Cloning and Characterization of Drosophila Topoisomerase IIIβ

RELAXATION OF HYPERNEGATIVELY SUPERCOILED DNA*S

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We cloned cDNA encoding Drosophila DNA topoisomerase III. The top3 cDNA encodes an 875-amino acid protein, which is nearly 60% identical to mammalian topoisomerase IIIβ enzymes. Similarity between the Drosophila protein and the topoisomerase IIIβs is particularly striking in the carboxyl-terminal region, where all contain eight highly conserved CXXC motifs not found in other topoisomerase III enzymes. We therefore propose the Drosophila protein is a member of the β-subfamily of topoisomerase III enzymes. The topβ gene is a single-copy gene located at 5 E-F on the X chromosome. P-element insertion into the 5′-untranslated region of this gene affects topoisomerase IIIβ protein levels, but not the overall fertility and viability of the fly. We purified topoisomerase IIIβ to near homogeneity and observed relaxation activity only with a hypernegatively supercoiled substrate, but not with plasmid DNA directly isolated from bacterial cells. Despite this difference in substrate preference, the degree of relaxation of the hypernegatively supercoiled substrate is comparable to relaxation of plasmid DNA by other type I enzymes. Drosophila topoisomerase IIIβ forms a covalent linkage to 5′ DNA phosphoryl groups, and the DNA cleavage reaction prefers single-stranded substrate over double-stranded, suggesting an affinity of this enzyme for DNA with non-double-helical structure.

Topoisomerases play important roles in many biological processes, such as DNA replication, transcription, and chromosome condensation (see Ref. 1 for review). They act by cleaving either one (type I enzymes) or both (type II enzymes) strands of DNA in order to allow strand-passage events to occur before rejoining the broken DNA ends. Type I topoisomerases are divided into two families, IA and IB, based on structural and mechanistic differences (2, 3). The type IA family is composed of archeabacterial reverse gyrase, bacterial topoisomerase (topo)I and topo III, and the eucaryotic topo III enzymes. All form covalent 5′-phosphotyrosine linkages with cleaved DNA. This is in contrast with type IB enzymes, which more readily relaxes a plasmid substrate when it contains a 29-nucleotide single-stranded loop (8). Furthermore, experiments monitoring cellular DNA supercoiling in a yeast strain under conditions where neither topo I nor topo II was active demonstrated that topo III does not play a major role in regulating DNA supercoiling (9).

Apart from these biochemical observations, one key toward understanding the biological function(s) of the topo III enzymes has come through the study of mutants. Neither bacteria nor yeast topo III is essential, although mutation in yeast results in a pleiotropy of phenotypes: slow growth, hyper-recombination between repeated sequences, increased mitotic and meiotic chromosome nondisjunction, and failure to sporulate (10, 11). The slow growth defect is most likely due to an extended S/G₂ transition (12). In addition, the chromosome nondisjunction and sporulation defects may be the result of an essential role for yeast topo III in meiotic recombination. This is supported by the fact that deletion of the spo11 gene bypasses recombination and allows the topo3 mutants to sporulate (13).

An extragenic mutation, sgs1, was found to suppress the slow growth and hyper-recombination phenotypes of the topo3 yeast mutant (12). Sgs1 is a member of a family of 3′-5′ helicases, which includes bacterial RecQ and five homologs in humans, including the Blm and Wrn helicases (14–19). Studies with bacteria have shown that RecQ helicase can both initiate homologous recombination and disrupt illegitimate recombination intermediates (20, 21). Mutations in the BLM and WRN genes result in Bloom’s syndrome and Werner’s syndrome, respectively (22, 23). These disorders are characterized by genomic instability and elevated rates of cancer, as well as the early onset of aging-related phenotypes in Werner’s patients. Furthermore, these two genes, BLM and WRN, are capable of suppressing hyper-recombination in the yeast sgs1 mutant (24). Sgs1 protein physically interacts with topo III (12), and possibly topo II as well (25). Studies in yeast revealed that sgs1 mutant alone eliminates recombination levels and results in decreased lifespan by the early onset of aging-related phenotypes (26, 27). This premature aging is thought to be due to an accumulation of extrachromosomal rDNA circles (up to levels equivalent to the genomic DNA), which then results in nucle-
olar fragmentation (28). The rDNA circle accumulation may be the result of increased recombination events between the tandem repeats within the rDNA gene cluster.

Recent interest in this field is underscored by the discovery of two topoisomerase III isozymes in mammals, topo IIIα and topo IIIβ (29–32). Human topo IIIβ encodes three alternatively spliced transcripts, and the largest of these gene products can interact with yeast Sgs1 protein (33). In mice, topo IIIα is essential, for knockouts die in utero (34). Therefore, the study of mutants provides a valuable tool for understanding the biological function(s) of the topo III enzymes. Given the power of Drosophila genetics and cell biology, we set out to identify topoisomerase III in this organism and report our initial findings in this paper.

