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Topoisomerase I has a strong binding preference for a conserved hexadecameric sequence in the promotor region of the rRNA gene from *Tetrahymena pyriformis*

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SUMMARY

Topoisomerase I is *in situ* associated with DNaseI hypersensitive sites located in the promotor and terminator regions of the extrachromosomal rDNA in Tetrahymena thermophila at sites with sequences fitting the motif AGACTTAGA(A/G)AAA(AAA/TTT). Reconstitution experiments with purified topoisomerase I and cloned fragments of rDNA demonstrate that the enzyme exhibits the same binding and cleavage properties on naked DNA. These observations are striking as topoisomerase I previously has been found to exhibit low sequence specificity. The specific binding of the enzyme has an absolute requirement for divalent cations with a preference for Ca^{2+}. The strong binding to the hexadecamer has been characterized by competition experiments, and it has been used to determine the molecular weight of the enzyme.

INTRODUCTION

DNaseI hypersensitive sites are usually found in regions flanking the 5' or 3' ends of genes that are active or can be activated (for review see refs. 2-3). This correlation between hypersensitivity and gene expression suggests that the hypersensitive sites may be of importance for the interaction between regulatory proteins and DNA[4-6]. We have in earlier reports demonstrated the presence of DNaseI hypersensitive sites in the promotor and terminator regions of the ribosomal RNA genes in nucleoli of exponentially growing cultures of *Tetrahymena*[7,8]. The hypersensitivity is not confined to a single point but rather involves a stretch of DNA of 100-200 bp. Recently we have studied the interaction of proteins with DNaseI hypersensitive regions and have found a strong binding of a topoisomerase I[9]. The enzyme binds specifically *in situ* to a highly conserved hexadecamer sequence motif AGACTTAGA(A/G)AAA(AAA/TTT) present within the hypersensitive sites investigated in the

rDNA[1]. This suggests that topoisomerase I might have a function in regulation of transcription by balancing the superhelicity of the template[1,9].

In this paper we describe that purified topoisomerase from Tetrahymena pyriformis in the presence of divalent cations binds sequence specifically to fragments of rDNA containing the hexadecamer sequence motif. The nucleotide sequence of the binding and cleavage sites in the promotor region of the rDNA from Tetrahymena pyriformis is determined. Likewise, the binding properties of topoisomerase I to the hexadecamer sequence as well as the molecular weight of the enzyme have been investigated.

MATERIALS AND METHODS

Isolation of topoisomerase I. Macronuclei were prepared from early log phase cultures (5 x 10^4 cells/ml) of Tetrahymena pyriformis, strain GL, as previously described[7-9]. Topoisomerase I was extracted from nuclear chromatin by 0.4 M NaCl and purified to a specific activity of 0.5-1 x 10^6 U/mg protein according to the method of Prell and Vosberg[10]. For control experiment topoisomerase was purified to homogeneity by the method of Liu and Miller[25].

Isolation and labelling of DNA fragments. Restriction of DNA was performed according to the suppliers' instructions (BioLabs, Amersham, Boehringer). The fragments were separated by electrophoresis in horizontal agarose gels submerged in Tris-phosphate buffer (36 mM Tris, 30 mM Na_2HPO_4, 1 mM EDTA, pH 7.8). Specific DNA fragments were isolated from the gel by electrophoretic transfer to DEAE membranes (Schleicher & Schüll NA-43). Isolated DNA fragments were 3'-end labelled using α^{32}P-dATP and the Klenow fragment of E.coli DNA polymerase I. 5'-End labelling was performed using γ^{32}P-ATP and T_4 polynucleotide kinase.

Labelling of plasmids. Plasmid DNA (pEG10 or pBR322) was digested with PvuII, denatured and primed with PstI clockwise primer (BioLabs) for filter retention assays or digested with PstI and primed with EcoRI clockwise primer for molecular weight determination. The primer was extended by the Klenow

fragment of E.coli DNA polymerase I in the presence of α^{32}P-dATP to get an approximate incorporation of 20 molecules dAMP per DNA molecule followed by a chase with cold dATP. Single stranded regions were digested by endonuclease S_1, and double stranded DNA separated from nucleotides by gel filtration.

