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Essential Functions of DNA Topoisomerase I in *Drosophila melanogaster*

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DNA topoisomerase I (topo I) is an essential enzyme involved in replication, transcription, and recombination. To probe the functions of topo I during *Drosophila* development, we used *top1*-deficient flies with heat-shock-inducible *top1* transgenes and were able to observe both zygotic and maternal functions of *top1*. A critical period for the zygotic function is in the late larval and early pupal stages. Topo I is required for larval growth and cell proliferation in imaginal disc tissues. The maternal functions consist of two aspects: oogenesis and early embryogenesis. During oogenesis, topo I is detected in the nuclei of early germ-line cells and follicle cells. The mutant ovary exhibits abnormal proliferation and defective nuclear morphology in these cells. There are extranumeral germ-line cells in individual egg chambers, while the follicle cells are underreplicated. Topo I is also stored maternally in early embryos. It localizes to the nuclei during interphase and prophase, but disperses into the cytoplasm at metaphase. Embryos from the mutant mother frequently show disrupted nuclear divisions with defects in chromosome condensation and segregation. The cytological and genetic analysis of the *top1* mutant demonstrates that in *Drosophila*, topo I plays critical roles in many developmental stages active in cell proliferation. © 2000 Academic Press

Key Words: **DNA topoisomerase I; *Drosophila melanogaster*; embryogenesis; oogenesis; chromosome condensation and segregation.**

INTRODUCTION

DNA topoisomerases are ubiquitous enzymes that resolve torsional tension and topological hindrance generated during major DNA transactions, e.g., transcription and replication (reviewed in Wang, 1996). There are two classes of DNA topoisomerases, type I and type II, which differ structurally and mechanistically. Type II DNA topoisomerases are dimeric. They form homodimers and generate a transient double-strand break for another DNA duplex to move across. Type I DNA topoisomerases (topo I) are monomeric and introduce a transient single-strand break in DNA, through which another strand can pass, thereby reducing DNA supercoiling. The single-strand cleavage/rejoining activity of topo I suggests that it may serve as a swivel for unwinding and rewinding of DNA helices associated with critical cellular processes. Indeed, topo I function has been implicated in DNA replication, transcription, repair, and recombination (reviewed in Hsieh, 1993; Gupta *et al.*, 1995; Wang, 1996).

Despite its involvement in replication and transcription, topo I is not essential in yeast (Thrash *et al.*, 1984; Uemura and Yanagida, 1984). Genetic analysis suggests that topo II has a function that overlaps with that of topo I in these essential processes (Goto and Wang, 1985; Uemura *et al.*, 1987), although topo I is probably the major replication swivel (Kim and Wang, 1989a). Moreover, the *top1* mutant in *Saccharomyces cerevisiae* shows elevated levels of rDNA recombination, whereas suppression of rDNA recombination requires both topo I and topo II (Christman *et al.*, 1988; Kim and Wang, 1989b). Topo I has a distinct function in chromosome condensation (Sadoff *et al.*, 1995). It also promotes illegitimate recombination in *S. cerevisiae* (Zhu and Schiestl, 1996). Recent work showed that topo I is essential in a pathogenic fungal strain of *Cryptococcus* (Del Poeta *et al.*, 1999). As for multicellular organisms, topo I is essential in both *Drosophila* (Lee *et al.*, 1993) and mice (Morham *et al.*, 1996). In *Drosophila*, topo I is actively involved in the transcription process (Fleischmann *et al.*, 1984; Gilmour *et al.*, 1986; Gilmour and Elgin, 1987), and the recruitment of topo I to transcribed regions can be mediated by its N-terminal domain (Shaiu and Hsieh, 1998). However, potential functions of *Drosophila* topo I in other processes remain to be investigated. Mice deficient in *top1* die as early embryos, between the 4- and the 16-cell stages. This suggests a critical role for mouse topo I in early embryogen-

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esis. However, the early embryonic lethality of the *top1*$^-$ mice hampers genetic analysis of *top1* function in other developmental stages.

We have previously isolated a *top1*-deficient mutant in *Drosophila* and have shown that topo I is essential for *Drosophila* development (Lee *et al.*, 1993). To further understand the biological function of topo I, we analyzed growth defects in the *top1* mutant. We introduced a *top1* transgene under the control of an inducible heat shock promoter, hsp 70, into the *top1* mutant. Therefore, the *top1* function in entire *Drosophila* development can be examined by limiting its expression to specific developmental stages. We report here that *Drosophila top1* is essential for stages actively engaged in cell proliferation, such as larval and pupal growth, oogenesis, and embryogenesis.

MATERIALS AND METHODS

Drosophila Strains

We have previously generated several *Drosophila top1* mutant strains, including *top1*^{77} (Lee *et al.*, 1993). We used *P*-element-mediated transformation to introduce transgenes containing either *top1* genomic DNA or cDNA and tested their ability to rescue the lethality of the *top1* mutant. A 13.7-kb genomic DNA fragment between the *Spe*I and *Eco*RI sites, which includes the entire *top1* transcribed region and 5.4 kb of flanking sequence, was subcloned into a CaSpeR *P* element vector to establish stable germ-line transformants (Brown *et al.*, 1998). In this study, two *top1* cDNA clones, ctop1-2 and ctop1-6, with the sizes of 5.2 and 3.8 kb, respectively, were subcloned into the CaSpeRhsp70 vector, a CaSpeR *P* element vector with the heat shock promoter hsp70 and actin 5C polyadenylation addition sites (Thummel *et al.*, 1988). The 3′ region of the actin gene stabilizes the encoded mRNA after heat shock treatment is ceased. The construction of the *P* element transformation vectors, germ-line transformation, and screening/maintenance of the transformant lines followed the established protocols (Robertson *et al.*, 1988; also Roberts, 1986; with details specified in our earlier work, Lee *et al.*, 1993). We obtained 16 independent lines of transformants, among which 12 lines had *ctop1* inserted on the second or third chromosome. These 12 lines, 6 carrying ctop1-2 and 6 carrying ctop1-6, were used for the rescue experiment since *top1* is on the X chromosome.

Single-Larva PCR

Isolation of DNA from single larvae was performed according to Gloor *et al.* (1993). PCR was used to identify the genotype of each larva. Primers specific for *top1*^{77} were 5′CAGAGAAACCTTGTAGCC3′ (nucleotides 8630–8647 in *top1* gDNA) and 5′GACGGGACCACCTTATGT3′ (in the terminal repeat of the *P* element). A 700-bp PCR fragment was generated only in the presence of *top1*^{77}. Primers specific for *Drosophila* Y chromosome were 5′CGCAGTCACTGCACCTGGTAATGG3′ and 5′CCAACGGTTGTGCGCAAAGC3′, both of which reside in the gene encoding *Drosophila* dynein-related heavy chain (Gepner and Hays, 1993). PCRs with these primers generated a 330-bp fragment from the Y chromosome.

Northern and Western Blot Analysis

Total RNA of 100 flies from each strain was prepared and 5–10 μg of total RNA for each sample was loaded on a 1.1% agarose gel containing formaldehyde for Northern blots as described earlier (Lee *et al.*, 1993). Protein extracts were made from a single fly or ovaries by homogenizing the sample directly in SDS sample buffer. They were loaded on a 7% SDS polyacrylamide gel and subjected to Western analysis as described by Lee *et al.* (1993).

Heat Shock Experiments

Female flies *top1*^{77}/*FM7; ry*^{506} were crossed with *w*$^-$/Y; *P*[*ctop1-2*], *TM3, Sb/TM6, Tb* males to produce embryos. Overnight collections of embryos were transferred to vials with agar medium and incubated at 25°C for growth. Heat shock was performed in a 37°C incubator for 1 h every day to induce *top1* expression. A total of 5292 adult progeny were counted. The rescue efficiency for the *top1*^{77} mutants by *top1* cDNA expression was calculated by comparing the number of the rescued progeny, *top1*^{77}/Y; *P*[*ctop1-2*], *TM3, Sb/ry*^{506} to their siblings, *top1*^{77}/*w*$^-$; *P*[*ctop1-2*], *TM3, Sb/ry*^{506}. Any number exceeding 100% was entered as 100%.

