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Eukaryotic DNA topoisomerase I reaction is topology dependent

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ABSTRACT

The effects of supercoiling on the topoisomerization reaction by eukaryotic DNA topoisomerases I have been analyzed. The systems used were: DNA topoisomerase I from wheat germ, chicken erythrocyte and calf thymus on a 2.3 kb DNA fragment which encompasses the immunoglobulin κ-light chain (Lκ) promoter of the mouse plasmacytoma MPC11; S.cerevisiae DNA topoisomerase I on a 2.2 kb DNA fragment from the same organism which encompasses the regulatory and the coding region of the ADH II gene; wheat germ DNA topoisomerase I on the plasmid pUC18.

It was found in every system that lack of torsional stress prevents topoisomerization of the substrate.

A simple regulatory model of DNA topoisomerase I function, based on topological considerations, is presented.

INTRODUCTION

DNA topoisomerase I is an abundant and ubiquitous component of both pro- and eukaryotic chromatin (1). In spite of extensive purification, of detailed analysis of its in vitro reaction and of the isolation of mutants (recently reviewed: 2-4), the basic question of its physiological role remains unanswered. The fact that DNA topoisomerase I interacts in vivo with transcriptionally active genes but not with inactive ones (5) points to a role of this enzyme in transcription.

The bacterial type I enzyme relaxes only the negatively supercoiled form (6). Relaxed double-stranded DNA is not cleaved (7,8) by the bacterial DNA topoisomerase I which apparently requires a single stranded region for active reaction (9-11). These two properties (requirement for an unpaired region and preference of negatively supercoiled substrates) are presumably correlated (2,12). At the contrary, DNA topoisomerase I from several eukaryotic sources

in vitro relaxes both negatively and positively supercoiled DNA in the absence of a high energy cofactor (reviewed in 2). As for relaxed DNA, evidence has been provided -in contrast with earlier observations (13)-that it can be cleaved (14-17) and nick-closed (18) by the eukaryotic enzyme.

We have re-examined this question performing a kinetic analysis of the topoisomerization reaction by DNA topoisomerase I from various eukaryotic sources on single isolated topoisomers. We have found that relaxed closed circular DNA allows a topoisomerization reaction several orders of magnitude slower than torsionally stressed molecules.

MATERIALS AND METHODS

Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and Boehringer. EtdBr was purchased from Sigma, radiochemicals from NEN. Chicken erythrocyte DNA topoisomerase I was purified according to Martin *et al.* (19). The purified enzyme had a specific activity of 4 x 10^3 U/mg; units are defined as the amount of enzyme that relaxes completely 1 μg of pBR322 DNA in 30 min. at 37 ^{0}C in the low salt buffer reported below (this unit definition is similar to that described in 27). Wheat germ and calf thymus DNA topoisomerase I were purchased respectively from Promega Biotec, Madison Wisconsin, and New England Biolabs. *Saccharomyces cerevisiae* DNA topoisomerase I was purified in this laboratory by R. Negri from a protease$^-$ strain, according to (33) and had a specific activity of 10^4 U/mg.

The DNAs used in this study are: 1) A segment of an immunoglobulin gene: the $\varkappa$-light chain (L$\varkappa$) MPC11 2318 bp XbaI-XbaI fragment which encompasses 439 bps upstream of the RNA Initiation Site, the leader exon, the V region and part the second intron, including the three remaining J (recombinational signals) sequences. The MPC11 immunoglobulin gene is described in 20 and 21, the sequence is in 22 and in 23. 2) The *S.cerevisiae* alcohol dehydrogenase gene (ADH II), obtained from the recombinant plasmid ADHII.BS.pBR322 (32). This plasmid contains a BamHI-Sau3A 2.2 kb yeast fragment cloned in the BamHI site of pBR322. This fragment encompasses 1 kb of the upstream region and the complete coding sequence of the ADHII gene; the sequence is in (32). 3) pUC18 DNA, the standard vector, with one minor modification introduced in this laboratory (the HindIII site has been transformed in a EcoRI site).

