Covalent Attachment of Ethidium to DNA Results in Enhanced Topoisomerase II-Mediated DNA Cleavage†

Geoffrey Marx,‡ Hui Zhou,‡ David E. Graves,*,‡ and Neil Osheroff§

Department of Chemistry, University of Mississippi, University, Mississippi 38677, and Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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ABSTRACT: The classic DNA intercalator, ethidium, was used to probe the effects of (i) intercalation and (ii) covalent modification of the DNA on the catalytic activity of topoisomerase II. Ethidium bromide, which binds reversibly to DNA via intercalation, does not stimulate topoisomerase II-mediated DNA cleavage at concentrations up to 100 μM, indicating that the intercalative binding of this molecule to DNA is not sufficient to alter the activity of the enzyme. In contrast, covalent attachment of the photoreactive ethidium analog to DNA resulted in marked enhancement of topoisomerase II-mediated single- and double-stranded DNA cleavage. This increase in DNA cleavage was observed at very low drug binding densities (<1 drug per 10−80 base pairs) which correspond to nanomolar concentrations, as compared with other topoisomerase II poisons such as etoposide or *m*-AMSA which require micromolar concentrations to elicit comparable DNA cleavage levels. Over the past decade, topoisomerase II has been an important target for a variety of clinically relevant anticancer agents due to the abilities of these agents to convert this enzyme to a cellular toxin resulting in an increase in the levels of enzyme-mediated DNA breaks. Modification of DNA by covalently attaching a DNA-targeting intercalating agent (i.e., ethidium bromide) resulted in a marked shift of the cleavage/religation equilibrium of the enzyme toward the cleaved state "poison" topoisomerase II as observed by the enhancement in single- and double-stranded cleavage; thus, key insight was gained into the mechanism(s) through which DNA binding agents may influence the catalytic properties of topoisomerase II. These data demonstrate that conversion of a reversible ethidium−DNA complex to an irreversible adduct results in the transformation of an ineffective intercalating drug into a potent topoisomerase II-targeted agent. Finally, they provide support for the recently proposed "positional poisoning model" for the actions of DNA lesions and anticancer drugs on the type II enzyme.

Several leading anticancer agents, including *m*-AMSA, adriamycin, daunorubicin, ellipticine, and mitoxantrone, mediate their anticancer activity by enhancing the ability of topoisomerase II to generate double- and single-stranded breaks in the DNA of treated cells (*1*−*4*). The presence of elevated levels of covalent topoisomerase II-cleaved DNA complexes provokes mutagenic and cell death pathways (*5*−*7*). Thus, chemical agents which are effective in promoting enhanced topoisomerase II-mediated DNA cleavage are referred to as topoisomerase II "poisons" because of their ability to convert this enzyme to a potent cellular toxin. Information concerning the actual mechanism(s) through which these intercalating drugs exert this effect is limited; however, the ability of these compounds to interact with both DNA and topoisomerase II appears to be essential for nucleic acid cleavage. In 1979, Kohn and co-workers reported that treatment of intact cells with the intercalative drugs ellipticine or adriamycin resulted in limited fragmentation of the chromosomal DNA (*8*, *9*). The fact that this drug did not cleave purified DNA led to speculation that there might be a nuclear receptor for the drug that was responsible for the drug-induced DNA cleavage (*10*). Further studies revealed that DNA strand breaks produced in the presence of drugs were mediated by a salt-extractable nuclear protein that Kohn and co-workers later identified as topoisomerase II (*11*). Using purified enzyme, Liu and co-workers later demonstrated that a topoisomerase II−DNA complex was formed which could be correlated with drug-induced DNA cleavage (*12*−*14*). Since these original studies, relationships between anticancer agents and enhanced topoisomerase II-mediated DNA cleavage have been the focus of intense interest due to their relevance to cancer chemotherapy.

Initial studies with DNA intercalators such as adriamycin, *m*-AMSA, and ellipticine led many investigators to propose that topological changes associated with the unwinding of the DNA helix due to drug binding were responsible for the altered enzyme activity (*15*, *16*). However, the discovery that nonintercalative compounds such as the epipodophyllotoxins (etoposide and tenoposide) were also potent topoisomerase II poisons pointed toward alternate mechanisms for influencing enzyme activities (*17*). Indeed, not all intercalating agents induce topoisomerase II-mediated DNA cleavage. A classic example of this differentiation can be seen by comparing *m*-AMSA with *o*-AMSA. Although structural isomers, *m*-AMSA exhibits 5 times greater potency in stimulating topoisomerase II-mediated DNA strand breaks than does its structural conformer, *o*-AMSA, even though both compounds bind DNA via intercalation (*12*). Other

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* Author to whom correspondence should be addressed. Phone: (601) 232-7732. Fax: (601) 232-7300. E-mail: graves@graves.chem.olemiss.edu.

‡ University of Mississippi.

§ Vanderbilt University School of Medicine.

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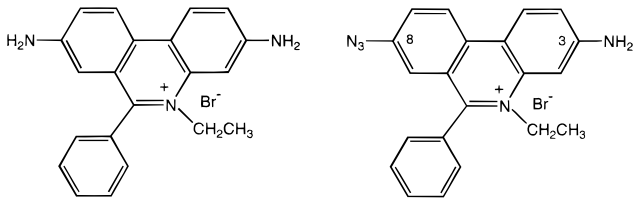

FIGURE 1: Structures of ethidium bromide (left) and the photoreactive analog, 8-azidoethidium (right).

examples of intercalators that have little effect on topoisomerase II activity include the classic intercalators ethidium bromide and 9-aminoacridine. Ross and co-workers demonstrated that addition of ethidium bromide could actually block etoposide-induced cleavage enhancement (*17*).

This paper explores the role of intercalative drug−DNA interactions on topoisomerase II-mediated strand cleavage. By design, this study dictates the formation of a well-characterized *irreversible* intercalative drug−DNA interaction prior to the addition of topoisomerase II; it also allows direct examination of the influence of a drug−DNA interaction on the catalytic cycle of topoisomerase II. In the earlier study by Ross and co-workers, ethidium was free to dissociate from the DNA helix during the topoisomerase II-mediated cleavage reaction. This limited direct observations of the influence of the drug on topoisomerase II activity (*17*). In studies presented here, photoaffinity labeling was used to render the drug−DNA complex irreversible prior to addition of the enzyme. Thus, the order in which the ternary complex was formed was dictated by (i) covalent attachment of the drug to the DNA in the absence of enzyme, followed by (ii) addition of the enzyme to the drug−DNA adduct to form the ternary complex.

