Many anticancer drugs “poison” topoisomerase II by enhancing its double-stranded DNA cleavage activity. To determine whether DNA lesions act as endogenous topoisomerase II poisons, we characterized the effects of position-specific apurinic sites on enzyme activity. Lesions located within the 4-base overhang generated by enzyme-mediated DNA scission stimulated cleavage —10–18-fold without altering the specificity of topoisomerase II. DNA breaks were double-stranded in nature, protein-linked, and readily reversible. In contrast, apurinic sites located immediately outside the cleavage overhang were inhibitory. Thus, apurinic sites, which are the most commonly formed lesion in DNA, are position-specific topoisomerase II poisons. A model is proposed that encompasses the actions of endogenous and exogenous topoisomerase II poisons and provides a pre-existing pathway for the cellular actions of topoisomerase II-targeted anticancer drugs.

To carry out a number of fundamental nuclear processes, including DNA replication, genetic recombination, and chromosome segregation, the cell requires an enzyme that can freely pass one DNA helix through another. The enzyme that has evolved to execute this essential function is topoisomerase II (1–5).

By virtue of its double-stranded DNA passage mechanism, topoisomerase II renders nucleic acids invisible to themselves (1–5). However, in order for the enzyme to confer this ethereal property on DNA, it must generate double-stranded breaks in the genetic material (6, 7). Normally, protein-associated DNA breaks formed by the type II enzyme are readily reversible and are fleeting intermediates in its catalytic cycle (3–5). Consequently, they are present at low steady-state levels and are tolerated by the cell. However, conditions that significantly increase the physiological concentration of these breaks trigger mutagenic events, such as insertions, deletions, translocations, and chromosomal breaks (2, 8–12). Furthermore, when present in sufficient numbers, these breaks initiate a programmed series of events that ultimately culminates in cell death.

Several clinically relevant anticancer drugs exploit the potentially lethal nature of topoisomerase II and convert this essential protein to a cellular toxin by increasing levels of enzyme-mediated DNA breaks (8, 9, 12–15). As a result of their actions, drugs that “poison” topoisomerase II (i.e. stimulate enzyme-mediated DNA scission) (16) are lethal to rapidly dividing cancer cells and are some of the most common and successful drugs used for the treatment of human malignancies (8, 9, 12, 14, 17–19).

The mechanism of action of exogenous topoisomerase II poisons is highly unusual. The remarkable ability of anticancer drugs to convert this essential enzyme to a cellular toxin suggests that these drugs take advantage of a pre-existing cellular pathway and argue for the existence of endogenous topoisomerase II poisons. This hypothesis is supported by considerable circumstantial evidence. For example, —80% of spontaneous infant leukemias share the same translocations at chromosome band 11q23 as those found in chemotherapy-induced secondary leukemias from patients previously treated with topoisomerase II-targeted agents (20–25). In addition, there is a strong correspondence between in vitro sites of topoisomerase II-mediated DNA cleavage and chromosome band 11q23 breakpoints observed in infant leukemias, even though the affected infants had no prior exposure to anticancer drugs (26, 27).

Since drugs are likely to function at the topoisomerase II/DNA interface (8, 12, 14, 28, 29), it is possible that DNA lesions act as endogenous topoisomerase II poisons. To address this issue, we previously studied the effects of abasic sites on the activity of Drosophila melanogaster topoisomerase II (30). In the presence of three abasic sites/pBR322 plasmid molecule (approximately one lesion/1500 base pairs), topoisomerase II-mediated DNA cleavage was stimulated —6-fold. Although abasic sites represent the first reported example of an endogenous topoisomerase II poison, conclusions from this study were limited by the fact that lesions were inserted randomly in the DNA and that the abasic sites generated by heat/acid treatment constituted a mixed population of apurinic and apyrimidinic lesions (31).

To move beyond the limitations of our previous study and to more fully assess the potential of spontaneous DNA lesions as endogenous topoisomerase II poisons, the effects of position-specific apurinic sites on topoisomerase II-mediated DNA cleavage were characterized. When apurinic sites were located within the 4-base 5-overhang generated by topoisomerase II-mediated DNA scission, levels of enzyme-associated DNA breaks increased —10–18-fold. Conversely, apurinic sites located immediately outside the cleavage overhang inhibited DNA scission. These results indicate that apurinic sites have a profound influence on the activity of the type II enzyme and act as position-specific topoisomerase II poisons.

