THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 47, pp. 39337–39345, November 25, 2005

Human Topoisomerase IIα Rapidly Relaxes Positively Supercoiled DNA

*IMPLICATIONS FOR ENZYME ACTION AHEAD OF REPLICATION FORKS**

Received for publication, March 25, 2005, and in revised form, September 26, 2005 Published, JBC Papers in Press, September 27, 2005, DOI 10.1074/jbc.M503320200

A. Kathleen McClendon[‡1], **A. Chapin Rodriguez**[§2], **and Neil Osheroff**[‡¶3]

From the Departments of [‡]*Biochemistry and* [¶]*Medicine (Hematology/Oncology), Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146 and* [§]*Bard High School Early College, New York, New York 10002*

Movement of the DNA replication machinery through the double helix induces acute positive supercoiling ahead of the fork and precatenanes behind it. Because topoisomerase I and II create transient single- and double-stranded DNA breaks, respectively, it has been assumed that type I enzymes relax the positive supercoils that precede the replication fork. Conversely, type II enzymes primarily resolve the precatenanes and untangle catenated daughter chromosomes. However, studies on yeast and bacteria suggest that type II topoisomerases may also function ahead of the replication machinery. If this is the case, then positive DNA supercoils should be the preferred relaxation substrate for topoisomerase IIα, the enzyme isoform involved in replicative processes in humans. Results indicate that human topoisomerase IIα relaxes positively supercoiled plasmids >10-fold faster than negatively supercoiled molecules. In contrast, topoisomerase IIβ, which is not required for DNA replication, displays no such preference. In addition to its high rates of relaxation, topoisomerase IIα maintains lower levels of DNA cleavage complexes with positively supercoiled molecules. These properties suggest that human topoisomerase IIα has the potential to alleviate torsional stress ahead of replication forks in an efficient and safe manner.

One of the most striking features of DNA is the intertwining of the two complementary strands of the double helix (1). Discovery of this characteristic led to the immediate recognition that biological processes such as replication would be severely affected by the topological state of the genetic material (2).

DNA in all species ranging from bacteria to humans is globally underwound (*i.e.* negatively supercoiled) (3–6). This underwinding makes it easier to separate complementary DNA strands from one another and greatly facilitates initiation of replication and the assembly of replication forks. Once the fork begins to travel along the DNA template, however, the deleterious effects of topology manifest themselves (Fig. 1). Because helicases separate, but do not unwind the two strands of the double helix, fork movement results in acute overwinding (*i.e.* positive supercoiling) of the DNA ahead of the replication machinery (Fig. 1*B*) (3, 5–7). This overwinding has two major consequences. First, it increases the difficulty of separating duplex DNA into individual strands. Therefore, accumulation of positive supercoils presents a formidable block to fork movement (5, 7–11). Second, DNA overwinding ahead of the fork leads to a compensatory underwinding behind the replication machinery. If the replisome rotates around the helical axis of the DNA, this underwinding allows some of the torsional stress in the prereplicated DNA to be translated to the newly replicated daughter molecules in the form of precatenanes (Fig. 1*C*) (6, 7, 11). If these precatenanes are not resolved, they ultimately lead to the formation of catenated duplex daughter chromosomes.

The topological state of DNA in the cell is modulated by enzymes known as topoisomerases (5, 7, 12–16). There are two classes of topoisomerases that are distinguished by their catalytic mechanisms. Type I enzymes act by generating a transient single-stranded break in the double helix, followed by either a single-stranded DNA passage event or controlled rotation about the break (14, 17, 18). As a result, these enzymes are able to alleviate torsional stress (*i.e.* remove superhelical twists) in duplex DNA.

Type II topoisomerases act by generating a transient double-stranded DNA break, followed by a double-stranded DNA passage event (14–16, 19). Consequently, enzymes in this latter class are able to remove superhelical twists from DNA and resolve knotted or tangled duplex molecules. Whereas lower eukaryotes such as yeast and *Drosophila* encode only a single type II topoisomerase (20, 21), bacteria and vertebrate species express two discrete forms of the enzyme (14, 22–24). Bacteria express DNA gyrase and topoisomerase IV. Although these two enzymes are closely related, they play distinct physiological roles. Gyrase is the only known topoisomerase that can actively underwind DNA (14, 23, 24). It functions to maintain the superhelical density of the bacterial chromosome and removes positive supercoils that accumulate ahead of DNA tracking systems. In contrast, topoisomerase IV is a potent decatenase that resolves knots in the genome and untangles newly replicated daughter chromosomes (14, 25). As discussed below, this type II enzyme also may play a role ahead of replication forks.

Vertebrates express two closely related isoforms of topoisomerase II, α and β (22, 26). These enzymes display a high degree of amino acid sequence identity and similar enzymological characteristics. However, they differ in their protomer molecular masses (170 *versus* 180 kDa, respectively) and are encoded by separate genes (14, 24, 27). Either topoisomerase IIα or β can complement the loss of topoisomerase II in yeast (28–30), but the two enzymes have distinct patterns of expression and physiological functions in vertebrate cells. The α isoform is up-regulated dramatically during cell proliferation and is tightly associated with mitotic chromosomes (13, 31–34). In contrast, expression of the β isoform is independent of proliferative status, and the enzyme dissociates from chromosomes during mitosis (13, 27, 31, 35). Thus, topoisomerase IIα is believed to be the isoform that functions in growth-dependent processes, such as DNA replication and chromosome

* This work was supported by National Institutes of Health Grant GM33944. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Trainee under Grant 5 T32 CA09582 from the National Institutes of Health.
[2] Supported by a predoctoral fellowship from the Howard Hughes Medical Institute and by the Medical Research Council (UK).
[3] To whom correspondence should be addressed: Dept. of Biochemistry, 654 Robinson Research Bldg., Vanderbilt University School of Medicine, Nashville, TN 37232-0146. Tel.: 615-343-4338; Fax: 615-343-1166; E-mail: neil.osheroff@vanderbilt.edu.

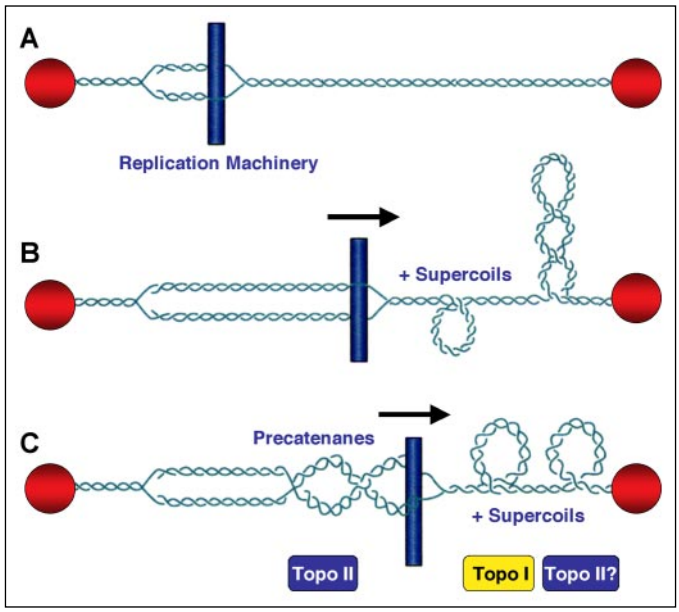

FIGURE 1. **Model for topoisomerase function and topological stress associated with DNA replication.** This figure was adapted from Wang (7). The replication machinery is represented by a rod moving through the double helix. DNA ends are anchored to hypothetical immobile structures existing in the nucleus. *A*, upon initiation of DNA replication, the two strands of duplex DNA are separated, and the replication fork is formed. *B*, movement of the replication machinery through the immobilized DNA template strands induces acute overwinding (*i.e.* positive supercoiling) ahead of the fork. *C*, if the replisome rotates around the helical axis of the DNA, compensatory underwinding (*i.e.* negative supercoiling) behind the replication machinery allows some of the torsional stress in the prereplicated DNA to be translated to the newly replicated daughter molecules in the form of precatenanes. If these precatenanes are not resolved, they ultimately lead to the formation of catenated duplex daughter chromosomes. Topoisomerase I is proposed to work ahead of the replication fork to remove positive DNA supercoils, whereas topoisomerase II is proposed to work primarily behind the fork to remove precatenanes. In contrast to replication models, drug models place topoisomerase II ahead of DNA tracking systems.

segregation (7, 13). Although cells can survive the absence of topoisomerase IIβ, this isoform cannot compensate for the loss of topoisomerase IIα (27, 36, 37).

