**Drosophila melanogaster** Topoisomerase IIIα Preferentially Relaxes a Positively or Negatively Supercoiled Bubble Substrate and Is Essential during Development*§

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Eukaryotic type IA topoisomerases are important for the normal function of the cell, and in some cases essential for the organism, although their role in DNA metabolism remains to be elucidated. In this study, we cloned *Drosophila melanogaster* topoisomerase (topo) IIIα from an embryonic cDNA library and expressed and purified the protein to >95% homogeneity. This enzyme partially relaxes a hypernegatively supercoiled plasmid substrate consistent with other purified topo IIIAs. A novel, covalently closed bubble substrate was prepared for this study, which topo IIIAs fully relaxed, regardless of the handedness of the supercoils. Experiments with the bubble substrate demonstrate that topo IIIAs has much different reaction preferences from those obtained by plasmid substrate-based assays. This is presumably due to the fact that solution conditions can affect the structure of plasmid based substrates and therefore their suitability as a substrate. A mutant allele of the *Top3α* gene, *Top3α*<sup>941</sup>, was isolated through imprecise excision mutagenesis of an existing P-element inserted in the first intron of the gene. *Top3α*<sup>941</sup> is recessive lethal, with most of the homozygous individuals surviving to pupation but never emerging to adulthood. Whereas this mutation can be rescued by a *Top3α* transgene, ubiquitous overexpression of *D. melanogaster* topo IIIβ cannot rescue this allele.

DNA topoisomerases are ubiquitous enzymes found in all cells and some viruses that regulate the topology of DNA in such cellular processes as replication, transcription, and recombination (1, 2). These enzymes work by making a transient covalent bond to the phosphodiester backbone of the DNA, creating a protein mediated DNA gate, which allows another strand (or strands) of DNA to pass through this reversible break. Type II topoisomerases create double-stranded breaks, and double strand passage, whereas type I topoisomerases break a single strand of DNA. The type I topoisomerases are further divided into the IA and IB subfamilies, based on structural and mechanistic differences (3, 4). Topoisomerase III (topo III)<sup>1</sup> is a member of the type IA subfamily that is conserved from bacteria to humans.

Topo III was originally purified by following superhelical relaxation activity from extracts of *Escherichia coli* containing a deletion for topo I (5). This enzyme was also independently purified based on its decatenating abilities in an assay utilizing plasmid DNA replication intermediates (6). Despite the strong sequence homology that this protein shares with *E. coli* topo I (7), purified topo III shows a weak ability to relax negatively supercoiled DNA (5). The relaxation activity of Topo III is strongly inhibited by single-stranded DNA (ssDNA) (5), and the enzyme was subsequently shown to preferentially bind ssDNA. The decatenase activity of topo III also depends on the presence of single-stranded regions in the catenated DNA (6). Recently, Nurse et al. showed that topo III serves as a decatenase in vivo by removing precatenanes that arise during replication of circular DNAs (8). These results, combined with others, have lead to the thought that topo III is responsible for decatenating replication and possibly recombination intermediates.

In *Saccharomyces cerevisiae*, TOP3 was discovered in a screen for mutants that enhanced the deletion of a marker flanked by repeated DNA sequences (9). Subsequently, it was shown that TOP3 deletions result in hyperrecombination, impaired sporulation, and a slow growth phenotype (9). Some of these phenotypes can be suppressed by mutations in SGS1, a member of the RecQ helicase family (10). This interaction between topo III and RecQ helicase proteins has since been shown to be a physical interaction that is conserved through evolution into higher eukaryotes (11–16).

In larger eukaryotes, there are two isoforms of topo III, α and β (17–21), differing primarily in their long carboxyl-terminal sequences. These two isoforms show very high homology with each other, as well as bacterial topo I, topo III, and yeast topo III, through the first 600 amino acids of the sequence, hereafter referred to as the type IA core region. This region contains the four type IA topoisomerase domains along with the active site tyrosine (4). Although both the α and the β carboxyl terminal regions contain several potential zinc finger motifs, there is very little homology between the two. The carboxyl terminus of *E. coli* topo I shows some similarity to the α isozymes and has been shown to possess five zinc ribbon domains with the first three binding zinc (22–24). This region in *E. coli* topo I binds ssDNA (25, 26) as well as interacting with RNA polymerase II (27), suggesting that these regions in the α and β isozymes may be multifunctional as well.

* The abbreviations used are: topo, topoisomerase; GST, glutathione S-transferase; ssDNA, single-stranded DNA; HNSC, hypernegatively supercoiled; PSC, positively supercoiled; GFP, green fluorescent protein.

