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I am submitting herewith a thesis written by Chia-Sin Hong entitled "Role of DNA Topoisomerase I in *Drosophila melanogaster* Male Meiosis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

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Role of DNA Topoisomerase I in *Drosophila melanogaster*

Male Meiosis

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

DNA topoisomerase I functions as a molecular swivel and regulates the topological states of DNA and chromatin by creating a transient break in a single DNA strand in a duplex and religation of the break after the passage of another strand. DNA topoisomerases play important roles in DNA replication, transcription, recombination and other aspects of DNA metabolism.

Previous studies have shown that the 240 base-pair repetitive sequence within the intergenic spacer of ribosomal DNA repeats functions in X-Y chromosome pairing in *Drosophila melanogaster* males. A 16 base-pair high-affinity topoisomerase I cleavage site has been found in the 240 base-pair repeats and a model for achiasmatic chromosome pairing involving topoisomerase I has been proposed. In this study, the yeast FLP-FRT site-specific recombination system was used to facilitate the study of the role of topoisomerase I in male meiosis using spermatocytes that are deficient in topoisomerase I.

Genetic studies of chromosome disjunction showed that the nondisjunction frequencies in the male and female experimental flies did not exhibit substantial increases over the wild-type frequencies and the heat-shock treated flies produced fewer progeny than the control flies. Immunocytological studies using anti-topoisomerase I antibody has not been able to identify any viable spermatocytes that are topoisomerase I-deficient. The results from both of these studies have suggested that DNA topoisomerase I is required for the development of spermatocytes in the male flies.

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CHAPTER 1

INTRODUCTION

Overview of topoisomerases and functions of DNA topoisomerase I

DNA topoisomerases are ubiquitous enzymes present in both prokaryotes and eukaryotes. These enzymes regulate and modulate the topological states of DNA and chromosomes through reversible breaking of DNA backbone bonds and permit changes in the linking number of the two DNA strands. In addition, they can catalyze various types of interconversions between topological isomers of DNA. Several examples of these topoisomerization reactions include supercoiling and relaxation, complete renaturation of complementary single-stranded DNA rings to form duplex rings, catenation and decatenation, and knotting and unknotting of single-stranded DNA rings (reviewed by Liu, 1983; Wang, 1985; Champoux, 1990). DNA topoisomerases are classified by their *in vitro* activities into two types. Type I topoisomerases break a single DNA strand of a duplex and pass another strand through this transient break followed by religation of the broken strand. Type II topoisomerases create a transient double-strand breakage in a duplex DNA and allow the passage of another duplex DNA segment (Wang, 1985).

DNA topoisomerases have important roles in many processes which require the topological restraints of two intertwined DNA strands to be resolved before such restrictions can impede the biological functions of DNA molecules. The enzymes function as molecular swivels in replication, transcription, recombination, and chromosome condensation and decondensation (Wang, 1985). Evidence from studies using yeast (Goto and

Wang, 1984) and prokaryotic mutants indicate that type II DNA topoisomerases are essential for viability. Mutations in type I enzymes are tolerated and cause no lethal phenotypes in these organisms (reviewed by Yanagida and Sternglanz, 1990). In multicellular organisms, recent evidence from *Drosophila melanogaster* indicates that DNA topoisomerase I is required for the growth of the fruit fly (Lee *et al.*, 1993). The functions of type I topoisomerase will be the focus below.

In 1969, James Wang observed the first DNA topoisomerase activity in a cell extract from *Escherichia coli* (Wang, 1969). An activity in the cell extract was capable of relaxing a highly coiled DNA. This enzyme was further purified and identified as the ω protein (Wang, 1971) and it was later renamed *Escherichia coli* DNA topoisomerase I. An enzyme with similar activity to *E. coli* topoisomerase I was found in mouse cell extracts (Champoux and Dulbecco, 1972). The mechanism of the enzyme activity has been studied using different reaction conditions. The nicking and reclosing reactions can be uncoupled by addition of SDS or alkali to trap the reaction intermediate. It is now known that a phosphodiester bond in the DNA backbone can be broken temporarily by a type I topoisomerase and a covalent enzyme-DNA intermediate formed simultaneously. An O⁴-phosphotyrosine bond is shown to be the linkage between the enzyme/DNA complex. Following strand passage, the enzyme dissociates from the DNA and the DNA backbone bond is reformed (Wang, 1985; Champoux, 1990). In contrast to type II topoisomerases, type I enzyme activities lack a requirement for a energy cofactor such as ATP. It was hypothesized that the energy required to catalyze the reformation of a continuous DNA strand is stored in the covalent bond formed between the broken DNA end and the

enzyme (Wang, 1971; Champoux and Dulbecco, 1972). Although prokaryotic and eukaryotic enzymes share some similarities in their activities, there are several differences. The prokaryotic enzyme is capable of unwinding negatively supercoiled DNA but it can only partially catalyze this relaxation (Wang, 1971). On the other hand, mouse DNA topoisomerase I can relax both positively and negatively supercoiled DNA efficiently (Champoux and Dulbecco, 1972; Pulleyblank *et al.*, 1975). There are also differences in the covalent linkages formed with the DNA. Eukaryotic topoisomerase I is covalently linked to a 3' phosphoryl end of the broken DNA strand (Champoux, 1978; Prell and Vosberg, 1980), whereas *E. coli* topoisomerase I is linked to the 5' phosphoryl group of the broken DNA strand (Depew *et al.*, 1978).

Additional type I topoisomerases have been identified in both yeast *Saccharomyces cerevisiae* (Wallis *et al.*, 1989) and *E. coli* (Dean *et al.*, 1983; DiGate and Marians, 1988, 1989). In *S. cerevisiae*, sequence analysis indicated that DNA topoisomerase III, which is encoded by *TOP3* gene, is homologous to the first two-thirds of *E. coli* topoisomerase I and is not homologous to any previously identified eukaryotic topoisomerases (Wallis *et al.*, 1989). *TOP3* (first known as enhanced delta recombination-*edr*) was identified because a mutation of this gene leads to higher frequency of recombination between the short repeated δ sequences of the yeast retrotransposon Ty. Recombination between δ sequences flanking the *SUP4-o* gene, a tyrosine tRNA ochre suppressor, results in the deletion of the gene (Wallis *et al.*, 1989). The *top3* null mutants are viable but they grow on rich medium at 50% of the wild-type rate. A complementation test showed that the *E. coli topA* gene can suppress the slow growth phenotype of yeast

top3 null mutation. In addition, mutations in either *TOP1* or *TOP2* severely affect the growth of *top3* null strains. The *top1/top3* double mutants grow more slowly than either of the single mutants (Wallis *et al.*, 1989). *In vivo* and *in vitro* studies using purified topoisomerase III also show that it has at most a weak activity in relaxing negative supercoils and links to the 5' end of the DNA strand (Kim and Wang, 1992). Purified *E. coli* topoisomerase III has similar enzymatic properties to other type I topoisomerases. It has efficient negative supercoil relaxation activity but does not relax positive supercoils. It is active at high temperature (52°C) instead of the normal 30°C for the enzyme activity assay, requires Mg²⁺ and binds to single-stranded DNA (DiGate and Marians, 1988). The amino acid sequence data show that topoisomerase III has homology with *E. coli* topoisomerase I over a stretch of 308 amino acids of both proteins (DiGate and Marians, 1989).

Studies on topoisomerase I mutants have revealed possible functions of the enzyme. For *E. coli*, it was demonstrated that although topoisomerase I is not essential for viability, mutants grow slower than wild type bacteria (Sternglanz *et al.*, 1981). It was found that the lethal phenotype of *E. coli topA* deletion mutations is sometimes compensated by acquiring a suppressor mutation in *gyrA* or *gyrB* that decreases the level of gyrase activity (type II topoisomerase) in the cell (Pruss *et al.*, 1982; DiNardo *et al.*, 1982). These findings suggest that DNA topoisomerase I and DNA gyrase regulate DNA supercoiling levels *in vivo*. In yeast *S. cerevisiae*, topoisomerase I is not required for growth. Similar to the *E. coli topA* and yeast *top3* mutants described above, these mutants also exhibit a slow growth phenotype (Thrash *et al.*, 1985). DNA topoisomerases have been found to be involved in the suppression of mitotic recombination in *S.*

cerevisiae (Christman *et al.*, 1988). *top1* null mutants or *ts top2* mutants grown at a semipermissive temperature show greatly elevated level of mitotic recombination within the rDNA cluster relative to *TOP*⁺ controls. The observed suppression of recombination is specific to the rDNA cluster; there is no increase in recombination frequency at other duplicated regions in either *top1* or *top2* mutants (Christman *et al.*, 1988). These results suggest that both DNA topoisomerases I and II are required to suppress mitotic recombination in rDNA in order to limit the possible deleterious effect on the growth of yeast resulting from amplification or deletions of the rDNA array (Christman *et al.*, 1988). Yeast *S. cerevisiae* topoisomerase III described earlier also has similar function in the suppression of recombination between the δ sequences.

Type I topoisomerase has been implicated in the process of transcription. Recent studies have identified the human DNA topoisomerase I as a cofactor necessary for efficient transcription (Merino *et al.*, 1993; Kretzschmar *et al.*, 1993). Various *in vitro* transcription assays using the GAL4-AH activated pMRG5 as reporter template have shown that topoisomerase I represses basal transcription in the absence of the acidic activator GAL4-AH. In the presence of the activator, net activation of transcription can be mediated by topoisomerase I. The activity of topoisomerase I as a transcription cofactor is also dependent on the presence of an activation domain on GAL4-AH (Kretzschmar *et al.*, 1993). The effect of topoisomerase I is specific for TATA-box containing promoters and is mediated by the TATA-binding protein (Merino *et al.*, 1993).

Observations from *in vitro* studies have shown that DNA topoisomerase I has a role in DNA pairing in conjunction with *E. coli* RecA

protein or *Ustilago maydis* Rec1 protein (Cunningham *et al.*, 1981; Kmiec *et al.*, 1983). *E. coli* RecA protein alone can promote pairing of single-stranded DNA with its complementary strand in a duplex DNA in a side-by-side fashion. Circular single-stranded DNA paired with homologous superhelical DNA or with linear duplex DNA that lacks homologous ends can be topologically linked by the addition of *E. coli* topoisomerase I (Cunningham *et al.*, 1981). Similarly, *U. maydis* Rec1 protein and topoisomerase I can catalyze pairing and topological linkage of circular DNA molecules (Kmiec *et al.*, 1983).