**EXPERIMENTAL PROCEDURES**

**Cloning the Drosophila Topoisomerase III Gene**—We synthesized several degenerate oligonucleotides based on the regions of high homology among type IA topoisomerase sequences and used them as primers for PCR amplification of *Drosophila* genomic DNA and cDNA. Two of these primers with the sequences of TATGGTGAGACTAATGTCTCACCATCATCACCAGCCA and GATCTAATGTCTCACCATCATCACCAGCCA give a specific amplification product of 167-bp DNA. These primers correspond to amino acids 323–337 (YPRTET) and 382–387 (HPPITP) in Fig. 1A. The PCR fragment was purified and sequenced to confirm that the cloned sequence is from the topoisomerase III gene. PCR was also used to prepare labeled DNA as a probe to screen a *Drosophila* embryonic cDNA library, which was prepared in our laboratory (35). A cDNA clone with a 3-kb insert was isolated, and sequences of both strands were determined.

**Expression Constructs and Generation of Topo III Antibody**—After removing 270 bp from the 5′-untranslated region, the top3 cDNA was ligated into a pET3a vector (Novagen) for isopropyl-1-thio-
-β-D-galactopyranoside-inducible expression in BL21(DE3)pLysS bacterial cells (36). The vector-topo IIIα was overproduced after induction, and it was purified by SDS-polyacrylamide gel electrophoresis. The gel-purified protein was used both as an antigen to immunize a rabbit and as a ligand for affinity purification of the rabbit antibody.

For yeast expression, 270 bp were first removed from the 5′-untranslated region of top3. Two oligonucleotides designed to introduce a new start codon and a 6-histidine tag were annealed and ligated immediately after the top3 start codon (5′-GGCCA-GATCTAAATGCTGCC/CTGTCAGAGCAGGGAATG/CTTAG/CTGCAGTGAGACATTAGATCT). The 6-His-65 and 5′-9 start codon and a 6-histidine tag were annealed and ligated immediately after the large region of purified by SDS-polyacrylamide gel electrophoresis. The gel-purified pyranoside-inducible expression in BL21(DE3)pLysS bacterial cells fluoride, and 1 mM zinc fingers, and 5′-AAAGGGACATCGGCC-5′ were annealed and labeled either at their 5′ ends using T4 polynucleotide kinase (U. S. Biochemical Corp.) and γ[32P]ATP or at their 3′ ends using T4 DNA polymerase (U. S. Biochemical Corp.) and α[32P]TP. Unincorporated nucleotide was removed by a G-25 Sephadex Quick Spin column (Roche Molecular Biochemicals). Label-transfer reactions with *Drosophila* topo I-ND423 contained 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 0.1 mM EDTA, and 10 μM camptothecin. *Drosophila* topo II reactions contained 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 10 mM MgCl2, 2.5 mM ATP, and 10 μM VM26. *Drosophila* topo III reactions were carried out under standard conditions lacking bovine serum albumin, with 1 mM MnCl2, substituted for MgCl2. Reactions were preincubated at either 30 °C (topo I and II) or 37 °C (topo III) for 5 min before adding topoisomerase to initiate cleavage. After mixing, the reactions were stopped immediately by adding an equal volume of 2× Laemmli sample buffer. The samples were boiled and electrophoresed on a polyacrylamide gel. The gel was either dried onto whatman paper or transferred to nitrocellulose before being subjected to autoradiography and Western blot analysis.

**RESULTS**

**Cloning and Sequencing the Drosophila top3 Gene**—Using regions of sequence similarity between the bacterial, yeast, and human type IA enzymes, we designed two degenerate oligonucleotides and used them as primers for PCR amplification of *Drosophila* genomic DNA (see “Experimental Procedures”). The PCR products were first cloned and sequenced to confirm their homologies with topoisomerase III sequences. The PCR reaction was then used to generate a radiolabeled probe for hybridization screening of a *Drosophila* embryo cDNA library. One of the positive clones was isolated, and both strands of the 2979-base pair insert were sequenced.