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Topo IIIα has been shown to be essential for embryogenesis in the mouse (28), whereas topo IIIβ deletions survive to adulthood, although they show a predisposition to tumors, a shortened life span, and reduced litter size (29, 30). Biochemically, the α and β isozymes purified thus far have shown relaxation activity consistent with the E. coli and yeast enzymes (17, 18, 21, 31, 32). Relaxation of negatively or hypernegatively supercoiled substrates normally occurs in conditions that would favor destabilization of the DNA helix, such as elevated temperatures, high glycerol concentrations, and low monovalent and divalent salt concentrations (17, 18, 21, 31, 32).

SGS1 also has homologs in mice and humans: RECQ4, RECQ5, WRN, and BLM (33–36). Mutations in these syndromes all display genomic instability with a predisposition to cancer, whereas Werner’s and Bloom’s syndromes, respectively (37–39). These syndromes are connected in that homologous recombination defects are found in the D. melanogaster Topo IIIα, a recessive lethal allele.

**EXPERIMENTAL PROCEDURES**

Cloning the D. melanogaster Topoisomerase IIIα Gene—The DmTop3α gene was discovered in the D. melanogaster genomic DNA database by homology searches. Oligonucleotides were designed to amplify a highly conserved region of the type IA core region of DmTop3α. This PCR product was used as a probe to screen a D. melanogaster embryonic cDNA library, which was prepared in our laboratory. A clone with a 3.9-kb insert that corresponded to the DmTop3α coding region was PCR-amplified from the above clone with a 5′ primer that included a Met-His6 coding sequence before the start codon. The product was digested and ligated into YEpG, placing it under the control of a galactose-inducible promoter, to make YEpGTop3α. Once the sequence was confirmed, YEpG and YEpGTop3α were transformed into JCW253, a Δtop3 derivative of FY251, using standard protocols. Transformants and the parental strains were grown in selective media overnight, and culture densities were adjusted to 1 × 10^7 cells/ml. Tenfold serial dilutions were made, and 1 μl of each was spotted onto nonselective plates containing either dextrose or galactose as the exclusive sugar source. The plates were incubated at 30 °C until the colonies were readily detectable (2–3 days). Purification of Topo IIIα from yeast transformed with YEpGTop3α was explored, but expression levels were too low to make it feasible for biochemical studies.

Preparation of an Anti-topo IIIα Antibody—Peptides TopoIII-1 (GI-602251) and TopoIII-2 (YEUVVDCRISVPP[N]-VYRAT) were synthesized using the multiple antigen peptide method (42) and injected into a rabbit using standard protocols. The peptides were covalently linked to Affi-Gel 15 (Bio-Rad) amine reactive cross-linking resin using the anhydride method provided with the resin. This column was used to affinity-purify the polyclonal antibody anti-TopoIII-1 from the rabbit’s serum using standard methods.

D. melanogaster Topo IIIα Expression and Purification—Topo IIIα was overexpressed using the Bac-to-Bac baculovirus expression system (Invitrogen) in Sf9 cells and purified using a cleavable N-terminal GST tag and a C-terminal decahistidine tag. The GST tag was PCR-amplified from pGEX-6P-1 (Amersham Biosciences) using oligonucleotides that amplified from the start codon of GST to just after the recognition sequence for PreScission Protease (Amersham Biosciences). DmTop3α was PCR-amplified from its start codon to its C terminus, excluding the stop codon. The decahistidine tag followed by a stop codon was synthesized as two complementary oligonucleotides, and the three pieces were sequentially cloned into pFastBac and the sequence was verified to create pBFG-T3α-H10. This plasmid was developed into a baculovirus (bgV-T3α-H10) using the Bac-to-Bac kit protocols. Supernatants of transfections or amplifications of the baculovirus were assayed by dot blot using an anti-6p (64 monoclonal antibody (eBioscience) to detect the recombinant baculovirus. Baculovirus titer was estimated by comparison of cleared supernatants with baculovirus of known titer.