DNA topoisomerase I of *Drosophila melanogaster* has been cloned and characterized (Hsieh *et al.*, 1992; Javaherian *et al.*, 1982). Topoisomerase I has been shown to be essential for the development of *D. melanogaster* (Lee *et al.*, 1993). The authors have isolated a mutant, *top1⁷⁷*, which contains a 3kb insertion at the *TOP1* gene located on the X chromosome and shows both female-homozygous and male-hemizygous lethal phenotypes. The *top1⁷⁷* phenotype can be rescued with an ectopic wild type topoisomerase I gene inserted on the second chromosome. The developmental expressions of topoisomerase I protein and mRNA transcript were found to be at the maximum level 6- to 12-hours after fertilization, which also corresponds to the stage of maximal DNA synthesis activity (Lee *et al.*, 1993).

A high affinity topoisomerase I binding sequence has been identified in the nontranscribed spacer flanking the ribosomal DNA (rDNA) genes in *Tetrahymena thermophila* (Bonven *et al.*, 1985). This highly conserved site is also present in the rDNA spacers of yeast, *Dictyostelium discoideum* and *D. melanogaster* (Bonven *et al.*, 1985). Other eukaryotic type I topoisomerases from calf, chicken, *Drosophila* and human recognize and preferentially cleave

the 16 base-pair (bp) repeat with the same specificity as does the *Tetrahymena* enzyme (Christiansen *et al.*, 1987). Based on the identification of rDNA spacers as the sites of male *Drosophila* X-Y chromosome pairing (McKee *et al.*, 1992), this study used a topoisomerase I null mutation to examine the function of the enzyme in X-Y chromosome pairing in spermatocytes.

Use of the yeast FLP-FRT recombination system to generate DNA topoisomerase I mutants by mitotic recombination

A mosaic organism is an individual with clones of cells of different genotypes. Mosaic organisms have been used extensively as a tool in the study of development. Developmental potential and determination of cell clones, cellular lineage analysis and restrictions in development, patterns of cell division, and study of mutant cell behavior are several problems that have been addressed by mosaic analysis (reviewed by Postlethwait, 1976; Ashburner, 1989). A cell is marked genetically and a group of progeny cells with the same genotype can be obtained in an individual of another genotype. For *Drosophila*, there are several methods that can be employed to generate mosaics (reviewed by Hall *et al.*, 1976; Lawrence *et al.*, 1986; Ashburner, 1989). One of the most widely used techniques is by X-ray induced mitotic recombination between homologous chromosomes in somatic cells. The ionizing radiation induces rare chromosomal breaks in a parent cell heterozygous for a mutant gene and leads to exchanges between homologous chromosome arms. Following chromosome segregation during the next mitosis, one of the two daughter cells often becomes homozygous for the mutation and the other is wild-type. Subsequently, proliferation of the

mutant cell will generate a clone of cells. This clone of cells can be distinguished from other cells if the parental mutant chromosome has either a marker mutation closely linked to the mutation or a recessive mutation that alters the morphology of the cuticle in a cell-autonomous manner (Hall *et al.*, 1976; Lawrence *et al.*, 1986). However, the frequency of X-ray induced mitotic recombination is low. X-rays also cause a significant extent of cell death and the lack of suitable markers for mosaic analysis of internal tissues have limited the effectiveness of this technique.

A new method similar to X-ray-induced recombination has been developed for the generation of mosaics in *Drosophila*. The technique makes use of the yeast *S. cerevisiae* site-specific recombinase FLP. This precisely defined recombination reaction is a more efficient and specific method and has none of the lethal or mutagenic effects associated with irradiation. FLP recombinase, a 43,000 dalton protein, is encoded by the yeast 2 μ plasmid and acts on the FLP recombination target, FRT (reviewed by Cox, 1988). The recombinase cleaves DNA at specific target sequence and ligates it to the cleaved DNA of a second site. Golic and Lindquist (1989) have transferred FLP recombinase and the FRT sequence into the *Drosophila* genome and have shown that the exogenous site-specific recombination system can function in flies. The FLP coding sequence is placed under the control of the *Drosophila hsp70* promoter in order to provide heat inducible expression. Golic and Lindquist (1989) showed that excision of a mini-*white* gene flanked by direct FRT repeats results in mosaic eye phenotypes. Golic (1991) has further demonstrated that FLP recombinase can catalyze mitotic recombination at a high frequency between FRTs located on homologous chromosome arms. In addition to somatic cells, this system also works in

both male and female germ cells (Golic and Lindquist, 1989; Golic, 1991; Chou and Perrimon, 1992). Recently, various *Drosophila* strains were constructed (Xu and Rubin, 1993; Xu and Harrison, 1994). In order to ensure a successful induction of mosaicism for a particular gene, the FRT sequence must be located closer to the centromere than the gene of interest. These strains were made in a way that each one has an FRT sequence inserted near the base of a single major chromosome arm. Many of the strains also carry a novel cell autonomous marker that expresses in the nucleus or cell membrane. These strains provide a powerful means of mosaic analysis and can be used to facilitate studies for more than 95% of *Drosophila* genes.

To generate DNA topoisomerase I mutant spermatocytes, a male that carries a topoisomerase I mutation on the X chromosome and is heterozygous for an autosomal topoisomerase I insertion is placed under heat shock to induce the production of FLP recombinase in the germ cells. Recombination between FRT sites and segregation of sister chromatids during the next cell division results in one cell that is homozygous for topoisomerase I insertion and one cell that is homozygous for the cell marker. The spermatocytes that are homozygous for the cell marker lack topoisomerase I and can be identified by the absence of staining with anti-DNA topoisomerase I antibody.

Overview of meiosis and spermatogenesis in male *Drosophila melanogaster*

Meiosis occurs in germ cells of sexually reproducing organisms; it involves halving of chromosome numbers to form haploid gametes and genetic recombination. During meiotic prophase I, chromosomes pair,

synapse and recombine. Homologous chromosomes align and synapse with the formation of synaptonemal complex, a linear, tripartite structure that holds the homologs in a zipper-like fashion during early prophase and is thought to be involved in meiotic recombination (Von Wettstein *et al.*, 1984). In the course of synapsis, small, dense recombination nodules appear to be associated with synaptonemal complex and are thought to contain recombination enzymes (Carpenter, 1975). Exchange between two non-sister chromatids occurs and results in recombination (crossover or gene conversion). When synaptonemal complex disappears during the late pachytene stage, the homologs that have recombined are held together and stabilized by chiasmata. Chiasmata are thought to be necessary for bivalent stability and to ensure proper chromosome segregation during anaphase (Jones, 1987; Hawley, 1988).

D. melanogaster males represent an example of an achiasmatic organism which does not undergo recombination but has regular chromosomal pairing and segregation. In *Drosophila* males, genetic recombination, synaptonemal complex and chiasmata are absent (Morgan, 1912; Meyer, 1960). However, despite the absence of chiasmata, homologs pair during meiotic prophase I and segregate during anaphase I.

The nucleolus organizers (NOs) have been implicated in the sex chromosome pairing in *Drosophila* males. NOs are located in the centric heterochromatin of the long arm of the X and near the base of the short arm of the Y (Ritossa, 1976) and each contains 200-250 tandem copies of the ribosomal DNA (rDNA) transcription units. Large deletions of the X heterochromatin that encompass the NO disrupt X-Y pairing and result in high frequencies of X-Y nondisjunction (Cooper, 1964; McKee and Lindsley,

1987). McKee and Karpen (1990) have shown that cloned rDNA genes ectopically inserted on the X can partially restore the pairing and disjoining of heterochromatically deficient X chromosomes from the Y. Recent evidence further showed that the 240 bp intergenic spacer repeats found in the rDNA could stimulate pairing, and the pairing ability was proportional to the copy number of the 240 bp repeats (McKee *et al.*, 1992).

Spermatogenesis is a complex developmental process leading to the formation of spermatozoa. This process has been studied extensively previously (reviewed by Cooper, 1950; Lindsley and Tokuyasu, 1980; Hackstein, 1987) and the cytological aspects are briefly described here. In male *D. melanogaster* adults, the structure of testis is a pair of coiled tubular sacs with closed apical ends and openings to the seminal vesicle where mature spermatozoa are stored. Within the lumen of the tubule, closely packed germ cells at various developmental stages, somatic tissues, cellular debris and fluid can be found (Cooper, 1950). A group of stem cells is located at the apex of the tubule where the process of spermatogenesis begins. These stem cells are firmly attached to the testicular apex and are believed to form part of the germinal center (Lindsley and Tokuyasu, 1980). The formation of primary spermatogonia is a result of an unequal mitotic division of the stem cells. A primary spermatogonium becomes invested by two cyst cells that form an envelope of the cyst as soon as it arises from the stem cell. Subsequent developmental stages occur in a defined cyst and all spermatogonia in a cyst are derived from the same progenitor. All members in a cyst of secondary spermatogonial cells undergo highly synchronous differentiation. In *D. melanogaster*, four spermatogonial divisions lead to a cyst containing sixteen primary spermatocytes. Primary spermatocytes

enter an intensive growth phase and their volume increases dramatically. When fully developed, primary spermatocytes undergo two meiotic divisions resulting in a cyst of 64 spermatids. Following spermatid elongation, individualization and coiling, mature sperms are formed.

Research proposal

The 240 base-pair repetitive sequence within the intergenic spacer of ribosomal DNA repeat has been demonstrated to play a major role in the X-Y chromosomal pairing in *Drosophila* males (McKee and Karpen, 1990; McKee *et al.*, 1992). Within the 240-bp repeat, a 16-bp high-affinity cleavage site for DNA topoisomerase I is present. Similar sequences have been detected in the rDNA of several organisms including yeast (Bonven *et al.*, 1985) and indicates the possible involvement of topoisomerase I in X-Y pairing. A model for achiasmatic chromosome pairing involving topoisomerase I has been proposed (McKee *et al.*, 1992).

In this study, the yeast FLP-FRT site-specific recombination system is used to facilitate the study of the role of topoisomerase I in male meiosis. Spermatocytes that are deficient in topoisomerase I can be generated by induction of mitotic recombination in males carrying a topoisomerase I null allele and heterozygous for an autosomal topoisomerase I gene insertion. Cytological studies using anti-topoisomerase I antibody and DAPI staining to examine the chromosome behavior in these topoisomerase I deficient cells are presented. In addition, genetic studies of chromosomal disjunction in progeny of male and female flies subjected to heat shock induction of mitotic recombination to generate topoisomerase I-deficient germ cells are also presented.

