a*, 0–3-h-old embryos; lanes *b*, 6–9-h-old embryos; lanes *c*, 12–15-h-old embryos; lanes *d*, 19–22-h-old embryos; lanes *e*, first instar larvae; lanes *f*, second instar larvae; lanes *g*, third instar larvae; lanes *h*, pupae; lanes *i*, male adults; lanes *j*, female adults.

Filson et al., 1985), coupled to glutaraldehyde-activated glass beads and used as a ligand for affinity purification of anti-topo II antibodies (Fig. 1 *C*). All subsequent immunochemical and immunocytochemical experiments were either performed or corroborated using anti-topo II antibodies affinity purified in this way.

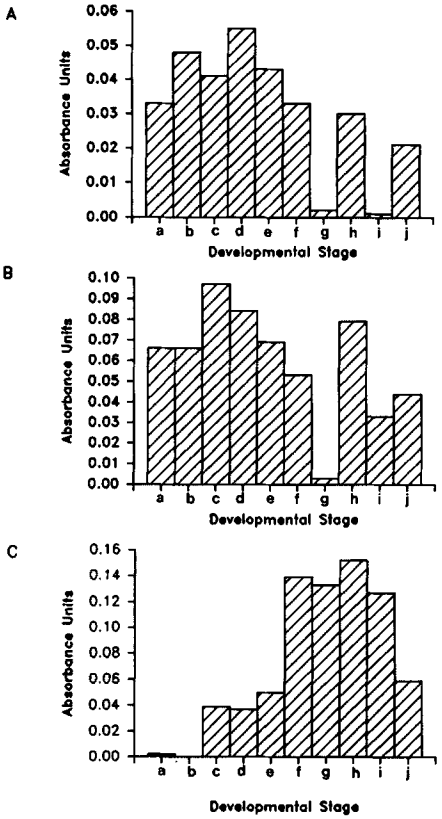

Figure 3. Quantitative analysis of immunoblots shown in Fig. 2. Immunoreactivity on blots shown in Fig. 2 was quantitated (Materials and Methods) by excision of individual bands from the nitrocellulose, dissolution in DMSO, and spectrophotometric analysis at 540 nm using an enzyme immunoassay microtitre plate reader (Bio-Tek Instruments Model EL-308). Designations (*a–j*) of developmental stage as indicated along the abscissa, correspond respectively, to lane headings (*a–j*) as indicated in legend to Fig. 2. *A*, *B*, and *C* correspond to *A*, *B*, and *C*, respectively, of Fig. 2; (*A*) topo II; (*B*) total lamin (all isoforms); (*C*) muscle myosin heavy chain.

Developmental Regulation of topo II Protein and mRNA Levels

topo II levels throughout *Drosophila* development were determined by quantitative immunoblot analysis (Smith and Fisher, 1984) and were compared with levels of lamin[2] and muscle myosin heavy chain. At first approximation, fluctuations in topo II levels (Fig. 2 *A*) were similar to those reported previously (Fairman and Brutlag, 1988). However, comparable fluctuations in lamin levels were also seen (Fig. 2 *B*). In contrast, muscle myosin heavy chain, a nonnuclear protein, showed an entirely different pattern of expression through development (Fig. 2 *C*).

The immunoblots shown in Fig. 2 were analyzed quantitatively (Materials and Methods) and the results are presented graphically in Fig. 3. In general, these results confirm im-

2. Unlike vertebrates where multiple, developmentally regulated lamins have been reported, *Drosophila* has only a single lamin gene encoding a single primary translation product (Smith et al., 1987; Gruenbaum et al., 1988). This single lamin gene is expressed throughout development and immunocross-reactive isoforms derived from the single primary translation product by posttranslational modification have been identified at all stages of development and in every cell and tissue type analyzed (Fisher, 1988).