Four liters of Sf9 cells were infected at a concentration of 1 × 10^6 cells/ml using an empirically determined amount of bgV-T3α-H10 (multiplicity of infection ~2). The cells were harvested at 65 h postinfection and spun down at 550 × g for 10 min. All of the following steps were performed on ice or at 4 °C, and all buffers were prechilled and contained 5 mM 2-mercaptoethanol, 1 μM 4-(2-aminoethyl)benzenesulfon- nyl fluoride, 80 mM aprotinin, 45 μM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, and 1.4 μM E-64. The cell pellet was resuspended in 200 ml of cytoplasmic lysis buffer (10 mM Tris, pH 8.0, 0.32 mM sucrose, 3 mM CaCl2, 2 mM MgCl2, 0.5% Nonidet P-40) and incubated on ice for 15 min. The nuclei were then pelleted at 1500 × g for 15 min, and the supernatant was decanted off of the nuclear pellet. The cell suspension was resuspended in 50 ml of nuclear resuspension buffer (20 mM Tris, pH 8.0, 1.5 mM MgCl2, 20 mM KCl, 25% glycerol). 50 ml of nuclear extraction buffer (80 mM Tris pH 7.6, 2 mM NaCl, 20% glycerol, 0.06% Triton X-100) was slowly added to the resuspended nuclei while stirring, and 50 ml of DNA precipitation buffer (18% polyethylene glycol 8000, 1 mM NaCl) was slowly added with stirring, and the solution was incubated for 30 min on ice with stirring. The insoluble material was pelleted at 20,000 × g for 20 min. The cleared supernatant was added to a prechilled bottle with 2 ml of glutathione resin (Amersham Biosciences). The GST-topo IIIα-H10 was allowed to batch bind at 4 °C for 1 h with gentle stirring.

The glutathione resin with bound GST-topo IIIα-H10 was gently pelleted and washed twice in batch with 20 ml of glutathione wash buffer (50 mM Tris pH 7.0, 1 mM NaCl, 10% glycerol) on ice. The insoluble material was pelleted at 20,000 × g for 20 min. The glutathione resin was incubated with 20 ml of glutathione wash buffer II (glutathione wash buffer I at 150 mM NaCl) and then resuspended in 2 ml of glutathione wash buffer II. 20 μl of PreScission Protease was added to the slurry, and the GST tag was digested off of the topo IIIα-H10 for 4 h at 4 °C. Following digestion, the slurry was loaded into a column, and the flow-through was collected. The column was washed with once with 2 ml of glutathione wash buffer II and then with 4 ml of GST elution dilution buffer (glutathione wash buffer II at pH 8.8). The final pH of the collected flow-through and washes is ~7.9, allowing it to be loaded directly onto equilibrated TALON Co2⁺ IMAC resin (Clontech). This column was washed with six volumes of IMAC wash buffer (glutathione wash buffer II at pH 7.9) and then eluted with IMAC elution buffer (IMAC wash buffer II with 100 mM imidazole). Peak fractions were pooled, and bovine serum albumin (New England Biolabs) was added to 0.25 mg/ml to help stabilize the Topo IIIα. A mock was prepared at this time using bovine serum albumin and IMAC elution buffer. The Topo IIIα and the mock were then dialyzed against storage buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 50% Glycerol, 0.5 mM dithiothreitol) and stored at -20 °C. Topo IIIα concentrations were determined using densitometry of stained SDS-polyacrylamide gels using β-galactosidase (EIA grade; Roche Applied Science) as a reference.

Immunoprecipitation of Endogenous Topo IIIα from S2 Cells—D. melanogaster S2 cells were grown in suspension using standard procedures. 3 × 10^6 S2 cells in midlog phase growth were pelleted at 550 × g for 10 min, and the media were discarded. The cell pellet was resuspended in 20 ml of ice-cold hypotonic lysis buffer (10 mM HEPES, pH 8.0, 10 mM KCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 5 mM 2-mercaptoethanol, and a protease inhibitor mixture consisting of 0.1 μM 4-(2-aminoethyl)-benzenesulfonfonyl fluoride, 80 mM aprotinin, 45 μM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, and 1.4 μM E-64) and incubated on ice for 15 min, and the insoluble cell debris was pelleted at 20,000 × g for 20 min. Topo IIIα was immunoprecipitated from 1 ml of this cleared supernatant with 10 μl of protein A-agarose (Roche Applied Science) and 7 μg of anti-TopoIII-1 using standard procedures.

Preparation of the Substrates—Hypernegatively supercoiled (HNSC) plasmid substrate was prepared as described previously (17).
RESULTS AND DISCUSSION

Cloning of D. melanogaster Topoisomerase IIIα—To isolate and study the Top3α gene from D. melanogaster, primers were designed from the genomic sequence to amplify a region from the highly conserved type IA core region of the gene, and this PCR product was used to screen a D. melanogaster embryonic cDNA library. A clone was isolated that contained a 3.9-kb insert. This cDNA was sequenced, and it encoded a short 5’ untranslated region, an open reading frame, and a short 3’-untranslated region with polyadenylation signal. The genomic structure of the gene is shown in Fig. 1A. The Top3α gene is located on the left arm of chromosome 2 at the cytological location 34E. The gene is very compact, containing three small introns, each of which is less than 60 bp in length, a short promoter region, and a short intergenic sequence to genes neighboring either its 5’-end or 3’-end. The largest open reading frame of the cDNA encodes a 1250-amino acid protein with high overall similarity to human and mouse topo IIIα.