Ethidium monoazide (8-azido-3-amino-6-phenyl-5-ethylphenanthradinium chloride) (structure shown in Figure 1) was chosen for these experiments due to the similarities in its DNA binding to that of the parent ethidium bromide (*18*, *19*). Prior to photolysis, ethidium monoazide binds DNA to form a complex that has geometry similar to that formed by the parent ethidium; however, upon photolysis with visible light the azido moiety is activated to the reactive nitrene and *in situ* covalent attachment of the ethidium is achieved. The intercalative nature of the drug−DNA adduct is maintained after photolysis (*20*, *21*).

The present work indicates that covalent attachment of ethidium to DNA results in enhanced topoisomerase II-mediated strand cleavage. Increases in double- and single-strand topoisomerase II-mediated breaks in the DNA were observed at covalent drug densities as low as 1 drug per 130 base pairs. This concentration is approximately 150 times lower than that required for comparable topoisomerase II-mediated DNA strand cleavage by *m*-AMSA and VP-16 (*22*, *23*). Thus, covalent attachment of an inactive DNA intercalator results in an extremely effective topoisomerase II mediating agent.

EXPERIMENTAL PROCEDURES

Enzyme and DNA. Topoisomerase II was purified from the nuclei of *Drosophila melanogaster* K_c tissue culture cells by the procedure of Shelton et al. (*24*). Negatively supercoiled plasmid pBR322 was obtained from *Escherichia coli* (HB101) by double banding in cesium chloride−ethidium bromide gradients (*25*).

Drug Preparations. Ethidium monoazide was synthesized by the method of Graves and co-workers under photographic safelight conditions (*26*). Approximately 0.1 mg of solid ethidium monoazide was dissolved in 0.5 mL of 0.01 M TE buffer (10 mM Tris, pH 7.9, and 1 mM EDTA).[1] After thorough mixing, this solution was filtered through a 0.45 μm filter. One hundred microliters of stock solution was diluted into 3 mL of buffer for experimental solutions. The concentration was determined spectrophotometrically using a molar extinction coefficient of 5220 M^{-1} cm^{-1} at 460 nm (*26*). All drug solutions were freshly prepared immediately prior to the experiment. Amsacrine (NSC-249992) was obtained from the National Cancer Institute. Ethidium bromide was purchased from Sigma Chemical Co. Purities and authenticities of these compounds were determined by proton NMR spectroscopy (Bruker DRX-500 spectrometer). Tris, SDS, proteinase K, and analytical reagent grade $CaCl_2 \cdot H_2O$ and $MgCl_2 \cdot 6H_2O$ were obtained from Sigma Chemical Co.

Plasmid−Ethidium Adducts. DNA samples with varying amounts of covalently attached ethidium were prepared in TE buffer under photographic safelight conditions. Ethidium monoazide was added to negatively supercoiled DNA, and the mixture was incubated for 60 min at 5 °C. The drug was covalently attached to the DNA via photolysis using two light boxes (Haake-Buchler Instruments), each equipped with two General Electric daylight No. F15T8-D lightbulbs delivering energy at a rate of ~20 J m^{-2} s^{-1} per bulb (*20*, *21*).

After photolysis, drug that was not covalently attached to the DNA was removed with a spin column packed with Chelex 100 (Bio-Rad). The spin column was prepared by packing 0.2 g of resin into the 0.5 mL microcentrifuge tube that was positioned inside a 1.5 mL microcentrifuge tube. The bottom of the 0.5 mL tube was punctured with an 18 gauge needle, allowing the DNA to elute into the 1.5 mL microcentrifuge tube. Excess buffer was removed from the column prior to loading the sample by centrifugation for 30 s. The DNA sample was layered on the top of the column and eluted by centrifugation at 8800*g* for 1 min (*20*, *21*). DNA−drug adducts collected in the 1.5 mL microcentrifuge tube were stored at 5 °C in the dark until their use. The amount of ethidium covalently attached to the DNA was quantitated by measuring the absorbance of the purified adduct at 500 nm using a molar absorptivity of 4100 M^{-1} cm^{-1} (*20*, *21*). DNA−ethidium adducts were prepared with binding densities ranging from 10 to 100 drugs per plasmid and used as substrates for the topoisomerase II activity assays.

Pre- and Post-Strand Passage Topoisomerase II-Mediated DNA Cleavage. DNA cleavage assays were performed as described by Corbett et al. (*27*). All DNA cleavage reactions employed 25 nM topoisomerase II and 5 nM (0.3 μg) negatively supercoiled DNA (or ethidium−DNA adduct) in a total volume of 20 μL of cleavage buffer (10 mM Tris, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.01 mM EDTA, and 2.5% glycerol) that contained 5 mM $MgCl_2$. For post-strand passage DNA cleavage reactions, 1 mM adenyl-5′-yl β,γ-imidodiphosphate [APP(NH)P], a nonhydrolyzable form of ATP, was added to the reaction mixture. Samples were

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid (disodium salt); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; DTT, dithiothreitol.

incubated for 6 min at 30 °C. Cleavage products were trapped by the addition of 2 μL of 10% SDS to a final concentration of 1%. One microliter of 250 mM EDTA and 2 μL of a 0.8 mg/mL solution of proteinase K were added, and the samples were incubated at 37 °C for 30 min to digest the topoisomerase II. Final products were mixed with 2.5 μL of loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol FF, and 10 mM Tris, pH 7.9), heated to 70 °C for 1 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate and 2 mM EDTA at 4 V/cm. Following electrophoresis, DNA bands were stained in a 1 μg/mL ethidium bromide solution and were visualized by transillumination with ultraviolet light (300 nm). The bands were photographed through Kodak 24A and 12 filters using Polaroid Type 55 positive/negative film. The amount of DNA was quantitated by scanning the negatives with a Model EC-910 scanning densitometer using Hoefer GS-370 Data System software. Under the described conditions, the intensity of the bands was directly proportional to the amount of DNA present. Double- and single-strand DNA breaks were monitored by the conversion of negatively supercoiled DNA to relaxed and linear molecules (*28*). A range of 0−100 μM noncovalent drug was used to examine the effects of the reversible DNA binding on the topoisomerase II-mediated DNA cleavage.