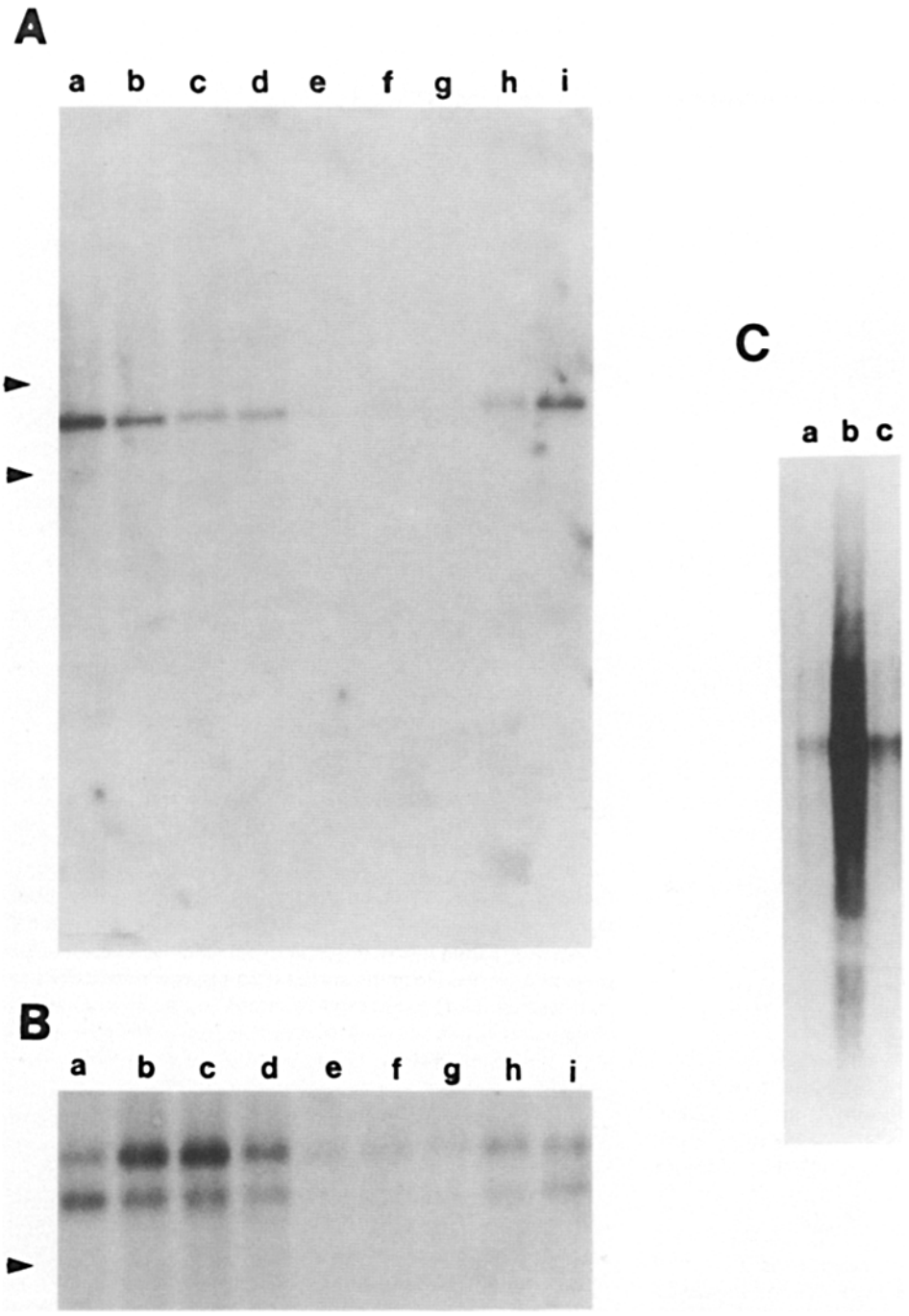

Figure 4. Northern blot analysis of topo II and lamin mRNAs during *Drosophila* development. RNA was prepared as described (Materials and Methods) and 30 μg of total RNA from each developmental stage was electrophoresed on a 0.8% agarose gel, transferred passively to Nytran, and probed with randomly primed ^{32}P-labeled cDNA fragments complementary to either *Drosophila* topo II mRNA (*A* and *C*) or *Drosophila* lamin mRNA (*B*). Arrowheads to the left of *A* designate the migration positions of 6.6- (*top*) and 4.4-kb (*bottom*) lambda Hind III fragments used as markers. Arrowhead to the left of *B* designates the migration position of the 2.3-kb lambda Hind III fragment used as a marker. RNA aliquots from identical developmental stages, loaded on the gels represented in *A* and *B*, were; lanes *a*, 0–3-h-old embryos; lanes *b*, 6–9-h-old embryos; lanes *c*, 12–15-h-old embryos; lanes *d*, 19–22-h-old embryos; lanes *e*, first instar larvae; lanes *f*, second instar larvae; lanes *g*, third instar larvae; lanes *h*, pupae; lanes *i*, total adults (males plus females). (*C*) Similar analysis to that shown in *A*; lane *a*, adult males; lane *b*, adult females; lane *c*, total adults (males plus females).

pressions obtained from visual inspection of the immunoblots in Fig. 2. The only significant deviation between levels of topo II and lamin noted in examining the quantitative data in Fig. 3 occurred with adult males. Whereas males and females had similar absolute levels of lamins (Fig. 3 *B*, compare *i* and *j*), males had markedly reduced levels of topo II relative to females (Fig. 3 *A*, compare *i* and *j*). That the low levels of topo II detectable in males were indeed reproducibly present was confirmed by repeated analyses (not shown).