**EXPERIMENTAL PROCEDURES**

**Materials**—Topoisomerase II was purified from D. melanogaster embryonic Kc cells as described by Shelton et al. (32). Hepes was obtained from Boehringer Mannheim; proteinase K and SDS were from Merck; bacteriophage T4 polynucleotide kinase was from New England Biolabs Inc.; [γ-32P]ATP (—6000 Ci/mmol) was from Amersham Corp.; Escherichia coli uracil-DNA glycosylase was from U.S. Biochemical Corp.; etoposide was from Sigma (stored at 4°C as a 10 mM stock solution in dimethyl sulfoxide); and deoxyuridine phosphoramide was from Glen Research Corp. All other chemicals were analytical reagent grade.

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Preparation of Oligonucleotides—A 40-base single-stranded oligonucleotide that corresponds to residues 87–126 of pBR322 (see Fig. 2) (33) and its complementary oligonucleotide were prepared on an Applied Biosystems DNA synthesizer. The sequences of top and bottom oligonucleotides were 5'-TGAATCTAACAATG CGCTCATCGTATCCTG-GCACTGGTTA -3' and 5'-ACGGTCCGAGTAGCATGCGTCGTTAGATTCCA -3', respectively. The points of topoisomerase II-mediated DNA cleavage are denoted by the arrows (6, 34). Single-stranded uracil-containing oligonucleotides were prepared in a similar manner utilizing the deoxyuridine phosphoramidite. Single-stranded oligonucleotides were radioactively labeled on their 5'-termini in reaction mixtures that contained 10 pmol of oligonucleotide, 5 units of polynucleotide kinase, and 75 pmol of [γ-32P]ATP in a total of 30 µl of kinase buffer. Labeled and unlabeled oligonucleotides were isolated by electrophoresis in 7% polyacrylamide gels. DNA bands were excised from gels and purified using the QIAGEN gel extraction protocol. The complementary oligonucleotides were annealed as described previously by Corbett et al. (34).

Prior to use, uracil bases were removed by incubating 2 µmol of double-stranded oligonucleotide/reaction (100 µl) with 0.1 unit of uracil-DNA glycosylase in 17 µl of 10 mM Hapes-HCl, pH 7.9, 0.1 mM EDTA, and 2.5% glycerol for 30 min at 37°C (35, 36). Following treatment with uracil-DNA glycosylase, samples were prepared for topoisomerase II assays by the addition of 1 µl of NaCl/NaKCl and 1 µl of 100 mM MgCl2. Wild-type oligonucleotides were treated by an identical procedure to control for the potential effects of this process on topoisomerase II activity.

Topoisomerase II-mediated DNA Cleavage—Topoisomerase II-mediated DNA cleavage reactions contained 100 nM oligonucleotide (treated with uracil-DNA glycosylase) in 19 µl of assay buffer (10 mM Hapes-HCl, pH 7.9, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, and 2.5% glycerol) and were initiated by the addition of 1 µl of topoisomerase II (100 nM final concentration). Reactions were incubated for 3 min at 30°C and stopped with 2 µl of 10% SDS followed by 1.5 µl of 250 mM EDTA. Cleavage products were digested with proteinase K (2 µl of 0.8 mg/ml solution) for 20 min at 37°C; ethanol precipitated twice; and resuspended in 5 µl of 40% formamide, 8.5 mM EDTA, 0.02% bromphenol blue, and 0.02% xylene cyanol FF. Products were resolved by electrophoresis in denaturing 7% gels, 14% polyacrylamide sequencing gels in 100 mM Tris borate, pH 8.3, and 2 mM NaEDTA. Alternatively, reaction products were resolved under nondenaturing conditions at 10°C in 20% polyacrylamide gels in 100 mM Tris borate, pH 8.3, and 2 mM NaEDTA. Gels were fixed by soaking for 30 min in a 10% methanol, 10% acetic acid solution and dried. Reaction products were visualized and quantified using a Molecular Dynamics PhosphorImager system.

Topoisomerase II-mediated DNA Religation—Religation assays were carried out by a modification of the procedure of Robinson and Osheroff (37). Cleavage/religation equilibria were established as described under "Topoisomerase II-mediated DNA Cleavage." Religation was initiated by shifting the reaction temperature from 30°C to 4°C and stopped by the addition of 2 µl of 10% SDS at the indicated times. Samples were prepared for electrophoresis and resolved in denaturing gels as described above. The apparent first-order rate of religation was determined by quantifying the loss of the 15-mer cleavage product.

