Communications of naturally occurring DNA lesions. That anticancer drugs mimic the cleavage-enhancing action of endogenous topoisomerase II poisons and implies that anticancer drugs mimic the cleavage-enhancing action of endogenous DNA lesions.

Several clinically relevant anticancer drugs induce genomic mutations and cell death by increasing topoisomerase II-mediated DNA breakage. To determine whether endogenous DNA damage also affects this cleavage event, the effects of abasic sites (the most commonly formed spontaneous DNA lesion) on topoisomerase II activity were investigated. The presence of 3 abasic sites/plasmid stimulated enzyme-mediated DNA breakage >6-fold, primarily by enhancing the forward rate of cleavage. This corresponds to a potency that is >2000-fold higher than that of the anticancer drug, etoposide. These findings suggest that abasic sites represent endogenous topoisomerase II poisons and imply that anticancer drugs mimic the cleavage-enhancing actions of naturally occurring DNA lesions.

Topoisomerase II is the cellular target for some of the most active agents currently used for the treatment of human cancers (1–4). These drugs, which include etoposide, doxorubicin, and mitoxantrone, elicit their antineoplastic effects by a mechanism that is markedly different from that of other enzyme-targeted agents. Rather than acting by inhibiting the catalytic activity of the enzyme, anticancer drugs dramatically increase levels of covalent topoisomerase II-cleaved DNA complexes that are normal, but fleeting intermediates in the catalytic cycle of the enzyme (1–4). When present at high concentrations, a portion of these transient enzyme-associated DNA breaks are converted to permanent untethered breaks during replication and transcription (5–7). Thus, these agents act as poisons and convert topoisomerase II, an essential enzyme, into a physiological toxin that produces protein-linked breaks in the genome of treated cells (1–4). Consistent with the ability to generate DNA breaks, topoisomerase II-targeted drugs produce mutagenic side effects. Insertions, deletions, and chromosomal aberrations have been observed in treated cells and animals (8, 9). Furthermore, levels of sister chromatid exchange and illegitimate recombination are increased significantly (8, 9). Finally, 11q23 chromosomal translocations that produce acute myelogenous leukemia have been reported in patients following treatment with etoposide-based regimens (8, 10–13).

Approximately 80% of infant leukemias share the same 11q23 chromosomal translocations as are found in the drug-induced leukemias despite the fact that the affected children have never been exposed to anticancer agents (14). Coupled with the fact that topoisomerase II poisons act by exploiting the natural ability of the enzyme to cleave DNA, this finding raises two possibilities. First, there may be cellular factors that induce topoisomerase II-mediated DNA breakage in vivo and trigger DNA recombination, mutagenesis, or cell death pathways. Second, anticancer agents targeted to this enzyme may actually represent exogenous counterparts of these factors.

Since anticancer agents presumably act at the topoisomerase II/nucleic acid interface (1–4, 5), we questioned whether specific lesions generated by spontaneous DNA damage function as these endogenous cleavage-enhancing factors. Abasic (apurinic/apyrimidinic) sites were chosen for this study because they are the most commonly formed endogenous lesion in DNA (~10,000/mammalian cell/day) (16–19). Results indicate that abasic sites are potent enhancers of double-stranded DNA cleavage mediated by Drosophila melanogaster topoisomerase II. This suggests that abasic sites represent cellular topoisomerase II poisons and implies that anticancer drugs mimic the actions of these endogenous DNA lesions.

EXPERIMENTAL PROCEDURES

Topoisomerase II was purified from D. melanogaster embryonic Kc cells as described by Shelton et al. (20). Negatively supercoiled pBR322 plasmid DNA was prepared as described previously (21). This was obtained from Sigma, protease K and SDS were from Merck, topoisomerase I was from Life Technologies, Inc., and [γ-32P]ATP (~3000 Ci/mmol) was from Amersham. Bacteriophage T4 endonuclease V was prepared by the procedure of Lloyd et al. (22) and was the generous gift of Dr. R. S. Lloyd (The University of Texas Medical Branch at Galveston). Etoposide was from Sigma and was prepared as a 10 mM stock solution in 100% dimethyl sulfoxide prior to use. All other chemicals were analytical reagent grade.

Generation of Abasic Sites in DNA—Abasic sites were generated in negatively supercoiled pBR322 plasmid DNA by incubation in 25 mM sodium citrate, pH 4.8, 250 mM KCl at 70 °C as described previously (23). Reactions were stopped by the addition of 1 mM Tris-HCl, pH 7.9, at 4 °C followed by buffer exchange into 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA in a BioSpin 6 chromatography column (Bio-Rad). The presence of abasic sites was confirmed by the loss of supercoiled DNA following digestion with bacteriophage T4 endonuclease V (22). The number of abasic sites incorporated was calculated from the equation: number of abasic sites/plasmid = [1% supercoiled plasmid remaining following digestion - 1% supercoiled plasmid remaining following digestion under the conditions employed] x 100.

