Stimulated Activity of Human Topoisomerases IIα and IIβ on RNA-containing Substrates*

(Received for publication, February 1, 1999, and in revised form, May 12, 1999)

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Eukaryotic topoisomerase II is a dimeric nuclear enzyme essential for DNA metabolism and chromosome dynamics. Central to the activities of the enzyme is its ability to introduce transient double-stranded breaks in the DNA helix, where the two subunits of the enzyme become covalently attached to the generated 5′-ends through phosphotyrosine linkages. Here, we demonstrate that human topoisomerases IIα and IIβ are able to cleave ribonucleotide-containing substrates. With suicide substrates, which are partially double-stranded molecules containing a 5′-recessed strand, cleavage of both strands was stimulated 8-fold when a ribonucleotide rather than a deoxyribonucleotide was present at the scissile phosphodiester of the recessed strand. The existence of a ribonucleotide at the same position in a normal duplex substrate also enhanced topoisomerase II-mediated cleavage, although to a lesser extent. The enzyme covalently linked to the 5′-ribonucleotide in the cleavage complex efficiently performed ligation, and ligation occurred equally well to acceptor molecules terminated by either a 3′-ribo- or deoxyribonucleotide. Besides the enhanced topoisomerase II-mediated cleavage of ribonucleotide-containing substrates, cleavage of such substrates could be further stimulated by ATP or antitumor drugs. In conclusion, the observed in vitro activities of the human topoisomerase II isoforms indicate that the enzymes can operate on RNA or RNA-containing substrates and thus might possess an intrinsic RNA topoisomerase activity, as has previously been demonstrated for Escherichia coli topoisomerase III.

DNA topoisomerases are ubiquitous enzymes that act to change the topological state of the DNA double helix (1). Two highly conserved classes of topoisomerases have been identified so far, which differ in their reaction mechanisms and physical properties (2, 3). Type I DNA topoisomerases alter DNA topology by making a transient single-stranded break in the DNA backbone, thereby allowing the passage of another DNA strand. In contrast, type II DNA topoisomerases introduce transient double-stranded DNA breaks and transfer an intact DNA duplex through the break before resealing it (4, 5). Based on the fundamental roles of DNA topoisomerases in manipulating DNA topology, it has been well established that these enzymes participate in virtually all aspects of DNA metabolism, including transcription, replication, recombination, and chromosome dynamics (6, 7).

Recent studies have shed light on a novel feature of DNA topoisomerases in RNA manipulation, in addition to their essential roles in DNA transactions (8). The prokaryotic type I topoisomerase, Escherichia coli topoisomerase III, has been shown to cleave RNA molecules to form a protein-RNA adduct (9). The enzyme has furthermore proven to be a true RNA topoisomerase, catalyzing topological changes on circular RNA as well as DNA substrates (10). More recently, it was manifested that also eukaryotic type I topoisomerases, including vaccinia virus and human topoisomerase I, recognize and cleave RNA-containing substrates. These two enzymes, however, possess a pronounced endoribonuclease activity, which results in self-displacement of the enzymes from the generated cleavage complexes due to a nucleophilic attack by the 2′-OH group of the ribose sugar on the phosphotyrosine linkages (11). Although the data existing so far exclusively deal with type I topoisomerases, they suggest a possible general feature of DNA topoisomerases as RNA processors.

Here, we have tested the ability of type II topoisomerases to operate on RNA-containing substrates. Advantage has been taken of an earlier developed partially double-stranded suicide substrate to investigate the ability of human topoisomerases IIα and IIβ to cleave and ligate substrates with a single ribonucleotide substitution (12). If the recessed strand of the suicide substrate contains a ribonucleotide rather than a deoxyribonucleotide at the scissile phosphodiester, cleavage of this strand was 8-fold stimulated by both topoisomerase II isoforms, and the same cleavage stimulation was seen on the complementary strand not holding a ribonucleotide. Topoisomerase II covalently linked to the ribonucleotide in the cleavage complex efficiently mediated ligation, and ligation occurred equally well to acceptors with a 3′-ribo- or deoxyribonucleotide. A stimulatory effect of the ribonucleotide on topoisomerase II-mediated cleavage was also observed when either topoisomerase IIα or IIβ was incubated with a duplex molecule having the ribonucleotide at the scissile phosphodiester in one strand. ATP and the antitumor drug VM-26 further stimulated cleavage of the ribonucleotide-containing duplex substrate, as they do of a normal DNA substrate. Taken together, our results indicate that human topoisomerase II, like type I topoisomerases, possesses the ability to operate on RNA as well as on DNA, and the possible functional implication of topoisomerase II in RNA processing is discussed.

