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The cleavage of DNA at phosphorothioate internucleotidic linkages by DNA gyrase

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ABSTRACT

We have constructed a plasmid which contains 22 copies of a 147 bp DNA fragment which contains the major DNA gyrase cleavage site from plasmid pBR322 (located at base-pair 990). We have found that this fragment is efficiently bound and cleaved by gyrase. The selectivity for the sequence corresponding to position 990 in pBR322 is maintained even when this site is located only 15 bp from one end of the 147 bp fragment. A strategy for the specific incorporation of a single thiophosphoryl linkage into the 147 bp fragment has been developed, and gyrase has been shown to catalyse efficient cleavage of fragments bearing phosphorothioate linkages at the gyrase cleavage site in one or both strands.

INTRODUCTION

The bacterial enzyme DNA gyrase can introduce negative supercoils into DNA using the free energy of ATP hydrolysis (reviewed in 1–3). The enzyme from *Escherichia coli* consists of two subunits, A and B, of molecular weights 97 and 90kDa respectively; the active enzyme is an A_2B_2 complex. The mechanism of DNA supercoiling by gyrase has been shown to involve the following steps (reviewed in 3,4): (i) the enzyme binds to DNA and a segment of ~120bp is wrapped around the protein; (ii) this wrapped segment is then cleaved in both strands at sites four bases apart, with covalent bonds being formed between the newly-formed 5'-phosphoryl groups at the break sites and tyr122 of the A subunits (these covalent bonds are thought to conserve the energy of the broken phosphodiester bonds); (iii) another segment of DNA (which may be close to or part of the wrapped segment) is then translocated through the double-strand break; this process reduces the linking number of a closed-circular substrate by two, ie. two negative supercoils are introduced; (iv) the broken phosphodiester bonds may now be resealed. Catalytic supercoiling requires the hydrolysis of ATP, but the non-hydrolysable analogue ADPNP (5'-adenylyl-β,γ-imido-diphosphate) will support limited supercoiling, suggesting that ATP binding permits one cycle of the supercoiling reaction but that hydrolysis is required for enzyme turnover.

A key step in the supercoiling reaction is the cleavage of DNA in both strands. It has been shown that drugs of the quinolone class can, under certain conditions, lead to the trapping of a cleaved product (reviewed in 3, 5). Quinolone drugs (eg. oxolinic acid, ciprofloxacin) are anti-bacterial agents whose intracellular target is DNA gyrase. *In vitro*, quinolones can be shown to inhibit DNA supercoiling by gyrase (6). Moreover, if a reaction containing gyrase, DNA and a quinolone drug is terminated by the addition of SDS, the DNA is found to be broken in both strands with the A proteins covalently bound at the 5'-phosphates (6). In addition to quinolone drugs, Ca^{2+} ions have also been shown to elicit this cleavage reaction (7).

Although DNA gyrase can be regarded as a relatively non-specific enzyme in terms of DNA sequence, the quinolone-induced cleavage sites have been shown to occur at preferred locations and many have been mapped at the sequence level (reviewed in 3). Indeed a consensus sequence has been derived (8):

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          ↓     T
5'-RNNNRNRTGRYC YNYNGNY
     (G)    G     (T)
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where R= purine, Y= pyrimidine, N= any nucleotide; T and G at the 13th position are equally preferred, and G and T in brackets are preferred secondarily to T and G respectively. The arrow indicates the site of cleavage by DNA gyrase. DNA cleavage by gyrase induced by Ca^{2+} appears to occur at the same sites as those induced by quinolone drugs (L.M. Fisher, M.H. O'Dea, M. Gellert, personal communication).

Plasmid pBR322 contains a preferred cleavage site for DNA gyrase located at base-pair 990 (9). Mutations at this site can reduce or abolish cleavage, and a fragment of 34 bp containing this site is not a cleavage substrate, whereas longer fragments (eg. 170 bp) are (10). These experiments indicate the importance of both the sequences surrounding the cleavage site and the length of the DNA molecule containing the site. Footprinting experiments have shown that the gyrase binding site is 100–150 bp long with the cleavage site located near the centre of the fragment (9, 11–13).

DNA molecules in which selected phosphorothioate internucleotidic linkages have been introduced have been prepared and exploited to probe DNA-protein recognition for a number of restriction endonucleases including *Eco*R I (14), *Nci* I (15), *Ban* II (16) and *Eco*R V (17), (for a review of earlier work see

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ref. 18). Introduction of a thiophosphate linkage at the cleavage site generally gives rise to DNA that is cleaved at a considerably reduced rate, if at all, compared to the phosphate-containing DNA. In this paper we describe studies of the cleavage of a DNA fragment containing the major gyrase site in pBR322, where the phosphodiester bonds at the cleavage site have been substituted with phosphorothioate linkages. A similar approach of incorporation of phosphorothioates into specific sites in DNA substrates has previously been applied to the λ Int protein (19–21), phage Mu transposase (21), and HIV-1 IN protein (22), however some of these studies utilised DNA in which all phosphoryl groups adjacent to a specified base or bases were replaced by thiophophates. In this study we have used methods that allow only the cleavage site to be substituted, minimising indirect effects arising from, for example, conformational changes.