Topoisomerase II-mediated DNA Cleavage—Cleavage assays were performed as described by Corbett et al. (21). Brieﬂy, 10 nM negatively supercoiled pBR322 DNA was incubated with 100 nM topoisomerase II in 20 μl of assay buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 2.5% glycerol) at 30 °C for 6 min.

*This work was supported by National Institutes of Health Grants GM39344 (to N.O.) and CA47479 (to L.J.M.) and American Cancer Society Faculty Research Award FRA-370 (to N.O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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DNA Lesions as Endogenous Topoisomerase II Poisons

**RESULTS**

The presence of abasic sites in pBR322 DNA stimulated double-stranded DNA cleavage mediated by D. melanogaster topoisomerase II (Fig. 1 and Fig. 2, inset). Cleavage was increased nearly 4-fold in plasmids that contained a single abasic site (statistical average) and more than 6-fold in plasmids that contained 3 lesions. At 10 nM plasmid/assay, 3 lesions correspond to an abasic site concentration of 30 nM.

All DNA cleavage assays shown were carried out in the absence of ATP and hence represent cleavage/reliigation equilibria established prior to strand passage (26). Abasic sites doubled levels of topoisomerase II-mediated DNA cleavage in the presence of a nonhydrolyzable ATP analog. Therefore, these lesions also enhance DNA cleavage that occurs in the ATP-bound form (i.e. post-strand passage conformation) of the enzyme.

To contrast the potency of abasic sites with that of a topoisomerase II-targeted anticancer agent, 100 μM etoposide (the most widely prescribed anticancer drug in clinical use (12, 13)) was required to produce an equivalent level of DNA cleavage as seen in the presence of 3 lesions/plasmid (Fig. 1, inset). As extrapolated from the concentration of abasic sites required to produce one-half maximal cleavage enhancement (10 nM abasic sites (Fig. 1) versus >25 μM etoposide (27, 28)), this DNA lesion appears to be at least 2,000 times more potent than etoposide under the conditions employed.

A number of control experiments were performed to ensure that the bulk of cleavage induced by abasic sites was due to the action of topoisomerase II rather than by chemical degradation or by a potential contaminating AP endonuclease (Fig. 2). First, cleavage required the presence of both topoisomerase II and Mg^{2+}. Second, the majority of double-stranded breaks were protein-linked (i.e. linear DNA molecules were not released unless samples were digested with proteinase K) and were readily reversed by the addition of salt or by chelation of the required divalent cation by EDTA. These characteristics are hallmarks of topoisomerase II-mediated DNA cleavage (29–31) and are incompatible with the mechanism of action of AP endonucleases (32, 33). A small proportion of double-stranded DNA breaks observed were not protein-associated. This cleavage may reflect the inherent instability of abasic sites (16–19) (as evidenced by the enzyme-independent increase in nicked (FII) DNA seen in Fig. 2, inset), which may be exacerbated by exposure to the large number of nucleophilic aminoaoyl residues in topoisomerase II (34). Since this latter class of breaks may not reflect events mediated by the active-site tyrosine of topoisomerase II, for all experiments shown (other than those in Fig. 2), double-stranded DNA breaks that were not protein-associated (i.e. plasmid molecules that were not digested with proteinase K, but migrated with the electrophoretic mobility of free linear pBR322 DNA) were subtracted as background from the total double-stranded cleavage observed.

As determined by electrophoresis in agarose gels containing the intercalating agent, chloroquine, the average linking number of pBR322 was not altered significantly by the presence of 3 abasic sites/plasmid. Therefore, the enhancement of topoisomerase II-mediated DNA cleavage by abasic sites was not
due to a global change in plasmid topology. Furthermore, abasic sites stimulated cleavage in linear DNA substrates, indicating that superhelicity is not needed for cleavage enhancement. Finally, the effect of abasic sites appears to be specific for type II topoisomerases, as no cleavage stimulation was observed in the presence of topoisomerase I.

Topoisomerase II establishes a DNA cleavage/religation equilibrium within a few seconds when control pBR322 molecules are used as substrates (35) (Fig. 3). This rapid attainment of equilibrium probably reflects the plethora of potential recognition/cleavage sites that are available to the enzyme in the plasmid. In contrast, cleavage of pBR322 that contained only 3 abasic sites was time-dependent and required 6 min to achieve maximal cleavage (Fig. 3). Beyond 6 min, levels of cleavage remained stable. This indicates that topoisomerase II does not initially bind its DNA substrate at abasic sites and suggests that it locates these lesions by a scanning mechanism. If this is the case, the enzyme may be able to locate isolated abasic sites, even if they are separated by long regions of DNA.

