Consequences of topoisomerase II inhibition in early embryogenesis of *Drosophila* revealed by in vivo confocal laser scanning microscopy

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SUMMARY

The regulation of DNA topology by topoisomerase II from *Drosophila melanogaster* has been studied extensively by biochemical methods but little is known about its roles in vivo. We have performed experiments on the inhibition of topoisomerase II in living *Drosophila* blastoderm embryos. We show that the enzymatic activity can be specifically disrupted by microinjection of antitopoisomerase II antibodies as well as the epipodophyllotoxin VM26, a known inhibitor of topoisomerase II in vitro. By labeling the chromatin of live embryos with tetramethylrhodamine-coupled histones, the effects of inhibition on nuclear morphology and behaviour was followed in vivo using confocal laser scanning microscopy. Both the antibodies and the drug prevented or hindered the segregation of chromatin daughter sets at the anaphase stage of mitosis. In addition, high concentrations of inhibitor interfered with the condensation of chromatin and its proper arrangement into the metaphase plate. The observed effects yielded non-functional nuclei, which were drawn into the inner yolk mass of the embryo. Concurrently, undamaged nuclei surrounding the affected region underwent compensatory division, leading to the restoration of the nuclear population, and thereby demonstrating the regulative capacity of *Drosophila* blastoderm embryos.

Key words: topoisomerase II, confocal microscopy, microinjection, *Drosophila*, chromatin, mitosis

INTRODUCTION

The regulation of DNA topology is essential for a number of cellular processes such as replication, transcription and mitosis. This function is performed primarily by DNA topoisomerases that are able to transiently break the phosphodiester bonds of the DNA backbone (for reviews, see Vosberg, 1985; Wang, 1985). Topoisomerases of the class II cleave both strands of double-stranded DNA, pass another helix or a part of the same helix through the break, and re-ligate the two strands. Eucaryotic topoisomerase II catalyses in vitro the: (i) relaxation of negatively and positively supercoiled DNA molecules; (ii) knotting and unknotting of a closed DNA circle; and (iii) catenation and decatenation of closed DNA circles. In addition to these enzymatic properties, topoisomerase II has been isolated as a major component of the nuclear matrix (Berrios et al., 1985) and is implicated in chromatin assembly and maintenance (Earnshaw et al., 1985; Gasser et al., 1986). In yeast, topoisomerase II is encoded by a single-copy, essential gene. Yeast cells bearing a temperature-sensitive mutation in the top2 gene are blocked at the segregation step of mitosis at the restrictive temperature (DiNardo et al., 1984; Uemura et al., 1987), indicating that the decatenation function of topoisomerase II is needed at this phase of cell division. Topoisomerase II was shown to be essential for replication of SV40 virus chromosomes in vitro (Ishimi et al., 1992), but it is probably topoisomerase I that serves to modulate DNA topology during replication of the yeast chromosomes (Kim and Wang, 1989). Topoisomerase activity is also involved in rRNA transcription in yeast, a requirement that can be met by either topoisomerase I or II (Brill et al., 1987).

The *Drosophila* genome contains a single topoisomerase II gene (Wyckoff et al., 1989) for which no mutation has been identified so far. The active enzyme is a homodimer of two 165 kDa polypeptides and exhibits the enzymatic properties of topoisomerases II described above. In addition, the enzyme has recently been shown to preferentially bind left-handed Z-DNA (Arndt-Jovin et al., unpublished; Glikin et al., 1991). Maximal expression of *Drosophila* topoisomerase II can be detected during embryogenesis and the prepupal stage (Fairman and Brutlag, 1988; Nolan et al., 1991). These periods correlate well with the maxima in DNA synthesis and mitotic activity. Regardless of the data from expression studies, the in vivo roles of topoisomerase II in *Drosophila* have not been elucidated. To test the analogy to the lower eucaryote yeast we need to investigate steps in DNA replication and mitosis. The early embryogenesis period of *Drosophila* development provides an excellent system for analysis of these steps. The transparent egg carries out 13 rapid cycles of nuclear division and
DNA replication until gastrulation. These cycles lack the G1 and G2 phases. Development is maintained and regulated by maternal products. After migration of the majority of nuclei from the yolk mass to the egg periphery between nuclear divisions 8 and 10, the embryo forms a syncytial blastoderm that is characterized by four nearly synchronous and rapid (20 min) mitotic cycles.

