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Base mutation analysis of topoisomerase II-idarubicin-DNA ternary complex formation. Evidence for enzyme subunit cooperativity in DNA cleavage

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ABSTRACT

Antitumor drugs, such as anthracyclines, interfere with mammalian DNA topoisomerase II by forming a ternary complex, DNA – drug – enzyme, in which DNA strands are cleaved and covalently linked to the enzyme. In this work, a synthetic 36-bp DNA oligomer derived from SV40 and mutated variants were used to determine the effects of base mutations on DNA cleavage levels produced by murine topoisomerase II with and without idarubicin. Although site competition could affect cleavage levels, mutation effects were rather similar among several cleavage sites. The major sequence determinants of topoisomerase II DNA cleavage without drugs are up to five base pairs apart from the strand cut, suggesting that DNA protein contacts involving these bases are particularly critical for DNA site recognition. Cleavage sites with adenines at positions −1 were detected without idarubicin only under conditions favouring enzyme binding to DNA, showing that these sites are low affinity sites for topoisomerase II DNA cleavage and/or binding. Moreover, the results indicated that the sequence *5′*-(A)TA/(A)-*3′* (the slash indicates the cleaved bond, parenthesis indicate conditioned preference) from −3 to +1 positions constitutes the complete base sequence preferred by anthracyclines. An important finding was that mutations that improve the fit to the above consensus on one strand can also increase cleavage on the opposite strand, suggesting that a drug molecule may effectively interact with one enzyme subunit only and trap the whole dimeric enzyme. These findings documented that DNA recognition by topoisomerase II may occur at one or the other strand, and not necessarily at both of them, and that the two subunits can act cooperatively to cleave a double helix.

INTRODUCTION

DNA topoisomerases are ubiquitous enzymes that regulate DNA topology (1). Type II DNA topoisomerases transiently cleave a DNA segment, catalyse the passage of a second DNA duplex through the double-stranded break and finally reseal strand cuts (1). Eukaryotic DNA topoisomerase II is required at mitosis for chromosome condensation and chromatid disjunction (2–6), may be involved in the removal of DNA supercoils (7) and, possibly, in the attachment of DNA loops to chromosomal scaffolds and nuclear matrix of interphase cells (8–10).

Mammalian topoisomerase II is the target of antitumor agents, such as anthracyclines, (11–14) that interfere with the breakage–reunion reaction of the enzyme apparently by stabilizing a DNA–enzyme complex in which DNA strands are broken and their 5′ termini covalently linked to the protein (1,12). The drug action thus results in DNA cleavage stimulation either in cultured tumor cells and with purified enzymes (1,11–14). These antitumor drugs stimulate DNA cleavage in a sequence selective manner yielding drug-specific cleavage intensity patterns in sequencing gels (12,13,15).

Cleavage consensus sequences were proposed for the bacterial and eukaryotic topoisomerase II (16–20), but they are only partially successful in the prediction of new cutting sites. Nevertheless, some knowledge was gained recently with the consensus sequence approach when drug-stimulated cleavage sites of murine topoisomerase II were clustered depending on the drug used and then analysed by statistical means (20). These investigations disclosed that specific nucleotides flanking the strand cut are required for drug stimulation of cleavage. The anthracyclines, doxorubicin and idarubicin, require an adenine at least at one of the two 3′ termini (positions −1) of a cleavage site (20). Different drugs highly prefer other specific bases immediately flanking the cleaved phosphodiester bond (21–24).Thus, drugs may form ternary complexes by directly interacting with these nucleotides at the DNA–protein interface,

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resulting in inhibition of DNA religation and in cleavage enhancement (15,20). This model is also supported by previous observations that a radiolabeled quinolone binds to DNA–gyrase complexes, but not to either component alone (25), and that mutant topoisomerases II selected for resistance to one compound are often resistant to other unrelated drugs (26–29).

Recently, we showed that oligonucleotides of 30–40 base pairs contain the same information of larger fragments for enzyme recognition of cleavage sites, because cleavage intensity patterns were similar in oligomers and in larger DNA fragments (23,30). This allows the study of drug sequence specificities with mutational analyses in short oligonucleotides (23,30,31). In previous studies, however, removing drug-required base pairs markedly reduced or even abolished cleavage stimulation by *m*AMSA and mitoxantrone (23,30), whereas VM-26 stimulation persisted (23). Since VM-26 is the least sequence selective drug, one should use thus the most sequence selective agent in a mutational study to identify the structural requirements of effective drug interactions.

Doxorubicin shows a high sequence selectivity in stimulating topoisomerase II DNA cleavage (20,24), nevertheless base requirements were shown to be at one position only (15,20). This contrasts with theoretical and chemico-physical studies on the sequence specificity of DNA–anthracycline interactions (32–36). In these works, anthracyclines were shown to prefer sites composed of three base pairs. Therefore, the present investigation was undertaken to define the complete base sequence preferred by anthracyclines and to elucidate aspects of the DNA cleavage–reunion reaction.

MATERIALS AND METHODS

Materials

Idarubicin was obtain from Pharmacia-Farmitalia (Milan), and freshly prepared in deionized water. DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures (37,38). Strand passing activity was determined with the P4 DNA unknotting assay, as described (37).