Like the human and mouse homologs, D. melanogaster topo IIIα has a short, unconserved region at the N terminus of the protein, which contains a putative mitochondrial localization sequence (46). Analysis using the algorithm developed by Claros and Vincens (47) gives this sequence an 88% probability of mitochondrial import, with a signal cleavage site following the import sequence. Consistent with the human and mouse homologs, a second methionine follows this region, and an nuclear localization signal lies in the remaining C-terminal portion of the protein. It is unclear at this time if this second methionine is used as an alternative start codon to create two populations of topo IIIα, one localized to the mitochondria and the other to the nucleus, although human topo IIIα has been shown to be targeted to two different compartments in this manner (46).

The type IA topoisomerase core region follows the potential mitochondrial localization sequence. This region shows high overall similarity to other type IA topoisomerases and is homologous to domains I-IV of E. coli topo I and topo III (4, 48), including the active site tyrosine at amino acid 356 (Fig. 1B). After the type IA core region, the topo IIIα signature C terminus begins.

The C-terminal sequence of D. melanogaster topo IIIα is conserved among C-terminal regions of various metazoans, with the exception of a large (260-amino acid) region “inserted” about one-third of the way into the typical C terminus. A detailed sequence alignment is shown in Supplemental Fig. 1. The C-terminal domain is divided into four segments (A–D) based on sequence comparison (Fig. 1B). Segment A of the D. melanogaster C-terminal region contains the first one-third of the typical topo III C terminus, with two of the conserved putative zinc finger motifs, whereas segment D is similar to the latter two thirds, containing the remaining two putative zinc fingers and putative zinc knuckle. Segments B and C compose the inserted sequence that is unique to D. melanogaster topo IIIα, with segment B being a glycine-rich region and segment C showing little homology to any proteins of known function.

D. melanogaster Topoisomerase IIIα Can Partially Rescue the Slow Growth Phenotype of Yeast Δtop3 Mutant—Once the cDNA clone was obtained, we used a heterologous expression system to test the biological function of D. melanogaster Top3α. YeGpGTop3α was constructed from the YeGp vector, in which DmTop3α is placed under the control of a galactose-inducible promoter (GAL1). YeGpGTop3α was tested for its ability to rescue the slow growth phenotype of the S. cerevisiae strain JCW253, a Δtop3 derivative of FY251. Fig. 1C illustrates the slow growth phenotype of JCW253 when compared with FY251 grown on either dextrose or galactose. The dextrose plate shows...
that transforming JCW253 with YEpGTop3α or empty vector does not change its slow growth phenotype. When these yeast strains are grown on galactose to activate the expression of DmTop3α, JCW253 with YEpGTop3α shows an accelerated growth rate when compared with either JCW253 or JCW253 with empty vector. Although the D. melanogaster topo IIIα does not bring the growth rate back to the wild-type level (FY251), it is clear that topo IIIα from the fruit fly has a biological function in yeast. The highly conserved core domain carries the same basic and conserved function across the species, since partial rescue of the slow growth phenotype of Δtop3 yeast strain. Yeast strains were grown overnight in selective media and diluted to $1 \times 10^6$ cells/ml. 10-fold serial dilutions were made, and 1 μl of each was spotted onto nonselective plates containing either dextrose or galactose as the sole sugar source.

**Expression and Purification of Topoisomerase IIIα—**For further biochemical studies, topo IIIα was expressed and purified using a baculovirus expression system. The Top3α cDNA, with a cleavable glutathione S-transferase N-terminal tag and a decahistidine C-terminal tag, was placed under the control of the polyhedron promoter and developed into a baculovirus. This virus was used to infect early log phase S99 cells, which were harvested 65 h later. The GST-topo IIIα-H10 was extracted out of the nuclei of these cells and bound in batch to glutathione-Sepharose (Fig. 2A, lane 3). The GST tag was digested off of the GST-topo IIIα-H10 and the free topo IIIα-H10 was washed off of the resin (Fig. 2A, lane 4). This material was then loaded onto a Co²⁺-chelating column (IMAC), the column was washed, and the topo IIIα-H10 was eluted with imidazole (see Fig. 2A, lane 5). The fraction from the IMAC column has a single polypeptide of about 140 kDa, with >95% purity as judged by SDS-PAGE.