MATERIALS AND METHODS

Enzymes

The A and B subunits of *E.coli* DNA gyrase were prepared from strains JMtacA and JMtacB as described previously (23). Restriction enzymes *Hga* I, *Hinf* I, *Hpa* II and *Bsm* I, T4 DNA ligase and polynucleotide kinase were purchased from New England Biolabs. *Bst*N I was from Boehringer, *Ava* I and *Hha* I from Anglian Biotec and *Eco*R V was a gift from S.E. Halford (Univ. Bristol). The Klenow fragment of DNA polymerase I was from Pharmacia.

Cloning

Plasmid pSTD147, which contains 22 copies of a 147bp DNA fragment derived from plasmid pBR322, was constructed using a procedure based on the method of Hartley and Gregori (24). A 123 bp fragment containing the major gyrase cleavage of plasmid pBR322 was excised by a *Bgl* I/*Bst*N I double digest. (A contaminating 121 bp *Bgl* I-*Bst*N I fragment was further cleaved by digestion with *Taq* I.) The gel-purified 123 bp fragment was ligated to two double-stranded oligonucleotides:

I-5′-CCGCCGGAATTCGCA, and II-5′-AGAATTCGGGGCGGGG
GGGCGGCCTTAAG-5′ CTTAAGCCCCGCCC-5′

The right end of oligonucleotide I bears a *Bgl* I 'sticky end' and the left end of oligonucleotide II bears a *Bst* NI sticky end, both oligos contain internal *Eco*R I recognition sequences. The resulting fragment was digested with *Eco*R I and the gel-purified product was cloned into the *Eco*R I site of plasmid pARA1 (24), in which this site is flanked by two non-equivalent *Ava* I sites. The resultant plasmid was digested with *Ava* I to yield a 147 bp fragment containing the gyrase cleavage site and bearing non-complementary *Ava* I sticky ends. Linear concatamers of this fragment were produced by ligation, and concatamers containing greater than 20 monomer units were gel purified and cloned into the *Ava* I site of plasmid pBRD1168, which comprises plasmid pBR322 from which an *Eco*R V-*Bsm* I fragment (1168 bp), containing part of the *tet* gene, had been removed. The resulting products were transformed into *E.coli* strain JM109, and one clone was found to harbour a plasmid containing 22 copies of the 147 bp fragment. This plasmid was named pSTD147.

DNA preparation

Supercoiled plasmid DNA was prepared as described previously (7). To prepare the 147 bp fragment, plasmid pSTD147 was digested with restriction enzyme *Ava* I, and the 147 bp fragment was resolved from the residual large fragment (3195 bp) by chromatography on an FPLC Superose 6 column (Pharmacia). Peak fractions were pooled and concentrated by ethanol precipitation.

To construct the 147 bp fragment bearing phosphorothioate linkages at the gyrase cleavage site, the fragment was first cut with *Hga* I and *Hinf* I and fragments *a*, *b* and *d* purified from a polyacrylamide gel (25), see Fig 1. Oligonucleotides corresponding to fragment *c* and bearing either phosphate or phosphorothioate diester linkages at the gyrase cleavage site were synthesised using an Applied Biosystems 380B oligonucleotide synthesiser by Debra Langton (Univ. of Leicester). ^{31}P-nmr spectra of the sulphur-containing oligonucleotides showed that they were mono-substituted with a phosphorothioate group in two diastereomeric forms, and that the phosphorothioate group was not significantly lost after 12 months storage at −20°C.

The 147 bp fragment was re-constructed by ligation of the gel-purified oligonucleotides to the gel-purified fragments *a*, *b* and *d* (Fig 1), and digested with *Ava* I. The 147 bp product was further purified from a polyacrylamide gel. The 147 bp fragments were ^{32}P-end-labelled using T4 polynucleotide kinase (PNK) and purified using a Sephadex G-50 (Pharmacia) spin column (25). The fragments were precipitated with ethanol and resuspended in 10mM Tris.HCl (pH 7.5), 1mM EDTA prior to use in cleavage reactions. The presence of a sulphur atom at the appropriate site in the 147 bp fragments was verified by the method of Gish and Eckstein (26).

