

# Effect of netropsin, distamycin A and chromomycin A<sub>3</sub> on the binding and cleavage reaction of DNA gyrase

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Received 26 May 1994; revised version received 30 August 1994

**Abstract** The influence of netropsin (Nt), distamycin A (Dst-3) and chromomycin A<sub>3</sub> (CHR) on the binding of gyrase from *Streptomyces noursei* to an 162 bp-fragment of pBR 322 containing a strong gyrase cleavage site and on the gyrase mediated cleavage of this fragment was analyzed. Binding of the enzyme to the fragment is effectively inhibited by the GC-specific drug CHR, but poorly influenced by Dst-3, while Nt is ineffective. Cleavage of the fragment catalysed by the enzyme is inhibited by all three ligands but to different extent. Dst-3 and Nt inhibit the enzyme cleavage reaction at 20- or 250-fold higher concentration than that required for CHR. The inhibitory mechanism of CHR on gyrase-DNA binding and cleavage may be related to a competitive interaction of the ligand to GC sequences located at and around the gyrase cleavage site. The fact that AT-specific minor groove binders Dst-3 and Nt poorly inhibit the binding of gyrase to the fragment due to the low amount of the AT basepair sequences contained in the fragment and their inhibitory influence on the cleavage step underlines the role of the DNA minor groove during enzyme action.

**Key words:** Minor groove binder; Inhibition; DNA gyrase; Enzyme–DNA binding; Cleavage

## 1. Introduction

DNA gyrase is a prokaryotic type II topoisomerase which catalyses the negative supercoiling of DNA [1–3]. The mechanism of DNA supercoiling by gyrase involves cleavage of DNA double strands, an ATP-driven passage of a DNA segment through the cleavage site and a religation step [4]. It has been shown that Gyr A is responsible for cleavage and religation, whereas Gyr B is involved in the ATP-dependent strand passage step [4–6].

Gyrase is a target for two classes of antibiotics – the quinolones and the coumarins. Quinolones (e.g. ciprofloxacin) act on Gyr A [7] or on DNA in the enzyme complex [8] and coumarins act on Gyr B [7]. It has been recognized that the high antigyrase activity of quinolones observed for the *E. coli* enzyme is not the same for other bacteria [9]. A very weak or no antigyrase effect on quinolones was observed for the enzymes from *Streptomyces noursei* [10] and *Micrococcus luteus* [11]. It was further shown that drugs which are DNA minor groove binders represent inhibitors of DNA gyrase in vitro [12]. Since a sequence specificity is known for several minor groove binders [13] these agents can be regarded as useful probes in the elucidation of gyrase binding and cleavage sites on DNA. Differences in drug sensitivity of gyrases and the molecular mechanism of their interference with the enzyme interaction on DNA is still an unsolved problem. In the present work we used purified subunits and the reconstituted enzyme from *Streptomyces noursei*. For the analysis of the effect of minor groove binders on the binding and cleavage reaction of DNA gyrase a DNA fragment from pBR 322 was used as a substrate.

## 2. Materials and methods

### 2.1. Enzymes, DNA and ligands

Isolation and purification of DNA gyrases was performed according to Staudenbauer and Orr [14]. In case of gyrase from *Streptomyces*

*noursei* a distinct modification of the procedure was required as described elsewhere [15].

The 162 bp fragment from pBR 322 consisting of the base pairs 936 to 1097 was obtained by digestion with *Bgl*I and *Sau*3a. It contains a strong cleavage site for *E. coli* gyrase at base pair 990 [16].

Netropsin (Nt) was used as described previously [13], distamycin A (Dst-3) and chromomycin A<sub>3</sub> (CHR) were purchased from Serva (Heidelberg) and Fluka, respectively. For chemical structures see [13].

### 2.2. Gel retardation assay

Binding of gyrase to the 162 bp fragment was detected by the mobility-shift assay using a <sup>32</sup>P-end labelled DNA fragment in solution containing 40 mM Tris (pH 7.5), 19 mM KH<sub>2</sub>PO<sub>4</sub>, 34 mM KCl and 3 mM MgCl<sub>2</sub> to achieve enzyme binding to DNA. Ligands were dissolved in distilled water and added to the assay mixture before enzyme addition. The mixture was incubated for 30 min at 30°C. Samples were electrophoresed in 5% polyacrylamide gels (3% cross-linking with bisacrylamide) in 90 mM Tris-borate, pH 7.0, buffer containing 3 mM MgCl<sub>2</sub>.