Topoisomerase II-Mediated DNA Relaxation. Relaxation reactions were performed as described by Corbett et al. (*27*). These studies employed 5 nM (0.3 μg) negatively supercoiled DNA or ethidium−DNA adduct in 19 μL of relaxation buffer (1 mM ATP in magnesium cleavage buffer). The enzyme was diluted in topoisomerase II diluent (10 mM Na_2HPO_4, 50 mM NaCl, 100 mM EDTA, 10% glycerol, 0.5 mg/mL BSA, and 1 mM DTT). To the DNA samples were added serial dilutions of enzyme ranging from 1 to 0.16 nM, and the resulting mixtures were incubated at 30 °C for 15 min. A volume of 3 μL of stop solution (0.77% SDS and 77.5 mM EDTA) and 2.5 μL of loading buffer were added to each of the samples. The DNA samples were subjected to electrophoresis and were visualized as described above.

Topoisomerase II-Mediated DNA Religation. The religation catalytic step can be separated from the cleavage forward reaction by taking advantage of the ability of calcium chloride to trap cleavage products in a reversible complex (*29*). To induce cleavage, 5 mM $CaCl_2$ was added to a mixture of 25 nM topoisomerase II and 5 nM (0.3 μg) negatively supercoiled DNA (or ethidium−DNA adduct) in a total volume of 20 μL of cleavage buffer (10 mM Tris, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.01 mM EDTA, and 2.5 % glycerol). Topoisomerase II-mediated DNA cleavage was carried out as described earlier, and cleavage products were trapped by the addition of 0.8 μL of 250 mM EDTA. Salt (0.6 μL of 5 M NaCl) was added to prevent further DNA cleavage. Samples were placed in a 0 °C ice bath to slow the reaction, and religation was initiated by the addition of 2 μL of ice-cold $MgCl_2$ to a final concentration of 8.5 mM. Reactions were terminated by the addition of 2.5 μL of 10% SDS at various times up to 30 s. Samples were subjected to electrophoresis and were visualized as described earlier. The rate of religation was determined by quantitating the amount of double-strand DNA breaks remaining at each time point of the reaction, with cleavage at time 0 (seconds) being arbitrarily set at 100%. Religation of plasmid DNA with and without covalently attached ethidium were plotted as the log of the percent of remaining cleavage product vs religation time.

Determination of Topoisomerase II−DNA Binding Isotherms. (Binding isotherms for the interaction of topoisomerase II with supercoiled pBR322 were obtained by the gel mobility shift method described by Carey (*30*). All DNA binding reactions utilized 2.5 nM plasmid and were carried out in a total volume of 20 μL of cleavage buffer. Varying amounts of topoisomerase II were added, resulting in final enzyme concentrations of 0, 18, 27, 36, 45, 54, 72, and 90 nM, respectively. Samples were incubated at 30 °C for 10 min, and then 2 μL of loading buffer was added to each sample. Electrophoresis was carried out in TAE buffer at 4 V/cm for 4 h. Following electrophoresis, the enzyme-bound DNA and free DNA could be quantitated on the basis of their altered mobilities under the conditions employed. The enzyme-bound DNA remained in the well, while the free supercoiled DNA migrates in the running lane (*31*). The gel was photographed using Polaroid Type 55 film. The amount of free DNA was quantitated by scanning the negative with an EC-901 densitomer as described earlier.

A decrease in the amount of supercoiled DNA (form I) in the presence of topoisomerase II was used to estimate the amount of bound DNA at a given concentration of topoisomerase II. The binding isotherms were plotted as the concentration of topoisomerase bound (C_b) vs the concentration of free enzyme (C_f) and were analyzed using a nonlinear least-squares method to determine the apparent DNA binding constants (K_a).

Similarly, DNA with covalently bound ethidium was used as a topoisomerase II binding substrate. The effect of covalent modification of DNA on this binding reaction was examined by comparing the binding constants (K_a) of DNA with varying amounts of covalently attached drug.

Topoisomerase I-Mediated Nick Assay. Topoisomerase I and reaction buffer were obtained from TopoGen (Columbus, OH). Each reaction used 1 unit of topoisomerase I with 5 nM (0.3 mg) negatively supercoiled pBR322 or 5 nM DNA adduct in a total of 20 μL of reaction buffer (50 mM Tris, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, and 30 μg/mL BSA). Reaction mixtures were incubated at 37 °C for 30 min. A volume of 2 μL of 10% SDS was added to trap single-stranded DNA breaks. To this was added 2 μL of 0.8 mg/mL solution of proteinase K, and the sample was incubated an additional 30 min at 45 °C to digest the topoisomerase I. Loading buffer (described earlier) was added, and the sample was loaded on a 1% agarose gel and subjected to electrophoresis at 4 V/cm in TAE buffer for 3 h. Visualization and analyses were carried out as described above.

RESULTS

Topoisomerase II-Mediated DNA Cleavage—Pre-Strand Passage DNA Cleavage. Covalent attachment of ethidium to DNA resulted in a marked enhancement of topoisomerase II-mediated DNA cleavage (Figure 2). At a binding density of 20 covalently attached ethidium molecules per plasmid (corresponding to 1 drug per 218 base pairs), enzyme-mediated single-strand and double-strand DNA cleavage was observed to increase 20- and 10-fold, respectively (compare lanes 5 and 7). In contrast, addition of noncovalent ethidium to yield ∼20 molecules bound per plasmid produced no