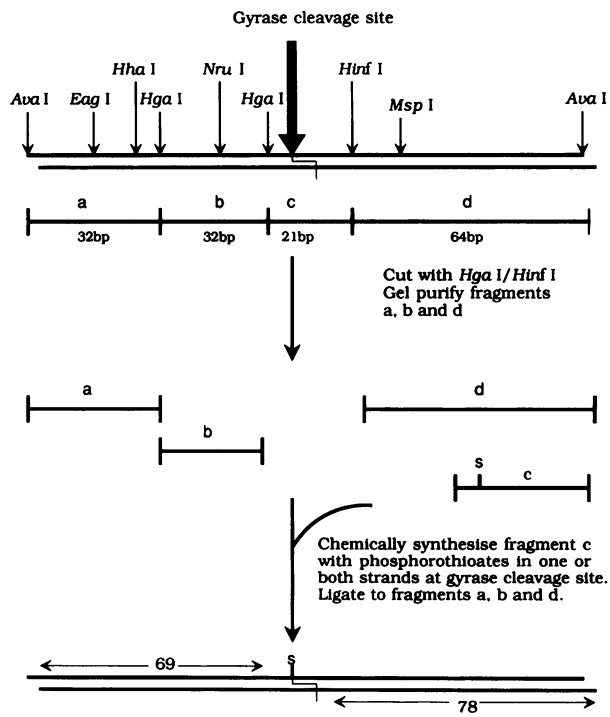

Fig. 1. The 147 bp DNA fragment. This fragment contains the major gyrase cleavage site from plasmid pBR322. Unique restriction enzyme sites, the two sites for *Hga* I, and the position of the gyrase cleavage site are shown in the upper part of the figure. Cleavage with *Hga* I and *Hinf* I yields four fragments. To construct 147 bp fragments with phosphorothioate linkages, fragments *a*, *b* and *d* were purified and ligated to fragment *c* containing phosphorothioates as shown. Also identified in the lower part of the figure are the fragments 3′ to the gyrase cleavage site released following cleavage.

Gyrase assays

Cleavage reactions (10μl) contained ^{32}P-labelled 147 bp fragment (about 0.02 pmole) 50mM Tris.HCl (pH7.5), 55mM KCl, 4mM $MgCl_2$, 5mM DTT, 5% (w/v) glycerol, 0.36mg/ml BSA and quinolone drug. Where indicated, $CaCl_2$ was substituted for $MgCl_2$ and quinolone drug. The reaction was initiated by the addition of DNA gyrase (0.1 pmole) and incubated for up to 4h at 25°C. SDS and proteinase K (Sigma) were then add to final concentrations of 0.14% and 1.4 mg/ml respectively, and the incubation continued for a further 30 min at 37°C. Reactions were terminated by extraction with an equal volume of chloroform/isoamyl alcohol (24:1). Then either 5μl of 40% sucrose, 100mM Tris.HCl (pH7.5), 1mM EDTA, 0.05% bromophenol blue was added for application of samples to an 8% native polyacrylamide gel, or 5μl of 98%(w/w) formamide, 10mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol was added for application of samples to an 8% denaturing polyacrylamide gel containing 42% (w/v) urea.

Gel retardation assays using DNA gyrase and the 147 bp fragment were performed as described previously (27).