MATERIALS AND METHODS

Purification of Recombinant Human Topoisomerases IIα and IIβ—The recombinant human topoisomerase IIα and IIβ enzymes were purified from the yeast strain BF201, which carries an expression vector containing either the human topoisomerase IIα or IIβ cDNA under control of the yeast Gal1 promoter. The recombinant enzymes are fused to a c-Myc tag and a hexahistidine tail at the C-terminal ends. The initial purification step using the Ni2+-nitrilotriacetic acid matrix was
as described previously (13). For further purification of the recombinant proteins to near homogeneity, the fractions pooled from the Ni²⁺-column were loaded on a 5-ml heparin-Sepharose column, and elution was performed with a 75-ml linear gradient of 200 mM to 1 mM NaCl. Topoisomerase II-containing fractions were further applied to a 0.4-ml SOURCE S column. The enzyme was finally eluted with a 5-ml linear gradient of 100–600 mM NaCl, and peak fractions were pooled and stored at –80 °C until use.

Oligonucleotides—DNA oligonucleotides as well as the oligonucleotides containing a single ribonucleotide substitution were synthesized on a Model 394 DNA synthesizer by DNA Technology Corp. and purified by preparative polyacrylamide gel electrophoresis. The 25-mer used as the bottom strand in the suicide or duplex substrate is modified at the 3'-end by the amino link —O-P₂—O—CH₂—CHOH—CH₂—NH₂ to inhibit ligation to this end.

Preparation of Cleavage Substrates—The suicide and duplex substrates were prepared by hybridizing 10 pmol of the top strand (16-mer and 24-mer, respectively) to 10 pmol of the complementary 28-mer bottom strand in 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, and 50 mM NaCl. The mixture was heated to 70 °C for 2 min and allowed to cool slowly to room temperature. After hybridization, the top strand of the substrates was 3'-end-labeled with [γ-³²P]ATP and Sequenase (U. S. Biochemical Corp.). resulting in a 17-mer top strand in the suicide substrate and a 25-mer top strand in the duplex substrate. For studies of bottom strand cleavage, the 28-mer bottom strand was 5'-end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase (New England Biolabs Inc.) before hybridization. The hybridized substrates were purified by native polyacrylamide gel electrophoresis.

Topoisomerase II-mediated Cleavage—A standard suicide cleavage reaction was set up by incubating 2.5 pmol of topoisomerase II with 0.1 pmol of labeled substrate in 50 μl of 10 mM Tris-HCl, pH 7.0, 2.5 mM MgCl₂, 25 mM CaCl₂, 20 mM NaCl, 15 μg/ml bovine serum albumin, and 0.1 mM EDTA (cleavage buffer) at 37 °C for 60 min. SDS (1% final concentration) was then added to stop the reaction, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis. Covalent complex formation was revealed by transfer of the radiolabeled oligonucleotide to the topoisomerase II polypeptide. Alternatively, the samples were subjected to phenol extraction after SDS treatment, and the protein-linked complexes were recovered from the phenol/water interface as described previously (12). The complexes were subsequently ethanol-precipitated and digested with proteinase K (1 mg/ml, 2 h, 37 °C). Following proteinase K digestion, the samples were equally divided into two tubes: one was further treated with NaOH (0.5 M, 60 min, 55 °C) followed by ethanol precipitation, and the other was left untreated. One volume of gel loading buffer (50% formamide, 0.03% bromphenol blue, 0.03% xylene cyanol, and 5 mM EDTA) was added to all the samples, and they were subjected to electrophoresis on 12% denaturing polyacrylamide gels. The level of cleavage was quantified using a PhosphorImager (Molecular Dynamics, Inc.) Reversibility of topoisomerase II-mediated cleavage was investigated by addition of 0.4 M NaCl to cleavage samples instead of 1% SDS. Samples were otherwise treated as described above. Cleavage of duplex substrates was performed by incubating 0.1 pmol of the substrate with 2.5 pmol of topoisomerase II in cleavage buffer. After 3 min at 37 °C, SDS was added to 1% to freeze the reaction. The protein-linked complexes were recovered from the phenol/water interface and analyzed by 12% denaturing polyacrylamide gel electrophoresis. The effect of ATP or the antitumor drug VM-26 (teniposide) on the cleavage activity of topoisomerase II was assayed by including either 1 mM ATP or 100 μM VM-26 in the reaction mixture.