Developmental immunoblot analyses of topo II and lamin protein levels were complemented by Northern blot experiments to determine relative mRNA levels. topo II mRNA was detected at all developmental stages although barely so in larvae (Fig. 4 *A*). Longer exposure of the Northern blot shown in Fig. 4 *A* confirmed the existence of the faint bands of radioactivity in lanes *e*, *f*, and *g* (not shown, but see Fig. 5 *A*). Comparison of topo II mRNA levels with lamin mRNA levels (Fig. 4 *B*) is complicated by the fact that there are two developmentally regulated lamin messages both coding for the same primary translation product (Gruenbaum et al., 1988). Nevertheless, it can be seen from the data in Fig. 4 that topo II mRNA levels varied through development in roughly the same way as did total lamin mRNA levels. The most conspicuous difference was early in embryogenesis when lamin mRNA levels increased over the first several hours before beginning to decrease at the final time point,

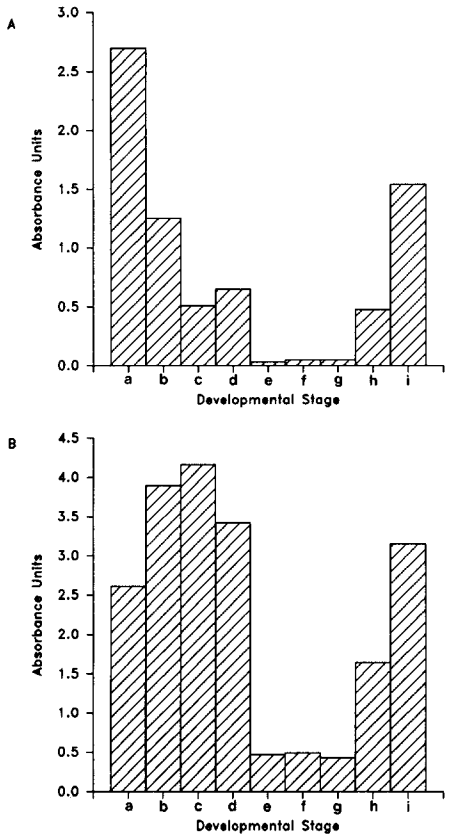

Figure 5. Quantitative analysis of Northern blots shown in Fig. 4. Quantitative densitometric analysis of Northern blots shown in Fig. 4 was performed on an LKB ULTROSCAN XL Laser Densitometer (Materials and Methods). Units of absorbance are arbitrary. Designations (*a–i*) of developmental stage as indicated along the abscissa, correspond respectively, to lane headings (*a–i*) as indicated in Fig. 4. *A* and *B* correspond to *A* and *B*, respectively, of Fig. 4; (*A*) topo II mRNA; (*B*) total lamin mRNA (both 2.8- and 3.0-kb species).

whereas topo II mRNA levels were highest at the earliest time point of embryogenesis analyzed. Quantitative densitometric analysis of the Northern blots shown in Fig. 4, *A* and *B* is shown in Fig. 5. These results confirmed impressions obtained from visual inspection of the respective autoradiograms.

The preponderance of topo II mRNA early in embryogenesis was consistent with the notion that large amounts of this message were accumulated and stored during oogenesis. We previously reported that this was apparently the explanation for the predominance of the 2.8 kb (as opposed to the 3.0 kb) lamin mRNA early in embryogenesis (Gruenbaum et al., 1988). To evaluate this possibility for topo II, adult *Drosophila* males and females were segregated and total RNA from each group analyzed separately. Results from this experiment (Fig. 4 *C*) demonstrated that while topo II mRNA was detectable in both males and females, much more of the message was found in females.

In Situ Identification of topo II in Various Drosophila Cells and Tissues

Developmental immunoblot and Northern blot experiments were complemented by indirect immunofluorescence and immunoperoxidase analyses performed on selected larval and adult tissues. Results of some of these experiments are shown in Figs. 6 and 7. Initially, examination of third instar larval cryosections that were labeled with affinity-purified anti-topo II IgG revealed specific nuclear staining in virtually all cells; a representative field is shown in Fig. 6, *a–c*. All nuclei identified by DAPI staining (Fig. 6 *c*) were also stained to greater or lesser degree with anti-topo II antibodies (Fig. 6 *b*). This was consistent with results obtained previously with unfractionated anti-topo II antiserum (McConnell et al., 1987).

Squash preparations of various third instar larval tissues and organs, including salivary gland, imaginal disc, and neural ganglion, were also examined by indirect immunofluorescence analysis using affinity-purified anti-topo II antibodies. As in cryosections, and consistent with results obtained previously with unfractionated anti-topo II antiserum (Berrios et al., 1985), virtually all nuclei identifiable by DAPI staining were labeled with affinity-purified anti-topo II antibodies. An example of results obtained with neural ganglion tissue is shown in Fig. 6, *d–f*.

Recently, we reported the identification and purification of *Drosophila* PCNA; *Drosophila* PCNA is structurally and functionally homologous to mammalian PCNA, and was detectable by immunofluorescence analysis in only a small subset of third instar larval neural ganglion cells (Ng et al., 1990).[3] This staining pattern, an example of which is shown in Fig. 6, *g–i*, is in marked contrast to that seen with anti-topo II antibodies (Fig. 6, *d–f*).