### 2.3. Cleavage assay

Cleavage of the <sup>32</sup>P-end labelled 162 bp fragment was assayed in a buffer containing 50 mM Tris (pH 7.5), 55 mM KCl, 5 mM DTT, 4 mM MgCl<sub>2</sub> and 5% glycerol. 100 μM ciprofloxacin (Bayer AG, Leverkusen) was added to prevent religation of the cleaved fragment. Samples were incubated 50 min at 30°C. SDS and proteinase K (Sigma) were added and incubated 30 min at 37°C. The reaction was terminated by EDTA and samples were deproteinized with an equal volume of phenol. The aqueous phase was applied to electrophoresis in a 7.5% (3% cross-linking) polyacrylamide gel in a 90 mM Tris-borate buffer, pH 7.0.

## 3. Results

### 3.1. Interaction of *Streptomyces noursei* gyrase with an 162 bp fragment

DNA gyrase interaction with the 162 bp DNA fragment was demonstrated by using gel retardation assays. In presence of Mg<sup>2+</sup> a discrete band is formed on polyacrylamide gels, which indicates complex formation between the fragment and gyrase. Fig. 1 shows the ability of gyrase from *Streptomyces noursei* to bind to this fragment, which contains the preferred gyrase cleavage site around nucleotide 990 known for *E. coli* gyrase [16,17]. Complex formation with native gyrase (A<sub>2</sub>B<sub>2</sub>) is achieved at a 10–12-fold molar excess of the enzyme-to-DNA

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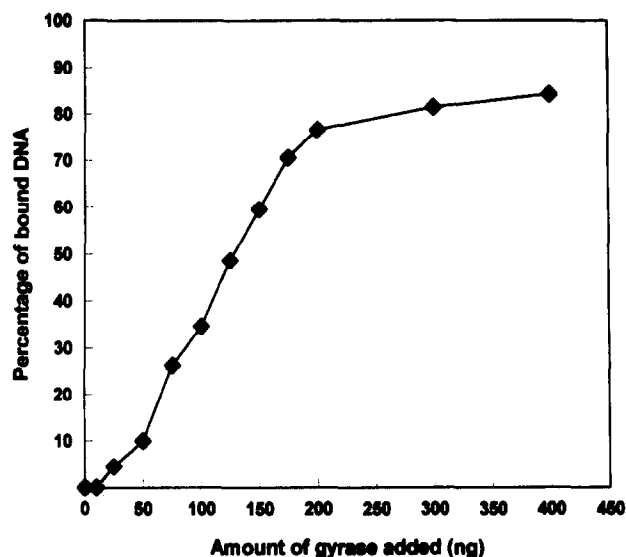


Fig. 1. Binding of *Streptomyces* gyrase to the 162 base pair fragment using the gel retardation assay (see section 2); DNA = 1 ng  $^{32}\text{P}$ -labelled 162 bp fragment; \* = amount of DNA from densitometrically integrated peak areas.

whereas at 60-fold molar excess of the enzyme binding approaches the saturation level (Fig. 1).

Comparison of the binding of gyrases from *Streptomyces noursei* and *E. coli* shows that a 30-fold molar excess of the *Streptomyces* enzyme is necessary to bind 50% of the DNA, whereas already a 15-fold molar excess of the *E. coli* gyrase binds 50% of this DNA fragment. Therefore we can conclude that the strong binding site at nucleotide 990 of the fragment from pBR 322 is also a site for *Streptomyces noursei* gyrase although the affinity of the *E. coli* gyrase to this site is two times higher. For comparison, completion of the transition of relaxed pBR 322 plasmid DNA into its supercoiled form mediated by *Streptomyces* gyrase requires a molar ratio of about 570–600:1 of DNA per enzyme (not shown). The Gyr A subunit alone, which is a very pure fraction [15], also forms a DNA–protein complex but at higher concentration (Fig. 2, lanes 3–6). Addition of Gyr B to Gyr A favours binding of Gyr A to the fragment (Fig. 2, lanes 10–13), while Gyr B alone does not form a complex.

