The EMBO Journal vol.6 no.6 pp.1817–1823, 1987

Sequence specificity of DNA topoisomerase I in the presence and absence of camptothecin

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Communicated by U.K.Laemmli

Previously, we have demonstrated that in *Tetrahymena* DNA topoisomerase I has a strong preference *in situ* for a hexadecameric sequence motif $A^A_G ACTTAGA^A_G AAA^{AAA}_{TTT}$ present in the non-transcribed spacers of r-chromatin. Here we characterize more extensively the interaction of purified topoisomerase I with specific hexadecameric sequences in cloned DNA. Treatment of topoisomerase I–DNA complexes with strong protein denaturants results in single strand breaks and covalent linkage of DNA to the 3′ end of the broken strand. By mapping the position of the resulting nicks, we have analysed the sequence-specific interaction of topoisomerase I with the DNA. The experiments demonstrate that: (i) the enzyme cleaves specifically between the sixth and seventh bases in the hexadecameric sequence; (ii) a single base substitution in the recognition sequence may reduce the cleavage extent by 95%; (iii) the sequence specific cleavage is stimulated 8-fold by divalent cations; (iv) 30% of the DNA molecules are cleaved at the hexadecameric sequence while no other cleavages can be detected in the 1.6-kb fragment investigated; (v) the sequence specific cleavage is increased 2- to 3-fold in the presence of the antitumor drug camptothecin; (vi) at high concentrations of topoisomerase I, the cleavage pattern is altered by camptothecin; (vii) the equilibrium dissociation constant for interaction of topoisomerase I and the hexadecameric sequence can be estimated as $\sim 10^{-10}$ M.

Key words: camptothecin/protein–DNA interaction/topoisomerase I/recognition sequence

Introduction

Type I and II DNA topoisomerases introduce transient breakage and reunion of the DNA backbone. During this process the enzymes become covalently linked to the nicked DNA and consequently it is possible to reveal the preferential binding sites of topoisomerases by irreversible arrest of this covalent intermediate by treatment with a protein denaturant (for review see Vosberg, 1985; Wang, 1985). The sequence specificity for eukaryotic type I topoisomerases has been subject to extensive studies. Although a weak bias in the nucleotide composition 5′ to the breakage point was observed, no unique recognition sequence could be demonstrated (Edwards *et al.*, 1982; Been *et al.*, 1984).

We have shown that eukaryotic type I topoisomerases preferentially cleaved the specific hexadecameric sequence $A^A_G ACTTAGA^A_G AAA^{AAA}_{TTT}$ present in DNaseI-hypersensitive regions in the non-transcribed spacers of the rDNA in *Tetrahymena* (Andersen *et al.*, 1985; Bonven *et al.*, 1985). Here we report a more extensive analysis on the interaction of topoisomerase I isolated to homogeneity from *Tetrahymena* with a hexadecameric sequence in the presence and absence of the antitumor drug camptothecin. This drug stabilizes the topoisomerase I–DNA covalent intermediates that form during relaxation of torsionally strained DNA and has recently been used for mapping the association of topoisomerase I with DNA *in vitro* (Hsiang *et al.*, 1985) and *in vivo* (Gilmour and Elgin, 1987). We find topoisomerase I to be highly sequence specific and that addition of camptothecin results in an altered cleavage pattern. Similar data have been obtained with other eukaryotic type I topoisomerases, e.g. the human enzyme, demonstrating a common mechanism of interaction between this type of enzyme and DNA.

Results

Purification of topoisomerase I

Topoisomerase I was purified from nuclei of exponentially growing cultures of *Tetrahymena* by a modification of the method of Ishii *et al.* (1983). Briefly, nuclei were isolated and extracted by 0.15 M sodium phosphate pH 7.5 followed by centrifugation at 200 000 *g* for 1 h. The topoisomerase I activity in the supernatant containing >90% of the total activity was thereafter purified to homogeneity by heparin- and phenyl-Sepharose column chromatography. The results of the purification are summarized in Table I. Starting from the nuclear extract the topoisomerase I is purified ~250-fold with an overall yield of 17%. The nuclear extract accounts for 60–80% of the cellular topoisomerase I activity suggesting that the enzyme totally has been purified ~400-fold. The specific activity of the preparation is 5×10^6 U/mg protein, corresponding to the values obtained for other homogeneous preparations of eukaryotic type I topoisomerases. Figure 1 shows a SDS–polyacrylamide electrophoretic analysis of the enzyme preparation. Two polypeptides with M_r ~80 and 76 kd, respectively, are observed. These M_r values correspond to those observed for several other eukaryotic type I topoisomerases (Ishii *et al.*, 1983; Vosberg, 1985). Both polypeptides react in Western blots with polyclonal antibodies directed against topoisomerase I (data not shown).