Circularization of DNA fragments was performed according to minor modifications (reported in 24) of the procedure developed by Schon *et al.* (25). Circularization was carried out at 4 ^{0}C in the presence of the specified concentrations

of EtdBr. The most supercoiled topoisomer was obtained by ligation in the presence of 1.2 µg/ml EtdBr. For the Ig κ light chain DNA in preparative gel electrophoresis, this topoisomer has a ΔLk of $-\overline{24}$, as determined by a reference ladder of topoisomers obtained by partial relaxation of the highly supercoiled form (as described in 24 and 26). Topoisomers characterized by low ΔLk were obtained by ligation at low concentration of EtdBr (24 and 26), as detailed in the legend to figure 1, and were recovered from the gel as pure forms (see below).

For the Ig DNA, the XbaI extremities of the purified 2318 bp fragment were terminally labeled at the 5' end with (γ-32P) ATP and T4 polynucleotide kinase before circularization. The ligated products were separated by gel electrophoresis at 2 V/cm for 18 hours in a vertical apparatus (37x18x0.3) on 1.4% agarose, at 20 °C unless otherwise specified, with buffer composed of 40mM Tris, 20mM Sodium Acetate, 1mM EDTA, pH 7.9. These conditions resulted in good resolution of the 2318 bp DNA fragment and allowed isolation by cutting out bands of each single uncontamined topoisomer. Identification of the positions of bands was achieved by brief autoradiographic exposure of the wet gel. After electroelution and purification, the isolated topoisomers were treated with DNA topoisomerase I,in 20 µl of 10mM NaCl, 20mM Tris-HCl pH 7.9, 10mM MgCl2 at 20 °C. Time of reaction and units of DNA topoisomerase I are specified where appropriate. Reactions were stopped by addition of SDS and EDTA (final concentrations 1.0% and 10mM, respectively) and analyzed on 1.4% agarose gel electrophoresis.

The other DNAs used were treated according to the same procedure. The 2.2 kb yeast DNA was labeled and ligated at the BamHI extremities (see above). pUC18 was opened, labeled and ligated at the BamHI site. Given the similarity of the sizes of the DNA used (2.2, 2.3 and 2.7 kb), the writhing of the products of ligation were very similar and are shown only for the Ig DNA (fig.1 panel E).