Examination of top1 Function in Larvae and X-gal Staining of Imaginal Discs

Females of *top1*^{77}; *P*[*gtop1*], *TM3, Sb/P*[*313*], *TM6, Tb* were crossed to *top1*^{77}/Y; *P*[*ctop1-2*], *TM3, Sb/ry*^{506}. *P*[*gtop1*] contains a genomic copy of the *top1* gene. *P*[*313*] contains NF315, a fusion product of the *top1* N-terminal region and the *lacZ* gene (Shaiu and Hsieh, 1998). This *top1–lacZ* fusion protein has no catalytic activity of *top1* and cannot replace *top1* function in *Drosophila*. Its presence does not affect normal topo I function (Shaiu and Hsieh, unpublished results). The *top1* mutant progeny from the above cross, females with *top1*^{77}; *ry*^{506}/*P*[*313*], *Tb*; males with *top1*^{77}/Y; *ry*^{506}/*P*[*313*], *Tb*; females with *top1*^{77}; *P*[*ctop1-2*], *Sb/P*[*313*], *Tb*; and males with *top1*^{77}/Y; *P*[*ctop1-2*], *Sb/P*[*313*], *Tb* can be readily identified by the *Tb* marker. Furthermore, they all carry the *top1–lacZ* fusion protein. The mutant larvae were staged according to their mouth hook and spiracle morphology. Imaginal discs from the third-instar larvae were dissected, fixed, and stained with X-gal according to Shaiu and Hsieh (1998). Images were recorded by a Leitz DMIL inverted microscope equipped with a Hamamatsu C5810 camera.

Immunofluorescence Microscopy

Ovaries were dissected from 3- to 4-day-old female flies and stained as described by Lin *et al.* (1994). Zero- to three-hour or overnight collections of embryos were prepared according to Zhang *et al.* (1996). The rabbit anti-topo I antibody (Lee *et al.*, 1993) was used at 1:20 dilution and the FITC-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used at 1:600 dilution. The goat anti-topo II antibody was used at 1:5 dilution and the Cy3-conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch Laboratories) was used at 1:500 dilution. DAPI (4,6-diamidino-2-phenylindole; Sigma Chemicals) was used at 1 μg/ml and fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR) was used at 1:15.

Live Embryo Video Image Analysis

A third-chromosome GFP–histone H2A fusion transgene (Clarkson and Saint, 1999) was introduced into homozygous $top1^{77}$ females with *P[ctop1-2]*. Regular heat shock treatment was used to maintain the viability of *top1* mutant females. The embryos were collected from a population of *top1* mutant mothers mated with P2 wild-type fathers. The dechorionated embryos were imaged using a Bio-Rad MRC600 confocal scanning microscope according to Kiehart *et al.* (1994) and the video images were analyzed by NIH Image 1.62.

RESULTS

top1 Expression Is Required for Larval Growth

We previously isolated a lethal mutation in DNA topoisomerase I, $top1^{77}$, during *P*-element-mediated mutagenesis (Lee *et al.*, 1993). To understand why *top1* is essential for growth, we determined the lethal phase for the $top1^{77}$ fly. Female flies of $top1^{77}$/*FM7* were crossed with *FM7*/Y to produce mutant progeny, $top1^{77}$/Y. All the embryos produced in this cross could develop into larvae, and they showed a normal nuclear morphology as ascertained by fluorescence microscopy (data not shown). When larval development was studied, we observed necrotic tissues in some of the second-instar larvae. Rather than occurring at specific loci, these necrotic tissues could be found throughout the larval body (data not shown). These abnormal larvae all died before entering the third-instar stage, suggesting a growth defect caused by $top1^{77}$. PCR of DNA from single larva (see Materials and Methods) confirmed that they were $top1^{77}$/Y (data not shown). In contrast, all the larvae carrying an *FM7* chromosome developed to the third-instar stage and continued to grow normally into adulthood. Since maternal storage of the *top1* gene has been observed in early embryos before zygotic transcription is turned on (Brown *et al.*, 1998), it is possible that contribution from the heterozygous mother, $top1^{77}$/*FM7*, permits embryonic and early larval development of the $top1^{77}$ fly.

The above mutant analysis indicates that the earliest critical window for zygotic function of *top1* is in the second-instar larva. We also took a different approach to investigate the critical times for zygotic function during *Drosophila* development. In these experiments, *top1* expression was turned on at various developmental stages and its effects were studied. We used two versions of *top1* cDNA, differing in the 3′ untranslated region, to generate two forms of transgenes controlled by a heat shock promoter, hsp70 (Fig. 1A). The long form, ctop1-2, contains a cDNA of 5.2 kb in size and its corresponding mRNA is found in all the developmental stages (Lee *et al.*, 1993). The short form, ctop1-6, is 3.8 kb long. It is specifically detected in female ovaries and early embryos, serving as maternal storage material (Brown *et al.*, 1998). We examined topo I expression in 12 independent lines by comparing *top1* expression in transgenic flies with and without heat shock induction. Since these transformants all have the wild-type X chromosome, the *top1* expression in flies without heat treatment is mostly from the endogenous gene. For ctop1-2, two of the transformants showed significant increase in *top1* expression after heat shock. After normalizing against RP49 messages and actin protein levels, these transgenic flies had a 1.5-fold increase in *top1* mRNA and an increase of 1.6- to 3-fold in protein level (one of the lines is shown in lanes 1–4, Figs. 1B and 1C). For ctop1-6, none of the 6 lines showed significant *top1* induction after heat shock (one line is shown in lanes 5–8, Figs. 1B and 1C). A shorter 3′ UTR in ctop1-6 may have resulted in a reduced stability of the induced *top1* message. There is an excellent correlation between the heat-induced *top1* expression and the genetic activity of the transgenes. None of the ctop1-6 transgenes showed significant *top1* expression after heat shock and they did not have any activity in rescuing *top1* mutant (see next section).

All these *ctop1* transgenes were introduced into the $top1^{-}$ ($top1^{77}$/Y) flies by crossing the female flies $top1^{77}$/*FM7*; ry^{506} with w^{-}/Y; *P[ctop1]*/+ males. The progeny were treated with heat shock throughout fly development and the resulting $top1^{77}$/Y; *P[ctop1]*/ry^{506} flies were scored. Among the 12 lines, 2 aforementioned lines exhibiting significant *top1* induction after heat shock completely rescued the lethality of $top1^{77}$/Y. Their ability to rescue was dependent on heat treatment (data not shown). The numbers of progeny with either $top1^{77}$/Y; *P[ctop1]*/ry^{506} or $top1^{77}$/+; *P[ctop1]*/ry^{506}, which contains a wild-type copy of *top1* in addition to the transgene, are comparable. Furthermore, female mutants with $top1^{77}$/$top1^{77}$, generated by another cross, can also be rescued by the induction of the transgene. Therefore, the genetic function of the heat-inducible *top1* transgene is equivalent to endogenous *top1*. For the remaining 10 lines of *ctop1* transgenes which showed no significant *top1* expression upon heat shock, no rescue was observed after heat treatment. These results indicate that rescue of the *top1* mutant phenotype depends on the heat-inducible *top1* expression.

To determine the developmental stages at which *top1* function is essential, we performed heat treatment covering various periods during fly development (Fig. 2). When heat shock was carried out for the first 7 days of development, up to 81 and 100% of the mutants were rescued in two independent experiments (Fig. 2). When the heat shock window was further narrowed, we found that heat treatment during days 4 to 7 resulted in very efficient rescue, with up to 79% in rescue rate. This period corresponds to the development of third-instar larvae into pupae, suggesting a critical function for *top1* at these stages. Therefore, using two different approaches, we have demonstrated that *top1* is essential for larval and pupal growth.

To understand *top1* function in larval development, we analyzed the growth defects in the *top1* mutant larvae whose chromosomes were properly marked. Females of $top1^{77}$; *P[gtop1]*, *Sb*/*P[313]*, *Tb* were crossed with $top1^{77}$/Y; *P[ctop1]*, *Sb*/ry^{506}, and no heat shock was performed on their progeny (see Materials and Methods for details). Progeny which carry *P[gtop1]* develop normally because the trans-