Purified topoisomerase I binds to a specific sequence element in the spacer of rDNA

To investigate if the purified topoisomerase I had maintained the sequence specificity observed *in situ*, the enzyme was incubated with a 2.7-kb *Hin*fI restriction fragment of rDNA spanning the DNaseI-hypersensitive regions (Bonven *et al.*, 1985) of the central spacer (cf. Figure 2A). The interaction of topoisomerase I with the DNA fragment was detected by trapping the intermediate with SDS. After proteinase K treatment, the sample was analyzed on a sequencing gel (Figure 2B, lane 4) in parallel with Maxam–Gilbert reactions of the same fragment (lanes 1–3). The sub-band pattern reveals cleavages at sites a and b. The cleavages appear as triplets with ~30 bp spacing between the individual cleavages. Comparison of the cleavages at site b with the Maxam–Gilbert tracks demonstrates that base-specific

Table I. Purification of topoisomerase I from *Tetrahymena thermophila*

	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Yield (per cent)
Nuclear extract	1.5×10^6	2.0×10^4	1	100
Heparin-Sepharose column (H1)	6.7×10^5	1.0×10^6	50	45
Heparin-Sepharose column (H2)	5.1×10^5	1.5×10^6	75	34
Phenyl-Sepharose column	2.5×10^5	5.1×10^6	255	17

cleavage in each case is generated between nucleotides six and seven in sequences fitting the motif $A^A_GACTTAGA^A_GAAA^{AAA}_{TTT}$. An identical result is obtained when the cleavages at site a are analyzed using a 294-bp *XbaI*−*SphI* fragment (cf. Figure 2A, result not shown). The data clearly demonstrate that the purified topoisomerase I exhibits the same binding properties as we previously observed *in situ* (Bonven *et al.*, 1985). Cleavage is observed on the non-coding strand only (data not shown). Dissociation of enzyme−DNA complexes by addition of 0.8 M NaCl prior to SDS suppresses the cleavage reaction completely (Figure 2B, lane 5). Thus, the data demonstrate that clusters of the hexadecameric recognition sequences also function *in vitro* as a topoisomerase I-attraction site.

Topoisomerase I binds strongly to a single recognition sequence

The topoisomerase I-recognition sequences occur as demonstrated above in the non-transcribed spacers of the rDNA as triplets with an approximate spacing of 30 bp (Figure 2). This arrangement might indicate interaction between individual topoisomerase I molecules binding within a cluster. In order to study the interaction between the enzyme and a single recognition motif we synthesized a 27-bp oligonucleotide containing the hexadecameric sequence AGACTTAGAAAAATTT. The oligonucleotide was inserted between the *Eco*RV and *Sph*I sites of pBR322 (cf. Figure 3A, pNC1). The cleavage reaction was investigated on a gel-purified 1.6-kb *Hin*dIII−*Pvu*II fragment, 3′ end-labelled at the *Hin*dIII site. The DNA fragment was incubated for 15 min with topoisomerase I at an enzyme to DNA molar ratio ~10 in the presence of 1 mM $MgCl_2$ and 3 mM $CaCl_2$. Cleavage was induced with SDS and the deproteinized samples analyzed on a sequencing gel in parallel with Maxam−Gilbert degradation reactions of the same fragment (Figure 3B). As seen by comparison with the sequencing reactions in lanes 1−3, cleavage is observed between nucleotides 6 and 7 in the consensus sequence. No other cleavages appear even after longer time of exposure (Figure 3B, lane 7). The weak bands seen in the upper part of the lane result from pre-existing nicks in the DNA fragment. Thus, the hexadecameric sequence *per se* functions as a high-affinity topoisomerase I attraction site independent of structural features of the flanking sequences in the rDNA. The observed specificity is striking as eukaryotic type I topoisomerases usually are considered not to exhibit any significant sequence specificity (Edwards *et al.*, 1982; Been *et al.*, 1984). The cleavage at the sequence is stimulated by divalent cations as shown in Figure 4. Thus, concentrations of 3 mM Ca^{2+} or Mg^{2+} cause an 8-fold increase in the specific cleavage reaction (cf. Figure 4, lanes 2 and 4).

Comparison of the extent of cleavages in the cluster at site b in Figure 2 has shown that the frequency of the topoisomerase I-cleavage reaction varies with single nucleotide changes in the hexadecameric recognition sequence (Bonven *et al.*, 1985). To investigate if a single base substitution could cause complete inhibition of the reaction, oligonucleotides with single base-pair

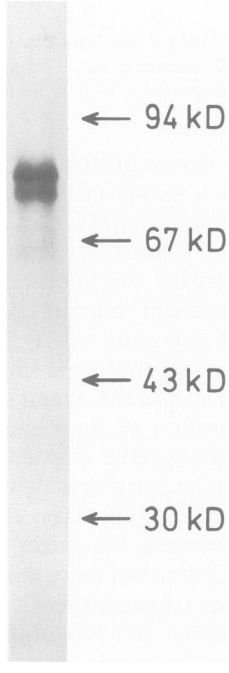

Fig. 1. SDS−polyacrylamide gel of purified topoisomerase I.

substitutions were synthesized and tested for their ability to form specific cleavage complexes with topoisomerase I. Figure 5, lane 1, shows a comparison between the hexadecameric sequence of pNC1 (cf. Figure 3A) and a sequence in which the T in position 6 is substituted with an A (pNC5) in lane 2. The substitution causes a 95% reduction in cleavage. The strong inhibitory effect of this base substitution is probably correlated with high specificity in the formation of the transient phosphodiester bond between the 3′ phosphoryl group and a tyrosyl residue of the enzyme (Champoux, 1981).