End labelling of DNA oligomers

DNA oligomers were synthesised with a 380B DNA synthesiser (Applied Biosystems, Milan, Italy), purified by gel electrophoresis, recovered by soaking gel slices in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, 0.1 % SDS. Their base sequences were confirmed by purine sequencing with the Maxam–Gilbert method (39). In order to obtain similar specific activity, in each experiment wild type and mutated strands were ^{32}P-labelled concurrently with T4 kinase (Life Technologies, Basel, Switzerland) and [γ-^{32}P] ATP (5000 Ci/mmol; Amersham, Milan, Italy) in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 1 mM spermidine for 60 min at 37°C. 1.5-fold higher amounts of unlabeled complementary strands were added to labelled strands in 10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA. Oligomers were then heated at 65°C for 5 min, slowly chilled at room temperature, ethanol precipitated, resuspended in deionized water and stored at −20°C.

Analysis of topoisomerase II DNA cleavage

Cleavage reactions were with 106 units of topoisomerase II in a volume of 20 μl in H buffer (10 mM Tris–HCl, pH 6.0, 10 mM $MgCl_2$, 50 mM KCl and 1 mM ATP) with or without drug at 37°C for 20 min. Experiments performed to evaluate salt effects were in L buffer (10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20 μg/ml BSA, 1 mM ATP) containing 10 or 100 mM NaCl. Reactions were stopped by adding 0.1 % SDS and 0.1 mg/ml proteinase K, and incubated at 42°C for 30 min.

DNA was then ethanol precipitated, resuspended in 2.5 μl of 80% formamide, 10 mM NaOH, 1 mM EDTA, and 0.1% dyes, heated at 95°C for 2 min, chilled on ice, and loaded onto a 17% polyacrylamide denaturing gel. Gels were run at 70 W for 2 hours, transferred to Whatmann 3MM sheets, dried, and autoradiographed with Amersham Hyperfilm MP. Cleavage sites were located by comparison to Maxam–Gilbert purine markers of the same oligomer. Since topoisomerase II DNA cleavage generates 3′-OH termini instead of the 3′-P ends generated by Maxam–Gilbert reactions, a shift of about 1.5 nucleotides toward higher molecular weights is expected for topoisomerase II cleavage products of 36-bp oligomers (40). The amount of cleaved DNA was determined by cutting out gel slices and scintillation counting (Kontron MR 300 β counter). For each site analyzed, the drug stimulative effect was measured as R defined by:

$$R = \frac{\text{cleaved DNA with drug}}{\text{\% cleaved DNA without drug}}$$

RESULTS

Sequence specific DNA cleavage with idarubicin in a 36-bp oligomer

The wild-type oligomer corresponded to SV40 DNA from genomic positions 4235 to 4270 (Figure 1), where major anthracycline-stimulated cleavage sites are present (20,41). DNA

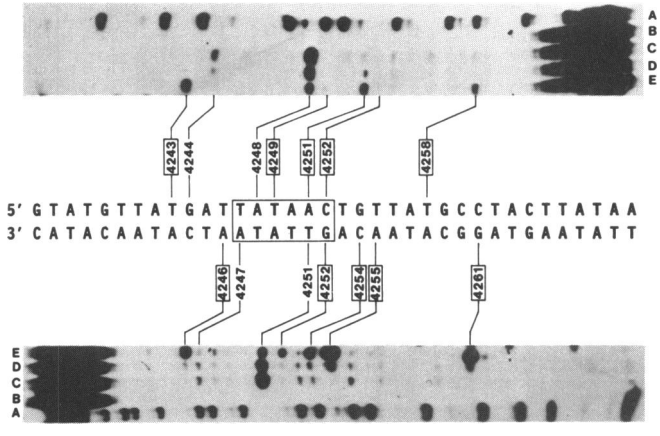

Figure 1. Topoisomerase II DNA cleavage with and without idarubicin in a 36-bp oligomer corresponding to SV40 DNA from nucleotides 4235 to 4270, shown in the middle. Top and bottom strands correspond to the coding strands of late and early messages, respectively. Upper panel (top strand): the run was from right to left; lanes are from top to bottom: purine markers; control DNA; topoisomerase II alone; with 0.1 and 1 μM idarubicin. Lower panel (bottom strand): the run was from left to right; lanes are from bottom to top: purine markers; control DNA; topoisomerase II alone; with 0.1 and 1 μM idarubicin. Numbers indicate the nucleotide at position +1; boxed numbers indicate drug-stimulated sites. The base pairs mutated in this work are boxed. Cleavage of the top strand at the 4252/4255 site is not detectable in this autoradiogram, but is easily observed in Figures 2–4.

cleavage was evaluated independently in the top and bottom strands corresponding to the coding strands of late and early messages of SV40, respectively. Since each strand break could be paired to a four-base staggered cut on the complementary strand, we indicate a cleavage site with the position numbers, with reference to the SV40 genome, of the two bases at the 5′ termini of the double-strand cut (Figure 1).

Topoisomerase II without drug introduced a strong cleavage at the 4248/4251 site of the wild type oligomer and minor cleavage at other sites (Figure 1). Idarubicin stimulated cleavage at sites 4243/4246, 4251/4254, 4252/4255, 4258/4261 and, weakly, at 4249/4252 in a dose-dependent manner (Figure 1), whereas cleavage at other sites was only reduced by the drug. These results are agreement with findings on SV40 DNA fragments (see Figure 2A in (20)), showing that the sequence specificity of anthracycline stimulation of DNA cleavage is conserved in short synthetic oligomers. Interestingly, drug stimulation at the 4251/4254 and 4252/4255 sites was greater at the top and bottom strands, respectively, showing a different strand preference of the drug effect at these two sites (Figures 1–4).