CHAPTER 2

MATERIALS AND METHODS

***Drosophila* strains and culture**

Flies were grown on standard yeast-cornmeal-molasses-agar media at 18° or 25°C. The w^- ; $p[top^+, w^+]$ strains, i. e. w^- ; $p[topBRa, w^+]$ and w^- ; $p[topBRb, w^+]$, are fly strains with topoisomerase I gene insertions on the second chromosome. These flies exhibit apricot-colored eye phenotype. Two top^- strains, top^{-77} and top^{-113} , carry the topoisomerase I null mutation. Both top^+ and top^- strains were donated by T. Hsieh. Most of the experiments described below were conducted with top^{-113} and $topBRa$. *Drosophila* FRT strains carrying the FRT sequence on either the left or right arm of the second chromosome and the MKRS strain carrying the FLP recombinase source were obtained from Bloomington Stock Center, Bloomington, Indiana. Xu and Rubin (1993) placed two copies of the *hs-neo* gene, which confers G418 (neomycin) resistance under the control of a heat shock promoter, into the $p[ry^+, hs-neo, FRT]$ construct to enable genetic selection for this element. Balancer chromosomes and genetic markers of *Drosophila* strains are described by Lindsley and Zimm (1992).

Neomycin resistance selection

During the course of constructing the *FRT*, $p[top^+]$ stock (in the F1 generation of cross B described below), neomycin resistance selection was applied in order to identify F2 male flies that carry a recombinant *FRT*, $p[top^+]$ chromosome. This recombinant chromosome was a result of crossing over in the F1 female *FRT*/ $p[top^+]$ parent. Two F2 male genotypes are

possible: *FRT*, *p[top⁺]/+* or *FRT/+*. F2 males with the *FRT*, *p[top⁺]/+* genotype can be distinguished from the other genotype by the apricot-colored eye phenotype due to the *w⁺* gene in the *p[top⁺]* construct.

Flies with *p[ry⁺, hs-neo, FRT]* elements are resistant to G418 (Geneticin, GIBCO laboratories). G418 selection was performed as follows: a few holes were made in a vial with fresh fly medium using a toothpick, and 100-150 μ l of 50 mg/ml freshly made G418 solution was added for every 10 ml of medium. The vial was allowed to air-dry for a few hours. 20-30 first or second instar larvae were transferred into the vial and the flies were collected upon eclosion. For crosses cultured at 25°C, no heat-shock treatment of the larvae was required. If crosses were cultured at a lower temperature, early-stage larvae were incubated twice at 38°C for 60 minutes for selection of *neo^R* flies (Xu and Rubin, 1993).

***Drosophila* crossing schemes for generating topoisomerase I deficient germ line clones**

Male germ line clones deficient in topoisomerase I were constructed in the following way. Initially, both of the *p[top⁺]* insertions were determined by *in situ* hybridization and were found to be located on the right arm of the second chromosome. *Drosophila* strains with a chromosome arm carrying an *FRT* element at 42D (on the right arm of second chromosome) were chosen for preparing the male germ line clones. First, a stock with males of the *FRT*, *p[top⁺]/FRT*, *p[top⁺]* genotype was made as described in cross B. Another strain with a chromosome arm carrying an *FRT* element at 42D and the cell-autonomous marker NM at 46F was used to construct the females with *top⁻/FM6; FRT-NM/FRT-NM; MKRS/+* genotype as described in cross C. The

P $w^- / w^-; p[top^+, w^+] / p[top^+, w^+] \quad \mathbf{X}$

$y, w / Y; FRT, p[ry^+, y^+] / FRT, p[ry^+, y^+]$



F1 $y, w / w^-; p[top^+, w^+] / FRT, p[ry^+, y^+] \quad \mathbf{X}$

$y, w / Y$



select for neomycin resistance

F2 $y, w / Y; FRT, p[ry^+, y^+], p[top^+, w^+] / + \quad \mathbf{X}$

$y, w / y, w; Cy / Sp$



F3 $y, w / y, w; FRT, p[ry^+, y^+], p[top^+, w^+] / Cy \quad \mathbf{X}$

$y, w / Y; FRT, p[ry^+, y^+], p[top^+, w^+] / Sp$



F4 $y, w / Y; FRT, p[ry^+, y^+], p[top^+, w^+] / FRT, p[ry^+, y^+], p[top^+, w^+]$

(C) Generating $top^-; FRT-NM; FLP$ stock

$p[ry^+, hs-neo, FRT], p[mini-w^+, hs-NM]$ is abbreviated as $FRT-NM, w^+$

(I) Preparing *y, w, top⁻/FM6; Cy/+* flies

P $top^-/FM7$ x $y, w/Y$

Df(1)X-1/FM6 x *Cy/Sp; Δ2,3/TM3*



F 1 $top^- / y, w$

X

FM6/Y; Cy/+; Δ2,3/+



F2 *y, w, top⁻ / FM6; Cy/+* (singles) **X**

X *FM6/Y*



(Note: Only vials selected were those that did not have any $y w$ male progeny which is an indication that the X chromosome from the mother is $y w^{top^-}$ and not $y w$)

F3 $y, w, top^- / FM6; Cy/+$

(II) Preparing *FM6/Y; FRT-NM/+; MKRS/+* males

P	$w/w; FRT-NM, w^+/FRT-NM, w^+$	X
----------	--------------------------------	----------

X/Y; +/+; MKRS/TM6



F1 *w/Y; FRT-NM, w⁺/+; MKRS/+* **X**

$w/w; FRT-NM, w^+/FRT-NM, w^+$



F2 $w/Y; FRT-NM, w^+/FRT-NM, w^+; MKRS/+$ **X**
 Df(1)X-1 / FM6



F3 $FM6/Y; FRT-NM, w^+/+; MKRS/+$

(III) F3 generations of (I) and (II) above were crossed together to obtain the $top^-; FRT-NM; FLP$ stock

P $y, w, top^- / FM6; Cy/+$ **X**
 $FM6/Y; FRT-NM, w^+/+; MKRS/+$



F1 $y, w, top^- / FM6; FRT-NM, w^+/Cy; MKRS/+$ **X**
 $FM6/Y; FRT-NM, w^+/Cy; MKRS/+$



F2 $y, w, top^- / FM6; FRT-NM, w^+/FRT-NM, w^+; MKRS/+$

(D) Generating *top*⁻ germ line clones

P *y, w, top*⁻ / *FM6; FRT-NM, w*⁺ / *FRT-NM, w*⁺; *MKRS* / + **X**
y, w / *Y; FRT, p[ry*⁺, *y*⁺], *p[top*⁺, *w*⁺] / *FRT, p[ry*⁺, *y*⁺], *p[top*⁺, *w*⁺]



heat-shock larvae

F 1 *y, w, top*⁻ / *Y; FRT, p[ry*⁺, *y*⁺], *p[top*⁺, *w*⁺] / *FRT-NM, w*⁺; *MKRS* / +

The following steps were performed on these males

(1) Dissect adult testis

(2) Immunofluorescent staining with anti-topoisomerase I
antibody, counter-stain with fluorescein labeled secondary antibody
and DAPI

(3) Analyze spermatocytes

The generation of *top*⁻ germ line clones by FLP-mediated mitotic recombination is shown in Figure 1.

***Drosophila* crossing schemes for nondisjunction studies in male and females**

Genetic analysis involving nondisjunction studies of sex chromosomes in both male and female flies were used to determine whether the topoisomerase I mutation induced by mitotic recombination has any effect on normal chromosome segregation.

Crosses were set up as follows to generate the appropriate flies .

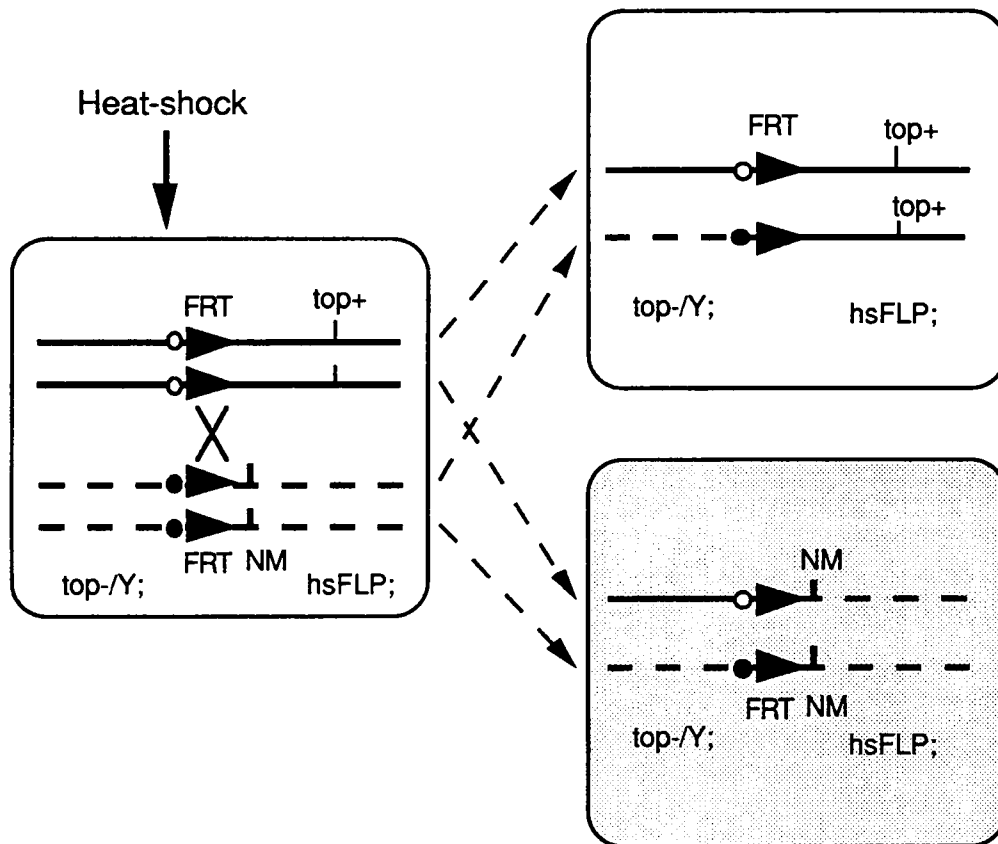


Figure 1.

Generation of clones of spermatocytes with homozygous DNA topoisomerase I mutation. Heat-shock treatment of the cell on the left (from the F1 generation of cross D) induces production of FLP recombinase and leads to FLP-mediated mitotic recombination between the FRT sites on different chromosome arms. After segregation of the chromatids, one of the daughter cells will become homozygous for the topoisomerase I mutation (shaded cell on right).