As the *Hin*dIII−*Pvu*II restriction fragment of pNC1 contains only one specific cleavage site, it is possible to obtain an estimate of the equilibrium dissociation constant for the binding of topoisomerase I to the recognition sequence. For this purpose a titration experiment was performed. Increasing amounts of topoisomerase I were incubated with a fixed amount of 3′ end-labelled DNA fragment. Interaction between topoisomerase I and DNA was detected by cleavage induction with SDS and the samples were analyzed on sequencing gels (Figure 6A). The extents of cleavage were quantitated by densitometry of the autoradiogram and depicted in Figure 6C (lower curve). Maximal cleavage of ~30% of the molecules is obtained at a molar ratio of enzyme to DNA of ~5. Based on the observations and the assumption that cleavage is a measure for binding of the enzyme to the substrate, an equilibrium dissociation constant of $\sim 10^{-10}$ M can be estimated according to Riggs *et al.* (1970).

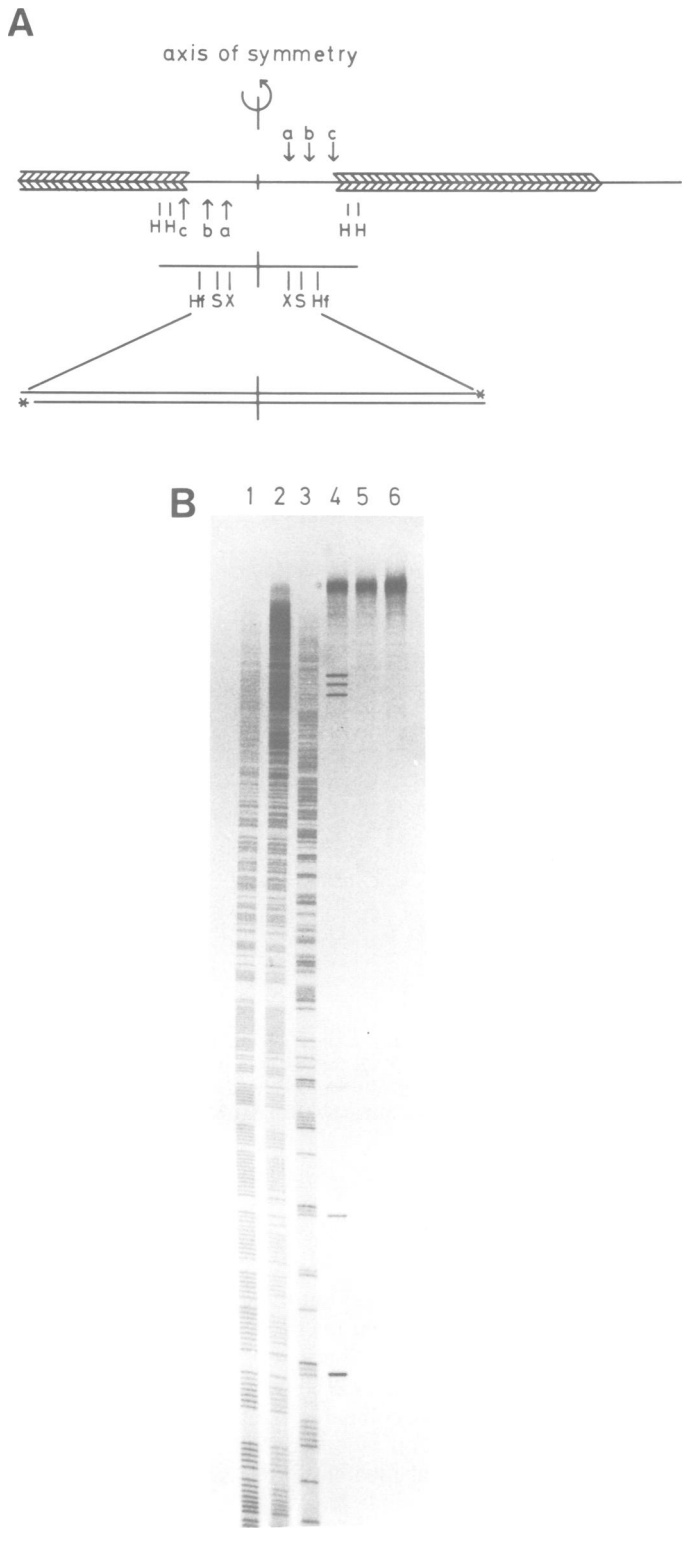

Fig. 2. Cleavage induction *in vitro* using purified topoisomerase I. **(A)** Restriction map of the central spacer of the palindromic rDNA molecule from *Tetrahymena*. Abbreviations: H = *Hin*dIII, Hf = *Hin*fI, S = *Sph*I, X = *Xba*I. **(B)** A 2.7-kb fragment encompassing the topoisomerase I-cleavage sites a and b was isolated from plasmid pTtr1 after restriction with *Hin*fI. The recessed 3′ ends were labelled with [α-^{32}P]dATP and the Klenow DNA polymerase and subjected to Maxam–Gilbert sequencing reactions: **(lane 1)** G+A reaction; **(lane 2)** A>C reaction; **(lane 3)** C>T reaction. The end-labelled fragment was incubated with purified topoisomerase I. Cleavage was induced with SDS + EDTA and the samples digested with proteinase K: **(lane 4)** SDS + EDTA followed by NaCl; **(lane 5)** NaCl + EDTA before SDS; **(lane 6)** the untreated 3′ end-labelled *Hin*fI fragment.

