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DNA minor groove-binding ligands: A different class of mammalian DNA topoisomerase I inhibitors

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ABSTRACT **A number of DNA minor groove-binding ligands (MGBLs) are known to exhibit antitumor and antimicrobial activities. We show that DNA topoisomerase (Topo) I may be a pharmacological target of MGBLs. In the presence of calf thymus Topo I, MGBLs induced limited but highly specific single-strand DNA breaks. The 3′ ends of the broken DNA strands are covalently linked to Topo I polypeptides. Protein-linked DNA breaks are readily reversed by a brief heating to 65°C or the addition of 0.5 M NaCl. These results suggest that MGBLs, like camptothecin, abort Topo I reactions by trapping reversible cleavable complexes. The sites of cleavage induced by MGBLs are distinctly different from those induced by camptothecin. Two of the major cleavage sites have been sequenced and shown to be highly A+T-rich, suggesting the possible involvement of a Topo I–drug–DNA ternary complex at the sites of cleavage. Different MGBLs also exhibit varying efficiency in inducing Topo I-cleavable complexes, and the order of efficiency is as follows: Hoechst 33342 and 33258 ≫ distamycin A > berenil > netropsin. The lack of correlation between DNA binding and cleavage efficiency suggests that, in addition to binding to the minor grooves of DNA, MGBLs must also interact with Topo I in trapping Topo I-cleavable complexes.**

Many DNA-binding agents are important therapeutic drugs. Recent studies have demonstrated that a large number of DNA intercalative agents exert their major pharmacological action through interference with the catalytic cycles of DNA topoisomerase (Topo) II to produce Topo II-linked DNA breaks (reviewed in refs. 1–4). Nonintercalative DNA-binding ligands, which show various degrees of sequence specificity for binding to the minor groove of DNA, represent another major class of compounds with broad-spectrum antiviral, antibacterial, antitumor, and antiprotozoal activity (5, 6). Their modes of interaction with DNA have been studied extensively (reviewed in refs. 7 and 8). Some of the most commonly studied minor groove-binding compounds, such as distamycin A, netropsin, and the bisbenzimidazole dyes (Hoechst dyes) [e.g., 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride trihydrate (Hoechst 33342) and 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperizinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride pentahydrate (Hoechst 33258)] are known to bind to the minor groove of DNA with A+T specificity and to cause widening of the minor grooves (7, 8). Despite extensive investigation into the DNA-binding mode of these compounds, the cellular targets of these compounds other than DNA have not been identified.

Most DNA minor groove-binding ligands (MGBLs) are positively charged (see Fig. 1 for some examples) and cannot readily diffuse through the cellular membrane. The strongly fluorescent dye Hoechst 33342, which differs from its parent compound Hoechst 33258 by a 4-ethoxy substitution on the phenyl ring and has enhanced membrane permeability, is readily taken up by living cells and has been used extensively for histochemical staining and flow cytometric analysis of DNA content of viable cells (9). Curiously, Hoechst 33342 induces protein–DNA crosslinks and DNA strand breaks in cultured mammalian cells (10, 11). G_2-phase arrest and chromosome endoreduplication are also prominent effects of Hoechst 33342 treatment (12, 13). These cellular effects are reminiscent of those induced by Topo poisons and other DNA-damaging agents (reviewed in ref. 3). To investigate the possible involvement of Topos in the action of DNA minor groove-binding drugs, we assayed the ability of these agents to induce DNA cleavage in the presence of purified calf thymus Topos I and II. We show here that a number of DNA MGBLs can interrupt to a variable extent the breakage/reunion cycle of Topo I, but not Topo II, by stabilizing a reversible Topo I-cleavable complex. Since Topo I-cleavable complexes are known to mediate the antitumor action of the prototypic Topo I-specific inhibitor camptothecin (reviewed in ref. 3), our results may suggest DNA Topo I to be an important pharmacological target of MGBLs.