Additional immunocytochemical experiments were performed with adult tissues. Hadlaczky et al. (1988) reported the presence of topo II in fly heads as determined by immunoblot analysis with monoclonal antibodies. To complement these immunochemical data and to establish further the presence of topo II in nonproliferating cells, we used affinity-purified polyclonal anti-topo II antibodies for immunocytochemical analysis of adult head cryosections. Because of intense autofluorescence, these sections were probed with a peroxidase-coupled secondary reagent (Materials and Methods). Results of this analysis demonstrated the presence of topo II in most if not all nuclei identifiable (not shown). No specific staining was seen either in control specimens or in specimens probed with affinity-purified anti-PCNA antibodies (not shown). Results similar to those obtained with anti-topo II antibodies were noted when specimens were probed with affinity-purified anti-lamin antibodies (not shown) except that with anti-lamin antibodies, nuclear rim staining was demonstrable.

In contrast with third instar larval tissues (Fig. 6) and adult head cryosections, topo II was not identifiable in most adult *Drosophila* muscle cell nuclei (Fig. 7, *d–f*). When similar muscle preparations were stained with affinity-purified anti-lamin antibodies, muscle cell nuclear staining was readily

3. Immunocytochemical detection of mammalian PCNA is complicated by the observation that in non-S-phase cells under certain conditions of fixation (methanol), it is undetectable in situ, despite the fact that it can be readily identified by immunoblot analysis of the same cells or tissue. Other fixation conditions apparently facilitate in situ detection of mammalian PCNA whenever it is present (see, for example, Bravo and Macdonald-Bravo, 1987). In our analyses, we have used only those fixation conditions under which PCNA is detectable, regardless of cell cycle position.

