DNA Topoisomerase VI Generates ATP-dependent Double-strand Breaks with Two-nucleotide Overhangs*

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A key step in the DNA transport by type II DNA topoisomerase is the formation of a double-strand break with the enzyme being covalently linked to the broken DNA ends (referred to as the cleavage complex). In the present study, we have analyzed the formation and structure of the cleavage complex catalyzed by Sulfolobus shibatae DNA topoisomerase VI (topoVI), a member of the recently described type IIB DNA topoisomerase family. A purification procedure of a fully soluble recombinant topoVI was developed by expressing both subunits simultaneously in Escherichia coli. Using this recombinant enzyme, we observed that the formation of the double-strand breaks on supercoiled or linear DNA is strictly dependent on the presence of ATP or AMP-PNP. This result suggests that ATP binding is required to stabilize an enzyme conformation able to cleave the DNA backbone. The structure of cleavage complexes on a linear DNA fragment have been analyzed at the nucleotide level. Similarly to other type II DNA topoisomerases, topoVI is covalently attached to the 5'-ends of the broken DNA. However, sequence analysis of the double-strand breaks revealed that they are all characterized by staggered two-nucleotide long 5' overhangs, contrasting with the four-base staggered double-strand breaks catalyzed by type IIA DNA topoisomerases. While no clear consensus sequences surrounding the cleavage sites could be described, interestingly A and T nucleotides are highly represented on the 5'-extensions, giving a first insight on the preferred sequences recognized by this type II DNA topoisomerase.

Type II DNA topoisomerases are ubiquitous enzymes that catalyze the ATP-dependent transport of one DNA duplex through a second DNA segment via a transient double-strand break (1). This ability to modulate the topological state of DNA is essential in major biological processes such as replication, recombination, and transcription (2). Until recently, these enzymes were thought to form a single family of homologous proteins. The discovery of DNA topoisomerase VI (topoVI) in hyperthermophilic archaea has modified this classification. Type II DNA topoisomerases are now subdivided into two subfamilies, type IIA and IIB DNA topoisomerases (Fig. 1) (3). The type IIA subfamily contains three cellular representatives: eucaryotic DNA topoisomerase II (topoII), bacterial DNA gyrase, and DNA topoisomerase IV (topoIV). DNA gyrase and topoIV are heterotetramers composed of two subunits GyrA and GyrB, and ParC and ParE, respectively, while the eucaryotic enzyme is a homodimer. Despite this difference in quaternary structure, protein sequences comparison revealed that GyrB and ParE subunits are homologous to the N-terminal part of the eucaryotic enzyme while GyrA and ParC are homologous to the C-terminal half. These similarities were further confirmed by structural analysis of several fragments of Saccharomyces cerevisiae DNA topoII and Escherichia coli DNA gyrase (4–7).

Archaeal topoVI is the prototype of the recently described type IIB DNA topoisomerase subfamily (3). This enzyme was first characterized from the hyperthermophilic archaeon Sulfolobus shibatae (8) and further identified in other archaeal species (see below). Like bacterial type II DNA topoisomerases, topoVI is a heterotetramer composed of two subunits, A and B, with molecular masses of 45 and 60 kDa, respectively. This enzyme is able to decatenate intertwined DNA and to relax both positively or negatively supercoiled DNA in the presence of ATP and divalent cations. These reactions, shared with the type II DNA topoisomerases implicated in chromosome segregation (i.e. eucaryotic topoII and bacterial topoIV), suggest that topoVI is the enzyme responsible for chromosome decatenation at the end of the replication in archaeal cells.

Surprisingly, initial sequence analysis of the genes encoding the two subunits of the S. shibatae enzyme revealed no evident similarities with classical type II DNA topoisomerases (3), apart from a few amino acids of the ATP binding/hydrolysis domain of type IIA DNA topoisomerases located near the N terminus of the topoVI B subunit. These conserved residues, which are distributed into three small motifs, are key elements of an ATP binding fold (referred to as the Bergerat fold) present in three other protein families: the heat-shock proteins of the Hsp90 family, the mismatch repair proteins of the MutL family, and a histidine kinase family (3, 9). Further analysis of the topoVI B subunit sequences by protein secondary structure prediction suggests that the first 215 residues adopt a similar fold.2 Whereas topoVI A subunits are not homologous to the cleavage domains of type IIA DNA topoisomerases (referred to as the A' domain in Fig. 1), significant similarities with the eucaryotic proteins, Spo11 from S. cerevisiae, Rec12 from Schizosaccharomyces pombe, and a putative Spo11 homolog in Caenorhabditis elegans have been described (3). Spo11 is one of the essential genes involved in the initiation of meiotic recombination (10, 11). Two different studies have clearly identified Spo11 as the