Several technical advances have enabled the observation of processes in living organisms. Digital imaging microscopy, either with raster confocal laser scanning or by full-field optical imaging and 3-dimensional deconvolution, allows the optical sectioning of thick, transparent specimens (Agard et al., 1989; Jovin and Arndt-Jovin, 1989; Pawley, 1989; Shotton, 1989; Hiraoka et al., 1991). In conjunction with fluorescence labeling of macromolecules and microinjection techniques, these instruments enable the study of specific aspects of a living organism with high temporal and spatial resolution. In the experiments reported here, we studied the role of topoisomerase II during early embryogenesis of Drosophila by microinjection of inhibitors of the enzyme. We showed that topoisomerase II action can be specifically disrupted in vitro and in vivo both by a monoclonal anti-topoisomerase II (anti-topo II) antibody and the epipodophyllotoxin, VM26. Inhibition of the enzyme in living embryos caused a block of nuclear division before anaphase as is the case for the simple eucaryote yeast. In addition, the enzyme appears to be necessary for some step in chromatin condensation before formation of the metaphase plate.

**MATERIALS AND METHODS**

**Drosophila strains and cell lines**

*Drosophila melanogaster* wild-type strain Oregon R was used throughout this study. The *Drosophila* cell line S3 was used for the preparation of topoisomerase II from nuclear extracts.

**Purification of topoisomerase II**

The purification of topoisomerase II from *Drosophila* S3 cells was according to Arndt-Jovin et al. (unpublished). The enzyme prepared in this way was used in relaxation and decatenation assays (see below) for immunization of rabbits, and for affinity purification of the topoisomerase II antibodies (see below).

**Topoisomerase II relaxation assay**

The assay was performed as described by Hsieh and Brutlag (1980) with slight modifications. Enzyme fractions were diluted in 10 mM sodium phosphate, pH 7.1, 50 mM NaCl, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 0.5 mg/ml bovine serum albumin (BSA), 10% glycerol and 0.2 mM dithiothreitol. Relaxation assays were performed in 10 µl reaction buffer containing 0.15 µg supercoiled pUC18 in 50 mM KCl, 100 mM NaCl, 10 mM MgCl2, 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 50 µg/ml BSA and 1 mM ATP (40 minutes at 30°C). The reactions were stopped by adding 1/10 of the reaction volume of Proteinase K (150 µg/ml in 1% SDS, 100 mM EDTA, 4% sucrose, 0.1% bromophenol blue) and incubation for 15 minutes at 50°C. Topoisomeres were resolved electrophoretically on an 0.7% agarose gel (50 V, 2 hours) and visualized by ethidium bromide staining.

**Antibodies**

Rhodamine- or peroxidase-coupled goat-anti-rabbit IgGs were obtained from Dianova (Hamburg, FRG). The two anti-topoisomerase II IgGs, W6 and H2, were produced by immunizing two rabbits with chromatographically purified *Drosophila* topoisomerase II.

**Protein electrophoresis and immunoblotting**

Extracts from *Drosophila* S3 cell nuclei, purified topoisomerase II, and marker proteins were run on a 7% SDS denaturing polyacrylamide gel according to O’Farrell et al. (1977) for 4 hours at 100 V. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA) according to the manufacturer’s instructions. The membrane was blocked for 2 hours in a 5% solution of dried milk powder in Tris-buffered saline (TBS) and incubated overnight at 4°C with W6 or H2 IgG at a concentration of 5-10 µg/ml (in TBS containing 0.05% Tween 80 and 50 µg BSA per ml). After washing (3x20 minutes TBS, 0.1% Tween 20, room temperature), a goat anti-rabbit IgG coupled with peroxidase was applied for 2 hours at room temperature. The membranes were washed again and an enzymatic colour precipitate was developed with diaminobenzidine (0.5 mg/ml in phosphate-buffered saline, + 0.01% H2O2). Control lanes of the blot were stained with colloidal gold (Aurodye, Amersham, Braunschweig, FRG) for visualization of the total protein.

**Immunostaining of whole-mount Drosophila embryos**

Immunostaining of *Drosophila* embryos was carried out as described by Mitchison and Sedat (1983), with slight modifications. Briefly, embryos (0-3 h) were dechorionated in 1:1 hypochlorite/buffer A for 2 minutes (buffer A: 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA, 15 mM PIPES, pH 7.4). The embryos were fixed in a heptane/buffer A/formaldehyde (10:10:12; by vol.) mixture for 15 minutes and devitellinized by thermal shock. The anti-topo II antibodies W6 or H2 were applied overnight at 4°C at a concentration of 20 µg/ml in buffer A. The rhodamine-coupled goat anti-rabbit IgG antibody was reacted at 4°C for 4 h. DNA was counter-stained for 15 minutes with 50 µM mithramycin, in 7.5 mM MgCl2, 25 mM Tris-HCl, pH 7.5. The same concentrations of buffered mithramycin were applied through the graded glycerol series. The embryos were mounted in the last glycerol solution (80% glycerol, 50 µM mithramycin).

**Preparation of molecules for microinjection**

**Histones**

Core histones from chicken erythrocytes were labeled with tetramethylrhodamine according to a method described by Minden et al. (1989). The labeling ratio of the H2A/H2B pool used for microinjection was about 0.1 (rhodamine:histone).