Beyond their critical physiological functions, prokaryotic and eukaryotic topoisomerases are the targets for some of the most successful antibacterial (23, 38–41) and anticancer (15–17, 42–44) drugs currently in clinical use. The common feature of all of these drugs is that they act by increasing levels of covalent topoisomerase-cleaved DNA complexes (*i.e.* cleavage complexes) that are requisite catalytic intermediates (15–17, 23, 40, 45). When DNA tracking systems, such as replication forks, collide with these complexes, transient enzyme-associated DNA breaks are converted to permanent breaks in the genome (15–17, 23, 40, 45). As a result, antibacterial and anticancer agents convert topoisomerases from essential enzymes to potent cellular toxins.

Topoisomerases play critical roles during DNA replication in eukaryotic cells (5, 7, 12–16). Because type I topoisomerases can relax positive or negative superhelical twists (14, 17, 18, 46), it has been assumed that these enzymes act ahead of replication forks to alleviate torsional stress. Conversely, because type II topoisomerases can untangle duplex DNA molecules (12, 14–16, 19), these enzymes are believed to act exclusively behind forks to resolve precatenanes or later in replication to unlink catenated daughter chromosomes. In marked contrast to this proposed segregation of function, models for anticancer drug action place topoisomerase II ahead of approaching replication forks (15, 16, 45, 47–49). This discrepancy raises the question of whether eukaryotic type II topoisomerases have normal physiological functions ahead of the DNA replication machinery.

Two lines of evidence suggest that this may be the case. First, yeast topoisomerase II can compensate for the loss of topoisomerase I in *Saccharomyces cerevisiae*, but loss of both enzymes abruptly halts DNA synthesis (8, 9). This finding indicates that the type II enzyme can assume the role of topoisomerase I ahead of the replication machinery. Second, *Escherichia coli* topoisomerase IV (whose functions in bacteria appear to parallel those of topoisomerase II in eukaryotes) can partially compensate for the loss of DNA gyrase during replication elongation (50, 51). Moreover, topoisomerase IV relaxes positive DNA supercoils ~20-fold faster than it does negative supercoils (52, 53).

If type II topoisomerases play a role ahead of replication forks in vertebrate species, then positively supercoiled DNA should be the preferred relaxation substrate for topoisomerase IIα, the isoform that is involved in replicative processes. Results indicate that human topoisomerase IIα relaxes positively supercoiled plasmids >10-fold faster than negatively supercoiled molecules. In contrast, human topoisomerase IIβ, which is not required for DNA replication, shows no such preferential relaxation. Finally, topoisomerase IIα maintains lower levels of DNA cleavage complexes with positively supercoiled molecules. These enzymological properties are consistent with those of a topoisomerase that functions ahead of the replication machinery.

EXPERIMENTAL PROCEDURES

Enzymes and Plasmid DNA—Human topoisomerase IIα and topoisomerase IIβ were expressed in *S. cerevisiae* (54) and purified as described previously (55). Based on Coomassie- or silver-stained protein gels, both enzymes were at least 95% homogeneous. *Archaeoglobus fulgidus* reverse gyrase was expressed in *E. coli* C41(DE3) and purified according to Rodriguez (56). Negatively supercoiled pBR322 DNA was prepared from *E. coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer.

Preparation of Positively Supercoiled Plasmid DNA—Positively supercoiled plasmid DNA was prepared by treating negatively supercoiled molecules with reverse gyrase (56). Reaction mixtures contained 35 nM negatively supercoiled pBR322 DNA and 420 nM reverse gyrase in a total of 500 μl of 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM $MgCl_2$, and 1 mM ATP. Reactions were incubated at 95 °C for 5 min, halted by the addition of 13 μl of 375 mM EDTA, and cooled on ice. Proteinase K was added (10 μl of 4 mg/ml), and the reactions were incubated at 45 °C for 30 min to digest the enzyme. Samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and positively supercoiled DNA was precipitated with cold ethanol. Plasmids were resuspended in 100 μl of 5 mM Tris-HCl (pH 7.4) containing 500 μM EDTA. To make certain that differences between negatively and positively supercoiled substrates were not influenced by the temperature or other conditions used in the preparation protocol, negatively supercoiled plasmids were treated in a parallel fashion, except that reverse gyrase was omitted from reaction mixtures.

The average number of superhelical twists present in DNA substrates and the resulting σ values were determined by electrophoretic band counting relative to fully relaxed molecules. For negatively supercoiled substrates, time courses for the relaxation of pBR322 by topoisomerase I were resolved by electrophoresis in 1% agarose gels in TBE (100 mM Tris borate (pH 8.3), 2 mM EDTA) containing 1–2 μg/ml chloroquine (Sigma) as running buffer. Optimal resolution was observed in 1 μg/ml chloroquine. For positively supercoiled substrates, time courses for the generation of positive superhelical twists by reverse gyrase were resolved by electrophoresis as above in TBE containing 5–15 μg/ml netropsin B (Roche Applied Science). Optimal resolution was observed in 7.5 μg/ml netropsin B. Positively supercoiled bands also were

counted in time courses of relaxation of positively supercoiled plasmid by topoisomerase I. Calculated σ values were consistent with those obtained from the reverse gyrase time courses.

DNA Relaxation—DNA relaxation assays were based on the procedure of Fortune and Osheroff (57). Unless stated otherwise, reaction mixtures contained 1 nM human topoisomerase IIα or topoisomerase IIβ, 5 nM negatively or positively supercoiled pBR322 DNA, and 1 mM ATP in a total of 20 μl of relaxation buffer (10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 175 mM KCl, 5 mM $MgCl_2$, and 2.5% glycerol). Samples were incubated at 37 °C, and DNA relaxation was stopped by the addition of 3 μl of 0.77% SDS, 77 mM EDTA (pH 8.0). Samples were mixed with 2 μl of agarose gel loading buffer (60% sucrose in 10 mM Tris-HCl (pH 7.9)), heated at 45 °C for 5 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris borate (pH 8.3), 2 mM EDTA.

Alternatively, samples were analyzed by two-dimensional gel electrophoresis. The first dimension was run for 2 h as described in the preceding paragraph. The gel was soaked for 2 h with gentle shaking in 200 ml of TBE containing 4.5 μg/ml chloroquine and run in the orthogonal dimension (90° clockwise) for 2 h in fresh TBE containing 4.5 μg/ml chloroquine. All of the gels were stained with 1 μg/ml ethidium bromide, and DNA bands were visualized with ultraviolet light and quantified using an Alpha Innotech digital imaging system.

DNA Binding—The ability of human topoisomerase IIα to bind negatively and positively supercoiled DNA was assessed using a competitive nitrocellulose filter binding assay. Binding mixtures contained 400 nM topoisomerase IIα, 5 nM linear pBR322 DNA that was cleaved with Hind III and terminally labeled with [^{32}P]phosphate and 0–20 nM negatively or positively supercoiled DNA in a total of 20 μl of binding buffer (10 mM Tris-HCl (pH 7.9), 100 or 175 mM KCl, 0.1 mM EDTA, and 2.5% glycerol). Samples were incubated at 37 °C for 6 min. Under the conditions of the assay, a DNA binding equilibrium was established in less than 1 min. Nitrocellulose membranes (0.45 μm HA, Millipore) were prepared by incubation in binding buffer for 10 min. Samples were applied to the membranes and filtered *in vacuo*. Membranes were washed three times with 1 ml of binding buffer, dried, and submerged in 8 ml of scintillation fluid (Econo-Safe Research Products International). Radioactivity remaining on the membranes was quantified using a Beckman LS 5000 TD scintillation counter. The percentage of linear DNA bound to topoisomerase IIα was determined based on the ratio of radioactivity on the membranes *versus* that of the input DNA.

It should be noted that DNA binding experiments were performed in the absence of ATP and Mg^{2+}. This was done to prevent the formation of concatenated DNA multimers, which are too large to pass through the filter, or the generation of covalent enzyme-DNA cleavage complexes during the course of the assay.

ATP Hydrolysis—ATPase assays were performed as described by Osheroff *et al.* (58). Reaction mixtures contained 1 nM human topoisomerase IIα, 55 nM negatively or positively supercoiled pBR322 DNA, and 1 mM [γ-^{32}P]ATP in a total of 40 μl of relaxation buffer. Mixtures were incubated at 37 °C, and 2-μl samples were removed at time intervals up to 12 min and spotted on polyethyleneimine-impregnated thin layer cellulose chromatography plates (EMD Chemicals). Plates were developed by chromatography in freshly made 400 mM NH_4HCO_3 and analyzed using a Bio-Rad molecular imager FX. ATP hydrolysis was monitored by the release of free phosphate.