Topoisomerase I reconstitution experiments. Reconstitution was done adding 30 U of topoisomerase I to approximately 1 ng of ^{32}P-labelled DNA in 10-30 µl of 10 mM Tris-HCl, pH 7.2, 0.1 M sucrose, 3 mM $CaCl_2$ and 1 mM $MgCl_2$. The mixture was incubated on ice for 30 min unless otherwise stated. Cleavage was induced by the addition of 1% SDS and 10 mM EDTA followed by incubation for 5 min at 25°C. Finally NaCl was added to 1.0 M. In control experiments 1.0 M NaCl was added prior to SDS in order to dissociate the DNA-topoisomerase complex.

DNA sequence determinations. DNA samples from reconstitution experiments were extracted with phenol and DNA-protein complexes recovered from the interphase[9]. The DNA was then deproteinized by incubation with proteinase K at a concentration of 200 µg/ml for 2 hrs at 60°C followed by sequential extraction with phenol:chloroform:isoamylalcohol (25:24:1) and chloroform:isoamylalcohol (24:1). The ^{32}P-labelled DNA was after ethanol precipitation applied on a 6% sequencing gel according to the method of Maxam and Gilbert[11].

Filter retention assays. Samples from reconstitution experiments were made to 330 mM NaOH and 10 mM EDTA in a final volume of 400 µl. The solutions were passed through a preequilibrated nitrocellulose filter (Schleicher & Schüll BA85) at a flow rate of 0.4 $ml \cdot min^{-1} \cdot cm^{-2}$. The filter was washed 3 times with 100 mM NaOH and 1 mM EDTA, dried and processed for autoradiography as previously described[9] or liquid scintillation photometry.

Determination of the molecular weight of topoisomerase I. The 5'-end ^{32}P-labelled oligonucleotide ^{32}pAGACTTAGAGAAAATT / GTACTCTGAATCTCTTTTAA was incubated for 30 min with 30 units of topoisomerase I extract from T.pyriformis according to the procedure described above. After cleavage induction with SDS, the oligonucleotide-protein complexes were precipitated with 5% trichloroacetic acid in the presence of carrier protein (50 µg cytochrome c/ml), and ana-

lysed on a 10% polyacrylamide gel of the Laemmli type[12]. The complex was after electrophoresis visualized in the dried gel by autoradiography. In experiments applying ^{32}P-labelled pEG10, the DNA-protein complex was incubated with DNaseI (50 U/ml, 30 min at 37°C) before analysis.

RESULTS

Previously we have shown that topoisomerase I appears tightly associated *in situ* with the minichromosome containing the gene coding for rRNA in *T.thermophila*[9]. Upon addition of strong detergents to isolated nuclei the enzyme introduces single strand nicks at the hypersensitive sites in the promotor