Topoisomerase II-DNA Binding—Topoisomerase II-DNA binding assays were carried out by a modification (34) of the nitrocellulose filter method of Higgins and Cozzarelli (38). Nitrocellulose filters (0.45 µm) were pretreated with 2% acetic acid in assay buffer. Binding reactions contained 100 nM topoisomerase II and 100 µM wild-type or apurinic oligonucleotide in 20 µl of assay buffer. Reactions were incubated at 30°C for 3 min, diluted to 500 µl with assay buffer, and rapidly applied to the center of nitrocellulose filters at a flow rate of ~5 ml/min. Filters were washed three times with 1 ml of cold assay buffer, dried, and quantified by liquid scintillation counting. Data were corrected for the ~1% DNA binding to filters observed in the absence of enzyme.

Topoisomerase II-catalyzed DNA Relaxation—Relaxation assays were carried out as described previously (30, 39). Briefly, 2 nM topoisomerase II was incubated with 1 µM ATP and 5 µM negatively supercoiled pBR322 plasmid DNA that contained zero or three abasic sites (generated by heat/acid treatment) in 20 µl of assay buffer. Reaction was carried out by varying etoposide concentrations, and products were resolved in 1% agarose gels in 100 mM Tris borate, pH 8.3, and 2 mM EDTA and stained with ethidium bromide. Relaxation was quantified by monitoring the removal of the supercoiled substrate as determined by scanning densitometry.

RESULTS

To identify endogenous lesions that can act as topoisomerase II poisons and to determine the location of these lesions relative to the points of enzyme-mediated DNA scission, we characterized the effects of position-specific apurinic sites on the activity of Drosohila topoisomerase II. An oligonucleotide-based system was employed for this study. The double-stranded 40-mer substrate contained a single, centrally located topoisomerase II cleavage site (6, 34). Enzyme-mediated scission of the top and bottom strands resulted in the formation of radioactive 15- and 21-mer products, respectively, when the DNA was labeled on its 5'-termini (see Fig. 2) (34). As is typical for the type II enzyme, the points of cleavage on opposing strands of the double helix are staggered such that scission yields 4-base 5'-overhanging cohesive ends (6, 7).

Position-specific apurinic sites were incorporated by replacing single nucleosides with deoxyuridine during synthesis of the oligonucleotide and then removing the uracil base with uracil-DNA glycosylase. Oligonucleotides contained either two wild-type strands (as a control) or a wild-type strand annealed to a complementary strand that contained a single apurinic site. To ensure that cleavage was due to enzyme-mediated DNA scission rather than spontaneous degradation of the apurinic site, cleavage of all lesion-containing oligonucleotides was monitored on the complementary wild-type strand. The positions of apurinic sites are designated relative to the point of cleavage on that strand. Bases upstream (i.e., 5') from the point of cleavage are designated with negative numbers, and those downstream (i.e., 3') are designated with positive numbers (40). The points of cleavage on both the top and bottom strands are located between the −1 and +1 bases.

Apurinic Sites Located within the 4-Base Cleavage Overhang Generated by Topoisomerase II Stimulate DNA Scission—To analyze the effects of apurinic sites on topoisomerase II-mediated DNA cleavage, each purine between the −3-positions on the top and bottom strands was converted to an apurinic site (see Fig. 2). This region corresponds to the 10 bases spanning the topoisomerase II-DNA cleavage site. As shown in Figs. 1 and 2, every apurinic site located within the 4-base overhang (i.e., the +1- to +4-positions) of this cleavage site greatly enhanced DNA scission. Cleavage was stimulated ~10-fold when
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An apurinic site was located at the +2-position on the top strand and from ~12- to 18-fold when the lesion was located at the +1-, +2-, or +4-position on the bottom strand (Fig. 2). Moreover, the site of topoisomerase II-mediated DNA cleavage was not altered by the presence or the position of the apurinic site (Fig. 1). Therefore, topoisomerase II does not cleave DNA at the position of the apurinic site. Rather, it appears that this DNA lesion enhances scission by altering interactions between the enzyme and its normal site of action.