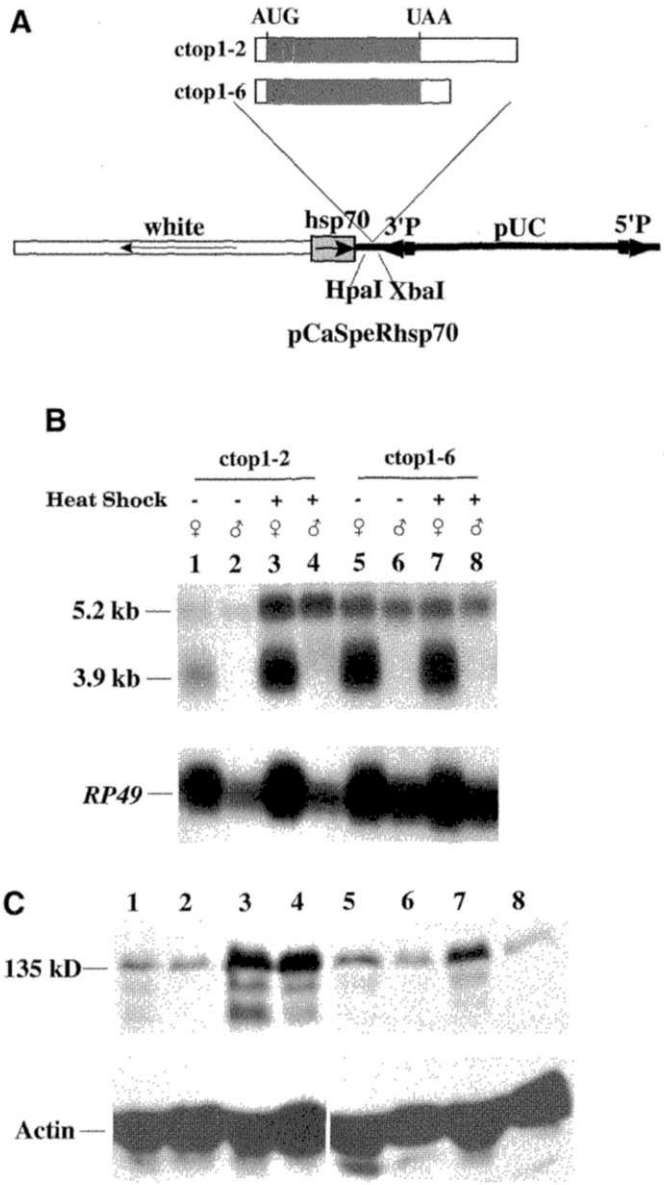

FIG. 1. *top1* expression under hsp70 promoter. (A) Two *top1* cDNA clones (ctop1-2 and ctop1-6) were inserted downstream of a heat shock promoter (*hsp70*) between the *Hpa*I and *Xba*I sites in the *p*CaSpeRhsp70 *P* element vector for germ-line transformation. The translational start codon, AUG, and stop codon, UAA, are indicated on a cDNA map. The *P* element vector contains a marker gene, *mini-white* (shown as *white* with an arrow marking its direction of transcription), two *P* element terminal repeats (3'P and 5'P) for transposition, and pUC for high-copy plasmid replication in *Escherichia coli*. (B) Heat shock induction of *top1* transcription in flies with both an endogenous *top1* gene and an ectopic copy of *top1* cDNA was examined by Northern blot analysis. Two populations of *top1* mRNA, 5.2 kb and approximately 3.8 kb, were detected and *RP49* served as a loading control. Lanes 1–4 show *top1* expression in flies with a copy of ctop1-2 (5.2 kb) while lanes 5–8 show flies with ctop1-6. Lanes 1, 3, 5, and 7 are from female flies, and lanes 2, 4, 6, and 8 are from males. Samples shown in lanes 3,4, 7, and 8 underwent heat treatment, while samples from lanes 1, 2, 5, and 6 did not. Heat shock induced 1.5-fold increase in *top1* mRNA expression in ctop1-2 flies, after being normalized to *RP49*. No induction was observed in ctop1-6 flies. (C) Protein expression of *top1* in the same set of flies was examined by Western blot analysis. Topo I migrates as a 135-kDa protein and the lower bands are products from partial proteolysis. Actin was used to normalize the amount of proteins loaded in each lane. In ctop1-2 flies, heat treatment induced an increase of 2.5-fold in females and 3-fold in males. There was a 0.4-fold increase after induction in ctop1-6 females while no induction was seen in males.

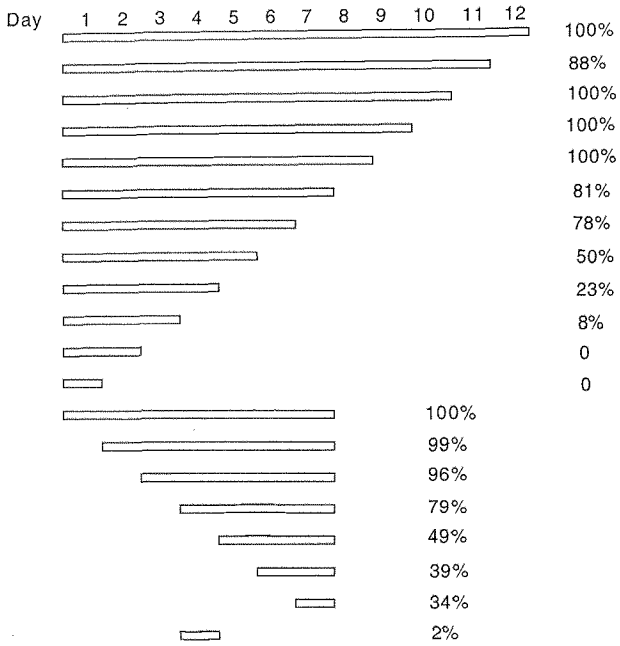

FIG. 2. Rescue efficiencies of ctop1-2 for the *top1* mutant with different durations of heat treatment. The number on the top marks the days in fly development after oviposition. The number on the right shows the percentage of rescue comparing the number of the mutant flies to that of their siblings carrying a copy of wild-type *top1*. We performed heat shock once a day every day over the period indicated by the bars. Heat shock induction of *top1* from day 4 to 7 or longer can efficiently rescue mutant lethality.

gene contains a genomic copy of *top1*. The rest of the progeny, with or without *P*[*ctop1*], lack sufficient *top1* expression and can be identified by the *Tb* marker. Many of these mutant larvae develop necrotic tissues and die at the second-instar stage, suggesting that $top1^{77}/top1^{77}$ develop the same defects as $top1^{77}$/Y. However, the presence of *P*[*ctop1*] allows some of the *top1* mutants to develop into third instar, but with a smaller size (Fig. 3B) compared with the $top1^{+}$ larvae (Fig. 3A). This result suggests that there could be a basal level of *top1* transcription under the *hsp70* promoter. The low *top1* expression allows mutant larvae to develop from second instar to third instar before they die in

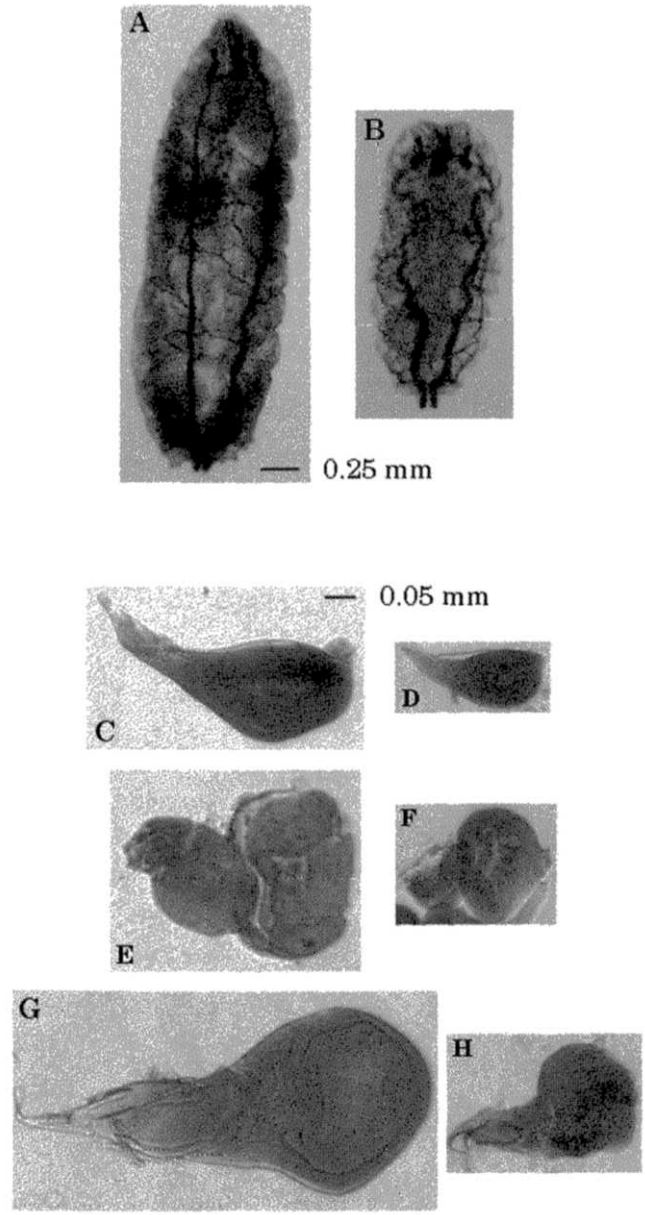

FIG. 3. Defects of the *top1* mutants during larval development. Third-instar larvae containing either wild-type *top1* (A) or mutant *top1* (B) are shown here. Both of them contain *Sb/P*[313], *Tb* on their third chromosome. Larval tissues were dissected and stained with X-gal. Imaginal discs of the *top1* mutant (D, F, and H) are compared to those of the $top1^{+}$ control (C, E, and G). C and D show haltere discs. E and F show eye and antenna discs. G and H show wing discs. The imaginal discs in mutant larvae are underdeveloped. All the mutant samples are shown in the same magnification as wildtype.

the larval–pupal transition. Except for the imaginal discs, most of the larval tissues grow by endoreplication. The reduced size of overall larvae suggests that *top1* affects endoreplication. We were interested in examining imaginal discs because these cells continue to undergo mitotic divisions The $top1^{-}$ larvae which carry *P*[*313*], *Tb* instead of *P*[*gtop1*] can be readily identified by the presence of the *lacZ* transgene on the *P*[*313*], *Tb* chromosome. Mutant tissues were dissected and stained with X-gal for β-galactosidase activity. Most of the mutant larvae do not contain imaginal discs. In mutants which do have imaginal discs, those discs either degenerate (data not shown) or are smaller than the wildtype (Figs. 3C to 3H). Microscopic examination of DAPI-stained samples shows that the mutant cells on imaginal discs are not significantly smaller than the wildtype cells (data not shown). Therefore, defects in the mutant imaginal discs clearly demonstrate a critical function for topo I in the division of these cells.

top1 Function in Oogenesis

We previously reported the expression of the *top1* gene in ovaries and early embryos (Lee *et al.*, 1993; Brown *et al.*, 1998), suggesting a maternal function for the topo I protein.