MATERIALS AND METHODS

Materials. Hoechst 33342 and 33258, DAPI (4′,6-diamidino-2-phenylindole), berenil, and distamycin A were purchased from Sigma; netropsin was from Boehringer Mannheim. All compounds were dissolved in 10 mM dimethyl sulfoxide and kept frozen in aliquots at −20°C. DNA Topos I and II were purified from calf thymus glands by using published procedures (14, 15). YEpG is a derivative of YEP24, which has the regulated yeast *GAL1* promoter inserted between the *Bam*HI and *Sal* I sites of the tetracycline-resistance gene (16). The Sequenase kit was purchased from United States Biochemical.

DNA Topos I and II Cleavage Assays. DNA Topos I and II cleavage assays were done as described (17, 18). The procedure for end-labeling of plasmid DNA has been described (19).

Mapping and Sequencing the Cleavage Sites. The mapping of cleavage sites induced by Hoechst 33342 and 33258 was done at two levels. First, the single-strand breaks were located on YEpG DNA by using uniquely end-labeled DNA and alkaline agarose gel (1.0%) electrophoresis. Second, according to the rough locations of sites mapped, two synthetic oligonucleotide 18-mers (5′-CTGACGCTCAGTGGAACG-3′ and 5′-GTTCTTCCTTCTGTTCGG-3′), which are complementary to the DNA sequences around 60–100 bases upstream of the cleavage sites (c and d sites in Fig. 2),

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Abbreviations: MGBLs, minor groove-binding ligands; Topo, topoisomerase; Hoechst 33342, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride trihydrate; Hoechst 33258, 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperizinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride pentahydrate; DAPI, 4′,6-diamidino-2-phenylindole.

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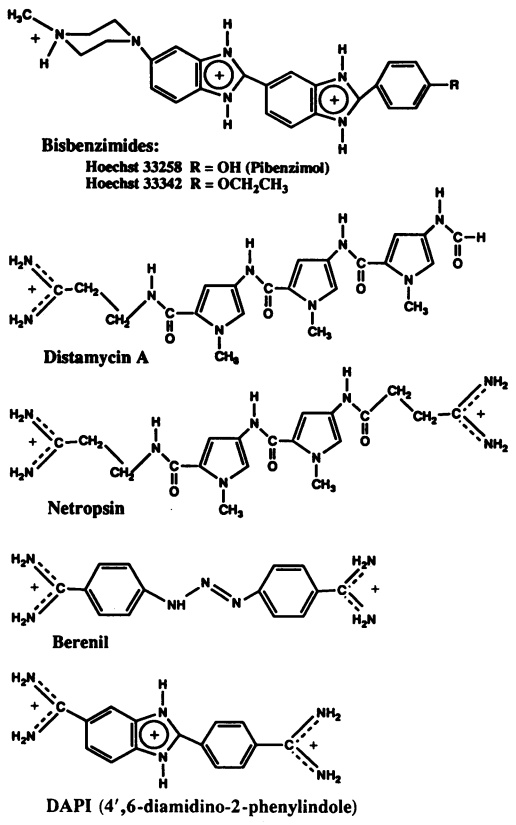

FIG. 1. Chemical structures of Hoechst 33342 and 33258 and other DNA MGBLs used in this study.

were synthesized and used in primer extension reactions as described (20). For the primer extension analysis, Hoechst 33258-induced Topo I-cleaved YEpG DNA was primer extended in the presence of [α-^{32}P]dCTP and other three unlabeled deoxynucleotides. The primer-extended products were then compared with the sequencing ladders generated by the dideoxy sequencing method using the same primers on uncleaved YEpG DNA.