Plasmid DNA Cleavage—DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (57). Reaction mixtures contained 0–800 nM topoisomerase IIα, 10 nM negatively or positively supercoiled pBR322 DNA, and 5 mM $MgCl_2$ or $CaCl_2$ in a total of 20 μl of cleavage buffer (10 mM Tris-HCl (pH 7.9), 100 mM KCl, 0.1 mM EDTA, and 2.5% glycerol). Mixtures were incubated at 37 °C for 6 min, and enzyme-DNA cleavage complexes were trapped by the addition of 2 μl of 5% SDS followed by 1 μl of 375 mM EDTA (pH 8.0). Proteinase K (2 μl of 0.8 mg/ml) was added, and the samples were incubated at 45 °C for 30 min to digest the type II enzyme. Samples were mixed with 2 μl of agarose gel loading buffer, heated at 45 °C for 5 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris acetate (pH 8.3), 2 mM EDTA containing 0.5 μg/ml ethidium bromide. DNA bands were visualized and quantified as described above, and cleavage was monitored by the conversion of supercoiled plasmid DNA to linear molecules.

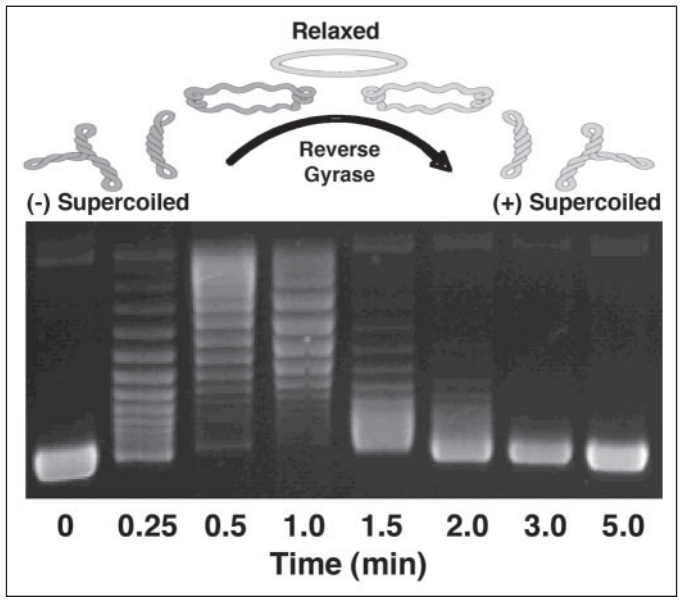

FIGURE 2. **Generation of positively supercoiled DNA by *A. fulgidus* reverse gyrase.** The positions of negatively (−) supercoiled, relaxed, and positively (+) supercoiled DNA are indicated. Negatively supercoiled pBR322 plasmid DNA was incubated with reverse gyrase for the indicated times. The extent of positive supercoiling was monitored by agarose gel electrophoresis.

DNA cleavage reactions were performed in the absence of ATP so that the topological state of the DNA did not change during the course of the reaction. It should be noted that the nucleotide cofactor does not influence the mechanism of topoisomerase II-mediated DNA scission (15).

Site-specific DNA Cleavage—DNA sites cleaved by human topoisomerase IIα in negatively and positively supercoiled DNA were mapped using a modification of the procedure of O'Reilly and Kreuzer (59, 60). DNA cleavage mixtures contained 2.2 μM human topoisomerase IIα, 10 nM negatively or positively supercoiled pBR322 DNA, and 1 mM $MgCl_2$ or $CaCl_2$ in a total of 160 μl of cleavage buffer. Samples were incubated at 37 °C for 6 min, and enzyme-DNA cleavage complexes were trapped by the addition of 16 μl of 1% SDS followed by 8 μl of 375 mM EDTA (pH 8.0). Proteinase K (16 μl of 0.8 mg/ml) was added, and the mixtures were incubated at 45 °C for 30 min to digest the type II enzyme. DNA products were purified by passage through Qiaquick spin columns (Qiagen) as described by the manufacturer. DNA cleavage products were linearized by treatment with HindIII. Terminal 5′-phosphates were removed by treatment with calf intestinal alkaline phosphatase and replaced with [^{32}P]phosphate using T4 polynucleotide kinase and [γ-^{32}P]ATP. Samples were treated with EcoRI, and the singly end-labeled DNA products were purified by passage through a CHROMA SPIN+TE-100 column (Clontech). Loading buffer (40% formamide, 8.4 mM EDTA, 0.02% bromphenol blue, and 0.02% xylene cyanole FF) was added, and the samples were subjected to electrophoresis in 6% sequencing gels. Gels were fixed in 10% methanol, 10% acetic acid and

dried *in vacuo*. DNA cleavage products were visualized with a Bio-Rad molecular imager FX.

RESULTS

Generation of Positively Supercoiled DNA Substrates—Steady-state interactions between eukaryotic topoisomerases and positively supercoiled DNA molecules have been examined previously (46, 58, 61–64). However, in most cases, positive superhelical twists were introduced in the DNA by the addition of intercalating agents such as ethidium bromide or chloroquine (58, 61–63). Because these chemicals locally underwind the double helix, they generate a compensatory global overwinding elsewhere in covalently closed circular DNA substrates. Although previous studies have provided useful information, they suffer from a common deficiency; it is not always possible to dissociate the effects of DNA topology on topoisomerase action from those of the intercalating agent. Thus, these studies have not been able to assess interactions between eukaryotic topoisomerases and positively supercoiled DNA in a quantitative manner.

To overcome this critical deficiency, we prepared positively supercoiled molecules by incubating pBR322 plasmid DNA with *A. fulgidus* reverse gyrase. This type I topoisomerase is the only enzyme unique to hyperthermophiles and the only known topoisomerase that can actively introduce positive superhelical twists into DNA (56, 65–68).

A time course for the conversion of negatively to positively supercoiled pBR322 DNA is shown in Fig. 2. The initial plasmid substrate contained ~15–17 negative superhelical twists/molecule ($\sigma \approx -0.035$ to -0.039) as determined by electrophoretic band counting in agarose gels containing chloroquine. This superhelical density is typical of plasmids isolated from *E. coli*. Reverse gyrase rapidly relaxed pBR322, and positive superhelical twists began to appear within the first minute of the reaction. As determined by electrophoretic band counting in gels containing netropsin B, positively supercoiled plasmids generated following a 5-min incubation with reverse gyrase contained ~15–17 positive superhelical twists/molecule ($\sigma \approx +0.035$ to $+0.039$). The handedness of positively supercoiled DNA was confirmed by two-dimensional gel electrophoresis (Fig. 3, *left panel*). Thus, the substrates employed for the experiments in this study contained equivalent numbers of superhelical twists but were of opposite handedness.

When resolved by one-dimensional gel electrophoresis, relaxation mixtures were run in the absence of intercalating dyes and subsequently stained with ethidium bromide. Despite the opposite handedness of the DNA substrates employed, similar resolution of negatively and positively supercoiled plasmids was observed. However, it is notable that positively supercoiled substrates bind less ethidium bromide than negatively supercoiled plasmids. Therefore, to ensure that equal amounts of initial substrates were used in all of the experiments, the DNA concentration was independently assessed by spectrophotometric analysis and

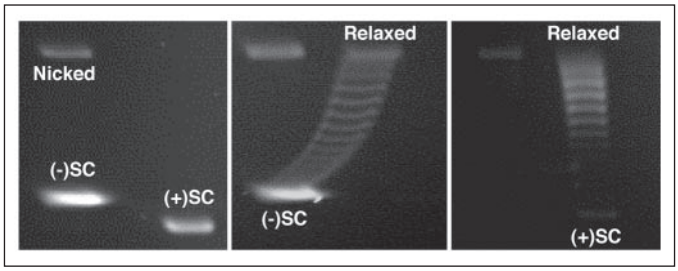

FIGURE 3. **Two-dimensional gel electrophoresis of negatively and positively supercoiled DNA relaxation by human topoisomerase IIα.** Ethidium bromide-stained agarose gels displaying representative relaxation reactions of negatively supercoiled (*(−)SC*) or positively supercoiled (*(+)SC*) pBR322 plasmid DNA by human topoisomerase IIα are shown. Assay mixtures contained 1 nM enzyme and 5 nM plasmid. The positions of negatively supercoiled, positively supercoiled, and nicked circular DNA in the absence of enzyme are shown in the *left panel*. Relaxation of negatively supercoiled DNA after 30 min is shown in the *center panel*. Relaxation of positively supercoiled DNA after 5 min is shown in the *right panel*. The positions of fully relaxed DNA products are indicated (*Relaxed*).

