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Regulation of the function of eukaryotic DNA topoisomerase I: Topological conditions for inactivity

(DNA topology/DNA supercoiling/mouse immunoglobulin κ light-chain promoter)

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ABSTRACT **The effects of supercoiling on the cleavage reaction by eukaryotic DNA topoisomerases I (wheat germ, chicken erythrocyte, and calf thymus) have been analyzed on DNA fragments (0.96 and 2.3 kilobases) encompassing an immunoglobulin κ light-chain promoter. In one topological condition of the substrate, the absolutely relaxed state, cleavage was found to be impeded. This finding defines the topology-dependent step of the eukaryotic DNA topoisomerase I reaction and shows that for the cleavage reaction topology is more critical than sequence effects. These findings suggest a simple model for the regulation of the DNA topoisomerase I reaction based on topological factors, which may explain the regulatory function of the enzyme in *in vivo* eukaryotic transcription.**

The enzymology of the DNA topoisomerase I reaction is known in detail (1), but the biological function of this reaction is not known. Isolation of conditional mutants has allowed the study of DNA topoisomerase I effects on transcription in both prokaryotes (1) and eukaryotes (2). The fact that expression of transfected DNA in eukaryotes depends on DNA topology (3) and that a topological "swivel" is needed in transcription supports the involvement of DNA topoisomerases I as regulators of DNA topology in this process (4). Nonsupercoiled closed circular duplex molecules are assumed to be substrates for DNA topoisomerase I (5), and even linear DNA has been commonly observed as a substrate for the nicking reaction (6–8). Therefore, one faces a paradox when addressing the function of DNA topoisomerase I: how can this enzyme regulate or be part of the regulation of DNA topology when its reaction has no apparent topological requirements? We report our analysis of the cleavage-step dependence of the DNA topoisomerase I reaction upon the topology of the DNA substrate. The study was performed with three different DNA topoisomerases I (wheat germ, chicken erythrocyte, and calf thymus) on topologically programmed forms of a 2.3-kilobase (kb) DNA segment encompassing the promoter and part of the coding sequences of the mouse MPC-11 cell line immunoglobulin κ light-chain (L_κ) gene and on a 0.96-kb subclone centered on the promoter region. The results show a strict topological requirement for the reactivity of the three enzymes and reveal that a distinct topological condition, the complete absence of torsional stress, impedes the function of DNA topoisomerases I.

MATERIALS AND METHODS

Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and Boehringer Mannheim. Ethidium bromide (EtdBr) and camptothecin were purchased from Sigma, and radiochemicals were purchased from NEN. Chicken erythrocyte DNA topoisomerase I was purified as reported (9); wheat germ and calf thymus DNA topoisomerases I were, respectively, from Promega Biotec and New England Biolabs. The DNAs used in this study are (*i*) 2318-base-pair (bp) *Xba* I–*Xba* I fragment encompassing 439 bp upstream of the RNA initiation site, the leader exon, the first intron, including the three remaining joining (J) (recombinational) sequences of the L_κ MPC-11 cell-line gene [this gene is a well-characterized system both for its conformational behavior under torsional strain (10) and for its sequence and functional properties (11, 12)]; and (*ii*) a 961-bp DNA subclone (the *Xba* I–*Hpa* II 904-bp segment inserted in the *Sma* I site of the polylinker of pUC18M) (9).

Circularization of DNA Fragments. Circularization was performed as described (9, 13) and was done with the specified concentration of EtdBr. Highly supercoiled topoisomers were obtained by ligation in the presence of EtdBr at 1.2 μg/ml. For the 2318-bp DNA, the resulting product has a $\Delta Lk = -\overline{24}$, where Lk is the linking number. Low ΔLk topoisomers were obtained by ligation at low concentration of EtdBr (9), illustrated in Fig. 4, and recovered from the gel as pure forms. For the 961-bp DNA the highest ΔLk obtained was equal to −12.