Since apurinic sites can spontaneously degrade to nicks (41–44), it is possible that the enhanced cleavage within lesion-containing oligonucleotides was due to the formation of nicks rather than the presence of apurinic sites per se. When topoisomerase II cleaves DNA in the vicinity of a nick, the resulting loss of continuity allows one of the newly generated 3'-hydroxyl DNA termini to dissociate from the active site of the enzyme (45–47). This dissociation uncouples the reversible DNA cleavage/religation equilibrium of the enzyme (3–5) and results in a nonreversible “suicide” cleavage event (46, 47). Therefore, to determine whether cleavage enhancement was due to the presence of nicks formed at apurinic sites, the reversibility of enzyme-mediated DNA breaks was characterized for all oligonucleotides that contained apurinic sites within the cleavage overhang. Following the addition of high salt or chelation of the essential divalent cation with EDTA, >90% of all lesion-stimulated breaks were reversed (Table I). These results indicate that the enhancement of topoisomerase II-mediated cleavage by DNA lesions was not due to the conversion of apurinic sites to nicks.

Since we generated apurinic sites by the enzymatic removal of position-specific uracil residues, all apurinic oligonucleotides contained a single base mismatch prior to treatment with uracil-DNA glycosylase. A recent study by Bigioni et al. (48) indicated that some base pair mismatches located within the cleavage overhang stimulated DNA scission. Although levels of enhancement (i.e. 2–4-fold) reported for mismatches were modest compared with levels observed with apurinic oligonucleotides, the present stimulation of cleavage could result from the incomplete removal of uracil-containing mismatches.

Two experiments were performed to address this critical issue. First, the conversion of uracil (located at the bottom +2-position) to an apurinic site was quantitated by labeling the 5’-terminus of the apurinic strand and inducing chemical cleavage of the lesion with 0.1 N NaOH (49). As determined by this treatment, ~95% of the oligonucleotide substrate contained an apurinic site. Second, the effect of a uracil:cytosine mismatch (with the uracil located at the +2-position on the bottom strand) on topoisomerase II-mediated DNA cleavage was assessed (Fig. 3). Consistent with the work of Bigioni et al. (48), the uracil-containing mismatch stimulated cleavage ~3-fold as compared with the ~18-fold stimulation observed following treatment with uracil-DNA glycosylase (see Figs. 1 and 2). Thus, the enhancement of DNA cleavage seen in the present study was due solely to the presence of apurinic sites in the oligonucleotide substrate.

Apurinic Sites Located Immediately outside the 4-Base Cleavage Overhang Inhibit Cleavage Complex Formation—In marked contrast to the cleavage enhancement by apurinic sites located within the 4-base cleavage overhang, those located immediately (i.e. 1–3 bases) outside this region abolished cleavage complex formation (see Figs. 1 and 2). This inhibition decreased as apurinic sites were moved farther outside the cleavage overhang, with those located ~6 bases away showing little effect on cleavage (data not shown). Thus, depending on the location of the apurinic site, these lesions have the potential to act as potent topoisomerase II poisons or as strong inhibitors of cleavage complex formation.

Topoisomerase II-mediated DNA Cleavage Is Stimulated by Randomly Inserted Apurinic Sites—To further characterize the stimulation of DNA cleavage by DNA lesions, apurinic sites were randomly inserted in the bottom strand of a double-stranded 59-mer oligonucleotide that contained five sites of topoisomerase II-mediated DNA cleavage (Fig. 4). This was accomplished by including a low level of deoxyuridine along with the indicated base (base:deoxyuridine = 94:6) at each of the 26 underlined sequence positions, followed by treatment with uracil-DNA glycosylase. Due to a slightly decreased efficiency of incorporation of deoxyuridine, each oligonucleotide contained an average of ~0.7 apurinic sites. Thus, a lesion should be represented at any given sequence position in approximately one-fortieth of the total oligonucleotide population.