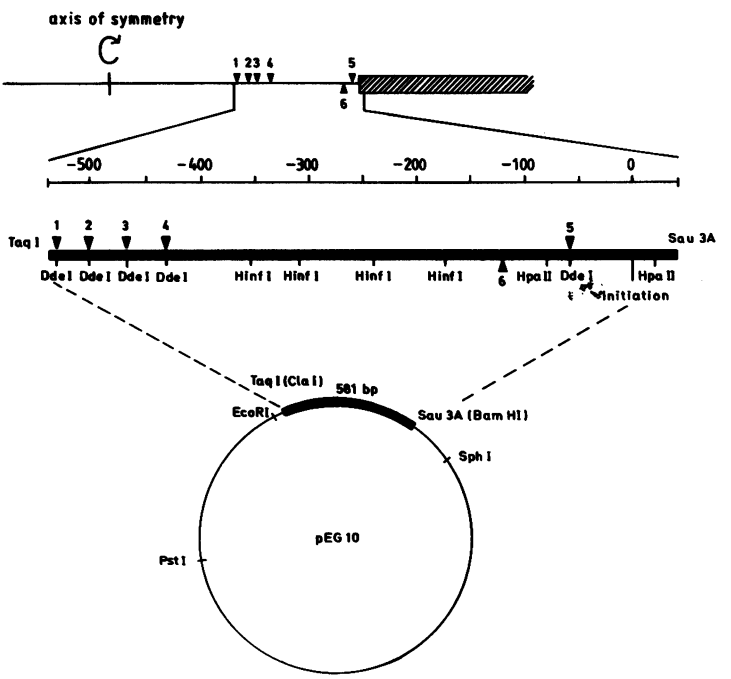


Figure 1. Restriction endonuclease map of pEG10.
A TaqI-Sau3A 581 bp fragment from the promotor region of *T.pyriformis* rDNA is inserted into the ClaI and BamHI sites of pBR322. The solid bar designates rDNA sequences. An excerpt of the palindromic rDNA molecule is shown in the top part. Coordinates relate to point of inititation of transcription. ▼ & ▲ Topoisomerase I binding sites; the numbers refer to the cleavage site numbers in Table 1.

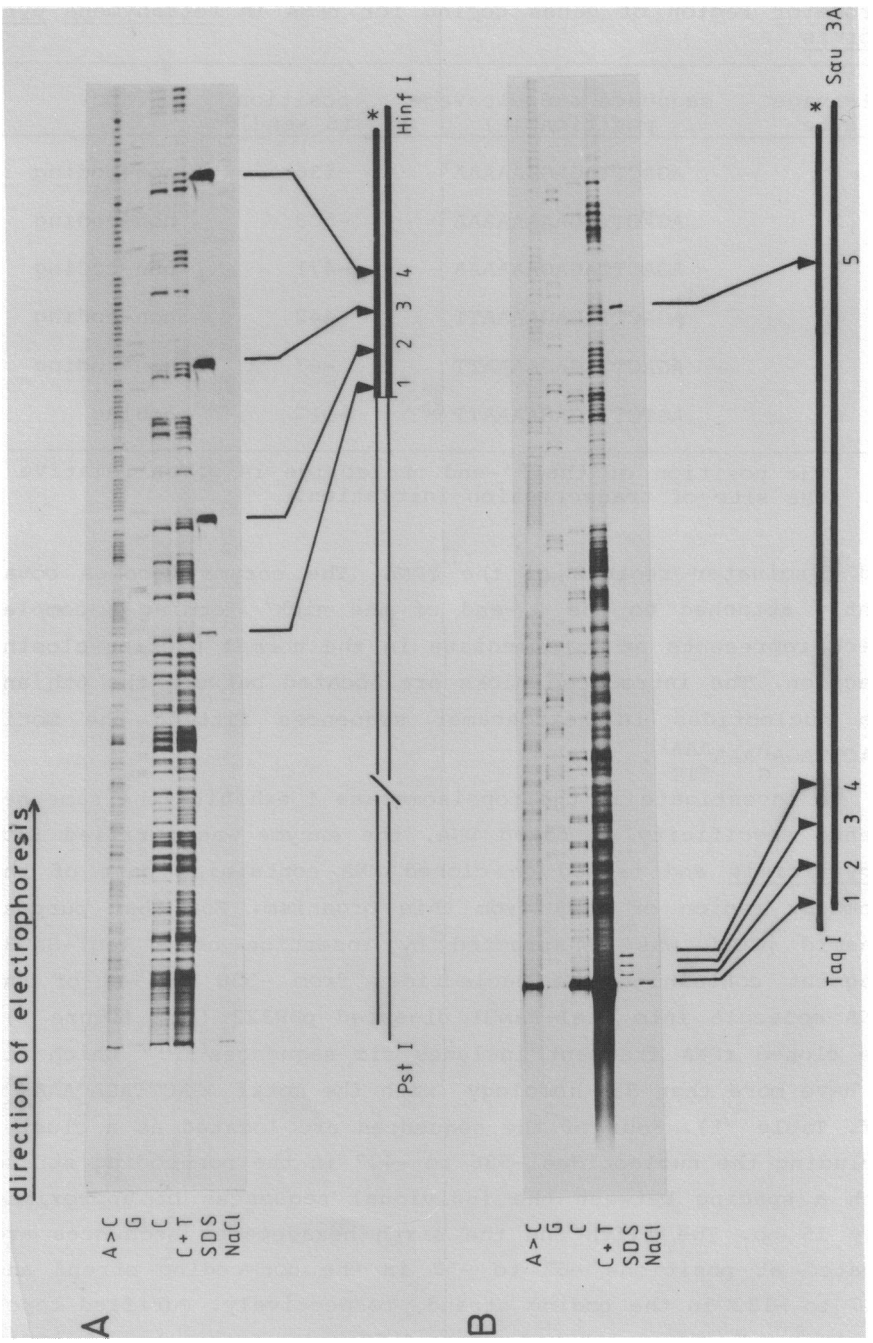
Table 1. Sequences at topoisomerase I cleavage sites in the promotor region of genes coding for rRNA in Tetrahymena pyriformis