RESULTS

DNA MGBLs Stimulate Site-Specific Topo I-Mediated DNA Cleavage *in Vitro*. Unlike camptothecin, which induced extensive Topo I-linked DNA single-strand breaks (Fig. 2, lanes C), Hoechst 33342 induced limited but highly specific single-strand breaks on YEpG DNA in the presence of calf thymus DNA Topo I (Fig. 2, lanes H). Interestingly, DNA breakage occurred over a limited range of ligand concentrations. Concentrations of Hoechst 33342 above 1 μM severely inhibited DNA cleavage. This dose dependence of the cleavage reaction is highly reminiscent of intercalator-induced DNA double-strand cleavage in the presence of DNA Topo II (18). Lower concentrations of DNA intercalators induce limited but highly specific double-strand DNA breaks in the presence of Topo II, while higher concentrations of DNA intercalators severely inhibit DNA cleavage (18). Inhibition at higher concentrations of intercalators has been attributed to reduced access of Topo II to the ligand-saturated DNA template. Inhibition of Topo I-mediated DNA cleavage by DNA MGBLs can probably be similarly explained. In addition to Hoechst 33342, a number of other DNA MGBLs have been tested. Some of them also induced Topo I-mediated DNA cleavage with similar cleavage-site specificity (Fig. 2, lanes D–H; bands labeled a, b, c, and d are commonly induced). However, the cleavage efficiency at various sites varied significantly. The efficiency of cleavage, which is characterized by band intensities of cleaved plasmid, is as follows: Hoechst 33342 and 33258 $\gg$ distamycin A > berenil > netropsin. DAPI is inactive in our assay. It is noteworthy that Hoechst 33258 and 33342 are at least as efficient as camptothecin in inducing Topo I-mediated DNA breakage when compared at lower concentrations (Fig. 2). The less efficient cleavage at higher concentrations of Hoechst 33342 and 33258 may be due to reduced access of Topo I to drug-saturated DNA. Neither calf thymus DNA Topo II nor *Escherichia coli* Topo I could substitute for calf thymus DNA Topo I in the cleavage reaction (data not shown).

DNA Single-Strand Breaks Induced by DNA MGBLs Reflect Topo I-Cleavable Complexes. Like camptothecin-induced breaks, DNA breaks induced by these DNA MGBLs in the presence of Topo I are primarily single-strand breaks, consistent with the mechanism of action of DNA Topo I. The cleavage products shown in Fig. 2 were analyzed after alkali denaturation of the DNA. When the cleavage products were analyzed without alkali treatment, no DNA fragments were observed (data not shown). To test whether Hoechst 33342-induced single-strand breaks are covalently linked to Topo I, the reaction mixtures were precipitated by a potassium salt/sodium dodecyl sulfate (K^+–SDS) coprecipitation procedure that selectively precipitates DNA covalently linked to protein (19). Like camptothecin, Hoechst 33342 induced covalent protein–DNA complexes, as evidenced by the effective precipitation of labeled DNA in the presence of calf

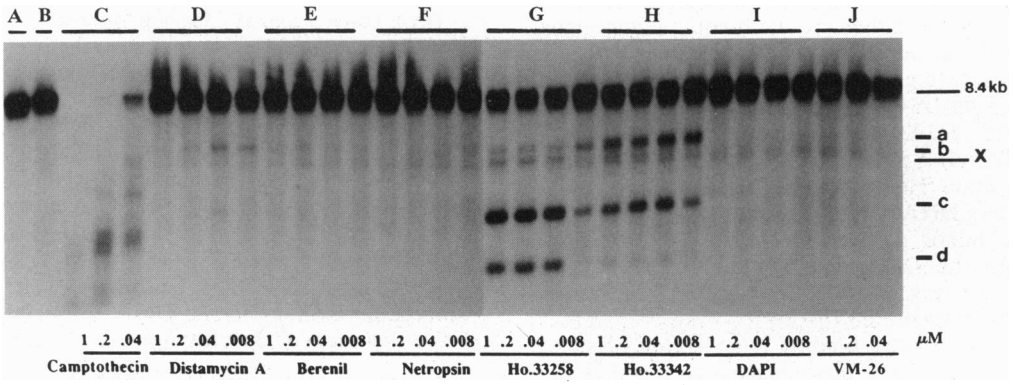

FIG. 2. DNA MGBLs induce site-specific DNA cleavage in the presence of mammalian DNA Topo I. Topo I-cleavage assays were done as described in text. Reactions were terminated by treatment with SDS and proteinase K and alkali-denatured prior to loading onto 1.0% agarose gel in neutral TBE (0.089 M Tris borate/0.008 M EDTA, pH 8.0) electrophoresis buffer. Lanes: A, DNA control, no drug, no enzyme; B, Topo I, no drug; C–J, Topo I plus camptothecin, distamycin, berenil, Hoechst 33258, Hoechst 33342, DAPI, and VM-26, respectively. Bands labeled a, b, c, and d are commonly induced by these MGBLs; the band labeled x marks a Topo I-induced background cleavage site that is present in all but lane A. All reactions contained 1% dimethyl sulfoxide.