Topoisomerase II-mediated Ligation—A topoisomerase II-mediated suicide cleavage reaction was performed as described above. After incubation at 37 °C for 60 min, the cleavage reaction was stopped by addition of NaCl to 0.4 M, thereby preventing further cleavage during the ligation reaction. Ligation was initiated by addition of a 12-mer ligation substrate in a 200-fold molar excess relative to the cleavage substrate. After further incubation (with incubation times as indicated in the figure legends), the reaction was stopped by addition of SDS to 1%. The samples were ethanol-precipitated, proteinase K-digested, and analyzed by electrophoresis on a 12% denaturing polyacrylamide gel.

RESULTS

A Ribonucleotide at the Scissile Phosphodiester of the Recessed Strand in a Suicide Substrate Stimulates Topoisomerase II- and III-mediated Cleavage—To investigate if human topoisomerase II can act as a potential RNA topoisomerase, we have taken advantage of a suicide cleavage substrate that contains a strong topoisomerase II recognition sequence (12, 14). The substrate consists of a 16-base-long 5'-recessed top strand with only three nucleotides 5’ to the cleavage position and a 28-base-long bottom strand. Use of the suicide substrate has been demonstrated to cause an uncoupling of the cleavage and ligation half-reactions due to the release of the trinucleotide 5’ to the cleavage position on the top strand, as schematically illustrated in Fig. 1A (12). In this study, the suicide substrate was modified by substituting the deoxyribonucleotide (A) at the normal scissile phosphodiester of the top strand with a ribonucleotide (rA), and the ability of topoisomerases IIα and IIβ to cleave this substrate was investigated. The substrate was labeled at the 3'-end of the recessed top strand and incu-
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Human topoisomerase II (Topo II) is a protein enzyme that can mediate the cleavage of DNA and RNA substrates. In the presence of a suitable acceptor strand, the enzymes become covalently linked to the cleaved substrate and can mediate ligation. This process is known as the suicide cleavage reaction.

The enzyme's activity is demonstrated by the formation of a covalently linked cleavage product, which can be detected by gel electrophoresis. The mobility of the cleavage product is determined by PhosphorImager scanning of the gel, and the level of cleavage is quantified by the amount of radioactive labeling of the enzymes due to their covalent linkage to the labeled top strand.

The suicide cleavage reaction can be used to study the specificity of topoisomerase II. In the study described in the document, the enzymes were found to have a strong preference for cleavage of the R-substrate as compared with the suicide substrate composed entirely of deoxyribonucleotides (D-substrate). This was demonstrated using a covalent linkage between the enzyme and the inserted ribonucleotide, as well as by the presence of a covalently linked undigested protein.

The authors also examined the ligation abilities of topoisomerase II on RNA-containing substrates. They found that the enzyme can mediate ligation on both the R- and D-substrates, in accordance with the suicidal nature of these substrates. The rate of the forward cleavage reaction was relatively constant after the first 15 min of incubation with the enzyme/substrate ratio used in the experiment. However, with both isoforms, the cleavage rate obtained with the R-substrate was ~8-fold higher than that obtained with the D-substrate. Thus, not only do human topoisomerases IIα and IIβ recognize and cleave the RNA-containing suicide substrate, the presence of a ribonucleotide at the scissile phosphodiester also stimulates the cleavage reaction dramatically.