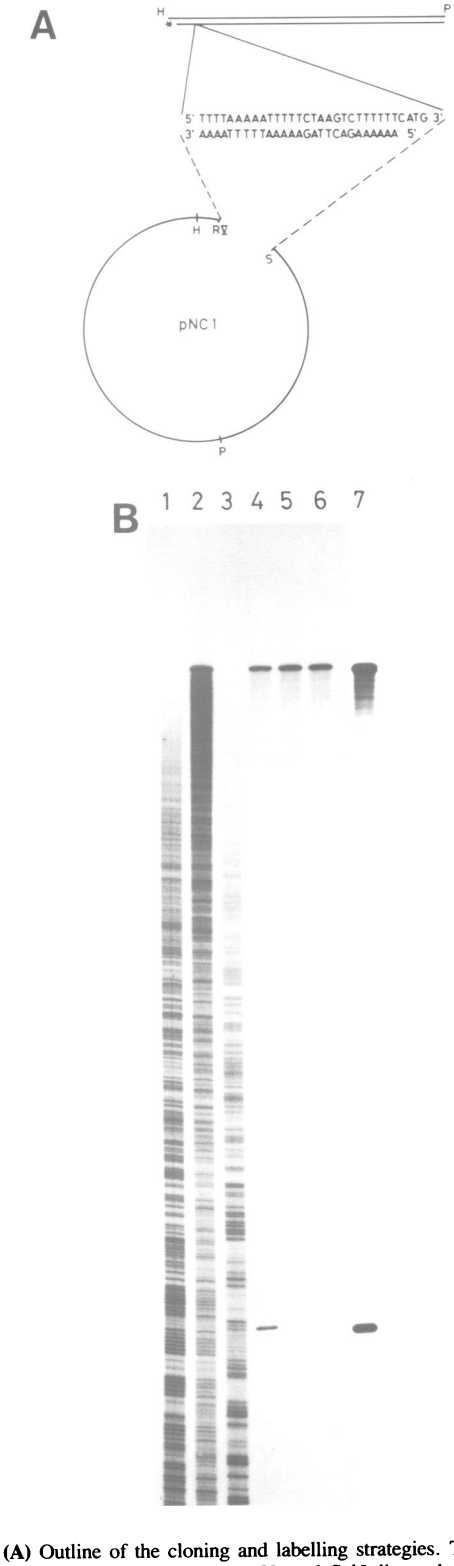

Fig. 3. (A) Outline of the cloning and labelling strategies. The synthetic oligonucleotide was cloned into *Eco*RV- and *Sph*I-digested pBR322, giving rise to pNC1. The DNA fragment used as substrate in the reconstitution experiment was excised with *Hin*dIII and *Pvu*II. After gel-purification the 1.6-kb fragment was 3′ end-labelled at the *Hin*dIII site with Klenow DNA polymerase and [α-^{32}P]dATP. Abbreviations: H = *Hin*dIII, RV = *Eco*RV, S = *Sph*I, P = *Pvu*II. **(B)** The isolated, 3′ end-labelled fragment was subjected to Maxam–Gilbert sequencing reactions: **(lane 1)** G+A reaction; **(lane 2)** A>C; **(lane 3)** C>T. The end-labelled fragment was incubated with topoisomerase I; the incubation was terminated with SDS and the samples digested with proteinase K: **(lane 4)** SDS + EDTA; **(lane 5)** NaCl + EDTA before SDS; **(lane 6)** the untreated 3′ end-labelled fragment. **Lane 7** represents a longer exposure of lane 4.

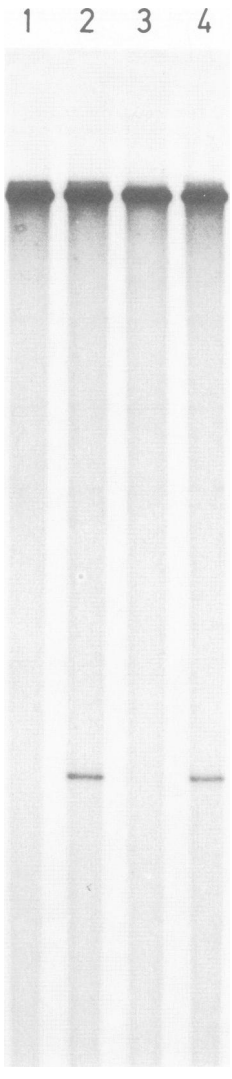

Fig. 4. Topoisomerase I–DNA cleavage reaction in the presence of different divalent cations. The *Hin*dIII–*Pvu*II fragment of pNC1 (cf. Figure 3), end-labelled at the *Hin*dIII site was incubated with topoisomerase I as described in Materials and methods with the following modifications: **(lane 1)** no topoisomerase present; **(lane 2)** 3 mM Ca^{2+} present; **(lane 3)** 5 mM EDTA present; **(lane 4)** 3 mM Mg^{2+} present.

Fig. 5. The effect of the change of a single base in the topoisomerase I recognition sequence. *Hin*dIII–*Pvu*II fragments of pNC1 and pNC5 (cf. text) were end-labelled at the *Hin*dIII site and incubated with topoisomerase I as described in Materials and methods. **(Lane 1)** pNC1; **(lane 2)** pNC5.

