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In the presence of subunit A inhibitors DNA gyrase cleaves DNA fragments as short as 20 bp at specific sites

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ABSTRACT

A key step in the supercoiling reaction is the DNA gyrase-mediated cleavage and religation step of double-stranded DNA. Footprinting studies suggest that the DNA gyrase binding site is 100–150 bp long and that the DNA is wrapped around the enzyme with the cleavage site located near the center of the fragment. Subunit A inhibitors interrupt this cleavage and resealing cycle and result in cleavage occurring at preferred sites. We have been able to show that even a 30 bp DNA fragment containing a 20 bp preferred cleavage sequence from the pBR322 plasmid was a substrate for the DNA gyrase-mediated cleavage reaction in the presence of inhibitors. This DNA fragment was cleaved, although with reduced efficiency, at the same sites as a 122 bp DNA fragment. A 20 bp DNA fragment was cleaved with low efficiency at one of these sites and a 10 bp DNA fragment was no longer a substrate. We therefore propose that subunit A inhibitors interact with DNA at inhibitor-specific positions, thus determining cleavage sites by forming ternary complexes between DNA, inhibitors and DNA gyrase.

INTRODUCTION

DNA gyrase (EC 5.99.1.3), a prokaryotic topoisomerase II enzyme, consists of two subunits, A and B, and the active enzyme is an A_2B_2 tetrameric complex (reviewed in 1–5). The enzyme can introduce negative supercoils into DNA using the free energy derived from ATP hydrolysis. Footprinting studies have shown that DNA gyrase protects ~100–150 bp of DNA from nuclease attack, with a most strongly protected central region of ~40–50 bp (6–9). The DNA is wrapped around the tetrameric protein in a single positive supercoil. After binding, DNA gyrase cleaves each strand at sites separated by 4 bp and forms a covalent phosphotyrosine bond between the 5′-phosphate groups of the cleaved DNA and a tyrosine (Tyr122 in *Escherichia coli*) of the A subunits (10,11). A segment of DNA is translocated through the break and presumably through the protein complex and the broken phosphodiester bonds are resealed. Binding of DNA gyrase to DNA is probably sufficiently stable to allow processive supercoiling before the enzyme dissociates from the DNA (12). At some point in the reaction an ATP molecule binds to each B subunit and hydrolysis of ATP is required for further catalytic cycles (13–15). Binding of ATP promotes a conformational change of the tetramer and it is thought that this change brings the DNA segment to be translocated into near proximity to the double-stranded DNA break (8).

A key step in the supercoiling reaction is DNA gyrase-mediated cleavage of DNA and it has been shown that both classes of subunit A inhibitors, the quinolones and the pyrimido[1,6-*a*] benzimidazoles, interrupt the cleavage and resealing cycle at the cleavage step (16–18). Cleavage in the presence of these inhibitors does not require ATP, occurs at preferred sites and it is assumed that these sites represent the physiological sites of action of DNA gyrase (6,19–22). *In vivo* analysis of cleavage sites and their flanking regions in the pBR322 plasmid generated in the presence of the quinolone oxolinic acid has suggested a consensus sequence (shown below) where R = purine, Y = pyrimidine, N = any nucleotide; T and G are equally preferred at the position 13 of the consensus sequence and the G and T in brackets are preferred secondarily to T and G respectively.

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          [G]     G    [T]
5′-RNNNRNRT↓GRYCTYNYNGNY-3′ consensus sequence
5′-GGCTGGAT↓GGCCTTCCCCAT-3′ preferred cleavage site
            ●
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DNA cleavage occurs at the site indicated by the arrow and also shown is the major cleavage site (between thymidine and guanosine) at position 990 (black dot) on this plasmid and the surrounding 20 bp sequence (19). The mismatch between the preferred cleavage site and the consensus sequence is underlined. It has been shown that a 34 bp DNA fragment containing this 20 bp cleavage sequence is not a substrate for the cleavage reaction in the presence of oxolinic acid (23). However, we and others could show that fragments of 70 bp or longer containing this 20 bp sequence at different positions were accepted as substrate and that these DNA fragments were positioned onto the enzyme in such a way that cleavage occurred at the expected site (18,23). Therefore, both the 20 bp cleavage sequence and the length of the flanking DNA on either one side of the cleavage site seem to be critical for cleavage reactions carried out in the presence of inhibitors. To further investigate the role of the 20 bp cleavage sequence and the relevance of the length of the flanking regions we have examined the DNA gyrase-mediated cleavage reaction in the presence of subunit A inhibitors with DNA fragments of 10–122 bp each containing the 20 bp cleavage sequence or part of it.

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MATERIAL AND METHODS

Enzymes for cloning, isolation and labeling of DNA fragments were purchased from Boehringer and the methods used were essentially as previously described (24).