**Antibodies**

IgG fractions of the anti-topo II sera were prepared by chromatography on Protein G-Sepharose (Sigma, St.Louis, MO). Anti-topo II and control antibodies were labeled with fluorescein isothiocyanate (FITC) to a ratio of about 3 fluorescein molecules per antibody. For affinity purification of anti-topo II a glutaraldehyde-activated membrane (actidisc, FMC, Rockland, ME) was loaded with about 90 µg purified topoisomerase II according to the manufacturer’s instructions. The IgG was circulated over this actidisc in a low salt buffer (0.15 M NaCl, 10 mM sodium-phosphate, pH 7.3) for 20 minutes. After washing non-specific antibodies from the membrane with phosphate buffer containing 1 M NaCl, the purified anti-topo II was eluted with 0.1 M glycine, pH 2.8, and dialysed against PBS. All antibodies were concentrated for injection by centrifugation in Centricon 10 tubes.
Drug
The topoisomerase II-specific drug VM26 (the kind gift of Brystol/Meyers) was dissolved in dimethylsulphoxide (DMSO) to a concentration of 10 mM and stored in small samples at –80°C. For injection this stock solution was diluted in PBS to a final concentration of 0.2-0.4 mM.

Microinjection
Microinjection of embryos was carried out using standard methods (see for example, Santamaria, 1986). Before injection all solutions were filtered through a 0.22 µm microfilter (Millipore). The injection was performed under 10S-Voltaire oil (Atochem, Pierre-Bénite, France) on an inverted microscope IM 35 (Zeiss, Göttingen, FRG) using a micromanipulator (Leica, Heidelberg, FRG) and a nitrogen pressure microinjection system (5242, Eppendorf, Hamburg, FRG). Needles were heat pulled from 10 µl glass capillaries (Brand, Wertheim, FRG) and the tip was broken with a fine tungsten needle to an opening of 2-4 µm. Embryos were injected at about 50% egg length at a dorsal or lateral position. The injected volume was between 200 and 500 pl (or 2-5% of the embryo volume).

For studies of fixed material, injected embryos were incubated in a humid chamber for 20-30 minutes and then fixed with 8% formaldehyde as described by Warn et al. (1987). After fixation, the embryos were washed several times in PBS containing 0.1% Tween 20, stained with 0.1 µg/ml Hoechst 33342 in PBS, washed again, and mounted in Gey’s Magic Mountant (1.5 g canada balsam per 1 ml methylsalicylate). The preparations were cleared for 1 day before analysis.

Microscopy and image analysis
A confocal laser scanning microscope (CLSM; Zeiss model LSM 10) was used for collecting digital images of optical sections through living as well as fixed embryos using either a 25x, NA 0.8, Plan-Neofluar or a 40x, NA 0.9, Plan-Neofluar immersion objective equipped with correction collars. The instrument was supplemented with an external, tunable, 3 W argon-ion laser (Spectra Physics, Mountain View, CA) aligned coaxially to the 2 internal lasers (5 mW argon-ion and He-Ne). Fluorescein and tetramethylrhodamine were excited at 488 nm and 514 nm, respectively, by the internal argon ion laser. The DNA dyes Hoechst 33342 and mithramycin were excited at 488 nm and 514 nm, respectively, by the internal argon ion laser. The following emission filters were used: BP525 for fluorescein and mithramycin, LP610 or LP550 for tetramethylrhodamine and LP408 for Hoechst. Images (8 bit) were acquired with an appropriate scanning time and frame averaging (see figure legends). They were transferred via an IEEE interface to a DE MicroVAX II, and in some cases subsequently to a Macintosh II fx computer, for image enhancement and processing using NIH-Image 1.4g (National Institutes of Health, Bethesda, Maryland), TCL-Image (Multihouse, Delft, NL), and specialized macros and programs written in TCL-command format or FORTRAN. Images were background subtracted, contrast stretched, and uniform filtered when necessary. Three-dimensional stereo pair images were generated according to the method of Brakenhoff et al. (1986).