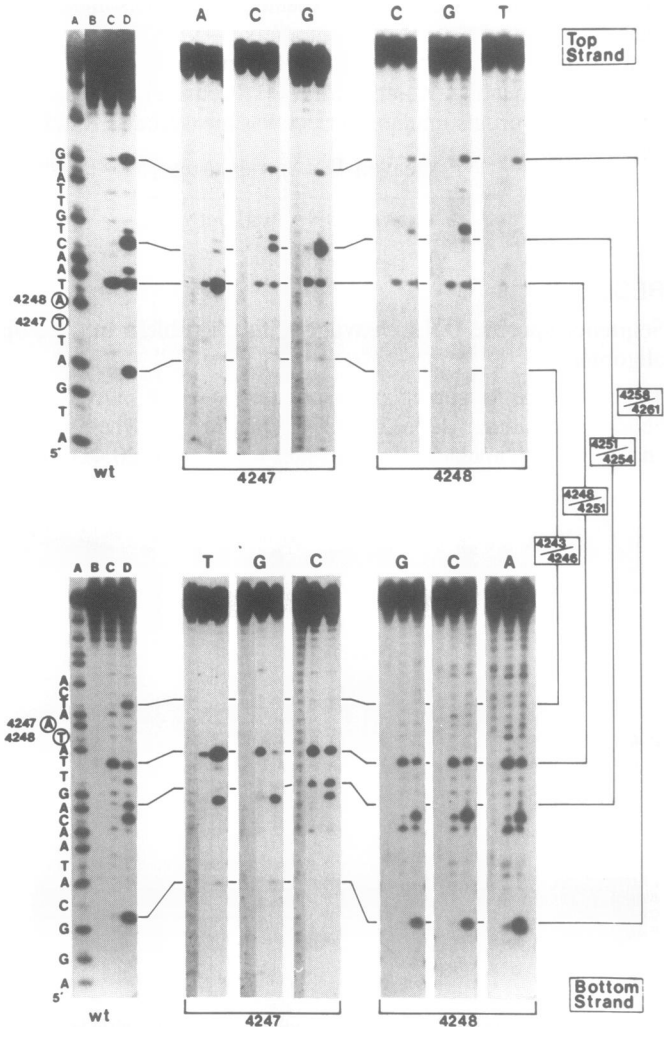

Figure 2. Topoisomerase II DNA cleavage in oligomers with base pair mutations at 4247 or 4248 (see the sequence in Figure 1). Upper and lower panels correspond to top and bottom strands, respectively. Numbers at the gel bottom indicate the position which was mutated; letters at the top of sets of three lanes indicate the base present at that position. wt indicates wild-type oligomer. For each set of three, lanes are from left to right: control DNA, no drug and 1 μM idarubicin, respectively. The purine marker lane is shown for wt oligomer; circles indicate mutated bases. Numbers on the right indicate cleavage sites selected to determine the degree of drug stimulation (see Figures 5–8).

Sites with adenines at −1 positions are low affinity sites for topoisomerase II

In this report, negative and positive numbers indicate nucleotides at the 3′ and 5′ termini of the top strand break with respect to the top strand sequence (...,−2,−1,+1,+2,...) and of the bottom strand break with respect to the bottom strand sequence (...,−2′,−1′,+1′,+2′,...). Topoisomerase II without drug was able to cleave DNA oligomers at sites that were stimulated by idarubicin and had adenines at positions −1 (Figure 1), and that were not detected in larger SV40 DNA fragments (20). In contrast to these early studies, in the experiment shown in Figure 1 the DNA oligomer was reacted with the enzyme in a buffer (H) that favored enzyme binding to DNA (Table I) (42). Therefore, to understand whether experimental conditions might influence the enzyme site selectivity, we compared H buffer to L buffer that contained either 10 or 100 mM NaCl. Levels of DNA cleavage were globally 2- to 5-fold higher in H buffer than in L buffer with 100 mM NaCl (Table I). In this case, DNA cleavage was not detectable at sites with adenines at positions −1 such as 4251/4254 and 4258/4261 (Table I and not shown). DNA cleavage levels were appreciable at these sites also in L buffer by lowering the NaCl concentration to 10 mM. However, drug stimulative effects were always observed at the same sites (Table I and not shown). Therefore, the results suggest that sites having adenines at positions −1 may be low-affinity sites for enzyme binding to and/or cleavage of DNA.

Effects of single base-pair mutations on DNA cleavage intensity patterns

In the remaining part of this study, we used the experimental conditions of Figure 1, since: i) fractions of cleaved DNA oligomers were higher and then more easily determined and ii) the sequence specificity of drug stimulation was the same. Then, drug stimulation of DNA cleavage was investigated in synthetic oligomers carrying single base-pair mutations from nucleotides 4247 to 4252 (shown in a box in Figure 1).

Table I. Cleavage amounts of wild type DNA oligomer in different buffers[a].

Site	H buffer[b]		L buffer[c]			
			10 mM NaCl		100 mM NaCl	
	−	+	−	+	−	+
4248/4251	2.5	1.0	2.7	0.8	0.5	0.3
4251/4254	0.3	2.5	0.4	1.4	nd[d]	0.4
4258/4261	0.3	1.5	0.3	1.2	nd	0.5

[a]The numbers are percentages of the cleaved top strand at the indicated sites, and are mean values of 2 to 3 independent experiments. Cleavage reactions were without (−) or with (+) 1 μM idarubicin.
[b]H buffer: 10 mM Tris−HCl, pH 6.0, 10 mM $MgCl_2$, 50 mM KCl, and 1 mM ATP.
[c]L buffer: 10 mM Tris−HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20 μg/ml BSA, 1 mM ATP.
[d]nd, not detectable.