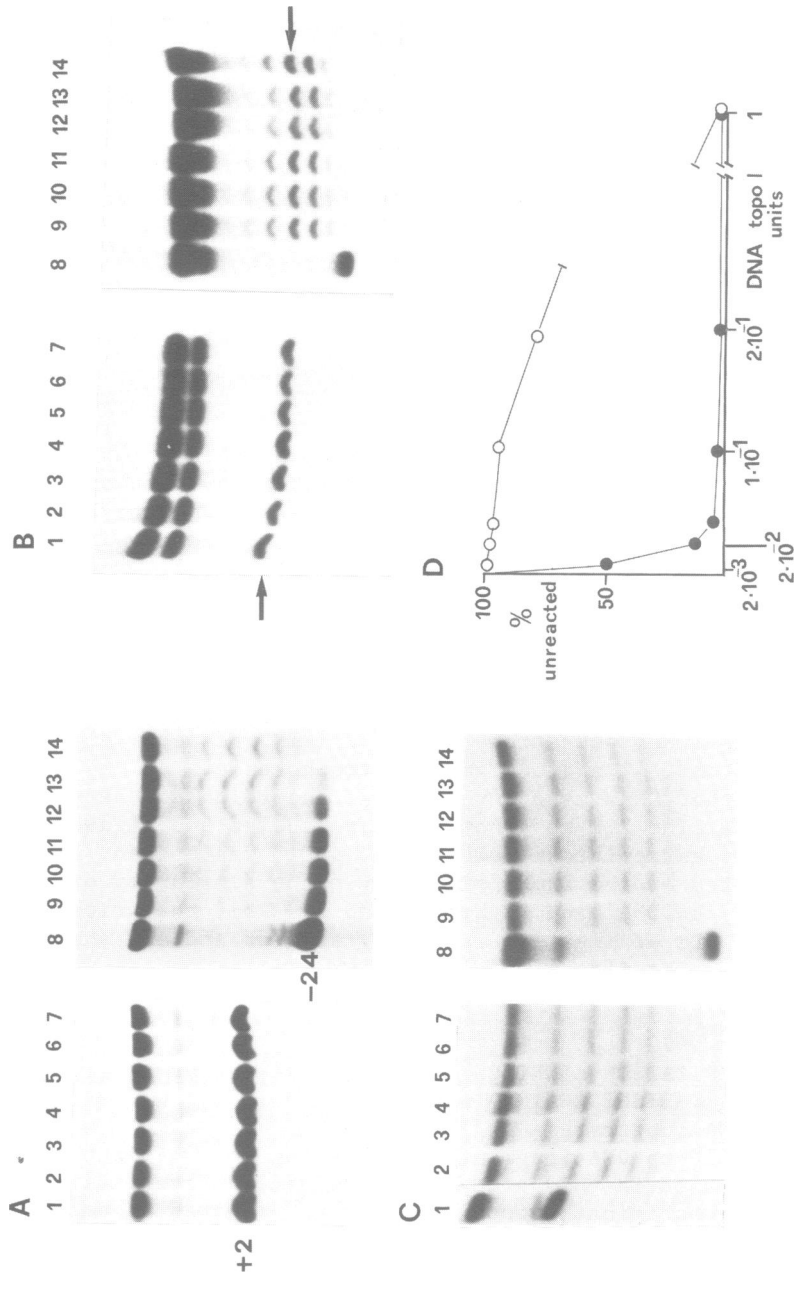
RESULTS

TOPOISOMERIZATION KINETICS OF RELAXED VERSUS SUPERCOILED DNA

1) THE Ig κ-LIGHT CHAIN

i) The DNA substrate

The 2318 bp DNA fragment encompassing the κ-light chain promoter used in this study is described in Materials. Single uncontaminated topoisomers were prepared as described (see Methods and legend to figure 1, panel E) and reacted with DNA topoisomerase I. Ligation at 4 °C (in the absence of intercalating agent) yields a family of topoisomers which


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
+2
−24
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14
D
100
50
% unreacted
2·10⁻³
2·10⁻²
1·10⁻¹
2·10⁻¹
1
DNA topo I units

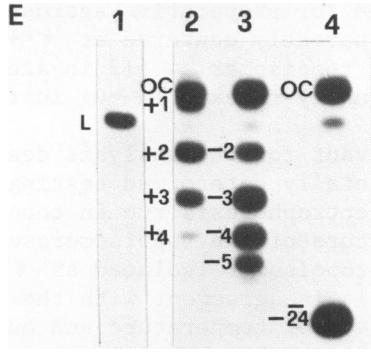


Figure 1: Kinetics of topoisomerization of $\tau=0$ and $\tau=-\overline{26}$ at various DNA topoisomerase I/DNA ratios (Ig κ-light chain DNA). Panel A: 2000 cpm (= 10 ng) of internally labeled purified closed circular DNA (isolated as $\tau=+2$) from preparative gel electrophoresis ($\tau=0$ in DNA topoisomerase buffer at 20 ^{0}C) were reacted with 2×10^{-2} U of chicken DNA topoisomerase I as specified (see Methods) for 0.5, 1, 2, 5, 20 and 90 min (lanes 2-7). Lane 1, no DNA topoisomerase I. Analysis in 1.4% agarose in the presence of 0.015 µg/ml EtdBr (to achieve better resolution of the final product of the topoisomerization reaction). Lanes 8-14, same as above for $\tau=-\overline{26}$. Panels B and C: as for panel A, with 1×10^{-1} and 1 DNA topoisomerase U, respectively. Panel D: % (ordinate) of the unreacted topoisomer after 5 min of treatment with the reported U of DNA topoisomerase I (abscissa). Data from panels A,B and C and from unreported experiments. Open symbols: relaxed; filled symbols: supercoiled. Panel E: The starting material. The products of ligation of the 2318 bp XbaI-XbaI fragment are shown. Lane 1: unligated linear (L). Lane 2: product of ligation at 4 ^{0}C in the absence of EtdBr. OC= open circular, +1, +2 etc.., positive topoisomers, numbered according to writhing number. Topoisomer +1 is visible where indicated between OC and L in underexposed autoradiograms (not shown). Lanes 3 and 4: products of ligation in the presence of 0.2 and 1.2 µg/ml EtdBr respectively. The sign of writhing is the expected one, taking into consideration the variation of temperature and buffer from the ligation reaction to the agarose gel electrophoresis. This was, in addition, verified by 2D gel electrophoresis (not detailed). The value of $\tau=-\overline{24}$ for the product of ligation at high EtdBr concentration has been determined by agarose gel electrophoresis run in the presence of EtdBr, with a reference ladder composed of products of partial topoisomerization (not detailed, see 24 and 26).

in the conditions used for preparative agarose gel electrophoresis is apparently centered at τ=+2 (figure 1, panel E, lane 2). The topoisomer τ =+2 in acetate buffer is the form which is actually relaxed (τ =0) in topoisomerase buffer at 20 °C.