Although insertion of a ribonucleotide at the normal scissile phosphodiester heavily stimulates topoisomerase II-mediated cleavage, the presence of the ribonucleotide might change the cleavage specificity of the enzyme. To determine if topoisomerases IIα and IIβ cleave the R-substrate at the same position as they cleave the D-substrate, cleavage reactions were performed in which the protein-linked complexes were isolated from a phenol/water interphase and analyzed on a 12% denaturing polyacrylamide gel. Both isoforms gave rise to one prominent cleavage product with the mobility of a 14.5-mer, as demonstrated previously (12, 17). The bands marked with an asterisk represent cleavage products with a longer protein fragment covalently linked, due to partial proteinase K digestion (12, 17).

Materials and Methods:
- Materials and Methods were described under the appropriate section.
- The suicide cleavage reaction was performed in which samples were withdrawn from the suicide cleavage reaction at different time points (0–75 min) and analyzed as described above.
- The rate of the forward cleavage reaction was relatively constant after the first 15 min of incubation with the enzyme/substrate ratio used in the experiment. However, with both isoforms, the cleavage rate obtained with the R-substrate was ~8-fold higher than that obtained with the D-substrate.
- The results show that the stimulated cleavage mediated by topoisomerases IIα and IIβ takes place at the ribonucleotide, and the enzymes become covalently linked to the ribonucleotide during complex formation.

Examination of the Ligation Abilities of Topoisomerase II Covalently Linked to a Ribonucleotide:
- Topoisomerase II covalently linked to a deoxyribonucleotide in the cleavage complex generated upon cleavage of a suicide substrate is known to be kinetically active. It can therefore act as a donor in ligation if a suitable ligation substrate is added to the reaction mixture after cleavage complex formation.

Results and Discussion:
- The results show that the stimulated cleavage mediated by topoisomerases IIα and IIβ takes place at the ribonucleotide, and the enzymes become covalently linked to the ribonucleotide during complex formation.

Conclusions:
- The study demonstrates the versatility of topoisomerase II in cleaving and ligating RNA substrates, which is important for understanding the role of these enzymes in the regulation of gene expression and DNA replication.

The abbreviations used are: R-substrate, ribonucleotide-containing substrate; D-substrate, deoxyribonucleotide-containing substrate.

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Fig. 3. Examination of the donor and acceptor capabilities of the ribonucleotide-containing substrates. A, schematic illustration of the ligation reaction performed by topoisomerase II (topoII) covalently linked to the cleaved suicide substrate. The boldface line illustrates the incoming ligation substrate (acceptor), which is complementary to the single-stranded region of the bottom strand. The asterisk represents radioactive labeling. B, time course of topoisomerase II-mediated ligation using the R- or D-substrate. A ligation reaction was set up as described under “Materials and Methods” in the presence of a 12-mer DNA oligonucleotide acceptor. Samples were withdrawn at the indicated time points, treated with SDS, and subjected to electrophoresis on a 12% denaturing polyacrylamide gel. Lanes 2–8 show the ligation products obtained with topoisomerase II covalently linked to the D- and R-substrates, respectively. Lanes 1 and 10 show 3'-end-labeled D- and R-substrates, respectively. Lanes 9 and 18 show DNA size markers increasing in steps of 2 bases. L, S, and Cl indicate the ligation product, cleavage substrate, and cleavage product, respectively. C, graphic illustration of the ligation time course presented in B. Cleavage and ligation products were quantified by PhosphorImager scanning of the gels, and the extent of ligation, expressed as the percentage of initial cleaved material, is plotted versus ligation time. D, graphic illustration of the time course of a ligation reaction performed as described for B and C, except that the acceptor was a 12-mer with a ribonucleotide (rG) at the 3'-end as shown. Cleavage and ligation products were quantified by PhosphorImager scanning of the gel, and the extent of ligation, expressed as the percentage of initial cleaved material, is plotted versus ligation time.

(lanes 2–8). The results are depicted graphically in Fig. 3C, where the levels of ligation at the different time points are given as a percentage of initial cleaved material to take into account differences in the cleavage level at the start of ligation. The initial rate of topoisomerase II-mediated ligation and the final percentage of ligated material are comparable for the enzyme linked to a ribo- or deoxyribonucleotide, indicating that the two complexes have similar donor capabilities, at least when the acceptor is DNA. Analogous results have been obtained with topoisomerase IIβ (data not shown).