Cloning, isolation and labeling of DNA fragments

A gel-purified *Bgl*I–*Bst*NI DNA fragment from position 933 to 1061 from the pBR322 plasmid was blunt-ended with the Klenow enzyme, *Eco*RI linkers were attached and the resulting *Eco*RI-digested 140 bp fragment ligated into the desphosphorylated *Eco*RI site of pUC18. The orientation of the inserted fragment was determined by restriction enzyme analysis and is shown in Figure 1. For the construction of deletions, the plasmid was digested with *Bam*HI and *Sph*I, the recessed 3′-termini of the *Bam*HI site were removed by incubation of 10 µg DNA with 20 U exonuclease III for 2–5 min at 37°C and the DNA was ethanol precipitated and dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Single-stranded DNA was digested with S1 nuclease, the plasmids were blunt-ended with Klenow enzyme and ligated with T4 DNA ligase. Competent *E.coli* HB101 cells were prepared by the calcium chloride method and transformed with the constructs described above. Plasmid DNA from the transformants was prepared and the DNA sequenced using Sequenase (US Biochemicals) and the primer 5′-CAGGAAACA-GCTATGAC-3′. The resulting DNA fragments are shown in Figure 1A.

*Pvu*II–*Hin*dIII fragments from the above-mentioned constructs were isolated from agarose gels, dephosphorylated with calf intestinal alkaline phosphatase, labeled at the 5′-ends with [γ-^{32}P]ATP with polynucleotide kinase and purified by centrifugation through a Sephadex G-50 spun column. The fragments were digested with *Msp*I, *Hin*fI or *Eco*RI, separated on a 10% acrylamide gel and the labeled fragments eluted from gel slices by a 2-fold incubation in 0.5 M ammonium acetate, pH 8.0, 1 mM EDTA at 37°C.

Oligonucleotides (Genosys) of 30, 20 and 10 bp, as shown in Figure 1B, were labeled at the 5′-ends of the top strand (marked *) with [γ-^{32}P]ATP and polynucleotide kinase and, after heating at 100°C for 2 min, annealed to the complementary unlabeled oligonucleotides for 35 min at 37°C, purified on a 20% acrylamide gel and eluted from gel slices as described above.

Expression and purification of the A and B subunits of DNA gyrase

The A and B subunits of DNA gyrase were expressed and purified as previously described (18). For some experiments the subunits were further purified on a novobiocin–Sepharose column. Novobiocin–Sepharose was prepared as described (14). After loading of the subunit A fraction, the column was washed with TGED buffer [50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 10% (w/v) glycerol] and the protein eluted with 1 M NaCl in TGED. After loading of the subunit B fraction, the column was washed with TGED, 1 M NaCl and 2 M urea in TGED and the protein eluted with 6 M guanidine hydrochloride. The B subunit was then renatured by dialysis against 50 mM Tris–HCl, pH 8.0, 100 mM KCl, 5 mM DTT, 1 mM EDTA, 10% (w/v) glycerol.

Stock solutions of inhibitors

Stock solutions of the subunit A inhibitors (Table 1) at 1 mM were made as follows. Fleroxacin was dissolved in 25% ethanol and 15 mM NaOH, ciprofloxacin in H_2O and Ro 46-2825, Ro 46-6962, Ro 46-7864 and Ro 47-3359 in 5 mM HCl. The stock solutions were diluted with H_2O and added to the reaction mixtures as indicated in the figures.

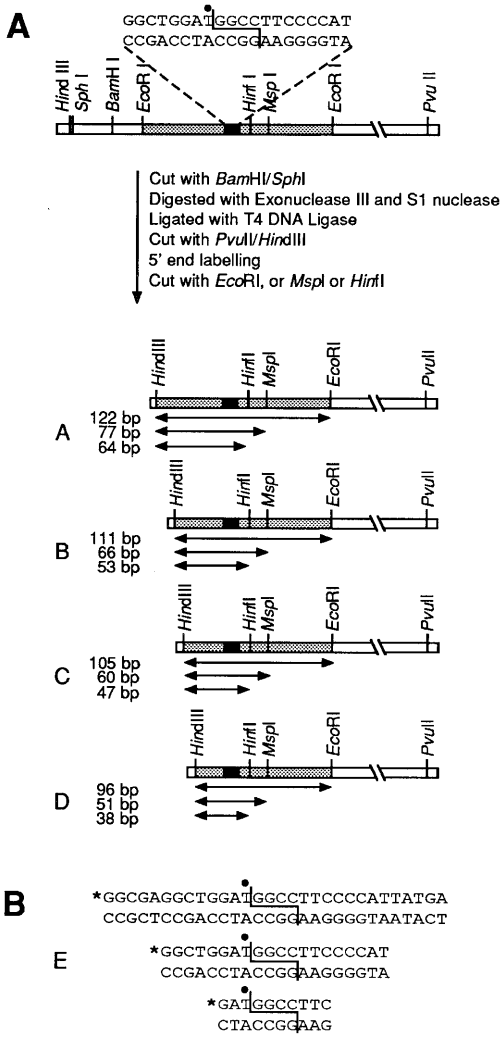
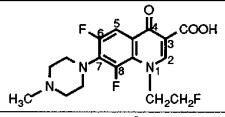
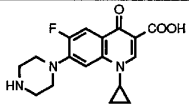
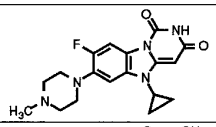
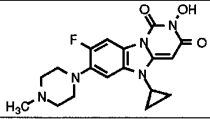
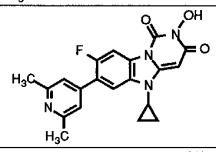
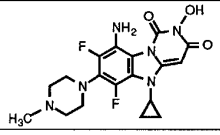