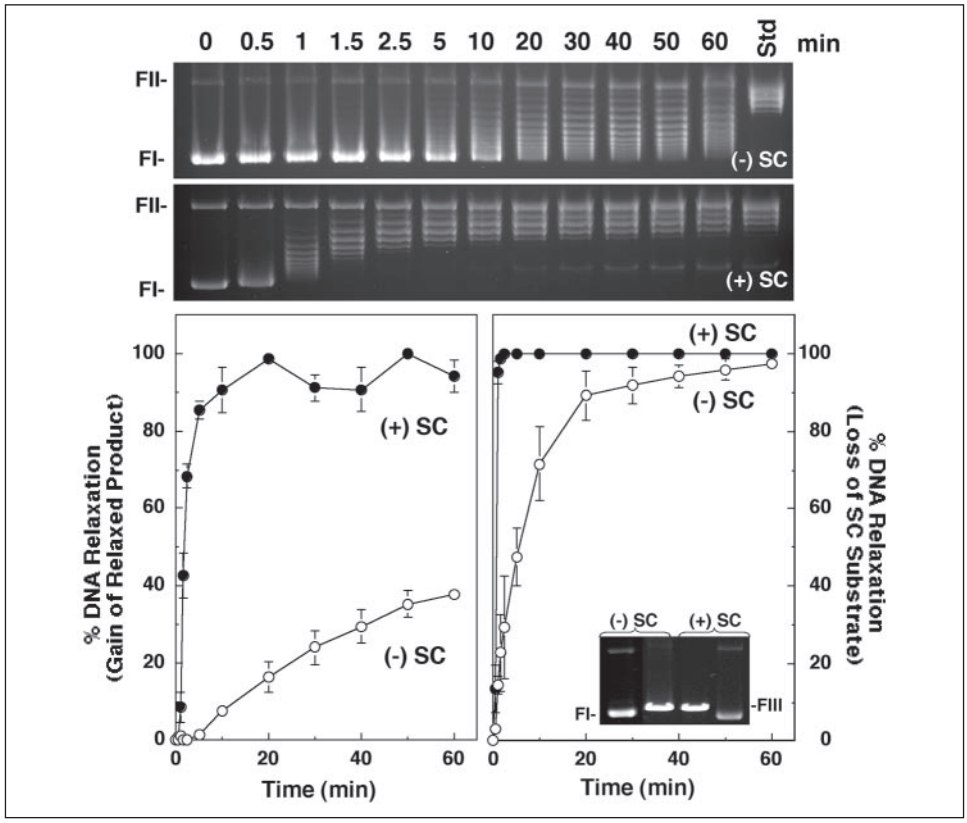

FIGURE 4. **Human topoisomerase IIα relaxes positively supercoiled DNA faster than negatively supercoiled molecules.** Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled (*(−) SC; top gel*) or positively supercoiled (*(+) SC; bottom gel*) pBR322 plasmid DNA by human topoisomerase IIα are shown. Assay mixtures contained 1 nM enzyme and 5 nM plasmid. Relaxed DNA standards (*Std*) were generated by incubation with 20 nM enzyme for 60 min. The positions of supercoiled plasmid DNA (form I, *FI*) and relaxed DNA (form II, *FII*) are indicated. DNA relaxation was quantified from either the gain of fully relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). *Error bars* represent the standard errors of the mean of two independent assays. Positively supercoiled DNA binds less ethidium bromide than does negatively supercoiled DNA. To demonstrate that equal amounts of the two substrates were employed in assays, linear digests of the plasmids are shown in the *inset*. Supercoiled plasmid DNA (form I, *FI*) and linear molecules (form III, *FIII*) are indicated.

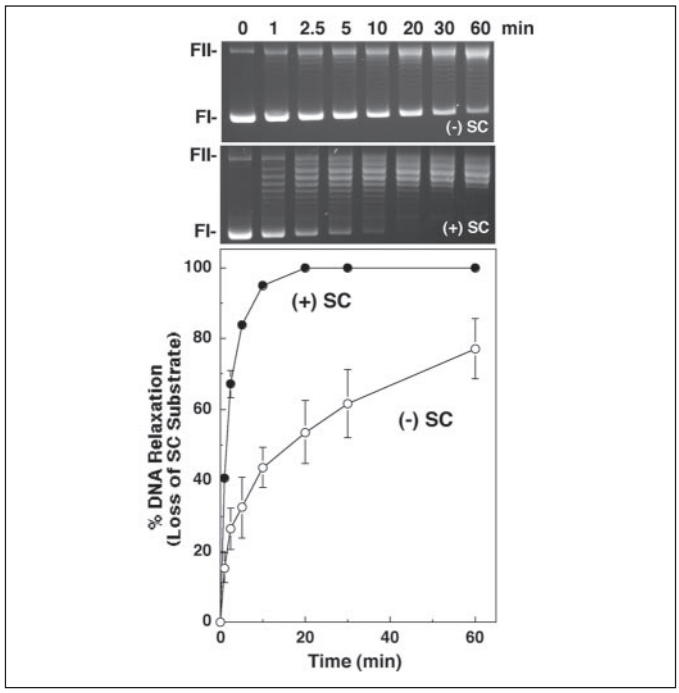

FIGURE 5. **Human topoisomerase IIα relaxes positively supercoiled DNA faster than negatively supercoiled DNA under processive conditions.** Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled (*(−) SC; top gel*) or positively supercoiled (*(+) SC; bottom gel*) pBR322 plasmid DNA by human topoisomerase IIα in 100 mM KCl are shown. Assay mixtures contained 1 nM enzyme and 5 nM plasmid. The positions of supercoiled plasmid DNA (form I, *FI*) and relaxed DNA (form II, *FII*) are indicated. Because reactions with both substrates were processive, DNA relaxation was quantified by the loss of supercoiled substrate. *Error bars* represent the standard deviations of four independent assays.

by ethidium bromide staining of linearized plasmid substrates (see *insets* in Figs. 4 and 9).

Human Topoisomerase IIα Preferentially Relaxes Positively Supercoiled DNA—If type II topoisomerases play a role ahead of replication forks in vertebrates, then positively supercoiled DNA should be the preferred relaxation substrate for topoisomerase IIα, the isoform that is involved in replicative processes. Therefore, to characterize interactions between human topoisomerase IIα and DNA substrates found ahead of replication forks, we assessed the ability of the enzyme to relax positively supercoiled molecules (Figs. 3 and 4).

Compared with relaxation of negatively supercoiled substrates, the enzyme removed positive superhelical twists at a much higher rate. In addition, topoisomerase IIα relaxed positive supercoils in a more distributive fashion. Because of this difference, relaxation rates were quantified by one-dimensional gel analysis using two complementary methods: gain of fully relaxed product or loss of fully supercoiled substrate (Fig. 4). As determined by the former method, the rate of relaxation of positively supercoiled DNA by topoisomerase IIα is >30-fold higher than the rate of relaxation of negatively supercoiled molecules. As determined by the latter method, it is nearly 10-fold higher.

Similar differences in relaxation rates were observed when reaction mixtures were resolved by two-dimensional gel electrophoresis (Fig. 3). The *center panel* shows the results of a 30-min relaxation assay with negatively supercoiled plasmid, and the *right panel* shows a 5-min assay with positively supercoiled substrate. It should be noted that as the DNA molecules approached their fully relaxed state, underwound plasmids remained slightly negatively supercoiled, and overwound plasmids remained slightly positively supercoiled. However, because the buffer conditions of the DNA relaxation assays and the gel electrophoresis are not identical, it is possible that the final distribution of reaction products differs somewhat.

The degree of DNA overwinding that occurs ahead of DNA replication forks in human cells is not known. However, single molecule experiments suggest that DNA ahead of tracking systems can reach a σ value as high as +0.110 (69). DNA buckling (*i.e.* the transition to non-B form structures) was not observed in these experiments below a σ value of +0.058 (69). Therefore, we believe that the positively supercoiled substrates used in our DNA relaxation assays ($\sigma \approx +0.035$ to +0.039) should be free of unusual non-B structures. However, to make certain that this was the case, additional DNA relaxation experiments were carried out using positively supercoiled substrates with a lower superhelical density ($\sigma \approx +0.022$). Similar high rates of DNA relaxation were observed with this substrate (data not shown). Thus, preferential relaxation by human topoisomerase IIα does not require extensive overwinding of the DNA.