Cleavage site	sequence and cleavage position (↓)	position (5'-end)[a]	strand
1	AGACTT↓AGAGAAAAAA	-536	non-coding
2	AGACTT↓AGAAAAAAAA	-503	non-coding
3	AGACTT↓AGAGAAAAAA	-471	non-coding
4	AGACTT↓AGAGAAAATT	-442	non-coding
5	AGACTT↓AGAGAAAATT	-67	non-coding
6	AGTCTT↓GAGAAAATT	-117	coding

a The position of the 5'-end nucleotide is given relative to the site of transcription initiation.

and terminator regions of the rDNA. The enzyme becomes covalently attached to the 3'-end of the nick[9] forming a complex which represents an intermediate in the normal nicking-closing reaction. The introduced nicks are located between the 6th and 7th nucleotides in hexadecamer sequences fitting the motif AGACTTAGA$^{A}_{G}$AAA$^{AAA}_{TTT}$[1].

To investigate if the topoisomerase I exhibits the same sequence specificity on naked DNA, the enzyme was purified from T.pyriformis and tested on cloned DNA containing part of the promotor region of rDNA from this organism. For that purpose plasmid pEG10 was constructed by insertion of a TaqI-Sau3A fragment containing the nucleotides from -538 to +43 of the rDNA molecule into ClaI-BamHI digested pBR322 (cf. figure 1). The cloned rDNA fragment includes six sequences[13,14] which fit or have more than 85% homology with the motif AGACTTAGA$^{A}_{G}$AAA$^{AAA}_{TTT}$ (cf. Table 1). Four of the sequences are located as a cluster including the nucleotides -536 to -427 in the non-coding strand with a spacing between the individual sequences of approximately 15 bp. The fifth and the sixth hexadecamer sequences are located at positions -67 to -52 in the non-coding strand and -117 to -132 in the coding strand, respectively. Purified topoisomerase I was incubated with different fragments of pEG10


direction of electrophoresis
A
A>C
G
C
C+T
SDS
NaCl
Pst I
Hinf I
1 2 3 4
*
B
A>C
G
C
C+T
SDS
NaCl
Taq I
Sau 3A
1 2 3 4 5
*

under conditions which simulate the in situ buffer conditions. A 964 bp PstI-HinfI fragment containing the cluster of four hexadecamer sequences was isolated, 3'-end labelled at the HinfI-site, incubated with topoisomerase I, activated with SDS, and electrophoresed on sequencing gels in parallel to the four sequencing reactions (Figure 2A). Four distinct cleavage products are seen after exposure to SDS (lane 1), while only the uncleaved fragment is observed in a sample made to 1 M NaCl prior to the addition of SDS (lane 2). The topoisomerase cleaves the non-coding strand in the four hexadecamer sequences shown in Table 1 (sites 1-4) between the 6th and 7th nucleotides. There is a high homology between these four hexadecamers. Thus, the sequences around cleavage sites 1 and 3 are identical. At cleavage site 2, a G in position 10 has been substituted by an A, while the A's in positions 15 and 16 have been substituted by T's at site 4. Apparently, the cleavage occurs with a lower frequency at site 1, than at the sites 2-4. This may be a consequence of the flanking pBR322 sequences which have a higher GC content than the otherwise flanking rDNA sequences.