To determine whether the purified protein was of the correct size, we performed a Western blot with an anti-topo IIIα antibody (see “Experimental Procedures”) comparing the purified protein with whole cell extract from D. melanogaster S2 cells. However, the endogenous levels of topo IIIα in S2 cells are too low to be detected in this manner (data not shown). The endogenous topo IIIα was further concentrated by immunoprecipitation from S2 extracts with the anti-topo IIIα antibody. Fig. 2B shows that the purified protein does co-migrate with the major species from the immunoprecipitate. At this time, it is unknown whether the slower migrating species apparent in the immunoprecipitate is topo IIIα modified in some manner or if it is simply a cross-reacting protein.

**Topoisomerase IIIα Partially Relaxes a HNSC, but Not a Positively Supercoiled (PSC), Plasmid Substrate—**We assayed the purified topo IIIα in vitro for relaxation activity on a HNSC plasmid substrate. The reaction products were analyzed by gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide. The ethidium bromide intercalates into the DNA, causing it to relax from a superhelical density of about –0.07, which is slightly more negatively supercoiled than plasmid DNA isolated from bacteria. Incubating these reactions for a longer period of time will partially relax all of the substrate, but it does not noticeably change the superhelical density of the product (data not shown). These results are similar to those obtained with human topo IIIα, human topo IIIβ, and D. melanogaster topo IIIβ (17, 18, 31).
To test the activity of topo IIIα on a PSC plasmid substrate, relaxed plasmid was incubated with ethidium bromide to shift the substrate to a positively supercoiled state and then reacted with topo IIIα. After the reactions were stopped, the substrate was gel electrophoresed. We note that there is less single-stranded DNA remaining after the reaction for the PSC plasmid substrate (Fig. 3B, lane 2) as compared to the HNSC plasmid substrate (Fig. 3B, lane 1). This is presumably due to further annealing of the single-stranded DNA resulting in a negatively supercoiled bubble substrate.

Preparation of a Bubble Substrate—Whereas the HNSC plasmid substrate provides a convenient assay for topo IIIα activity, it is potentially only a topo III substrate under certain limited conditions. Topo III may function on this substrate due to the presence of unwound DNA regions induced by supercoiling. Solution conditions can help stabilize or destabilize a DNA helix, however, so the optimized reaction conditions determined for a topo III protein with this substrate are likely to be the best compromise between the preference of the enzyme and the effect of the reaction conditions on the DNA structure.

The solution to this problem would be to use a covalently closed DNA circle with single-stranded regions regardless of the solution conditions. A substrate had previously been made that was a covalently closed circle with an extraneous, single-stranded bubble (50). Whereas this substrate satisfied our requirement, it was difficult to make in large quantities, limiting the number of assays that could be performed and necessitating detection with autoradiography. To address this issue, we endeavored to make a new substrate that would be a covalently closed circular DNA with a single-stranded “bubble” region in large enough quantities to make routine analysis feasible.

We created this substrate by annealing and linking purified single-stranded circles that were created by a helper phage from pBlueScript SK+ and pBlueScript SK- (Fig. 3A). Because these two plasmids are exactly the same except for the orientation of the f1 origin, the resulting single-stranded circles are complementary except for the f1 origin. In this region, both of the circles have the exact same sequence rather than complementary ones, so once the two circles are annealed and linked together by a topoisomerase, the f1 region will remain a single-stranded bubble. Hyperthermophilic reverse gyrase with ADP was used to anneal and link the two purified single-stranded circles in one step. When the single-stranded circles (Fig. 3B, lane 1) are incubated at 80°C in a mock reaction, the two complementary DNA sequences anneal (Fig. 3B, lane 2). Reaction of the annealed circles with reverse gyrase results in relaxed, covalently closed bubble substrate (Fig. 3B, lane 3), which is then negatively supercoiled with the same technique used to create the HNSC plasmid substrate. It is interesting to note that there is less single-stranded DNA remaining after reaction with reverse gyrase than in the mock reaction (Fig. 3B, compare lanes 2 and 3). This is presumably due to further annealing and linking of the single-stranded DNA promoted by reverse gyrase.

Topoisomerase IIIα Fully Relaxes the Bubble Substrate, Regardless of the Handedness of the Supercoils—After reacting the NSC bubble substrate with topo IIIα, almost all of the substrate ran at the position of the nicked or covalently closed open circle (Fig. 4A, lane 3). Some topoisomerasers are present in this lane just below the nicked/open circle position, suggesting the presence of fully relaxed DNA. To confirm this, another aliquot of the same reaction was analyzed by electrophoresis in the presence of ethidium bromide. Covalently closed substrate will become positively supercoiled with the intercalation of the ethidium bromide and migrate much faster than the nicked circle. In this electrophoretic condition, it is apparent that there is no detectable increase in the amount of nicked material.
when the NSC bubble substrate is reacted with topo IIIα, whereas the intensity of the relaxed product band is comparable with the NSC starting material (Fig. 4B). This result confirms that a negatively supercoiled substrate with a single-stranded region can be fully relaxed by topo IIIα.