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1 The abbreviations used are: topoVI, DNA topoisomerase VI; topoII, DNA topoisomerase II; topoIV, DNA topoisomerase IV; DTT, dithiothreitol; AMP-PNP, adenosine 5'-(β,γ-imino)triphosphate; bp, base pairs.

2 C. Buhler, unpublished observation.
proteolytic domains of the GyrB subunit (17, 18). A domain is an \( \alpha \)-\( \beta \) structure that resembles the "Rossmann fold," present in a variety of metal-binding phosphotransfer proteins, including nucleases, topoisomerases, and response regulators (19). Secondary structure predictions suggested that topoVI also contains a Rossmann fold located, however, on the A subunit, instead of the B subunit, as in the case of Type IIA enzymes. (Fig. 1) (20). This prediction has been confirmed with the resolution of the crystal structure of the archaeal Methanococcus jannaschii topoVI A subunit (spanning residues 69–369 and referred to as topoVI-A' fragment) (21). This structural analysis has shown that the Rossmann-fold like domain of topoVI lies next to a CAP-like domain, giving an overall structure unique among type II DNA topoisomerases, and confirming that topoVI belongs to a new family of type II DNA topoisomerases. This study has also revealed the presence of a \( \text{Mg}^{2+} \) ion within the Rossmann-fold domain, supporting the idea of a metal-assisted cleavage reaction by type II DNA topoisomerases (21, 22).

Therefore, all the catalytic domains (Rossmann-fold and CAP) known to be involved in the DNA cleavage reaction by type IIA DNA topoisomerases are located within the same subunit for both topoVI A subunit and Spo11 protein. This particular organization could suggest that DNA cleavage is performed without the help of an additional protein. Hence this would explain the absence of a topoVI B subunit homolog in most eucaryotes, suggesting that Spo11 can cleave DNA by itself at the onset of the meiotic recombination. However, we failed to detect DNA cleavage using \( S. \ shibatae \) topoVI A subunit alone, while recombinant \( S. \ shibatae \) topoVI reconstituted from both subunits separately overexpressed in \( E. \ coli \) generates double-strand breaks (23). Furthermore, eucaryotic Spo11 proteins have not yet been purified, precluding the possibility to test their ability to perform DNA cleavage in the absence of a companion protein.

To further analyze the formation of the double-strand breaks catalyzed by type IIB DNA topoisomerase, we have overproduced and purified a new recombinant topoVI in \( E. \ coli \). Cloning both \( S. \ shibatae \) topoVI subunits in the same expression vector results in the overproduction of a soluble and active heterotetrameric enzyme. Analysis of the DNA cleavage reaction on supercoiled or linear DNA reveals a strict dependence on ATP binding, contrasting with DNA cleavages catalyzed by type IIA DNA topoisomerases (24–26).

We have also analyzed the structure of topoVI double-strand breaks on a 529-bp linear fragment. We observed that, similarly to type IIA DNA topoisomerases and \( S. \ cerevisiae \) Spo11 protein (12, 27), topoVI is covalently attached to the 5' DNA ends of the cleaved DNA. Characterization of several double-strand breaks at the nucleotide level reveals unusual features. All breaks generated have staggered two-nucleotide overhangs, contrasting with the four-base overhangs known for type IIA DNA topoisomerase (25, 26). Moreover, the 5' overhangs are mainly composed of the nucleotides A and T, giving the first insight of a consensus sequence recognition for this particular type II DNA topoisomerase.