To investigate the possible *top1* function in oogenesis, we first examined topo I localization in ovaries by confocal immunofluorescence microscopy and compared its distribution with that of topo II (Fig. 4). In the germarium, topo I and topo II are present in the nuclei of both germ-line cells and follicle cells. When germ-line cells develop and move to the posterior region of the germarium, topo I protein level increases while the topo II protein level decreases. In early egg chambers, topo I protein is detected in the nuclei of both germ-line cells and follicle cells, while topo II is mainly found in follicle cell nuclei. After stage 2 egg chamber, oocyte chromosomes condense into a karyosome (indicated by arrows in Fig. 4), while topo I distributes throughout the entire nucleus. The nuclear topo I level in germ-line cells decreases at stage 5 of egg chamber development and disappears quickly thereafter. In an interesting contrast, topo I remains in follicle cell nuclei throughout oogenesis, similar to topo II. The staining pattern of topo I suggests its function in the development of germ-line cells at early stages and in follicle cell development throughout oogenesis. On the other hand, topo II may be involved only in follicle cell development.

To understand if topo I is critical for oogenesis, we have used heat shock as a means to control *top1* transgene expression in adult flies. Females of $top1^{77}/FM7$; ry^{506} were crossed with $top1^{77}$/Y; *P*[*ctop1*]/*ry506* to produce $top1^{77}$ male and female progeny carrying *P*[*ctop1*] transgene (Fig. 5A). Heat shock was carried out for the first five days after oviposition to allow the development of the mutant into adulthood. Adult flies of $top1^{77}$ were viable and maintained without further heat shock. After crossing these mutant progeny with wild-type counterparts, we discovered that male flies, $top1^{77}$/Y; *P*[*ctop1*]/ry^{506}, are fertile, while $top1^{77}$; *P*[*ctop1*]/ry^{506} females are sterile and lay very few eggs. Furthermore, immunofluorescence microscopy revealed that the testes from the mutant males have no gross defects compared with the wild-type flies (data not shown). This

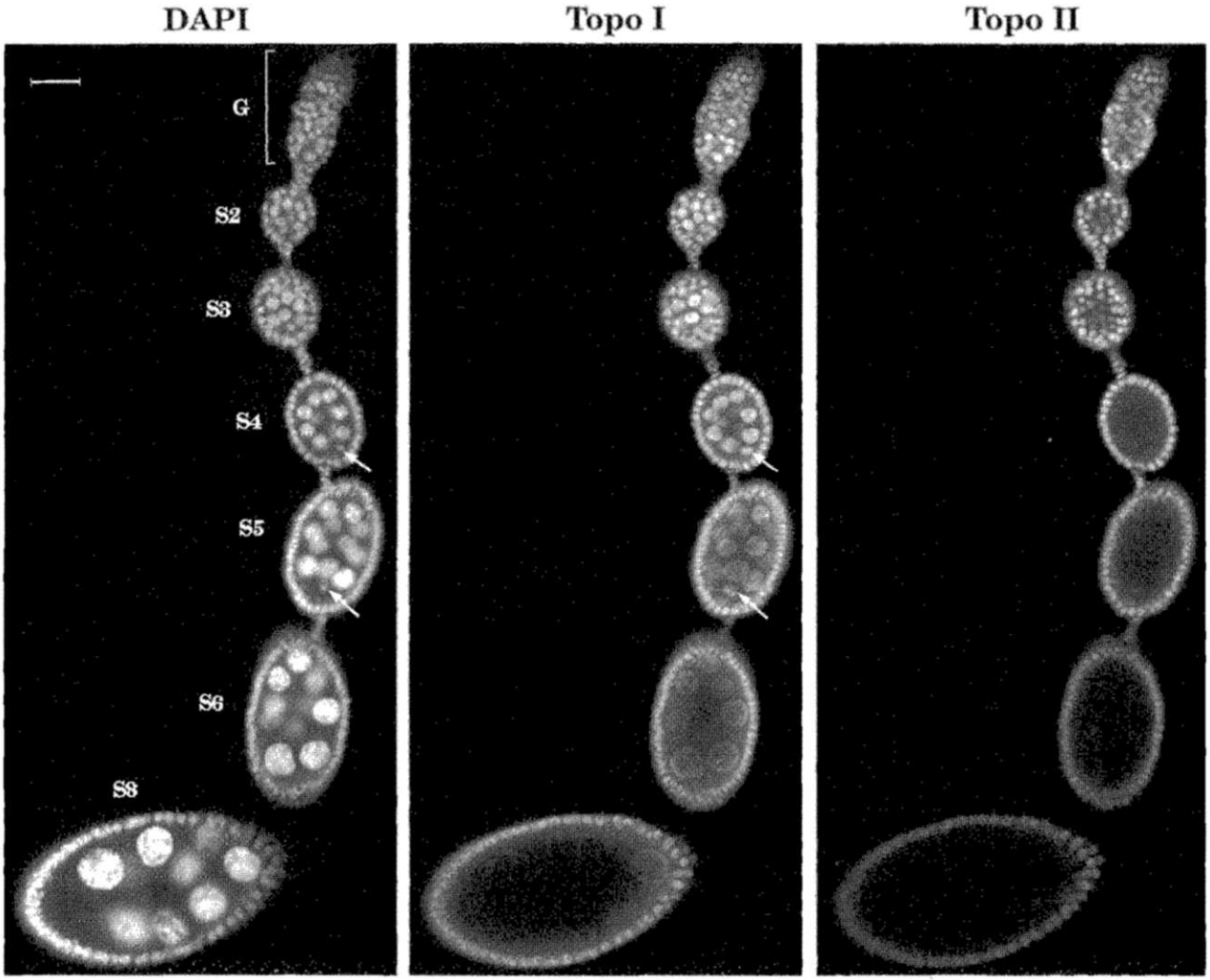

FIG. 4. Topo I and topo II localization in ovary. Ovaries of wild-type females were dissected and triple-stained with topo I antibody, topo II antibody, and the DNA dye DAPI. The germarium (G) and egg chambers from stage 2 (S2) to stage 8 (S8) are indicated on the left. Topo I signal is more concentrated in the nuclei of the germ-line cells in the germarium and early stage egg chambers (S2–S5) and in follicle cell nuclei. Oocyte nuclei in S4 and S5 egg chambers are indicated by arrows. The chromosomes are condensed into karyosomes at these stages while topo I is dispersed throughout the nuclei. Bar, 25 μm.

result suggests that topo I plays an essential role in oogenesis and has a less critical role in spermatogenesis.