As shown in Fig. 4, even at this low level of incorporation of apurinic sites, a significant cleavage enhancement was observed at three of the five topoisomerase II cleavage sites. Furthermore, no novel sites of cleavage were detected in the apurinic substrate. These results lend strong support to the

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**Table I**

| Position of apurinic site | Relative cleavage |
|-------------------------|------------------|
|                        | SDS  | +EDTA | +Salt |
| Bottom strand           |      |       |       |
| None                    | 1.0  | <0.05 | <0.05 |
| +1                      | 12.1 | ±0.4  | ±0.1  |
| +2                      | 18.3 | ±4.5  | ±0.4  |
| +4                      | 16.5 | ±0.8  | ±0.2  |
| Top strand              |      |       |       |
| +2                      | 9.5  | ±0.7  | ±0.1  |

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1 Upon overexposure of the gel, the minor DNA band located directly above the cleavage product of the apurinic substrates was also detected in similar relative proportions in the wild-type cleavage product. This minor band was also detected in a 15-mer standard with a sequence identical to the cleavage product. Thus, it appears to be an artifact of the gel electrophoresis rather than an alternative site of scission.
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**FIG. 3.** Stimulation of topoisomerase II-mediated DNA scission is due to the presence of apurinic sites rather than base mismatches. Cleavage reactions contained the wild-type oligonucleotide (None), bottom +2-position apurinic oligonucleotide (AP), or bottom +2-position uracil-containing oligonucleotide (prepared in the absence of uracil-DNA glycosylase; Uracil). DNA substrates were labeled on the 5'-termini of the top (wild-type) strand. A control reaction that contained the wild-type substrate in the absence of topoisomerase II is shown (Std).

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**FIG. 4.** Topoisomerase II-mediated DNA cleavage is stimulated by randomly inserted apurinic sites. Apurinic sites were randomly inserted (see "Results") in the bottom strand of a double-stranded 59-mer oligonucleotide that contained five sites of topoisomerase II-mediated DNA cleavage. Each oligonucleotide contained an average of ~0.7 apurinic sites. Points of scission are designated by arrows, and DNA cleavage sites are denoted on the top and bottom strands by a letter and its corresponding prime (e.g., A and A'). DNA substrates were labeled on the 5'-termini of the top strand. Levels of topoisomerase II-mediated DNA cleavage were determined relative to the wild-type substrate. Data represent the averages of three independent experiments. Standard deviations are indicated.

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**FIG. 5.** Apurinic sites stimulate topoisomerase II-mediated double-stranded DNA cleavage. Reactions contained the wild-type substrate (None) or substrate that contained an apurinic site at the +2-position of the bottom strand (AP). Cleavage products were resolved in non-denaturing polyacrylamide gels following incubation in the presence (+ProK) or absence (−ProK) of proteinase K. Data represent the averages of two independent experiments. Standard deviations are indicated by error bars. A representative non-denaturing polyacrylamide gel showing the double-stranded DNA cleavage products is shown (inset). DNA substrates were labeled on the 5'-termini of the top (wild-type) strand. A control reaction that contained the wild-type substrate in the absence of topoisomerase II is shown (Std).

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conclusion that apurinic sites stimulate DNA scission at intrinsic sites of topoisomerase II-mediated cleavage.

**Topoisomerase II-mediated DNA Scission Induced by Apurinic Sites Is Double-stranded in Nature**—Topoisomerase II creates double-stranded breaks by generating two coordinated nicks on opposite strands of the DNA helix (50–53). The data presented above monitored cleavage only on the DNA strand opposite the apurinic site. Therefore, to determine whether these lesions stimulate enzyme-mediated DNA scission on both strands, products from a cleavage reaction that utilized the 40-mer oligonucleotide substrate were analyzed on a nondenaturing polyacrylamide gel. As shown in Fig. 5, the presence of the bottom +2-position apurinic site enhanced double-stranded DNA cleavage >12-fold. Therefore, apurinic sites stimulate topoisomerase II-mediated DNA cleavage on both strands of the double helix.

The covalent linkage between topoisomerase II and cleaved DNA is a hallmark of the enzyme (1–5). Despite this linkage, if an apurinic site present in the cleavage overhang degraded to a nick, the 5'-terminal 15-mer of the top strand (which contained the radioactive label; see Fig. 2) would be able to disso-
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Fig. 6. Apurinic sites do not inhibit topoisomerase II-mediated religation of cleaved DNA. Religation of the wild-type oligonucleotide (○) or the apurinic oligonucleotide containing a lesion at the bottom +2-position (●) was initiated by shifting reaction temperatures from 30 to 4°C and was stopped at the indicated times by the addition of SDS. Data represent the averages of three independent experiments.