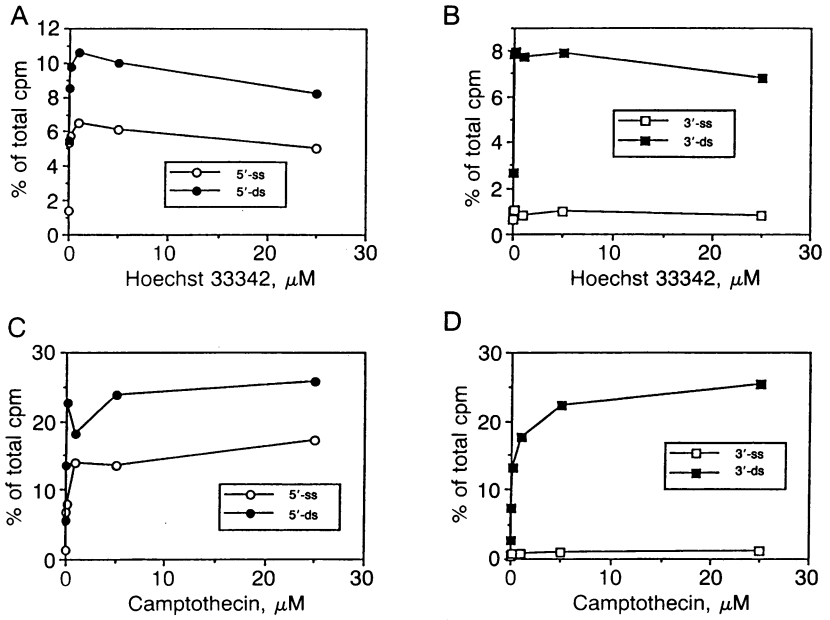

FIG. 3. Topo I is covalently linked to the 3' ends of the broken DNA strands induced by Hoechst 33342. Calf thymus Topo I and end-labeled DNAs were used in the cleavage reactions in the presence of either camptothecin (*C* and *D*) or Hoechst 33342 (*A* and *B*). The reaction products were precipitated by using either the neutral (● and ■) or the alkaline K^+–SDS coprecipitation procedure (○ and □) (19). 5'-ss identifies 5'-end-labeled DNA precipitated with the alkaline K^+–SDS procedure. 5'-ds is 5'-end-labeled DNA precipitated with the neutral K^+–SDS procedure. 3'-ss is 3'-end-labeled DNA precipitated with the alkaline K^+–SDS procedure. 3'-ds is 3'-end-labeled DNA precipitated with the neutral K^+–SDS procedure.

thymus DNA Topo I (Fig. 3). The polarity of the covalent linkage between Topo I and the broken DNA strand was also determined by using 3'- and 5'-end-labeled DNA and an alkaline K^+–SDS coprecipitation procedure (Fig. 3) (19). Like camptothecin-induced protein–DNA complexes (see Fig. 3 *C* and *D*), Hoechst 33342-induced protein–DNA complexes were precipitated only with the 5'-end-labeled DNA (Fig. 3*A*) but not with the 3'-end-labeled DNA (Fig. 3*B*). These results indicate that Hoechst 33342 traps a covalent Topo I–DNA complex in which Topo I is covalently linked to the 3' end of the broken DNA single strand.

The formation of Topo I-linked DNA breaks could reflect the formation of a reversible Topo I-cleavable complex. To test this possibility, two standard "reversing procedures" (21) were used to "reverse" Hoechst 33258- and 33342-induced DNA breaks (Fig. 4). DNA fragmentation due to camptothecin (lane C), Hoechst 33258 (lane D), or Hoechst 33342 (lane E) was dramatically reduced (compare in Fig. 4 lanes B–E with lanes F–I) when the incubated reaction mixtures were heated to 65°C for 10 min prior to stopping the reactions with SDS and proteinase K. Another reversing procedure involved challenging the preincubated reactions with 0.5 M NaCl. Again, DNA fragmentation was dramatically reduced (compare lanes B–E with lanes J–M). These results indicate that Topo I-linked DNA breaks induced by MGBLs most likely reflect trapped Topo I-cleavable complexes, which were converted to Topo I-linked single-strand breaks upon treatment with a strong protein denaturant such as SDS.