RESULTS
Characterization of the polyclonal anti-topoisomerase II antibodies W6 and H2
For our experiments we used IgG fractions of two polyclonal antisera, W6 and H2, against topoisomerase II purified from nuclear extracts of Drosophila S3 culture cells (Arndt-Jovin et al., unpublished). The antisera were directed specifically against topoisomerase II according to the following observations. (i) On Western blots of unfractionated S3-nuclear extracts, using the IgGs at a concentration of 0.5 µg/ml, W6 (exclusively) and H2 (predominantly) detected a protein band with an apparent molecular mass of 165 kDa, corresponding to the purified Drosophila topoisomerase II (Fig. 1A, lanes 2-6 for W6 and 7-11 for H2). Although H2 IgG also detected a minor band of about 70 kDa, there was no difference between the effects of the two antibodies when injected into Drosophila embryos. On dot blots of purified topoisomerase II, the IgGs recognized as little as 0.2 ng of the enzyme (data not shown). (ii) Both IgG fractions were localized exclusively to the nuclear chromatin in methanol-fixed or non-fixed S3 cells as determined by indirect immunofluorescence (data not shown). (iii) Both antisera inhibited the enzymatic activity of topoisomerase II in vitro. Two standard topoisomerase assays are the relaxation of supercoiled plasmid DNA (sc-DNA) and the decatenation of catenated DNA from Crithidia. We performed these assays using purified Drosophila topoisomerase II in the absence and presence of anti-topo II and control antibodies. Fig. 1B shows the relaxation assay. Various amounts of anti-topo II (W6) and control antibodies (ranging from 40 ng to 1 µg) were added to 1 ng topoisomerase II and incubated for 30 minutes at 4°C. A sample of the solution after centrifugation (5 minutes at 14000 revs/min in an Eppendorf centrifuge) was then tested for topoisomerase II activity using 0.15 µg of supercoiled pUC18. Whereas control antibodies (i.e. IgG from non-immunized rabbits) did not show any effect on the relaxation of sc-DNA by the enzyme (Fig. 1B, lanes 4-6), the enzyme activity was clearly inhibited by the anti-topo II antibodies (Fig. 1B, lanes 2 and 3). Similarly, the decatenation of Crithidia DNA by topoisomerase II was inhibited by addition of anti-topo II antibodies (data not shown).

W6 and H2 recognize their antigen in the living Drosophila embryo
Our goal was to study the role of topoisomerase II in embryogenesis by inhibition of the enzyme in vivo. For successful protein inhibition experiments with antibodies it is necessary that the antibody be stably bound to its antigen after microinjection. However, the reaction of a monospecific antibody in vivo could be different from the in vitro situation, due to altered accessibility of the antigen and the presence of cellular proteases. We therefore tested if anti-topo II antibodies could also bind to their target antigen in the living Drosophila embryo by microinjection of directly fluorescently labeled anti-topo II.

Polyclonal anti-topo II (W6) was fluorescently labeled with FITC and then affinity purified on matrix-linked topoisomerase II as described in Materials and Methods. Embryos of nuclear cycles 8-13 were injected with this antibody (8 mg/ml in the needle) at 50% egg length. The embryos were then allowed to develop further for 20-30 minutes, after which they were fixed and washed several times to remove unbound antibody. The distribution of the directly labeled antibodies was analyzed by CLSM and compared with the staining pattern derived from indirect immunofluorescence with anti-topo II antibodies on pre-
Fig. 1. (A) Immunoblots using anti-topo II antibodies W6 and H2. Proteins from a 350 mM (NH₄)₂SO₄ extraction of S3 nuclei and purified Drosophila topoisomerase II were separated by denaturing SDS-PAGE, transferred to a PVDF membrane and either stained for protein by Aurodye staining (lane 1), or immunostained with W6 (lanes 2-6), or H2 (lanes 7-11). After incubation with peroxidase-coupled goat anti-rabbit IgG, antigenic bands were visualized by diaminobenzidine staining. The number of bands detected with W6 and control antibody were incubated for 30 minutes with purified topoisomerase II in enzyme dilution buffer. A sample of each solution was used to relax supercoiled pUC18. Topoisomerases were separated by gel electrophoresis and visualized by ethidium bromide staining. Lane 1: control, no antibody (ab) present. Lanes 2 and 3: incubation with W6 antitopo II, 100 and 40 ng, respectively. Lanes 4-6: incubation with non-immune control antibody, 90, 360 and 900 ng, respectively.

(A) Immunoblots using anti-topo II antibodies W6 and H2. Denaturing SDS-PAGE, transferred to a PVDF membrane and either stained for protein by Aurodye staining (lane 1), or immunostained with W6 (lanes 2-6), or H2 (lanes 7-11). After incubation with peroxidase-coupled goat anti-rabbit IgG, antigenic bands were visualized by diaminobenzidine staining. The molecular mass (in kDa) is indicated on the left. (B) Inhibition of topoisomerase II relaxation activity by W6. Various amounts of W6 and control antibody were incubated for 30 minutes with purified Drosophila topoisomerase II in enzyme dilution buffer. A sample of each solution was used to relax supercoiled pUC18. Topoisomerases were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Lane 1: control, no antibody (ab) present. Lanes 2 and 3: incubation with W6 antitopo II, 100 and 40 ng, respectively. Lanes 4-6: incubation with non-immune control antibody, 90, 360 and 900 ng, respectively. rx, relaxed; sc, supercoiled plasmid DNA.

fixed embryos. As shown in Fig. 2, both procedures gave very similar staining patterns. The antibodies were preferentially concentrated on chromatin. Before gastrulation at least 40% of the antibody signal was detected in the cytoplasm in both injected and indirectly stained embryos, indicating the presence of a sizeable cytoplasmic enzyme pool at this stage.