To quantitate the defect caused by an insufficient supply of topo I protein, we compared the number of *top1*^{77}; *P*[*ctop1*]/*ry*^{506} eggs laid to that of wildtype. The mutant females were recovered after heat shock and the duration of heat shock treatment was varied to control topo I level. The first group of mutant flies was generated by heat treatment for the first 5 days during the development, and the second group received 10 days of heat shock (Fig. 5A). Fly vials each with 10 wildtype or mutant females and 7 wildtype males were examined for the number of eggs collected during a 5-day period. For wild-type females, more than 1000 eggs were recovered in each vial. In contrast, we recovered only 7 and 16 eggs, respectively, from the two vials with mutant females that received 5-day heat shock. With longer heat shock for the second group, 68 and 75 eggs were recovered, suggesting that prolonged topo I expression during development can improve oogenesis. Similar results were obtained by mating the female flies to their sibling males (data not shown). To monitor topo I protein levels in mutant ovaries, we prepared ovarian extracts and performed Western blot analysis (Fig. 5B). The mutant females have less than 10% topo I protein compared to wildtype. However, Western blot analysis does not reveal any significant difference in *top1* expression between two groups of the mutant females that received 5- or 10-day heat shock. It is likely that the topo I protein produced during heat shock induction is mostly degraded by the time of ovary collection. The low level of topo I protein comes from the residual topo I after

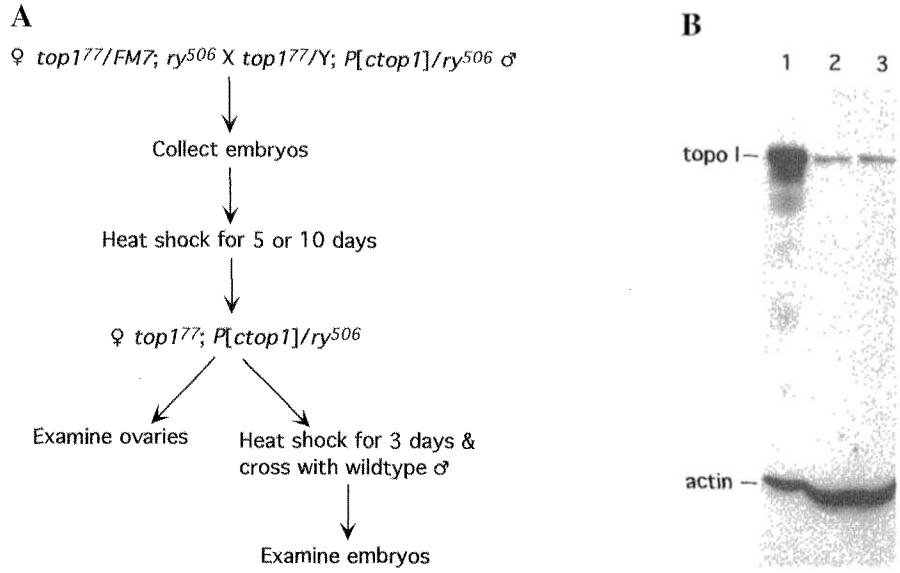

FIG. 5. *top1* expression in wild-type and mutant ovaries. (A) A genetic scheme used to generate the flies from which the mutant ovaries and embryos were obtained. (B) Fly ovaries were lysed directly in SDS sample buffer and 50 μg of total protein was loaded for each sample on a 7% SDS–polyacrylamide gel. Western blot analysis was performed with topo I antibody and actin antibody. Lane 1 is from the wild-type ovaries. Lanes 2 and 3 are from the ovaries of mutant females after 5 and 10 days heat treatment, respectively. Actin serves as a loading control. Levels of topo I are greatly reduced in mutant ovaries (8.8% of the wildtype).

the heat shock induction or from the basal expression of *top1* under the *hsp70* promoter. Since the ovary starts differentiation at late third-instar stage and continues to develop during pupal stage, the improved oogenesis by longer heat shock treatment suggests that topo I has an important function in the early steps of ovary development.

We analyzed oogenesis defects of the *top1* mutant by immunofluorescence microscopy. A wide range of defects was observed as early as in the germarium (Fig. 6B) and as late as in matured oocytes (Fig. 7). Normally, a germarium contains three major regions: region 1 holds stem cells, cystoblasts, and developing cysts; region 2 has about six cysts of 16 cells; and region 3 has a single stage 1 egg chamber (Spradling, 1993; also see Fig. 6A). In a mutant germarium (Fig. 6B), no regional development of cysts can be clearly ascertained and the abnormal nuclear morphology reveals extensive nuclear lysis.

In addition to the defect in the germarium, we found extranumeral germ-line cells in postgermarial egg chambers. In each wild-type egg chamber (Figs. 6C and 6F), a single cyst containing one oocyte and 15 nurse cells is surrounded by a layer of follicle cells. However, in mutant egg chambers, extra numbers of nurse cells, ranging from around 20 to more than 40, along with multiple oocyte-like cells were observed (Figs. 6D, 6E, 6G, and 6H). To understand if the extra germ-line cells were caused by abnormal cystocyte division, we examined the organization of ring canals by actin staining. During cystocyte divisions, incomplete cytokinesis leaves daughter cells interconnected by specialized cleavage furrows called ring canals. The number of ring canals in one cell indicates how many divisions it went through, and a wild-type oocyte has four ring canals. In a mutant egg chamber with 3 oocyte-like cells (indicated by arrows in Figs. 6D and 6E, which were the images at different focal planes), four ring canals are found in each of them. Since there are fewer than 45 nurse cells in this egg chamber, they cannot be generated by simply combining three cysts after four cycles of complete cystocyte divisions. It is possible that some of those cystocytes fail to divide while the oocyte-like cells completed four divisions. Therefore, in a situation in which follicle cells surrounding the developing egg chambers are underreplicated, a fusion of several cysts may occur, and the divisions of cystocyte are not regulated in these cysts. Further experiments will be needed to determine how the extra oocytes and nurse cells are generated in the mutant egg chambers. The nuclear morphology in mutant nurse cells is apparently abnormal compared to wildtype (Figs. 6D, 6E, 6G, and 6H vs 6C and 6F). DNA staining in the mutant has an uneven distribution and lower density, suggesting that endoreplication and condensation in mutant nurse cells are defective. This nuclear defect has also been observed in mutant egg chambers with normal numbers of germ-line cells. Interestingly, although chromosomes in all the oocyte-like cells from the mutant condensed into karyosomes (arrows in Figs. 6D, 6E, 6G, and 6H), only the posterior oocyte-like cell took up yolk particles during vitellogenesis (observed by light microscopy, data not shown). Furthermore, nurse cell nuclei in the anterior are usually smaller than those in the posterior in mutant egg chambers, similar to what is observed in wild-