Topoisomerase II-targeted anticancer agents enhance enzyme-mediated DNA breakage by one of two mechanisms (1). Drugs such as etoposide (Fig. 4) and amsacrine act primarily by inhibiting the ability of topoisomerase II to religate its cleaved DNA substrate. Alternatively, agents such as quinolones, genistein, nitroimidazoles, pyrimido benzimidazoles, and ellipticine have little effect on religation and presumably act by enhancing the forward rate of cleavage (1, 36, 37). Abasic sites did not inhibit the apparent first order rate of DNA religation (Fig. 4). In fact, the religation rate of plasmid molecules containing 3 abasic sites was nearly twice that of control DNA. This finding provides strong evidence that abasic sites enhance nucleic acid breakage primarily by increasing the forward rate of cleavage.

Although the cytotoxic potential of topoisomerase II poisons reflects their ability to enhance DNA breakage, these agents also inhibit the catalytic strand passage reaction of the enzyme (38). This was not the case for DNA containing abasic sites (Fig. 5A). At a catalytic enzyme to plasmid ratio (i.e., 1:3), topoisomerase II relaxed negatively supercoiled abasic DNA at a rate that was ~25% faster than that of control plasmid. It is notable, however, that catalytic DNA relaxation is limited by the rate at which the enzyme releases its fully relaxed DNA substrate (39). This slow dissociation rate could potentially mask the stimulatory effects of abasic sites on DNA cleavage or other reaction steps prior to enzyme recycling. Therefore, the experiment was repeated utilizing a stoichiometric ratio of enzyme to plasmid (i.e., 1:3:1) (Fig. 5B). Under these conditions, which do not require enzyme dissociation, negatively supercoiled substrates containing 3 abasic sites were relaxed with an initial velocity that was ~2.5 times higher than that of control molecules.

The enhancement of DNA relaxation by abasic sites is in marked contrast to the inhibitory effects of anticancer drugs, even those that stimulate the forward rate of enzyme-mediated DNA cleavage (1). However, in all cases examined, these drugs impaired interactions between topoisomerase II and its ATP cofactor (24) (which is required for DNA strand passage (29–31)). In contrast, abasic sites did not affect the rate of enzyme-catalyzed ATP hydrolysis (Fig. 6). This lack of inhibition may explain why abasic sites, which enhance the forward rate of cleavage, are able to stimulate the catalytic activity of the enzyme.

**DISCUSSION**

Abasic sites are the first endogenous DNA lesion or modification found to increase topoisomerase II-mediated DNA cleavage. Previous studies examined the effects of ultraviolet-induced cyclobutane pyrimidine dimers (21) or DNA methylation (40) on topoisomerase II activity. Pyrimidine dimers had no effect on DNA cleavage, but rather inhibited overall activity by specifically inhibiting the DNA strand passage step of the topoisomerase II catalytic cycle. Furthermore, methylation suppressed DNA cleavage up to 7 bases from the modified cytosine.

Abasic sites are produced in vivo by a myriad of DNA damaging events. Among these are oxidation (including attack by NO· produced as part of the macrophage immune response),

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1. P. S. Kingma and N. Osheroff, unpublished results.
ionizing radiation, DNA-reactive chemicals (including alkylating agents used for the treatment of human cancers), and spontaneous hydrolysis (17, 19, 33, 41). Similar to topoisomerase II-targeted agents, these events induce mutagenesis, recombination, and under excessive conditions, trigger pathways that lead to cell death. Due to the rapid removal of abasic sites by the cellular repair machinery (17, 19), physiological interactions with topoisomerase II under normal conditions probably are rare. However, the results of the present study indicate that when these interactions occur, they may lead to the production of protein-associated double-stranded DNA breaks. Furthermore, the potential for these interactions is increased under conditions that induce DNA damage or compromise repair systems (42).

On the basis of the studies presented above, it is proposed that abasic sites are a conduit for numerous cellular insults that ultimately enhance topoisomerase II-mediated double-stranded DNA cleavage. Although DNA scissions produced by this process are transient in nature, they are frequently converted to permanent double-stranded breaks during replication and transcription (5–7). Unlike the readily repaired single-stranded DNA nicks normally produced at abasic sites by the actions of AP endonucleases, lyases, and spontaneous elimination reactions (17, 19), double-stranded breaks are potential hot spots for genomic alterations, chromosomal translocations, and the induction of apoptotic events (8, 9). This suggests that anticancer agents targeted to the enzyme exert their mutagenic and antineoplastic effects by utilizing a mechanism that mimics the cleavage-enhancing actions of endogenous DNA lesions, thus allowing these drugs to exploit existing cellular pathways.

Acknowledgments—We are grateful to Dr. R. S. Lloyd for the generous gift of bacteriophage T4 endonuclease V and Drs. S. J. Froelich-Ammon and D. A. Burden for helpful discussions.

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