The largest open reading frame of the obtained clone is predicted to encode a protein of 575 amino acids (calculated molecular mass 97.0 kDa), the sequence of which is shown in Fig. 1A. This protein is within the same size range as the *E. coli* topo I and human and murine topo III enzymes. Residue 332 is the predicted active-site tyrosine, as it is contained within a highly conserved GYISYPRTET sequence. One potential bipartite nuclear localization signal is found in the amino terminus (38). In addition, four potential zinc fingers of the tetracysteine motif are located in the carboxyl terminus of the protein. While the smaller bacterial and yeast topo III enzymes appear to lack zinc fingers, *E. coli* topo I has been shown to coordinate three zinc atoms, and the COOH-terminal domain containing these tetracysteines may have an extranuclear role in DNA binding (39, 40). In addition to the tetracysteine motifs, the COOH-terminal portion of the *Drosophila* open reading frame is also characterized by clusters of glycine and arginine residues (Fig. 1A). In the COOH-terminal 62 amino acids of this protein, there are 23 glycines and 6 arginines, which accounts for 47% of the residues.
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- **Drosophila top3β Is A Single-copy Gene**—A biotin-labeled top3β DNA probe was used for *in situ* hybridization with polytene chromosomes from the salivary glands of 3rd instar larvae (41). The probe hybridizes between regions 5E and 5F on the X chromosome (data not shown). Hybridization is observed only at this locus, suggesting top3β exists as a single-copy gene on the X chromosome. Furthermore, genomic Southern hybridization also suggests that top3β is a single-copy gene (data not shown). These experiments were carried out under stringent hybridization conditions, which cannot exclude the possibility that *Drosophila* may possess another top III isoform. Indeed, submission of human topoisomerase IIIα cDNA for BLAST search against the Berkeley Drosophila Genome Project data base results in several matches with an 87.8-kb P1 genomic clone located at 37E1–37E2 on the left arm of the 2nd chromosome (GenBank accession no. AC005428). Translation of these sequences identifies a putative new protein, *Drosophila* topoisomerase IIIα. Experiments are currently under way to isolate and analyze this new gene.

- **Expression of Topoisomerase IIIβ during Drosophila Development**—To probe its biological function(s), we investigated the expression pattern of top IIIβ protein throughout *Drosophila* development. Extracts made from *Drosophila* at various stages of development were analyzed by Western blot using affinity-purified top IIIβ antibody (Fig. 2; see “Experimental Procedures” for source of this antibody). The antibody recognizes a protein of approximately 97 kDa. In contrast to top I and top II proteins, which peak during the 6–12-h period of development (42, 43), top IIIβ protein levels peak during the first 6 h of embryogenesis. Throughout this time, the top IIIβ levels are fairly constant (data not shown). The protein levels decline during the later stages of embryogenesis, the larval stages, and the pupal stage, but increase again during adulthood. It is interesting to note that top3α-knockout mice die during embryogenesis (34). Our developmental Western blot suggests that *Drosophila* top IIIβ may also play an important role during the first few hours of the fruit fly life.

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**Table I**

| Type IA enzyme | Identity | Similarity | Size |
|----------------|----------|------------|------|
| S. acidocaldarius reverse gyrase | 21% | 39% | 1248 |
| E. coli top I | 25 | 41 | 865 |
| E. coli top III | 22 | 39 | 653 |
| H. influenzae top III | 23 | 40 | 651 |
| S. cerevisiae top III | 33 | 49 | 656 |
| S. pombe top III | 34 | 53 | 522 |
| C. elegans top III | 36 | 53 | 759 |
| M. musculus topo IIIα | 38 | 54 | 1003 |
| H. sapiens top IIIα | 38 | 54 | 1001 |
| A. thaliana topo IIIβ | 43 | 58 | 836 |
| M. musculus topo IIIβ | 59 | 71 | 862 |
| H. sapiens topo IIIβ | 58 | 71 | 862 |
| D. melanogaster topo III | 75 | 77 | 875 |

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FIG. 1. Protein sequence of *Drosophila* topoisomerase III. A, the largest open reading frame of top3 encodes a 97-kDa protein. Residue 332 is the predicted active-site tyrosine (bold). The amino terminus contains one potential bipartite nuclear localization signal (double underline). The carboxyl terminus contains eight CXXC motifs (bold underline), which may encode as many as four zinc fingers. The COOH-terminal domain is characterized by 4 tetracysteines; both the eight CXXC sequences and the intervening spacers are highly conserved (Fig. 1B). This is in contrast with the comparison between *Drosophila* top III and mammalian top IIIαs or between mammalian top IIIβ and top IIIαs, where most of the homologies lie in the NH2-terminal half (a PILEUP comparison of type IA topoisomerases is contained in the online version of this article). Another hallmark for the top IIIβ sequences is the presence of GR-rich clusters in their COOH termini. We therefore propose that the cloned *Drosophila* top3 sequence belongs to the β-subfamily.