**EXPERIMENTAL PROCEDURES**

**Materials**—The phosphocellulose P-11 was obtained from Whatman. Polyethyleneimine, ATP, AMP-PNP, phenylmethylsulfonil fluoride, and protein molecular weight markers were purchased from Sigma. Leupeptin and pepstatin A were purchased from Roche Molecular Biochemicals. Sephacryl S300 from Amersham Pharmacia Biotech. Sequagel-6 from National Diagnost (Hesse, United Kingdom). \( [\gamma-^{32}\text{P}]\text{ATP}, \ [\gamma-^{32}\text{P}]\text{ATP}, \ \text{and} \ [\alpha-^{32}\text{P}]\text{dATP} \) from Amersham Pharmacia Biotech. The pair of DNA oligonucleotides, seqEcoRI and seqScaI, used for the am-

**FIG. 1. Schematic alignment of amino acid sequences of type IIA DNA topoisomerases and Spo11 protein.** Numbers refer to amino acids of \( S. \ cerevisiae \) topoII, \( E. \ coli \) DNA gyrase, \( S. \ shibatae \) DNA topoVI, and \( S. \ cerevisiae \) Spo11. Solid boxes correspond to homologous regions within type II A enzymes and between topoVI A subunit and Spo11. Gray boxes correspond to the Rossmann-fold-like domain described in yeast topoII (4) and \( M. \ jannaschii \) topoVI A subunit structures (21). CAP corresponds to a fold similar to the DNA-binding domain of \( E. \ coli \) catabolite activator protein containing the active site tyrosine (TYr) (TYr^{78}) of yeast topoII (36) and Spo11 protein (TYr^{135}) (3). Vertical bars correspond to conserved motifs present in the ATP binding/hydrolysis domain of both subfamilies (3). The loop in GyrB corresponds to an insertion found in some \( \gamma \) proteobacteria. ATPase and B' refers to the proteolytic domains of the GyrB subunit (17, 18). A' corresponds to the DNA breakage-reunion domain of type IIA enzymes.
plification of a 529-bp pBR322 (comprising EcoRI and ScaI restriction sites), 5′-CATGAGAACTCTGGAACAAAGGGCCCT-3′ and 5′-GTGACATCTAAACCAGCTTACG-3′, were purchased from MWG Biotech (Ebersberg, Germany). Sequence reactions were performed using the fmol DNA sequencing system from Promega (Madison, WI).

Enzymes—Proteinase K, restriction enzymes, T4 polynucleotide kinase, E. coli DNA polymerase I Klunow fragment, and Tli DNA polymerase were purchased from Promega. T4 DNA polymerase and DNA ligase were purchased from New England Biolabs (Beverly, MA). Shrimp alkaline phosphatase was purchased from Roche Molecular Biochemicals.

Construction of pET3btop6BA Expression Vector—The pET3btop6BA expression vector was constructed from plasmids pET3b-SsuB and pET25-SsuA (23). pET3b-SsuB was digested with BamHI restriction enzyme and the sticky ends were made blunt using T4 DNA polymerase. pET25-SsuA plasmid was digested with XhoI and BamHI. The fragment containing translation initiation signals and the gene encoding the A subunit was purified and made blunt with T4 DNA polymerase. This DNA fragment was then ligated into the linearized pET3b-SsuB vector. Clones containing the A subunit gene in the proper orientation were identified by restriction analysis.