4247 and 4248 mutations. The largest effects of mutations at 4247 were on the 4243/4246 and 4248/4251 sites (Figure 2). At the former site drug stimulation was always abolished by mutations, and at the last site drug stimulation was only observed with the A/T base pair (Figure 2). The 4247 base pair corresponded to $-1'$ and -1 positions for these cleavage sites, respectively. Moreover, at the 4251/4254 site a G/C base pair at 4247, corresponding to the -4 position, increased cleavage without drug (Figure 2). Mutations of the 4248 base pair abolished idarubicin stimulation of DNA cleavage at the 4243/4246 site and markedly reduced cleavage stimulation at 4251/4254 (Figure 2). The 4248 base pair correspoded to $-2'$ and -3 for these cleavage sites, respectively. Moreover, DNA cleavage level at 4252 in the top strand was higher when a guanine was at 4248, corresponding to -4 position (Figure 2). The minor drug stimulation of the 4249/4252 site was also abolished by mutations of the 4247 and 4248 base pairs (-2 and -1 positions, respectively). DNA cleavage the 4261 nucleotide (lower strand) was reduced, but drug stimulation was not abolished at this site (Figure 2).

4249 and 4250 mutations. Cleavage at the 4251/4254 site was hardly visible in oligomers bearing mutations at 4249 (Figure 3), which corresponded to its -2 position. Cleavage was also markedly reduced at the 4243/4246 site, and some DNA cleavage with drug could be observed in the top strand only in autoradiograms exposed longer than that shown in Figure 3. A much stronger cleavage without drug was observed at the 4253/4256 site when a guanine was at 4249, which corresponded to its -4 position (Figure 3). In contrast to mutations at 4247, 4248 and 4249, mutations at 4250 did not reduced drug stimulation at the 4243/4246 site (Figure 3). However, they had a marked effect at the 4251/4254 site: this was much more cleaved by the enzyme without drug, whereas no drug stimulation was observed (Figure 3). The 4250 nucleotide corresponded to the -1 position of the 4251/4254 site. Cleavage of the top strand at the 4252/4255 site was increased when a thymine or guanine was present at its -2 position (4250 lanes, Figure 3). Mutations at 4250 that provided a guanine at positions -4 and $4'$ of the 4254/4257 and 4243/4246 sites, respectively, allowed a stronger cleavage without drug at these sites (Figure 3).

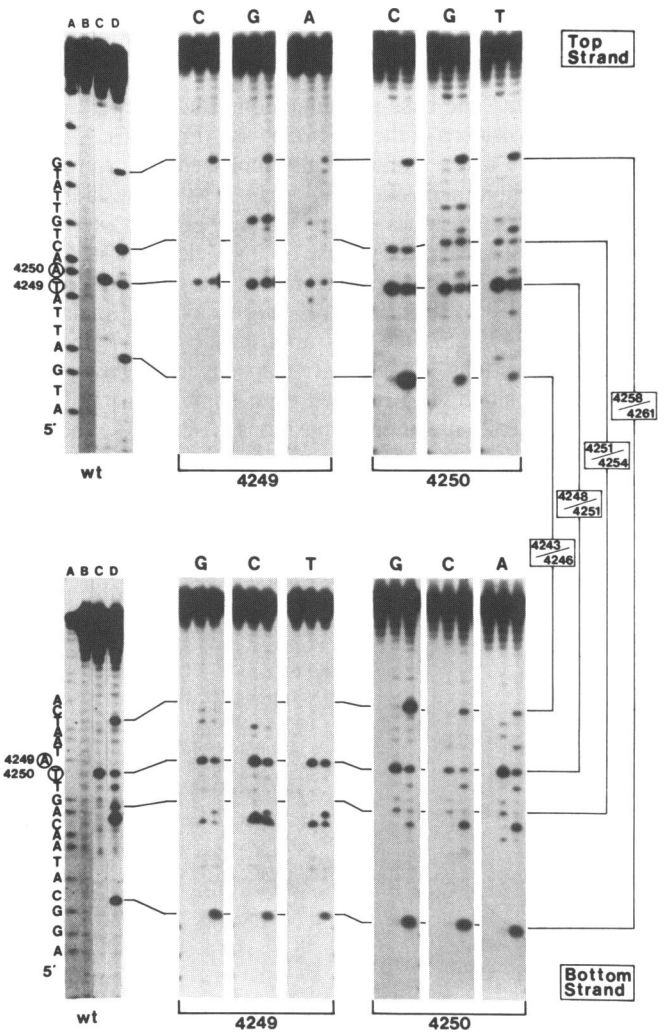

Figure 3. Topoisomerase II DNA cleavage in oligomers with base pair mutations at 4249 or 4250 (see the sequence in Figure 1). See legend to Figure 2 for further details.