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**Note:** 466 BVDIGYKTKMAETXHYXY 695 for *arabidopsis* top IIIβ

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**B**

**Drosophila top IIIB**

| Drosophila top IIIB | 610 | CDSKNNVWFYGKLNLWYDLDYNVYLWSTF/KLWSSN 658 |
| Drosophila top IIIB | 624 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 664 |
| human top IIIB | 624 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 664 |
| arabidopsis top IIIB | 660 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 666 |
| Drosophila top IIIB | 669 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 675 |
| mouse top IIIB | 665 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 675 |
| human top IIIB | 705 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 704 |
| arabidopsis top IIIB | 705 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 706 |
| Drosophila top IIIB | 719 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 725 |
| human top IIIB | 719 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 725 |
| arabidopsis top IIIB | 719 | CDSKNNVWFYGKLNLWYDLDYNVQLSSLQ 725 |
| Drosophila top IIIB | 774 | LQEESQKDRQDKTAKLKLVLHQQWAKQVSKKA 790 |
| human top IIIB | 774 | LQEESQKDRQDKTAKLKLVLHQQWAKQVSKKA 790 |
| arabidopsis top IIIB | 774 | LQEESQKDRQDKTAKLKLVLHQQWAKQVSKKA 790 |
| Drosophila top IIIB | 783 | KVEFSEKTVKKSPC 797 |
| human top IIIB | 783 | KVEFSEKTVKKSPC 797 |
| arabidopsis top IIIB | 783 | KVEFSEKTVKKSPC 797 |
| Drosophila top IIIB | 787 | KEPKDIVQCHV 803 |
| human top IIIB | 787 | KEPKDIVQCHV 803 |
| arabidopsis top IIIB | 787 | KEPKDIVQCHV 803 |
| Drosophila top IIIB | 790 | TVENFVAVRQHDM 806 |

**Amino acid homology comparisons between Drosophila topo III and other members of the type IA family**

Comparisons were done using the BLAST algorithm.
sequence and is located 29 bp upstream of the translation initiation codon. Therefore, while the P-element insertion is expected to affect the expression of top3β, it would not necessarily result in a null mutation.

We obtained this fly stock and used PCR to confirm the presence and location of the P-element insertion. We then used Western blot analysis to compare topo IIIβ levels in male and female transgenic flies to their wild type counterparts. While topo I and topo II levels are approximately the same in the wild type and mutant flies, the topo IIIβ protein level is greatly diminished in the EP(X)1432 mutant (Fig. 3). These mutant flies are both viable and fertile. However, we did not examine whether they have reduced fertility/viability, or whether they have an altered recombination frequency. This result suggests that the overall viability and fertility of the fruit fly are not sensitive to the levels of topo IIIβ.

_Drosophila top3β Suppresses the Yeast top3Δ Slow Growth Phenotype—_Mutation of the Saccharomyces cerevisiae TOP3 gene is known to result in several phenotypes, including a growth rate which is only 50% that of wild type (11). In order to assess whether _Drosophila top3β_ possesses functional similarity to the yeast TOP3 gene, we cloned the _top3β_ cDNA into a YEpG vector to generate pTWtop3. In this construct, 270 bp have been removed from the 5′-untranslated region to facilitate heterologous expression. In addition, a new initiation codon, followed by a 6-histidine tag, has been inserted just upstream of the original _top3β_ start codon. Expression of this construct is under control of the galactose-inducible GAL1 promoter. This expression construct was transformed into JCW253, a yeast strain deleted for TOP3. The _Drosophila top3β_ cDNA can rescue the slow growth of the _top3Δ_ mutant when grown in media containing galactose (Fig. 4). This improved growth rate is not observed when YEpG vector lacking the _top3β_ insert is transformed into the yeast mutant, or when the strains are grown on media containing glucose (data not shown). Therefore, _Drosophila top3β_ can be functionally expressed in yeast, and it shares functional similarity with the yeast TOP3 gene. Recent results have shown that human _top3β_ can also rescue the _top3Δ_ growth defect in yeast (33).

Relaxation of Hypernegatively Supercoiled, but Not Negatively Supercoiled, DNA by Topoisomerase IIIβ—_The functional expression of Drosophila topo IIIβ_ in yeast allowed us to purify this protein for biochemical studies. To eliminate any potential contamination of major type I topoisomerase activity, namely that from yeast topo I, we expressed _Drosophila_ topo IIIβ in _top1− yeast_ (46). JEL1 (top1) yeast transformed with the pTWtop3 expression vector were grown in 8 liters of rich medium containing 2% galactose. The cells were harvested and lysed, and nuclear extract was prepared with an extraction buffer containing 1 M NaCl. The nuclear extract was fractionated over a nickel-NTA column, followed by a single-stranded DNA affinity chromatography, and the 97-kDa protein is the predominant species in the purified fraction.