(E) Nondisjunction study of males

P $w^-/w^-; p[top^+, w^+]/p[top^+, w^+]$ **X**

$y, w/Y; FRT, p[ry^+, y^+]/FRT, p[ry^+, y^+]$



F1 $y, w/w^-; p[top^+, w^+]/FRT, p[ry^+, y^+]$ **X**

bb^2/B^sY



select for neomycin resistance

F2 $y, w/B^sY; FRT, p[ry^+, y^+], p[top^+, w^+]/+$ **X**

$y, w/y, w; Cy/Sp$



F3 $y, w/y, w; FRT, p[ry^+, y^+], p[top^+, w^+]/Cy$ **X**

$y, w/B^sY; FRT, p[ry^+, y^+], p[top^+, w^+]/Sp$



F4 $y, w/B^sY; FRT, p[ry^+, y^+], p[top^+, w^+]/FRT, p[ry^+, y^+], p[top^+, w^+]$

X $y, w, top^-/FM6; FRT-NM, w^+/FRT-NM, w^+; MKRS/+$



heat shock male larvae

F5 $y, w, top^- / B^sY; FRT-NM, w^+ / FRT, p[ry^+, y^+], p[top^+, w^+]; MKRS/+$
X $y, cv / y, cv$



Score flies

(F) Nondisjunction study of females

P generation males were from cross E, F4 generation and females were from cross C(III), F2 generation.

P $y, w, top^- / FM6; FRT-NM, w^+ / FRT-NM, w^+; MKRS/+$ **X**
 $y, w / B^sY; FRT, p[ry^+, y^+], p[top^+, w^+] / FRT, p[ry^+, y^+], p[top^+, w^+]$



F1 $y, w, top^- / B^sY; FRT-NM, w^+ / FRT, p[ry^+, y^+], p[top^+, w^+]; MKRS/+$
X $y, w, top^- / FM6; FRT-NM, w^+ / FRT-NM, w^+; MKRS/+$



heat shock female larvae

F2 $y, w, top^- / y, w, top^-; FRT, p[ry^+, y^+], p[top^+, w^+] / FRT-NM, w^+;$
 $MKRS/+$
X bb^2 / B^sY



score flies

Heat-shock induction of mitotic recombination in larvae

Third instar larvae were collected from vials. Larvae were rinsed in water and transferred into a new vial. The vial was placed in a 38°C water bath for 60 to 90 minutes to induce the production of FLP recombinase that will catalyze mitotic recombination events. After heat shock was completed, larvae were placed in fresh medium until eclosion. Hatched flies were used for nondisjunction studies or immunofluorescent studies.

***In situ* hybridization**

In situ hybridization to salivary gland polytene chromosomes using a biotin-labeled DNA probe was carried out as described in Ashburner (1989a). Larvae were cultured in standard yeast-cornmeal-molasses-agar media at 25°C under noncrowding conditions. Well-fed third instar larvae were rinsed in 0.9% saline to remove debris. Salivary glands were dissected in 45% acetic acid on a subbed slide (subbing solution: 0.1% gelatin and 0.01% chromium potassium sulfate) and fixed in a drop of fresh 45% acetic acid for three minutes. The glands were squashed with a rubber mallet under siliconized (Sigmacote, Sigma Chemical) coverslips. The slides were kept at 4°C in acetic acid vapor chamber overnight. The edges of coverslips were marked with a glass etcher. Slides were immersed in liquid nitrogen until bubbling stopped and coverslips were removed with a razor blade. The slides were fixed in methanol-acetic acid (3:1 v/v) for 10 minutes followed by dehydration in 50% ethanol, 70% ethanol, 95% ethanol for 10 minutes each and air dried. The slides were washed in 2X SSC at 65°C for 30 minutes and transferred to

2X SSC at room temperature for 2 minutes. The slides were suspended in a beaker containing 2 liters of 100 mM TEA (triethanol amine pH 8.0). 10 ml acetic anhydride were added while stirring the solution vigorously. When acetic anhydride was dissolved, stirring was stopped and the slides were left in the solution for 10 minutes in the chemical hood. The slides were washed twice with 2X SSC for 5 minutes each time, and dehydrated twice in 70% ethanol and 90% ethanol for 5 minutes each. Slides were air dried for 10 minutes and denatured in 70 mM NaOH for 3 minutes. Slides were washed in 2X SSC for 5 minutes after denaturation and dehydrated twice for 5 minutes each time in 70% ethanol and 90% ethanol. Air dried slides were ready for hybridization. The DNA probe, a pDw vector which contains a cloned *white* gene sequence, was prepared by using BioNick labeling system (Gibco-BRL) and purified by using column chromatography with a Sephadex G-50 column. The biotinylated probe was stored in hybridization solution at -20°C for up to a year. The probe was denatured by boiling for 5 minutes and cooled quickly on ice. Slides were placed flat on a bench and 10 µl of denatured probe was pipetted onto slides where the coverslips were marked. Siliconized cover slips were placed on the slides with care not to trap bubbles and the edges were sealed with rubber cement. After rubber cement was dried, slides were placed in a slide chamber with moist filter paper. The chamber was placed in a 58°C water bath overnight to allow hybridization of the probe with polytene chromosome. On the next day, the rubber cement was peeled off without removing coverslips. Slides were immersed in 2X SSC at 53°C briefly to let coverslips fall off. Slides were washed three times in 2X SSC at 53°C for 20 minutes each, followed by 2 washes in 1X PBS for 10 minutes at room temperature, 1 wash in 2% BSA in TMN1, Triton X-100 at 42°C for 20

minutes, and 1 wash in 2% BSA in TMN1, Triton X-100 at room temperature for 10 minutes. The biotinylated probes that had hybridized with the chromosomes on the slides were detected with BluGENE nonradioactive nucleic acid detection system (Gibco-BRL). One slide at a time was placed on the bench, 50 μ l of SA/AP (2 μ l streptavidin/alkaline phosphatase conjugate in 1 ml TMN1 with Triton X-100) was pipetted onto slide where the coverslip was marked. Another 50 μ l of SA/AP was added to each slide after 5 minutes. After another 5-minute wait, each slide was rinsed with 3-4 ml of TMN1, Triton X-100 over a beaker to let the solution drain into the beaker. The slides were washed twice in TMN1, Triton X-100 for 3 minutes each time and twice in TMN2 for 3 minutes each. 30 μ l of NBT/BCIP dilution was pipetted onto the center of a plastic coverslip. One slide was drained and was held over the plastic coverslip with the chromosomes facing down so that the droplet would adhere to the slide. The slide was flipped over carefully so that the coverslip would not fall off. The slides were placed flat on a tray and stored in dark for 2 hours. Coverslips were rinsed off by swishing in a beaker of distilled water. Slides were stained with orcein solution for about 10 seconds, immediately rinsed with water and air dried. To look at a slide, one drop of sterile water was added and a coverslip was placed on the slide. To photograph, the edges of coverslips were sealed with rubber cement to prevent oil from going under the coverslips and ruining the slide. The slides were examined on a Zeiss Universal Axioplan microscope and the *in situ* photographs were taken with Kodak TMX 100 film.

Immunofluorescent staining

Immunofluorescent staining of the adult testis using anti-topoisomerase I antibody was carried out using a modified procedure based on the method described in Ashburner (1989a). Testes were dissected in testis buffer on slides. Testes were transferred to one drop of fresh testis buffer on poly-L-lysine coated slides (poly-L-lysine, Sigma Chemical) and two cuts were made to divide it into three approximately equal pieces. Siliconized coverslips were placed over the slides. The slides were left on the bench for a few minutes to allow the testis buffer to evaporate but never let the tissue dry out completely. The coverslips were tapped gently to allow the contents within testis tubules to spill out just before freezing in liquid nitrogen. The edges of the coverslips were marked with a glass etcher. The slides were immersed in liquid nitrogen until bubbling stopped and coverslips were removed with a razor blade. Before the slides were defrosted, 100 μ l of 4% paraformaldehyde, 0.03% SDS and 2% glutaraldehyde solution were pipetted onto the slides for 15 minutes and drained gently. Slides were placed in a Coplin jar containing 50 ml of 4% paraformaldehyde for 10 minutes and rinsed in 1X PBS twice for 5 minutes each wash. Testes were permeabilized in cold methanol (-20°C) for 1 minute and air dried for 30 seconds to allow evaporation of methanol. The slides were immersed in 1% BSA in 1X PBS for 30 minutes at room temperature. 20 μ l of rabbit-anti-*Drosophila* topoisomerase I antibody (provided by T. Hsieh) at a 1: 100 dilution in MBS (10 mM MOPS, 150 mM NaCl) were added onto the slides and slides were incubated for 45 minutes at room temperature in a moist chamber. Slides were washed two times for 5 minutes each in 1X PBS and incubated with fluorescein-conjugated goat-anti-rabbit IgG (Pierce) at a 1:100 dilution in

MBS for 45 minutes in a moist chamber under darkness. Slides were rinsed in 1X PBS for 2 minutes and 20 μ l of 0.5 μ g/ml DAPI (Boehringer Mannheim) in 180 mM Tris-HCl, pH 7.5 were added for 7 minutes. The slides were dipped in 1X PBS for a few times and mounted with 8 μ l VECTASHIELD mounting medium (Vector Laboratories) under coverslips. The coverslips were sealed with nail polish and slides were stored in the dark at 4°C. Slides were viewed on a Zeiss Universal Axioplan fluorescence microscope and photographed using Kodak Gold 400 film.

X-ray induction of mitotic recombination

X-ray irradiation of adult *y, w, top⁻/Y; FRT, p[ry⁺, y⁺], p[top⁺, w⁺]/FRT-NM, w⁺; +/-* flies (adult males which were not heat-shocked from the F1 generation of cross D) was performed as a parallel experiment to determine whether topoisomerase I-deficient spermatocytes were generated in the testis. The males were irradiated with X-rays (2000 rad) which should kill all spermatocytes present during the time of irradiation and also cause mitotic recombination in spermatogonia. The males were kept in a fresh vial for 4 to 6 days to allow formation of new spermatocytes. Testes from these males were dissected as described above and stained with the same antibodies.