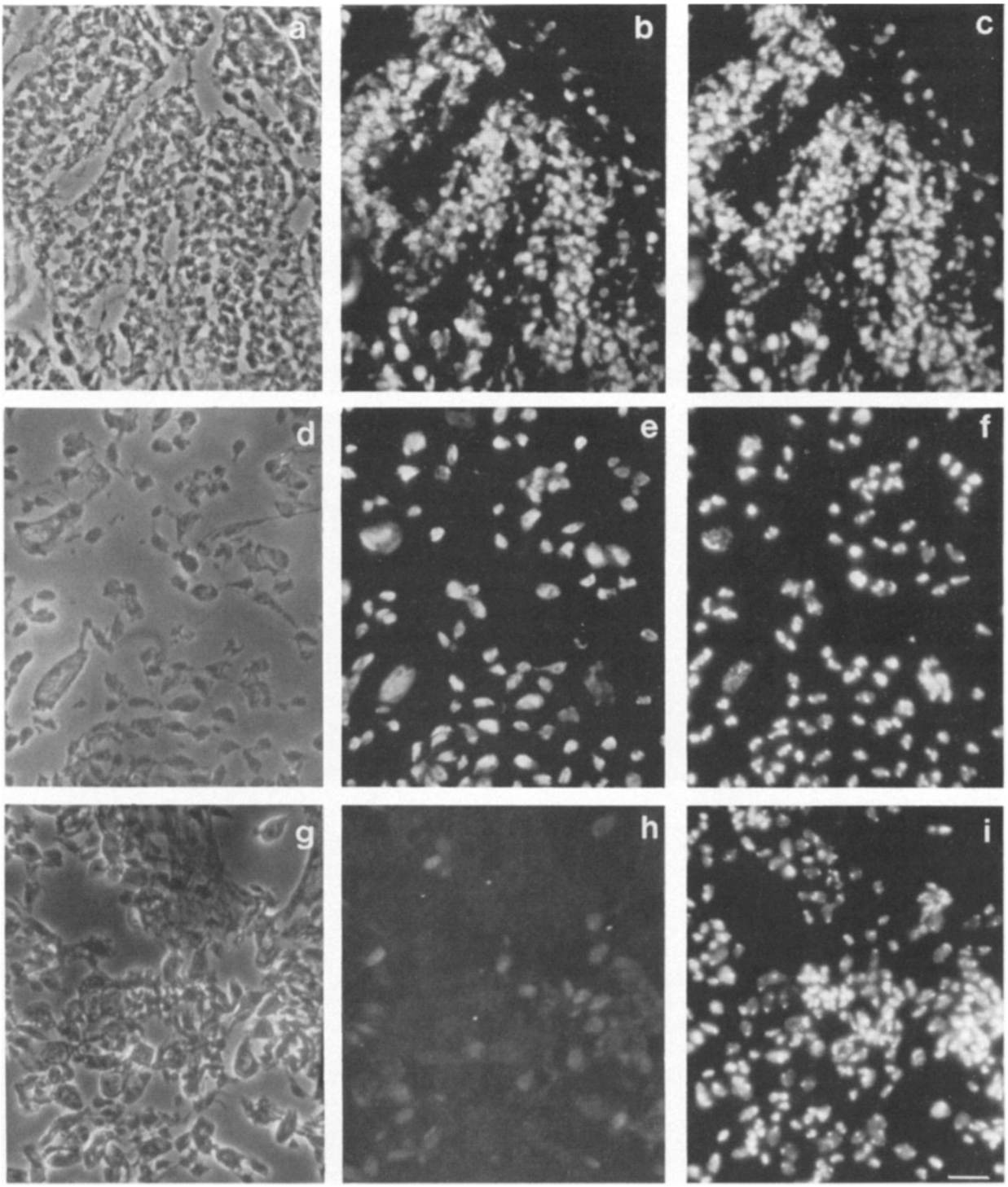

Figure 6. In situ localization of topo II and PCNA in *Drosophila* third instar larval tissues. Phase contrast (*a*, *d*, and *g*), immunofluorescence (*b*, *e*, and *h*), and DAPI fluorescence (*c*, *f*, and *i*) micrographs. Specimen in *a–c*, a cryosection through the third instar larval imaginal disc tissue, was probed with affinity-purified anti-*Drosophila* topo II IgG at a final concentration of about 1 μg/ml followed by rhodamine-conjugated donkey anti-rabbit IgG. Specimen in *d–f*, a squash preparation from third instar larval neural ganglion tissue, was probed similarly to the specimen in *a–c*. Specimen in *g–i*, a squash preparation identical to that shown in *d–f*, was probed with affinity-purified anti-*Drosophila* PCNA IgG at a final concentration of ~2 μg/ml followed by rhodamine-conjugated donkey anti-rabbit IgG. Preimmune serum from both rabbits gave no detectable immunofluorescence on these specimens (not shown; see McConnell et al., 1987; Ng et al., 1990). Bar, 10 μm.