Apurinic sites enhance topoisomerase II-DNA binding. Binding mixtures contained 100 nM wild-type oligonucleotide (None) or 100 nM oligonucleotide that contained an apurinic site at the +2-position or the −3-position on the bottom strand. Data represent the averages of two independent experiments. Standard deviations are indicated by error bars. In all cases, levels of enzyme-DNA binding were at least 5–10-fold higher than levels of scission in corresponding DNA cleavage reactions. Therefore, the data shown represent primarily noncovalent enzyme-DNA binding rather than the formation of covalent topoisomerase II-DNA cleavage complexes.

Fig. 7. Apurinic sites enhance topoisomerase II-DNA binding. Binding mixtures contained 100 nM topoisomerase II and either 100 nM wild-type oligonucleotide (None) or 100 nM oligonucleotide that contained an apurinic site at the +2-position or the −3-position on the bottom strand. Data represent the averages of two independent experiments. Standard deviations are indicated by error bars.

Fig. 8. Apurinic sites dominate the effects of etoposide. A, relaxation assays contained 2 nM topoisomerase II and 5 nM plasmid that contained zero (○) or three (●) abasic sites/plasmid. Levels of activity at 0 μM etoposide were set to 100% for each substrate. B, cleavage reactions contained the wild-type oligonucleotide (None), the wild-type oligonucleotide and 200 μM etoposide (Etop), or an oligonucleotide with a cleavage-enhancing apurinic site at the bottom +2-position in the absence (AP) or presence (AP+Etop) of 200 μM etoposide. Levels of relative cleavage enhancement by the bottom +2-position apurinic site appear slightly lower (~13-fold versus ~18-fold; see Fig. 2) due to the presence of dimethyl sulfoxide in the reaction mixture. C, cleavage reactions contained 100 nM double-stranded oligonucleotide and 200 μM etoposide. The ratio of the wild-type oligonucleotide to substrate that contained a cleavage inhibitory apurinic site at the −3-position on the bottom strand was varied. For all experiments, data represent the averages of three to four independent experiments. Standard deviations are indicated by error bars.

Apurinic sites do not inhibit topoisomerase II-mediated religation of cleaved DNA. Religation of the wild-type oligonucleotide (○) or the apurinic oligonucleotide containing a lesion at the bottom +2-position (●) was initiated by shifting reaction temperatures from 30 to 4°C and was stopped at the indicated times by the addition of SDS. Data represent the averages of three independent experiments.

Position-specific apurinic sites on topoisomerase II-DNA binding was determined using a nitrocellulose filter binding assay. While this assay does not represent a true equilibrium system, the ability of a protein to retain DNA on the filter generally correlates with the affinity of that protein for its DNA substrate. Therefore, values obtained from this assay were used for comparative purposes.

When an apurinic site was located in the cleavage overhang at the bottom +2-position, topoisomerase II-DNA binding was ~3-fold higher as compared with the wild-type oligonucleotide (Fig. 7). Thus, it appears that cleavage enhancement by apurinic sites is due at least in part to an increased affinity of the enzyme for the lesion-containing DNA. In contrast, a lesion at the bottom −3-position, which is outside of the cleavage overhang, had little effect on enzyme-DNA binding (Fig. 7). While this result is consistent with the lack of cleavage enhancement by apurinic sites located external to the overhang, it does not explain the inhibition of cleavage complex formation induced by these sites. However, it suggests that external apurinic sites do not attenuate enzyme-mediated DNA scission by drawing topoisomerase II away (i.e. hijacking the enzyme) from its productive site of action.

Interactions between Apurinic Sites and Topoisomerase II Dominate the Effects of Etoposide.—To compare the DNA cleavage-enhancing activity of apurinic sites with that of a topoisomerase II-targeted anticancer drug, we determined the effects of etoposide (the most widely prescribed anticancer drug in clinical use (22, 23)) on cleavage of the wild-type oligonucleotide. At a 2000-fold molar excess of drug to oligonucleotide (i.e. 200 μM etoposide), this agent stimulated DNA scission ~3-fold (Fig. 8B). This is in contrast to the ~10–18-fold cleavage enhancement observed for apurinic sites at a lesion:oligonucleotide ratio of 1:1. Although drug-induced cleavage stimulation varies widely from site to site and the oligonucleotide sequence utilized in this study does not conform to the consensus sequence for optimal etoposide efficacy (i.e. C at the −1-position) (19), these results suggest that relative to anticancer drugs, apurinic sites are formidable topoisomerase II poisons.