CHAPTER 3

RESULTS

Cytological locations of DNA topoisomerase I insertions

DNA topoisomerase I transgene chromosomal insertion sites were mapped by *in situ* hybridization to salivary gland polytene chromosomes. The P element constructs used in the germ line transformation experiments to generate the *top*⁺ strains carry both the DNA topoisomerase I gene and a *white*⁺ gene marker (data provided by T. Hsieh). The DNA probe for these insertions was pDw which is a plasmid carrying a *white* gene sequence. pDw DNA probe provides an internal control to indicate whether the hybridization reactions are working correctly. In addition to hybridization to topoisomerase I transgene insertion sites, pDw also hybridizes to the genetic locus for the *white* gene on the tip of the X chromosome. Both of the topoisomerase I insertions are found on the right arm of the second chromosome. The location of p[*topBRa*] was mapped to 53E-F (Fig. 2) and p[*topBRb*] was inserted at 50C (Fig. 3).

Non-disjunction study of X-Y chromosomes in male *Drosophila melanogaster*

Heat-shocked FRT/FLP males carrying the *top*⁻ mutation on their X-chromosome and a single *top*⁺ transgene on chromosome 2 were assayed for sex chromosome nondisjunction. In male nondisjunction studies, two types of males were used as the heat-shocked parents in cross E, F5 mated with *y, cv* females. The genotypes of these males were either *top*⁻⁷⁷/*B*^S*Y*; *topBRa*/*NM*; *MKRS*/+ or *top*⁻¹¹³/*B*^S*Y*; *topBRa*/*NM*; *MKRS*/+, the only difference being the

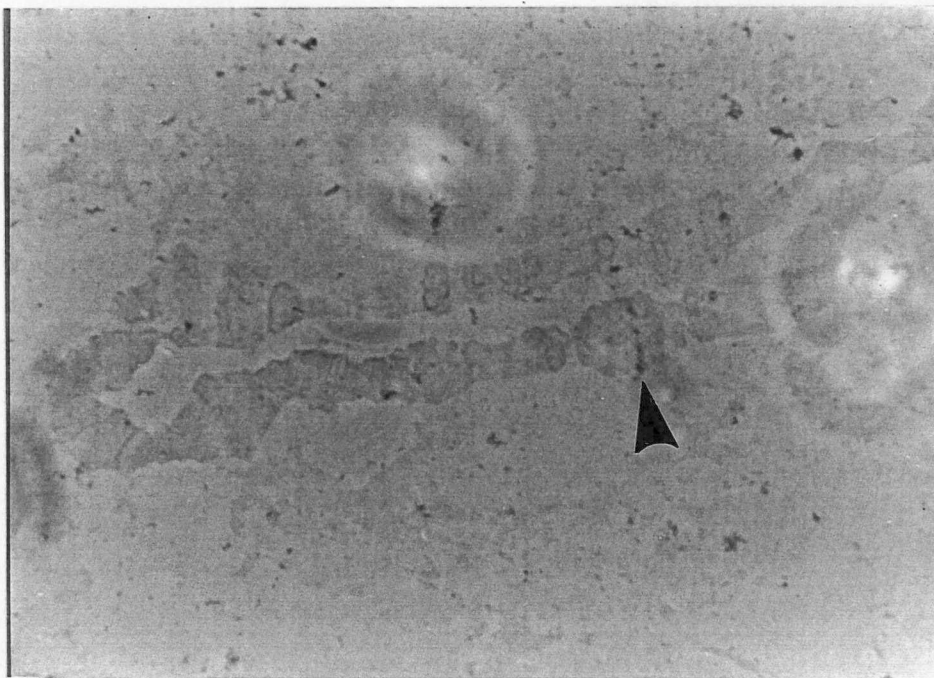


Figure 2. *In situ* hybridization of biotin labeled pDw to p[*topBRa*].
Arrow indicates insertion at 53E-F.

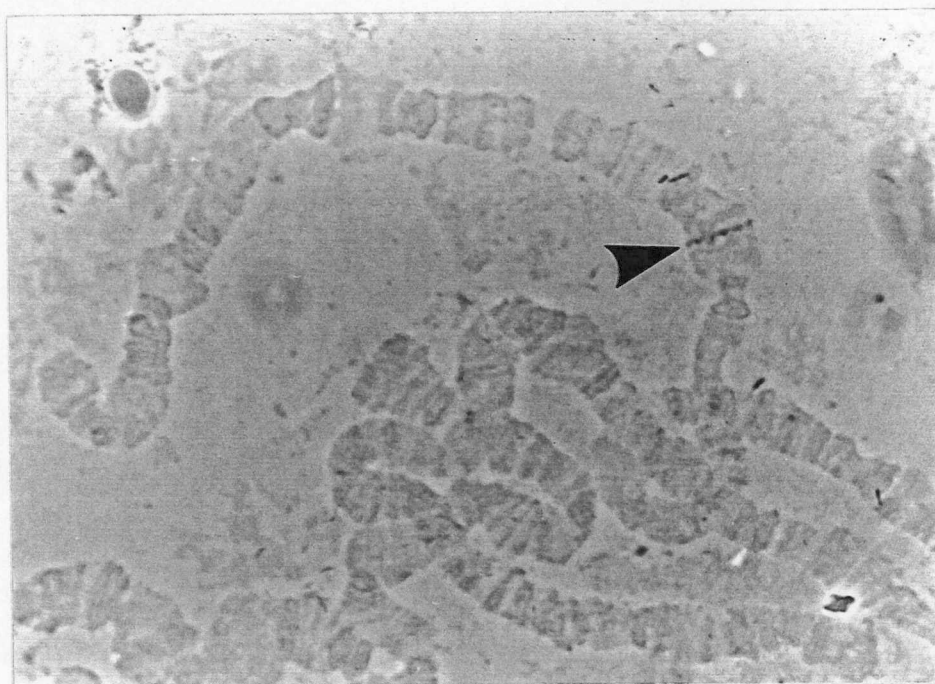


Figure 3. *In situ* hybridization of biotin labeled pDw to p[*topBRb*].
Arrow indicates insertion at 50C.

top⁻ allele present on the X chromosome. The male flies used as controls had +/+ instead of *MKRS*/+ and thus did not have a source of FLP recombinase to induce mitotic recombination but were otherwise identical to the experimental males. Both control and experimental males were heat-shocked as described in Materials and Methods. The normal progeny from these crosses were *cv* (crossveinless wings) males with bar-eyes (*B*, narrow eyes) and normal-winged (*cv*⁺) females with non-bar eyes (*B*⁺, wild-type round shaped). Non-bar-eyed *cv* male and bar-eyed *cv*⁺ female progeny resulted from a nondisjunction event involving the X and Y chromosomes in the male parent. Nondisjunction in the mothers also produces bar-eyed females and non-bar-eyed males but in this case the daughters are *cv* and the sons are *cv*⁺.

Tables 1 and 2 present the numbers of progeny scored in each category from *top*⁻113/*B*^S*Y* and *top*⁻77/*B*^S*Y* males respectively. In both types of males, the percentages of nondisjunction of progeny in the experimental groups did not show a substantial increase over the nondisjunction frequencies of the control groups. The average numbers of progeny produced by the *top*⁻113/*B*^S*Y* males are 239 for the heat-shocked experimental flies and 312 for the control flies. For the *top*⁻77/*B*^S*Y* males, each heat-shocked male produced an average of 146 progeny and the non-heat-shocked controls produced an average of 242 progeny per male.

Non-disjunction study of X-X chromosomes in *Drosophila melanogaster* females

In female nondisjunction studies, *top*⁻113/*top*⁻113; *topBRa*/NM; *MKRS*/+ females were the heat shocked parents in cross F, F2 mated with

Table 1. Nondisjunction (NDJ) study of *top⁻¹¹³/B^SY* males. Progeny scored from *top⁻¹¹³/B^SY; topBRa/NM* males X *y cv* females.

(a) Experimental flies

	Number scored
B cv males (Normal)	5568
B ⁺ cv ⁺ females (Normal)	6586
B ⁺ cv males (Paternal NDJ)	10
B ⁺ cv ⁺ males (Maternal NDJ)	9
B cv ⁺ females (Paternal NDJ)	8
B cv females (Maternal NDJ)	2
Total number of flies	12183

Total percentage of paternal NDJ: $(18/12183) \times 100\% = 0.15\%$

Total percentage of maternal NDJ: $(11/12183) \times 100\% = 0.09\%$

(b) Control flies

	Number scored
B cv males (Normal)	3074
B ⁺ cv ⁺ females (Normal)	3640
B ⁺ cv males (Paternal NDJ)	5
B ⁺ cv ⁺ males (Maternal NDJ)	6
B cv ⁺ females (Paternal NDJ)	1
B cv females (Maternal NDJ)	0
Total number of flies	6726

Total percentage of paternal NDJ: $(6/6726) \times 100\% = 0.09\%$

Total percentage of maternal NDJ: $(6/6726) \times 100\% = 0.09\%$

Table 2. Nondisjunction (NDJ) study of *top-77/B^SY* males. Progeny scored from *top-77/B^SY*; *topBRa/NM* males X *y cv* females.

(a) Experimental flies

	Number scored
B cv males (Normal)	1082
B ⁺ cv ⁺ females (Normal)	1407
B ⁺ cv males (Paternal NDJ)	3
B ⁺ cv ⁺ males (Maternal NDJ)	2
B cv ⁺ females (Paternal NDJ)	0
B cv females (Maternal NDJ)	0
Total number of flies	2494

Total percentage of paternal NDJ: $(3/2494) \times 100\% = 0.12\%$

Total percentage of maternal NDJ: $(2/2494) \times 100\% = 0.08\%$

(b) Control flies

	Number scored
B cv males (Normal)	1205
B ⁺ cv ⁺ females (Normal)	1452
B ⁺ cv males (Paternal NDJ)	1
B ⁺ cv ⁺ males (Maternal NDJ)	2
B cv ⁺ females (Paternal NDJ)	1
B cv females (Maternal NDJ)	0
Total number of flies	2661

Total percentage of paternal NDJ: $(2/2661) \times 100\% = 0.08\%$

Total percentage of maternal NDJ: $(2/2661) \times 100\% = 0.08\%$

bb²/B^sY males. Female flies used as controls were *+/+* instead of *MKRS/+* and did not undergo mitotic recombination mediated by FLP when heat shocked. Progeny resulting from nondisjunction events in the mothers were *B⁺, w⁺* (wild-type red-colored eyes) males and *B, w^a* (apricot-colored eyes) females, whereas progeny arising from paternal nondisjunction events were *B⁺, w^a* males and *B, w⁺* females. Progeny arising from normal segregation events were *B* males and *B⁺* females.