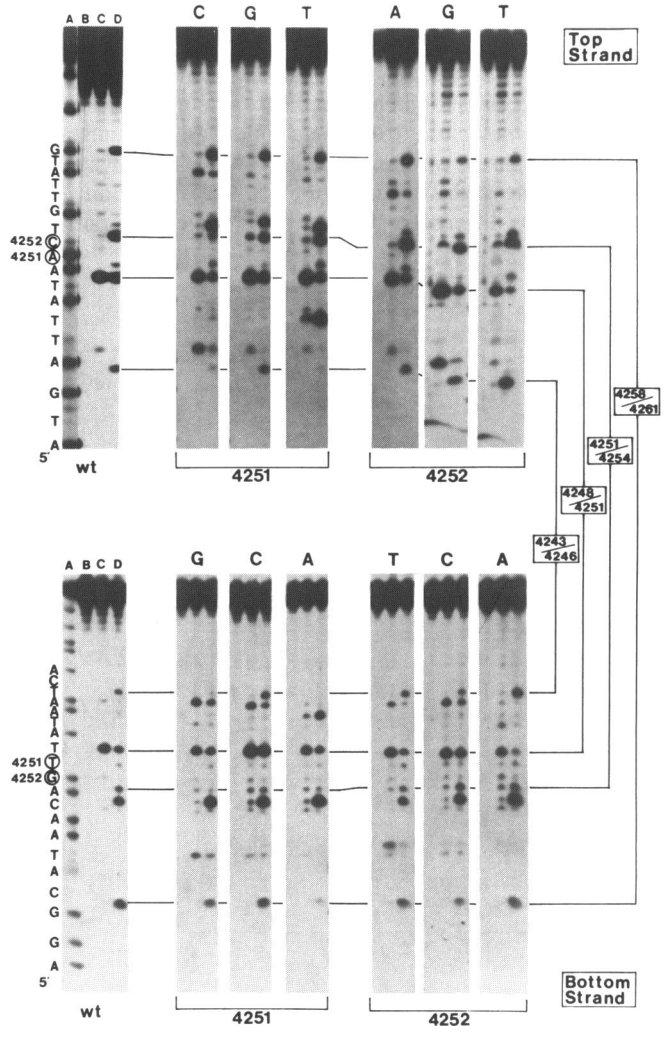

Figure 4. Topoisomerase II DNA cleavage in oligomers with base pair mutations at 4251 or 4252 (see the sequence in Figure 1). See legend to Figure 2 for further details.

We also studied the effect of an inosine at 4250 in the top strand paired to a cytosine in the bottom strand. Inosine base has a hydroxyl group at C6 of the purine ring instead of an amino group of adenines. Drug stimulation was not observed at the 4251/4254 site, whereas it was present at the 4252/4255 site (not shown).

4251 and 4252 mutations. With base mutations at 4251 and 4252, cleavage stimulation at the 4243/4246 site was partially influenced but never abolished (Figure 4). Mutations at 4252 were without influence at 4251/4254 and 4252/4255 sites. However, removing the 4251 adenine slightly decreased drug stimulation of the bottom strand cleavage at the 4251/4254 site, whereas drug stimulation was still present at the 4252/4255 site (Figure 4). For this site, the 4251 base corresponded to the −1 position. Interestingly, cleavage stimulation by idarubicin was observed at the 4245/4248 site when an adenine was at its −3′ position (see mutations at 4251 in Figure 3). It should be noted that this site has an adenine at position −1′, but not −1. Other main modulations of DNA cleavage without drug were at: i) 4256/4259, cleavage was favored by a cytosine at 4251, which corresponded to its position −5; ii) 4255/4258, an adenine, instead of a cytosine, at 4252 allowed a higher cleavage level (4252 corresponded to its position −3) (Figure 4).

Altogether, these observations showed that single base mutations affected the drug stimulative action depending on the identity and position of the mutated base: the influence was large on cleavage sites close 1 to 5 nucleotides to the mutated base pair. The apparent strong reduction of DNA cleavage at the 4258/4261 site with base mutations at 4247 (position −11) is in part due to differences in exposure time of autoradiograms in Figure 2. Moreover, for each mutated nucleotide a cleavage site was always increased without drugs when a guanine was at its −4 or −4′ position.

Base mutations effects on the degree of DNA cleavage stimulation by idarubicin

The degree of drug stimulation was also determined and expressed as the ratio (R) of the fraction of cleaved DNA with the drug over that without drugs of three drug-stimulated sites, 4243/4246, 4251/4254 and 4258/4261, and the 4248/4251 site, not stimulated by idarubicin in the wild-type oligomer (Figures 5−8).

4243/4246 site. The mean cleavage stimulation by idarubicin was higher in the top than in the bottom strands (R=36.4 and 11, respectively) (Figure 5). Cleavage stimulation was abolished in both strands when either the adenine at position −1′ or the thymine at position −2′ was replaced by either one of other bases. A slight stimulation was detected only in the bottom strand with a cytosine at position −2′ (Figure 5). The absence of cleavage stimulation was invariably accompanied by cleavage suppression. Interestingly, cleavage stimulation was always observed with any base at positions −3′ to −6′ with the exception of a cytosine at −3′ (Figure 5). A guanosine at the −4′ position favored cleavage preferentially at the bottom strand.

4251/4254 site. The base pair mutations studied corresponded to −4 to +2 positions of the 4251/4254 site (Figure 6). Mutations at positions −1 and −2 dramatically abolished drug stimulation, whereas mutations at positions −4 and +2 still allowed the drug action (Figure 6). Positions −3 and +1 may be in an intermediate situation, since a marked decrease of DNA cleavage stimulation was observed in the mutated oligomers, but without a complete lost of it (Figure 6). Consistently, drug cleavage stimulation at the minor 4249/4252 site was abolished by 4247 and 4248 mutations (positions −2 and −1, respectively), whereas 4249 mutations (position +1) reduced drug stimulation, and 4250, 4251 and 4252 mutations (positions +2, +3 and +4, respectively) did not alter drug effects (Figures 1−4).