To investigate if ligation also takes place to a ribonucleotide-terminated acceptor, another ligation experiment was performed utilizing a 12-mer ligation substrate identical to the one used in Fig. 3B and C, except that a ribonucleotide (rG) is present at the 3'-end. Cleavage and ligation were performed as described above, and a time course of the ligation reaction for topoisomerase IIα is presented in Fig. 3D. As seen from the ligation curves, the ribonucleotide-terminated ligation substrate efficiently acted as an acceptor whether the donor was topoisomerase II linked to a ribo- or deoxyribonucleotide. From a comparison of the ligation reactions presented in Fig. 3 (C and D), it is furthermore evident that the two 12-mer ligation substrates acted with equal efficiency in the ligation reaction, indicating that there is no major difference in the ability of a ribonucleotide and a deoxyribonucleotide to perform the required nucleophilic attack on the phosphotyrosine linkage during ligation. Although it is likely that the nucleophilic attack performed by the ribonucleotide-containing ligation substrate is mediated through the 3'-OH group of the ribose sugar, we cannot exclude the possibility that the 2'-OH group of the sugar may act to form a 2'-5' linkage during ligation in addition to the canonical 3'-5' linkage. The results suggest that topoisomerase II, besides its activities on DNA, can operate on RNA substrates or even at the link between RNA and DNA in substrates made up partly by RNA. The enzyme is therefore a likely candidate for performing different topological changes on DNA-containing nucleic acids in the cell.

Presence of a Ribonucleotide at the Scissile Phosphodiester in the Top Strand of the Suicide Substrate Stimulates Topoisomerase II-mediated Cleavage of the Bottom Strand—So far, we have used the R-substrate to investigate how insertion of a ribonucleotide at the scissile phosphodiester influences cleavage of the strand holding the ribonucleotide. However, the dimeric topoisomerase II enzyme functions by creating a dou-
ble-stranded break 4 base pairs apart on the DNA backbone, with one subunit acting on each strand (4, 18). It was therefore interesting to investigate how a ribonucleotide at the scissile phosphodiester of the top strand in the suicide substrate would influence cleavage mediated by the other subunit on the bottom strand. To address this question, the 28-mer bottom strand was labeled at the 5′-end before hybridization to either the ribo- or deoxyribonucleotide-containing top strand to generate the bottom strand labeled R- and D-substrates, respectively. The substrates were incubated with topoisomerase IIα or IIβ for 3 min at 37 °C before each sample was divided in two: one was stopped by SDS, and the other was treated with NaCl. DNA was isolated from the SDS and NaCl samples by ethanol precipitation, and cleavage products were loaded directly on 12% denaturing polyacrylamide gels. Both isoforms cleaved the R- and D-substrates at the same position on the bottom strand, creating a protein-free cleavage product of 12 bases as expected from the 4-base staggered fashion of topoisomerase II-mediated DNA cleavage (data not shown). The bottom strand cleavage levels for topoisomerase II were further quantified by PhosphorImager scanning of the gels and are graphically presented in the histogram in Fig. 4A. As a control, a similar experiment was performed using the top strand 3′-end of the top strand. The results are presented graphically in Fig. 4B. A comparison of the SDS samples obtained with the R- and D-substrates shows that the enzyme had a strong preference for the R-substrate also concerning bottom strand cleavage. Cleavage of this strand was stimulated 8-fold (Fig. 4A), which is similar to the cleavage stimulation seen on the top strand holding the ribonucleotide (Fig. 4B). The same observations have been obtained for topoisomerase IIβ (data not shown). Thus, the presence of a ribonucleotide at the scissile phosphodiester of the top strand not only stimulates cleavage of this strand, but also has a tremendous influence on the cleavage mediated by the other subunit on the bottom strand. The observation that cleavage of both strands is equally stimulated indicates a strong coordination between the two topoisomerase II subunits during substrate binding and/or cleavage.