Overexpression and Purification of Recombinant DNA Topoisomerase VI—E. coli cells BI21(DE3) were transformed with the plasmids pET3b-SsuB and pET25-SsuA (a plasmid expressing SsuB). Cells were grown at 37°C in 0.5 liter of LB medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin until O.D.600 reached 0.5. Isopropyl-β-D-thiogalactopyranoside (200 μM) was then added to the culture and cells were further grown for 2 h at 37°C. Unless stated otherwise all enzyme purification steps were conducted at 4°C. Cells were harvested by centrifugation and resuspended in buffer A (HEPES-NaOH, 20 mM; pH 7.5, KCl, 1 mM; DTT, 1 mM; EDTA, 1 mM; EGTA, 1 mM; phenylmethylsulfonyl fluoride, 1 mM; 1 μg/ml leupeptin; and 1 μg/ml pepstatin). Cells were broken by sonication and the resulting solution was centrifuged at 15,000 × g for 15 min. The clarified lysate was then heated 15 min at 65°C and centrifuged at 20,000 × g for 15 min. Nucleic acids were precipitated by slow addition of polyethyleneimine with continuous stirring to a final concentration of 0.1%. After 15 min of additional stirring, the solution was centrifuged at 20,000 × g for 1 h. Proteins were precipitated by addition of ammonium sulfate to a final concentration of 70% saturation and centrifuged at 10,000 × g for 1 h. Precipitated proteins were dissolved in 400 ml of buffer B (HEPES-NaOH, 20 mM; pH 7.5, DTT, 1 mM; KCl, 50 mM) and adsorbed onto a 1 ml phosphocellulose column equilibrated with buffer B. The column was washed with 15 ml of buffer B and subjected to a 20-ml linear gradient of 50–800 mM KCl in buffer B. Fractions were pooled and passed over a Sepharac S300 gel-filtration column (Amersham Pharmacia Biotech) equilibrated with buffer C (HEPES-NaOH, 20 mM; pH 7.5, DTT, 1 mM; KCl, 350 mM). Peak fractions were pooled and stored at −80°C. One unit of topVI is defined as the amount of enzyme required to relax 50 ng of pBR322 in 4 min at 75°C under optimal conditions (25). DNA was finally purified using micropure EZ and resuspended into 10 mM Tris, pH 8.5, and an equal volume of a loading buffer (100 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol). In parallel, similar purifications of the topVI-cleaved DNA were performed and further incubated with E. coli DNA polymerase I Klunow fragment and each dNTP as described by the manufacturer. Reactions were stopped by heating at 75°C for 10 min before adding the loading buffer. The position of the 3′-ends of topVI cleavage sites on both strands were determined by an enzymatic sequencing approach: the length of these fragments were determined by comparison with the enzymatic sequencing of the initial DNA substrate using the primer sequOri or sequScaI. TopVI cleavage products were loaded on a 6% sequencing gel (6% polyacrylamide, 19:1 acrylamide/bisacrylamide, containing 7 μl urea), along with the sequencing reactions products. Electrophoresis were performed at 2500 V (45 watts) in TBE buffer with different migration times to gel map the topVI induced DNA breaks. Gels were dried and analyzed as before.

Protein Analysis—Polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue dye staining. Protein concentrations were estimated by Bradford assay using bovine serum albumin as a standard (28).

RESULTS

Expression and Purification of Recombinant DNA Topoisomerase VI—We have previously constructed the plasmids pET3b-SsuB and pET25-SsuA for the expression of both topVI subunits separately in E. coli (23). These plasmids were used as starting materials for the construction of the pET3btop6BA expression vector (see “Experimental Procedures”). The top6A gene and translational initiation signals were subcloned behind the top6B gene from the pET3bSsuB plasmid. The resulting vector contains both top6 genes under the same T7 promoter. Isopropyl-β-D-thiogalactopyranoside induction resulted in the production of significant amounts of two polypeptides with apparent molecular masses of 45 and 60 kDa, consistent with the sizes of topVI A and B subunits, respectively (Fig. 2, lane 3). Noteworthy, both overexpressed subunits are now present in the soluble protein fraction (Fig. 2, lane 4) whereas the expression of the A subunit alone in E. coli resulted in the
production of an insoluble polypeptide (23). A purification scheme was developed based on the thermostability expected for S. shibatae topoVI and its heterotetrameric state. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2, lanes 4-8). As a final step, the heterotetrameric form of the recombinant enzyme was selected on a Sephacryl S300 gel filtration column. This purification procedure yielded 0.7 mg of recombinant topoVI from 500 ml of culture, with an estimated purity of about 90%. The estimated specific activity (7 × 10⁴ units/mg of protein) is consistent with the specific activity of the native enzyme (8).