This point is relevant for the analysis described below and has been experimentally determined testing isolated topoisomers in gel electrophoresis run in topoisomerase buffer at the temperature of the topoisomerase reaction: in these conditions the topoisomer isolated as τ =+2 runs as τ =0. This behaviour is in agreement with the original evaluation of the effect of temperature and buffer variations on DNA topology (2, 28) and has been experimentally verified also for the DNA used in the present study.

A direct proof that the τ =+2 in the electrophoretic Tris-Acetate buffer is the τ =0 in topoisomerase buffer is obtained also from relaxation experiments: see figure 1, panel B. The arrow on the left indicates the topoisomer τ =+2, considered to be the relaxed one in topoisomerase buffer at 20 °C. The arrow on the right indicates the center of the gaussian distribution of topoisomers produced by the topoisomerization reaction. The two positions coincide.

ii) Kinetics of topoisomerization of τ =0 and τ =-$\overline{26}$ at various E/DNA ratios.

Relaxed DNA (that is: the topoisomer isolated as τ =+2 in the described conditions) was treated for various times with the indicated amounts of chicken DNA topoisomerase I (figure 1, lanes 1-7 in panel A, B and C). The kinetics of topoisomerization have revealed that at low E/DNA ratio (up to $1x10^{-1}$ U of DNA topoisomerase I/10 ng DNA) relaxed DNA does not react appreciably. Only at higher ratios relaxed DNA becomes substrate and is eventually topoisomerized (see figure 1, panels C and D).

Negatively supercoiled DNA (τ = -$\overline{26}$) (figure 1, lanes 8-14 in panels A, B and C) is at the contrary promptly relaxed, even at the lowest E/DNA ratio tested ($2x10^{-3}$ U/10 ng). Panel D compares the reactivity of the two topoisomers.

In the conditions used (low salt) the topoisomerization is completely processive (29); this property is confirmed in our system, as shown by the absence of intermediate topoisomers. The final product is a gaussian family resulting from the thermal instability of the strand extremities at the time of the ring closure (28). The composition of this gaussian family at equilibrium is the complex result of the sum of several different kinetics, characterized by the low reactivity of the central topoisomer (fig.2 panel B), by the relatively higher reactivity of the lateral ones (ΔLk=$\pm$1 and $\pm$2), by a reactivity which increases with the increasing