We tested the purified topo IIIβ protein for relaxation activity toward plasmid DNA isolated directly from bacterial cells. The protein showed no activity toward this negatively supercoiled substrate in the range of temperature tested, from 30 to 65 °C (Fig. 6A, lanes 1–6). The observation that type IA enzymes have an affinity for single-stranded DNA (8, 47) led us to test the relaxation activity of topo IIIβ toward highly unwound, or hypernegatively supercoiled, plasmids. These hypernegatively supercoiled substrates were generated by incubating the plasmid DNA with an excess of ethidium bromide, followed by relaxation with _Drosophila_ topo I. Upon phenol extraction to remove the ethidium, the topo I-relaxed DNA becomes hypernegatively supercoiled. When we assay for relaxation with this substrate, a slight but definite reduction in mobility is observed, with relaxation at an optimal between 37 and 45 °C (Fig. 6A, lanes 7–12). Relaxation of this highly undervound substrate by topo IIIβ is only partial. The observed shift in

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**Fig. 2.** The greatest expression of _Drosophila_ topo IIIβ occurs during the first 6 h of embryogenesis. Extracts made from _Drosophila_ at various stages of development were analyzed by Western blot, probing with affinity-purified topo IIIβ antibody (upper panel) and with actin antibody (lower panel). Actin serves as a control to verify an equal amount of protein was loaded in each lane (determined to be 100 μg/lane by Bradford assay).

**Fig. 3.** TOP3β expression is greatly reduced in an EP(X)1432 mutant. A, protein levels for topoisomerases I, II, and IIIβ were analyzed by Western blot in both wild type (lanes 2 and 4) and EP(X)1432 mutant (lanes 1 and 3) flies. The female samples are in lanes 1 and 2, and males in lanes 3 and 4. The left panel was probed with antibodies against topo I and topo II, while the right panel was probed with antibody against topo IIIβ. The lower molecular weight species in the topo I and II blot correspond to the proteolytic products of topo I in the extracts. B, Coomassie staining confirms that approximately the same amount of total protein was loaded in lanes 1–4.

**Fig. 4.** _Drosophila top3β_ suppresses the slow growth phenotype of a yeast top3Δ null mutant. Expression of _Drosophila top3β_ is under control of a GAL1 promoter. The plate shows growth after 4 days at 30 °C on synthetic media containing galactose. Clockwise from the top: FY251 parental strain (wild type), JCW253 (isogenic with FY251, except for top3Δ), JCW253 transformed with _Drosophila_ top3β (pTW-top3), and JCW253 transformed with vector control (YEpG).
mobility appears to terminate at a definite point, with the bulk of the topoisomers migrating with approximately the same mobility as the negatively supercoiled plasmid marker. In addition, topo IIIβ relaxation does not appear to be sequence-specific, since we have observed this same phenomenon with four different plasmids ranging in size from 4 to 13 kb.

This partial relaxation of hypernegatively supercoiled DNA by topo IIIβ is not due to an insufficient amount of enzyme in the reaction. The relaxation reaction is essentially complete within 1 h; either prolonged incubation for another 12 h or addition of a second aliquot of enzyme does not result in further shift in mobility (Fig. 6B, lanes 1–4). Topo IIIβ remains active in the reaction mixture since addition of another hypernegatively supercoiled substrate to the reaction results in similar relaxation of the larger substrate (Fig. 6B, lane 5). This suggests that the topo IIIβ enzyme is specific for highly underwound substrates. Consistent with this idea is the observation that Drosophila topo IIIβ does not relax positively supercoiled DNA (data not shown).

We further investigated the effect of divalent cations and monovalent salt on the activity of Drosophila topo IIIβ. Relaxation activity can be observed in the absence of added divalent cation, but addition of EDTA abolishes this activity, demonstrating a requirement for divalent cations (Fig. 7A, lanes 1 and 5). Activity can be restored when a molar excess of Mg2+, Mn2+, or Ca2+, but not Co2+, Cu2+, or Zn2+, are included in addition to EDTA (Fig. 7A, lanes 6–11). The optimal Mg2+ concentration is about 1 mM and at higher concentrations the reaction is inhibited (Fig. 7A, lanes 2–4). The preference of topo IIIβ for low ionic strength can also be seen in the observation that the optimal monovalent salt concentration is below 50 mM (Fig. 7B). This is possible that an extended region of single strand DNA in the circular DNA substrate is requisite for topo IIIβ reaction and the presence of a higher concentration of divalent cations reduces this single-strandedness in the DNA.