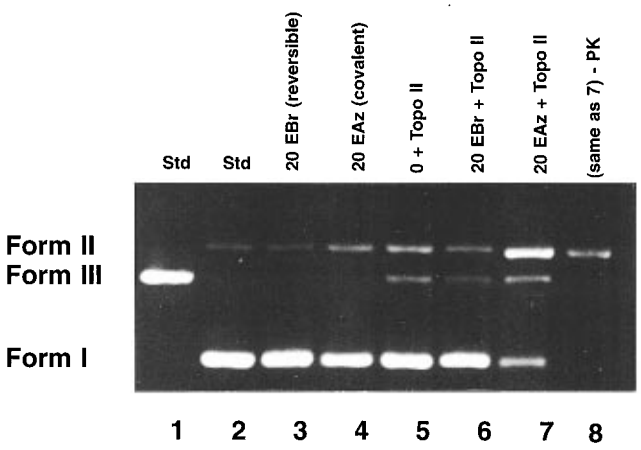

FIGURE 2: Single- and double-stranded DNA cleavage stimulated by covalent modification of pBR322 by ethidium. DNA cleavage assays were carried out in the presence of either reversibly bound ethidium or covalently attached ethidium at a binding density of 20 drugs per plasmid. Lanes 1 and 2 are linear and supercoiled DNA standards, respectively. Lanes 3 and 4 are pBR322 in the presence of reversible binding ethidium and covalently attached ethidium azide, respectively, with no topoisomerase II present. Lane 5 contains DNA plus topoisomerase II with no drug present. Lane 6 contains DNA and topoisomerase II in the presence of reversibly binding ethidium. Lane 7 is equivalent to lane 6 except the drug was covalently attached to the DNA at a binding density of 20 covalently bonded drugs per plasmid prior to addition of topoisomerase II. The DNA cleavage products in lanes 5−7 were digested with proteinase K prior to electrophoresis. Lane 8 is equivalent to lane 7 with no proteinase K digestion.

increase in topoisomerase II-mediated DNA cleavage and actually inhibits the reaction (compare lanes 5 and 6).

The number of drugs covalently attached to the DNA influenced the level of topoisomerase II-mediated single- and double-strand breaks (Figure 3). In this assay, lanes 1, 2, and 8 provide visual markers for linear and supercoiled DNAs. Lane 3 shows the effect of addition of topoisomerase II to the DNA in the absence of drug. Lanes 4 and 5 show the effects of covalently attached ethidium to the DNA at a binding density of 10 drugs per plasmid in the absence and presence of topoisomerase II, respectively. Comparison of lane 4 with lane 5 clearly demonstrates that even at a binding density of 10 drugs per plasmid (corresponding to one drug per 400 base pairs) considerable enhancement in topoisomerase II-mediated cleavage is observed. As the binding density of the covalently attached drug is increased (lane 7, 20 drugs per plasmid; lane 10, 40 drugs per plasmid; lane 12, 60 drugs per plasmid; and lane 14, 100 drugs per plasmid), the amount of single- and double-strand DNA is shown to increase markedly while the DNA adduct lanes that are not treated with topoisomerase II (lanes 6, 9, 11, and 13) are relatively unchanged. A decrease in the amount of DNA loaded onto the gel is observed in lanes 11−14. This decrease is due to binding of the ethidium-bonded DNA to the Chelex-100 resin at the higher drug concentrations.

Densitometric scans of these data are tabulated and presented in Figure 4, which correlates topoisomerase II-mediated single- and double-strand cleavage with the amount of drug covalently bonded to the DNA (closed circles). The amount of ethidium (open circles) and noncovalently attached ethidium azide (closed squares) added to the samples to result in analogous ratios of noncovalently bound drugs were estimated on the basis of a DNA binding affinity of 3×10^5 M^{-1} (*19*). This graph demonstrates significant enhancement of topoisomerase II-mediated cleavage of the drug-modified DNA, reaching a maximum amount at a binding density of approximately 40−60 drugs per plasmid. This level of binding corresponds to 1 drug per 70−100 base pairs, a value which is well below binding densities that would interfere with protein interaction or saturation of the DNA. At this drug concentration, more than half of the negatively supercoiled DNA was cut, with greater than 25-fold stimulation of single-strand breaks and a 15-fold stimulation in double-strand breaks. The concentration of covalently attached ethidium (0.2−0.3 μM) is approximately 300 times lower than the concentrations of *m*-AMSA (*32*) or etoposide (*33*) (60−100 μM) required to elicit comparable cleavage enhancement effects.

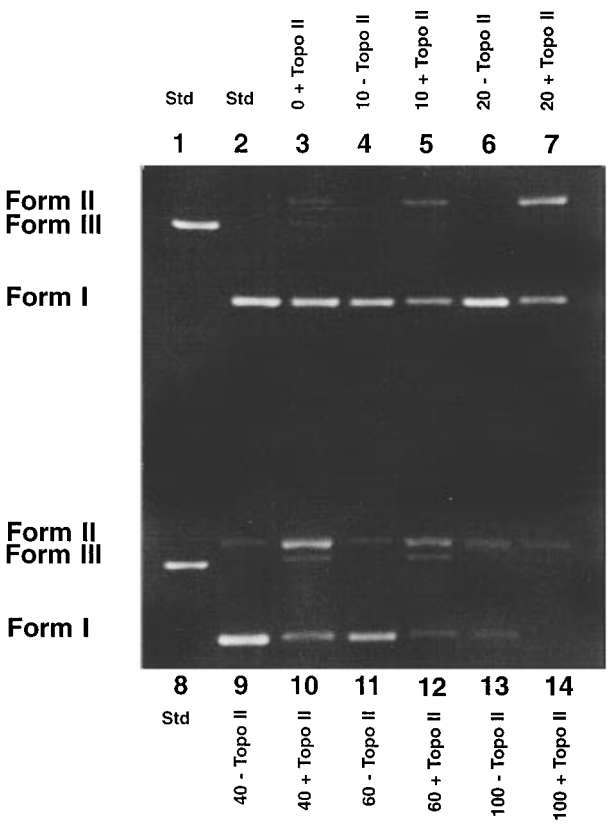

FIGURE 3: Comparisons of the amount of covalently attached ethidium on topoisomerase II-mediated DNA cleavage. Lanes 1, 2, and 8 are linear and supercoiled controls. Lane 3 is unmodified plasmid in the presence of topoisomerase II. Lanes 4 and 5 show the effects of modified plasmid (10 covalently attached drugs per plasmid) in the absence and presence of topoisomerase II. Lanes 6 and 7 show the effects of modified plasmid (20 covalently attached drugs per plasmid) in the absence and presence of topoisomerase II. Lanes 9 and 10 show the effects of modified plasmid (40 covalently attached drugs per plasmid) in the absence and presence of topoisomerase II. Lanes 11 and 12 show the effects of modified plasmid (60 covalently attached drugs per plasmid) in the absence and presence of topoisomerase II. Lanes 13 and 14 show the effects of modified plasmid (100 covalently attached drugs per plasmid) in the absence and presence of topoisomerase II.