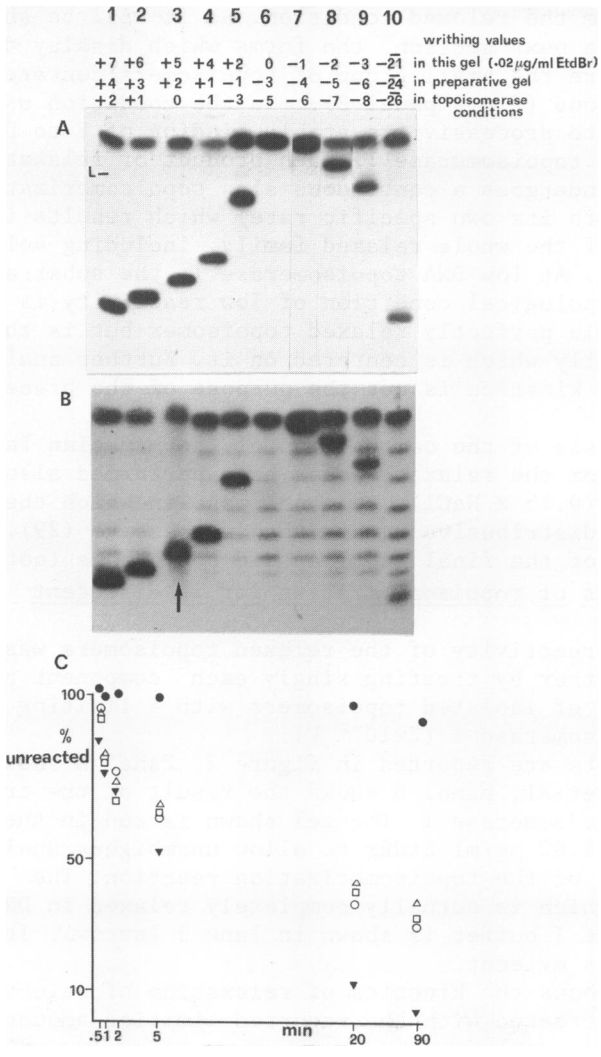


Figure 2: Topoisomerization of single topoisomers (Ig κ-light chain DNA). A: starting material. The writhing values indicated on top of each lane refer (upper line): to the writhing in the analytical gel shown (the gel is run in 0.02 μg/ml EtdBr). Middle line: to the original writhing in the preparative gel. Lower line: to the actual writhing value in the DNA topoisomerase buffer. B: the result of 2 min of topoisomerization by 2×10^{-2}U of DNA topoisomerase I. C: relaxation kinetics for selected topoisomers. Ordinate : % unreacted topoisomer; abscissa: time (min); ●: τ=0, ○: τ=+1, △: τ=+2, □: τ=-6, ▼: τ=-$\overline{26}$.

distance from the relaxed condition. As it will be shown in detail in the next section, the forms which display decreased reactivity are the small group of topoisomers centered around the relaxed one (fig.2 panel B). In the condition used (low salt= complete processivity= stable binding of E to DNA, limiting DNA topoisomerase I) each product of relaxation presumably undergoes a continuous slow topoisomerization (each one with its own specific rate) which results in the production of the whole relaxed family, including self-reproduction. At low DNA topoisomerase I, the substrate which is in the topological condition of low reactivity is not one single perfectly relaxed topoisomer but is the gaussian family which is centered on it. Further analysis of this complex kinetics is not the purpose of the present study.

The analysis of the composition of the gaussian family resulting from the relaxation has been performed also at higher salt (0.15 M NaCl), in conditions in which the reaction is distributive rather than processive (29). The composition of the final gaussian did not change (not shown).

iii) Kinetics of topoisomerization for 10 different topoisomers.

The poor reactivity of the relaxed topoisomers was analyzed further by treating singly each component of a whole series of isolated topoisomers with a limiting amount of DNA topoisomerase I ($2x10^{-2}$ U).

The results are reported in figure 2. Panel A shows the starting material, panel B shows the result of the treatment with DNA topoisomerase I. The gel shown is run in the presence of 0.02 μg/ml EtdBr to allow unambigous analysis of the products of the topoisomerization reaction. The topoisomer which is actually completely relaxed in DNA topoisomerase I buffer is shown in lane 3 (arrow). Its lower reactivity is evident.

Panel C shows the kinetics of relaxation of selected topoisomers treated with the reported limiting amount of DNA topoisomerase I.

2) THE S.cerevisiae ADH II GENE

Kinetics of relaxation

The highly supercoiled form and a relaxed topoisomer (the topoisomer isolated as τ =+3, actual writhing in topoisomerase buffer=+1) were treated in parallel for various times with a limiting dose ($2x10^{-2}$ U) of yeast DNA topoisomerase I. Fig.3 shows that also for the yeast system the same strong kinetic difference between different topological forms exists. The topoisomers which are components of the relaxed family were analyzed further. Fig. 4 shows the relaxation kinetics of three different