Nuclear division can be followed in living embryos by confocal laser scanning microscopy

For real-time observations of nuclei embryos of nuclear cycles 4-8 were injected with tetramethylrhodamine-labeled histones (rh-histones) H2A/H2B at 0.5 mg/ml in PBS. These subsequently become incorporated into embryonic nucleosomes, allowing a direct visualization of chromatin by fluorescence microscopy (Minden et al., 1989). After an incubation of 20-30 minutes in a humid chamber (1-2 nuclear cycles at 20°C) most of the embryonic nuclei contained sufficient rh-histones to be visualized by CLSM using the 514 nm line of an argon-ion laser. Under these conditions, normal nuclear division could be followed throughout the blastoderm stage in most embryos, indicating that repeated laser illumination was compatible with survival of the embryos. In order to rule out long-term toxic effects of the rh-histones, we incubated a number of histone-injected embryos in a humid chamber containing oxygen atmosphere and determined the hatching rate. A total of 51% (n = 69) of the embryos that were injected before the completion of nuclear migration hatched within 30 hours at room temperature. Embryos injected during the blastoderm stage showed an even higher hatching rate of 72% (n = 39). These values were not significantly different from the hatching rates for control embryos incubated with physiological buffer (58% and 74%, respectively), showing the absence of toxic effects of rh-histones up to the first larval stage.

Anti-topoisomerase II antibodies inhibit mitosis

In order to achieve in vivo inhibition of the topoisomerase II, we injected the antibodies W6 and H2, described above, into embryos that were developing normally by about 30 minutes after rh-histone injection. The effects of the antibody injections on nuclear structure and behaviour were analysed by CLSM over a period of 3-4 nuclear divisions in blastoderm.

Control embryos (i.e. embryos which had been injected with non-immune IgG) did not show any changes in nuclear morphology or behaviour (data not shown). In contrast, injection of anti-topo II antibodies clearly led to a localized disruption of normal mitosis. The antibodies were injected at interphase and the effect was usually visible at the first nuclear division (i.e. 5-10 minutes) after the injection. Fig. 3 shows regions of a non-injected embryo (A-D) and an embryo that had been injected with anti-topo II antibody H2 (E-J) at subsequent time points of mitosis. In both cases the chromatin condensed at prophase (Fig. 3A, E, F). Whereas in normal mitosis it was possible to discern chromosomes in the metaphase plate (Fig. 3B; the chromosomes are oriented perpendicular to the image plane), condensation proceeded up to a state of tightly packed chromatin in the antibody-injected embryos (Fig. 3G), but the affected nuclei did not show a clear metaphase plate. Separation of daughter sets at anaphase was severely affected in these nuclei. In some cases there was no segregation at all, in others the anaphase was characterized by chromatins, which stayed at the midspindle level and was only slowly resolved (Fig. 3H, I). The affected daughter nuclei were still connected by chromatin bridges at telophase, thereby forming dumbbell-shaped nuclei, which then proceeded to decondense much like the non-affected nuclei but failed to enter further condensation and division cycles. These effects spread out from the injection site into larger surrounding regions during subsequent nuclear cycles. The affected nuclei decondensed fur-
ther and eventually formed a mass of chromatin in which it was no longer possible to distinguish individual nuclei. Concurrently with the decondensation, the defective chromatin detached from the surface of the embryo and sank down into the yolk mass as depicted from 2 confocal sections through a representative embryo (Fig. 4).

The topoisomerase-specific drug VM26 inhibits mitosis

The epipodophyllotoxine VM26 has been shown to inhibit topoisomerase II in vitro (Chen et al., 1984) as well as in a variety of cell types, including Drosophila Kc cells (Udvardy and Schedl, 1991). We studied the effects of such an inhibition in living embryos. Rh-histone-injected embryos that were developing normally were injected either with VM26 (0.20 mM, 5% DMSO in PBS) or with control solution (5% DMSO in PBS) at 40-60% egg length. CLSM images were acquired in intervals ranging from 60 seconds to 30 minutes over a time period of up to 2 hours (or 4 nuclear divisions at 20°C).