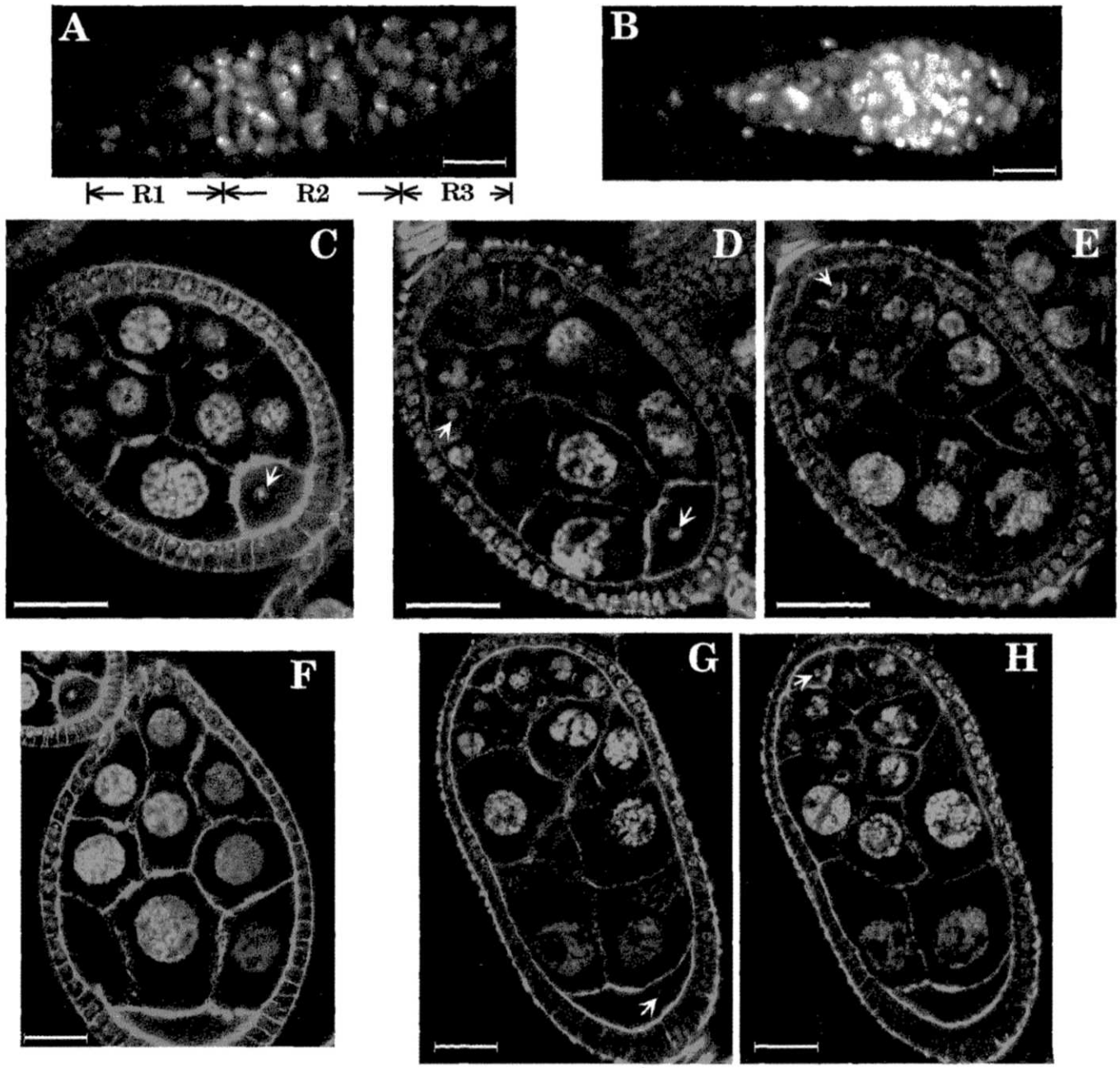

FIG. 6. Defects in germ-line cells from mutant ovaries. Germaria from wild-type (A) and mutant (B) ovary were analyzed by fluorescence microscopy of DAPI staining. Three major regions of the germarium, R1, R2, and R3, are indicated in the wild-type sample (A). This stereotypic pattern is disrupted in the mutant sample (B). Bars in A and B represent 12.5 μm. Egg chambers (C–H) were stained with phalloidin–FITC for actin filaments (green) and DAPI (red, in false color). All the egg chambers are oriented with the anterior end at the top. D and E, also for G and H, are confocal images taken at two successive focal planes for the same mutant egg chamber to visualize different cells. The arrows point to oocyte karyosomes. In each wild-type egg chamber (C and F), there were 15 nurse cells and 1 oocyte (out of the focal plane in F). In the mutant egg chamber shown in D and E, up to 3 oocyte-like cells and 29 nurse cells could be counted. However, only the posterior oocyte-like cell in a stage 8 mutant egg chamber, shown in G and H, underwent normal development. Bars in C–H represent 25 μm.

type (Figs 6C to 6H). It seems that anterior–posterior polarity is established normally, even though multiple oocytes may exist in one egg chamber.

In addition to defects in the germarium and germ-line cells, the *top1* mutant also exhibits underreplication of follicle cells. Egg chambers with fewer follicle cells were

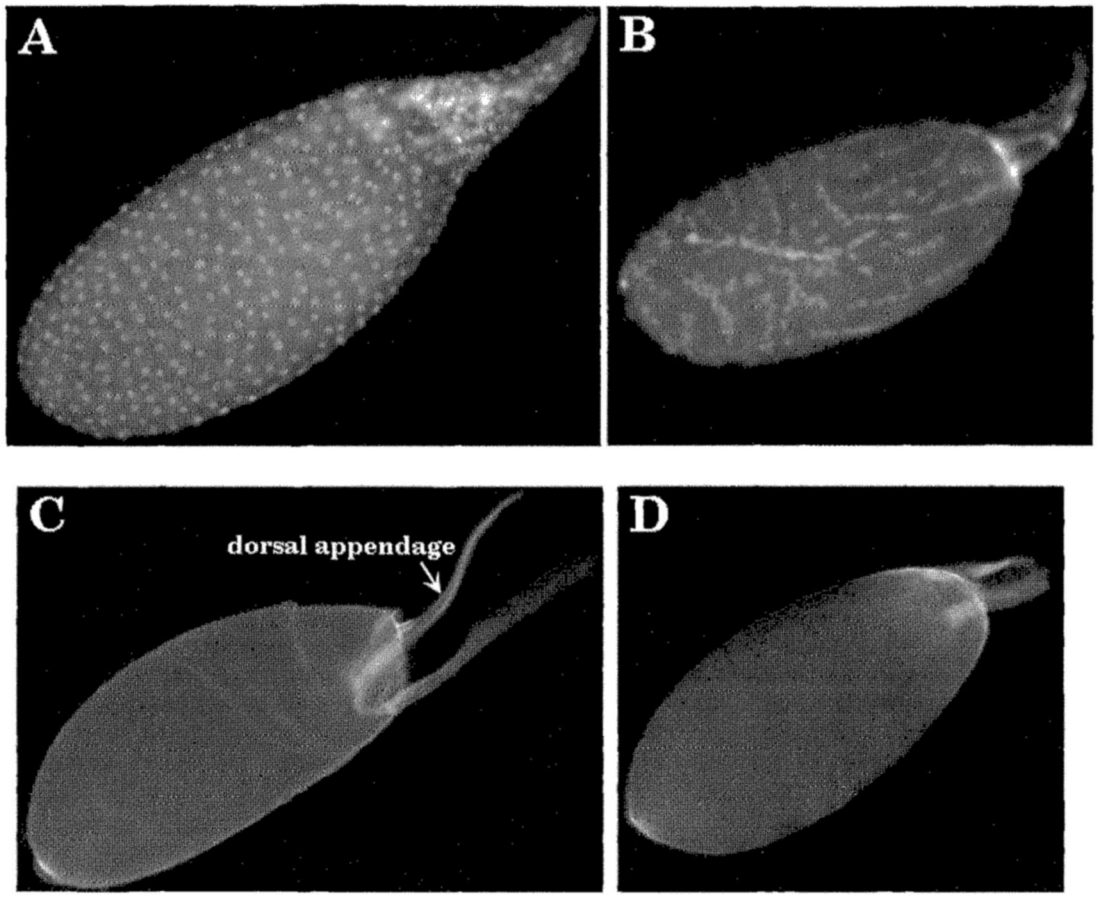

FIG. 7. Defective follicle cells in mutant ovaries. DAPI staining (A and B) shows that the mutant egg chamber has a lower number of follicle cells (B), compared to the wildtype (A). Dark-field photos (C and D) reveal that dorsal appendages of the mutant (D) are shorter than those of wildtype (C).

observed (data not shown), indicating a defect in cell division. While most of the mutant egg chambers were degraded during the process of oogenesis, a few mutant egg chambers completed vitellogenesis and formed a chorion membrane outside the mature oocyte (Figs. 7B and 7D). We did not detect any defects during the migration of follicle cells toward the oocyte (data not shown). However, the follicle cells are fewer than normal and they are irregularly distributed over the mature oocyte (Fig. 7B). Moreover, the pair of dorsal appendages in the mutant (Fig. 7D) are much shorter than those of wildtype (Fig. 7C), suggesting that there is insufficient chorion protein, most likely caused by a smaller number of follicle cells.

We have also examined mutant ovaries from female flies receiving different durations of heat treatment. Ovaries from mutant females that had 3-day heat shock treatment at the beginning of the development show more extensive defects in germaria and germ-line cells than those that received 5-day heat shock. Ten-day heat treatment from embryonic to pupal stages almost eliminates the defects in germaria and germ-line cells, while follicle cells are still defective and those females remain sterile. These results confirm that a high level of topo I is required throughout oogenesis.

top1 Function in Embryogenesis

Drosophila early embryogenesis is completely under maternal control (Zalokar, 1976; Merrill *et al.*, 1988; Wieschaus and Sweeton, 1988). Embryos produced by *top1* mutant females cannot develop into larvae, suggesting that the *top1* gene has a maternal function in early embryonic development.

Topo I expression in early embryos has previously been detected by Western blot analysis (Lee *et al.*, 1993). We examined topo I localization in wild-type embryos by immunofluorescence analysis. The first 13 nuclear divisions of *Drosophila* embryogenesis occur in a syncytium without cytokinesis and are programmed by the maternal machinery. During this period, topo I distributes throughout the interphase nuclei (top middle, Fig. 8A), similar to topo II (Swedlow *et al.*, 1993; top right, Fig. 8A). When the

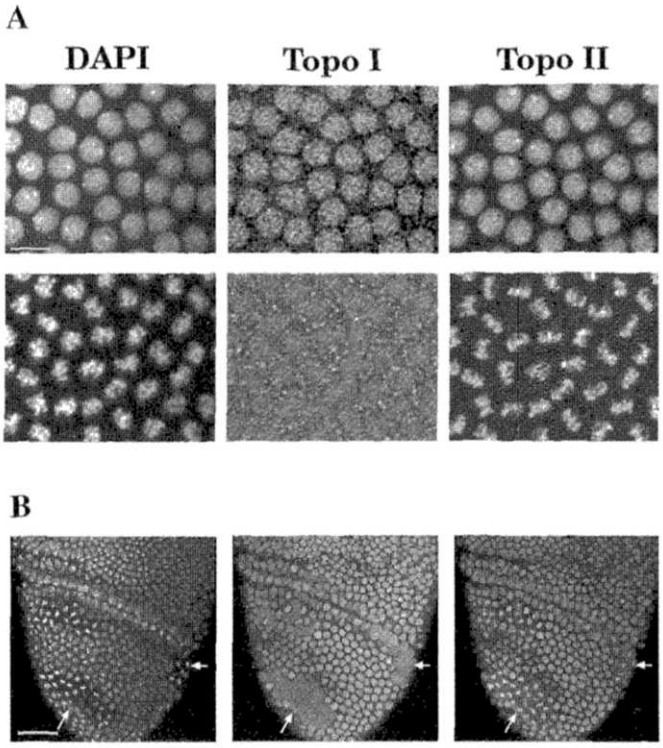

FIG. 8. Topo I and topo II localization during embryogenesis. Wild-type embryos were triple-stained for topo I, topo II, and DAPI. (A) Two cycle 13 embryos in either interphase (top row) or metaphase (bottom row) are shown here. The top row shows nuclear localization of topo I and topo II in an interphase embryo. The bottom row shows cytoplasmic localization of topo I and chromosomal association of topo II in a metaphase embryo. Bar, 10 μm. (B) The anterior region of a gastrula is shown here. Arrows point to two of the mitotic domains. Topo I protein localizes in interphase nuclei but disperses into the cytoplasm of the cells in the mitotic domains. Bar, 20 μm.