In order to detect one of the two expected topoisomerase I cleavage sites downstream of the HinfI-site, the TaqI-Sau3A fragment was isolated, 3'-end labelled at the Sau3A-site, incubated with topoisomerase I and activated with SDS. Electrophoresis on sequencing gels revealed one cleavage event within

Figure 2. Topoisomerase I cleavage sites in the promotor region of rDNA analysed on sequencing gels.
A. The 964 bp PstI-HinfI fragment from pEG10 was isolated, 3'-end labelled with α^{32}P-dATP at the HinfI-site and incubated with topoisomerase I (see Materials and Mathods for details). The SDS-induced cleavages were analysed on a sequencing gel. A NaCl treated control sample was electrophoresed in parallel. The sequences around the cleavage sites can be read from the four lanes with the sequencing reactions performed by the chemical modification method of Maxam and Gilbert[11].
B. The 581 bp TaqI-Sau3A fragment was isolated from pEG10, 3'-end labelled with α^{32}P-dATP at the Sau3A site and processed as above. The numbers 1-5 refer to the cleavage site numbers in Table 1.
It should be noticed that the chemically cleaved DNA fragments have a slightly higher mobility than topoisomerase I cleaved fragments due to the presence of 5'-phosphate groups in the former and 5'-hydroxyl groups in the latter (cf. ref. 24).

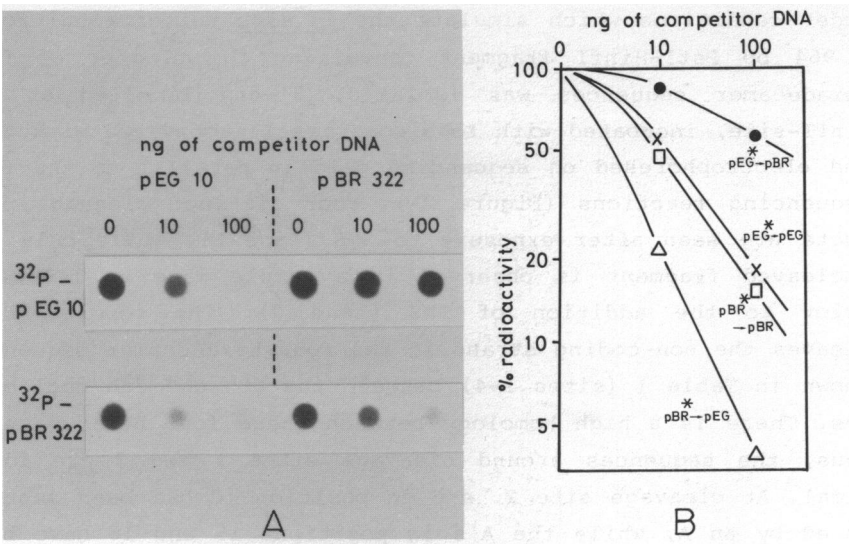


Figure 3. Topoisomerase I binding preference. Competition experiments.
^{32}P-labelled DNA and varying amounts of cold competitor DNA (0, 10, and 100 ng, respectively) were incubated with topoisomerase I. Cleavage and covalent binding of the topoisomerase to DNA was induced with SDS and monitored by filter retention as decribed in Materials and Methods. DNA containing (pEG10) and lacking (pBR322) hexadecamer topoisomerase recognition sequences were used in the experiment. The results were visualized by autoradiography (A) and scintillation counting (B).

the non-coding strand (Figure 2B) between the 6th and 7th nucleotide of the hexadecamer sequence shown in Table I (site 5). This sequence is identical to that at site 4. For detection of cleavage in the coding strand a 405 bp SphI-HinfI fragment was 3'-end labelled at the HinfI-site. One cleavage site was determined (result not shown) in the hexadecamer sequence with more than 85% homology to the consensus (site 6, Table I). Thus, the purified topoisomerase I recognizes and cleaves the five hexadecamer sequences fitting the consensus as observed *in situ*[1]. The fact that the hexadecamer sequence with >85% homology also is cleaved indicates some tolerance for sequence variation from the consensus.