Hoechst 33342-Induced Protein–DNA Crosslinks in Human KB Cells Exhibit Properties Characteristic of Topo-Cleavable Complexes. The specific trapping of Topo I as reversible cleavable complexes by DNA MGBLs *in vitro* may explain the protein–DNA crosslinks and DNA strand breaks observed in Hoechst 33342-treated mammalian cells (10, 11). To test whether these strand breaks reflect the formation of reversible cleavable complexes in cells, we have measured their reversibility by briefly heating the Hoechst 33342-treated cells to 65°C for 10 min, a condition known to reverse cleavable complexes *in vitro* as well as *in vivo* (21). We have used the K^+–SDS coprecipitation procedure to measure protein–DNA crosslinks in drug-treated cells. Like camptothecin, Hoechst 33342 induced a high level of protein–DNA crosslinks in human KB cells treated with drug for 45 min. Hoechst 33258, distamycin A, and netropsin did not produce a significant amount of protein–DNA crosslinks. The lack of protein–DNA crosslinks in Hoechst 33258-treated cells is probably due to the lower membrane permeability of Hoechst 33258 (22). Using the K^+–SDS coprecipitation procedure, we also tested the reversibility of protein–DNA crosslinks formed in Hoechst 33342-treated human KB cells. Brief heating (65°C) of 5 μM Hoechst 33342-treated human KB cells for 10 min prior to lysis with SDS reduced amounts of the protein–DNA crosslinks to the background level (see footnote † in Table 1), suggesting that the majority of the protein–DNA crosslinks in Hoechst 33342-treated cells are due to cleavable complexes.

DNA Single-Strand Breaks Induced by DNA MGBLs Occur in Local A+T-Rich Regions. Four highly specific Topo I-mediated DNA cleavage sites (see Fig. 2, sites a, b, c, and d) in the presence of Hoechst 33342 have been identified by agarose gel electrophoresis in neutral electrophoresis buffer. The approximate locations of these cleavage sites on both strands of YEpG DNA were further determined by using uniquely end-labeled DNA and alkaline agarose gel electrophoresis. The strategy for mapping these sites is shown in

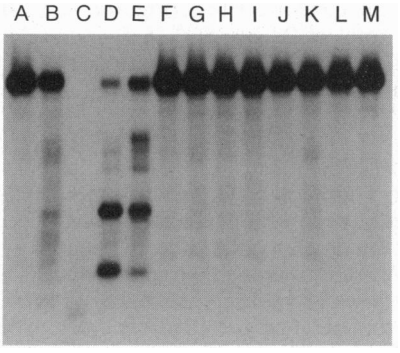

FIG. 4. Hoechst 33258- and 33342-induced DNA breaks are "reversible." The DNA cleavage assay was done as described in the legend of Fig. 2 except that the amount of calf thymus DNA Topo I in each assay was doubled. All reaction mixtures contained 1% dimethyl sulfoxide. Lanes: A, DNA control; B, Topo I only; C–E, Topo I plus 1 μM camptothecin, Hoechst 33258, or Hoechst 33342, respectively; F–I, reactions as in lanes C–E, respectively, except that the preincubated reactions (15 min at 37°C) were heated to 65°C for another 10 min prior to termination with SDS and proteinase K; J–M, reactions as in lanes C–E, respectively, except that the preincubated reactions (15 min at 37°C) were brought to 0.5 M NaCl for another 10 min prior to termination with SDS and proteinase K.

Table 1. Hoechst 33342 induces reversible DNA–protein crosslinks in human KB 3-1 cells

Drug	% of radioactivity precipitated by K^+–SDS procedure with increasing drug concentrations			
	0.0 μM	0.2 μM	1.0 μM	5.0 μM
Camptothecin	0.0	2.3 ± 1.8*	12.0 ± 2.0	22.6 ± 2.8
Hoechst 33342	0.0	2.7 ± 0.7	13.7 ± 1.4	16.4 ± 3.5
		(0.1 ± 0.1)†	(0.0 ± 0.1)†	(0.0 ± 0.2)†
Hoechst 33258	0.0	0.0 ± 1.1	0.1 ± 1.4	1.3 ± 1.6
Distamycin A	0.0	0.6 ± 1.1	0.0 ± 2.3	0.0 ± 1.4
Netropsin	0.0	0.7 ± 1.0	0.5 ± 1.4	0.0 ± 0.7