The number of progeny scored in each category from *top⁻¹¹³/top⁻¹¹³* females are presented in Table 3. The average numbers of progeny produced are 190 by each heat-shocked *top⁻¹¹³/top⁻¹¹³* female and 221 by the control flies. Seven flies of the unknown nondisjunction categories were collected during early stage of this study and their eye-color phenotypes were not recorded carefully. There is no adequate data for identification of the paternal or maternal origin of nondisjunction events in these flies. In order to account for the nondisjunction frequencies accurately, these flies were divided into maternal and paternal groups based on the ratio of maternal to paternal events when the percentages were calculated. Similar to the results observed in studies of male nondisjunction, the percentages of nondisjunction progeny in the experimental groups did not show a substantial increase over the nondisjunction frequencies observed in the control groups.

The following alternative assumptions can explain the observations obtained from male and female nondisjunction studies: (1) DNA topoisomerase I is necessary for normal germ cell development and deficiencies in topoisomerase I lead to the disruption of normal sperm and egg formation. In this case, it would not be possible to tell if topoisomerase I is required for meiotic segregation, since germ cells lacking topoisomerase I

Table 3. Nondisjunction (NDJ) study of *top-113/top-113* females.
Progeny scored from *top-113/top-113*; *topBRa/NM* females X
bb²/B^SY males.

(a) Experimental flies

	Number scored
B males (Normal)	2769
B ⁺ females (Normal)	5691
B ⁺ males (Maternal NDJ)	6
B ⁺ males (Paternal NDJ)	8
B ⁺ males (Unknown NDJ)	6
B females (Maternal NDJ)	3
B females (Paternal NDJ)	0
B females (Unknown NDJ)	1
Total number of flies	8484

Total percentage of maternal NDJ: $(13/8484) \times 100\% = 0.15\%$

Total percentage of paternal NDJ: $(11/8484) \times 100\% = 0.13\%$

(b) Control flies

	Number scored
B males (Normal)	931
B ⁺ females (Normal)	2188
B ⁺ males (Maternal NDJ)	1
B ⁺ males (Paternal NDJ)	1
B females (Maternal NDJ)	0
B females (Paternal NDJ)	0
Total number of flies	3121

Total percentage of maternal NDJ: $(1/3121) \times 100\% = 0.03\%$

Total percentage of paternal NDJ: $(1/3121) \times 100\% = 0.03\%$

would die prior to meiosis. (2) DNA topoisomerase I is required neither for germ cell development nor for chromosome segregation; thus homozygous topoisomerase I mutations generated by mitotic recombination would have no phenotype in germ cells. The results of the nondisjunction studies are consistent with either explanation.

Immunocytological studies of spermatocytes in heat-shock treated males with anti-topoisomerase I antibody

To determine whether germ cell development can occur in topoisomerase I⁻ clones in testes, heat-shocked *top⁻¹¹³/Y; topBRa/NM; MKRS/+* males were assayed for the presence of topoisomerase I⁺ spermatocytes using anti-topoisomerase I antibody. Antibody staining was assayed in squashed testes by fluorescence microscopy. Squashes were counter-stained with DAPI to visualize DNA. The *top⁻¹¹³/Y; topBRa/NM; MKRS/+* males used in this study were heat shocked during late third instar larval stage and usually eclosed about 6 to 8 days after heat shock.

The results were that all 1642 spermatocyte nuclei with normal DAPI staining were positive for topoisomerase I staining. Typical spermatocyte staining patterns scored for experimental and control testes are shown in Table 4.

Cytological observations showed that DNA topoisomerase I is found to be present throughout the nucleus but in greatest quantity around the nucleolus (Fig. 4). Previous immunofluorescent studies of polytene chromosomes of *Drosophila* salivary glands showed that DNA topoisomerase I is associated with transcriptionally active loci (Fleischmann *et al.*, 1984). In primary spermatocytes before entry into meiosis, chromatin usually

Table 4. Scoring of spermatocytes from heat-shock treated *top⁻¹¹³/Y* males. Spermatocytes were obtained from heat-shocked *top⁻¹¹³/Y; topBRa/NM; MKRS/+* males.

	Number of testis	DAPI positive	Topo I positive	Topo I negative
Experimental	19	1642	1642	0
Control	4	455	455	0

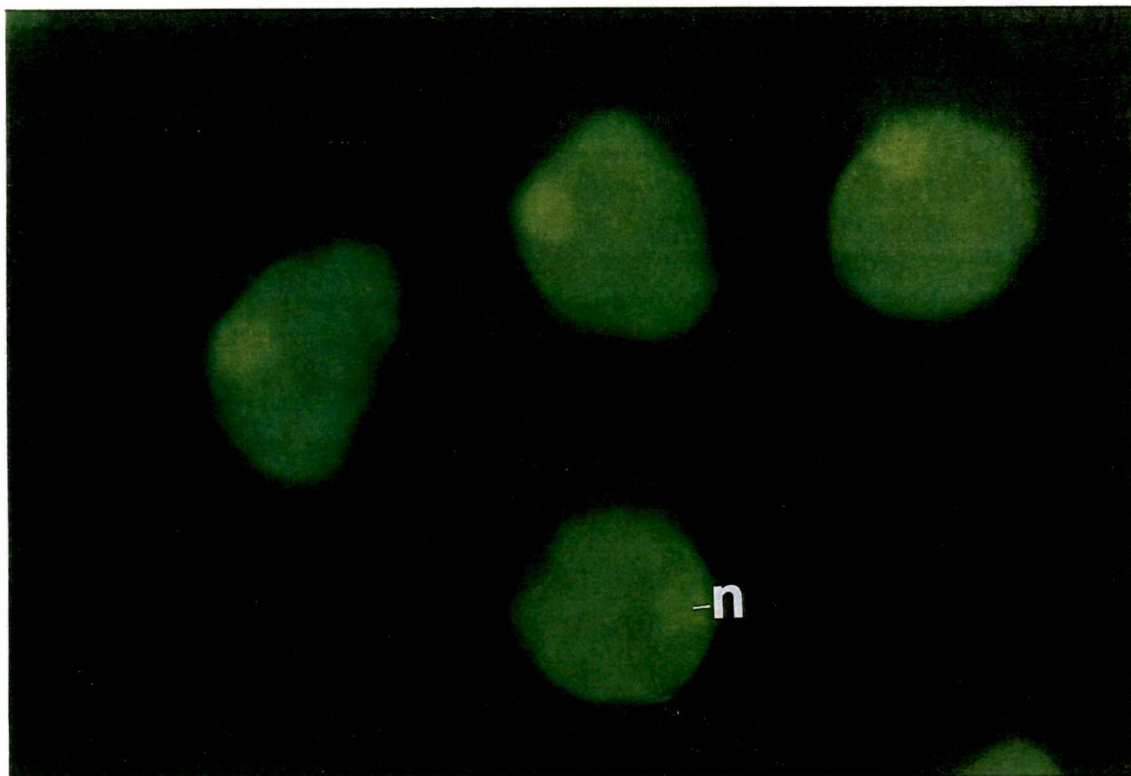


Figure 4. Spermatocytes stained with anti-topoisomerase I antibody and fluorescein-conjugated secondary antibody. These primary spermatocytes came from heat-shocked *top⁻¹¹³/Y* males. Nucleolus is indicated by the letter n.

appeared as not very well defined lobes when stained with DAPI (Fig. 5). Elongating spermatids stained well with DAPI but did not stain with anti-topoisomerase I antibody (picture not shown). A few spermatocytes appeared to be negative with topoisomerase I staining were found in both experimental and control testes. These spermatocytes were probably dead because the chromatin structure appeared to be abnormally diffuse when stained with DAPI.

This experiment showed that topoisomerase I antigen is present in all viable spermatocytes in males that have been heat-shocked to generate clones of topoisomerase I⁻ cells, this result implies that topoisomerase I is essential for spermatocyte development.

Immunocytological studies with anti-topoisomerase I antibody of spermatocytes in X-ray irradiated males

To confirm the results of heat-shock experiment, the immunocytological study was repeated using X-ray instead of heat-shock to generate the topoisomerase I⁻ clones. X-rays have been used extensively to induce mitotic recombination in the past. In this experiment, the *top⁻¹¹³/Y*; *topBRa/NM*; *MKRS/+* males were irradiated within 6 days after eclosion to induce mitotic recombination. Upon irradiation, all spermatocytes that were present were eliminated. The irradiated flies were left in a vial for 5 to 6 days to allow regeneration of spermatocytes before they were dissected. The numbers of spermatocytes scored are presented in Table 5. Antibody staining of 1575 experimental spermatocytes did not identify any cell that was topoisomerase I-deficient; thus, in the cytological studies of both heat-shocked and X-ray irradiated males, the inability to detect any topoisomerase

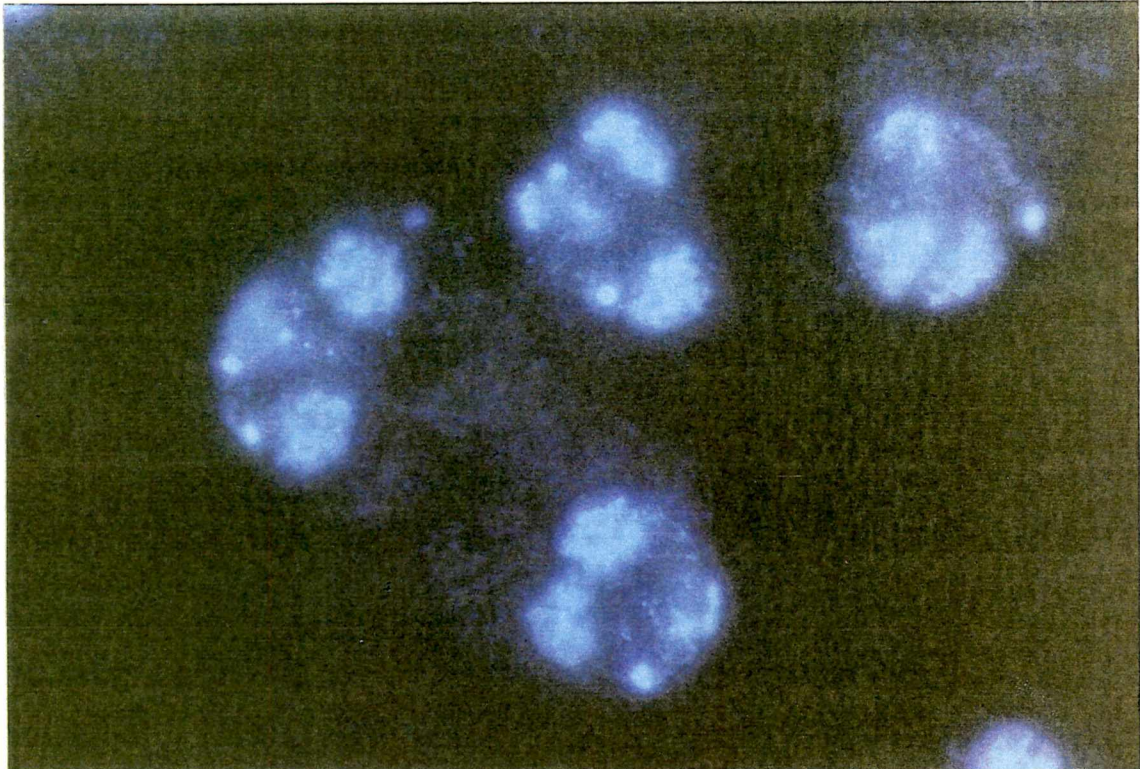


Figure 5. Spermatocytes stained with DAPI. Spermatocytes from heat-shocked males stained with DAPI to show chromatin (blue).