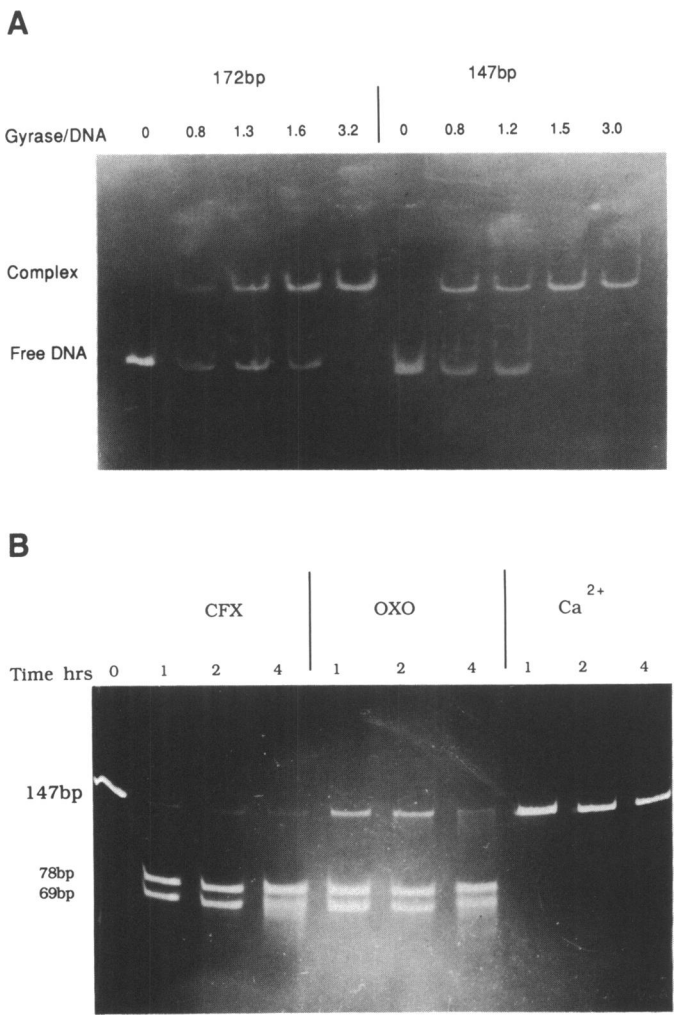

Fig. 2. Interaction of DNA gyrase with the 147 bp fragment. **(A)**. Gel retardation assay. Samples containing the 147 bp fragment (100nM) or a 172 bp fragment (90nM) were incubated with DNA gyrase at the molar ratios indicated for 1h. at 25°C. Samples were then loaded onto a 5% polyacrylamide gel (acrylamide: bis, 29:1) and subjected to electrophoresis in 90mM Tris/borate, 5mM $MgCl_2$, and the gel stained with ethidium bromide. Positions of free DNA and gyrase-DNA complex are indicated. **(B)**. DNA cleavage assay. Samples containing 147 bp fragment (84 nM) were incubated at 25°C with gyrase (780 nM) in the presence of ciprofloxacin (CFX; 3μg/ml), oxolinic acid (OXO; 70 μg/ml) or $CaCl_2$ (3 mM) as indicated in the figure. (Where $CaCl_2$ was included in reaction mixtures, $MgCl_2$ was omitted.) Aliquots were removed at the times indicated and SDS and proteinase K were added , and the incubation continued for a further 30 mins. at 37°C. Samples were loaded onto an 8% polyacrylamide gel (acrylamide: bis, 19: 1) in 90mM Tris/borate, 5mM EDTA, and the gel stained with ethidium bromide. The positions of the substrate (147 bp) and product (78 and 69 bp) bands are indicated.

RESULTS

Interaction of gyrase with a 147 base-pair fragment

DNA gyrase can be shown to cleave DNA in the presence of quinolone drugs (3,5). In this reaction the DNA sequences 5ó to the cleavage site on each strand are covalently attached to the protein, but the sequences 3ó to the cleavage site are released. It has previously been shown that, in such a reaction, gyrase cleaves plasmid pBR322 at a preferred site at base pair 990 (8−10):

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      990↓
5′−GGCTGGATGGCCTTCCCAT
3′−CCGACCTACCGGAAGGGTA
                  ↑
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This cleavage site is consistent with the consensus sequence described above. In order to study the interaction of DNA gyrase with this site, we have cloned a 147 bp fragment containing this site in 22 copies in plasmid pSTD147 (see Materials and Methods). This allows the production of milligram amounts of this fragment by FPLC chromatography. We have used this DNA molecule to study the quinolone-induced gyrase cleavage reaction. Figure 2A shows a gel-retardation experiment comparing the binding of gyrase to the 147 bp fragment with that of a 172 bp fragment previously used in the studies of gyrase-DNA interaction (13,28). This experiment shows that gyrase forms a DNA-protein complex with the 147 bp fragment, and that this complex is formed more efficiently relative to that formed with the 172 bp fragment . For example, at comparable molar ratios of gyrase to DNA (eg. 0.8), more DNA-protein complex is apparent with the 147 bp fragment than with the 172 bp fragment (Fig. 2A). (That a molar excess of gyrase over DNA is required to completely bind these fragments may indicate a proportion of inactive protein in the gyrase preparation.)

Incubation of the 147 bp fragment with gyrase and quinolone drug, and termination of the reaction with SDS and digestion with proteinase K leads to the production of two products (78 bp and 69 bp) consistent with a single cleavage occurring at the site indicated above (Fig 2B). The extent of cleavage was found to be virtually 100% after four hours of incubation (Fig 2B). The quinolone drugs oxolinic acid and ciprofloxacin show identical cleavage patterns indicating that cleavage is occurring at the same site with these two drugs. [The exact site of cleavage has been determined by DNA sequencing (Fig. 4A), and found to be identical to that determined by Fisher *et al.* (9,10), and given above.] Under the conditions of the experiment described in Fig. 2B, we found no evidence of cleavage stimulated by Ca^{2+}. DNA cleavage stimulated by Ca^{2+} has been documented previously (7), under reaction conditions which differ from those employed in this work, *viz.* 24 mM KCl (cf. 55mM) and the inclusion of spermidine. Modification of our reaction conditions to those employed in ref. 7 generated cleaved products in the