Analysis of the DNA Topoisomerase I Cleavage Sites. *The 2318-bp fragment.* The *Xba* I extremities of the purified 2318-bp fragment were terminally labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase before circularization. The fragments were circularized with EtdBr at 20°C. The ligated products were purified as described (9). After electroelution and purification, ≈2 ng of each topological form was treated with the indicated units of DNA topoisomerase I (defined in ref. 9) with 0.1 mM camptothecin (unless otherwise stated). Reactions were usually carried out in 150 mM NaCl/20 mM Tris·HCl, pH 7.9/10 mM $MgCl_2$ at 20°C, stopped with 1% SDS/10 mM EDTA (final concentration), and processed (8). Mapping of the DNA topoisomerase I cleavage sites was obtained by secondary restriction with *Bgl* II, located at position 2286 (Fig. 1). The DNA fragments produced by DNA topoisomerase I and restriction were identified on a sequencing gel (reference ladder *Eco*RI plus *Hin*dIII bacteriophage λ digest plus partial *Hae* III digest of pUR250). In the 2318-bp *Xba* I–*Xba* I fragment, the RNA initiation site is at position 439, and the *Bgl* II site is at position 2286; numbering is relative to the RNA initiation site (+1). In the reaction interrupted by camptothecin or by SDS, eukaryotic DNA topoisomerase I remains covalently bound to the 3′ extremity of the DNA (14). This bonding impedes migration of the DNA fragment in the sequencing gel and limits the analysis of cleavage sites on a circular, internally labeled large domain. Given that our analysis of the DNA topoisomerase I cleavage sites is performed on circularized DNA molecules, only cleavage sites on the strand indicated

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Abbreviations: EtdBr, ethidium bromide; L_κ, immunoglobulin κ light-chain gene.

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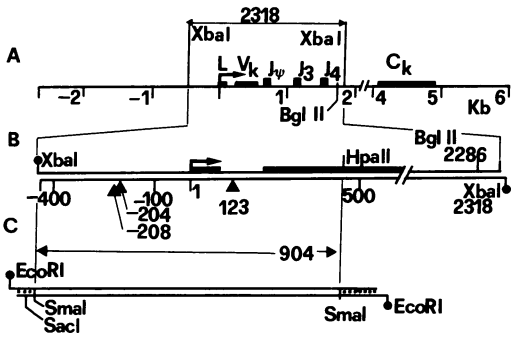

FIG. 1. Schematic maps of the DNA fragments. (*A*) General scheme of the MPC-11 L_κ gene. Joining (J) series equals recombination sequences. The 2318-bp *Xba* I–*Xba* I fragment used in the study is indicated. L, light chain; V_κ, variable region of κ; C_κ, constant region of κ. (*B*) Magnification of the 2318-bp region (5×). The *Bgl* II site is at position 2286 relative to the upstream *Xba* I site. ▲, DNA topoisomerase I cleavage sites (−208 ± 1; −204 ± 1; and +123 ± 3, numbering the RNA initiation site as 1). End-labeling (●) was at *Xba* I. (*C*) Segment subcloned in the pUC18M polylinker.

as lower (Fig. 1) were necessarily detected. A symmetrical analysis on the other strand revealed no predominant cleavage sites.

The 961-bp fragment. The same logic and procedures were applied. Labeling and ligation were at the *Eco*RI sites.

RESULTS

We have reported that the relaxation kinetics of supercoiled DNA differs from that of relaxed DNA (as measured by production of a Boltzman distribution from a single relaxed topoisomer) (15). The study of the kinetics of the relaxation reaction and its relation with DNA topology is complex because the several steps involved (binding, cleavage, resealing) (4) might all be influenced by the state of the substrate; the processive nature of the reaction adds to the complexity. To analyze the dependence of the DNA topoisomerase I reaction on the topology of the substrate, we have focused our analysis on the cleavage step.

Dependence of the Cleavage Reaction upon DNA Topology; the Frequency of Cleavage as a Function of the ΔLk. *The 961-bp DNA fragment.* The initial reaction (binding) of DNA topoisomerase I with its target site is poorly defined; strong binding sites have been described (16) that are not strong cleavage sites. Nevertheless (1), a cleavage position is necessarily a binding one, and both the sequence of the site and the position of the cleavage reflect the way in which a DNA topoisomerase I interacts with its substrate. Mapping of these cleavages has been performed in several systems. The results may be grouped in two categories: an aspecific cutting pattern seen in several systems that suggests that the DNA sequence alone places few limits on the access of the enzyme to DNA (6,7) and a highly specific localization of cuts (8) in *Tetrahymena* ribosomal genes.