From earlier observations, it was anticipated that cleavage of the bottom strand in the suicide substrate predominantly occurs as an equilibrium process, with most cleavage complexes being reversed upon addition of NaCl, whereas cleavage of the top strand is suicidal and salt-irreversible (12). The same holds true for the R-substrate, as bottom strand cleavage was largely reversed by addition of NaCl (Fig. 4A), whereas top strand cleavage was not (Fig. 4B). The fact that religation took place on the bottom strand under conditions where no religation occurred on the top strand suggests that the two topoisomerase II subunits are uncoupled in the ligation reaction, as demonstrated previously (12), although it cannot be excluded that some of the cleavage events result from topoisomerase II-mediated single-stranded cleavage (19, 20).

A Ribonucleotide in a Duplex Substrate Stimulates Topoisomerase II-mediated Cleavage, and Cleavage Can Be Further Stimulated by ATP or VM-26—The results presented above indicate that topoisomerase II has an intrinsic ability to recognize and cleave RNA-containing substrates as well as to use such substrates both as donors and acceptors in its catalytic cycle. However, all the results were obtained using suicide substrates, which are ideal for delineating mechanistic aspects of topoisomerase II, but are different from the enzyme’s normal duplex substrates. To confirm that topoisomerase II has an ability to act on an RNA-containing substrate also when the substrate has a duplex nature, two other substrates were generated that were identical to the R- and D-substrates, except they were fully extended at the 5′-end of the top strand. The two substrates, denoted by R′ and D′, respectively, were labeled at the 3′-end of the top strand and incubated with topoisomerase IIα or IIβ. After 3 min at 37 °C, SDS was added to freeze the equilibrium. Cleavage complexes were recovered from a phenol/water interphase and analyzed on a denaturing polyacrylamide gel after proteinase K digestion. As shown in Fig. 5A (lanes 2, 4, 7, and 9), the cleavage products obtained with the R′- and D′-substrates migrated to the same position, demonstrating that both isoforms recognize and cleave at the ribonucleotide phosphodiester also when the ribonucleotide is present in a duplex substrate. In addition, more cleavage was observed with the R′-substrate than with the D′-substrate (compare lanes 4 and 2 and lanes 9 and 7), although cleavage stimulation was not as pronounced as with the suicide substrate. The fact that topoisomerase II has an increased activity on ribonucleotide-containing duplex substrate demonstrates that the stimulatory effect of the ribonucleotide on cleavage is not just an intrinsic characteristic of the suicide substrate, and the result lends further support to the possible action of human topoisomerase II as an RNA processor.

Several antitumor agents as well as ATP are known to stimulate topoisomerase II-DNA cleavage complex formation (14, 21, 22). To investigate if these agents also have a stimulatory effect on complex formation between topoisomerase II and
RNA-containing substrates, a cleavage experiment was performed in which either ATP or the antitumor drug VM-26 was added to a cleavage reaction containing the duplex R\textsuperscript{9}- or D\textsuperscript{9}-substrate. Following gel electrophoresis, cleavage products were isolated from a phenol/water interphase and loaded on a 12% denaturing polyacrylamide gel after proteinase K treatment. Lanes 1–4, topoisomerase II\textalpha-mediated cleavage; lanes 6–9, topoisomerase II\textbeta-mediated cleavage; lanes 5 and 10, DNA size markers increasing in steps of 2 bases. S and Cl indicate substrate and cleavage products, respectively. The asterisks to the left of the gel indicate cleavage products with a longer protein fragment covalently linked due to partial proteinase K digestion (12, 17).

**DISCUSSION**

Eukaryotic DNA topoisomerase II catalyzes a repertoire of DNA transactions, which all depend on the ability of the enzyme to introduce transient double-stranded breaks in the DNA backbone (4). The DNA cleavage and ligation reactions of topoisomerase II have been investigated in great detail using linear duplex (19, 23, 24) or suicide (12, 25) substrates, and the latter allow studies of the two reactions independently (26). The analyses have demonstrated an ability of topoisomerase II to take part in both inter- and intramolecular ligation reactions, and a more detailed characterization has been performed of the various DNA substrates that can act as donors and acceptors in the DNA transfer reactions (27).