Eukaryotic topoisomerases have not previously been found to exhibit any significant sequence specificity[15,19]. Thus, topo-

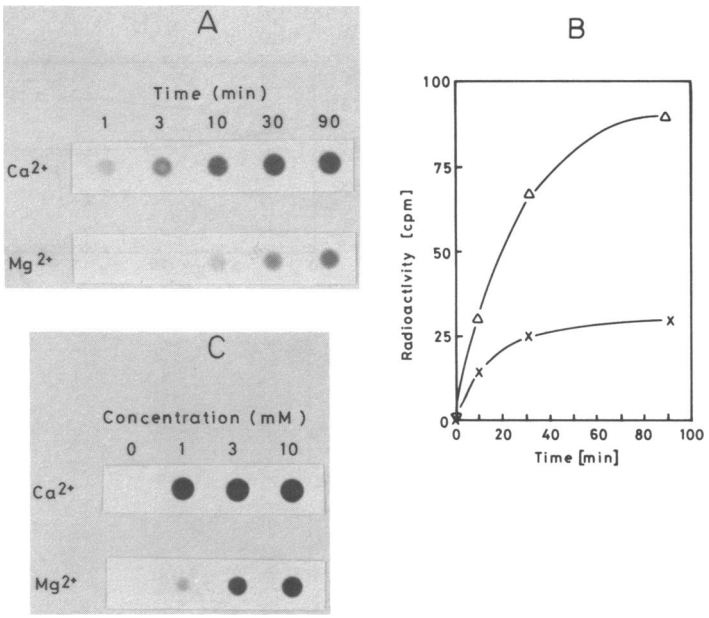


Figure 4. Effect of divalent cations on binding of topoisomerase I to DNA.
^{32}P-labelled pEG10 and topoisomerase I were incubated for different periods of time in the presence of 3 mM Ca^{2+} or 3 mM Mg^{2+} (A and B) or incubated for 30 min in the presence of varying concentrations of Ca^{2+} and Mg^{2+} (C). Samples were processed as described in Figure 3.

isomerase I cleavage sites have been observed for every one to two turns of the DNA helix with a slight preference for the quadromer sequence motif $\overset{ACT}{TGA}T$. To quantify the preference of topoisoemrase I for the specific hexadecamer sequence over the tetramer,. competition experiments between DNA carrying and lacking the topoisomerase I recognition sequences, pEG10 and pBR322, respectively, were performed. Aliquots of ^{32}P-labelled DNA and varying amounts of unlabelled competitor DNA were incubated with purified topoisomerase I. After cleavage induction the distribution of topoisomerase I was monitored by filter retention assays (cf. Materials and Methods). The results presented in Figure 3 show that 5-10 times more pBR322 than pEG10 is needed to reduce the amount of filterbound radioactivity to a certain level. Similar filter retention was obtained using