The salt concentration utilized for DNA relaxation experiments, 175 mM KCl, represents the optimal condition for the relaxation of negatively supercoiled substrates. To determine whether the switch from a processive to a distributive reaction contributed to the preferential relaxation of positively supercoiled plasmids, additional assays were carried out at lower ionic strengths. The relaxation of positively supercoiled DNA switched to a fully processive reaction at 100 mM KCl (Fig. 5). Even under fully processive relaxation conditions, positively supercoiled DNA substrates were relaxed at a rate that was ~10-fold faster than that observed with negatively supercoiled plasmids. Therefore, the processivity of DNA relaxation by human topoisomerase IIα does not appear to be the underlying basis for the preferential removal of positive superhelical twists.

Human Topoisomerase IIβ Does Not Preferentially Relax Positively Supercoiled DNA—Although topoisomerase IIα and β differ dramatically in their physiological regulation and functions, the two isoforms display only minimal differences in their enzymatic properties (15, 16, 22, 27, 70). Because topoisomerase IIβ is not believed to play a role in DNA replication, it was of interest to determine whether this isoform also displayed a preference for positively supercoiled molecules. As seen in Fig. 6, topoisomerase IIβ relaxed positive and negative DNA supercoils at similar rates (less than 2-fold difference). Once again, relaxation of positively supercoiled molecules appeared to be more distributive. The recognition of positively supercoiled DNA by topoisomerase IIα represents the first major enzymological difference between the two isoforms. Furthermore, it is consistent with the proposal that topoisomerase IIα, and not topoisomerase IIβ, functions in DNA replication.

Effects of the Geometry of DNA Supercoils on Substrate Binding by Human Topoisomerase IIα—Previous studies indicate that the ability of human topoisomerase IIα to recognize DNA sequence or damage is controlled by chemical steps in the catalytic cycle of the enzyme rather than by alterations in substrate binding affinity (71–73). In contrast, the ability of eukaryotic type II topoisomerases to distinguish negatively supercoiled DNA from relaxed substrates appears to be governed primarily by binding interactions (61, 74–76). These enzymes differentiate between supercoiled and relaxed DNA topoisomers by preferentially interacting with DNA cross-overs, which are more prevalent in supercoiled molecules (61, 75). However, because both negatively and positively supercoiled molecules contain DNA nodes, albeit of opposite handedness, it is not known how topoisomerase IIα discriminates between underwound and overwound DNA.

As a first step toward addressing this critical issue, the relative affinity of the human enzyme for negatively and positively supercoiled plasmids

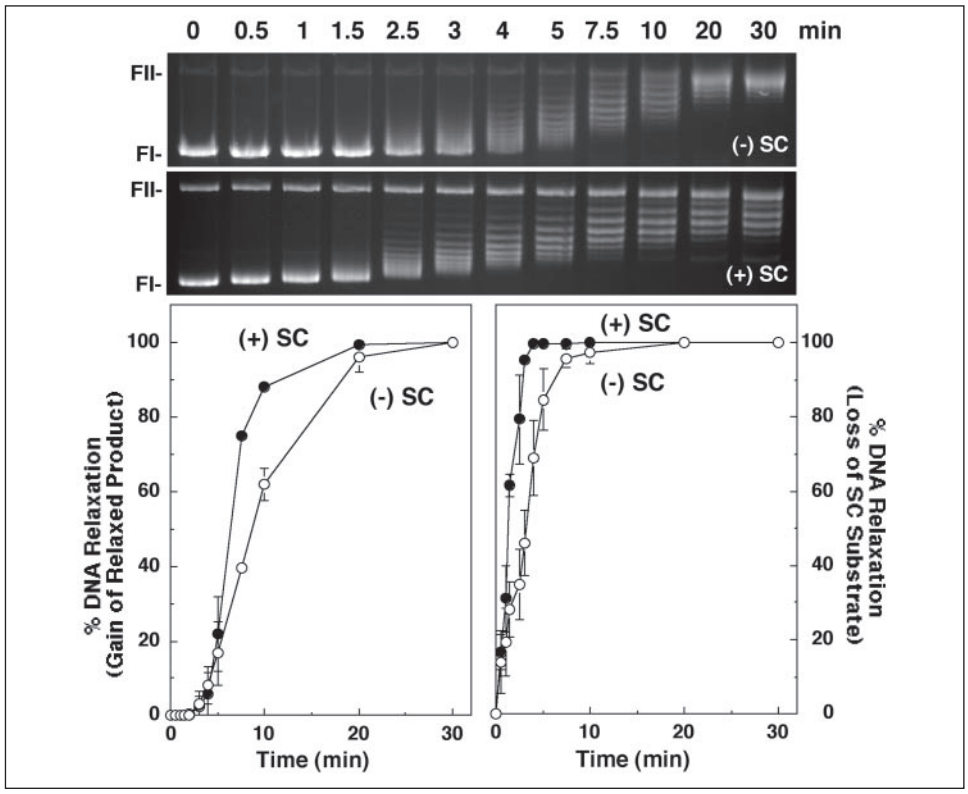

FIGURE 6. **Human topoisomerase IIβ does not preferentially relax positively supercoiled DNA.** Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled (*(−) SC*; *top gel*) or positively supercoiled (*(+) SC*; *bottom gel*) pBR322 plasmid DNA by human topoisomerase IIβ are shown. Assay mixtures contained 1 nM enzyme and 5 nM plasmid. The positions of supercoiled DNA (form I, *FI*) and relaxed DNA (form II, *FII*) are indicated. DNA relaxation was quantified from either the gain of fully relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). *Error bars* represent the standard errors of the mean or standard deviations of two or three independent assays, respectively.

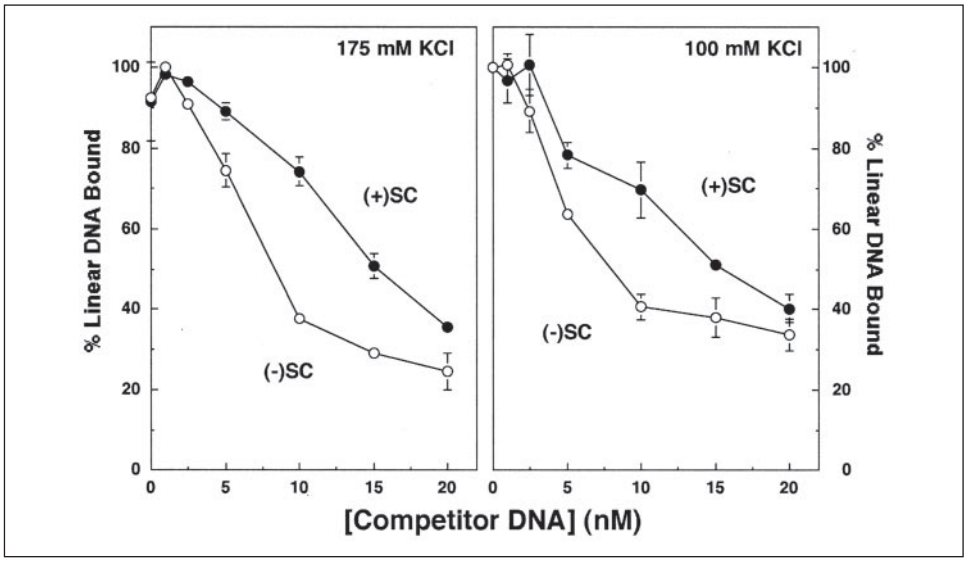

FIGURE 7. **Binding of human topoisomerase IIα to negatively and positively supercoiled DNA.** The ability of 0–20 nM negatively supercoiled (*(−) SC*) or positively supercoiled (*(+) SC*) pBR322 plasmid DNA to compete with the binding of 5 nM ^{32}P-labeled linear pBR322 DNA by human topoisomerase IIα is shown. The percentage of linear DNA bound was determined by the ratio of cpm retained on a nitrocellulose filter *versus* the input amount of radioactivity. *Error bars* represent the standard deviations of three independent assays.

was determined using a competitive DNA binding assay (Fig. 7). In this system, the ability of supercoiled plasmids to compete with radiolabeled linear pBR322 molecules for binding to topoisomerase IIα was monitored on nitrocellulose filters. The enzyme was exposed to both DNA substrates simultaneously, and the 6-min time point used for the assay ensured that a binding equilibrium was established (data not shown). DNA binding was analyzed at the two salt conditions, 175 and 100 mM KCl, used for DNA relaxation assays.

At either ionic strength, topoisomerase IIα displayed a slightly higher affinity (~2-fold) for negatively supercoiled plasmids as compared with positively supercoiled substrates (Fig. 7). This finding indicates that the rapid relaxation of positively supercoiled molecules by human topoisomerase IIα is not due to a higher binding affinity of the enzyme for its initial DNA substrate.