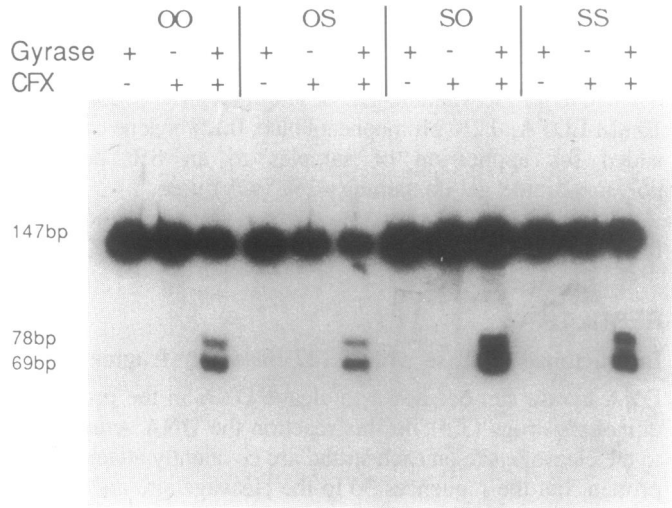

Fig. 3. Cleavage of phosphorothioate-substituted DNA by gyrase. Native and substituted 147 bp fragments (0.7 nM) ^{32}P-labelled at the 5'-termini with PNK, were incubated with gyrase (3.3 nM) and/or ciprofloxacin (CFX, 100μg/ml) for 1h., as described in the legend to Fig. 2B. Following electrophoresis, the gel was subjected to autoradiography. OO, indicates native (unsubstituted) 147 bp fragment; OS indicates 147 bp fragment bearing a phosphorothioate linkage in the lower strand as shown in Fig. 1; SO indicates 147 bp fragment bearing a phosphorothioate in the upper strand; SS, indicates phosphorothioates in both strands.

presence of 2mM or 7mM $CaCl_2$ which were found to be of the same mobility as those in Fig. 2B, consistent with cleavage at the same site (data not shown).

The 147 bp fragment contains six unique sites for restriction enzymes (Fig. 1). Cleavage of the multimer of the 147 bp fragment derived from plasmid pSTD147 at these restriction enzyme sites produces a set of circularly-permuted DNA fragments, ie. comprising the same DNA sequences but with ends located at different positions in the sequence. Using the method of Wu and Crothers (29), we have found, by polyacrylamide gel electrophoresis, no appreciable differences in mobility among the six fragments (data not shown), suggesting that there may not be a significant bend in this DNA molecule (see Discussion). When these circularly-permuted fragments were used as substrates in the gyrase/quinolone cleavage reaction we found that cleavage occurred predominantly at the preferred site, although secondary cleavages were apparent when the preferred site was close to the end of the molecule (eg. *Nru* I-cut DNA) (data not shown). It is surprising given the proposed structure of the gyrase-DNA complex (3) and the large associated footprint (9, 11−13), that cleavage still occurred predominantly at the same site even when this was only 16 bp from one end of the DNA fragment (*Nru* I-cut DNA) or 15bp from the other (*Hinf* I-cut DNA).

Construction of 147bp fragments substituted with phosphorothioate linkages at the gyrase cleavage site

One method for probing the interaction of DNA-specific enzymes with DNA is to analyse the interaction of these enzymes with DNA substituted with phosphorothioate linkages. This approach has been used to probe both the specificity and/or the stereochemistry of DNA cleavage for a wide range of such enzymes, including snake venom phosphodiesterase (30), spleen exonuclease (31), topoisomerases I and II (32), *Eco*R I (14), *Eco*R V (17) and other restriction endonucleases (33). Using oligonucleotides containing phosphorothioates at the sites indicated by the arrows in the sequence shown above, we have constructed 147 bp molecules bearing phosphorothioate linkages at the DNA gyrase cleavage site in one or both strands as illustrated in Fig. 1. This scheme takes advantage of the fact that the two restriction enzymes used to fragment the 147 bp molecule (*Hga* I and *Hinf* I) generate unique cohesive ends and thus the production of the desired fragment will be strongly favoured upon ligation (Fig. 1). Thus four different 147 bp species were made, with either no phosphorothioates, with a phosphorothioate linkage in one or the other strand, or with phosphorothioate linkages in both strands. We have found that these molecules show identical electrophoretic mobility by polyacrylamide gel electrophoresis (data not shown). That the sulphur is at the gyrase cleavage site was determined by the method of Gish and Eckstein (26). This method involves the specific cleavage of the phosphorothioate-substituted DNA at sulphur by 2-iodoethanol. We have found that the phosphorothioate-substituted DNA is cleaved only once at the expected site (see Fig. 4B).