Figure 1. Cloning and isolation of the DNA fragments. (**A**) The *Bgl*I–*Bst*NI DNA fragment from position 933 to 1061 from the pBR322 plasmid was blunt-ended, *Eco*RI linkers were attached and the resulting *Eco*RI-digested 140 bp fragment ligated into the desphosphorylated *Eco*RI site of pUC18. The plasmid was digested with *Bam*HI and *Sph*I, the recessed 3′-termini of the *Bam*HI site were removed by incubation with exonuclease III and single-stranded DNA digested with S1 nuclease. The resulting plasmids were blunt-ended and religated. Competent *E.coli* HB101 cells were transformed with the above described constructs. Plasmid DNA from the transformants was prepared and sequenced using the primer 5′-CAGGAAACAGCTATGAC-3′. *Pvu*II–*Hin*dIII fragments were isolated from agarose gels and labeled at the 5′-ends with [γ-^{32}P]ATP. The fragments were digested with *Msp*I, *Hin*fI or *Eco*RI and the labeled fragments gel purified. The 140 bp pBR322 sequence is shown as a shadowed box and the cleavage sequence where DNA gyrase cleaves preferentially in the presence of subunit A inhibitors is shown as a black box. To keep the original orientation of the pBR322 plasmid, the pUC18 plasmid map is reversed. (**B**) Oligonucleotides of 30, 20 and 10 bp were labeled at the 5′-end of the top strand with [γ-^{32}P]ATP (marked *), annealed to the complementary unlabeled oligonucleotides and purified by electrophoresis on polyacrylamide gels. The lines denote the earlier reported 4 bp staggered cleavage site and the thymidine at position 990 from the pBR322 plasmid is marked with a black dot.

Table 1. Structures of quinolones and pyrimido[1,6-*a*] benzimidazoles

Compound	Structure
Fleroxacin	
Ciprofloxacin	
Ro 46-6962	
Ro 46-2825	
Ro 47-3359	
Ro 46-7864	

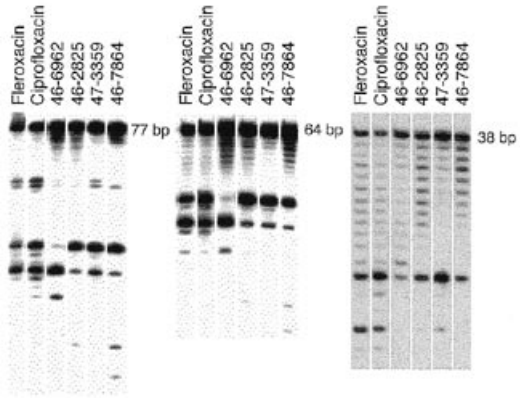


Figure 2. Determination of the cleavage sites on the 77, 64 and 38 bp DNA fragments generated by DNA gyrase in the presence of quinolones and pyrimido[1,6-*a*]benzimidazoles. The DNA fragments, labeled at the 5′-ends of the top strand with [γ-^{32}P]ATP, were incubated at an estimated concentration of 20–40 fmol at 37°C for 60 min in a total volume of 20 µl with ~2.5 pmol DNA gyrase in the presence of 100 µM subunit A inhibitors in 35 mM Tris–HCl, pH 8.0, 24 mM KCl, 2 mM spermidine and 4 mM $MgCl_2$. Reactions were stopped by the addition of SDS to a final concentration of 1% and the enzyme was digested for 2 h at 37°C with proteinase K at a final concentration of 500 µg/ml. The DNA was purified and redissolved in 5 µl TE, pH 8.0. 5 µl loading buffer were added, the samples heated for 4 min at 90°C, chilled on ice, loaded on a 12% sequencing gel containing 7 M urea and electrophoresed in 1× TBE buffer. Reaction products were visualized by autoradiography and scanned with a BioRad G-700 imaging densitometer.