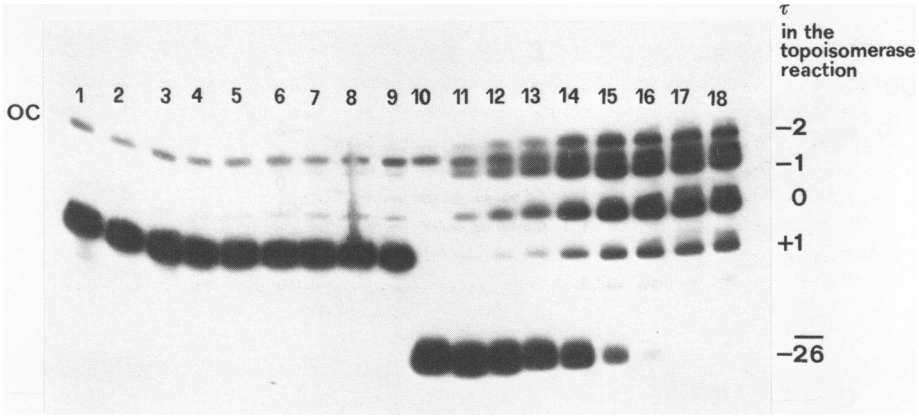


Figure 3: The S.cerevisiae system. Kinetics of topoisomerization of τ =+1 and τ =-$\overline{26}$ at low DNA topisomerase I. Experimental details as in fig.1. S.cerevisiae DNA topoisomerase I: 2x 10^{-2} U. Reaction times: 0, 0.5, 1, 2, 5, 20, 60, 120 and 180 min. (lanes 1 to 9 for τ =+1, lanes 10 to 18 for τ =-$\overline{26}$

topoisomers (τ =0, +2 and +3, actual writhing in topoisomerase buffer -2, 0 and +1) by a dose of topoisomerase 10-fold higher. As for the Ig DNA, the closer a DNA is to the relaxed state, the lower is its reactivity.

3) RELAXATION OF pUC18 DNA BY WHEAT GERM DNA TOPOISOMERASE I

Also this system displays a topology-dependent rate of relaxation. Fig.5 shows the relaxation of several topoisomers of the labeled pUC18 DNA (prepared and isolated as the other DNAs) as a function of the E/DNA ratio. Panel E quantitatively describes these data (compare this panel with fig.1 panel D).

DISCUSSION

We have shown that eukaryotic DNA topoisomerase I has a kinetic preference for nicking and closing torsionally stressed over relaxed DNA. Relaxed DNA is a poor substrate for DNA topoisomerase I: it becomes reactive to the enzyme only at high E/DNA ratios. The difference in reactivity among different topological forms is evident even when relaxed DNA is compared with DNA molecules differing only for 1 linking (figures 2, 4, 5). These analyses show that the perfectly relaxed state is the topological condition of lowest reactivity.

The data reported on Ig DNA have been obtained with chicken erythrocyte DNA topoisomerase I: identical behaviour

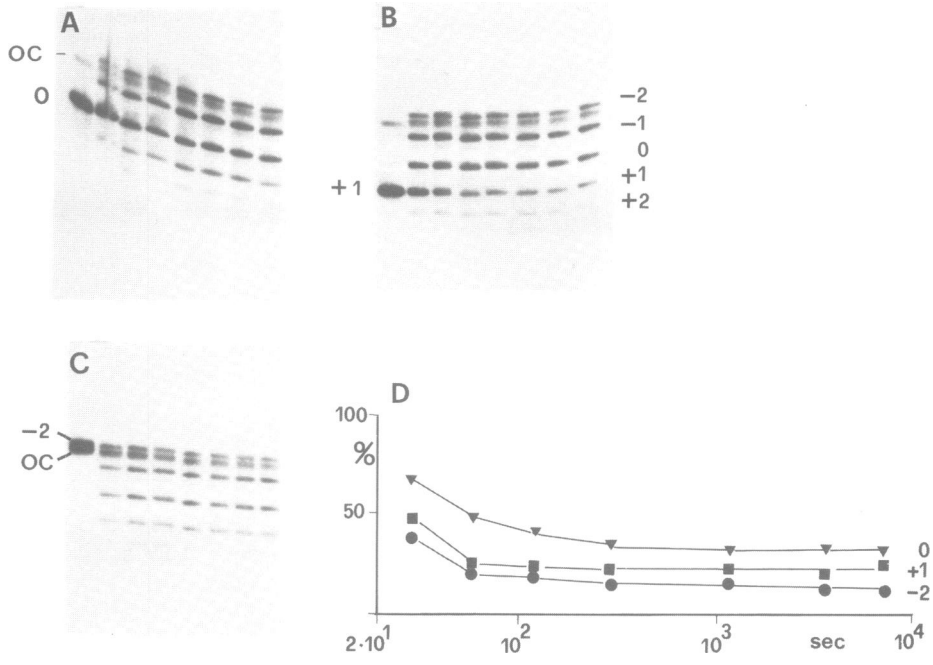


Figure 4: The S.cerevisiae system. Kinetics of topoisomerization at high DNA topoisomerase I of τ =+1, 0 and -2. S.cerevisiae DNA topoisomerase I: 2×10^{-1} U. Reaction times (from left to right in each panel): 0, 0.5, 1, 2, 5, 20, 60, 120 min. Panel A: topoisomer 0, Panel B: topoisomer +1, Panel C: topoisomer -2. The graph shows the kinetic of desappearance of each topoisomer (reported as % of the whole population, subtracted of the O.C. form).
τ =-2 reacts faster, followed by +1 and 0.