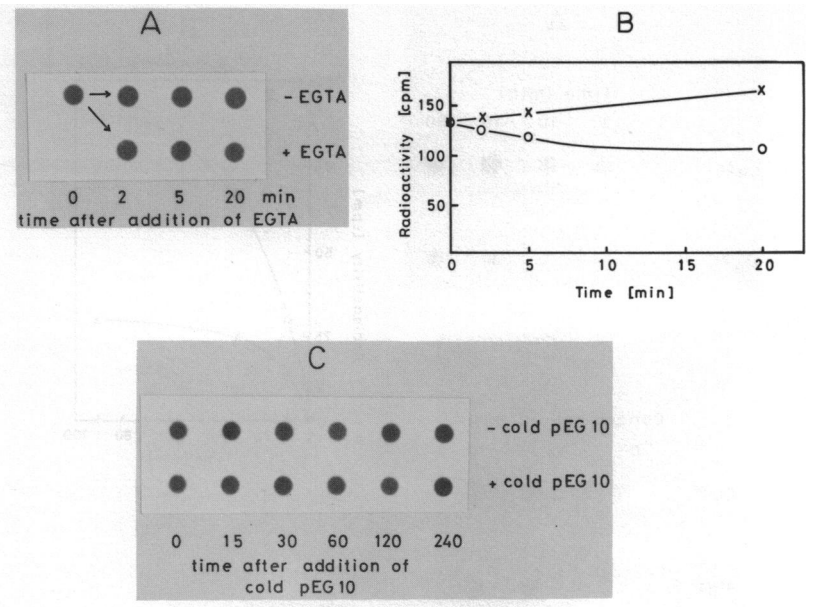


Figure 5. Stability of topoisomerase I-DNA complexes. ^{32}P-labelled pEG10 was preincubated with topoisomerase I for 30 min in the presence of 3 mM Ca^{2+}. The sample was then divided into four and incubated in the absence and presence of 10 mM EGTA (A and B) and 200 ng cold pEG10 (C), respectively. Samples were then processed as described in Figure 3.

topoisomerase I purified to homogeneity. As the tetramer sequence motif appears more than a hundred times in pBR322, the preference for the hexadecamer over the tetramer is more than 100 fold.

The specific binding of topoisomerase I to the recognition sequence requires the presence of divalent cations as seen in Figure 4. A study of the time course for this reaction in which the time of incubation of topoisomerase I and DNA before cleavage induction was varied from 1 to 90 min shows a preference for Ca^{2+} over Mg^{2+} (Figure 4A+B). A preference for Ca^{2+} is also observed keeping the incubation time at 30 min but varying the concentration of Ca^{2+} and Mg^{2+}, respectively (Figure 4C). Once the topoisomerase I-DNA complex is formed in the presence of Ca^{2+} it is stable. Thus, addition of EGTA to already established complexes has no drastic effect on their number (Figure 5A+B); incubation for 90 min in the presence of EGTA led to a

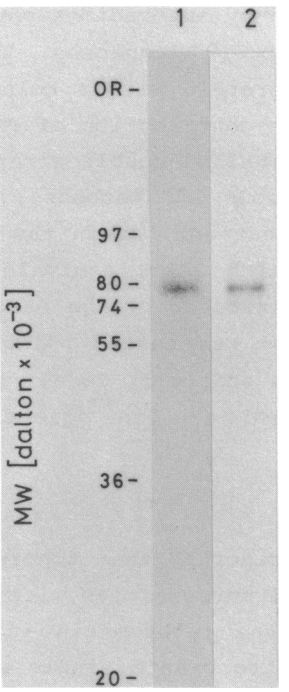


Figure 6. Determination of the molecular weight of topoisomerase I from T.pyriformis.
Topoisomerase I was incubated with ^{32}P-labelled pEG10 (A) and a ^{32}P-labelled double stranded hexadecamer oligonucleotide (B), respectively. After activation with SDS the samples were processed for SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The topoisomerase protein bands are visualized by autoradiography.

reduction of only 20% (Figure 5B). The high stability of the established complexes are also confirmed by incubation with a large excess of competitor DNA for up to 240 min (Figure 5C).

The strong affinity of topoisomerase I for the hexadecamer sequences has made it possible to determine the molecular weight of the enzyme prior to extensive purification. Two alternative methods have been applied (Figure 6). In one case the topoisomerase was incubated with ^{32}P-labelled pEG10 (cf. Materials and Methods) and covalently linked to the DNA by activation with SDS. After DNase treatment the proteins were separated by SDS-polyacrylamide gel electrophoresis and the

topoisomerase visualized by autoradiography due to the presence of protected labelled pEG10 sequences. The topoisomerase-DNA complex migrates as a protein with a molecular weight ~78 kD. In order to minimize the contribution of DNA, the topoisomerase was reacted with a ^{32}P labelled double stranded hexadecamer oligonucleotide (see Materials and Methods), and handled as above except for the DNase treatment. Again the molecular weight was found to be 78 kD of which 2 kD, equivalent to 6 nucleotides, are estimated to be contributed by the oligonucleotide. The reliability of this method was tested using homogenous topoisomerase I from Drosophila and calf. In all three cases the molecular weights determined are in agreement with previously published values.