Protein–DNA crosslinks in drug-treated cells were measured by using the K^+–SDS coprecipitation procedure (23). The total trichloroacetic acid-precipitable cpm for each sample averaged about 8.3×10^3. Proteinase K treatment (400 μg/ml at 65°C for 2 hr) of the cell lysates reduced the precipitable counts to the background level.

*Data are arithmetic means (±SD) of three determinations, and the background counts have been subtracted.

†Hoechst 33342-treated cells were heated to 65°C for 10 min prior to SDS lysis.

Fig. 5. Sites a and c were mapped to the lower strand of YEpG DNA by using end-labeled DNA that was uniquely end-labeled at the 3′ end of the lower strand (*Nhe* I was used to cut off the short piece of DNA containing the 3′ end of the upper strand) (Fig. 5*B Left*). Sites b and d were mapped to the upper strand by using end-labeled DNA that was uniquely end-labeled at the 3′ end of the upper strand (*Sal* I was used to cut off the short piece of DNA containing the 3′ end of the lower strand) (Fig. 5*B Right*). A minor site was also detected that was mapped between sites b and d (Fig. 5*B Right*).

To determine the exact sequence at the cleavage sites, several primers that are complementary to the sequences ≈60–100 bp downstream from the roughly located cleavage sites were made. These primers were then used in primer extension reactions to map the precise location of the cleavage sites. The primer-extended products were then compared with the sequencing ladders generated by the dideoxy sequencing method using the same primers. The precise sites of cleavage for two of the sites (c and d) were determined by this method, and the local DNA sequences surrounding the sites are shown in Fig. 6. The local DNA sequence surrounding the cleavage site c (Fig. 6*A*) consists of 25 A·T bp out of a total of 27 bp (93% A·T content). The local DNA sequence surrounding the cleavage site d (Fig. 6*B*) consists of 24 A·T bp out of a total of 28 bp (86% A·T content). A common sequence of 5′-TCATTTTTT-3′ was found at the sites of cleavage. In both cases, cleavage occurs between 5′-TC-3′.

DISCUSSION

Our studies have demonstrated that Hoechst 33342 and 33258 are effective Topo I poisons *in vitro*. Other DNA MGBLs tested proved to be less effective in trapping Topo I-cleavable complexes. Similar to camptothecin, a prototypic Topo I poison, DNA MGBLs probably trap the same Topo I reaction intermediate, the cleavable complex. This is evidenced by the covalent linking of Topo I to the 3′ end of the broken DNA strand and the characteristically reversible nature of the Topo I-linked DNA breaks. However, unlike camptothecin, DNA MGBLs induce limited but highly site-specific single-strand breaks in the presence of Topo I. This is in contrast to the single-strand breaks induced by camptothecin, which are more pronounced but less specific (17). In addition, the majority of breaks induced by camptothecin are also present as background cleavage sites (24). One possible explanation for this difference is that DNA MGBLs do not stabilize Topo I–ligand–DNA ternary complexes; however, by binding and therefore blocking certain specific sites on DNA, DNA MGBLs "squeeze" Topo I molecules to certain ligand-free