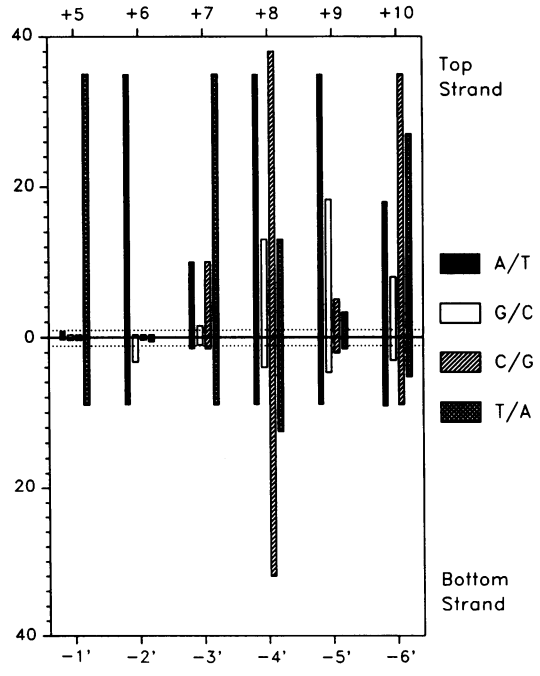

Figure 5. Base mutation effects on idarubicin stimulation of DNA cleavage at the 4243/4246 site. Panel above zero line, top strand; panel below zero line, bottom strand. Numbers in X-axis indicate the positions of mutated bases relative to this site in the two strands. The Y-axis represents the ratio R between the fraction of cleaved DNA without drug over that with 1 μM idarubicin. The dashed line corresponds to R = 1 (no drug effect). R > 1 indicates cleavage stimulation.

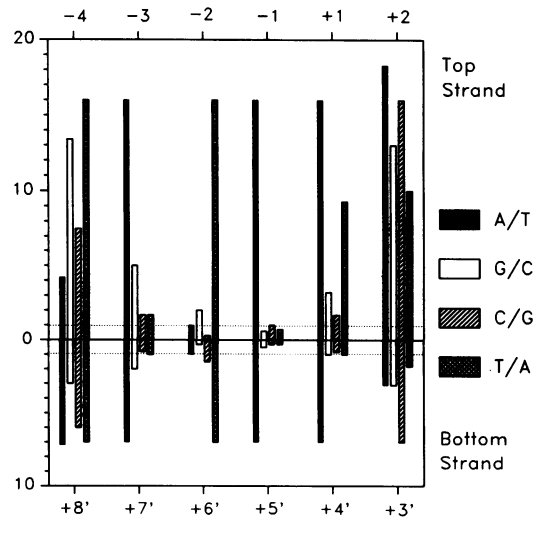

Figure 6. Base mutation effects on idarubicin stimulation of DNA cleavage at the 4251/4254 site. See legend to Figure 5 for further details.

4258/4261 site. R values for the 4258/4261 site, relative to which base pair mutations were at positions −11 to −6, showed that drug cleavage stimulation was never abolished in all the mutated oligomers (Figure 7). Some differences in R values could be observed: one of the lowest stimulation was observed when an adenine was at position −11, which corresponded to an adenine at position −1 of the 4248/4251 site. Thus, the reduced stimulation at the 4258/4261 site might be due to competition with the major 4248/4251 site, markedly drug-stimulated with that particular base mutation.

4248/4251 site. The drug suppressive effect at the major 4248/4251 site was the same regardless of base mutation, indicating a lack of influence of DNA sequence on the cleavage suppression by anthracyclines (Figures 1−4 and 8). Only when an adenine was present at position −1 (Figure 8), drug-stimulated DNA cleavage (R = 13) was observed in both strands. Interestingly, when an adenine was at the analogous position −1′, drug cleavage stimulation was not detected (Figures 4 and 8). Thus, the adenine requirement at −1 positions (20) was sufficient for cleavage stimulation by idarubicin when it was in the top, but not in the bottom strands.

Drug stimulation in a oligomer bearing two base pair mutations

Since both an adenine at positions −1 and a thymine at positions −2 are critical for drug stimulation of the 4249/4252, 4243/4246 and 4251/4254 sites (Figures 5 and 6), we noticed that in the case of the 4248/4251 site this condition is satisfied only with the adenine mutation at 4247 (position −1), but not with that at 4252 (position −1′; see the base sequence in Figure 1). Therefore, we studied the effects of a double mutation at 4252 and 4253 positions, where the dinucleotide 5′-CpT-3′ was replaced by 5′-TpA-3′ in the top strand. These mutations corresponded to an A at −1′ and a T at −2′ positions of the 4248/4251 site. Surprisingly, the overall cleavage pattern was not dramatically changed (Figure 9), although novel effects could be observed. A) Cleavage at the 4248/4251 site was not stimulated by the drug, however neither it was suppressed (Figure 9). Since DNA cleavage in the presence of anthracycline is a balance between suppressive and stimulative effects (14,41) and the absence of cleavage stimulation was always coupled to cleavage suppression at the 4248/4251 site (Figure 8), this observation suggested that a low degree of drug stimulation is present in this case (Figure 9). Nevertheless, we could not achieve the strong stimulation observed with an adenine at position −1 (Figure 2, left panels). B) The drug stimulated cleavage at a new