As detailed below, in the L_κ gene fragments a large number of cleaved sites, produced by the three different eukaryotic DNA topoisomerases I, were detected. This cleavage shows that the I_κ promoter region behaves in this respect like the other aspecifically cleaved DNAs (6, 7) and not like the exceptionally selective system (8). The frequent occurrence of cleavage sites decreases interest in determining their surrounding sequences but facilitates study of the dependence upon topology for their induction. Fig. 1 shows a map of the whole light-chain gene, of the 2318-bp region analyzed, and of the 961-bp subclone derived from it. The topological dependence of the cleavage reaction was studied as follows. DNA molecules with different linking numbers were obtained by ligation of DNA fragments in the presence of defined concentrations of EtdBr. Individual topoisomers were prepared as described (9) and analyzed for cleavage by DNA topoisomerase I.

Fig. 2 shows that relaxed DNA (*A*, lanes 2–5) is cut much more poorly than the supercoiled form (lanes 6–9), as revealed both by the appearance of cut fragments (higher on supercoiled than on relaxed forms) and by the disappearance (faster on supercoiled than on relaxed forms) of the uncleaved full-length molecules (top band). Fig. 2*B* shows preferential cleavage of the supercoiled form as a function of the DNA topoisomerase I concentration. The same results were obtained analyzing the cleavage profiles induced by calf thymus DNA topoisomerase I on the other strand (data not shown) and by chicken erythrocyte (data not shown) or wheat germ DNA topoisomerase I on both strands. Fig. 2*C* (lanes 2–6) shows the cleavage pattern by wheat germ DNA topoisomerase I on the same strand reported for the calf thymus enzyme (*A*) as a function of enzyme concentration. This experiment shows that the cutting pattern is similar, although not identical, between phylogenetically distant enzymes, as has been reported (7). The fact that the amount

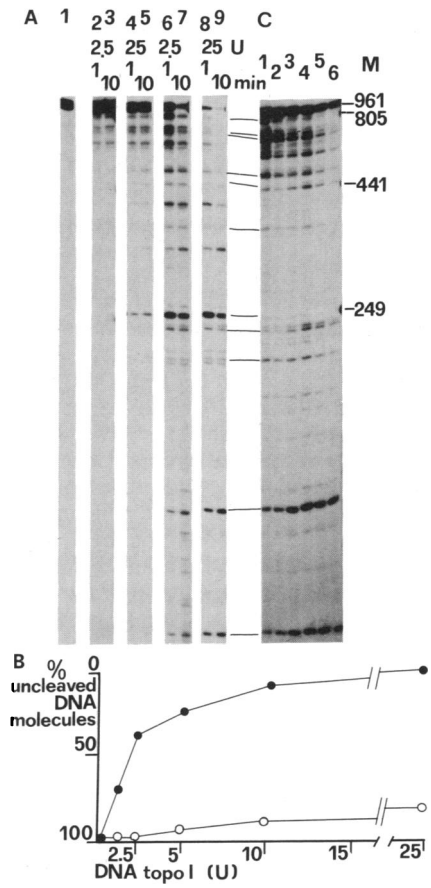

FIG. 2. Efficiency of cleavage of the 961-bp relaxed and supercoiled DNA domain. (*A*) Lanes 2–5. Relaxed DNA was treated with 2.5 or 25 units of calf thymus DNA topoisomerase I for 1 or 10 min. The relaxed form was the one isolated from preparative gel as $\Delta Lk = +1$. Secondary restriction was with *Sac* I. Lanes: 1, control with no DNA topoisomerase I; 6–9, identical treatments on the supercoiled form ($\Delta Lk = -12$). M, size markers. (*B*) Residual uncleaved molecules as a function of DNA topoisomerase I concentration on relaxed (○) and supercoiled (●) DNA. Enzymatic treatment (1 min) with the indicated amount of DNA topoisomerase (topo) I (abscissa). The residual uncleaved material is indicated (ordinate) as % of untreated sample. Data from *A* and similar experiments. (*C*) Supercoiled DNA treated as above with 0.8, 1, 2, 4, 6, and 8 units of wheat germ topoisomerase I.

of small fragments increases as a function of the concentration of DNA topoisomerase I shows that the DNA molecules undergo multiple cleavages.