The generality of this conclusion is supported by two additional results. First, in a plasmid-based system, a 10,000-fold molar excess of etoposide (i.e. 100 μM etoposide versus 10 nM plasmid) was required to induce similar levels of cleavage that were generated at an abasic site/plasmid ratio of 3:1 (30). Second, in an oligonucleotide that contained three etoposide consensus cleavage sites (see Fig. 4), the highest level of cleavage enhancement observed at a 1000-fold molar excess of etoposide (i.e. 100 μM drug versus 100 nM oligonucleotide) (site E) was only 2-fold greater than that observed with an apurinic oligonucleotide population in which <10% of the substrate contained a lesion within this cleavage overhang.

To further investigate the relative strengths of etoposide versus apurinic sites, additional experiments were performed to determine whether interactions between topoisomerase II and these lesions dominate those of the anticancer drug. In the first, the ability of etoposide to enhance cleavage of an oligonucleotide containing a stimulatory apurinic site (i.e. the bottom +2-position) was characterized. Three possible results could be expected. If apurinic sites dominate, cleavage stimulation should be similar to that observed in the absence of drug. If etoposide dominates, cleavage stimulation should fall to levels observed with 200 μM drug and wild-type oligonucleotide. Finally, since apurinic sites and etoposide enhance cleavage primarily by opposite mechanisms (i.e. stimulation of cleavage complex formation versus inhibition of religation (57)), DNA...
scission should be dramatically stimulated if the drug and lesion act in concert. As shown in Fig. 8B, levels of cleavage of the apurinic oligonucleotide generated in the presence of etoposide were similar to those observed in the absence of drug.

In the second experiment, the effects of an inhibitory apurinic site (located at the bottom –3-position) on etoposide-enhanced cleavage were assessed. As shown in Fig. 8C, drug-induced cleavage was abolished by this DNA lesion. The above results indicate that although etoposide and apurinic sites both affect topoisomerase II-mediated DNA cleavage, their actions appear to be mutually exclusive, with the effects of apurinic sites dominating those of the drug.

To extend these results to a more global environment, competition between abasic sites and etoposide was examined in a plasmid-based system. Abasic sites randomly generated by heat/acid treatment slightly enhanced catalytic relaxation of pBR322 DNA by topoisomerase II (30). In contrast, etoposide markedly inhibited this reaction (Fig. 8A). When plasmid that contained three abasic sites/molecule was utilized as a relaxation substrate, these lesions significantly protected against drug-induced inhibition (the IC50 value rose from ~35 to >100 μM drug) (Fig. 8A). These results indicate that randomly generated abasic sites (which are primarily apurinic lesions (31)) dominate the effects of etoposide in a plasmid-based system.

**DISCUSSION**

The unusual mechanism of action of topoisomerase II-targeted drugs (i.e. stimulation of cleavage rather than inhibition of enzyme activity) (8, 9, 12, 14, 17), coupled with evidence linking the enzyme to genomic mutagenesis (10, 11), argues for the existence of endogenous topoisomerase II poisons. Our previous study on DNA lesions suggested that abasic sites could act as such endogenous poisons (30). To define interactions between topoisomerase II and specific DNA lesions, the effects of position-specific apurinic sites on enzyme-mediated DNA cleavage were characterized. Results indicate that apurinic sites, depending on their position within an oligonucleotide substrate, can act either as potent enhancers or inhibitors of DNA scission. DNA lesions located within the topoisomerase II cleavage overhang enhanced scission ~10–18-fold, while those located directly outside the points of cleavage abolished scission.

Apurinic sites did not affect the rate of enzyme-mediated DNA religation, despite the fact that they eliminated at least 25% of the base pairing within the cleavage overhang. This result indicates that base pairing within the cohesive ends may not be the driving force behind religation and implies that the enzyme directs the spatial alignment of the two DNA termini during religation. It further indicates that apurinic sites enhance scission primarily by stimulating the formation of topoisomerase II-DNA cleavage complexes. This is due at least in part to an enhanced affinity of the enzyme for the apurinic substrate.

The structural alterations in the apurinic DNA that underlie cleavage enhancement are not clear. The absence of base pairing at an apurinic site may contribute to this effect. Supportive evidence comes from the work of Bigioni et al. (48) (also see Fig. 3), which indicated that base mismatches stimulated topoisomerase II-mediated DNA cleavage with a similar position specificity as observed in this study. However, the fact that apurinic sites are considerably more effective at enhancing DNA cleavage than either base mismatches or apyrimidinic sites strongly suggests that the simple loss of base pairing may not be responsible for the effects of this DNA lesion. Although little information exists regarding the spatial orientation of DNA containing apurinic sites, it appears that these lesions induce greater distortion in the double helix than do either mismatches or apyrimidinic sites (58–63). More detailed structural studies may be required to determine the precise alterations in DNA conformation that ultimately lead to enhancement of topoisomerase II-mediated DNA cleavage by apurinic sites (and potentially other DNA lesions).