Injection of the control solution did not interfere with normal development of the embryos. In contrast, injection of VM26 clearly disrupted normal mitosis. With the injection of VM26 at a concentration of 0.2-0.4 mM in the needle, between 100 and 300 nuclei showed defects in the first mitosis after injection. Fig. 5 shows stereo images of a living embryo at 4 different time points after drug injection. Each picture was reconstructed from 10 optical sections taken by CLSM covering about one half of the embryonic diameter. The first picture was taken in nuclear cycle 11, 20 minutes after the VM26 injection (Fig. 5A, the site of injection is marked by the arrowheads). Whereas the pole regions showed a normal nuclear density at this time, the drug had completely prevented mitosis 10/11 in the midregion of the embryo reaching from 20% to about 70% egg length. Fig. 5B, C and D shows the embryo in nuclear cycles 12, 13 and 14, respectively. Two effects are evident from these pictures as well as from similar drug-injected embryos not shown here. First, the nuclei in the region of VM26 injection produced aberrant mitoses and appeared more intensely stained and somewhat larger than the normal nuclei surrounding them. Second, the affected region became successively smaller and was eventually restricted to the site of drug injection (Fig. 5D). This regulative process was accomplished by loss of the defective nuclei from the surface to the inner yolk mass and repopulation of the affected area by daughter nuclei from the non-affected regions. By taking pictures from drug-injected embryos with intervals as short as 60 seconds we were able to observe this regulative process in a time-lapse mode. The healthy nuclei appeared to “push” the defective ones towards the interior, eventually taking their place on the surface. In some cases, regulation was particularly successful. For example, the embryo shown in Fig. 5 went through gastrulation and developed a cuticle with all segments but, however, it did not hatch.

Fig. 2. Anti-topo II antibodies (W6) in injected and immunostained embryos. (A and B) Surface view of a blastoderm embryo that had been injected with fluorescein-coupled W6, allowed to develop for 30 minutes, and then fixed and stained for DNA. (A) Antibody fluorescence. (B) DNA/Hoechst fluorescence. (C and D) Surface view of an embryo at a similar stage. This embryo has been immunostained with W6, i.e. it was fixed before applying the anti-topo II antibody. (C) Antibody fluorescence. (D) DNA/mithramycin fluorescence. Objective: 63×, NA1.4, Planapochromat. Scanning time: 16 s/frame, 4-8 frame averaging. Bars, 10 μm.
VM26 affects chromatin condensation and separation

For more detailed information on the effects of VM26 in vivo it was necessary to inject the drug at a fixed point of the nuclear cycle and to acquire images with higher magnification at short time intervals. We chose the onset of interphase as the time of injection, allowing approximately 10 minutes before the next mitosis for drug diffusion and binding. Fig. 6 shows nuclei in an embryo that was injected with VM26 (0.38 mM) at the onset of interphase of cycle 11. Whereas we did not detect any abnormalities in nuclear morphology during interphase 11, the following mitosis was inhibited in a concentration-dependent manner. At prophase 11 all the nuclei, irrespective of their location, performed at least a partial condensation of chromatin as judged by their decrease in size and increase in fluorescence intensity (Fig. 6, compare A and B). In the region immediately adjacent to the site of injection, i.e. where the highest drug concentration was present, mitosis was blocked at this stage without further changes in chromatin morphology (Fig. 6B-D, top). In fact, these nuclei remained in this state for more than 50 minutes. At no time were chromosomes or chromatin fibers detached from these nuclei. Farther away from the injection site with presumably lower concentrations of VM26, further condensation of chromatin into individual chromosomes and abortive formation of the metaphase plate was observed. At anaphase the subsequent segregation of the chromatin daughter sets was severely affected. Unequal amounts of chromatin were pulled towards the spindle poles and chromosomal material remained at the metaphase plate (Fig. 6D). Such nuclei were still connected by chromatin bridges after decondensation and became similar to the dumbbell-shaped nuclei which were observed in embryos injected with anti-topo II (see Fig. 3). It is worthwhile to note that decondensation of chromatin took place in normal and dumbbell-shaped nuclei but not in the region of highest drug concentration. Further away from the injection site mitosis appeared to proceed normally (Fig. 6,

Fig. 3. Effect of anti-topo II IgG on mitosis. Embryos were injected with rh-histones before nuclear migration, to visualize chromatin. (A-D) Normal mitosis, pro-, meta-, ana- and telophase, respectively. (E-J) Mitosis of an embryo that had been injected with anti-topo II IgG (2 mg/ml in PBS). The nuclei in this field close to the site of injection failed to separate normally. The time in minutes relative to E is given at the lower left of each panel. Objective: 40×, NA 0.9, Plan Neofluar immersion with correction collar. Scanning time: 4-8 s. Bar in A, 10 μm; magnification for E-J, approximately the same.
Fig. 4. Long-term effect of anti-topo II IgG on the chromatin. The embryo shown here was treated as described in the legend to Fig. 4 (microinjection of the antibody was at cycle 11). (A) shows a surface and (B) a sagittal section 60 minutes after antibody injection (nuclear cycle 14). A mass of decondensed chromatin is lying toward the inner yolk mass of the embryo. Obj.: as in Fig. 3; 64 s/section. Bar, 100 µm.

bottom). The effect of the drug on chromatin condensation and separation is illustrated at higher resolution in Fig. 7 where nuclei from other drug-injected as well as control embryos are shown at six time points through mitosis. As in the embryo shown in Fig. 5 the affected nuclei were removed from the embryo periphery toward the yolk mass and the affected region was repopulated by normal nuclei.