Topo IIIα was also tested for its ability to relax positively supercoiled bubble substrate. Relaxed bubble substrate was reacted in the presence of ethidium bromide to generate positively supercoiled bubble substrate. The PSC bubble substrate was relaxed by topo IIIα, generating negative supercoils upon the removal of the ethidium bromide (Fig. 4A, lane 6). This result was repeated, with the same outcome, using PSC bubble substrate created with reverse gyrase (data not shown). These results show that the handedness of the supercoils in the substrate is inconsequential to topo IIIα activity; only the presence of single-stranded DNA matters.

Topoisomerase IIIα Relaxes HNSC Plasmid and NSC Bubble Substrates with Similar Temperature Preferences—We tested the temperature preferences for the relaxation of the two substrates, since temperature plays a role in helix stability. While testing this variable, as well as the ones that follow, we shortened the reaction times in order to stop the reaction before it reached completion, allowing us to detect smaller differences in activity. Consistent with many other topo IIIIs, topo IIIα has a temperature preference for the partial relaxation of HNSC plasmid substrate elevated above the physiological temperature of the source organism. Fig. 5A shows that the preferred temperature for this reaction is 45 °C. With the NSC bubble substrate at various temperatures. Reactions were incubated at the indicated temperature for 6 min before stopping. The products of the reactions were analyzed by electrophoresis in the presence of 0.5 μg/ml ethidium bromide. B, relaxation of NSC bubble substrate at various temperatures. Reactions were incubated at the indicated temperature for 6 min before stopping. The products of the reactions were analyzed by electrophoresis without ethidium.

FIG. 3. Formation of a novel, covalently closed bubble substrate. A, an illustration of bubble substrate formation. Purified single-stranded circles created from pBlueScript SK+ and pBlueScript SK− using the helper phage M13K07 are annealed and linked at 50 °C in the presence of reverse gyrase. The substrate is then made NSC with standard methods. B, the various intermediates of the formation of the bubble substrate. Lane 1, the purified ssDNA+ and ssDNA− circles; lane 2, the annealed ssDNA circles formed during a reverse gyrase mock reaction; lane 3, the linked ssDNA circles formed by reverse gyrase. The positions of the various topological states of the DNA are shown to the left: nicked circle/open circle (NC/OC), linked and relaxed (L & R), annealed (Ann), single-stranded circles (ssCircles).

FIG. 4. Topo IIIα can relax both negatively and positively supercoiled forms of a novel bubble substrate. The positions of the various topological states of the plasmid are indicated to the left of the gels: relaxed Circle (Rel), nicked circle (NC), open circle (OC), negatively supercoiled (NSC). A, topo IIIα can relax NSC and PSC bubble substrate. NCSC bubble substrate (lane 1) was treated with a mock sample (lane 2) or topo IIIα (lane 3). PSC bubble substrate was made by the preincubation of ethidium bromide with relaxed bubble substrate (lane 4), followed by the addition of mock (lane 5) or topo IIIα (lane 6). After the reaction, a phenol extraction removed the ethidium from the DNA, restoring it to a relaxed state if the linking number was unchanged by the reaction. The products of the reactions were analyzed by gel electrophoresis without ethidium. B, the product of the topo IIIα reaction is fully relaxed bubble substrate. An aliquot of the reactions shown in A, lanes 1–3, was analyzed by gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide to separate nicked substrate from covalently closed open circle (fully relaxed substrate).
substrate, the relaxation activity is maximal between 40 and 45 °C (Fig. 5B). This result demonstrates that the temperature preferences determined for the relaxation of plasmid substrates by topo IIIα are not significantly influenced by temperature-induced destabilization of the DNA helix.

Fig. 5 also illustrates the difference in the reaction rates between the HNSC plasmid substrate and the NSC bubble substrate. Densitometry analysis of the 45 °C reaction products in Fig. 5A shows that about 30% of the HNSC plasmid substrate has been relaxed in 6 min. In the same amount of time, all of the NSC bubble substrate has been relaxed at the same incubation temperature (Fig. 5B). This demonstrates that DNA with a denatured, single-stranded region is a preferred substrate for the relaxation activity of D. melanogaster topo IIIα.