Effect of camptothecin on the sequence specificity of topoisomerase I cleavage

The antitumor drug camptothecin is known to stabilize the topoisomerase I–DNA covalent intermediates that form during relaxation of torsionally strained DNA (Hsiang *et al.*, 1985). To monitor the effect of this drug on the interaction of topoisomerase I with the specific attraction sequence, the titration experiment presented in Figure 6A was repeated in the presence of 50 μM camptothecin. This concentration corresponds to that used in a number of *in vivo* experiments (Kessel, 1971; Abelson and Penman, 1972). Camptothecin has, as seen in Figure 6B, a marked influence on the cleavage reaction. Thus, up to 70% of the DNA molecules are cleaved in the recognition sequence (Figure 6C, upper curve), while no other sites can be detected. This demonstrates that the sequence specificity of the enzyme is maintained under these conditions where the molar ratio between enzyme and substrate is ~10. It is interesting that the equilibrium dissociation constant remains unaltered under these conditions, suggesting that camptothecin favors the formation of cleavable complexes, but does not change the binding properties of the enzyme.

If the amount of enzyme is increased to 60 units, corresponding to a molar ratio of 30, cleavage in the absence of camptothecin is observed only in the recognition sequence (Figure 7A, lane 1), while camptothecin present in concentrations of 50 and 100 μM, respectively (Figure 7A, lanes 2,3), causes cleavages at a number of additional sequences. The intensities of these cleavage products are rather weak compared with that originating from cleavage at the recognition sequence. Since the fragment used for the study is 3′ end-labelled close to this sequence, cleavage at this site will mask possible cleavages at the distal cleavage sites. The effect of camptothecin on cleavage at these additional sequences is therefore better visualized using the DNA fragment from pNC5, containing the mutated recognition sequence (Figure 7B). The experiment shows that increasing the concentration of camptothecin from 0 to 100 μM (lanes 1–5) leads to cleavage of >80% of the DNA fragments at these alternative sequences. Control experiments show that the cleavages are not caused merely by interaction between camptothecin and the DNA (cf. Figure 7A, lane 4). Likewise, cleavage is abolished if the enzyme–DNA complex is dissociated with NaCl prior to addition of SDS (results not shown).