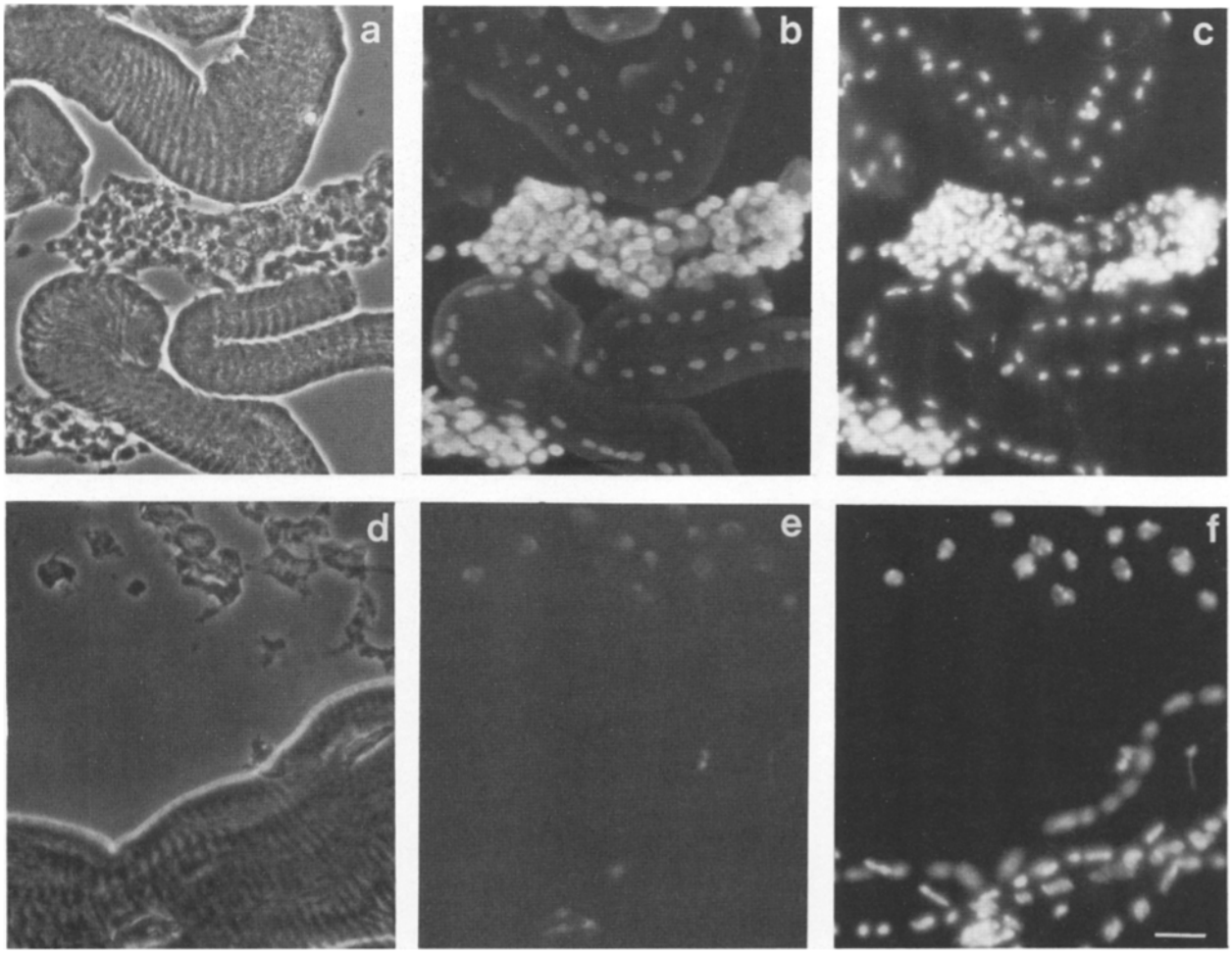

Figure 7. In situ localization of topo II and lamin in *Drosophila* adult muscle squashes. Phase-contrast (*a* and *d*), immunofluorescence (*b* and *e*), and DAPI fluorescence (*c* and *f*) micrographs. The specimen in *a–c* was probed with affinity-purified anti-*Drosophila* lamin IgG at a final concentration of ~1 μg/ml followed by rhodamine-conjugated donkey anti-rabbit IgG. The specimen in *d–f* was probed with a similar concentration of affinity-purified anti-*Drosophila* topo II IgG followed similarly by rhodamine-conjugated donkey anti-rabbit IgG. Bar, 10 μm.

demonstrable (Fig. 7, *a–c*). However, muscle cell anti-lamin antibody nuclear staining was relatively weak compared with staining of other nonmuscle cell nuclei in the same specimen (Fig. 7 *b*). This may be particularly significant in that both types of nuclei stained similarly with DAPI (Fig. 7 *c*).

Identification of a Soluble Pool of topo II Early in Embryogenesis

Recently, we reported that a soluble pool of nuclear lamin was present in early embryos that was apparently derived from germinal vesicle breakdown at the final stage of oogenesis (Smith and Fisher, 1989). To determine whether a similar pool of topo II was present in early embryos, immunoblot analyses were performed after cell fractionation to separate nuclear pellets from post-nuclear supernatants at different time points of embryogenesis. The results of these experiments (Fig. 8) indicated that for both topo II (Fig. 8 *A*) and lamin (Fig. 8 *B*), there was a soluble pool of antigen present at the earliest time point when no nuclear antigen was detectable (compare Fig. 8, *A* and *B*, lanes *e* with lanes *i*). In the case of lamin, this antigen was in the single lamin Dm_{mit} isoform, characteristic of cells that have undergone nuclear envelope breakdown either during meiosis or mitosis (Smith and Fisher, 1989; Lin and Fisher, 1990).

At later time points of embryogenesis, topo II as well as lamin appeared in the nuclear pellet fraction (Fig. 8, *A* and *B*, lanes *j*, *k*, and *l*). Both interphase lamin isoforms Dm_1 and Dm_2 (Smith et al., 1987) were present (Fig. 8 *B*, lanes *j*, *k* and *l*). In the case of lamin, accumulation of lamins Dm_1 and Dm_2 in the nuclear pellet fraction through development correlated with loss of soluble lamin Dm_{mit} (Fig. 8 *B*, compare lanes *i–l* with lanes *e–h*, respectively). In contrast, for topo II, both nuclear and apparently nonnuclear forms were identified at later time points (Fig. 8 *A*, compare lanes *j*, *k*, and *l* with lanes *f*, *g*, and *h*, respectively). In situ analyses (not shown) suggest that in vivo, all topo II is nuclear in late embryonic cells during interphase. We therefore think it likely that the apparently nonnuclear pool of topo II present at later time points of embryogenesis (Fig. 8 *A*, lanes *g* and *h*) is in reality, an extractable nuclear pool.