nuclear envelope breaks down during metaphase, topo I protein diffuses into the cytoplasm while a fraction of the topo II protein remains associated with condensed chromosomes (bottom, Fig. 8A). Starting at cycle 14, the zygotic machinery takes over embryogenesis and individual cells are formed by membrane invagination. The cell-cycle-dependent localization of topo I remains the same in these mitotic cells (Fig. 8B). At this stage, cell divisions become asynchronized and mitotic domains are observed during gastrulation (arrows, Fig. 8B). Topo I protein is detected in the cytoplasm of mitotic cells while topo II remains on the chromosomes. Both topo I and topo II staining can be detected throughout embryogenesis (data not shown).

To analyze the mutant phenotype during embryonic development, we treated female adults of *top1*^{77}; *P*[*ctop1*]/*ry*^{506} with additional heat shock (Fig. 5A) to improve oogenesis. As a result, the mutant females laid more eggs but more than 90% of the embryos still died before larval stage. Mutant embryos were triple stained with DAPI, and antibodies against topo I and topo II, and then compared to wild-type embryos (Fig. 9). Various nuclear defects have been detected in the mutant. In a wild-type syncytial blastoderm, a monolayer of nuclei divides in the cortex and yolk nuclei undergo endoreplication in the center of the embryo (left, Fig. 9A). In a mutant embryo, multiple layers of nuclei beneath the cortex are observed (right, Fig. 9A). Since abnormal cortical nuclei usually sink into the interior of the embryo, the above observation suggests a possible defect in nuclear divisions. Nuclear sinking was also reported with the microinjection of topo II antibodies or a topo II inhibitor, both of which caused abnormal chromosome condensation and segregation (Buchenau *et al.*, 1993). In the same *top1*$^{-}$ embryo, we also find both interphase nuclei (short arrow, Fig. 9A) and metaphase nuclei (long arrow), suggesting that the early program of synchronous nuclear division has been disrupted.

Low levels of *top1* expression also lead to a greatly reduced number of nuclei in early embryos. For example, nuclei are barely seen in two regions of a mutant embryo and they are different in size and morphology (Fig. 9B). Telophase nuclei are found in addition to interphase nuclei (inset, Fig. 9B). We also detect nuclei with an extra amount of DNA in the mutant embryo, most likely coming from nondisjunction or fusions after abnormal nuclear divisions.

Among all the mutant embryos we have examined, most of them showed degenerated nuclei, and neither topo I nor topo II signal was detected (data not shown). Interestingly, we have also found embryos which have a nearly normal nuclear distribution but display chromosomal defects during mitosis (Fig. 9C). These embryos have a relatively normal level of topo I expression (data not shown), compared to those shown in Figs. 9A and 9B. In a mutant embryo at the metaphase of cycle 10, chromosomes are not as fully condensed as wildtype (top, Fig. 9C). We can see diffuse chromosome arms extending away from the metaphase plate (arrow in top right, Fig. 9C). These decondensed chromosomes are frequently observed in the mutant embryos. Another mitotic defect results from unsuccessful chromosome segregation (bottom right, Fig. 9C). Chromosome bridges are found between pairs of daughter nuclei or even between three neighboring nuclei. This failure of chromosome separation may be responsible for nuclear fusions observed in the same embryo. The mitotic defects in the *top1* mutant embryos can be observed by live embryo videos (Fig. 10). (Please see supplementary material at http://www.academicpress.com/www/journal/db/dbsupp.htm.) The dynamic behavior of the chromosomes in either wildtype (left) or mutant (right) embryos was revealed by the histone 2A–green fluorescent fusion protein. In the mutant embryos the nuclear division cycles are prolonged and asynchronous. It takes about twice as long for the mutant embryos to traverse through the cell cycle compared with the wild-type embryos. However, the mutant embryos do not seem to lag behind at any specific point in the cell cycle.

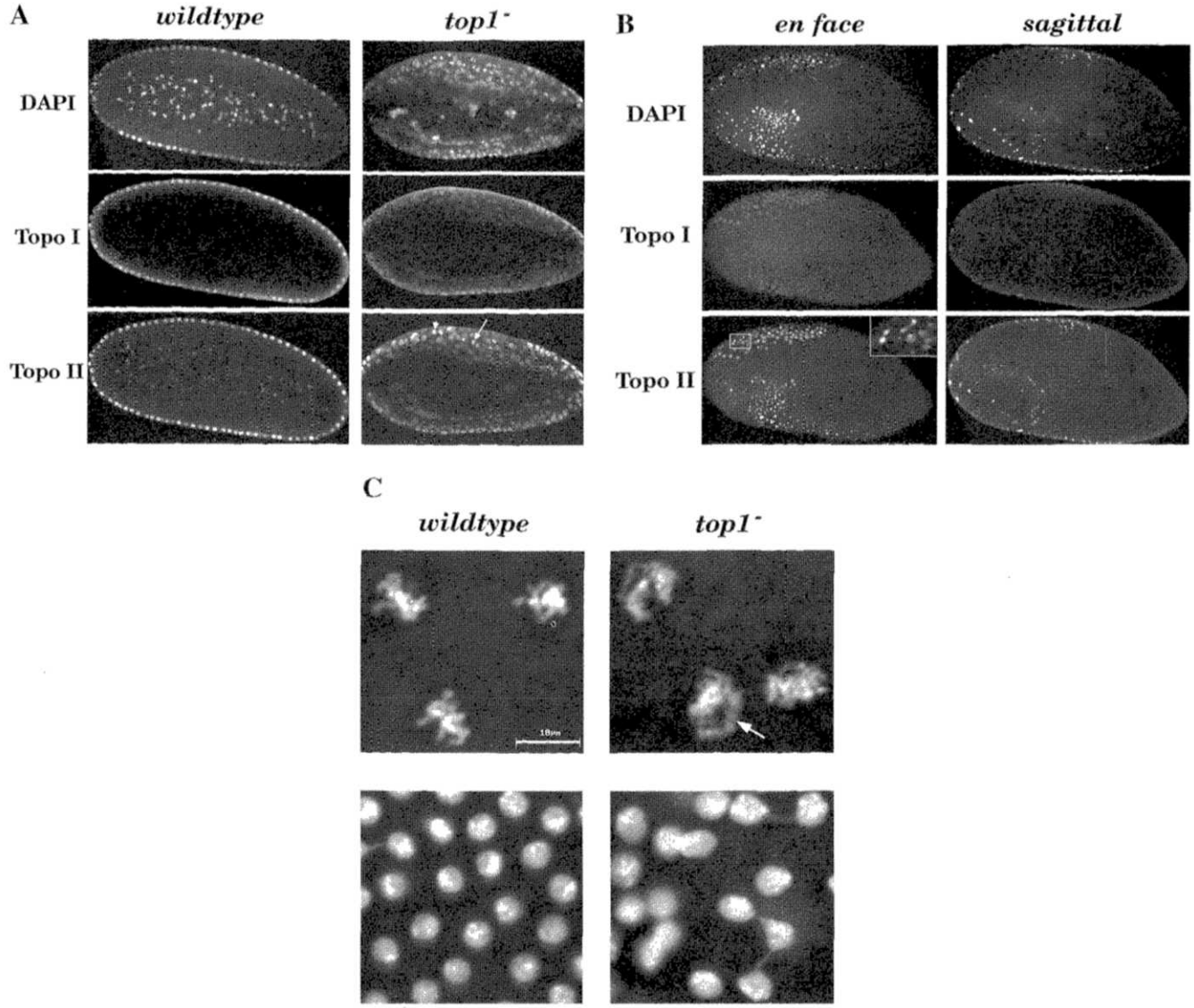

FIG. 9. Defects in *top1* mutant embryos. Embryos were prepared as in Fig. 8. (A) The sagittal view of a mutant embryo (right column) was compared to that of a wild-type embryo (left column). A major fraction of the nuclei was found in the interior of the mutant embryo and both interphase nuclei (short arrow) and metaphase nuclei (long arrow) could be observed. (B) The *en face* view (left column) and sagittal view (right column) of the same mutant embryo are shown here. Two clusters of nuclei were found in the anterior end of the cortex and many nuclei were also in the interior of the embryo. The nuclei were of different sizes and morphology. Inset, 3× original magnification. (C) DAPI staining shows abnormal nuclear morphology of mutant embryos (right column), in comparison with the wildtype (left column). Chromosomes in a cycle 10 mutant embryo were not properly condensed at metaphase (arrow). Nuclear fusions were frequently observed in a cycle 12 mutant embryo at late telophase (lower right).