While the agarose gel electrophoresis employed here provides a convenient method to monitor the relaxation of hypernegatively supercoiled DNA by topo IIIβ, its limitation in the resolution of the negatively supercoiled DNA precludes us from determining the change in linking number that occurred in this reaction. To this end, we have used a series of DNAs with different linking numbers as a reference for gel electrophoresis in the presence of ethidium bromide (Fig. 8). This series of topoisomers was generated to cover the range of supercoiling from the hypernegatively supercoiled substrate to the fully relaxed species. The pieces of the ladder were made by incubating plasmid DNA with varying amounts of ethidium bromide (DNA bp:ethidium ratios of 2:1 to 80:1), followed by relaxation with Drosophila topo I, and phenol extraction. The pieces of the topoisomer ladder, along with the hypernegatively supercoiled substrate (H), topo III reaction product (T), negatively supercoiled plasmid (N), and the fully relaxed species (R), were arranged in the order of linking number changes and the trends in mobility shifts. Identical sets of DNA samples were resolved by electrophoresis through agarose gels either in the presence of 0.75 or 0.05 μg/ml ethidium (Fig. 8, A and B). It is interesting to notice that, while there is only a small shift in the mobilities between the hypernegatively supercoiled substrate and its topo IIIβ reaction product when analyzed by gel electrophoresis in the absence of ethidium (Figs. 6 and 7) or in the presence of a low concentration of ethidium (Fig. 8B), there is a much greater shift in the mobilities at a higher ethidium concentration (Fig. 8A). Under such an electrophoretic condition, the hypernegatively supercoiled DNA remains slightly negatively supercoiled and the topo IIIβ product is converted...
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Effect of divalent and monovalent cation on Drosophila topo IIIβ. A, relaxed (R), negatively supercoiled (N), and hypernegatively supercoiled (H) plasmid markers are run in the leftmost lanes. Reaction conditions contain 40 mM Hepes-KOH (lane 1) plus 1 mM MgCl₂ (lane 2), 5 mM MgCl₂ (lane 3), 10 mM MgCl₂ (lane 4), or 2 mM EDTA (lane 5). Lanes 6–11 are the same as lane 5, except they contain 4 mM MgCl₂, CaCl₂, CoCl₂, CuSO₄, MnCl₂, or ZnSO₄ (lanes 6–11). B, reactions were performed under standard conditions (lane 1) plus increasing amounts of monovalent cation from 50–200 mM, either NaCl (lanes 2–5) or KCl (lanes 6–9).

FIG. 7. Effect of divalent and monovalent cation on Drosophila topo IIIβ. A, relaxed (R), negatively supercoiled (N), and hypernegatively supercoiled (H) plasmid markers are run in the leftmost lanes. Reaction conditions contain 40 mM Hepes-KOH (lane 1) plus 1 mM MgCl₂ (lane 2), 5 mM MgCl₂ (lane 3), 10 mM MgCl₂ (lane 4), or 2 mM EDTA (lane 5). Lanes 6–11 are the same as lane 5, except they contain 4 mM MgCl₂, CaCl₂, CoCl₂, CuSO₄, MnCl₂, or ZnSO₄ (lanes 6–11). B, reactions were performed under standard conditions (lane 1) plus increasing amounts of monovalent cation from 50–200 mM, either NaCl (lanes 2–5) or KCl (lanes 6–9).

FIG. 8. Quantitating the linking number change induced by topo IIIβ. The hypernegatively supercoiled substrate (H), topo IIIβ reaction product (T), negatively supercoiled plasmid (N), and fully relaxed species (R) are as indicated. Various pieces of the topoisomer ladder are labeled 1–19. The samples were electrophoresed in the presence of either 0.75 μg/ml (A) or 0.05 μg/ml (B) ethidium bromide. The samples are arranged in order of increasing linking number, from the most hypernegatively supercoiled species on the left, to the fully relaxed species on the right.

Topoisomerase IIIβ Binds Covalently to DNA 5’ Phosphoryl Ends—To further confirm we had identified a type IA enzyme, we investigated whether topo IIIβ could link covalently to DNA 5’ phosphoryl groups. In this experiment, the topoisomerase can be trapped in a covalent complex with labeled DNA when the reaction is stopped by a strong denaturant like SDS. In effect, the label on the DNA is transferred to the protein, and the reaction is stopped by a strong denaturant like SDS. In effect, the label on the DNA is transferred to the protein, and the reaction is stopped by a strong denaturant like SDS.

Topoisomerase IIIβ binds covalently to DNA 5’ phosphoryl ends. When we used a larger oligomer, however, an upward shift in mobility was observed, as would be expected for a protein bound to a larger DNA fragment (data not shown).