In conclusion, analysis of the cleavage pattern by the various DNA topoisomerases I on topologically different forms of the 961-bp DNA fragment has shown that (*i*) the pattern of cleavage sites on the supercoiled DNA is qualitatively similar to that of the closed relaxed DNA [on supercoiled DNA, the reaction is kinetically favored (Fig. 2 *A* and *B*)] and (*ii*) at a given enzyme concentration localization of cleavage in the relaxed closed form of this DNA is very similar to that produced on open circles and on linear DNA (data not shown).

The 2318-bp DNA fragment. The low reactivity to cleavage of the relaxed DNA species is, in principle, unexpected because it has been reported that both positively and negatively supercoiled DNAs are substrate for eukaryotic DNA topoisomerase I (17) with about equal efficiency (18). However, our present observation agrees with our previous finding (15) that perfectly relaxed DNA is a poor substrate for topoisomerization. To discriminate between active and inactive substrates for both the cleavage and topoisomerization reactions, topological parameters of the DNA must be carefully identified and the system should allow programmed topological variations. These conditions are met in large DNA domains that allow programmed changes of their writhe without changes of their linking numbers.

We have therefore focused our analysis of the topology-dependence of the cleavage reaction on the 2318-bp domain. Fig. 3 shows an analysis of cleaving this DNA, tested in its supercoiled and relaxed forms. The experiment measures the disappearance of full-length molecules (arrow) caused by the first hit of the enzyme. This is the only parameter measurable in cleavage reactions performed in large DNA domains with camptothecin, because in the 2318-bp DNA domain the large number of cleaved sites seen in the 961-bp DNA increases so much as to smear the distribution. The dependence of cleavage upon topology is evident (Fig. 3*B*).

Perfectly Relaxed Closed Circles Are Substrates that Are Cleaved by DNA Topoisomerase I at a Slower Rate. The basic topological property of a closed circular DNA molecule is its constant linking number (*Lk*). This topological entity was originally described as the sum of the number of twists (*Tw*) that either strand forms around the central axis of the molecule and of the writhing (*Wr*) number that measures the shape of the central axis in the equation $Lk = Tw + Wr$ (19).

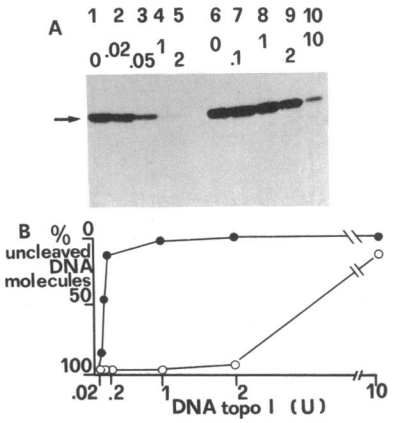

FIG. 3. Efficiency of cleavage of the 2318-bp relaxed and supercoiled DNA domain. (*A*) Supercoiled (lanes 1–5) and relaxed (lanes 6–10) were treated (conditions as for Fig. 2, 1 min) with the indicated units (U) of calf thymus DNA topoisomerase I. Arrow, full-length molecules. (*B*) Residual uncleaved material (%; ordinate) as a function of enzyme concentration (abscissa). ●, Supercoiled DNA; ○, relaxed DNA; topo, topoisomerase.

Closed DNA molecules change the partitioning of *Lk* between *Tw* and *Wr* according to thermal and ionic conditions. We find that this conformational variation is relevant for the reactivity of the DNA toward DNA topoisomerase I (see below) and should therefore be carefully defined in each experimental system.

Definition of topological parameters. Fig. 4*A* shows the products of ligation at 2°C without (lane 1) or with different amounts of EtdBr (lanes 2–7). Fig. 4*B* shows the topoisomers obtained as a function of the temperature of ligation from 4°C to 40°C (topoisomers +1 and 0 are evident only in underexposures or in gels run in the presence of EtdBr) (data not shown). Fig. 4*C* is the scanning analysis of the distribution of topoisomers shown in *B*; the calculated ΔLk is tabulated in *D*. It can be easily observed that the calculated untwisting [(0.012° per °C)·(*n*) where *n* = number of bp] due to the temperature at the moment of ring closure (5, 20) is also valid for this system. For a fragment of 2318 bp, untwisting of 360° will be caused by an increase of 14°C.