**ATP Binding Is Required for DNA Topoisomerase VI Induced DNA Cleavage**—The amounts of nicked or linear DNA molecules produced by this new recombinant enzyme, in the presence of calcium or magnesium cations and the cofactors ATP or AMP-PNP, were analyzed by agarose gel electrophoresis after standard cleavage assay (Fig. 3). TopoVI relaxes a negatively supercoiled DNA only in the presence of ATP and Mg²⁺ (Fig. 3, lane 3). When ATP is substituted by its nonhydrolyzable analog AMP-PNP, the enzyme generates important nicked and linear DNA products, indicating that SDS treatment efficiently traps the cleavage complexes (Fig. 3, lane 4). However, in the absence of AMP-PNP, no cleavage was observed (Fig. 3, lane 2). When Mg²⁺ is substituted by Ca²⁺, topoVI linearizes nearly all the supercoiled plasmid, in contrast to type IIA enzymes which produce a mixture of single- and double-strand breaks (29, 30). Ca²⁺-dependent DNA cleavage by S. shibatae topoVI also requires the presence of ATP or AMP-PNP (Fig. 3, compare lane 6 with lanes 7 and 8). Since this new recombinant enzyme generates important DNA breaks, this result shows a direct involvement of ATP binding in the DNA cleavage reaction, contrasting with the same reaction catalyzed by type IIA DNA topoisomerases (25, 26). Similar results were obtained in the presence of 30 and 80 mM KCl with no variations in quality or quantity of DNA products, except a diminution of the relaxation activity at 80 mM KCl (data not shown). In the following experiments, cleavage reactions were performed in optimal conditions, i.e. 10 mM CaCl₂ and 1 mM AMP-PNP.

**Distribution of DNA Topoisomerase VI Double-strand Breaks on pBR322**—In order to analyze double-strand breaks distribution on pBR322, we labeled two linear fragments covering the entire plasmid sequence. The plasmid was first linearized with EcoRI and labeled at its 5′-ends using T4 polynucleotide kinase and [γ-³²P]ATP. This DNA was further digested with the restriction enzyme ScaI leading to two singly end-labeled fragments of 515-bp (“small”) and 3848-bp (“large”) (Fig. 4, panel A). Both DNA substrates were used in a standard DNA cleavage assay and the resulting cleaved molecules were separated by electrophoresis on a 1% agarose gel (Fig. 4, panel B) or a nondenaturing acrylamide gel (Fig. 4, panel C) for the small and large molecules, respectively. As previously observed with a supercoiled DNA substrate (see above), no DNA cleavage occurred in the absence of ATP on these linear DNA (Fig. 4, B, lane 2, and C, lane 2). TopoVI efficiently generates double-strand breaks at multiple sites when ATP is added to the reaction (Fig. 4, B, lane 3, and C, lane 3). Noteworthy, a strong topoVI cleavage site near the SspI restriction site of pBR322 is observed (Fig. 4C, compare lanes 3 and 5). The topoVI induced DNA breaks are different in efficiency, indicating that a limited sequence specificity does exist for the interaction of the enzyme with DNA. When using AMP-PNP instead of ATP, a slight increase in the efficiency of the double-strand cleavage is observed, with no variation in the overall cleavage pattern (Fig. 4, B, lane 4, and C, lane 4).

**DNA Topoisomerase VI Is Covalently Linked to the 5′-Ends of the Cleaved DNA**—A common feature of type IIA DNA topoisomerases is a covalent fixation to the 5′-ends of the cleaved DNA via the active site tyrosine. Similar results were obtained for S. cerevisiae Spo11 protein which is also covalently attached to the 5′-ends of the meiotic double-strand breaks via a phosphotyrosine linkage (12, 27). In order to identify which strand is covalently linked to S. shibatae topoVI, we labeled the 515-bp linear fragment, either at its 5′- or 3′-end with ³²P. These substrates were used in a standard DNA cleavage assay, and covalent fixation of the enzyme to the cleaved DNA was analyzed regarding further proteinase K treatment (Fig. 5). When 5′-labeled DNA was used as a substrate, cleaved DNA fragments were recovered even in the absence of proteinase K treatment (Fig. 5, lanes 2 and 3). In contrast, when using a 3′-end-labeled DNA fragment, cleavage products enter the gel only after the proteolytic treatment (Fig. 5, compare lanes 5 and 6). Thus, similarly to type IIA DNA topoisomerase and Spo11, S. shibatae topoVI is covalently linked to the 5′-ends of the cleaved strands.

**Sequence Analysis of DNA Topoisomerase VI Cleavage Sites**—The purification of a soluble and fully active recombinant topoVI offers the possibility to characterize in vitro the structure of the double-strand breaks generated by a member of the type IIB DNA topoisomerase family. The structure of several topoVI cleavage sites were analyzed by mapping the 3′-ends of the cleaved DNA fragments on each strands (named upper and lower strands). The precise length of these fragments were determined by comparison with an enzymatic sequencing of the initial DNA substrate (Fig. 6).