The label-transfer experiment provides another assay for the interactions between topo IIIβ and its DNA substrates. We examined this reaction under different divalent cation conditions and with both single- and double-strand DNA substrates (Fig. 8). The single-stranded substrates were generated by heat denaturation of the double-stranded oligonucleotides used above. Under all conditions tested, the single-stranded DNA is cleaved to a greater extent than the double-stranded DNA. In some cases, a doublet is observed, indicating that the oligonucleotide sequence contains at least two cleavage sites for topo IIIβ. Cleavage is observed in the absence of added divalent cation. However, it is stimulated when a divalent cation like Mg²⁺ or Mn²⁺ is added. The presence of Mn²⁺ seems to support the cleavage reaction at least as well as Mg²⁺, which is also the case for the relaxation of hypernegatively supercoiled DNA. It will be interesting to examine if other eucaryotic topo III also have a preference for Mn²⁺, just like the Drosophila enzyme.

DISCUSSION

BLAST sequence alignments suggest Drosophila topo III is a member of the β-subfamily of type IA topoisomerases, being nearly 60% identical to mammalian topo IIIβ enzymes. The homology among the topo IIIβ enzymes is particularly striking.

into a highly positively supercoiled species. Based on analysis from these data and from other gel electrophoretic conditions, we can estimate that the linking difference between H and T DNA is between 30 and 35, T and N between 5 and 10, and N and R around 30. Therefore, the linking change from H to T is nearly equivalent to that from N to R, which occurs in the reaction catalyzed by most of the DNA topoisomerases. Drosophila topo IIIβ is as proficient in reducing the linking number deficit as other type I enzymes, except it seems to operate in a different supercoiling range.

Topoisomerase IIIβ binds covalently to DNA 5’ phosphoryl ends. To further confirm we had identified a type IA enzyme, we investigated whether topo IIIβ could link covalently to DNA 5’ phosphoryl groups. In this experiment, the topoisomerase can be trapped in a covalent complex with labeled DNA when the reaction is stopped by a strong denaturant like SDS. In effect, the label on the DNA is transferred to the protein, and the labeled protein can be detected by autoradiography. Two 16-mers, each with a 4-nucleotide 5’-protruding end, were annealed prior to radiolabeling at either their 5’ ends with T4 polymerase, or their 3’ ends with T4 DNA polymerase. As controls for the experiment, we incubated our labeled oligonucleotides with Drosophila topo II (which is known to bind to 5’ phosphoryl groups) and an amino-terminally truncated form of Drosophila topo I (which is known to bind to 3’ phosphoryl groups). An autoradiograph signal is seen for topo IIIβ only when it is incubated with the 3’ end-labeled substrate, indicating that it binds to 5’ phosphoryl groups (Fig. 9A). This result is expected for a type IA topoisomerase. Western blot on the same samples shows that the autoradiograph signal due the label transfer coincides with the protein signal for the three topoisomerases. It also demonstrates that the same amount of protein was incubated with both the 5’ and 3’ end-labeled substrates. When we used a larger oligomer, however, an upward shift in mobility was observed, as would be expected for a protein bound to a larger DNA fragment (data not shown).

The label-transfer experiment provides another assay for the interactions between topo IIIβ and its DNA substrates. We examined this reaction under different divalent cation conditions and with both single- and double-strand DNA substrates (Fig. 8B). The single-stranded substrates were generated by heat denaturation of the double-stranded oligonucleotides used above. Under all conditions tested, the single-stranded DNA is cleaved to a greater extent than the double-stranded DNA. In some cases, a doublet is observed, indicating that the oligonucleotide sequence contains at least two cleavage sites for topo IIIβ. Cleavage is observed in the absence of added divalent cation. However, it is stimulated when a divalent cation like Mg²⁺ or Mn²⁺ is added. The presence of Mn²⁺ seems to support the cleavage reaction at least as well as Mg²⁺, which is also the case for the relaxation of hypernegatively supercoiled DNA. It will be interesting to examine if other eucaryotic topo III also have a preference for Mn²⁺, just like the Drosophila enzyme.
in the COOH-terminal region where all contain eight highly conserved CXXC motifs, with the spacing between these motifs being highly conserved as well. These CXXC motifs suggest the topo III βs may possess as many as four zinc fingers. These motifs do not conform to the proposed zinc finger motif for E. coli topoisomerase I (Cys-X2-Cys-Gly-X6-Met-X7-13-Cys-X6-10-Cys) (39). There are three such zinc finger motifs in the COOH terminus of E. coli topo I, which has been shown to bind three zinc(I) ions and require zinc coordination for cleavable complex formation with DNA (40). Interestingly, the mammalian topo IIIαs contain zinc finger motifs similar to E. coli topo I (excluding the consensus for a glycine at the 5th position), but lack the eight CXXC motifs found in the topo IIIβs. Based on these sequence observations, we suggest the type IA enzymes may fall into three subfamilies: 1) enzymes that lack zinc finger motifs, such as reverse gyrase, bacterial topo III and yeast topo III; 2) enzymes containing the motif found in E. coli topo I, which includes the mammalian topo IIIαs; 3) enzymes containing eight CXXC motifs, as found in the topo IIIβs.