Ionic effects. Fig. 4*E* shows that one topoisomer isolated in Tris acetate buffer as $\Delta Lk = +2$ migrates as $\Delta Lk = 0$ in a gel run at the same temperature in DNA topoisomerase I buffer (and similarly a topoisomer isolated as $\Delta Lk = +3$ runs as +1, and $\Delta Lk = +4$ as +2). The same result is true when the second run is performed in ligase buffer (9). Thus, the topoisomer actually relaxed in ligase and/or topoisomerase buffer at 20°C is the one isolated as $\Delta Lk = +2$ in agarose gel in Tris acetate buffer at 20°C.

Temperature effects. Fig. 4*F* shows that a topoisomer isolated at 20°C as $\Delta Lk = +2$, when run in the same buffer at 27°C (lane 1) remains +2, but when the run is performed at 34°C (Δt = 14°C), its writhe changes by +1 (lane 4), as predicted. Similarly a $\Delta Lk = +3$ at 27°C (lane 3) has writhing = +4 at 34°C (lane 5). Thus, a topoisomer isolated in the position of $\Delta Lk = +2$ at 20°C goes back to the position of $\Delta Lk = 0$ (actually relaxed) in topoisomerase buffer; the increase in temperature causes strand untwisting in a predictable amount (5, 20).

Decreased reactivity as a function of the decreased distance from the topological zero. Camptothecin, a specific inhibitor that blocks resealing of the cleavage by a DNA-bound topoisomerase I molecule, was used to reveal the largest possible number of DNA sites active in the topoisomerization reaction. Reactions of eukaryotic DNA topoisomerase I, run without camptothecin, revealed fewer cleaved sites than with camptothecin (21). Accordingly, the smear-like appearance of the cleavage pattern of the 2318-bp domain with camptothecin (Fig. 3) is partially lost in reactions performed in its absence, and three sites slightly predominant over the background appear that map at +123 (±3), −208, and −204 (±1) on the lower strand (see Fig. 1, mapping not shown). Analysis of the variations of the intensity of these sites allows the study of the cleavage reactions in DNA forms characterized by minor topological variations.

The topoisomer that has been isolated as having an apparent $\Delta Lk = +2$ at 20°C assumes writhing 0 in topoisomerase buffer (see Fig. 4*E*); it does not behave as a substrate for the cleavage reaction at 20°C (Fig. 5, lane 1). At 27°C untwisting begins, and cleavage becomes detectable (lane 2); at 34°C the effect is clear (lane 3). On the other hand, the topoisomer that has been isolated as having an apparent $\Delta Lk = +3$ assumes writhing equal to +1 because of the same buffer variation, and the topoisomer is still reactive at 20°C (lane 5), as expected for a positive topoisomer (17, 18). However, because of the increased temperature, the topoisomer loses the capacity to be cleaved (lanes 6 and 7). In this case temperature untwists a slightly overtwisted conformation, therefore reducing its original reactivity. Lane 4 is a control run in parallel on a highly supercoiled topoisomeric family ($\Delta Lk = -24$); scans of these data also are reproduced.