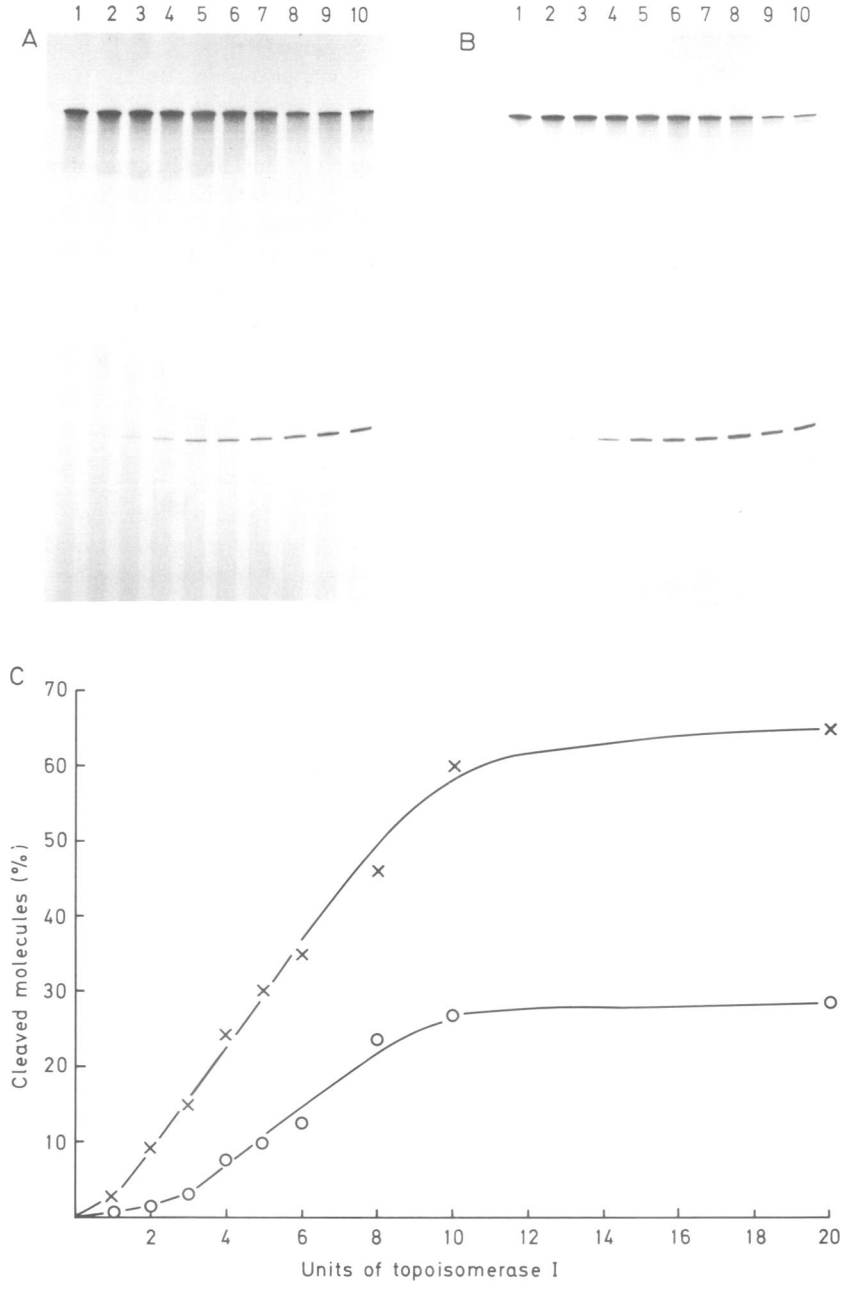

Fig. 6. Interaction between topoisomerase I and DNA in the presence and absence of camptothecin. **(A)** 5 fmol of labelled *Hin*dIII−*Pvu*II fragment from pNC1 was incubated with topoisomerase I in amounts from 0 to 20 units (1 unit ~2 fmol). The cleavage reaction was induced and the samples treated as described in Materials and methods. Amounts of topoisomerase I: **(lane 1)** 0 units; **(lane 2)** 1 unit; **(lane 3)** 2 units; **(lane 4)** 3 units; **(lane 5)** 4 units; **(lane 6)** 5 units; **(lane 7)** 6 units; **(lane 8)** 8 units; **(lane 9)** 10 units; **(lane 10)** 20 units. **(B)** As A, but the incubation was performed in the presence of 50 μM camptothecin. **(C)** Graph of the extents of specific cleavage in A **(lower curve)** and B **(upper curve)**. The quantitations are based on densitometric scanning of the autoradiograms shown in A and B.

Discussion

Topoisomerase I binds *in situ* preferentially to sequences fitting the hexadecameric sequence motif $A^{A}_{G}ACTTAGA^{A}_{G}AAA^{AAA}_{TTT}$ located at DNaseI-hypersensitive regions in the non-transcribed spacers of rDNA in *Tetrahymena thermophila* and *Tetrahymena pyriformis* (Andersen *et al.*, 1985; Bonven *et al.*, 1985). We have in the present paper by cleavage induction of complexes reconstituted with purified topoisomerase I from *Tetrahymena* and the cloned hexadecameric sequence AGACTTAGAAAAA-TTT shown that up to 30% of the DNA molecules are cleaved base-specifically between the sixth and the seventh nucleotide in the sequence, while no other specific cleavage products are observed. Our substitution of one nucleotide in the core of the recognition sequence reduces the cleavage by 95%. The strong inhibitory effect of this substitution in position 6 of the sequence is probably correlated with high specificity in formation of the transient phosphodiester bond between the 3′ phosphoryl group and a tyrosyl residue of the enzyme (Champoux, 1981). The specificity in formation of cleavable complexes enabled us to make an estimate of the equilibrium dissociation constant for the interaction of enzyme and DNA. The constant $\sim 10^{-10}$ M is comparable with those obtained for other sequence-specific DNA-

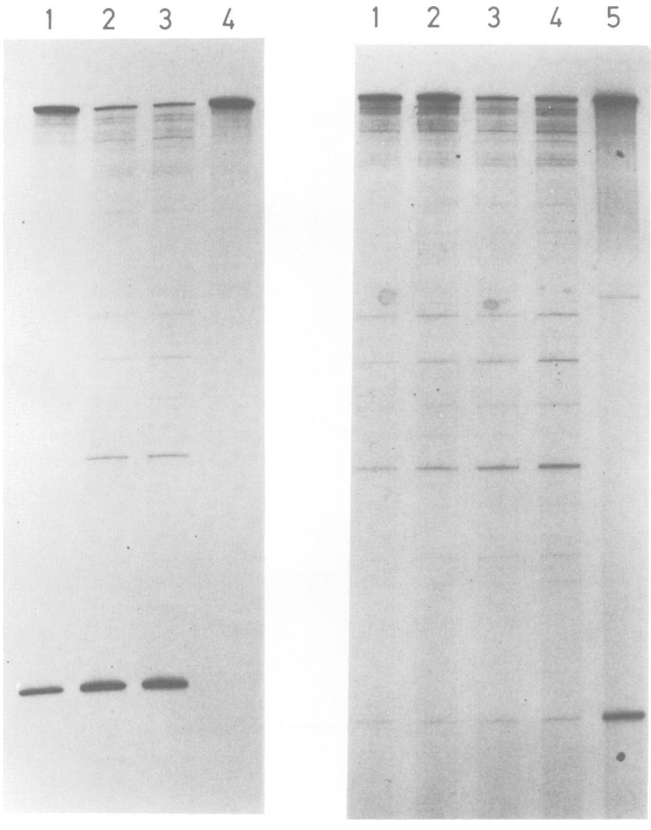

Fig. 7. Sequence-specific cleavage with topoisomerase I in the presence of camptothecin. **(A)** 5 ng of labelled *Hin*dIII−*Pvu*II fragment from pNC1 was incubated as follows: **(lane 1)** 60 units of topoisomerase I; **(lane 2)** 60 units of topoisomerase I and 50 μM camptothecin; **(lane 3)** 60 units of topoisomerase I and 100 μM camptothecin; **(lane 4)** 100 μM camptothecin. **(B)** 5 ng of labelled *Hin*dIII−*Pvu*II fragment from pNC5 was incubated with 60 units of topoisomerase I in the presence of the following concentrations of camptothecin: **(lane 1)** 5 μM; **(lane 2)** 10 μM; **(lane 3)** 50 μM; **(lane 4)** 100 μM. The fragment from pNC1 incubated with 60 units of topoisomerase I is electrophoresed in **lane 5**.

binding proteins, e.g. 10^{-10} M for gyrase (Higgins and Cozzarelli, 1982) and $10^{-10}-10^{-11}$ M for the Cro repressor (Takeda *et al.*, 1977).