Analysis of cleavage products on sequencing gels

The DNA fragments, labeled with [γ-^{32}P]ATP at the 5′-ends of the top strand, were incubated at an estimated concentration of 20–40 fmol at 37°C for up to 120 min in a total volume of 20 µl with ~2.5 pmol DNA gyrase in 35 mM Tris–HCl, pH 8.0, 24 mM KCl and 2 mM spermidine. $MgCl_2$ was added at a concentration of 4 mM and the inhibitor at the concentrations indicated in the figures (up to 500 µM). Reactions were stopped by the addition of SDS to a final concentration of 1%. Proteinase K was added to a final concentration of 500 µg/ml and the samples digested for 2 h at 37°C. The DNA was purified by phenol/chloroform extraction and ethanol precipitation and redissolved in 5 µl TE, pH 8.0. Five microliters loading buffer (50% formamide, 0.05% bromophenol blue, 0.03% xylene cyanol FF and 5 mM EDTA) were added, samples were heated for 4 min at 90°C, chilled on ice, loaded on a 12 or 15% sequencing gel containing 7 M urea and electrophoresed in 90 mM Tris-borate, 2 mM EDTA (1× TBE buffer). Reaction products were visualized by autoradiography and scanned with a G-700 imaging densitometer (BioRad). Sequencing of the DNA fragments were carried out by the Maxam–Gilbert sequencing method.

RESULTS

The pBR322 plasmid contains, around position 990, a 20 bp sequence where DNA gyrase preferentially cleaves in the presence of quinolones (19). In an earlier report we demonstrated that DNA fragments of 85 and 71 bp containing this cleavage sequence at different positions were accepted as substrate for cleavage reactions in the presence of subunit A inhibitors (18). To further explore the role of this 20 bp cleavage sequence and the necessary length of the flanking regions in more detail, we examined the DNA gyrase-mediated cleavage reaction in the presence of subunit A inhibitors with DNA fragments of 10–122 bp (Fig. 1A and B). The DNA fragments of 30–122 bp contained the 20 bp cleavage sequence at different positions. The 20 bp fragment consisted of this cleavage sequence and the 10 bp DNA fragment contained only part of it. Assuming that labeling occurred with similar efficiency, equal amounts of these DNA fragments were incubated with DNA gyrase and Mg^{2+} in the presence or absence of subunit A inhibitors. After incubation, the protein was denatured with SDS and digested with proteinase K. The DNA was purified and loaded onto a denaturing gel. For determination of the positions of the cleavage sites, the migration of the cleavage products was compared with the sequenced 122, 111, 105, 96, 30 and 20 bp DNA fragments.

Our earlier results showing that pyrimido[1,6-*a*]benzimidazoles have a mode of action similar to that of quinolones could be confirmed (18). Both classes of subunit A inhibitors induced DNA gyrase to cleave at the same sites and differences between these inhibitors could be observed only in the preferences of DNA gyrase for these cleavage sites (shown for the 77, 64 and 38 bp DNA fragments in Fig. 2 and Table 2). However, the results indicate that DNA gyrase preferred the cleavage site between positions 993 and 994 (TGGC↓CT, cleavage occurs at the site indicated by the arrow) in the presence of the pyrimido[1,6-*a*] benzimidazoles Ro 46-2825, Ro 46-7864 and Ro 47-3359, but in the presence of Ro 46-6962 the cleavage site between position 990 and 991 (T↓GGCCT) was preferred. It is worthwhile to mention that Ro 46-6962 is one of the pyrimido[1,6-*a*]benzimidazoles that do not chelate divalent cations but nevertheless inhibit the DNA gyrase-promoted supercoiling reaction as well as the quinolones and the chelating pyrimido[1,6-*a*] benzimidazoles (18).

Table 2. Quantification of DNA gyrase-mediated major cleavage sites between T↓GGCCT and TGGC↓CT in the presence of quinolones or pyrimido[1,6-*a*] benzimidazoles

Inhibitor	DNA (bp)	T↓GGCCT lower band (% of total)	TGGC↓CT upper band (% of total)
Fleroxacin	77	27	14
	64	31	23
	38	17	20
Ciprofloxacin	77	21	23
	64	20	29
	38	12	37
Ro 46-6962	77	26	0.5
	64	23	2
	38		9
Ro 46-2825	77	5	31
	64	8	34
	38		18
Ro 47-3359	77	13	33
	64	4	21
	38	3	39
Ro 46-7864	77	4	27
	64	8	35
	38		12

As previously reported, DNA gyrase produced high amounts of cleavage products at ~100 μM inhibitor concentration (18). For the 64 bp fragment no significant increase in cleavage products between 100 and 500 μM fleroxacin was detected and even longer incubation periods (up to 120 min) did not result in a significant increase in cleavage products (Fig. 3A and Table 3). Cleavage of the 38 bp fragment increased significantly between 100 and 500 μM at the TGGC↓CT site but not at the T↓GGCCT site (Fig. 3B and Table 3). Inhibitors together with Mg^{2+} in the absence of the enzyme did not cleave the DNA fragments (data not shown).