We identified 19 detectable fragments on both strands (Fig. 6, lane 1 and lane 4, for the upper and lower strand, respectively). Noteworthy, we were able to map the strong topoVI cleavage site previously observed near the SspI restriction site.
Comparison of the DNA breaks intensity on both strands suggests that they all correspond to each other. Moreover, since nearly all supercoiled pBR322 plasmids are linearized after SDS treatment in the presence of AMP-PNP and Ca\(^{2+}\) (see Fig. 2), these DNA cuts should correspond effectively to double-strand breaks and not to single-strand DNA cleavages. Interestingly, the precise resolution of all these breaks reveals that they are facing each other with a two-base stagger (Fig. 7).

To further confirm this unusual structure, we used the purified cleaved fragments as substrates for the Klenow fragment of E. coli DNA polymerase I, as previously performed to characterize the structure of the double-strand breaks catalyzed by type IIA DNA topoisomerases (25, 26). We observed that all the cleaved DNA fragments were extended by two nucleotides for both strands (Fig. 6, lanes 2 and 5), confirming that topoVI effectively generates double-strand breaks with two-nucleotide overhangs.

Comparison of the sequences of these double-strand breaks revealed an additional feature unique for a type II DNA topoisomerase (see Fig. 7). 15 out of the 19 cleavage sites contain A and T nucleotides on their 5′ protruding ends. Noteworthy, the four remaining cleavage sites (marked e, h, j, and o) correspond to minor cleavage sites. As for other type II DNA topoisomerases, no evident sequence similarities surrounding the cleavage site could be identified (2).

**DISCUSSION**

In this work, we used a fully soluble form of recombinant S. shibatae topoVI to analyze the double-strand DNA cleavage mechanism, on both linear and negatively supercoiled DNA. This new recombinant enzyme was obtained by expressing the two topoVI subunits simultaneously in E. coli from a single vector. We had previously reported the reconstitution of S. shibatae topoVI from its separately purified subunits (Fig. 2) (23). However, in that case, the A subunit was recovered in inclusion bodies, and could only be solubilized after denaturation and renaturation, raising the possibility that some data obtained with the renatured enzyme could have been misleading. The coexpression of the two subunit genes in tandem might be important for the in vivo reconstitution of the heterotetramer in a soluble form. It should be noticed that the two genes encoding topoVI subunits are indeed organized in tandem in several archaeal genomes, including S. shibatae.

DNA double-strand cleavage is a key step in the catalytic mechanism of type II DNA topoisomerase. We report here two major differences of the DNA cleavage reaction between both families of type II DNA topoisomerases. 1) ATP is absolutely required for the DNA cleavage reaction by topoVI (Figs. 3 and 4). 2) TopoVI cleavage sites are all characterized by staggered two-nucleotide long 5′ protruding ends, instead of four-nucleotide overhangs previously observed for type IIA DNA topoisomerase cleavage sites (Fig. 6).

The strict requirement of either ATP or its nonhydrolyzable analog AMP-PNP for trapping topoVI cleavable complexes is unique among type II DNA topoisomerases since several re-
ports indicate that ATP is not required in the DNA cleavage reactions catalyzed by type IIA enzymes. (i) DNA gyrase is able to relax a negatively supercoiled DNA in the absence of ATP (31). (ii) Eucaryotic topoII, or truncated enzymes lacking the ATPase binding/hydrolyzing domain, are still able to cleave DNA in the absence of ATP (4, 18). (iii) Finally, analysis of \textit{E. coli} DNA topoIV activities indicates also an ATP independent DNA cleavage reaction (24).

The recent crystallographic structure of the DNA-binding core of the \textit{M. jannashii} topoVI A subunit has revealed that a Rossman-fold like domain, which coordinates a magnesium ion through conserved acidic residues, lies next to the active site tyrosine, itself located within a CAP-like DNA-binding domain (21). Therefore the topoVI A subunit possesses, by itself, all the functional domains required for DNA cleavage by type IIA DNA topoisomerases (B' plus A' domain, see Fig. 1). However, since no cleavage activity using the \textit{S. shibatae} topoVI A subunit alone has been detected (23), these functional domains do not seem to be sufficient to generate DNA cleavage, at least not in the case of this enzyme. The data presented here indeed support the idea that topoVI-induced DNA cleavage requires the cooperation of both A and B subunits, since this cleavage is strictly ATP dependent and the ATP-binding site of topoVI is located on the B subunit.