NSC Bubble Substrate Relaxation Has a Higher Mg2⁺ and Monovalent Salt Optimum than HNSC Plasmid Substrate Relaxation—We also examined the optimal buffer conditions for topo IIIα relaxation of the NSC bubble substrate and HNSC plasmid substrate. Fig. 6A shows that the relaxation of the HNSC plasmid substrate requires Mg2⁺ but is exquisitely sensitive to its concentration in the reaction. The optimum Mg2⁺ concentration is 0.125 mM, and there is very little relaxation of the HNSC plasmid substrate at Mg2⁺ concentrations higher than 0.5 mM. Notice that the reaction buffer contains 0.1 mM EDTA, which attenuates the free Mg2⁺ concentration in the reaction. In the reactions using the NSC bubble substrate, for which the assay depends on the preference of the enzyme rather than the effect of the buffer on DNA structure, optimum activity of topo IIIα begins at 0.25 mM Mg2⁺ (Fig. 6B). Unlike the HNSC plasmid substrate reactions, the NSC bubble substrate reaction is insensitive to Mg2⁺ concentrations from 0.25 mM to at least 8 mM. This shows that topo IIIα itself is not inhibited by high concentrations of Mg2⁺ but that Mg2⁺ is probably stabilizing the HNSC plasmid substrate and eliminating the denatured regions upon which topo IIIα can act.

We next investigated the monovalent salt concentration preferences for the two reactions. Topo IIIα was assayed in the presence of several different salts, and the enzyme has a preference for sodium acetate over sodium chloride, potassium acetate, and potassium chloride (data not shown). The optimum amount of sodium acetate for the relaxation of HNSC plasmid substrate is 50 mM, as judged by the disappearance of the substrate, and the reaction was completely inhibited by 200 mM sodium acetate (Fig. 6C). Also interesting to note, the more salt that is present in the reaction, the more negatively supercoiled the final product is. This is probably due to the Na⁺ stabilizing the underwound DNA, eliminating the single-stranded regions upon which topo IIIα can work. Once the effects of counterions on DNA structure are removed by using the NSC bubble substrate, the monovalent salt optimum of topo IIIα is 250–300 mM sodium acetate (Fig. 6D).

These results demonstrate that the ionic strength of the reaction can have a dramatic effect on the preference of topo IIIα for HNSC DNA as a substrate. The reactivity of some of the relevant topo IIIα substrates can be affected by ionic
strength. Replication and recombination intermediates that topo IIIα may act upon contain single/double-stranded junctions and could have structures very sensitive to solution ionic strength. For example, synthetic Holliday junctions have also shown that the solution environment can change the shape of this structure (51).

**Generation of a Recessive Lethal Top3a Allele**—We acquired a P-element insertion mutant from the Bloomington stock center, Top3a^{EP2272}, which has an insertion just 7 base pairs after the 5’ splice site of the first intron (see Fig. 7A). This mutation is homozygous viable, with no detectable change in the levels of topo IIIα in the adult fly or embryo (data not shown). In order to create a more severe allele of Top3a, we mobilized the existing P-element with a genomic transposase source, and the part of the P-element that was excised contained the insertion site, albeit with a large internal deletion. The part that was sequenced (Fig. 7A), allowing it to pass the first set of criteria of our screen. The 5’ splice site of the first intron is still intact, although it is possible that the new context of the splice site, as a result of the deletion, might affect the expression or splicing of this mRNA in ways that are different from the parental line Top3a^{EP2272}.

**Top3a^{191} Dies as a Pupa**—The Top3a^{191} allele is maintained as a stock with CyO, a dominant balancer chromosome that contains a wild-type copy of Top3a. In order to determine when the homozygous Top3a^{191}/Top3a^{191} flies die, the homozygous mutant flies had to be separated from their Top3a^{191}/CyO siblings (filled squares, solid line) pupate almost 2 days later than their Top3a^{191}/Top3a^{+} siblings (filled squares, solid line). Whereas the Top3a^{+}/Top3a^{+} pupae (open squares, dashed line), the homozygous mutants never do, dying as pupae.

FIG. 7. Top3a^{191} is a recessive lethal mutation that dies during pupation. A, the generation of Top3a^{191} from Top3a^{EP2272}. Top3a^{EP2272} has a P-element (indicated in gray) inserted within the first intron of Top3a. An imprecise excision screen yielded Top3a^{191}, in which the denoted portion of the P-element was deleted with the w^{mc} marker (indicated in dark gray), but no other changes were detected. B, Top3a^{191} is delayed to pupate and never ecloses. Top3a^{191}/Top3a^{+} (filled circles, solid line) pupate almost 2 days later than their Top3a^{+}/Top3a^{+} siblings (filled squares, solid line). Whereas the Top3a^{+}/Top3a^{+} pupae (open squares, dashed line), the homozygous mutants never do, dying as pupae.
heterozygous mothers was insufficient for embryonic development for some of the homozygous mutant embryos. There is little cell division in the larval phase, although DNA endoreplication still occurs. We do not know whether the retarded development of the larvae is due to problems with DNA replication in these polytene cells or if the small amount of DNA replication and cell division of the imaginal disks creates these delays to pupation. Melanotic bodies are visible on some late third instar larvae, indicating that some tissues have already started dying before pupation begins. Once pupated, all homozygous individuals develop melanotic bodies and never complete this phase.