One intriguing aspect of this work and the study by Bigioni et al. (48) is that the same apurinic sites and mismatches that stimulate DNA scission when they are located within the cleavage overhang abolish scission when they are located directly outside this region. It is not clear how such positional differences have opposite effects on the same enzyme-mediated reaction. However, some DNA cleavage mapping studies indicate that topoisomerase II recognizes bases primarily outside the cleavage overhang and can tolerate alterations between the points of scission (40, 54). Thus, DNA lesions located outside the cleavage overhang may alter critical topoisomerase II-DNA contacts necessary for proper alignment of the enzyme and subsequent DNA cleavage.

How is it that DNA lesions as well as a number of structurally diverse anticancer drugs all enhance topoisomerase II-mediated DNA cleavage? To address this question, we propose...
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The positional poison model includes a number of features. First, consistent with a previous hypothesis on the actions of anticancer agents (64), it proposes that DNA lesions and drugs both enhance topoisomerase II-mediated DNA cleavage predominitely by altering the structure of DNA. However, for cleavage enhancement to take place, structural alterations must be positioned within the cleavage overhang. This aspect of the model is supported by the positional specificity of cleavage-enhancing DNA lesions as well as by cleavage specificity (40, 65, 66), DNA binding (9), and drug cross-linking (28) studies, all of which place exogenous topoisomerase II poisons on the DNA within the cleavage overhang.

Second, since most drugs interact with DNA in a relatively nonspecific fashion and are mobile (9), the model proposes that interactions with the enzyme are necessary to position the drug-induced alteration of DNA correctly within the cleavage site. This latter aspect of the model is supported by drug–enzyme binding and mutagenesis studies that provide strong evidence for interactions between topoisomerase II and anticancer agents (9, 12, 29, 67–69). In addition, a recent kinetic analysis indicates that drug–DNA interactions mediate the formation of cleavage complexes (70). Due to the immobility of DNA lesions, interactions with the enzyme are not required to position the lesion correctly within the DNA. Therefore, while the efficacy of lesions is predetermined by their location along the double helix, the specificity of drugs can be induced by the enzyme.

On the basis of the above results, two further postulates are implied. One is that anticancer drugs enhance enzyme-mediated DNA cleavage because they induce structural alterations in DNA that mimic those of endogenous topoisomerase II poisons. The other is that anticancer agents ultimately kill cells by exploiting pre-existing processes that result from the interactions of topoisomerase II with DNA lesions.

Apurinic sites are formed by spontaneous hydrolysis of DNA and are induced by a variety of genomic insults, including oxidation, ionizing radiation, DNA-reactive chemicals, and base excision repair pathways (42–44, 71, 72). They are the most commonly formed DNA lesion in mammalian cells (41) and appear to act as a conduit for many forms of DNA damage. Under normal circumstances, apurinic sites are rapidly removed by the cellular repair machinery (42, 44, 73). However, under conditions of acute genomic insult or compromised DNA repair, apurinic sites accumulate in the genetic material (74). Considering the high frequency of topoisomerase II cleavage sites in DNA (at least in vitro) (40, 75, 76), many of these lesions are likely to form within cleavage overhangs and act as topoisomerase II poisons.

Given the potential harm induced by the stimulation of topoisomerase II-mediated DNA cleavage, does the cell derive any benefit from the existence of endogenous topoisomerase II poisons? A final implication of the positional poison model is that when the genome becomes severely damaged, topoisomerase II-generated double-stranded DNA breaks induced by high levels of apurinic sites (or potentially other DNA lesions) trigger apoptotic pathways that ultimately culminate in cell death (Fig. 9). Thus, this model provides a novel physiological function for topoisomerase II and a mechanism by which the enzyme can selectively remove damaged cells from a population. Furthermore, it provides a teleological thread that links the chemotherapeutic actions of clinically relevant anticancer drugs to a natural cellular process.
Apurinic Sites Act as Topoisomerase II Poisons

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