Here, we have analyzed the potential of the human topoisomerase II isoforms, topoisomerases II\textalpha and II\textbeta, to operate on RNA-containing substrates. We found that both isoforms recognize and cleave either suicide or duplex substrates containing a single ribonucleotide at the scissile phosphodiester in one strand. Cleavage of the suicide substrate, in which the ribonucleotide is inserted at the scissile phosphodiester of the 5'-recessed strand, is stimulated ∼8-fold, whereas cleavage of the duplex is stimulated ∼2-fold. Studies of the ligation reaction have shown that topoisomerase II covalently linked to a ribo-

**Fig. 5.** Topoisomerase II-mediated cleavage of a ribonucleotide-containing duplex substrate in the absence and presence of ATP and VM-26. A, human topoisomerase II (TopoII)\textalpha- and II\textbeta-mediated cleavage of a ribonucleotide-containing duplex substrate. The cleavage reactions were performed as described under “Materials and Methods.” The top and bottom strand oligonucleotides composing the duplex substrates used in the cleavage reaction are shown at the top. The substrate with a ribonucleotide at the scissile phosphodiester is denoted by R\textsuperscript{9}, and the substrate with a deoxyribonucleotide at this position is denoted by D\textsuperscript{9}. The asterisk at the 3'-end of the top strand illustrates radioactive labeling. Cleavage products were isolated from a phenol/water interphase and loaded on a 12% denaturing polyacrylamide gel after proteinase K treatment. Lanes 1–4, topoisomerase II\textalpha-mediated cleavage; lanes 6–9, topoisomerase II\textbeta-mediated cleavage; lanes 5 and 10, DNA size markers increasing in steps of 2 bases. S and Cl indicate substrate and cleavage products, respectively. The asterisks to the left of the gel indicate cleavage products with a longer protein fragment covalently linked due to partial proteinase K digestion (12, 17). B, effect of ATP and VM-26 on topoisomerase II-mediated cleavage of the R\textsuperscript{9}- and D\textsuperscript{9}-substrates. Cleavage experiments were performed as described above, but in the absence or presence of 1 mM ATP or 100 \textmuM VM-26 as indicated. After gel electrophoresis, cleavage products were quantified by PhosphorImager scanning, and cleavage levels are presented in arbitrary units relative to the cleavage level obtained in the absence of ATP or VM-26. The left and right panels show cleavage obtained with the D\textsuperscript{9}- and R\textsuperscript{9}-substrates, respectively.
nucleotide in the cleavage complex has the same donor activity as topoisomerase II linked to a deoxyribonucleotide. Moreover, ligation substrates terminated with either a ribo- or deoxyribo-nucleotide at the 3′-end function equally well as acceptors in the ligation reaction. The stimulated activity of the human topoisomerase II isoforms on ribonucleotide-containing substrates can be further increased by either ATP or the antitumor drug VM-26. Together, our observations suggest that topoisomerase II can operate on RNA or RNA-containing substrates, so the enzyme, besides being a DNA topoisomerase, might also act as an RNA topoisomerase.

Different type I topoisomerases have previously been described to possess RNA-manipulating activity. Thus, E. coli topoisomerase III is capable of cleaving RNA molecules to produce a protein–RNA adduct (9), and also, this enzyme has directly been proven to hold RNA strand passage activity (10). Likewise, vaccinia virus topoisomerase I has been investigated for the ability to act on RNA or RNA-containing substrates. Although this enzyme does not cleave substrates in which either the scissile or the complementary strand is composed entirely of RNA (28), a duplex substrate in which the scissile strand is composed of DNA upstream and RNA downstream of the scissile phosphate is proportionally cleaved by the enzyme (29). Combined with our observations on human topoisomerase II, the ability to act on RNA or RNA-containing substrates seems to be a general characteristic for all topoisomerases.