Interaction of gyrase with phosphorothioate-substituted DNA

Incubation of the phosphorothioate-substituted DNA molecules with gyrase and ciprofloxacin shows that all four 147 bp species are substrates for cleavage by gyrase (Fig. 3). In all cases, following digestion of gyrase with proteinase K, only two products (78 bp and 69 bp) are evident, and these products have apparently identical electrophoretic mobility for the four different species. Moreover, when the variations in the amounts of radioactivity in the different fragments loaded on the gel in Fig. 3 is accounted for, the extent of cleavage in each of the four reactions in Fig. 3 is very similar, indicating that the efficiency of cleavage is the same at each site. Note that the intensity of the 69 bp product is greater than that for the 78 bp product. This can be accounted for by the greater efficiency of labelling of the upper strand of the 147 bp fragment than the lower strand (Fig. 1) by PNK (see below and Fig. 4B).

Using the native 147 bp fragment, we have determined the precise site of cleavage by gyrase using DNA sequencing. Fig. 4A is a DNA sequencing gel which shows that the sites of cleavage in each strand are exactly the same as had been previously determined by other workers (9,10). Note however that a second cleavage occurs at low efficiency, as indicated by the high molecular band in track X (lower strand) in Fig. 4A.

In Fig. 4B the digestion of the end-labelled 147 bp fragment by *Hpa* II (track H) produces two labelled products, 99 and 50 nucleotides in length, the former being more radioactive than the latter. This shows that the two strands are labelled with different efficiencies, as noted above in Fig. 3. Cleavage of the 147 bp fragment by gyrase releases 78 and 69 nucleotide products arising from the sequences 3' to the cleavage site (Fig. 1), while the 5' sequences remain covalently attached to the protein. The cleavage of the OO and SS fragments by gyrase in the presence of ciprofloxacin yields products of identical mobility, showing that the DNA molecule with sulphur in both strands is cleaved at exactly the same site as the native 147 DNA molecule, ie. the site of cleavage is identical irrespective whether there is a phosphodiester or phosphorothioate linkage at the gyrase cleavage site. Cleavage of the SS fragment with 2-iodoethanol by the method of Gish and Eckstein (26), yields products of the same size as those generated by quinolone-directed cleavage by gyrase, confirming sulphur substitutions at the correct sites. Cleavage by this reagent is very inefficient and the intensity of the products