The modeling of a DNA duplex into the TopoVIA' dimer DNA binding groove, showed that the CAP-like domains lie too far from the DNA backbone to cleave and generate a phosphotyrosine linkage (21). Therefore, a conformational change of the topoVI A subunits seems to be required to cleave the DNA backbone. Our results thus strongly suggest that the B subunit could induce this change (through ATP binding), shifting the catalytic domains into proper orientation to cut DNA. In the absence of ATP, the A subunit dimer conformation in the whole enzyme could be similar to the structure obtained for the \textit{M. jannashii} topoVI-A' dimer.

A “two-gate model,” in which a transported DNA segment enters and exits from two opposite sides of the enzyme, has been proposed for both subfamilies of type II DNA topoisomerases (2, 21, 32). While the transported duplex can be stored within the A' domain of type IIA DNA topoisomerases, the topoVI-A' dimer lacks such a storage area. As a consequence, to allow the crossing of the two DNA segments, a DNA gate should be created by dissociation of the topoVI-A dimer, after covalent fixation to the 5'-ends of the broken DNA segment. In this model, as well as capturing the DNA segment to be transported, the assigned function of B subunits should be to hold the separated A subunits in order to avoid enzyme dissociation and subsequent generation of free double-strand breaks (en-

![Sequence analysis of topoisomerase VI cleavage sites](http://www.jbc.org/). A, a 529-bp fragment was labeled at its 5'-end and digested with StuI (upper strand) or EcoRI (lower strand) restriction enzymes to generate singly end-labeled DNA. These substrates were used in a standard cleavage assay and the purified cleaved fragments were loaded onto sequencing gels along with sequencing reactions of their respective molecules. Lanes 1 and 4, purified DNA cleavage products; lanes 2 and 5, DNA cleavage product further treated with \textit{E. coli} DNA polymerase I Klenow fragment; lanes 3 and 6, control reactions without topoVI.
DNA Cleavage by Topoisomerase VI

S. cerevisiae Spo11 to the 5′-ends of meiotic double-strand breaks (12, 27). Sequence determination of topoVI induced double-strand breaks reveals an unusual feature for a type II DNA topoisomerase. All breaks are characterized by staggered two-nucleotide long 5′ protruding ends (Fig. 6), while earlier studies of the double-strand breaks generated by type IIA DNA topoisomerases revealed four-nucleotide overhangs (25, 26). On the other hand, contrasting results were obtained from in vivo analysis of the double-strand breaks structure induced by Spo11 during meiosis. While these breaks are characterized by two-nucleotide 5′ overhangs in the ARG4 promoter region (33), blunt DNA ends were observed in the CYS3 promoter region or upstream of the HIS4 gene (34, 35). However, since these analysis were performed in vivo, from a rad50Δ mutant in which meiotic double-strand breaks persist, these blunt structures could reflect filling in of the 3′ ends by the cellular repair machinery. 4

The sequence determination and mapping of the topoVI induced double-strand breaks revealed another feature unique among type II DNA topoisomerases. Whereas cleavage efficiency differs from site to site, their 5′ extensions are mainly composed of A and T nucleotides (Fig. 7). Interestingly, A and T nucleotides have also been found in the 5′ overhangs of the most prominent double-strand break generated by Spo11 protein in the ARG4 promoter region (33). While no obvious consensus sequence have been described for type IIA enzymes (2), the high occurrence of these nucleotides in the 5′ extensions generated by topoVI could represent a first insight in a preferred sequence recognized by a type IIB DNA topoisomerase.

While studies on the catalytic mechanism by type IIB DNA topoisomerases are still in their infancy, this study highlights unusual features for a type II DNA topoisomerase both on formation and structure of the cleavage complex. Moreover the strict requirement of both topoVI subunits in DNA cleavage catalysis would suggest that the eucaryotic Spo11 protein should be somehow assisted to generate the meiotic double-strand breaks.

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