Post-Strand Passage DNA Cleavage. In order to examine the influence of covalent modification of the DNA on topoisomerase II-mediated post-strand passage, a nonhydrolyzable form of ATP [APP(NH)P] was added to the cleavage assay mixture. Since DNA strand passage by topoisomerase II requires the binding (but not hydrolysis) of ATP, addition of 1 mM APP(NH)P allows direct examination of the

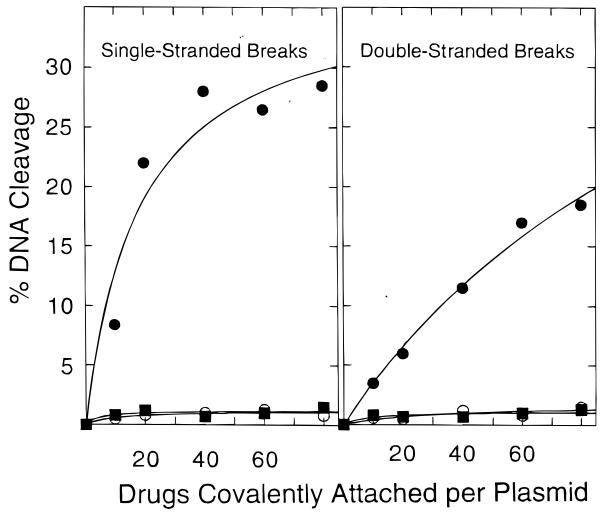

FIGURE 4: Influence of binding density of covalently attached ethidium on topoisomerase II-mediated single-stranded DNA breaks (left panel) and double-stranded DNA breaks (right panel). Covalent attachment of ethidium to substrate DNA results in the enhancement of both single-stranded and double-stranded DNA cleavage shown with closed circles. In contrast, the presence of appropriate concentrations of nonphotolyzed ethidium azide shown with open circles and ethidium bromide shown with closed squares show no cleavage enhancement effects.

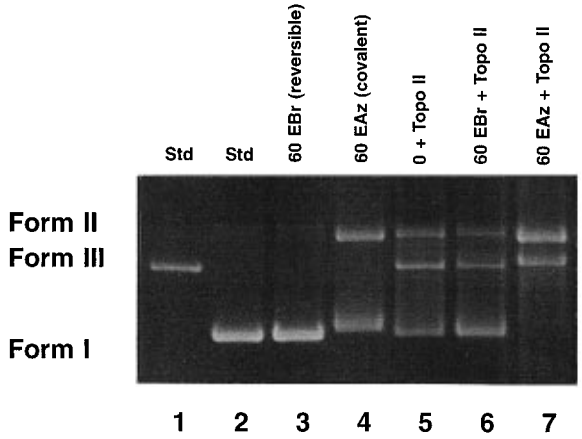

FIGURE 5: Stimulation of topoisomerase II-mediated post-strand passage breaks due to the presence of covalently attached ethidium. The binding density of ethidium is 60 drugs per plasmid. Lanes 1 and 2 show linear and supercoiled plasmid, respectively. Lane 3 shows the DNA in the presence of 60 ethidiums (reversible) per plasmid. Lane 4 shows the plasmid with 60 ethidiums covalently attached. Lane 5 shows the plasmid with no drug but reacted with topoisomerase II. Lane 6 shows the effects of reversible binding ethidium at 60 bound drugs per plasmid (reversible). Lane 7 shows the effects of 60 covalently attached ethidiums per plasmid on the topoisomerase II-mediated post-strand cleavage reaction.

cleavage/religation equilibrium of the enzyme in its post-strand passage conformation (*34*, *35*).

Effects of covalent adduct formation on topoisomerase II-mediated DNA cleavage in a post-strand passage position are shown in Figure 5. In this gel, lanes 1 and 2 show linear and supercoiled plasmid controls. Lanes 3 and 4 compare the topological states of DNA in the presence of reversibly bound and covalently attached ethidium, respectively. While covalent attachment of ethidium to the DNA does not induce spontaneous double-strand cleavage, an increase in the amount of nicked (form II) DNA is noted. In lane 5, topoisomerase II-mediated DNA cleavage is carried out in the absence of drug, resulting in a small amount of linear

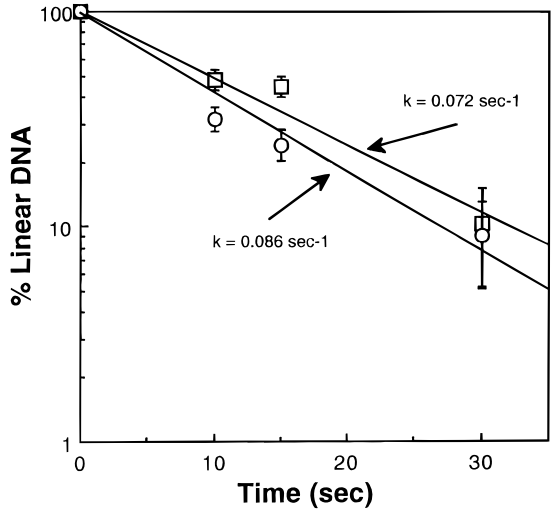

FIGURE 6: Effects of covalently attached ethidium on topoisomerase II-mediated religation of cleaved DNA. Data represent the average of two independent experiments. DNA religation was initiated by shifting the temperature from 30 to 55 °C and was stopped at indicated times. The open circles represent native pBR 322 DNA, while open squares represent DNA modified with 60 covalently attached ethidiums per plasmid. Religation rate constants are comparable for native and modified DNAs, 0.086 and 0.072 s^{-1}, respectively, indicating that covalent modification of the DNA by ethidium does not alter the religation process.

DNA. Lanes 6 and 7 show the effects of topoisomerase II cleavage in the presence of reversible binding ethidium (lane 6) and covalently attached ethidium (lane 7). In contrast to the effects of reversible binding ethidium, covalent attachment of the drug to the DNA results in significant amounts of both single- and double-stranded topoisomerase II-mediated DNA cleavage. As observed in the pre-strand passage DNA cleavage experiment, significant cleavage enhancement was observed at relatively low binding densities of covalently attached drug (<1 drug per 80 base pairs).