Table 5. Spermatocytes scored from X-rayed *top⁻¹¹³/Y; topBRa/NM* males.

	Number of testis	DAPI positive	Topo I positive	Topo I negative
Experimental	13	1575	1575	0
Control	4	672	672	0

I-deficient spermatocyte suggests that topoisomerase I is required for the normal development of spermatocytes.

Efficiency of the FLP-FRT recombination system

The efficiency of recombination reactions catalyzed by FLP-FRT system was determined by assaying the presence of large yellow dorsal bristles in *y, w, top⁻¹¹³/y, w; FRT, p[ry⁺, y⁺], p[top⁺, w⁺]/FRT-NM, w⁺; MKRS/+* females (females obtained in cross D, F1 generation), which were heat shocked during third instar larval stage. This yellow-bristle phenotype was due to yellow genotype (resulted from *y, w, top⁻¹¹³/y, w; FRT-NM/FRT-NM, w⁺*) in a background of wild type black bristles (from *y, w, top⁻¹¹³/y, w; FRT, p[ry⁺, y⁺], p[top⁺, w⁺]/FRT-NM, w⁺* genotype). Table 6 shows the number of yellow bristles scored from the heat-shocked females. 94% of female flies were observed to carry an average of 3.9 mutant yellow bristles per fly. This observation indicates that the recombination system is working effectively.

Table 6. Efficiency of the FLP-FRT site-specific recombination system. This is done by assaying the presence of yellow bristles in heat-shocked *top⁻¹¹³/y w; topBRa/NM; MKRS/+* females.

Number of flies	Flies with y bristles	Percent of flies with y bristles	Total number of bristles scored	Average bristles per fly
34	32	$(32/34) \times 100\% = 94\%$	127	$127/32 = 3.97$

CHAPTER 4

DISCUSSION

In this study, the yeast FLP site specific recombination system was used to generate germ line mosaics carrying spermatocytes that are deficient in DNA topoisomerase I. Analysis of large dorsal bristles in *y, w, top⁻¹¹³/y, w; FRT, p[ry⁺, y⁺], p[top⁺, w⁺]/FRT-NM, w⁺; MKRS/+* females (females obtained in cross D, F1 generation), which were heat shocked during third instar larval stage, indicated that the recombination system is working effectively. 94% of female flies were observed to carry an average of 3.9 mutant yellow bristles per fly. This yellow-bristle phenotype was due to yellow genotype (resulted from *y, w, top⁻¹¹³/y, w; FRT-NM/FRT-NM, w⁺*) in a background of wild type black bristles (from *y, w, top⁻¹¹³/y, w; FRT, p[ry⁺, y⁺], p[top⁺, w⁺]/FRT-NM, w⁺* genotype). In *y, w, top⁻¹¹³/Y; FRT, p[ry⁺, y⁺], p[top⁺, w⁺]/FRT-NM, w⁺; MKRS/+* males, yellow bristles were also observed. The yellow bristle cells in these males are *top⁻*. This result indicates that cuticle cells do not have an absolute requirement for topoisomerase I, at least in the late stages of development. In previous studies which applied the FRT-FLP system, mosaics were observed at a high frequency (95%) in embryos heat shocked at ages 180-240 minutes (Dang and Perrimon, 1992). Mosaics were also observed in somatic cells as well as in the germ line (Golic and Lindquist, 1989; Golic, 1991; Chou and Perrimon, 1992). Dang and Perrimon (1992) showed that the efficiency of mosaic induction increases when the embryos were heat shocked at younger stages (embryos at ages 180-240 minutes compared to those at 360-540 minutes). During the earlier stage of this study, it was observed that when first and second instar larvae were

heat shocked, most of them did not survive to eclosion. This observation suggests that there were probably too many topoisomerase I deficient cells in a single larva. Apparently, a great quantity of cells with the mutation may have caused irreversible lethal effects on the growth and development of the organism.

The presence of yellow bristles in the female and male flies mentioned above may indicate that the formation of cuticle has a less stringent developmental requirement for topoisomerase I than do cells in the early embryo. The deficiency in topoisomerase I resulting from heat shock treatments administered during the third instar larval stage had only little effect in bristle formation process. Yellow bristles found in a male suggested that topoisomerase I is dispensable in cuticle formation.

In contrast to cuticular tissue formation, spermatogenesis has an absolute requirement for DNA topoisomerase I. Spermatogenesis is a dynamic process that continually produces mature spermatozoa in the *Drosophila* testis and involves cysts of cells at various developmental stages. These cysts undergo mitotic- and meiotic divisions in which DNA topoisomerase I is likely to play an important role. Based on the observations from immunofluorescent staining of testes, the absence of mitotic-recombination-induced topoisomerase I deficient spermatocytes suggests that there is a requirement for the enzyme activity in spermatocytes or even prior to spermatocyte formation. X-ray induction of mitotic recombination also failed to produce any topoisomerase I-deficient cell detectable by antibody staining. Genetic studies of sex chromosome nondisjunction presented in Tables 1, 2 and 3 provided further evidence of the necessity of topoisomerase I in *Drosophila*. In all of the three studies, the

average number of progeny obtained from the heat-shocked males or females is fewer than that of the control flies. This result would indicate that heat-shock-induced topoisomerase I homozygous mutation has caused death in spermatocytes, eggs or even some of the stem cells. The frequencies of nondisjunction observed in progeny of both types of males and the female are very low and resembled those of wild type. In wild type flies, sex chromosome nondisjunction frequency in males is between 0.01-0.1% and X-X nondisjunction rate in females is 0.05-0.1% (Ashburner, 1989). It is suggested that the progeny obtained from the crosses arose from normal gametes, which possess wild type topoisomerase I activity, developed after the heat shock induction. Both cytological and genetic studies presented here suggest that the first assumption offered in the Results section is valid.

DNA topoisomerase I has been demonstrated to be essential for development of a multicellular organism, *Drosophila* (Lee *et al.*, 1992). In contrast, DNA topoisomerase I is not essential for eubacteria and yeast (Sternglanz *et al.*, 1981; Thrash *et al.*, 1984, 1985). An important biological function suggested for the enzyme is to produce a swivel at the fork of replication (Wang, 1971; Champoux and Dulbecco, 1972). Genetic analysis of yeast has implicated a role of DNA topoisomerase I in the process of DNA synthesis, but this function may be replaced by topoisomerase II. The absence of DNA topoisomerase I leads to a temporary delay in the extension of short DNA chains and elongation of these short chains requires one of the two types of topoisomerases (Kim and Wang, 1981). Topoisomerase I is an inhibitor of recombination as demonstrated by genetic studies of yeast. rDNA recombination is suppressed by concerted action of DNA topoisomerase I and topoisomerase II (Christman *et al.*, 1988) and

recombination between terminal repeats of Ty retrotransposon is suppressed by topoisomerase III (Wallis *et al.*, 1989).

The presence of a topoisomerase I cleavage site in the 240 bp intergenic spacer repeat within the rDNA gene is of interest as the rDNA intergenic spacer repeats have been shown to function as X-Y pairing sites in male *Drosophila* (McKee *et al.*, 1992). McKee *et al.* (1992) proposed a model for achiasmatic pairing involving DNA topoisomerase I. Although this study has not met its original goal to analyze meiotic pairing in spermatocytes, further studies to determine the role of topoisomerase I in X-Y pairing may include using topoisomerase I mutations that are temperature sensitive or affect the enzyme activity instead of the lethal mutation used in this study. Biochemical studies to isolate topoisomerase I/DNA or topoisomerase I/strand transferase intermediates in the pairing process may prove to be useful in the understanding of the process.

REFERENCES

REFERENCES

- Ashburner, M. (1989) *Drosophila A Laboratory Handbook*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Ashburner, M. (1989a) *Drosophila A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Bonven, B. J., Gocke, E., and Westergaard, O. (1985) A high affinity topoisomerase I binding sequence is clustered at DNase I hypersensitive sites in *Tetrahymena* R-chromatin. *Cell* 41: 541-551.
- Carpenter, A. T. C. (1975) Electron microscopy of meiosis in *Drosophila melanogaster* females. I. Structure, arrangement, and temporal change of the synaptonemal complex in wild-type. *Chromosoma* 51: 157-182.
- Champoux, J. J. (1978) Mechanism of the reaction catalyzed by the DNA untwisting enzyme: Attachment of the enzyme to 3' -terminus of the nicked DNA. *J. Mol. Biol.* 118: 441-446.
- Champoux, J. J. (1990) Mechanistic aspects of type-I topoisomerases. In: *DNA Topology and Its Biological Effects*, eds. Cozzarelli, N. R., and Wang, J. C. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) pp. 217-242.
- Champoux, J. J., and Dulbecco, R. (1972) An activity from mammalian cells that untwists superhelical DNA-a possible swivel for DNA replication. *Proc. Natl. Acad. Sci. USA* 69: 143-146.
- Chou, T.-B., and Perrimon, N. (1992) Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. *Genetics* 131: 643-653.
- Christiansen, K., Bonven, B. J., and Westergaard, O. (1987) Mapping of sequence-specific chromatin proteins by a novel method: topoisomerase I on *Tetrahymena* ribosomal chromatin. *J. Mol. Biol.* 193: 517-525.
- Christman, M. F., Dietrich, F. S., and Fink, G. R. (1988) Mitotic recombination in the rDNA of *S. cerevisiae* is suppressed by the combined action of DNA topoisomerases I and II. *Cell* 55: 413-425.
- Cooper, K. W. (1950) Normal spermatogenesis in *Drosophila*. In: *The Biology of Drosophila*, ed. Demerec, M. (Wiley & Sons, New York) pp. 1-61.