DISCUSSION

We have described the local disruption of topoisomerase II action in living Drosophila blastoderm embryos by the topo II-specific drug VM26 as well as anti-topo II antibodies. Microinjection of antibodies has been used successfully to disrupt the function of nuclear proteins including histones (Scheer et al., 1979), lamins (Benavente and Krone, 1986), RNA polymerase I (Benavente et al., 1987), and a mitotic spindle protein (Kallajoki et al., 1991). The method has also been applied to Drosophila embryos. Warn et al. (1987) reported the injection of monoclonal antibodies against α-tubulin into blastoderm embryos and demonstrated antigen labeling as well as protein inhibition in vivo. The antibodies we chose to use for studying topoisomerase II recognized the enzyme on Western blots (Fig. 1A) and cytological preparations. In addition, they clearly inhibited biochemical reactions catalysed by topoisomerase II in vitro (Fig. 1B). Although we recently tested a number of monoclonal antibodies against topoisomerase II, none of them was able to inhibit enzyme action and all of them bound preferentially to the denatured rather than to the native topoisomerase II (authors’ unpublished observations). We regarded the in vitro inhibition by the polyclonal antibodies as a prerequisite for the intended in vivo experiments.

In a second step we determined whether the injected anti-topo II localized similarly in the living embryo as the enzyme distribution observed in fixed material. This was indeed the case (Fig. 2). Microinjected anti-topo II bound preferentially to the chromat. This localization was specific for anti-topo II, since in control experiments microinjected non-immune antibodies distributed homogeneously (and therefore non-specifically) throughout the whole embryo periphery (unpublished observation). The presence of a cytoplasmic pool of topoisomerase II in early Drosophila embryogenesis revealed by our immunochromatographic experiments is in agreement with results obtained by Whalen et al. (1991) and Nolan et al. (1991). From our data we conclude that injected anti-topo II recognizes the enzyme in vivo.

Using the injection of tetramethylrhodamine-labeled histones for visualization of the chromatin and confocal laser scanning microscopy it was possible to study the disruption of topoisomerase II action in living embryos. Both anti-topo II and the drug VM26 showed similar inhibitory effects on the nuclear cycle of developing embryos. The inhibitor was introduced in interphase but no obvious disruption in nuclear morphology occurred until the next mitosis. The separation of chromosomes at anaphase was efficiently prevented or hindered (Figs 3, 6, 7), confirming the expectation from the studies of yeast mutants that topoisomerase II is essential for this process due to its unique capability of decatenating interwined DNA molecules. However, we cannot exclude the possibility that the mitotic block we observe is not the consequence of events compounded in interphase such as chromosome breakage.

There is growing evidence that topoisomerase II is also required for the final step in chromatin condensation (Uemura et al., 1987; Newport and Spann, 1987; Adachi et al., 1991). Wood and Earnshaw (1990) reported experiments on chromatin condensation in vitro using nuclei with variable levels of endogenous topoisomerase II incubated in mitotic cell extracts. Whereas an overall condensation of chromatin (accompanied by a decrease in nuclear volume) was independent of topoisomerase II, the final resolution of condensed chromatin into chromosomes appeared to require the enzyme. The authors proposed that condensation may be divided into a more general “compaction of chromatin”, which can take place without topoisomerase II, and a final “condensation into chromosomes” for which topoisomerase II is essential. Our data fit well to this model. We saw initial partial condensation of chromatin and decrease in nuclear volume in both the antibody- and the drug-injected embryos, but this did not proceed any further as was the case in non-injected embryos (Figs 3, 7). Anaphase was either then prevented completely or it started from a situation where the chromatin was only partially condensed but no individual chromosomes could be observed reaching the metaphase state. In normal embryonal mitosis the two con-
Densation processes are probably not separated. Hiraoka et al. (1989) presented high-resolution pictures of condensing *Drosophila* blastoderm nuclei in vivo. These authors did not observe a compaction-like process before formation of chromosomes in the “wild-type situation”. Thus, compaction may be a phenomenon that is observed only when topoisomerase II activity is blocked.