Effects of Covalent Attachment of Ethidium on the Religation Reaction. Calcium-promoted DNA cleavage was induced by trapping covalent enzyme−DNA complexes in active forms by addition of EDTA (*29*). The EDTA chelates all Ca^{2+}, leaving the enzyme covalently attached to the DNA but unable to religate. The samples are temperature-shifted to halt the forward cleavage reaction, and an excess of Mg^{2+} is added to allow for religation. Religation is then stopped by rapid addition of SDS at time points up to 30 s (standard religation time).

Effects of covalent attachment of ethidium on religation in the catalytic cycle of topoisomerase II are shown in Figure 6. Apparent first-order religation rates determined from this plot demonstrate that religation of the ethidium−DNA adduct is comparable to that of native supercoiled DNA. A religation rate constant equal to 0.072 ± 0.02 s^{-1} was obtained for native supercoiled DNA. Similarly, using ethidium−DNA adduct as the DNA substrate for the topoisomerase II-mediated religation reaction, a religation rate of 0.086 ± 0.01 s^{-1} was observed, comparable in magnitude to that for native supercoiled DNA. This value contrasts with other topoisomerase II inhibitors such as etoposide and *m*-AMSA, which exhibit religation rates of 0.024 s^{-1} (etoposide) and 0.014 s^{-1} (*m*-AMSA) (*32*, *33*). The decreased rate constants observed for etoposide and amsacrine

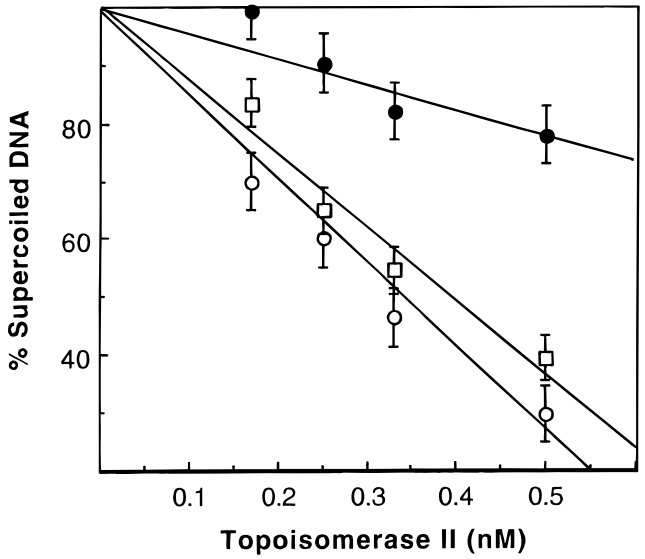

FIGURE 7: Effects of covalent modification of DNA by ethidium on topoisomerase II-mediated relaxation of supercoiled DNA. Data represent the averages of three independent experiments. Straight lines are linear least-squares analyses of the best fits through the data. Open circles and squares show the relaxation rates of native plasmid and plasmid plus reversible binding ethidium, respectively. The closed circles represent the relaxation of DNA modified with covalently bonded ethidium and shows a marked inhibition in relaxation as indicated by the smaller slope.

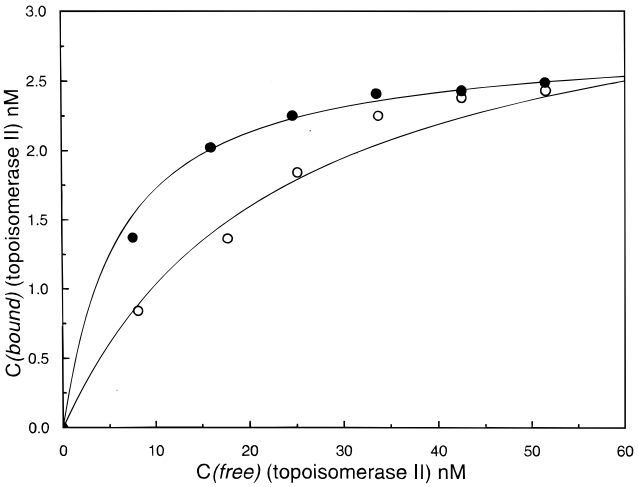

FIGURE 8: Binding isotherm of topoisomerase II to native and modified DNA. Data represent an average of three experiments using the mobility shift assay (*30*). Open squares represent modified DNA, 60 covalently attached ethidium molecules per plasmid, while the open squares represent the unmodified DNA. Binding isotherms were obtained by titrating stoichiometric amounts of topoisomerase II into fixed DNA concentrations and were quantitated by measuring the density of the free supercoil DNA (mobile band) relative to the topoisomerase II−DNA complex (immoble band). Curves represent best fits using nonlinear least-squares analyses of these binding isotherms.

demonstrate a significant decline in the ability of topoisomerase II to carry out the religation step of the catalytic cycle and account for the enhanced DNA cleavage observed with these agents.

Supercoiled DNA Relaxation. The effect of covalent attachment of ethidium on the removal of negative supercoils by topoisomerase II is quantitated and presented in Figure 7. As shown in this figure, topoisomerase II is capable of relaxing the ethidium-modified DNA; however, the relaxation was markedly inhibited. As can be seen by the open squares, the presence of reversible binding ethidium has no effect on the relaxation process. This plot reveals that an additional 3 times more enzyme would be required for the ethidium−DNA adduct to undergo an equivalent level of relaxation as compared with native supercoiled DNA.

Covalent Modification of the DNA Influences Topoisomerase II Binding. The first step in the topoisomerase II catalytic cycle is the noncovalent interaction of the protein with DNA. The influence of the covalent attachment of ethidium on this interaction was examined using gel mobility shift analysis (*31*, *36*). Isotherms for the binding of topoisomerase II to wild-type pBR322 or pBR322 modified at a binding density of 60 drugs per plasmid are shown in Figure 8. The apparent association constants were determined using nonlinear least-squares analyses. Binding of topoisomerase II to native supercoiled DNA in the absence of covalently attached ethidium resulted in an apparent binding constant (K_a) of 0.4×10^8 M^{-1}. However, using the ethidium-modified plasmid as a binding substrate, the affinity of topoisomerase II rose approximately 3.5-fold ($K_a \sim 1.4 \times 10^8$ M^{-1}).