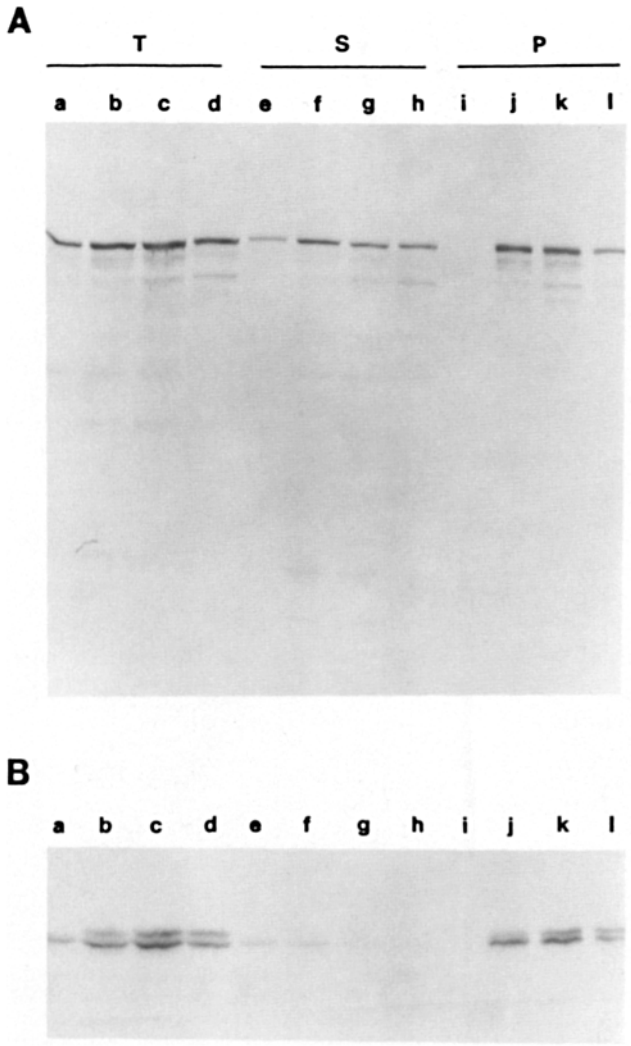

Figure 8. Identification of soluble and pelletable isoforms of topo II and lamin during *Drosophila* embryogenesis. SDS-7% PAGE and immunoblot analyses were performed as described (Materials and Methods) on total embryo homogenates (*T*, lanes *a–d*), 10,000 *g* homogenate supernatants (*S*, lanes *e–h*), and 10,000 *g* homogenate pellet fractions (*P*, lanes *i–l*). Equivalent aliquots, 4 U (Fisher et al., 1982), were loaded in each lane. Lanes *a*, *e*, and *i*, 0–3-h-old embryos; lanes *b*, *f*, and *j*, 6–9-h-old embryos; lanes *c*, *g*, and *k*, 12–15-h-old embryos; lanes *d*, *h*, and *l*, 19–22-h-old embryos. Immunoblot shown in *A* was probed with anti-*Drosophila* topo II antiserum diluted 1:1,000. The entire blot is shown. Identical results were obtained with affinity-purified anti-topo II antibodies (not shown). Immunoblot shown in *B* was probed with affinity-purified anti-*Drosophila* lamin antibodies. Only the region of primary immunoreactivity is shown.

Discussion

In vertebrates, topo II is apparently regulated during the cell cycle. After mitosis, much of it is rapidly degraded only to be resynthesized in actively proliferating cells in late G_1 or early S phase (Heck et al., 1988). In cells that stop proliferating, topo II disappears and resting cells, i.e., those in phase G_0 of the cell cycle, apparently lack topo II entirely (Heck and Earnshaw, 1986).[4] Despite earlier suggestions that *Drosophila* topo II behaved similarly as deduced from developmental analyses (Fairman and Brutlag, 1988), results reported in this article indicate otherwise.

Although in current experiments, wide fluctuations of topo II levels were seen through development in *Drosophila*, these fluctuations were paralleled by similar changes in nuclear lamin levels at all stages but in the adult males. An internal standard nuclear protein such as lamin was not analyzed in the earlier study (Fairman and Brutlag, 1988). *Drosophila* topo II mRNA levels also fluctuated more or less in parallel with lamin mRNA levels; the most significant difference between the two was seen early in embryogenesis. In this respect, we agree with the findings of Fairman and Brutlag (1988). Indeed, these authors suggested that the large pool of topo II message seen in early embryos was most likely maternally derived, yet they saw no evidence of this pool in adults. In contrast, we detected topo II mRNA in both male and female adults. Consistent with the suggestion of Fairman and Brutlag (1988) regarding the maternal origins of the topo II mRNA found in early embryos, we found that adult females contained much more topo II message than adult males (Fig. 4 *C*).

Also in contrast with the results of Fairman and Brutlag (1988), we found topo II protein present at all developmental stages and saw little evidence of the proteolytic breakdown products reported by them, particularly late in embryogenesis (compare Fig. 4 of Fairman and Brutlag (1988) with Fig. 8 *A*, lane *d*). In this respect, our results agree with those of Hadlaczky et al. (1988) who found using monoclonal antibodies, that topo II was detectable as an undegraded 166-kD polypeptide throughout embryogenesis, in pupae, adult fly heads, and Schneider S3 tissue culture cells. A single 38-kD polypeptide, identified by these authors and suggested to be a stable in vivo breakdown product of *Drosophila* topo II (Hadlaczky et al., 1988), was not noted in our experiments (see also Fig. 5 of Ng et al., 1990).