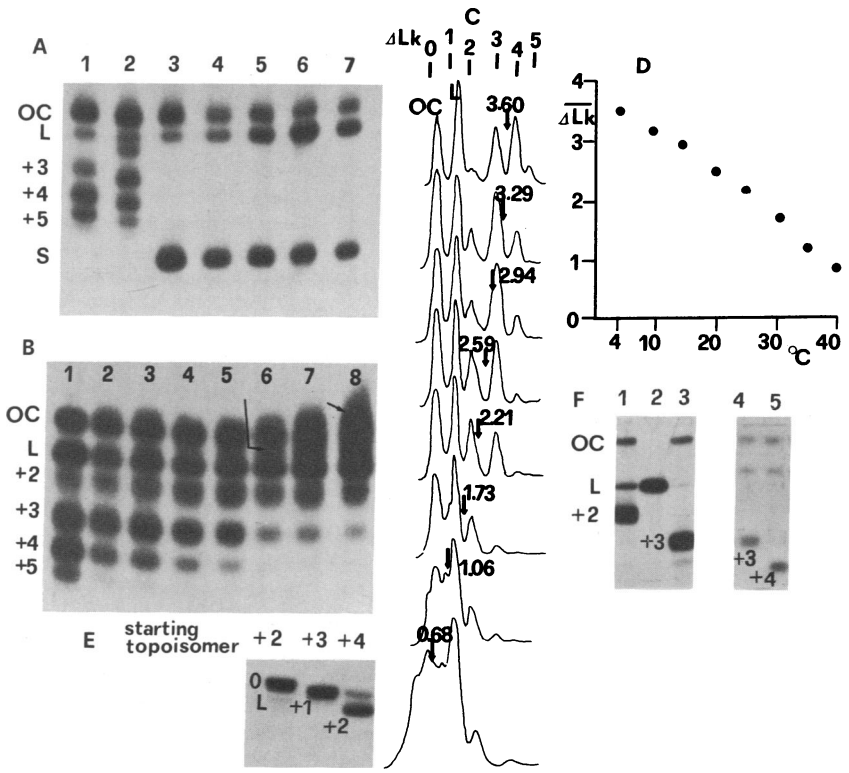

FIG. 4. Topological parameters of the system. (*A*) Products of ligation of the 2318-bp *Xba* I–*Xba* I fragment at 2°C without (lane 1) or with various concentrations of EtdBr (0.2, 0.5, 0.7, 1.0, 1.2, 1.5 μg/ml; lanes 2–7, respectively). S, supercoiled. (*B*) Products of ligation without EtdBr at 4, 10, 15, 20, 25, 30, 35, and 40°C (lanes 1–8, respectively). Arrows, topoisomers +1 and 0. (*C*) ΔLk calculated from scanning of the data reported in *B*. (*D*) Plot of the ΔLk reported in *C* as a function of the temperature of ligation. (*E*) Effect of variation of electrophoretic buffer on writhing. Topoisomers were isolated from gel run in Tris acetate buffer as ΔLk = +2, +3, or +4 and rerun in topoisomerase buffer. (*F*) Topoisomers isolated as ΔLk = +2 or +3 from gels at 20°C were rerun at 27°C (lanes 1 and 3) or at 34°C (lanes 4 and 5). OC, open circular; L, linear.

These results show that the important parameter for the reactivity of a given topoisomer is not its apparent writhing value in the conditions of preparative gel electrophoresis but, rather, the topological condition induced by the ionic and thermal environment of the DNA topoisomerase I assay. The difference between preparative and reaction conditions also explains the nonreactivity of topoisomers isolated as "apparently positive" forms, the conformations of which change in topoisomerase buffer. Truly positive topoisomers are also reactive toward DNA topoisomerase I (17, 18) in this system (data not shown).

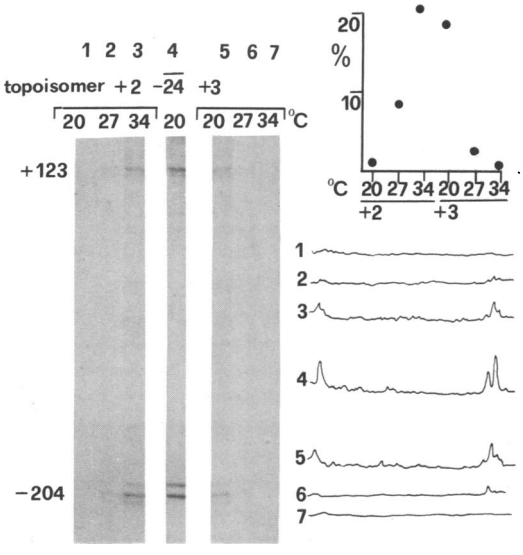

FIG. 5. Frequency of cleavage in the proximity of the relaxed condition. Lanes: 1–3, cleavage sites on the topoisomer isolated as +2 at 20°C and treated with DNA topoisomerase I at 20°C, 27°C, and 34°C; 4, on supercoiled DNA ($\Delta Lk = \overline{24}$) at 20°C; 5–7, cleavages on topoisomer +3; DNA topoisomerase I treatment at 20°C, 27°C, and 34°C. (*Lower right*) Scans. (*Upper right*) The graph shows the intensity of the band generated by cleavage at position −204 for each reported treatment (as % relative to intensity of the band produced on the topoisomer $-\overline{24}$).