A clear difference between the effects of the two inhibitors on nuclear appearance could be observed from about 30 minutes postinjection. Whereas in drug-injected embryos the nuclei in the region of highest drug concentration stayed in their partially condensed state, antibody-injected embryos clearly showed decondensation of affected nuclei (compare Figs 4 and 5). A difference between the actions of the drug and the antibody may explain the observed effects. VM26 stabilizes the so-called

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**Fig. 5.** Effect of VM26 on early embryogenesis. Stereo images of an embryo injected with rh-histones to visualize chromatin and then with the topo II-specific drug VM26 (0.20 mM, 5% DMSO in PBS). Each stereo pair was reconstructed from 10 confocal sections 6 µm apart. Dorsal is up and anterior to the right. (A) 20 minutes after drug injection (a.d.i.), nuclear cycle 11. The arrowheads indicate the site of injection. The region affected by the drug reaches about 20 to 70% egg length. (B) 50 minutes a.d.i., nuclear cycle 12. (C) 85 minutes a.d.i., nuclear cycle 13. Note the high fluorescence intensity and large size of affected nuclei. (D) 120 minutes a.d.i., nuclear cycle 14. Obj.: 25×, NA 0.8, Plan Neofluar immersion with correction collar. 2s/frame, 4 frame averaging/section. Bar, 100 µm.
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cleavable complex, i.e. the DNA 5'-phosphoryl-linked topoisomerase II, thereby immobilizing the enzyme on the DNA. In contrast, the antibody probably prevents the cleavage step itself and does not prevent dissociation of the enzyme from the DNA. Decondensation presumably requires removal of topoisomerase II and this cannot be achieved in the presence of VM26.

Fairman and Brutlag (1988) estimated that there are $1.5 \times 10^9$ to $3 \times 10^9$ topoisomerase II molecules per embryo. It is clearly possible to saturate the enzyme with small molecules like VM26. We have injected concentrations yielding $10^9$ to $10^{11}$ drug molecules per embryo, which provided a gradient of effects ranging from complete inhibition to no inhibition at all. In contrast, the effective concentration of topoisomerase II-specific antibodies is probably one-tenth of that of the enzyme concentration if calculated for the volume of the whole embryo. However, since the rate of diffusion of a 150 kDa macromolecule is much slower than that of the drug, we can assume that the antibody is saturating at the site of injection. Therefore, the difference between the action of the antibody and the drug is unlikely to be solely due to a concentration effect.

An interesting question arises from the observation that the non-dividing nuclei in VM26-injected embryos are larger and more intensely stained (and therefore presumably contain more histones) than normal nuclei even after several nuclear cycles. This suggests that continued DNA synthesis is possible in these non-mitotic nuclei. Although VM26 blocks initiation of DNA synthesis subsequent to mitotic arrest in differentiated mammalian cells (Charron and Hancock, 1990), other studies suggest that mitosis and initiation of DNA synthesis are not as tightly coupled or controlled in Drosophila pre-gastrulation embryos (Raff and Glover, 1988). Experiments are in progress to measure DNA synthesis directly in drug- and antibody-injected embryos by simultaneous incorporation of fluorochrome-labeled nucleotides to resolve this question.

A disruption of the yeast topoisomerase II gene is lethal for the organism. The ability to locally inhibit the enzyme in a multinucleate organism as described in this study has provided a means to investigate the control and regulation of cell division in early Drosophila embryogenesis. Although the affected region can initially be very large (compare Fig. 5) embryos demonstrated their dramatic regenerative capacities in removing the non-functioning nuclei from the periphery and replacing them with functional ones, underscoring the non-determined nature and multipotency of nuclei throughout the embryo prior to gastrulation. Regulation was accomplished by shifting nuclei from non-affected regions into the defective area. Warn et al. (1987) reported the repopulation of defective regions (induced by microinjection of anti-tubulin α) with functional micro-

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**Fig. 6.** Effect of VM26 on mitosis. Dorsolateral surface of an embryo injected with rh-histones and VM26 (0.38 mM, 5% DMSO in PBS). Each picture was reconstructed from 5 confocal sections, 7 µm apart. The site of injection was outside the field shown here. (A) 8 minutes a.d.i., early prophase of cycle 11. (B) 13 minutes a.d.i., nuclei at the bottom and the right end of the field have reached metaphase. Nuclei at the top have undergone initial condensation. (C) 15 minutes a.d.i., anaphase. (D) 17 minutes a.d.i., telophase. The 3 different regions described in the text are visible by this time. Obj.: as in Fig. 3; 2s/frame, 2 frame averaging/section. Bar, 10 µm.
tubule arrays and nuclei from surrounding areas. In other studies, additional mitoses were shown to be involved in the regulation of experimentally induced regions with low nuclear density (Edgar et al., 1986; Yasuda et al., 1991). A more complete understanding of the mechanism of this control may be achieved by observation of other important macromolecules in the embryo after topoisomerase II inhibition, such as actin, tubulin and lamin.

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REFERENCES


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