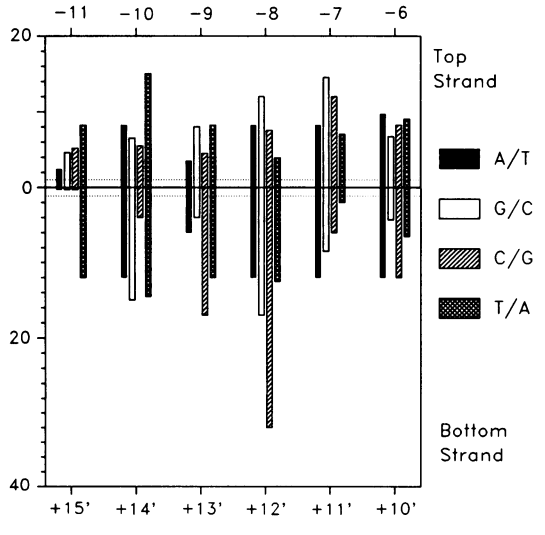

Figure 7. Base mutation effects on idarubicin stimulation of DNA cleavage at the 4251/4254 site. See legend to Figure 5 for further details. It has to be noted that R values for the bottom strand in the case of base mutations at 4247 (position −1′) was not determined and is then not reported.

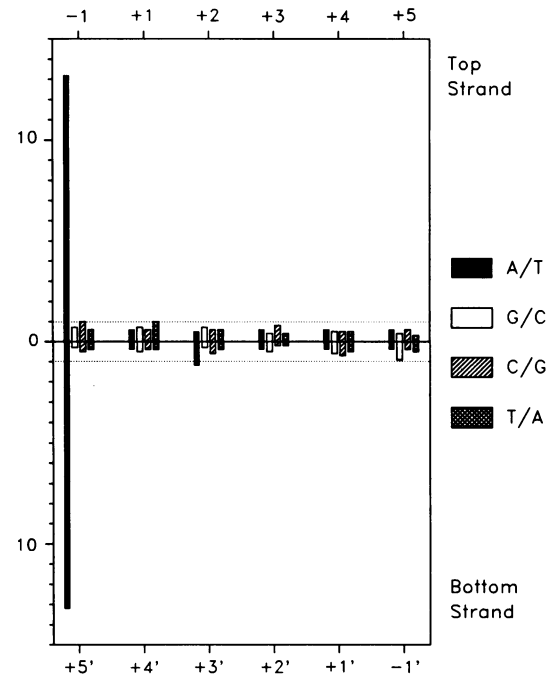

Figure 8. Base mutation effects on idarubicin stimulation of DNA cleavage at the 4251/4254 site. See legend to Figure 5 for further details.

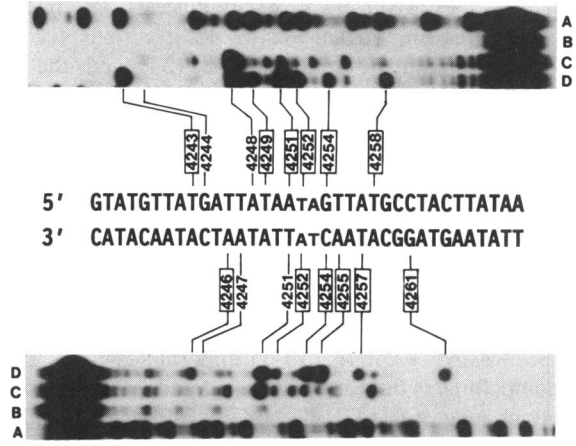

Figure 9. DNA cleavage with and without idarubicin in a oligomer bearing a double mutation, as indicated by lower case letters. See legend to Figure 1 for further details.

site, 4254/4257, which gained an adenine and a thymine at its −1 and −2 positions, respectively (Figure 9).

DISCUSSION

By using synthetic DNA oligomers bearing single base-pair mutations, we have directly demonstrated that important sequence determinants governing locally DNA cleavage by mammalian topoisomerase II are at the breaksite itself. The major sequence determinants are few base pairs (up to 5) apart from to the strand cut, nevertheless our present data also demonstrate that complex DNA sequence−enzyme interactions control DNA cleavage (see below). Our present findings are consistent with results obtained with the consensus sequence approach (18−20), and are in agreement with similar findings obtained with the bacterial topoisomerase II, DNA gyrase (43) and with the bacteriophage T4 topoisomerase II (31).

Anthracycline sequence specificity

The studied base mutations were very close to several cleavage sites. It is somewhat surprising that although competition among sites could affect cleavage efficiency, nevertheless mutation effects were rather consistent among several sites. This was due, at least in part, to the high sequence selectivity of idarubicin.

The present results provide new and independent evidence that idarubicin has the primary requirement of an adenine at position −1 and a thymine at position −2, whereas no drug-preferred bases are observed at positions +2 and from −11 to −4 positions. Positions −3 and +1 may significantly modulate drug stimulation when drug-required bases are present in the same strand, with adenines preferred at these positions. Thus, the complete sequence preferred by idarubicin at the cleavage site is: *5′*-(A)TA/(A)-*3′* from −3 to +1 positions with the slash and parenthesis indicating the strand cut and conditioned preference, respectively.