was observed for the wheat germ and calf thymus enzymes on the same DNA (not detailed). The results described for the Ig DNA have been faithfully duplicated in other heterologous (pUC18 DNA- W.G. DNA topoisomerase I) or homologous (both enzyme and DNA from S.cerevisiae) systems.

The observation that in each one of the three systems analyzed a ΔLk=1 is sufficient to stimulate the topoisomerization reaction (see figures 2, 4, 5) explains the apparent discrepancy between the data reported in this paper and previous observations (i.e. ref. 18 and reviewed in 2 and 3).

Given the kinetic preference for nicking and closing torsionally stressed over fully relaxed DNA, when the

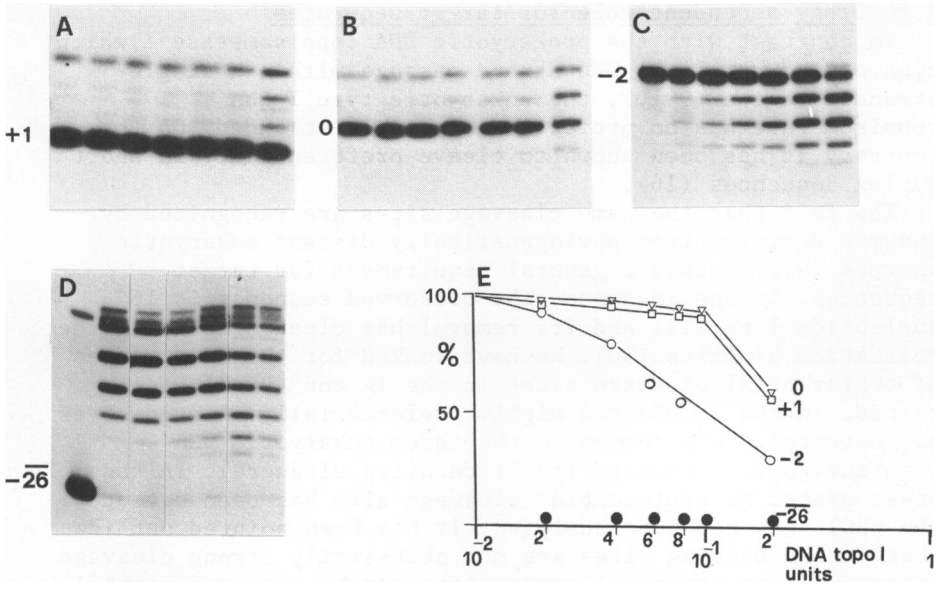


Figure 5: The pUC18 system. Relaxation of various topoisomers as a function of the DNA topoisomerase I/DNA ratio.
Panel A: τ =+1. B: τ =0. C: τ =-2. D: highly supercoiled (τ=-$\overline{26}$). For each panel the amount of Calf Thymus DNA topoisomerase I is (from left to right): 0, $2x10^{-2}$, $4x10^{-2}$, $6x10^{-2}$, $8x10^{-2}$, 10^{-1}, $2x10^{-1}$ U/assay. Panel E: graphical representation of the data reported in fig.5. Each line is the residual amount of indicated topoisomers reported as % of whole population (subtracted of the Open Circular).
□ =+1, △ =0, ○ =-2, ● =-$\overline{26}$.

thermal and ionic conditions used for the isolation differ from those used for the topoisomerization reaction (as it is commonly the case), the supposedly relaxed molecule undergoes a topological modification which is sufficient to activate the reaction and to modify an otherwise unreactive state. Other reasons for the previously unnoticed lack of reactivity of relaxed DNA are: 1) the excess of DNA topoisomerase I and 2) the long reaction periods which are normally used; these extreme conditions prevent the analysis of the dependence of topoisomerization on topology.

There is no way at present to distinguish among several possible mechanisms responsible for the observed topological sensitivity of DNA topoisomerase I. We hypotesize a central role of target DNA sequences in this process.