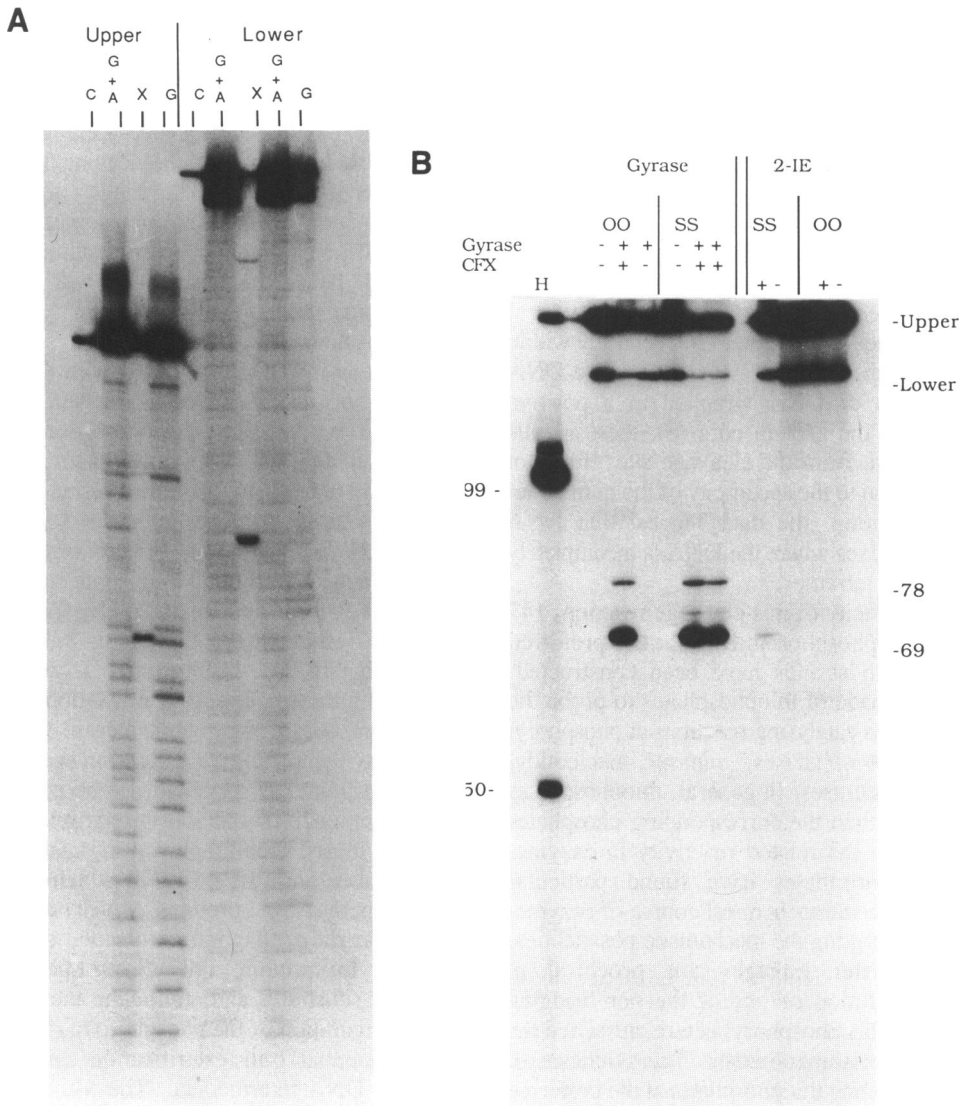

Fig. 4. Denaturing gels of gyrase-cleaved 147 bp fragments. **(A)**. Determination of cleavage site. Native 147 bp fragment was labelled with PNK, as described in the legend to Fig. 3. One aliquot was cleaved with *Hpa* II (yielding the upper strand as the larger labelled product) and another aliquot was cleaved with *Hha* I (yielding the lower strand as the larger labelled product). The products were subjected to gyrase/OXO cleavage as described in the legend to Fig. 2 (tracks marked X) and also to Maxam/Gilbert sequencing (tracks marked G+A and G) as described by Sambrook *et al.* (25). The tracks marked C indicate control (untreated) samples. **(B)**. Comparison of cleavage in OO and SS 147 bp fragments. Fragments substituted in both strands (SS) or unsubstituted (OO) were ^{32}P-labelled with PNK as described in the legend to Fig. 3. One sample of OO DNA was cleaved by *Hpa* II (track H) to give the products indicated. Other samples were treated with gyrase and CFX as described in the legend to Fig. 3. Further samples were subjected to cleavage by 2-iodoethanol (2-IE) as described by Gish and Eckstein (26).

are consequently faint compared with the gyrase cleavage products. It is also important to note from Fig. 4B that DNA gyrase is capable of cleaving the phosphorothioate-substituted substrate to greater than 50%. This can most easily be judged from the relative intensities of the lower strand (the least efficiently labelled) and its cleavage product (78 nt).

DISCUSSION

In order to study the cleavage of phosphorothioate-substituted DNA by DNA gyrase we have chosen a 147 bp DNA fragment containing the major gyrase cleavage site from plasmid pBR322. We have found that this fragment is bound efficiently by gyrase and is cleaved in the presence of quinolone drugs at the site previously determined by other workers (8,9). It is interesting to speculate why this DNA sequence constitutes a preferred cleavage site. One possibility, suggested previously (34), is that preferred gyrase cleavage sites occur in DNA sequences with an intrinsic curvature. However, we have found no evidence of anomalous gel mobility (indicative of curvature) when circularly-permuted 147 bp fragments were analysed by polyacrylamide gel electrophoresis. This result is consistent with that obtained by Stellwagen (35) and with the analysis of the frequency of the AA dinucleotide within this sequence (36), a feature associated with curvature. However, theoretical calculations of De Santis *et al.* (37) predict curvature for this sequence (based on dinucleotide wedge analysis), and electron microscopic studies of pBR322 also suggest curvature in the region of the 990 site (38). Thus it is indeed possible that the sequences surrounding this site do possess intrinsic curvature and that this feature contributes to the preference for DNA gyrase binding and cleaving at this site.

In order to probe the degree of specificity for the preferred gyrase cleavage site (position 990 in pBR322) within the context of the 147 bp fragment, use was made of the circularly-permuted fragments discussed above, which allow the preferred DNA cleavage site to be located either centrally or nearer to the ends of the fragment. When these fragments were used as substrates for the gyrase/quinolone cleavage reaction, cleavage occurred predominantly at the position corresponding to the 990 site in pBR322, even when this site was only 15 or 16 bp from the end of the DNA fragment. This implies that the factors responsible for the preference for this cleavage site are comparatively local and do not arise from the tendency to cleave DNA centrally within a bound fragment. Although the model for the gyrase-DNA complex has about 120 bp of DNA wrapped in a positive superhelical manner around the protein core (3), these results suggest that the DNA remote from the cleavage sites does not make a significant contribution to the specificity of the quinolone-promoted cleavage. In addition, the data suggest that DNA cleavage may occur in complexes where the DNA is incompletely wrapped around the gyrase tetramer.