- Cooper, K. W. (1964) Meiotic conjunctive elements not involving chiasmata. *Proc. Natl. Acad. Sci. USA* 52: 1248-1255.
- Cox, M. M. (1988) FLP site-specific recombination system of *Saccharomyces cerevisiae*. In: *Genetic Recombination*, eds. Kucherlapati, R., and Smith, G. R. (American Society for Microbiology, Washington, D. C.) pp. 429-443.
- Cunningham, R. P., DasGupta, C., Shibata, T., and Radding, C. M. (1981) Homologous pairing and topological linkage of DNA molecules by combined action of *E. coli* recA protein and topoisomerase I. *Cell* 24: 213-233.
- Dang, D. T. and Perrimon, N. (1992) Use of a yeast site-specific recombinase to generate embryonic mosaics in *Drosophila*. *Dev. Genetics* 13: 367-375.
- Dean, F., Krasnow, M. A., Otter, R., Matzuk, M. M., Spengler, S. J., and Cozzarelli, N. R. (1983) *Escherichia coli* type-I topoisomerases: Identification, mechanism, and role in recombination. *Cold Spring Harbor Symp. Quant. Biol.* 47: 769-784.
- Depew, R. E., Liu, L. F., and Wang, J. C. (1978) Interaction between DNA and *Escherichia coli* protein ω . Formation of a complex between single-stranded DNA and ω protein. *J. Biol. Chem.* 253: 511-518.
- DiGate, R. J., and Marians, K. J. (1988) Identification of a potent decatenating enzyme from *Escherichia coli*. *J. Biol. Chem.* 263: 13366-13373.
- DiGate, R. J., and Marians, K. J. (1989) Molecular cloning and DNA sequence analysis of *Escherichia coli* *topB*, the gene encoding topoisomerase III. *J. Biol. Chem.* 264: 17924-17930.
- DiNardo, S., Voelkel, K. A., Sternglanz, R., Reynolds, A. E., and Wright, A. (1982) *Escherichia coli* DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. *Cell* 31: 43-51.
- Fleischmann, G., Pflugfelder, G., Steiner, E. K., Javaherian, K., Howard, G. C., Wang, J. C., and Elgin, S. C. (1984) *Drosophila* topoisomerase I is associated with transcriptionally active regions of the genome. *Proc. Natl. Acad. Sci. USA* 81: 6958-6962.
- Golic, K. (1991) Site-specific recombination between homologous chromosomes in *Drosophila*. *Science* 252: 958-961.

- Golic, K., and Lindquist, S. (1989) The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell* 59: 499-509.
- Goto, T., and Wang, J. C. (1984) Yeast DNA topoisomerase II is encoded by a single-copy, essential gene. *Cell* 36: 1073-1080.
- Hackstein, J. H. P. (1987) Spermatogenesis in *Drosophila*. In: *Spermatogenesis: Genetic Aspects*, ed. Hennig, W. (Springer-Verlag, Berlin) pp. 63-116.
- Hawley, R. S. (1988) Exchange and chromosomal segregation in eucaryotes. In: *Genetic Recombination*, eds. Kucherlapati, R., and Smith, G. R. (American Society for Microbiology, Washington, D. C.) pp. 497-527.
- Hall, J. C., Gelbart, W. M., and Kankel, D. R. (1976) Mosaic Systems. In: *The Genetics and Biology of Drosophila*, eds. Ashburner, M., and Novitski, E. (Academic Press, New York) vol. 1a, pp. 256-314.
- Hsieh, T., Brown, S. D., Huang, P., and Fostel, J. (1992) Isolation and characterization of a gene encoding DNA topoisomerase I in *Drosophila melanogaster*. *Nucleic Acids Res.* 20: 6177-6182.
- Javaherian, K., Tse, Y.-C., and Vega, J. (1982) *Drosophila* topoisomerase I: Isolation, purification and characterization. *Nucleic Acids Res.* 10: 6945-6955.
- Jones, B. (1987) Chiasmata. In: *Meiosis*, ed. Moens, P. B. (Academic Press, New York) pp. 213-238.
- Kim, R. A., and Wang, J. C. (1989) Function of DNA topoisomerases as replication swivels in *Saccharomyces cerevisiae*. *J. Mol. Biol.* 208: 257-267.
- Kim, R. A., and Wang, J. C. (1992) Identification of the yeast *TOP3* gene product as a single strand-specific DNA topoisomerase. *J. Biol. Chem.* 267: 17178-17185.
- Kmiec, E. B., Kroeger, P. E., Brougham, M. J., and Holloman, W. K. (1983) Topological linkage of circular DNA molecules promoted by *Ustilago* rec1 protein and topoisomerase. *Cell* 34: 919-929.
- Kretzschmar, M., Meisterernst, M., and Roeder, R. G. (1993) Identification of human DNA topoisomerase I as a cofactor for activator-dependent transcription by RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 90: 11508-11512.

- Lawrence, P. A., Johnston, P., and Morata, G. (1986) Methods of marking cells. In *Drosophila: A practical Approach*, ed. Roberts, D. B. (IRL Press, Oxford) pp. 229-242.
- Lee, M. P., Brown, S. D., Chen, A., and Hsieh, T. (1993) DNA topoisomerase I is essential in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 90: 6656-6660.
- Lindsley, D. L., and Tokuyasu, K. T. (1980) Spermatogenesis. In: *The genetics and Biology of Drosophila*, eds Ashburner, M., and Wright, T. R. F. (Academic Press, New York) vol. 2d pp. 225-294.
- Lindsley, D. L., and Zimm, G. G. (1992) *The Genome of Drosophila melanogaster*. Academic Press, San Diego.
- Liu, L. F. (1983) DNA topoisomerases-enzymes that catalyze the breaking and rejoining of DNA. *CRC Crit. Rev. Biochem.* 15: 1-24.
- McKee, B. D., Habera, L., and Vrana, J. A. (1992) Evidence that intergenic spacer repeats of *Drosophila melanogaster* rRNA genes functions as X-Y pairing sites in male meiosis, and a general model for achiasmatic pairing. *Genetics* 132: 529-544.
- McKee, B. D. and Karpen, G. H. (1990) *Drosophila* ribosomal RNA genes function as an X-Y pairing site during male meiosis. *Cell* 61: 61-72.
- McKee, B. D. and Lindsley, D. L. (1987) Inseparability of X-heterochromatic functions responsible for X:Y pairing, meiotic drive, and male fertility in *Drosophila melanogaster* males. *Genetics* 116: 399-407.
- Merino, A., Madden, K. R., Lane, W. S., Champoux, J. J., and Reinberg, D. (1993) DNA topoisomerase I is involved in both repression and activation of transcription. *Nature (London)* 365: 227-232.
- Meyer, G. F. (1960) The fine structure of spermatocyte nuclei of *Drosophila melanogaster*. In: *Proceedings of the European Regional Conference on Electron Microscopy*, eds. Houwink, A. L., and Spit, B. J. (Die Nederlandse Vereniging voor Electronmicroscopie Delft, Delft, The Netherlands) pp. 951-954.
- Morgan, T. H. (1912) Complete linkage in the second chromosome of the male *Drosophila melanogaster*. *Science* 36: 719-720.
- Postlethwait, J. H. (1976) Clonal analysis of *Drosophila* cuticular patterns. In: *The Genetics and Biology of Drosophila*, eds. Ashburner, M., and Wright T. R. F. (Academic Press, New York) vol. 2c pp. 359-441.

- Prell, B., and Vosberg, H.-P. (1980) Analysis of covalent complexes formed between calf thymus DNA topoisomerase and single-stranded DNA. *Eur. J. Biochem.* 108: 389-398.
- Pruss, G. J., Manes, S. H., and Drlica, K. (1982) *Escherichia coli* DNA topoisomerase I mutants: Increased supercoiling is corrected by mutations near gyrase genes. *Cell* 31: 35-42.
- Pulleyblank, D. E., Shure, M., Tang, D., Vinograd, J., and Vosberg, H.-P. (1975) Action of nicking-closing enzyme on supercoiled and nonsupercoiled closed circular DNA: Formation of a Boltzmann distribution of topological isomers. *Proc. Natl. Acad. Sci. USA* 72: 4280-4284.
- Ritossa, F. (1976) The *bobbed* locus. In: *The Genetics and Biology of Drosophila*, eds. Ashburner, M., and Wright T. R. F. (Academic Press, New York) vol. 1b pp. 801-846.
- Sternglanz, R., DiNardo, S., Voelkel, K. A., Nishimura, Y., Hirota, Y., Becherer, K., Zumstein, L., and Wang, J. C. (1981) Mutations in the gene coding for *Escherichia coli* DNA topoisomerase I affect transcription and transposition. *Proc. Natl. Acad. Sci. USA* 78: 2747-2751.
- Thrash, C., Voelkel, K., DiNardo, S., and Sternglanz, R. (1984) Identification of *Saccharomyces cerevisiae* mutants deficient in DNA topoisomerase I activity. *J. Biol. Chem.* 259: 1375-1377.
- Thrash, C., Bankier, A. T., Barrell, B. G., and Sternglanz, R. (1985) Cloning, characterization, and sequence of the yeast DNA topoisomerase I gene. *Proc. Natl. Acad. Sci. USA* 82: 4374-4378.
- Von Wettstein, D., Rasmussen, S. W., and Holm, P. B. (1984) The synaptonemal complex in genetic segregation. *Ann. Rev. Genet.* 18: 331-414.
- Wallis, J. W., Chrebet, G., Brodsky, G., Rolfe, M., and Rothstein, R. (1989) A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. *Cell* 58: 409-419.
- Wang, J. C. (1969) Interaction between DNA and an *Escherichia coli* protein β . *J. Mol. Biol.* 43: 263-272.
- Wang, J. C. (1971) Interaction between DNA and an *Escherichia coli* protein ω . *J. Mol. Biol.* 55: 523-533.
- Wang, J. C. (1985) DNA topoisomerases. *Ann. Rev. Biochem.* 54: 665-697.

- Xu, T., and Harrison, S. D. (1994) Mosaic analysis using FLP recombinase. In: *Methods in Cell Biology*, vol. 44. (Academic Press, New York) pp. 655-681.
- Xu, T., and Rubin, G. M. (1993) Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* 117: 1223-1237.
- Yanagida, M. and Sternglanz, R. (1990) Genetics of DNA topoisomerases. In: *DNA Topology and Its Biological Effects*, eds. Cozzarelli, N. R., and Wang, J. C. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) pp. 299-320.

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