Furthermore, both chromosome condensation and segregation are also defective, consistent with the result obtained by fluorescence microscopy of the fixed samples (Fig. 9C).

DISCUSSION

Drosophila melanogaster provides an excellent genetic system to study eukaryotic topo I function during development. We have demonstrated that in *Drosophila,* topo I is essential for embryogenesis, larval and pupal growth, and oogenesis. Embryos with an insufficient level of maternal *top1* expression have abnormal nuclear divisions before the cellular blastoderm stage. Most of them have significantly reduced number of nuclei, suggesting that maternal topo I is essential for DNA replication at these stages. Yet in yeast, the function of topo I in replication can be largely substituted by topo II, except for a delay in the maturation of

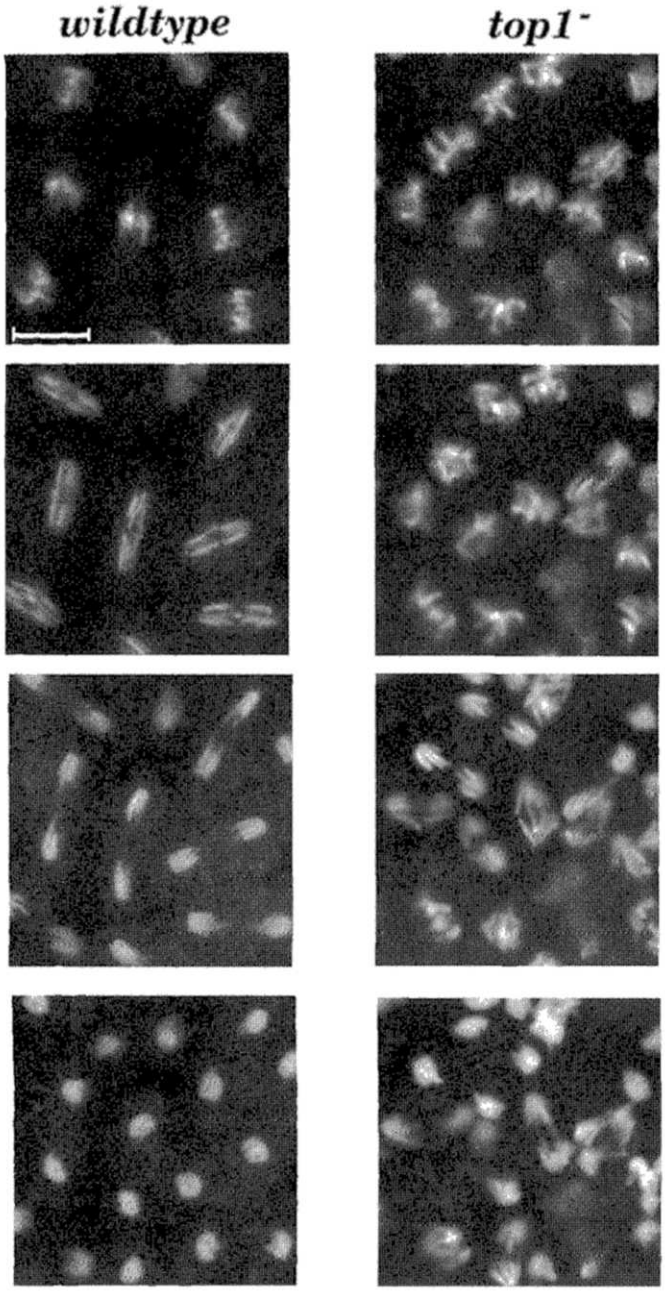

FIG. 10. Time-lapse images of live embryos. A histone H2A–GFP fusion transgene allowed the observation of live embryos by confocal fluorescence microscopy. The bar in the upper left represents 10 μm. The series of images for both the wild-type and the *top1* mutant are spaced 45 s apart and were taken from continuous frames of the live embryo video. The mutant embryo requires more time than the wildtype to undergo condensation and segregation, and most nuclei have nondisjunction.

Okazaki fragments in the absence of topo I (Kim and Wang, 1989a). It is possible that in a multicellular organism, especially during embryogenesis when synchronous nuclear divisions happen without either G1 or G2 phases, a delay in DNA synthesis will result in the disruption of such an ordered and rapidly paced developmental program. Indeed, our results show that nuclear divisions in *top1*-deficient embryos are frequently out of synchrony. In addition to an essential function in early embryos, both our mutant phenotype analysis and heat shock experiments point to a critical function for *Drosophila* topo I during late larval and early pupal development. At this period, the second major mitotic wave in *Drosophila* development occurs in imaginal disc cells. Mutations in many cell-cycle genes cause lethality at the larval–pupal transition, when the maternal supply necessary for mitosis runs out (reviewed in Gatti and Baker, 1989; Stern *et al.*, 1993). These mutants, as well as the *top1* mutant, all show either an absence of imaginal discs or a reduction in disc size, suggesting that these genes are essential for cell proliferation. While the late pupal development and adult fly viability do not depend on normal levels of topo I, topo I is essential for oogenesis. Defects in the germarium, where stem cells and cystoblasts divide, and underproliferation in follicle cells again show the importance of topo I in cell division. Surprisingly, low level of *top1* expression does not affect spermatogenesis, a process which also involves active transcription and successive rounds of mitosis and meiosis. It is possible that other members of the topoisomerase family, either topo II or topo III, may play major roles in spermatogenesis.

Studies with $top1^{77}$ and $top1^{77}$; $P[ctop1\text{-}2]/ry^{506}$ larvae provide evidence that topo I has an important function in nondividing cells, possibly through its effect on endoreplication and transcription. $top1^{77}$ larvae develop necrotic tissues throughout their body and later on die in second instar. $top1^{77}$; $P[ctop1\text{-}2]/ry^{506}$ can develop through the third-instar stage, however, with a smaller body size. When the salivary gland of the *top1* mutant was examined, we found that the mutant cells and their nuclei were both smaller than normal (data not shown). All these growth defects suggest that topo I plays a crucial role in nonproliferating larval tissues, in which cells undergo endoreplication and transcription to increase their size. During the process of transcription, topo I can provide DNA swivels to relieve torsional stress associated with the movement of transcription forks (reviewed in Wang, 1996). Topo I may also have a more direct role in the assembly of initiation complex (Stewart *et al.*, 1990; Kretzschmar *et al.*, 1993; Merino *et al.*, 1993; Shykind *et al.*, 1997). It is interesting that topo I may have an important function in endoreplication throughout the development. Both nurse and follicle cells undergo extensive endoreplication like the larval tissues and they show defective cell divisions in *top1* mutant ovaries. The mutant nurse cells have a lower DNA content and aberrant nuclear morphology, suggesting an impediment in DNA synthesis in these cells.

Genetic approaches in yeast have demonstrated that topo I is involved in chromosome condensation. Mutations that require *top1* expression for yeast viability map to four

groups of genes including *top2* and a gene (*trf4*) that interacts with *smc1* and *smc2* (Sadoff *et al.*, 1995; Castano *et al.*, 1996) Overexpression of *top1* can partially suppress an allele of *cut3*, which also belongs to the *smc* family (Saka *et al.*, 1994). Cell-free systems in yeast and *Xenopus* both require topo I activity for chromatin assembly (Almouzni and Mechali, 1988; Garinther and Schultz, 1997). Based on fluorescence microscopy of live embryos and fixed samples, *Drosophila* topo I may be required for chromosome condensation. In the *top1* mutant embryos, loosely packed chromosomes are apparent at metaphase. At the resolution of our current analysis, we cannot determine if the condensation defect is causal to other abnormalities like chromosome nondisjunction and unsynchronized nuclear divisions. Topo II has also been shown to play critical roles in chromosome condensation during *Drosophila* embryogenesis (Buchenau *et al.*, 1993). However, our immunolocalization data of topo I and topo II suggest that they have specific functions in different stages of chromosome condensation. Topo I may be involved in the initial steps of chromosome condensation at prophase, since it disperses into the cytoplasm during metaphase when chromosomes become highly condensed. Topo II may have a unique function in the final steps of chromosome condensation during the metaphase when a fraction of topo II proteins remains on the fully condensed metaphase chromosome. The overlapping and distinct functions of topo I and topo II in chromosome condensation and segregation will be an important issue for future studies.

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