A study by Sekiguchi and Shuman (11) has demonstrated that vaccinia virus and human topoisomerase I both hold an endoribonuclease activity. Thus, the phosphotyrosine linkage of the covalent RNA-3′-phosphoryl-enzyme intermediate is attacked by the vicinal 2′-OH of the ribose sugar, whereby the enzyme is released, and a free 2′,3′-cyclic phosphate product is generated. The reaction is highly efficient, with up to 80% of the input substrate being converted to the enzyme-free cleaved product. Another eukaryotic topoisomerase I-like protein, the site-specific DNA recombinase Flp, also possesses endoribonuclease activity (30). Such an activity has not been observed for E. coli topoisomerase III, which, in contrast to the others, becomes covalently linked to the 5′-phosphate of its substrate (9). Likewise, our investigations have not revealed any endoribonuclease activity for human topoisomerase II. In no case is there a release of an enzyme-free cleaved product seen upon topoisomerase II-mediated cleavage of the ribonucleotide-containing substrates. This is further supported by the results obtained from an experiment in which ligation was continued in the absence of an acceptor for another 30 min after the cleavage reaction had been stopped with NaCl. In this experiment, no change in the level of protein-linked cleavage product was observed during the extended ligation period, demonstrating that self-displacement of the enzyme does not take place under the employed conditions (data not shown). Thus, biochemically, the 2′-OH of the ribose sugar is probably structurally remote from the 5′-phosphotyrosine linkage in the topoisomerase II-ribonucleotide cleavage complex and is unable to perform a nucleophlic attack. Our demonstration of a lacking endoribonuclease activity of human topoisomerase II lends support to the earlier proposed suggestion that only topoisomerases forming covalent linkage to the 3′-phosphate of RNA can be released by nucleophilic attack from the vicinal 2′-OH of the ribose sugar (11).

The fact that topoisomerase II is highly efficient on ribonucleotide-containing substrates whether the substrates have a suicidal or duplex nature suggests that the observed in vitro activities are not just irrelevant side reactions. However, it is not yet evident why cleavage is stimulated ~8-fold on the suicide substrate and only 2-fold on the duplex substrate. To this end, it should be noted that topoisomerase II-mediated cleavage of suicide substrates in general is much less efficient than cleavage of duplex substrates (17). The lowered cleavage activity on the suicide substrates probably reflects a decreased affinity of topoisomerase II for these substrates. Based on this, a likely explanation for the differences observed in cleavage stimulation with the ribonucleotide-containing suicide and duplex substrates is that binding of topoisomerase II to the suicide substrate is relatively more stabilized by the presence of a ribonucleotide than binding of the enzyme to the duplex substrate. Another interesting observation seen here with the suicide substrate is that the same 8-fold cleavage stimulation is obtained on both strands, although only one of these holds a ribonucleotide. Taking the dimeric nature of topoisomerase II into consideration, where one subunit acts on each strand, the single ribonucleotide is expected to strongly influence binding of only the enzyme subunit directly contacting it. Our observation therefore indicates that the effect of the ribonucleotide somehow can be transmitted from one subunit to the other, suggestive of a strong coordination between the two enzyme subunits during substrate recognition and/or cleavage.

The results obtained in this study using substrates containing a single ribonucleotide strongly indicate that topoisomerase II can operate on RNA or RNA-containing substrates. Although DNA topoisomerase II has not yet been proven to act as an RNA operator within the cell, a number of possible in vivo functions can be suggested for the enzyme based on its observed in vitro activities. For example, topoisomerase II might actively recognize and cleave at ribonucleotides that have been misincorporated into DNA, as suggested earlier for type I topoisomerases (11). However, the fact that topoisomerase II has an increased cleavage activity as well as an efficient ligation activity on the ribonucleotide-containing substrates makes it more likely that the enzyme is a candidate for performing topological changes on RNA or RNA/DNA hybrids within the cell. This hypothesis is further supported by the observation that topoisomerase II lacks endoribonuclease activity, which otherwise would disturb the normal catalytic cycle by releasing the enzyme before normal ligation. Since the endoribonuclease activity is pronounced for eukaryotic topoisomerase I (11), this enzyme is less likely to be a topological monitor of RNA-containing substrates. So far, a function of topoisomerase II as a modulator of RNA or RNA/DNA topology is only speculative. A main issue for the future is therefore to investigate if topoisomerase II holds full RNA topoisomerase activity in such a way that, besides its cleavage/ligation activities, it has the ability to perform strand transfer reactions on RNA or RNA-containing substrates. A clear delineation of the biological roles of the RNA-manipulating activities of topoisomerase II, however, awaits further in vitro and in vivo experimentation.

Acknowledgments—We are grateful to Drs. Harald Biersack, Ole Frederik Nielsen, Mogens Kruhøffer, and Kent Christiansen for valuable discussions and to Kirsten Andersen for skillful technical assistance.

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