Immediately following the CXXC motifs in the topo IIIβs are clusters of glycine and arginine residues. While all of the type IA enzymes contain glycines and arginines in their COOH termini, only the topo IIIβ enzymes have them arranged in clusters. We first noticed this phenomenon in the Arabidopsis topo IIIβ paralogous (GenBank accession AAD15404), which has a stretch of 20 consecutive Gs and Rs near the COOH-terminal end of the protein. The significance of these GR clusters is not known. It is possible they may mediate nucleic acid binding through the positive charge of the arginines. Alternatively, these GR clusters may specify a protein-protein interaction domain. Similar GR clusters have also been identified in several RNA-binding and nucleolar-localizing proteins, such as nucleolin (48), mammalian protein C23 (49), and nucleolar snRNP antigen (50).

Like other topo III enzymes, Drosophila topo IIIβ appears to possess a weak activity in relaxing negatively supercoiled plasmid DNA isolated directly from bacterial cells. In fact, we were only able to observe relaxation activity with a highly underwound, or hypernegatively supercoiled, substrate. This suggests our assay may be useful for the identification of new members of the topo III family. In addition, it affects the idea that the topo III enzymes are not inefficient in supercoil relaxation, but rather that they work within an atypical linking number range. To this end, we were able to quantitate the level of hypernegatively supercoil relaxation induced by Drosophila topo IIIβ and found it to be comparable to the degree of relaxation of negatively supercoiled plasmid DNA by E. coli topo I. Our results also support the idea that the topo III enzymes possess a unique substrate requirement, namely one that has exposed single-stranded regions. These single-stranded regions can be generated either by heating the reaction to high temperature, as is the case for E. coli topo III (5), or through a hypernegatively supercoiling of the substrate. It is interesting to speculate on which biological processes may create such highly underwound DNA species. Strand separation events, such as DNA replication and transcription, could provide structures that are hot spots for topo III activity. Alternatively, topo III may act on the DNA intermediates created during the process of recombination.

Genetic experiments in yeast have demonstrated that TOP3 plays a role in suppressing mitotic recombination and in resolving recombinomed homologous chromosomes during meiosis I (11, 13). Furthermore, the combined action of either yeast or bacterial topo III and the DNA helicase RecQ can promote the formation of DNA catenanes (51). Similar strand passage reactions may be involved in the initiation and resolution steps during recombination. The unwinding action of a RecQ-type helicase appears to generate a DNA structure that can be recognized by a topo III enzyme (51). It will be interesting to determine whether our topo IIIβ interacts with the newly identified Drosophila RecQ homolog, Dmblm (52), and what role, if any, these enzymes play in the above mentioned processes.

The developmental Western blot demonstrates that the greatest expression of topo IIIβ occurs during the first 6 h of embryogenesis. This suggests an important function for topo IIIβ during the first few hours of the fly lifecycle. The topo IIIβ protein levels decrease during later stages of embryogenesis, the instar larval stages, and the pupal stage, then increase again during adulthood. This expression pattern suggests topo IIIβ may be important for some unique aspect of the the DNA replication and chromosome segregation process, given embryogenesis is characterized by rapid cycles of DNA replication and chromosome segregation in the absence of intervening G phases, while endoreplication is prolific in the larval stages (53).

While we do not know whether the EPX1432 mutant is hypomorph or null for topo IIIβ activity, our results indicate that the mutant flies are fertile and viable under greatly reduced levels of topo IIIβ. It is still possible that the topo3β mutants may contain defects in aspects yet to be tested, including recombination and repair. However, it appears the biological function of topo IIIβ may not be required for the viability and fertility of Drosophila. Through a search of the published data base for the Drosophila genome, we have identified DNA sequences that may encode topo IIIa. Drosophila therefore likely contains both forms of the topo III isozyme, just like the mammalian cells. The experiments using knock-out mice have
demonstrated that the function of topo IIIα is essential (34). It will be interesting to determine whether topo IIIβ is essential as well. The specific and overlapping functions of these two isozymes in Drosophila and mammalian cells will clearly be an important issue to address in the future.

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