Results of immunoblot analyses, which form the basis for many of our current conclusions, were corroborated and extended by in situ studies. In these experiments, we compared the distribution of topo II with that of both lamin and PCNA. topo II was detectable in virtually every cell in a variety of *Drosophila* larval and adult tissues by immunocytochemical analyses using affinity-purified anti-topo II antibodies. The exception to this was adult muscle where topo II was not detected. This pattern of distribution was relatively similar to that seen for lamin which was detected in every cell type examined, including muscle and was quite different from that of PCNA. PCNA was only seen in a small minority of those cells analyzed; as reported elsewhere, this correlated with an entirely different pattern of expression through *Drosophila* embryogenesis revealed by immunoblot analysis (see Fig. 5 of Ng et al., 1990). In this context, it may also be noteworthy that recent studies of cell cycle regulation of yeast topo II failed to uncover evidence for significant decreases in topo II levels in nondividing cells (Cardenas et al., 1990).

How can we reconcile the results reported in this article with those from comparable studies of vertebrate systems

4. These results are consistent with a number of other studies of vertebrate topo II (see, for example, Hsiang et al., 1988).

(Heck and Earnshaw, 1986; Heck et al., 1988)? In first considering trivial explanations, we think it highly unlikely that our results are attributable to lack of antibody specificity. Similar immunochemical and immunocytochemical results were obtained with each of two different anti-*Drosophila* topo II antisera, as well as with antibodies affinity purified using as the affinity ligand, either authentic topo II purified from *Drosophila* embryos or clone-encoded *Drosophila* topo II expressed in bacteria. This last population of antibodies was used for all of the in situ analyses presented in this article. In addition, our results regarding the identification of *Drosophila* topo II in nonproliferating cells are in agreement with those of Hadlaczky et al. (1988) obtained with monoclonal antibodies.

It is possible that despite our efforts to identify nonproliferating cells from *Drosophila*, none except those from adult muscle were truly in phase G_0 of the cell cycle. (In this context, it is perhaps noteworthy that the differentiating myoblast system was one in which vertebrate topo II was seen to disappear [Heck and Earnshaw, 1986].) This possibility cannot be excluded. However, the very different behavior of *Drosophila* PCNA as revealed both by immunoblot analyses (Ng et al., 1990) and in situ (Fig. 6), indicates that at the very least, topo II cannot be used as a specific marker for cell proliferation in *Drosophila* as has been proposed for vertebrates (Heck and Earnshaw, 1986).

We are left therefore with two possible explanations for the differences observed between *Drosophila* and vertebrates. The first which, though not very satisfying, must be considered, is that *Drosophila* is fundamentally different from vertebrates with respect to regulation of topo II and/or the cell cycle. The tendency of *Drosophila* chromosomes to polytenize in terminally differentiated tissues may be of relevance in this regard. The second possibility was proposed previously (Fisher, 1989) and is suggested by the recent identification of two immunologically and genetically distinct mammalian topo II isozymes (Drake et al., 1987, 1989; Chung et al., 1989). If these observations are generally applicable to vertebrates and the antibodies used by others (Heck and Earnshaw, 1986; Heck et al., 1988; Heck et al., 1989) to study cell cycle regulation of topo II were specific for only one of these isozymes, patterns of expression of the other isozyme may have gone unrecognized. *Drosophila* on the other hand, might have only one topo II isozyme, or alternatively, anti-*Drosophila* topo II antibodies might not distinguish among different isozymes. In either instance, a different pattern of topo II regulation in *Drosophila* relative to vertebrates might be revealed; such differences could well be more apparent than real.

Throughout this article, we compared topo II with nuclear lamin. An underlying motive in performing this comparison was to test the hypothesis that topo II was a karyoskeletal protein analogous with lamin. Although the results obtained do not address this question directly, we feel that the experiment documented in Fig. 8 is relevant. The presence of large soluble pools of both lamin and topo II early in embryogenesis, presumably derived from germinal vesicle breakdown at the end of oogenesis, suggests that the solubility of topo II in vivo can be regulated in a manner comparable to that of lamins. Similar behavior of *Xenopus* topo II has been reported (Luke and Bogenhagen, 1989).

Adachi et al. (1989) demonstrated the ability of *S. pombe* topo II to bind cooperatively to plasmids containing specific DNA-scaffold attachment site sequences and upon so doing, to form supramacromolecular aggregates. We as well as others demonstrated that purified lamins can assemble into filaments ("aggregate") in vitro (Aebi et al., 1986; Lin and Fisher, 1990). Moreover, we showed that only lamin isolated from interphase nuclei was capable of in vitro self-assembly; the soluble form found in early embryos was unable to do so (Lin and Fisher, 1990). By analogy, it may be that only the form of topo II isolated from interphase nuclei is able to participate in structural interactions necessary to form supramacromolecular aggregates. Direct comparison of the interphase nuclear form of *Drosophila* topo II with the soluble form found in early embryos may thus lead to explicit insights regarding the biological significance of aggregates such as those reported by Adachi et al. (1989) and hence, the potential role of topo II as a karyoskeletal protein.

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