The recognition sequences for topoisomerase I in the non-transcribed spacers of rDNA are clustered in direct repeats with a regular spacing of ~30 bp or ~3 helical turns apart. DNaseI protection studies show that topoisomerase I molecules from chicken erythrocytes cover the DNA helix over a linear span of ~30 bp (Trask and Muller, 1983). Thus, it is conceivable that the topoisomerase I molecules might bind adjacent to hexadecameric elements from the same face in an orientation allowing head-to-tail interaction. Such an arrangement might favor cooperativity in the binding of the enzyme molecules (Hochschild and Ptashne, 1986).

Antitumor drugs have recently been applied for mapping topoisomerase I- and topoisomerase II-cleavage sites *in vivo* and *in vitro*. These studies take advantage of the stimulating effect on formation of cleavable complexes exerted by these drugs. However, the cleavage patterns observed using different drugs are not identical (Ross *et al.*, 1984; Tewey *et al.*, 1984). We have tested the validity of using camptothecin in the localization of topoisomerase I sites *in vitro* and find the cleavage pattern to be dependent on the molar ratio between enzyme and DNA. At a low enzyme to DNA molar ratio of ~5, 70% of the DNA fragments are cleaved at the hexadecamer sequence. When the enzyme to DNA molar ratio is increased to ~30 a number of additional cleavages appear. Thus, addition of camptothecin to the reaction leads to a substantial increase in the cleavage frequency, although the equilibrium dissociation constant remains unaltered. This suggests that stimulation of cleavage by camptothecin reflects the formation of an altered enzyme−DNA complex that upon SDS treatment cleaves the DNA with higher efficiency, and that it does not influence the affinity of the enzyme for DNA at the recognition sequence. The latter possibility can, however, not be excluded for the interaction of the enzyme with the observed additional cleavage sites.

Ciliated protozoans including *Tetrahymena* diverged from the eukaryotic lineage early in the evolutionary history (Horowitz and Gorovsky, 1985). Despite this diversity, the specificity of the *Tetrahymena* topoisomerase I for the hexadecameric sequence motif recurs in type I enzymes isolated from a number of eukaryotic organisms (Bonven *et al.*, 1985; Christiansen *et al.*, 1987). Thus, type I topoisomerases from *Drosophila*, chicken, calf thymus, and Burkitts lymphoma cells gave a similar preferential sequence-specific cleavage at the hexadecameric sequence (Christiansen *et al.*, 1987). It has been questioned, however, whether strong cleavage sites necessarily are preferential sites in the catalysis of DNA breakage and rejoining (Wang, 1985). With the present system, we have shown that the hexadecameric consensus recognition sequences are strong cleavage sites both *in vivo* and *in vitro*. When these results are considered together with the observation that type I topoisomerases from both *Tetrahymena* and man preferentially relax plasmids containing the recognition motif (to be published), it seems plausible to assume that the sequence-specific topoisomerase I−DNA interaction also influences the DNA topology *in vivo*. The DNA substrates presented in this paper might therefore provide a useful tool for such studies.

Materials and methods

Purification

Cultures of *Tetrahymena thermophila* strain B1868-7 were grown to a density of ~10^5 cells/ml and macronuclei isolated as described by Gocke *et al.* (1978) except that 0.2 mM PMSF was added to the solutions. Nuclear proteins were extracted by the addition of one volume 0.30 M sodium phosphate pH 7.5, 1 mM EDTA, 0.2 mM PMSF, 2 mM DTT, 20% glycerol. The extract was centrifuged for 1 h at 55 000 r.p.m. in a Beckman Ti60 rotor. The supernatant was diluted 2.5 times in Elu-buffer (10 mM Tris−HCl pH 7.5, 0.5 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 10% glycerol), adjusted to 5 mM EDTA and applied onto a heparin-Sepharose column (H1). The column was washed with Elu-buffer containing 0.4 M NaCl and subsequently eluted with Elu-buffer containing 0.8 M NaCl.

Fractions containing topoisomerase I activity were pooled, diluted four times with Elu-buffer and applied to another heparin-Sepharose column (H2) pre-equilibrated with Elu-buffer containing 0.15 M NaCl. The column was washed with Elu-buffer containing 0.4 M NaCl and eluted with a gradient of 0.4−1.0 M NaCl in Elu-buffer.

Fractions containing the bulk of the activity were pooled, adjusted to 1.2 M $(NH_4)_2SO_4$ and applied onto a phenyl-Sepharose column, equilibrated with Elu-buffer containing 1.2 M $(NH_4)_2SO_4$. The column was washed with Elu-buffer containing 1.0 M $(NH_4)_2SO_4$ and subsequently eluted with a gradient of 1.0−0.2 M $(NH_4)_2SO_4$ in Elu-buffer. Active fractions were pooled, brought to 50% glycerol and stored at −20°C. Protein concentrations were determined according to the method of Lowry *et al.* (1951).