A topology dependent role for target sequences

In contrast with the prokaryotic DNA topoisomerase I which cleaves and catalyzes DNA strand passage within a single-stranded region (9-10), the eukaryotic type I DNA topoisomerase has no preference of single-stranded. On the contrary it has been shown to cleave preferentially in short duplex sequences (16).

The fact that the same cleavage sites are recognized by enzymes deriving from phylogenetically distant eukaryotic sources (9,17) shows a general requirement for target sequences. In one instance, the conserved sequence is 16 nucleotide long (31) and its removal has clear effects on the relaxation kinetics (30). We have looked for the occurrence of preferential cleavage sites in the Ig and ADH II DNA tested. In the Ig DNA two highly preferentially cleaved sites are detected, both common to the three eukaryotic DNA topoisomerases I studied (to be detailed elsewere). In the yeast system no preferential cleavage site has been detected; the pUC18 has not been analyzed. It has been pointed out (2) that strong binding sites are not necessarily strong cleavage sites and that strong cleavage sites may be not preferential sites in the catalysis of strand breakage and rejoining. The fact we have just mentioned (that is: similar kinetics are observed indipendently of the possibility of detection of cleavage sites) stresses the validity of this argument. In each one of the three systems analyzed here, the relationship topology/kinetics is always reproducibly the same, independently of the possibility of detection of preferential cleavage sites. Therefore, a simple and generally valid relationship between topology of specific sites of cleavage and rate of relaxation cannot be easely established. However the following consideration deserves attention:
both negatively and positively supercoiled forms are active substrates. The relaxed is not. In other words,it is the deformation (undertwisting or overtwisting) of target sequences which allows productive interaction with the enzyme whereas unstrained, normally-twisted sequences do not allow reaction. An interesting corollary of this functional scheme would be the double role exerted by target sequences: inhibitory when undeformed, activatory when deformed. Target sequences could therefore exert the role of "topological sensors" in the DNA domain under their control: when deformed by one of several possible causes acting on the domain (modification of the physico-chemical environment, interaction with proteins, transcription) they would become reactive to the ubiquitous, abundant DNA topoisomerase I and cause the DNA domain to return to the original conformation. When undeformed, they could prevent a futile continous

topoisomerization, otherwise unavoidable if the exposed (31) target sequences were uncontrollably and continuously reactive.

AN APPARENT PARADOX

Topoisomerization as a function of the enzyme/DNA ratio (summarized in fig.1, panel D for the Ig system, in fig.5 for pUC18 DNA) shows that at least 200 fold more topoisomerase I is needed to topoisomerize 50% of relaxed relatively to highly supercoiled molecules. Analysis of the kinetics of topoisomerization of the relaxed form of the Ig DNA (fig. 1 B and C) reveals an apparent paradox: i.e. 1U of DNA topoisomerase I appears to completely nick-close the relaxed topoisomer within 0.5 min., yet 0.1U of enzyme does not appear to have any affect even after 90 min. This could suggest that the extent of activity might not be proportional to time and/or enzyme concentration. The same has been observed in the analysis of relaxation of the yeast and pUC18 DNA. A closer inspection (keeping to the Ig system only; the other systems behave similarly) explains the paradox as follows:

i) the nicking-closing reaction is very fast (<30sec) both at low and high Δ Lk (fig. 1 C2,C9). The kinetically limiting step precedes therefore the cleavage reaction and is very likely the localization-interaction with active target sites on the DNA (see above for a possible role of target DNA sites). ii) the localization-interaction with the active DNA sites is proportional to time and enzyme concentration in supercoiled DNA at low enzyme/DNA ratios (fig. 1A 9-14 and D).

iii) the localization-interaction with the DNA sites is proportional to the concentration on relaxed DNA only at high enzyme/DNA ratios (fig. 1D, between 0.1 and 1 DNA topoisomerase units). As mentioned, the dose necessary to nick close 50% of the relaxed DNA is 250 fold > than that needed for supercoiled DNA. Given that at least 1:1 enzyme:DNA is necessary for supercoiled DNA because of the completely processive nature of the reaction, the nicking-closing of the relaxed DNA occurs at a completely saturating dose.

These quantitative observations suggest that on torsionally stressed DNA a mechanism is generated which preferentially drives the enzyme on active target DNA sites. This mechanism is absent on relaxed DNA.

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