Table 3. Quantification of DNA gyrase-mediated major cleavage sites between T↓GGCCT and TGGC↓CT in the presence of different concentrations of fleroxacin

Fleroxacin conc. (μM)	DNA (bp)	T↓GGCCT lower band (% of total)	TGGC↓CT upper band (% of total)
50	64	27	15
	38	17	7
100	64	28	21
	38	17	16
200	64	24	24
	38	17	27
500	64	17	26
	38	10	43

The results obtained with the DNA fragments of 30–122 bp show that DNA gyrase cleaved preferentially at two sites in the presence of 100 μM fleroxacin (Fig. 4). The sequencing data revealed that these sites are within the 20 bp cleavage sequence. In addition to the preferential cleavage site reported earlier within the sequence T↓GGCCT, efficient cleavage also occurred 3 bp downstream (TGGC↓CT), confirming our earlier observations obtained with DNA fragments of 71 and 85 bp (18). Further, even

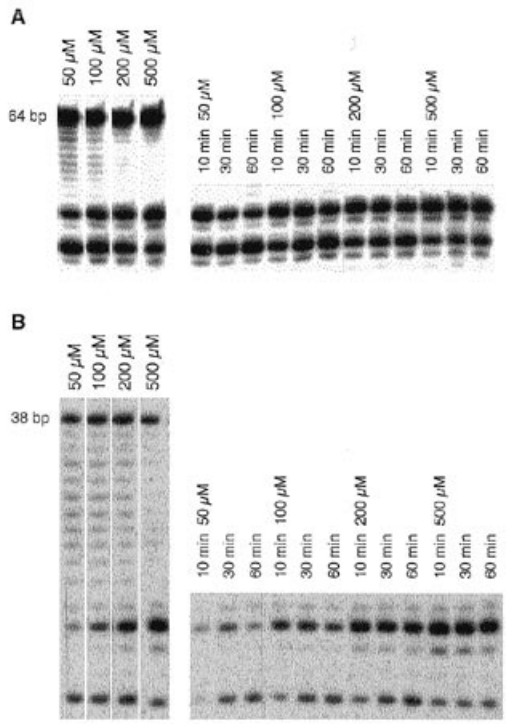


Figure 3. Determination of the cleavage sites on the 64 and 38 bp DNA fragments generated by DNA gyrase in the presence of fleroxacin. The DNA fragments were incubated at 37°C for up to 60 min in the presence of up to 500 μM fleroxacin. Reactions were carried out, stopped, purified and electrophoresed as indicated in Figure 2.

with the 20 bp, but not with the 10 bp fragment, small amounts of DNA fragments which arose from cleavage reactions within the sequence T↓GGCCT could be detected, indicating that even the 20 bp cleavage sequence *per se* is to some extent a substrate for the enzyme (Fig. 4E).

Densitometric quantification of the major cleavage products on the gels shows that the major cleavage products of the DNA fragments down to 30 bp consisted of ~5–50% of the total amount of all bands, with a tendency that smaller DNA fragments were poorer substrates for the DNA gyrase than longer ones. The data also suggest that the preference for the major cleavage sites shift from the T↓GGCCT cleavage site for the DNA fragments >96 bp to TGGC↓CT for the shorter DNA fragments (Table 4). The 20 bp DNA fragments were cleaved with reduced efficiency (~1% of the total of all bands) and this dropped to zero with the 10 bp DNA fragment as substrate.

The DNA fragments were cleaved in the presence of inhibitors, though less efficiently, at several additional sites (Figs 2–4). The electrophoretic mobilities of these cleavage products on the gels indicate that cleavage at these weaker sites occurred at the same positions in all DNA fragments, indicating again that the enzyme forms complexes at specific sites in the presence of quinolones.

Cleavage within the 20 bp cleavage sequence in the presence of Mg^{2+} but absence of inhibitors could not be observed, confirming our earlier results showing that DNA gyrase performed the religation reaction very efficiently in the absence of inhibitors (18). However, especially with DNA fragments <70 bp, a ladder of fragments deriving from cleavage reactions close to the end of the DNA fragments could be observed and cleavage at these sites was