Effects of the Geometry of DNA Supercoils on ATP Hydrolysis by Human Topoisomerase IIα—DNA-bound topoisomerase II hydrolyzes more ATP than does free enzyme (58, 77–81). It has been proposed that the stimulation of ATP hydrolysis by DNA represents a nonproductive cycling of the enzyme in the "closed clamp" form and actually decreases the efficiency of ATP utilization (81). Therefore, if topoisomerase IIα relaxes positively supercoiled substrates more efficiently than it does negatively supercoiled DNA, it might hydrolyze lower levels of ATP during the course of the reaction. As seen in Fig. 8, the rate of ATP hydrolysis in the presence of positively supercoiled plasmid was ~2-fold

lower than the rate observed in reactions that contained negatively supercoiled DNA.

Human Topoisomerase IIα Maintains a Lower Level of DNA Cleavage Complexes with Positively Supercoiled Substrates—As a prerequisite to the strand passage event, topoisomerase II creates transient double-stranded breaks in its DNA substrate. To maintain genomic integrity during this process, the enzyme forms covalent bonds between active site tyrosyl residues and the 5′-terminal phosphates of the cleaved DNA (5, 15, 16, 19, 82). However, two negative outcomes are possible if a DNA tracking system, such as the replication machinery, collides with one of these cleavage complexes. First, the collision can disrupt the cleavage complex, making it impossible for the enzyme to ligate the cleaved strands (49, 83). Second, the collision can arrest the progress of the replication fork, which triggers fork restart and recombination pathways (84). Both of these outcomes eventually lead to the formation of double-stranded breaks in the genome (15, 16, 47–49, 85, 86).

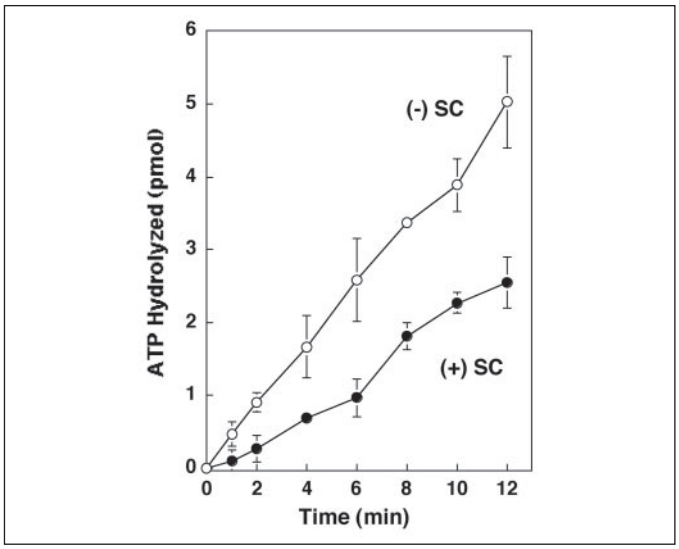

FIGURE 8. **Hydrolysis of ATP by human topoisomerase IIα in the presence of negatively and positively supercoiled DNA.** A time course for the hydrolysis of ATP in the presence of negatively supercoiled (*(−) SC*) or positively supercoiled (*(+) SC*) pBR322 plasmid DNA is shown. ATPase activity was monitored by quantifying the release of free phosphate from [γ-^{32}P]ATP. *Error bars* represent the standard deviations of three independent assays.

Because the DNA that precedes the replication fork is overwound, we characterized the ability of human topoisomerase IIα to cleave positively supercoiled plasmid molecules (Fig. 9). Over a range of enzyme:plasmid ratios, levels of DNA scission with positively supercoiled substrates were 3–4-fold lower than observed with negatively supercoiled plasmids. Similar results were seen when the physiological divalent cation, Mg^{2+}, was replaced with Ca^{2+} (Fig. 9, *inset*). This latter divalent cation supports higher levels of DNA cleavage (60, 87). Although DNA scission was reduced with overwound substrates, the site specificity of cleavage, as well as the relative site utilization, was identical with positively and negatively supercoiled DNA (Fig. 9).

DISCUSSION

The movement of the replication machinery on DNA results in the accumulation of positive superhelical twists ahead of the fork and precatenanes behind (3, 5–7, 11). Although the torsional stress of DNA overwinding can be alleviated by the actions of an enzyme that generates single-stranded breaks in the double helix, the untangling of daughter chromosomes can only be accomplished by an enzyme that creates double-stranded breaks (5, 7, 12, 14–16, 19). Thus, it has been assumed that topoisomerase I functions ahead of the replication fork, whereas topoisomerase II acts behind.

Previous *in vivo* studies on *S. cerevisiae* topoisomerase II (8, 9) and *E. coli* topoisomerase IV (50, 51) suggest a role for type II enzymes ahead of the replication fork. This suggestion is supported by *in vitro* studies, which indicate that topoisomerase IV preferentially relaxes positively

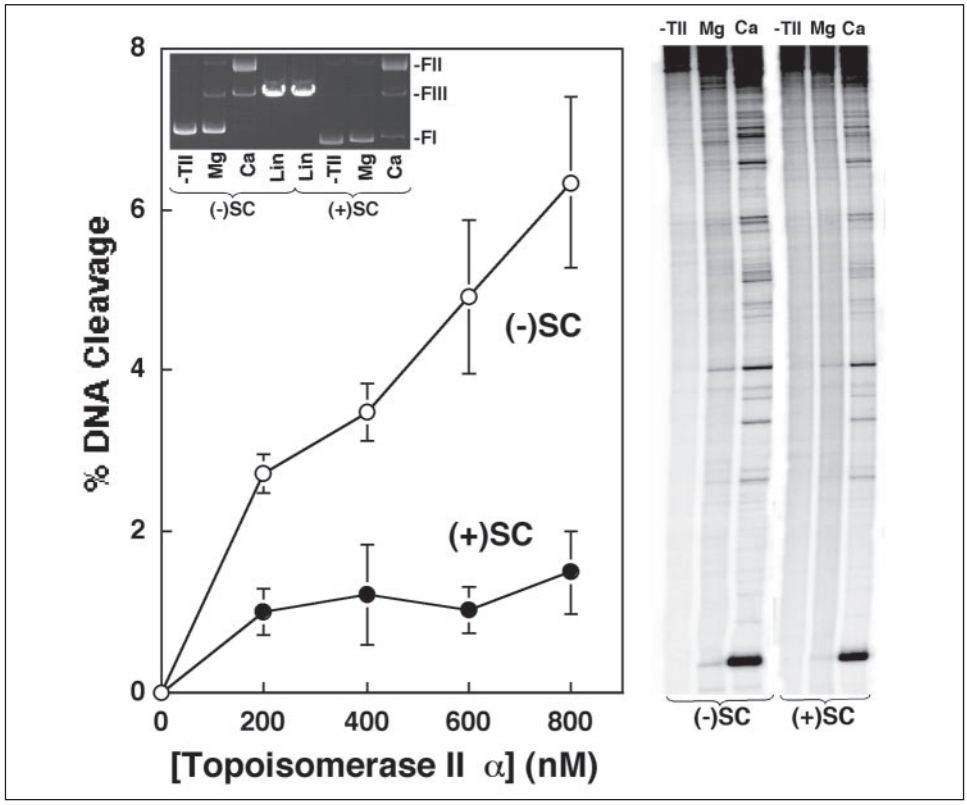

FIGURE 9. **Human topoisomerase IIα maintains lower levels of DNA cleavage complexes with positively supercoiled DNA than with negatively supercoiled molecules.** *Left panel*, the ability of 0–800 nM human topoisomerase IIα to cleave 10 nM negatively supercoiled (*(−) SC*) pBR322 plasmid DNA or positively supercoiled (*(+) SC*) molecules is shown. Assays employed Mg^{2+} as the divalent cation. *Error bars* represent the standard deviations of three to four independent experiments. The *inset* shows a representative ethidium bromide-stained agarose gel of DNA cleavage assays that contained 10 nM negatively supercoiled or positively supercoiled DNA plasmids and 600 nM topoisomerase IIα and utilized either Mg^{2+} or Ca^{2+} as the divalent cation. Supercoiled DNA from reactions that lacked topoisomerase IIα (*−TII*) and a linear (*Lin*) DNA standard are shown. The positions of supercoiled (form I, *FI*), nicked circular (form II, *FII*), and linear molecules (form III, *FIII*) are indicated. *Right panel*, DNA sites cleaved by human topoisomerase IIα were mapped in negatively supercoiled or positively supercoiled plasmid substrates. The products of DNA cleavage assays were linearized and singly end-labeled with [^{32}P]phosphate. The reaction products were visualized using a Bio-Rad molecular imager FX and are representative of three independent experiments. The reactions were carried out in the presence of 10 nM plasmid DNA and 2 μM enzyme and utilized either Mg^{2+} or Ca^{2+} as the divalent cation. DNA from reactions that lacked topoisomerase IIα (*−TII*) are shown.

supercoiled DNA (52, 53). To determine whether mammalian type II topoisomerases also display catalytic properties consistent with functions ahead of a replication fork, we characterized the ability of human topoisomerase IIα to relax positively supercoiled DNA.