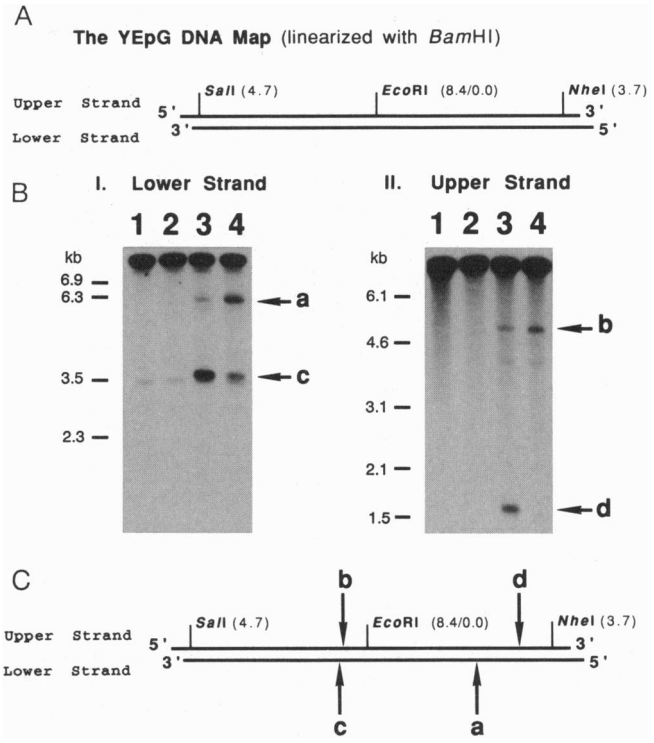

FIG. 5. Mapping of Topo I-mediated cleavage sites induced by Hoechst 33342 and 33258. Topo I-mediated cleavage sites induced by the MGBLs on YEpG DNA were mapped by alkaline gel electrophoresis as described in text. (*A*) Strategy for mapping. *Bam*HI-linearized YEpG DNA was uniquely end-labeled at the 3′ end of either the upper or the lower strand. For upper-strand labeling, *Bam*HI-linearized YEpG DNA was first labeled at both 3′ ends with Klenow polymerase and [α-^{32}P]dCTP, followed by *Sal* I digestion to remove the labeled 3′ end of the lower strand. For lower-strand labeling, *Bam*HI-linearized YEpG DNA was first labeled at both 3′ ends, followed by *Nhe* I digestion to remove the labeled 3′ end of the upper strand. (*B*) Mapping of the cleavage sites using the DNA with either the labeled lower strand (*Left*) or the labeled upper strand (*Right*). Lanes: 1, no enzyme, no ligand; 2, Topo I, no ligand; lane 3, Topo I and 1 μM Hoechst 33258; 4, Topo I and 1 μM Hoechst 33342. Four major ligand-induced Topo I-mediated cleavage sites on YEpG DNA are indicated by boldface arrows. (*C*) Approximate positions of these four sites on both strands of YEpG DNA.

regions on DNA, resulting in more specific cleavage in these regions. In this view, the cleavage sites reflect the sites of Topo I–DNA binary complexes rather than Topo I–ligand–DNA ternary complexes. Indeed, a number of DNA minor group binding ligands have been demonstrated to alter the Topo cleavage-site specificity induced by other Topo-specific poisons (25–27). However, our results suggest that the effect of DNA MGBLs on Topo I-mediated DNA cleavage at these major sites (sites a–d) is more likely to be due to the formation of Topo I–drug–DNA ternary complexes rather than Topo I–DNA binary complexes; first, as shown in Figs. 2 and 4, the amount of cleavable complexes as measured by both the cleavage assay and the K^+–SDS coprecipitation assay increased substantially in the presence of Hoechst 33342. This is not to be expected if Hoechst 33342 only alters the site specificity of the background (binary) cleavable complexes. Second, DNA strand breaks and "reversible" protein–DNA crosslinks in cultured mammalian cells also increased substantially in the presence of Hoechst 33342. However, this argument is clouded by the observation that DNA MGBLs stimulate the catalytic activity of Topo I at lower ligand concentrations (27). The lack of correlation between activity stimulation and cleavage stimulation by various DNA MGBLs suggests that activity stimulation by