Topoisomerase I relaxation assay

Topoisomerase I activity was measured by relaxation of supercoiled plasmid DNA. The assay contained 10 mM Tris−HCl pH 7.5, 0.5 mM EDTA, 1 mM DTT, 1 mM spermidine, 150 mM NaCl, 100 μg BSA per ml, 10% glycerol and 1 μg of supercoiled pBR322 in a final volume of 15 μl. Reactions were incubated for 30 min at 30°C, and terminated by addition of NaCl to 0.8 M. Subsequently the samples were loaded on a 1% agarose gel. One unit of activity is defined

as the amount of topoisomerase that relaxes 50% of 1 μg fully supercoiled pBR322 DNA in 30 min at 30°C.

Gel electrophoresis

Agarose gels (1%) were run overnight at 4°C submerged in 36 mM Tris−Base, 30 mM NaH_2PO_4, 1 mM EDTA. After staining with ethidium bromide the DNA was visualized by u.v. illumination.

Maxam−Gilbert (1980) sequencing reactions and topoisomerase I cleavage products from reconstitution experiments were analyzed on denaturing polyacrylamide gels [6% (w/v) acrylamide, 0.3% (w/v) *N,N*-methylene-bisacrylamide, 8 M urea] run for 3−4 h in 89 mM Tris−Base, 89 mM boric acid, 2 mM EDTA, pH 8.3.

Protein samples were analysed on SDS−polyacrylamide gels (15% acrylamide, 0.5% *N,N*-methylene-bisacrylamide, 0.1% SDS), run overnight in 10 mM Tris−Base, 0.2 M glycine, 0.1% SDS, pH 8.3, according to the method of Laemmli (1970). Subsequently the gels were silver-stained using the BioRad silver staining kit.

Plasmids

The plasmids pNC1 and pNC5 were constructed by insertion of a synthetic 27-bp oligonucleotide into pBR322 digested with the restriction enzymes *Eco*RV and *Sph*I as outlined in Figure 3A. The inserted sequences of pNC1 and pNC5 are 5′-AAAAAAGACTTAGAAAAATTTTTAAAA-3′ and 5′-AAAAAAGACTAA-GAAAAATTTTTAAAA-3′, respectively. Plasmid pTtr1 contains the central spacer of the rDNA from *Tetrahymena thermophila* cloned into the *Hin*dIII site of pBR322 (Challoner *et al.*, 1985).

Isolation and labelling of restriction fragments

Restriction digestions of plasmids were performed according to the suppliers' instructions (Bio Labs, Boehringer). The fragments were separated by agarose-gel electrophoresis and after ethidium bromide staining, the relevant fragments were isolated by electrophoretical transfer to DEAE membranes (NA-45 from Schleicher and Schuell). The DNA fragments were recovered from the membrane by elution with 1 M NaCl and precipitated with 2.5 volume of ethanol. Isolated fragments were labelled at recessed 3′ ends using [α-^{32}P]dATP (3000 mCi/mmol) and the Klenow fragment of *Escherichia coli* DNA polymerase I.

Cleavage induction

End-labelled restriction fragments (3−5 fmol) were incubated with 1−20 units of topoisomerase I in 30 μl Tris−HCl pH 8.0, 60 mM NaCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 M sucrose, 1 mM DTT, 100 μg BSA per ml for 15 min at 25°C. Cleavage was induced by addition of SDS and EDTA to final concentrations of 1% and 10 mM, respectively, followed by incubation for 5 min at 45°C. Finally NaCl was added to 0.8 M. In control samples NaCl and EDTA were added prior to SDS. Subsequently the samples were ethanol precipitated and after proteinase K digestion (300 μg/ml, 60 min at 45°C), 1 volume of deionized formamide, 0.05% bromophenol blue, 0.03% xylene cyanol, 5 mM EDTA, pH 8.5 was added and the samples were loaded on denaturing polyacrylamide gels. Deviations from this procedure with respect to the content of divalent cations and camptothecin are given in the text and figure legends.

Densitometry

The cleavage percentage was determined by densitometric scanning of the autoradiograms using a Shimadzu dual wavelength thin-layer chromato scanner, model CS 930.

Materials

Heparin-Sepharose 6B and phenyl-Sepharose 4B were purchased from Pharmacia. Bovine serum albumin (BSA) was purchased from Sigma. Camptothecin (lactone form, no. 94600) was kindly provided by the National Cancer Institute, Bethesda, Maryland, USA. Stock solutions of camptothecin, 10 mM in dimethyl sulfoxide (DMSO), were stored in aliquots at −20°C. DMSO was from Merck, Klenow fragment of DNA polymerase I from Bio Labs, and radionucleotides from ICN.

Acknowledgements

We wish to thank Drs Anni H.Andersen, Kent Christiansen and Eigil Kjeldsen for helpful discussions, Dr T.Andoh for critical reading of the manuscript, Ms K.Andersen for skilful technical assistance, and Ms E.Christensen for preparation of the figures. This study was supported by contract B-6-0170-DK (B) with CEC, Brussels, the Danish Cancer Society and the National Agency of Technology. B.J.B. was supported by the Danish Natural Science Research Council (grant 11-3797) and the Danish Cancer Society (grant 86-178).

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Received on February 20, 1987; revised on March 26, 1987