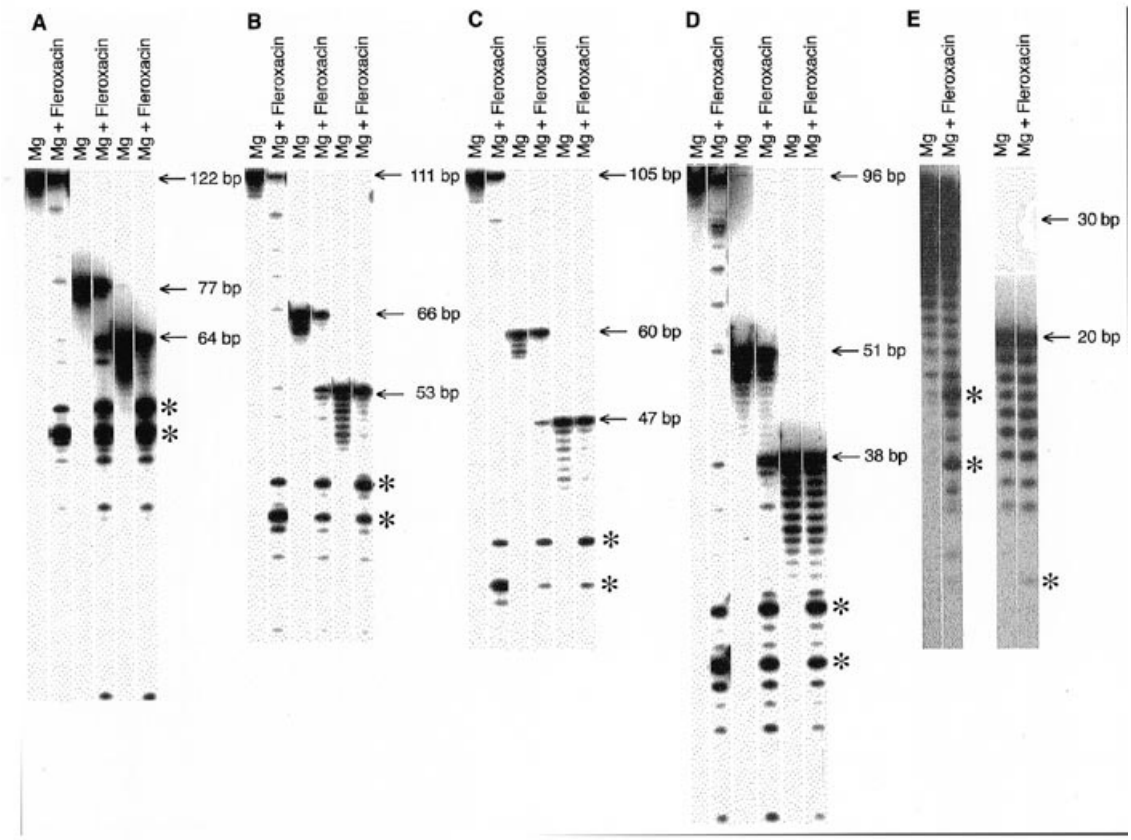


Figure 4. Determination of the cleavage sites on DNA fragments generated by DNA gyrase in the presence of fleroxacin. The DNA fragments were incubated at 37°C for 10 min with DNA gyrase as described in Figure 2. When fleroxacin was added the final concentration was 100µM. Reactions were carried out, stopped, purified and electrophoresed as indicated in Figure 2. The asterisks indicate the major cleavage sites. (**A**–**E**) The figures show the same groups of DNA fragments as in Figure 1.

even increased in the absence of inhibitors (Fig. 4). These cleavage products also appeared when the DNA fragments were incubated in the presence of Mg^{2+} either with the A subunit or with the B subunit alone (shown for the 38 bp DNA fragment in Fig. 5A) and the appearance of the cleavage products was time dependent (Fig. 5B). However, cleavage did not occur when the DNA fragments were incubated with the subunits or with the tetrameric complex in the absence of a divalent cation in the reaction buffer (Fig. 5). Because both proteins were purified after over-production on heparin and ion-exchange columns, the co-purification of an Mg^{2+}-dependent 3′→5′ DNA exonuclease cannot be excluded. However, it is also possible that both fractions still contained small amounts of the other subunit. Therefore, both subunits were further purified on a novobiocin–Sepharose-column (14). Eluting the subunit A fraction at high salt concentration should remove the remaining B subunit, which binds to novobiocin and can only be eluted under denaturing conditions. After loading the B subunit fraction, the column was washed with high-salt buffer to remove the A subunit. The B subunit was eluted with guanidine hydrochloride and renatured by dialysis. However, the ladder of DNA fragments also appeared after incubation of the DNA fragments with these purified subunits (Fig. 5C). Even after this highly specific affinity chromatography step, co-purification of a Mg^{2+}-dependent 3′→5′ DNA exonuclease cannot be entirely excluded, but another possible explanation is that both fractions still contained a small amount of the other subunit which was co-purified due to protein–protein interactions and that these complexes cleaved at the end of the DNA fragments. However, because cleavage at these sites also appeared in the presence of Mg^{2+} but absence of inhibitors, one can assume that they are not the result of specific enzyme–quinolone–DNA complexes but occurred at non-specific sites.

Table 4. Quantification of DNA gyrase-mediated major cleavage sites between T$^{\downarrow}$GGCCT and TGGC$^{\downarrow}$CT within DNA fragments of different length in the presence of fleroxacin

DNA (bp)	T$^{\downarrow}$GGCCT lower band (% of total)	TGGC$^{\downarrow}$CT upper band (% of total)
122	37	10
111	50	17
105	45	24
96	26	12
77	18	18
66	20	26
64	21	27
60	5	23
53	19	37
51	13	14
47	7	33
38	11	15
30	7	9
20	1	