A marked preference for thymines at position −2 was also detected in a set of 97 cleavage sites in SV40 (20), and this preference was even more evident when considering only sites most strongly stimulated by anthracyclines (unpublished data). Therefore, the local base sequence preferences of topoisomerase II-trapping drugs may extend beyond the −1/+1 position range, although primary requirements are at these two positions. Indeed, the sequence *5′*-AY/NG-*3′* (Y, pyrimidine; N, any base) was shown to be required from −2 to +2 positions for strong cleavage stimulation by mitoxantrone (23) and the triplet *5′*-AT/T-*3′* from −2 to +1 positions is highly preferred at both strands by genistein for strong cleavage stimulation (24). Therefore, drug-induced stabilization of ternary complexes probably involves more than one base pair at the cleavage site.

High and low affinity sites for topoisomerase II

The present results also demonstrate that two positions are critical for DNA cleavage without drugs: −4 and −1 positions, being guanines preferred and adenines excluded, respectively. Positions −5, −3, −2 and +1 may also be important but our data are not as compelling as they are for the −4 and −1 positions. The observations suggest that DNA−protein contacts involving these bases or, alternatively, base-dependent local helix structural variety, are particularly critical for site recognition. Close contacts between topoisomerase II and −1 position base seem crucial since the −1 base must be held in the right position for efficient break religation.

Sites with adenines at positions −1 likely represent low affinity sites for mammalian DNA topoisomerase II, since cleavage can be detected at those sites only when using conditions that favor enzyme binding to DNA and removing the −1 adenine at the 4251/4254 site markedly increased DNA cleavage without drugs. Therefore, this finding provides some explanation of why anthracycline-stimulated cleavage sites were not detected without drugs in a previous work (20), and is consistent with a sequence of molecular events during which topoisomerase II itself selects sites for cleavage first, and then idarubicin or other drugs bind to the pre-formed, transient binary complex DNA−enzyme.

Subunit cooperativity in the DNA cleavage−reunion reaction

A key finding of the present work is a heterogeneity of response to base mutations among some sites (4243/4246, 4249/4252 and 4251/4254 vs. 4252/4255) or between the two strands at the 4248/4251 cleavage site.

Removal of either the adenine or the thymine at positions −1 or −2, respectively, abolished drug stimulation of DNA cleavage at both strands of 4243/4246, 4249/4252 and 4251/4254 sites, whereas it did not at the 4252/4255 site. It is interesting that although idarubicin stimulated strand breaks at sites always paired with a four-base stagger, however, relative cleavage intensities were not similar for the corresponding cuts in the two strands at the 4251/4254 and 4252/4255 sites. The former site was more stimulated by the drug at the top strand than at the bottom one, whereas the last site was more stimulated at the bottom than at the top strands (Figures 1−4). A close examination of the oligomer base sequence reveals that at both cleavage sites drug stimulation was higher at the strand with the tetramer *5′*-ATA/A-*3′* from −3 to +1 positions (the complete idarubicin preferred sequence), whereas the corresponding weaker cleavage was at the tetramer *5′*-TAA/C-*3′* from −3 to +1 positions. The studied base mutations alter the two tetramers that are more and less cleaved at the 4251/4254 and 4252/4255 sites, respectively. Therefore, these observations altogether suggest that one of the two topoisomerase II subunit is mainly driving the cleavage from the strand that fulfills the complete sequence preferred by the drug, and that only mutations changing the drug-preferred sequence can abolish idarubicin stimulation of cleavage, whereas mutations at the other strand have minor effects.

A striking and interesting finding was also obtained at the 4248/4251 site. An adenine at its −1 position allowed drug cleavage stimulation at both strand cuts, whereas an adenine at −1′ position did not at either strand cuts (Figure 8). In the former case, the *5′*-ATA/A-*3′* tetramer was from −3 to +1 positions, whereas in the last case the tetramer *5′*-CAA/T-*3′* was from −3′ to +1′ positions. Only the former corresponds to the tetramer preferred by idarubicin. When the sequence *5′*-CTA/T-*3′* was present at the bottom strand, in the oligomer bearing two base-pair mutations, idarubicin had a cleavage stimulative effect at either strands since no suppression was observed (Figure 9; see sequence in Figure 1). These results, thus, strongly indicate that a drug molecule can effectively interact with and trap an enzyme subunit at the strand where the drug-preferred bases are present, and then force the other subunit to cleave the other strand even if drug-preferred bases are not present.

Recently, Roca and Wang (44) proposed an ATP-dependent protein clamp model for topoisomerase II: a DNA-bound dimeric protein can be in an open conformation, and upon ATP binding closes the clamp trapping the passing DNA duplex. In this model, cooperativity between the two subunits appears crucial for

efficient strand passing activity. Indeed, Lindsley and Wang (45) demonstrated that ATP binding to topoisomerase II is cooperative when the enzyme is bound to the DNA; moreover, the binding of one molecule of ATP to one enzyme subunit can induce a conformational change in the entire molecule, thus showing cooperativity between the two polypeptides (46,47). Our present data strongly suggest that DNA recognition by topoisomerase II may occur at one or the other strand and not necessarily at both of them, and that subunit cooperativity also occurs at the DNA cleavage step.

Therefore, an enzyme subunit may cleave a DNA strand due to cooperative effects rather than to its own recognition of the cleavage site. In this last case, base mutations at the strand recognized by the enzyme would have more pronounced effects than those at the other strand. Since different sites (and even the two halves of a site) can have different responses to the same point mutation, thus one should caution in generalizing mutational analysis results obtained with only one particular site.

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