DISCUSSION

Supercoiling of closed circular substrates was indicated in early studies (17) as a requirement for the action of eukaryotic DNA topoisomerase I. Conversely, results from a later analysis (5) were taken as definitive proof that closed relaxed topoisomers could generate species with higher and lower linking number and that therefore linear and nicked circular DNAs could also be considered as substrates for the enzyme. In that study, however, relaxed topoisomers were isolated from gel electrophoresis run with 5 mM Mg^{2+}, and treatment with DNA topoisomerase I was performed under different conditions (at 37°C for 24 hr, without Mg^{2+}) (5). Because of the known effects of ionic strength and temperature on superhelicity, caution should be exercised in establishing what actually is the relaxed state of DNA in the topoisomerization reaction. A measurement of avian topoisomerase I reactivity with DNA by a complex trapping method (22) has shown preferential cleavage of supercoiled DNA.

We show that both the nicking–closing reaction (15) and the cleavage reaction by eukaryotic DNA topoisomerases I are extremely sensitive to subtle topological variations (Figs. 2, 4, and 5)—both in the overtwisted and in the undertwisted

side of the relaxed conformation. When relaxed or close-to-relaxed DNA forms (as the ones studied in Fig. 5) undergo moderate torsional strain, the onset of writhing is not favored, and only alteration of twist occurs (23, 24). Therefore, the experiments reported in Fig. 5 show that variations of twisting are relevant for the activation or inactivation of the cleavage reaction and that unitary changes in writhing are not a mandatory requirement for changes of cleavage activity. We were unable to determine with our experimental system whether and how conditions of partial writhing affect reactivity. The reported data explain the discrepancy with the previously reported analysis (5) and establish that a condition exists in which DNA topoisomerase I is nonreactive. Accurate evaluation of the physicochemical and topological conditions of the assay is necessary (Fig. 4) to observe the narrow interval of topological conditions that causes nonreactivity (Fig. 5).

Nonreactivity is relative, not absolute: the cleavage reaction of perfectly relaxed circular DNA is several orders of magnitude slower than for supercoiled DNA (15). Interestingly, it is even slower than the cleavage of linear molecules (data not shown). The difference in the kinetics of nicking between linear and relaxed circular DNA suggests that a circle is torsionally locked in a way that a linear molecule is not. In addition to RNA polymerase II studies (9) this system is the only one that allows evaluation of a differential reactivity among different topological forms and opens to analysis the effects of twisting flexibility of linear molecules on their interaction with proteins in general. Chromatin structure does not limit the accessibility of DNA to this enzyme (25).

Given the physiological conditions of enzyme excess and accessibility of substrate sites, we suggest that one level of regulation of DNA topoisomerase I is topological, according to the following simple regulatory scheme—every time DNA changes its conformation by interaction with proteins, by the unwinding related to initiation of transcription, by environmental variations, by torsional stress associated with linking deficiency, by removal of a nucleosome, etc., DNA topoisomerase I recognizes the distortion and returns DNA to the relaxed conformation. According to our observations, relaxation causes topological inactivation of DNA topoisomerase I. This view is also supported by the topological, sequence-independent nature of the cutting pattern.

Such mechanism of topological feedback may serve the major purpose of keeping the structure of DNA constant and preventing a continuous futile activity of the enzyme on relaxed DNA. The relevance of the first effect is obvious; in eukaryotic promoters changes in conformation in positions relevant to their function (TATA sequence, RNA initiation site, etc.) have been seen (9). The promoter region analyzed here also undergoes major conformational changes as a function of superhelical density [as determined by the analysis of the distribution of DNA sites that become hypersensitive to the single-strand-specific endonuclease P1 (10)]. Because it is very unlikely that DNA-interacting proteins are indifferent to major variations of DNA conformation, DNA conformation must be regulated or kept constant.

Should the behavior of the enzyme that we have seen *in vitro* reflect its *in vivo* properties, the role of DNA topoisomerase I would be that of a topological sensor, serving the purpose of keeping constant the conformation of the DNA domain placed under its topological control. The recent observations on involvement of DNA topoisomerase I in eukaryotic transcription *in vivo* (26) lend support to this hypothesis.

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