In order to confirm that this enhanced binding affinity was due to covalent attachment of ethidium, a binding experiment was also carried out in the presence of reversible binding ethidium and nonphotolyzed ethidium monoazide (data not shown). In both cases, drug concentrations were adjusted to provide a binding density of 60 drugs per plasmid. The apparent binding constant for both reversible drugs ($K_a \sim 0.4 \times 10^8$ M^{-1}) was comparable to that observed in absence of drug. This result indicates that the reversible binding drugs had no effect on the protein−DNA interaction, while the covalent attachment of ethidium to the DNA resulted in enhanced binding of the topoisomerase II to the DNA.

DISCUSSION

The catalytic mechanism and/or cellular functions of topoisomerase II rely on the ability of this enzyme to create transient double-strand breaks in the DNA backbone (*1*, *5*, *37*). Over the past decade, this property has been exploited by a number of clinically important anticancer agents. The modes of action of these topoisomerase II-targeting agents have been demonstrated to correlate with the abilities of these chemical agents to stabilize the cleaved topoisomerase II−DNA complex through interference of specific steps within the catalytic cycle of the enzyme, including enhancement of the forward cleavage reaction or inhibition of relaxation or religation (*1*, *5*). Earlier studies have been limited to drugs such as amsacrine, adriamycin, and ellipticine which reversibly bind DNA, but are thought to exert their anticancer activity through their enhancement of topoisomerase II-mediated DNA scission. However, not all reversible DNA binding agents have a similar influence on this topoisomerase II activity. For example, ethidium bromide is a classic DNA intercalating agent and has been demonstrated to be ineffective in enhancing topoisomerase II-mediated DNA strand breaks; hence, DNA binding alone is not sufficient for the stimulation of topoisomerase II-mediated DNA cleavage (*12*, *17*).

In an effort to gain insight into the influence of DNA binding agents on topoisomerase II activities, a simple scenerio was proposed whereby an intercalative DNA binding ligand would be covalently attached to the DNA

prior to formation of the ternary complex. The ligand-modified DNA was then used as a substrate for the topoisomerase II-catalyzed reactions. This approach effectively eliminated any free ligand interactions with the enzyme except for the portion of the drug molecule in contact with the protein within the ternary complex. The objectives of this study were 2-fold: first, to determine whether a ligand that is covalently bonded to the DNA can be recognized by the enzyme, and secondly, to determine whether the covalently bonded ligand would influence topoisomerase II activity.

This study demonstrated that covalent attachment of ethidium to supercoiled DNA resulted in marked enhancement of topoisomerase II-mediated single- and double-stranded DNA cleavage. Cleavage enhancements of greater than 10−20-fold in double- and single-stranded DNA cleavage were observed when the ethidium was covalently bonded to the substrate DNA. This observation contrasts with the effects observed in the presence of reversibly binding ethidium. The amount of enzyme-mediated DNA cleavage observed was demonstrated to be dependent on the amount of ethidium that was covalently bonded to the DNA, showing a linear relationship between cleavage and drug density up to approximately 40−60 ligands per plasmid.

In dissecting the level at which the inhibition process was influenced by covalent modification of the DNA, various steps in the catalytic cycle were probed, including pre- and post-strand passage DNA cleavage, relaxation, and religation. Results of these studies reveal that covalent modification of the DNA by ethidium brings about a marked inhibition of the overall relaxation of supercoiled DNA, indicative of a highly selective influence on the catalytic properties of the enzyme by the covalently attached ligand. In contrast, relaxation of supercoiled plasmid in the presence of reversibly binding ethidium shows no inhibition. Additional studies revealed the binding affinity of topoisomerase II for native and modified DNAs to be distinct, with the observation of a 3-fold enhancement in the binding affinity of topoisomerase II for the modified DNA over that of native DNA and suggests a plausible mechanism for the enhanced DNA cleavage by topoisomerase II. These data suggest that perturbation of the DNA lattice by covalent attachment of ethidium was recognized by the enzyme, as evidenced by the enhanced binding affinity for the ethidium-modified DNA, and results in a concomitant alteration in the catalytic cycle. In the case of the covalently bonded ethidium, this effect is manifested by inhibiting the relaxation step of the catalytic cycle.

To better understand the influence of covalent modification of DNA by ethidium on topological states of supercoiled DNA and to confirm the cleavage enhancement imposed on topoisomerase II, topoisomerase I assays were performed. These reactions using the adduct as the substrate for the type I class enzyme showed no effect on this enzyme. Topoisomerase I was capable of total relaxation of our supercoiled adduct even at ratios up to 60 drugs per plasmid (where maximal cleavage enhancement was observed for topoisomerase II cleavage reaction). In the presence of covalently attached ethidium, no additional topoisomerase I-mediated single- or double-strand breaks were observed. This lack of influence on this related family of enzymes, while showing potent effects with topoisomerase II, indicates a large degree of specificity for topoisomerase II by the covalently attached ethidium.

Although the mechanistic basis for the DNA cleavage enhancement by covalently attached ethidium has yet to be detailed, the results of this study are consistent with the recently proposed "positional poison model" for the actions of topoisomerase II poisons (*38*). This model is based on studies that analyzed the effects of DNA lesions on topoisomerase II-mediated nucleic acid scission (*38*−*41*) as well as on kinetic and binding studies that characterized enzyme−drug interactions within the topoisomerase−DNA−drug ternary complex (*42*, *43*).

The positional poison model states that DNA lesions and drugs both enhance topoisomerase-mediated DNA cleavage predominately by altering the structure of DNA (*38*). However, in order for cleavage enhancement to take place, structural alterations must be positioned within the 4-base stagger created by topoisomerase II-mediated cleavage. In addition, since most DNA-binding drugs interact with nucleic acids in a relatively nonspecific fashion and are mobile, the model proposes that interactions with the enzyme are necessary to position drug-induced alterations of DNA correctly with the cleavage site. Thus, while the efficacy of lesions is predetermined by their location along the double helix, the specificity of anticancer agents is directed by the enzyme.