DISCUSSION

We have previously reported that topoisomerase I *in situ* is associated with DNaseI hypersensitive sites in the promotor and terminator regions of the r-chromatin in *T.thermophila*[9]. The enzyme can be activated to create single strand nicks by exposure to strong detergents. Sequence analysis showed that the cleavages are located between the 6th and 7th nucleotide in hexadecamer sequences fitting the motif AGACTTAGA$^{A}_{G}$AAA$^{AAA}_{TTT}$[1]. In the present report we demonstrate that purified topoisomerase I specifically binds to six sites in cloned promotor rDNA from *T.pyriformis*. These sites hold hexadecamer sequences which fit or are >85% homologous to the above mentioned motif. This sequence specificity is striking as eukaryotic topoisomerase I previously not has been found to exhibit any significant sequence specificity. Thus, experiments by Edwards et al.[15] and Been et al.[16] showed that topoisomerase I-binding sites on a 2 kbp fragment of SV40 DNA occurred for every 1 to 2 turns of the DNA helix. Only a slight preference for tetramer sequences fitting the motif $^{ACT}_{TGA}$T was observed. One of these tetramer sequences is included in the core of our hexadecamer motif. Competition experiments demonstrate, however, that the sequences flanking this tetramer strongly influence the recognition, as topoisomerase I has more than 100 fold higher affinity for the hexadecamers than for the tetramers. The high preference for

the hexadecamer sequences has been observed under conditions where divalent cations, preferentially Ca^{2+}, are present. In the absence of divalent cations only low specific binding is observed. We find that homogenous topoisomerases I from other eukaryotic organisms under these conditions also exhibit similar sequence specificity. Thus, topoisomerases I from Drosophila, chicken and calf are found to have a preference for the hexadecamer sequences in rDNA from Tetrahymena (Gocke, Bonven, Nielsen & Westergaard, unpublished).

The in situ topoisomerase cleavage sites in rDNA in T.thermophila are all located on the non-coding strand and appear in clusters of three with an approximate 30 bp repeat within the cluster[1]. A similar repeat with four hexadecamer sequences exists in the non-coding strand of rDNA from T.pyriformis. The significance of this conserved spacing is unclear, but a plausible explanation might be that the topoisomerase I binds and operates in a cooperative manner. However, the topoisomerase also binds in the presence of only one hexadecamer recognition sequence as in sites 5 and 6 (cf. Figure 3).

A computer search has revealed that many eukaryotic genes transcribed by either RNA polymerase I, II or III are flanked by sequences with high homology to the presented topoisomerase I recognition sequence motif (Gocke, Bonven, Nielsen & Westergaard, unpublished results). In many cases these sequences are located within DNaseI hypersensitive regions indicating that topoisomerase I may be involved in the regulation of transcription.

This notion is supported by additional data: (i) topoisomerase I has been found associated with actively transcribed rDNA genes in Xenopus[17], (ii) topoisomerase I is associated with nucleosomes in a fraction enriched in transcribed sequences[18,19], (iii) topoisomerase I accumulates in transcriptionally active regions of the polytene chromosomes in Drosophila[20].

We have previously hypotesized that the topoisomerase I recognition sequence represents points of relaxation of topological stress accumulated during transcription[9]. Stress will be accumulated in the central part of the extrachromosomal, palindromic rDNA molecule when RNA polymerases move outwards on both

arms of the template unless the polymerase-RNP complexes can travel freely around the DNA fiber. Also, in chromosomal genes points of relaxation will be needed if the longitudinal transport of stress is hindered for example by fixation of the DNA fiber in the nuclear matrix. As a correlation between transcription of DNA and superhelical density of the template previously has been indicated[21-23], it is not unreasonable to ascribe the hexadecameric sequences a function in gene expression by attracting topoisomerase I. The enzyme may thereby influence the balance between relaxation and supercoiling, which in turn may govern the chromatin structure within a domain.

The presence of putative topoisomerase recognition sequences in regions flanking many eukaryotic genes of various origin and the conserved sequence preference for topoisomerase I from lower to higher eukaryotes indicate a basic regulatory function within the cells. Here we have argued for a possible involvement of the hexadecamer topoisomerase I recognition sequences in transcription, although a function in replication and recombination cannot be excluded.

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