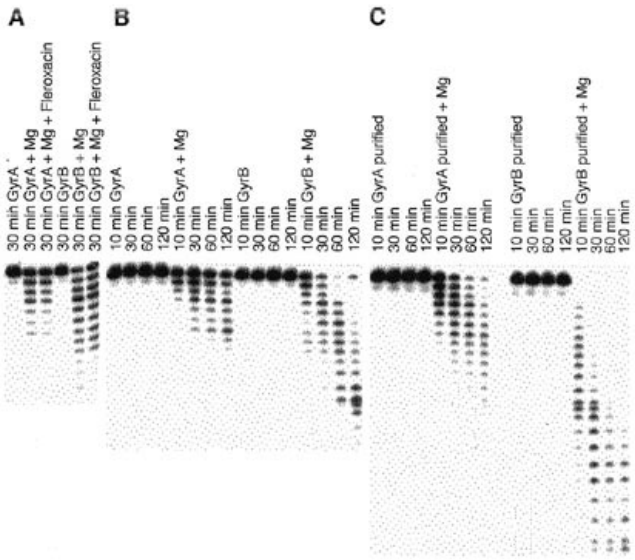


Figure 5. Cleavage sites at the end of the 38 bp DNA fragment generated by the DNA gyrase subunits in the presence of Mg^{2+}. (**A**) The DNA fragment was incubated with ~10 pmol DNA gyrase subunits at 37°C for 30 min in the presence or absence of magnesium ions and/or fleroxacin as indicated in the figure. (**B**) The DNA fragment was incubated with ~10 pmol DNA gyrase subunits at 37°C for up to 120 min in the presence or absence of Mg^{2+}. (**C**) The DNA fragment was incubated with ~10 pmol novobiocin–Sepharose-purified DNA gyrase subunits at 37°C for up to 120 min in the presence or absence of Mg^{2+}. Reactions were stopped, purified and electrophoresed as indicated in Figure 2.

Cleavage generated in the presence of Ca^{2+} and the absence of inhibitors occurs at the same sites as those obtained in the presence of oxolinic acid, but with different relative efficiencies (3). However, it seems probable that not only the sequence but also the length of the DNA fragments is critical for cleavage reactions carried out in the presence of Ca^{2+} (18). The results of this work confirmed these observations because the DNA fragments ≥96 bp were cleaved in the presence of Ca^{2+} and absence of inhibitors within the sequence T↓GGCCT but the shorter ones (≤77 bp) were not substrates (data not shown).

DISCUSSION

DNA gyrase cleaves in the presence of quinolones at preferred sites on DNA and this quinolone-induced cleavage reaction has been taken as a model for the double-stranded cleavage event during supercoiling. Footprinting experiments have shown that the DNA gyrase binding site is 100–150 bp long, with the cleavage site located near the center of the fragment (6–9). Cleavage sequences share homology around the breakage point and, based on analysis of cleavage sites and their flanking regions in the pBR322 plasmid, a 20 bp consensus sequence has been proposed (19). It was also shown that there is a major cleavage site at position 990 on this plasmid (19). However, a 34 bp DNA fragment containing the 20 bp cleavage sequence around this major cleavage site is not a substrate for the enzyme in the presence of oxolinic acid and flanking DNA is required for efficient DNA breakage (23). In a previous report we used restriction fragments of 85 and 71 bp from the plasmid pBR322 as model substrate DNA, each containing the preferred 20 bp cleavage sequence at a different position, and we could show that even these DNA fragments, despite their length being in principle insufficient to wrap around the tetrameric protein, were accepted as substrate for cleavage reactions in the presence of subunit A inhibitors (18). The DNA fragments were positioned onto the enzyme in such a way that cleavage occurred at the predicted site within the 20 bp cleavage sequence. In this report we show that even a 30 bp DNA fragment containing the 20 bp preferred cleavage sequence from the pBR322 plasmid is still a substrate for DNA gyrase. Even a 20 bp DNA fragment was cleaved, but with very low efficiency. Cleavage within a 10 bp DNA fragment, containing only part of the 20 bp cleavage sequence, could not be observed, indicating that a fragment of this length is no longer a substrate for the enzyme. Inefficient cleavage of short DNA fragments may reflect a lowered binding affinity because a minimum number of DNA–protein contacts are necessary for efficient cleavage.

Confirming our results obtained earlier with the 85 and 71 bp DNA fragments, DNA gyrase cleaved all the cleavable DNA fragments in the presence of subunit A inhibitors preferentially at two sites within the 20 bp cleavage sequence of the pBR322 plasmid.

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        – +   – +
5′-GGCTGGAT|GGC|CTTCCCCAT-3′
          0     3
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Bases that form covalent phosphodiester bonds with the enzyme are marked + and the free 3′-hydroxyl ends at the cleavage site are marked –. The sequencing data shows that cleavage occurred at the site earlier reported within the sequence T↓GGCCT between T at position 990 (numbered 0) and G at position 991, but in addition 3 bp downstream (TGGC↓CT) between C at position 993 (numbered 3) and C at position 994. Even if one considers that cleavage occurs on the other strand 4 bp away (6,7,25), no obvious sequence homology can be deduced from these major cleavage sites and one can only speculate that the quinolones may prefer a guanosine at position +1 in at least one strand. To confirm this speculation, further strong cleavage sites would have to be analyzed. However, it is known that some antitumor drugs target eukaryotic topoisomerase II and that these compounds also stimulate topoisomerase II-mediated DNA cleavage by interfering with the breakage–religation reaction (26–31). Different drug families show a variable degree of sequence specificity but cleavage sites are generally conserved within the same family (28,32–35). Also, quinolone derivatives that have been shown to induce eukaryotic topoisomerase II to cleave at specific sites prefer a specific base at the cleavage site (36). It is postulated that these antitumor drugs form a ternary complex by binding to preferred nucleotides adjacent to the cleavage site and to amino acid residues of the enzyme. It is thus a possibility that DNA gyrase subunit A inhibitors also prefer specific bases and induce DNA gyrase to cleave at inhibitor-specific sites.