Topoisomerase IIα displayed two important enzymological characteristics that would be beneficial to an enzyme that operates ahead of the replication machinery. First, the enzyme removed positive superhelical twists at a rate that was >10-fold faster than the rate for negative superhelical twists. Thus, topoisomerase IIα displays preferential activity with the DNA substrates that accumulate ahead of the fork. Furthermore, the rapid rate of catalysis with overwound substrates makes it less likely that the replication machinery would collide with a molecule of topoisomerase IIα acting on the prereplicated DNA. Second, the enzyme maintained lower levels of DNA cleavage complexes with positively supercoiled substrates. This decreases the probability that a collision with a replication fork would result in the formation of a topoisomerase II-associated double-stranded break in the genetic material. Taken together, these properties suggest that human topoisomerase IIα has the potential to alleviate torsional stress ahead of replication forks in an efficient and safe manner.

In contrast to the α isoform, topoisomerase IIβ, which is not believed to function in DNA replication, relaxed positively and negatively supercoiled substrates at similar rates. Relatively few studies on the enzymatic properties of topoisomerase IIβ have been reported. However, in every fundamental mechanistic aspect that has been compared, topoisomerase IIα and β are virtually identical (22, 27, 70). Results of the present study indicate a major difference between these two isoforms that is consistent with their proposed physiological roles. It is not clear how topoisomerase IIα, but not β, is able to distinguish the handedness of DNA supercoils. However, based on structural studies on DNA gyrase and topoisomerase IV, coupled with nucleic acid modeling and binding experiments, it has been proposed that this ability resides in the C-terminal domain of bacterial type II enzymes (64, 88–91).

The amino acid sequence of the C-terminal domain varies considerably from species to species. Although topoisomerase IIα and β possess a high degree of amino acid sequence identity in their catalytic cores (~79% identity), the two enzymes diverge significantly in their C-terminal domains (~31% identity) (92). The present observations make it tempting to speculate that the C-terminal domain of human topoisomerase IIα plays a role in sensing the geometry of DNA substrates. Studies with deletion mutants of the human enzyme currently are underway to test this hypothesis.

Lower eukaryotic species, such as *S. cerevisiae* and *Drosophila* contain only one isoform of topoisomerase II (20, 21). Single molecule experiments suggest that *Drosophila* topoisomerase II removes DNA supercoils of different handedness at similar rates (64). Steady-state experiments conducted as part of the present work (data not shown) support this conclusion. *Drosophila* topoisomerase II relaxed positively supercoiled plasmids at a rate that was marginally (<2-fold) higher than that obtained with negatively supercoiled molecules. Similar results were observed with *S. cerevisiae* topoisomerase II, which relaxed positively supercoiled plasmids ~2–3 times faster. It is not known why the ability to discern the handedness of DNA supercoils is not extended (or is only weakly extended) to eukaryotic organisms that contain only a single isoform of topoisomerase II. However, it may be related to the distinct separation of physiological functions of topoisomerase IIα and β in vertebrate species.

Human topoisomerase IIα and *E. coli* topoisomerase IV (52, 53) both preferentially relax positively supercoiled DNA. Although neither enzyme displays an increased affinity for positive DNA supercoils, they exhibit different attributes with overwound molecules with regard to DNA cleavage. Whereas topoisomerase IIα maintains lower levels of cleavage complexes with positively supercoiled substrates, topoisomerase IV displays higher amounts of DNA scission (52). Thus, there appears to be at least one fundamental difference between the modalities that these two enzymes use to distinguish the handedness of superhelical twists.

Numerous reports indicate that topoisomerase IIα is an important physiological target for anticancer drugs (15–17, 42, 43, 45). If the enzyme spends a significant period of time ahead of replication forks, the relevant nucleic acid substrate would be positively supercoiled DNA. However, previous studies used either negatively supercoiled or linear substrates to characterize the effects of drugs on the DNA cleavage activity of topoisomerase IIα. Because the handedness of superhelical twists has a strong influence on the ability of the human enzyme to cleave DNA, it will be of interest to re-examine drug actions when positively supercoiled molecules are used as cleavage substrates.

In summary, human topoisomerase IIα preferentially removes positive superhelical twists from DNA. This finding suggests that positively supercoiled DNA is the preferred physiological substrate for this enzyme and implies that topoisomerase IIα plays a role in relieving torsional stress that accumulates in front of the replication machinery or other DNA tracking systems. The high rates of DNA relaxation observed with positively supercoiled substrates, coupled with the low levels of DNA cleavage, makes topoisomerase IIα ideally suited to function ahead of replication forks.

Acknowledgments—We thank Jo Ann Byl for helpful discussions and for the human topoisomerase IIβ used in this study and Jennifer S. Dickey and Renier Vélez-Cruz for critical reading of the manuscript.

REFERENCES

1. Watson, J. D., and Crick, F. H. C. (1953) *Nature* **171,** 737–738
2. Watson, J. D., and Crick, F. H. C. (1953) *Nature* **171,** 964–967
3. Cozzarelli, N. R., and Wang, J. C. (eds) (1990) *DNA Topology and Its Biological Effects*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
4. Kanaar, R., and Cozzarelli, N. R. (1992) *Curr. Opin. Struct. Biol.* **2,** 369–379
5. Wang, J. C. (1996) *Annu. Rev. Biochem.* **65,** 635–692
6. Schvartzman, J. B., and Stasiak, A. (2004) *EMBO Rep.* **5,** 256–261
7. Wang, J. C. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 430–440
8. Brill, S. J., DiNardo, S., Voelkel-Meiman, K., and Sternglanz, R. (1987) *Nature* **326,** 414–416
9. Kim, R. A., and Wang, J. C. (1989) *J. Mol. Biol.* **208,** 257–267
10. Wang, J. C., and Liu, L. F. (1990) in *DNA Topology and Its Biological Effects* (Cozzarelli, N. R., and Wang, J. C., eds) pp. 321–340, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Peter, B. J., Ullsperger, C., Hiasa, H., Marians, K. J., and Cozzarelli, N. R. (1998) *Cell* **94,** 819–827
12. Osheroff, N. (1998) *Biochim. Biophys. Acta* **1400,** 1–2
13. Nitiss, J. L. (1998) *Biochim. Biophys. Acta* **1400,** 63–81
14. Champoux, J. J. (2001) *Annu. Rev. Biochem.* **70,** 369–413
15. Fortune, J. M., and Osheroff, N. (2000) *Prog. Nucleic Acids Res. Mol. Biol.* **64,** 221–253
16. Wilstermann, A. M., and Osheroff, N. (2003) *Curr. Top. Med. Chem.* **3,** 1349–1364
17. Pommier, Y., Pourquier, P., Fan, Y., and Strumberg, D. (1998) *Biochim. Biophys. Acta* **1400,** 83–105
18. Champoux, J. J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11998–12000
19. Wang, J. C. (1998) *Quart. Rev. Biophys.* **31,** 107–144
20. Wyckoff, E., and Hsieh, T. S. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 6272–6276
21. Goto, T., and Wang, J. C. (1984) *Cell* **36,** 1073–1080
22. Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T., and Mirabelli, C. K. (1989) *Biochemistry* **28,** 8154–8160
23. Levine, C., Hiasa, H., and Marians, K. J. (1998) *Biochim. Biophys. Acta* **1400,** 29–43
24. Velez-Cruz, R., and Osheroff, N. (2004) in *Encyclopedia of Biological Chemistry*, Vol. 1, pp. 806–811, Elsevier Science Publishing Co., Inc., New York
25. Ullsperger, C., and Cozzarelli, N. R. (1996) *J. Biol. Chem.* **271,** 31549–31555
26. Drake, F. H., Zimmerman, J. P., McCabe, F. L., Bartus, H. F., Per, S. R., Sullivan, D. M., Ross, W. E., Mattern, M. R., Johnson, R. K., and Crooke, S. T. (1987) *J. Biol. Chem.* **262,** 16739–16747