Top3\beta Cannot Rescue Top3\alpha\textsuperscript{191}—Whereas the generation of a recessive lethal Top3\alpha allele in a Top3\beta\textsuperscript{−/−} background shows that endogenous topo III\beta cannot fulfill an essential role of topo III\alpha, it is possible that this is simply due to the expression level or the expression pattern of the Top3\beta gene. To address this possibility, we used a heat shock-promoted Top3\beta transgene to attempt rescue of the Top3\alpha\textsuperscript{191} allele. This particular transgenic construct shows strong expression when checked with Western blot using an anti-topo III\beta antibody (data not shown). Table I shows that the Top3\beta transgene was unable to rescue the homozygous mutant flies, whereas two Top3\alpha transgenes rescued the Top3\alpha\textsuperscript{191} allele with 40–50% efficiency. The rescued flies were crossed inter se and were maintained as a stock for over 12 generations with no detectable phenotype.

The failure of topo III\beta to rescue the Top3\alpha mutant could be attributed to several factors. The most straightforward explanation is that these two proteins may have distinct functions. The C termini of the two isoforms contain a similar structural motif, multiple zinc fingers, but their differences may make the enzymes specific for recognition of different DNA structures or may specify a different set of protein-protein interactions.

It is also possible that the two proteins are functionally redundant, at least enough so for survival, but topo III\beta is simply not localized correctly to take the place of topo III\alpha in mutant cells. Topo III\alpha has a potential mitochondrial import sequence at the N terminus of the protein, but when topo III\beta is analyzed by the Claros and Vincens (47) algorithm, it is only given a 22% chance of mitochondrial import with no potential signal cleavage site. If there is a role for topo III\alpha in resolution of replicating mitochondrial chromosomes and this role is essential, then it would be unlikely that the ubiquitous overexpression of topo III\beta would rescue this phenotype.

Conclusions—Whereas there have been many studies on the genetics and biochemistry of topo III proteins, the important role or roles that this protein plays in DNA metabolism remain unclear. Here, we have found that D. melanogaster Top3\alpha is essential during development, as in the mouse. The mouse knockout is lethal at a much earlier stage in development (28), but this organism cannot maternally load the enzyme in the egg to the same degree that D. melanogaster can, perhaps prolonging the development of mutant fruit flies. Future studies utilizing defined knockouts of D. melanogaster Top3\alpha and disruption of the maternal loading of the enzyme may prove insightful for determining its importance in these early stages of development. With the genetically malleable D. melanogaster system, it would also be interesting to utilize domain deletions and chimeras of the α and β isozymes in null backgrounds to better define the different roles of these enzymes.

In this paper, we have also presented the expression of D. melanogaster topo III\alpha in a eukaryotic system and the purification of the enzyme to near homogeneity. Whereas this enzyme has activity and reaction condition preferences similar to other topo III\alpha studied on other topo III\alpha studied with DNA single-stranded regions, this substrate may appear artificial at first, but the action of other DNA-metabolizing enzymes, including the RecQ family of helicases, may present such DNA to topo III in vitro. It may be possible to create DNA substrates using the techniques presented here that would mimic DNA recombination and replication intermediates more closely than currently available substrates. With these biochemical and genetic tools, the biological function of topoisomerase III will be an active area for future investigation.

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TABLE I
Top3\alpha transgenes can rescue Top3\alpha\textsuperscript{−/−}, but a Top3\beta transgene cannot

| Transgenes         | Top3\alpha\textsuperscript{191} + (escapers) | Top3\alpha\textsuperscript{191} + (rescued) | Top3\alpha\textsuperscript{191} transgene | Top3\alpha\textsuperscript{191} transgene |
|---------------------|---------------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| P [HSP → cTop3\alpha]\textsuperscript{17} | 0                                           | 243                                      | 243                                      | 56                                        |
| P [HSP → cTop3\beta]\textsuperscript{13} | 0                                           | 221                                      | 224                                      | 44                                        |
| P [HSP → cTop3\beta]\textsuperscript{3}  | 0                                           | 222                                      | 217                                      | 0                                         |
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Drosophila melanogaster Topoisomerase IIIα Preferentially Relaxes a Positively or Negatively Supercoiled Bubble Substrate and Is Essential during Development

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