To probe further the specificity of this cleavage reaction, 147 bp fragments incorporating phosphorothioates at the preferred cleavage site in one or both strands have been constructed. Considerable use has been made of thiophosphates to probe the mechanisms of many enzymes catalysing reactions at phosphoryl centres such as kinases, phosphatases, mutases, nucleotidyl transferases and phosphodiesterases. In general, thiophosphates are chemically less reactive than the corresponding phosphates and this is often reflected in the related reactivity in enzyme-catalysed reactions. Thiophosphates have found particular application in determining the stereochemical course of enzyme-catalysed reactions thereby limiting the mechanistic possibilities. Internucleotide phosphodiester linkages are prochiral at phosphorus such that substitution of one of the non-bridging oxygens by sulphur renders this phosphoryl centre chiral and the possibilities for two diastereoisomers exists. The syntheses of the oligonucleotides incorporating thiophosphates at the preferred gyrase cleavage site gave rise to an approximate 1:1 mixture of the two diastereoisomers (confirmed by ^{31}P nmr spectroscopy) and no attempt at separation has been made.

The strategy for the construction of the 147 bp fragment with thiophosphates selectively located in either or both of the strands, at the preferred gyrase cleavage site is worthy of comment. Restriction sites were chosen such that the double-stranded synthetic oligonucleotides can only be ligated in one orientation. Assembly and ligation of the modified 147 bp fragments could be achieved in good yield and the products shown to contain the desired thiophosphorylated sites by cleavage using the method of Gish and Eckstein (26).

By analogy with other enzyme systems, modification of the preferred gyrase cleavage site by incorporation of a thiophosphoryl residue might be expected to give rise to any of the following: (i) prevention of cleavage, thus generating a gyrase inhibitor; (ii) shifting of the cleavage site either locally or to more remote secondary cleavage sites; (iii) selective cleavage of one of the diastereoisomers at reduced rates; (iv) similar cleavage properties to the parent 147 bp fragment. The results presented above show that the thiophosphorylated 147 bp fragments appear all to be cleaved with equal efficiency as the parent 147 bp DNA fragment. Sizing gels have shown unequivocally that cleavage occurs at the same site indicating that gyrase can catalyse the cleavage of a thiophosphoryl linkage. Importantly, the ^{31}P-nmr of the two thiophosphorylated oligonucleotides had shown the expected intensity ratios of thiophosphate to phosphate. However, to exclude the possibility that the observed bands arise from cleavage of small contaminating amounts of the unthiophosphorylated 147 bp fragments, experiments were conducted in which the extents of cleavage were greater that 50% which confirms that gyrase is indeed cleaving the thiophosphoryl group. The latter experiments also suggest that gyrase is capable of cleaving ***both*** of the diastereoisomers of the thiophosphate diesters. In many other systems a significant stereoselectivity is observed, hence the observation that gyrase can cleave both diastereomers is an intriguing one.

Darby and Vosberg (32) have analysed the interaction of calf thymus topoisomerases I and II with thiophosphate-substituted DNA. The substrates used in this study were double-stranded DNA circles comprising one unsubstituted strand and one strand substituted at virtually every position of a specified base or bases. (However, a short stretch of unsubstituted double-stranded DNA was present in all cases.) In these experiments they found significant inhibition of the relaxation reactions of both topoisomerases, and, with a substrate containing one almost fully substituted strand, no relaxation by topoisomerase II could be detected. Interestingly this latter substrate was cleaved as efficiently as the unsubstituted DNA by topoisomerase II suggesting that inhibition of relaxation may occur at the DNA religation step of the topoisomerase reaction. (However, the possibility that the cleavage reaction occurred at the short stretch of unsubstituted DNA could not be completely excluded.)

The approach of specific thiophosphate substitution of DNA has previously been employed in studies of λ Int protein, Mu transposase and HIV-1 IN protein (19−22). These are topoisomerase-like proteins which normally catalyse intermolecular rather than intra-molecular strand transfer processes. With λ Int protein, only S_p-phosphorothioate linkages are efficient substrates and, following the recombination reaction, the configuration of the phosphorothioate is retained, suggesting two sequential trans-esterification steps involving a covalent protein- DNA intermediate. The Mu transposase, by contrast, shows inversion of configuration of the phosphorothioate, supporting a one-step trans-esterification mechanism in this case. Similarly , the reactions of the HIV-1 IN protein also proceed with inversion, again suggesting a one-step mechanism.

The observation that gyrase can catalyse the cleavage of a thiophosphoryl linkage as efficiently as a phosphoryl linkage is, on first consideration, rather surprising, but is consistent with the observations made with the other topoisomerases and topisomerase-like proteins described above. It must be remembered however,that the drug-induced cleavage reaction of gyrase is a disruption of the normal catalytic cycle and the rate-limiting step in this abnormal— cycle is not known. It is likely that the chemical cleavage step is not the rate-limiting step and therefore a difference in rate of the cleavage step with the thiophosphorylated fragments may not show up in the overall rate.

The results of this study have demonstrated that DNA gyrase can cleave thiophosphorylated DNA, thus, in principle, it will be possible to determine the stereochemical course of this reaction.

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