In light of the positional poison model, it is proposed that noncovalent ethidium does not stimulate topoisomerase II-mediated DNA scission because it has no specific interactions with the enzyme. As a result, despite its intercalative properties, ethidium is unable to form an appropriate enzyme−drug−DNA ternary complex. In contrast, upon covalent attachment to DNA, a proportion of the bound ethidium becomes fixed within pre-existing sites of topoisomerase II cleavage. These molecules alter the DNA structure within the cleavage site and consequently require no specific interactions with the enzyme to enhance scission. In support of these conclusions, as was found for covalently attached ethidium, abasic lesions in DNA enhance topoisomerase II−DNA binding and do not inhibit enzyme-mediated DNA religation (*39*).

REFERENCES

1. Corbett, A., and Osheroff, N. (1993) *Chem. Res. Toxicol. 6*, 585−597.
2. Froelich-Ammon, S. J., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 21429−21432.
3. Chen, Y. A., and Liu, L. F. (1994) *Annu. Rev. Pharmacol. Toxicol. 34*, 191−218.
4. Pommier, Y., Fesen, M. R., and Goldwasser, F. (1996) in *Cancer Chemotherapy and Biotherapy: Principles and Practice* (Chabner, B. A., and Longo, D. L., Eds.) pp 435−461, Lippincott-Raven, Philadelphia.
5. Wang, J. C. (1996) *Annu. Rev. Biochem. 65*, 635−692.
6. Nitiss, J. L., and Beck, W. T. (1996) *Eur. J. Cancer 32A*, 958−966.
7. Ferguson, L. R., and Baugley, B. C. (1993) *Environ. Mol. Mutagen. 24*, 245−261.
8. Ross, W. E., Glaubiger, D. L., and Kohn, K. W. (1979) *Biochim. Biophys. Acta 562*, 41−50.
9. Ross, W. E., Glaubiger, D. L., and Kohn, K. W. (1979) *Biochim. Biophys. Acta 519*, 23−30.
10. Ross, W. E., and Bradley, M. O. (1981) *Biochim. Biophys. Acta 654*, 129−134.
11. Filipski, J., Yin, J., and Kohn, K. W. (1983) *Biochim. Biophys. Acta 741*, 116−122.

12. Nelson, E. M., Tewey, K. M., and Liu, L. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1361−1365.
13. Tewey, K. M., Chen, G. L., Nelson, E. M., and Liu, L. F. (1984) *J. Biol. Chem. 259*, 9182−9187.
14. Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., and Liu, L. F. (1984) *Science 226*, 466−468.
15. Zwelling, L. A., Kerrigan, D., and Michaels, S. (1982) *Cancer Res. 42*, 2687−2691.
16. Zwelling, L. A., Michaels, S., Erickson, L. C., Ungerleider, R. S., Nichols, M., and Kohn, K. W. (1981) *Biochemistry 20*, 6553−6563.
17. Rowe, T., Kupfer, G., and Ross, W. E. (1985) *Biochem. Pharmacol. 34*, 2483−2487.
18. Garland, F., Graves, D. E., Yielding, L. W., and Cheung, H. C. (1980) *Biochemistry 19*, 3221−3226.
19. Graves, D. E., Watkins, C. L., and Yielding, L. W. (1981) *Biochemistry 20*, 1887−1892.
20. Gilbert, P. L., Graves, D. E., and Chaires, J. B. (1991) *Biochemistry 30*, 10925−10931.
21. Gilbert, P. L., Graves, D. E., Britt, M., and Chaires, J. B. (1991) *Biochemistry 30*, 10931−10937.
22. Pommier, Y., Schwartz, R. E., Zwelling, L. A., and Kohn, K. W. (1985) *Biochemistry 24*, 6406−6410.
23. Ross, W. E., Rowe, T., Glisson, B., Yalowich, J., and Liu, L. R. (1984) *Cancer Res. 44*, 5857−5860.
24. Shelton, E. R., Osheroff, N., and Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9530−9535.
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
26. Graves, D. E., Yielding, L. W., Watkins, C. L., and Yielding, K. L. (1977) *Biochim. Biophys. Acta 479*, 98−104.
27. Corbett, A. H., Zechiedrich, E. L., Lloyd, R. S., and Osheroff, N. (1991) *J. Biol. Chem. 266*, 19666−19671.
28. Robinson, M. J., Corbett, A. H., and Osheroff, N. (1993) *Biochemistry 32*, 3638−3643.
29. Osheroff, N., and Zechiedrich, E. L. (1987) *Biochemistry 26*, 4303−4309.
30. Carey, J. (1991) in *Methods in Enzymology* (Sauer, R. T., Ed.) Vol. 208, pp 103−117, Academic Press, New York.
31. Osheroff, N. (1986) *J. Biol. Chem. 261*, 9944−9950.
32. Robinson, M. J., and Osheroff, N. (1990) *Biochemistry 29*, 2511−2515.
33. Osheroff, N. (1989) *Biochemistry 28*, 6157−6160.
34. Osheroff, N., Shelton, E. R., and Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9536−9543.
35. Robinson, M. J., and Osheroff, N. (1991) *Biochemistry 30*, 1807−1813.
36. Osheroff, N., and Brutlag, D. L. (1983) *UCLA Symp. Mol. Cell. Biol., New Ser. 10*, 55−64.
37. Berger, J. M., and Wang, J. C. (1996) *Curr. Opin. Struct. Biol. 6*, 84−90.
38. Kingma, P. S., and Osheroff, N. (1997) *J. Biol Chem. 272*, 1148−1155.
39. Kingma, P. S., Corbett, A. H., Burcham, P. C., Marnett, L. J., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 21441−21444.
40. Kingma, P. S., and Osheroff, N. (1997*) J. Biol. Chem. 272*, 7488−7493.
41. Kingma, P. S., Greider, C. A., and Osheroff, N. (1997) *Biochemistry 36*, 5934−5939.
42. Froelich-Ammon, S. J., Patchan, M. W., Osheroff, N., and Thompson, R. B. (1995) *J. Biol. Chem. 270*, 14998−15005.
43. Burden, D. A., Kingma, P. S., Froelich-Ammon, S. J., Bjornsti, M.-A., Patchan, M. W., Thompson, R. B., and Osheroff, N. (1996) *J. Biol. Chem. 271*, 29238−29244.

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