Hence, DNA gyrase cleaved DNA fragments in the presence of subunit A inhibitors preferentially within the 20 bp cleavage sequence, but the cleavage pattern also shows cleavage products derived from cleavage reactions at the end of the DNA fragments. These cleavage products may be the result of a Mg^{2+}-dependent 3′→5′ DNA exonuclease which was co-purified with novobiocin affinity column-purified DNA gyrase subunits. The specific and very different purification steps for the two subunits are expected to remove potential exonuclease contamination at least from either one of the two subunits. The purified subunits, however, even

after several purification steps, might still be cross-contaminated with small amounts of the other subunit due to their tight interaction in the A_2B_2 tetrameric DNA gyrase complex. Because cleavages at the end of the DNA fragments also appeared in the presence of Mg^{2+} but absence of inhibitors, cleavage at these sites by DNA gyrase, resulting in DNA fragments shortened by some bases, would not be the effect of specific enzyme–quinolone–DNA complexes but would occur at non-specific sites. However, the mechanism of such a suicide type of cleavage at the very ends of DNA fragments is not clear.

Based on our results we propose the following model. The enzyme attempts to bind to DNA by wrapping the DNA around the tetrameric protein. If DNA gyrase interacts, in the presence of an inhibitor, with DNA fragments which are long enough to be wrapped around the enzyme (>100 bp), they are preferentially cleaved at the T↓GGCCT site. Whether DNA gyrase also cleaves at this position in the presence of Mg^{2+} but absence of inhibitors cannot be determined, because DNA gyrase performs the religation reaction very efficiently in the absence of an inhibitor (18). Because DNA fragments shorter than ~100 bp cannot be reasonably positioned onto the enzyme, it may attempt, in the absence of an inhibitor, to establish a maximum number of DNA–protein contacts by binding such a DNA fragment to at least one side of the enzyme complex. Thus asymmetrical binding of the DNA fragments occurs and the enzyme may even bind to the end of the DNA fragments. However, if DNA gyrase interacts, in the presence of an inhibitor, with DNA fragments of a length that is in principle insufficient, it cleaves at the same inhibitor-specific sites as with longer fragments but probably with a higher preference for the TGGC↓CT site. Therefore, the enzyme is trapped at inhibitor-specific positions by forming a ternary complex between DNA, inhibitor and DNA gyrase. Whether it is the conformation or the sequence which determines these inhibitor-specific positions is not clear. With the shorter DNA fragments it cannot be determined whether DNA gyrase also cleaves at these inhibitor-specific positions in the presence of Mg^{2+} but absence of inhibitors. However, earlier experiments performed with Ca^{2+} instead of Mg^{2+} indicated that a 85 bp fragment containing the 20 bp cleavage sequence was not cleaved within this cleavage sequence but at a site that can be explained by an asymmetrical wrapping of the DNA fragment around the enzyme. About 70 bp of one end of the DNA fragment were wrapped around one side of the enzyme and ~15 bp around the other (18). We have observed that the 122, 111, 105 and 96 bp DNA fragments were cleaved in the presence of Ca^{2+} within the sequence T↓GGCCT (data not shown) and these results support our hypothesis, because the distance from the cleavage site to the *Eco*RI site is ~70 bp. DNA fragments ≤ 77 bp were not substrates for the cleavage reaction in the presence of Ca^{2+} and probably could not be positioned correctly onto the enzyme.

In this work we have shown that even a 20 bp DNA fragment containing a 20 bp preferred cleavage sequence from plasmid pBR322 was a substrate for the DNA gyrase-mediated cleavage reaction in the presence of inhibitors. Although such fragments are too short to be wrapped around the enzyme or at least around one side, the 30 bp DNA fragment was cleaved, although with reduced efficiency and with different preferences, at the same sites as the 122 bp DNA fragment. The 20 bp DNA fragment was cleaved with very low efficiency at one of these sites and only a 10 bp DNA fragment was not a substrate. Whether DNA gyrase in the presence of subunit A inhibitors either, by analogy with eukaryotic topoisomerase II inhibitors, interacts with preferred nucleotides adjacent to the cleavage site or whether it is the DNA conformation which determines the inhibitor-specific cleavage sites requires further investigation. However, we propose that the subunit A inhibitors interact with DNA at inhibitor-specific positions thus determining cleavage by forming a ternary complex between DNA, inhibitors and DNA gyrase, but it remains an open question whether these sites are also the physiological sites.

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