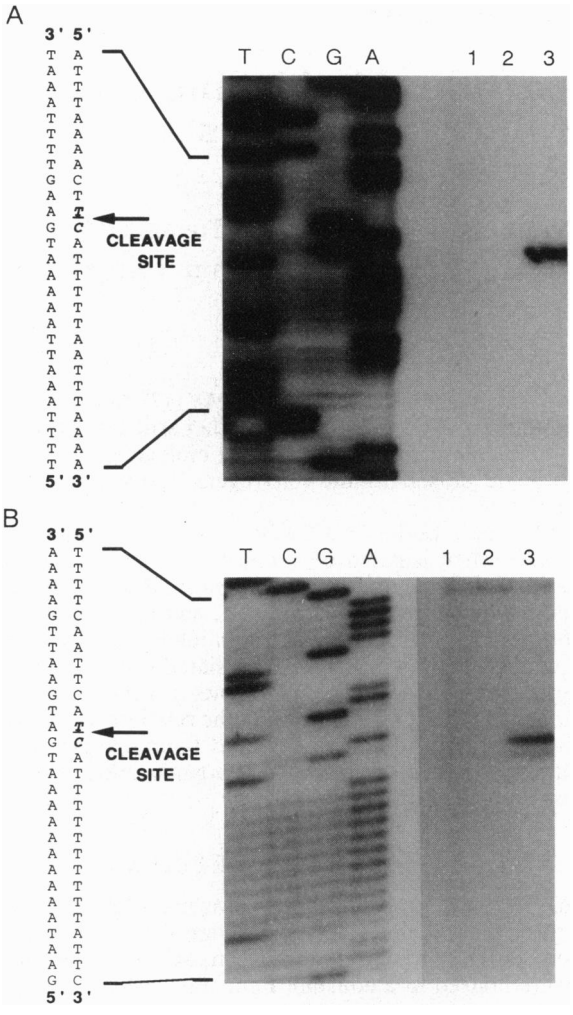

FIG. 6. Nucleotide sequences of the two major cleavage sites induced by Hoechst 33258. Primer extension analysis of the nucleotide sequences of the two major sites, c (*A*) and d (*B*), was performed as described in text. Lanes: 1, no enzyme, no Hoechst 33258; 2, Topo I, no Hoechst 33258; 3, Topo I and 1 μM Hoechst 33258. The arrows indicate the exact DNA breakage sites induced by Hoechst 33258 in the presence of mammalian DNA Topo I. In both cases, cleavage occurs between T and C as shown.

DNA MGBLs cannot fully account for cleavage stimulation by these ligands. Second, if Topo I molecules are squeezed into ligand-free regions on DNA, these regions are likely to be less A+T-rich. However, two of the cleavage sites that have been sequenced are located in regions with extremely high A+T contents. In fact, a common sequence of 5′-TCATTTTT-3′ is found at the sites of cleavage for two of the major cleavage sites (sites a and c, cleavage occurs between T and C). The third site (site d) was also mapped in a region with the same consensus (unpublished results). The T track downstream from the cleavage site may be a potential binding site for these ligands. Analysis of drug cleavage-inhibition patterns (footprinting) is necessary to establish the exact position of the drug in the vicinity of the cleavage sites. Third, if one assumes that various DNA MGBLs interact with DNA at the same sites, the DNA-binding strength of the ligand should be the sole determining factor for altering Topo I-cleavage specificity unless Topo I also interacts with DNA-bound ligands. However, the large difference in cleavage efficiency among DNA MGBLs does not correlate with their DNA-binding strength (28).

We are uncertain whether binding to the minor grooves of DNA is responsible for trapping of Topo I-cleavable complexes by DNA MGBLs. For example, Hoechst 33342 and 33258 probably have two modes of DNA binding. At higher concentrations, both Hoechst 33342 and 33258 also unwind DNA and therefore may have an intercalative mode of DNA binding (29). However, all DNA binding ligands that are known to stimulate Topo I-mediated DNA cleavage have a DNA minor groove-binding mode. For example, actinomycin D is known to stimulate both Topo I- and Topo II-mediated DNA cleavage (30, 31). Although actinomycin D is a classical intercalator, the pentapeptide rings of actinomycin D are known to form hydrogen bonds with the minor groove of DNA while the phenoxazone ring is intercalated into DNA. In addition, we have shown that many other known DNA minor groove-binding antibiotics including nogalamycin, mithramycin, and chromomycin A_3 also poison Topo I but not Topo II (unpublished results). It is possible that the two modes of DNA binding, intercalation and minor groove binding, may be responsible for trapping Topo II- and Topo I-cleavable complexes, respectively. Such a dichotomy in the mode of DNA binding for trapping Topo-cleavable complexes may be a general phenomenon and reflects the fundamental difference in the structure of the putative covalent reaction intermediates of Topo reactions. Further studies are necessary to establish the mechanism of trapping Topo I-cleavable complexes by DNA MGBLs.

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