27. Austin, C. A., and Marsh, K. L. (1998) *BioEssays* **20,** 215–226
28. Wasserman, R. A., Austin, C. A., Fisher, L. M., and Wang, J. C. (1993) *Cancer Res.* **53,** 3591–3596
29. Jensen, S., Redwood, C. S., Jenkins, J. R., Andersen, A. H., and Hickson, I. D. (1996) *Mol. Gen. Genet.* **252,** 79–86
30. Meczes, E. L., Marsh, K. L., Fisher, L. M., Rogers, M. P., and Austin, C. A. (1997) *Cancer Chemother. Pharmacol.* **39,** 367–375
31. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) *Cell Growth & Differ.* **2,** 209–214
32. Heck, M. M., and Earnshaw, W. C. (1986) *J. Cell Biol.* **103,** 2569–2581
33. Hsiang, Y. H., Wu, H. Y., and Liu, L. F. (1988) *Cancer Res.* **48,** 3230–3235
34. Bauman, M. E., Holden, J. A., Brown, K. A., Harker, W. G., and Perkins, S. L. (1997) *Mod. Pathol.* **10,** 168–175
35. Isaacs, R. J., Davies, S. L., Sandri, M. I., Redwood, C., Wells, N. J., and Hickson, I. D. (1998) *Biochim. Biophys. Acta* **1400,** 121–137
36. Dereuddre, S., Delaporte, C., and Jacquemin-Sablon, A. (1997) *Cancer Res.* **57,** 4301–4308
37. Grue, P., Grasser, A., Sehested, M., Jensen, P. B., Uhse, A., Straub, T., Ness, W., and Boege, F. (1998) *J. Biol. Chem.* **273,** 33660–33666
38. Khodursky, A. B., Zechiedrich, E. L., and Cozzarelli, N. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11801–11805
39. Gootz, T. D., and Brighty, K. E. (1996) *Med. Res. Rev.* **16,** 433–486
40. Maxwell, A. (1997) *Trends Microbiol.* **5,** 102–109
41. Hooper, D. C. (1998) *Clin. Infect. Dis.* **27,** S54-S63
42. Hande, K. R. (1998) *Biochim. Biophys. Acta* **1400,** 173–184
43. Hande, K. R. (1998) *Eur. J. Cancer* **34,** 1514–1521
44. Takimoto, C. H., Wright, J., and Arbuck, S. G. (1998) *Biochim. Biophys. Acta* **1400,** 107–119
45. Li, T. K., and Liu, L. F. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41,** 53–77
46. Champoux, J. J., and Dulbecco, R. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 143–146
47. Holm, C., Covey, J. M., Kerrigan, D., and Pommier, Y. (1989) *Cancer Res.* **49,** 6365–6368
48. D'Arpa, P., Beardmore, C., and Liu, L. F. (1990) *Cancer Res.* **50,** 6919–6924
49. Howard, M. T., Neece, S. H., Matson, S. W., and Kreuzer, K. N. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12031–12035
50. Hiasa, H., and Marians, K. J. (1994) *J. Biol. Chem.* **269,** 16371–16375
51. Khodursky, A. B., Peter, B. J., Schmid, M. B., DeRisi, J., Botstein, D., Brown, P. O., and Cozzarelli, N. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 9419–9424
52. Crisona, N. J., Strick, T. R., Bensimon, D., Croquette, V., and Cozzarelli, N. R. (2000) *Genes Dev.* **14,** 2881–2892
53. Stone, M. D., Bryant, Z., Crisona, N. J., Smith, S. B., Vologodskii, A., Bustamante, C., and Cozzarelli, N. R. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8654–8659
54. Worland, S. T., and Wang, J. C. (1989) *J. Biol. Chem.* **264,** 4412–4416
55. Kingma, P. S., Greider, C. A., and Osheroff, N. (1997) *Biochemistry* **36,** 5934–5939
56. Rodriguez, A. C. (2002) *J. Biol. Chem.* **277,** 29865–29873
57. Fortune, J. M., and Osheroff, N. (1998) *J. Biol. Chem.* **273,** 17643–17650
58. Osheroff, N., Shelton, E. R., and Brutlag, D. L. (1983) *J. Biol. Chem.* **258,** 9536–9543
59. O'Reilly, E. K., and Kreuzer, K. N. (2002) *Biochemistry* **41,** 7989–7997
60. Baldwin, E. L., Byl, J. A., and Osheroff, N. (2004) *Biochemistry* **43,** 728–735
61. Madden, K. R., Stewart, L., and Champoux, J. J. (1995) *EMBO J.* **14,** 5399–5409
62. Fortune, J. M., Velea, L., Graves, D. E., and Osheroff, N. (1999) *Biochemistry* **38,** 15580–15586
63. Roca, J. (2001) *J. Mol. Biol.* **305,** 441–450
64. Charvin, G., Bensimon, D., and Croquette, V. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 9820–9825
65. Kikuchi, A., and Asai, K. (1984) *Nature* **309,** 677–681
66. Bouthier de la Tour, C., Portemer, C., Nadal, M., Stetter, K. O., Forterre, P., and Duguet, M. (1990) *J. Bacteriol.* **172,** 6803–6808
67. Forterre, P. (2002) *Trends Genet.* **18,** 236–237
68. Rodriguez, A. C., and Stock, D. (2002) *EMBO J.* **21,** 418–426
69. Mielke, S. P., Fink, W. H., Krishnan, V. V., Gronbech-Jensen, N., and Benham, C. J. (2004) *J. Chem. Phys.* **121,** 8104–8112
70. Leontiou, C., Lightowlers, R., Lakey, J. H., and Austin, C. A. (2003) *FEBS Lett.* **554,** 206–210
71. Kingma, P. S., and Osheroff, N. (1997) *J. Biol. Chem.* **272,** 1148–1155
72. Bromberg, K. D., Hendricks, C., Burgin, A. B., and Osheroff, N. (2002) *J. Biol. Chem.* **277,** 31201–31206
73. Velez-Cruz, R., Riggins, J. N., Daniels, J. S., Cai, H., Guengerich, F. P., Marnett, L. J., and Osheroff, N. (2005) *Biochemistry* **44,** 3972–3981
74. Osheroff, N., and Brutlag, D. L. (1983) in *Mechanisms of DNA Replication and Recombination* (Cozzareli, N. R., ed) Vol. 10, pp. 55–64, Alan R. Liss, Inc., New York
75. Zechiedrich, E. L., and Osheroff, N. (1990) *EMBO J.* **9,** 4555–4562
76. Roca, J., Berger, J. M., and Wang, J. C. (1993) *J. Biol. Chem.* **268,** 14250–14255
77. Miller, K. G., Liu, L. F., and Englund, P. T. (1981) *J. Biol. Chem.* **256,** 9334–9339
78. Halligan, B. D., Edwards, K. A., and Liu, L. F. (1985) *J. Biol. Chem.* **260,** 2475–2482
79. Lindsley, J. E., and Wang, J. C. (1993) *J. Biol. Chem.* **268,** 8096–8104
80. Oestergaard, V. H., Giangiacomo, L., Bjergbaek, L., Knudsen, B. R., and Andersen, A. H. (2004) *J. Biol. Chem.* **279,** 28093–28099
81. Vaughn, J., Huang, S., Wessel, I., Sorensen, T. K., Hsieh, T., Jensen, L. H., Jensen, P. B., Sehested, M., and Nitiss, J. L. (2005) *J. Biol. Chem.* **280,** 11920–11929
82. Berger, J. M., Gamblin, S. J., Harrison, S. C., and Wang, J. C. (1996) *Nature* **379,** 225–232
83. Hiasa, H., Yousef, D. O., and Marians, K. J. (1996) *J. Biol. Chem.* **271,** 26424–26429
84. Kaufmann, S. H. (1998) *Biochim. Biophys. Acta* **1400,** 195–211
85. Baguley, B. C., and Ferguson, L. R. (1998) *Biochim. Biophys. Acta* **1400,** 213–222
86. Hong, G., and Kreuzer, K. N. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5046–5051
87. Osheroff, N., and Zechiedrich, E. L. (1987) *Biochemistry* **26,** 4303–4309
88. Corbett, K. D., Shultzaberger, R. K., and Berger, J. M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 7293–7298
89. Hsieh, T. J., and Chan, N. L. (2004) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **60,** 564–566
90. Hsieh, T. J., Farh, L., Huang, W. M., and Chan, N. L. (2004) *J. Biol. Chem.* **279,** 55587–55593
91. Corbett, K. D., Schoeffler, A. J., Thomsen, N. D., and Berger, J. M. (2005) *J. Mol. Biol.* **351,** 545–561
92. Caron, P. R., and Wang, J. C. (1994) *Adv. Pharmacol.* **29B,** 271–297