Evidence for a specific complex formation of the enzyme with DNA can be shown to occur by applying competitor DNAs. As expected the native plasmid pBR 322 strongly inhibits binding of gyrase to the 162 bp fragment whereas other DNA fragments, for instance the Epstein-Barr-Virus nuclear antigen binding region, were ineffective (data not shown). Using pBR 322 DNA devoid of the 162 bp fragment as a competitor, a 50-fold higher concentration of the unlabelled DNA over the labelled 162 bp fragment was required to achieve initial inhibition, a 100-fold higher concentration for 50%, and a 200-fold higher concentration for the complete inhibition of the binding of *Streptomyces noursei* gyrase to the 162 bp fragment. Data for the *E. coli* enzyme are 6-, 15- and 56-fold higher concentrations of this competitor DNA for initial, 50% and complete inhibition, respectively. Since native pBR 322 is much more effective as competitor DNA the role for the affinity of *Streptomyces* gyrase to the 162 bp fragment is underlined.

Using poly(dI-dC)·poly(dI-dC) as a competitor we found that a 200-fold higher concentration of this synthetic polymer over the labelled 162 bp fragment was required to achieve initial inhibition whereas a complete inhibition occurred only at 400-fold excess of the polymer (data not shown). These results suggest that a specific interaction is involved in the *Streptomyces* gyrase complex formed with the 162 bp fragment.

### 3.2. Effects of minor groove binders on gyrase activity and enzyme–DNA binding

Based on indirect evidence nonintercalating agents may inhibit gyrase activity by interaction in the minor groove of the DNA which probably blocks binding of the enzyme at the cleavage and religation site [12]. To obtain further insight into the mechanism of the interference of groove binders with the gyrase reaction we examined the effect of three sequence specific DNA-ligands: Nt and Dst-3, which both prefer AT base-pairs [13] and CHR A<sub>3</sub> which strongly prefers GC basepairs [18,19]. We have studied the influence of these DNA specific ligands on the enzyme binding to the 162 bp fragment containing a gyrase site and on the gyrase mediated cleavage of this fragment that occurs in presence of ciprofloxacin. The preferred cleavage site at basepair 990 known for the *E. coli* enzyme must not be the same for the *Streptomyces* gyrase.

However, from parallel assays with both enzymes analyzed on the same gel and from a gel electrophoretic analysis of the labelled ciprofloxacin induced cleavage fragment we assume that the cleavage site for *Streptomyces* gyrase must be located at or near basepair 990 (Fig. 3A). The quinolone-induced gyrase cleavage reaction requires a much higher ciprofloxacin concentration for the *Streptomyces* enzyme [10] compared to that of *E. coli*.

For comparison the influence of Nt, Dst-3 and CHR on the ciprofloxacin-induced linearization of pBR 322 DNA as well as the effect of these ligands on the supercoiling reaction are included in Table 1. In agreement with previous data [12] these

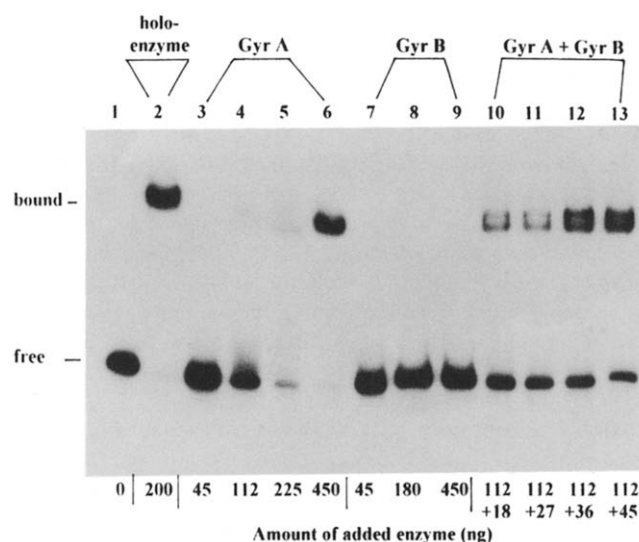


Fig. 2. Binding of DNA gyrase subunits to the 162 base pair fragment using the gel retardation assay (see section 2); DNA = 1 ng  $^{32}\text{P}$ -labelled fragment; subunits of gyrase from *Streptomyces noursei*. The concentrations in lanes 10–13 correspond to constant amount of GyrA (upper row) and increasing amounts of GyrB (lower row).

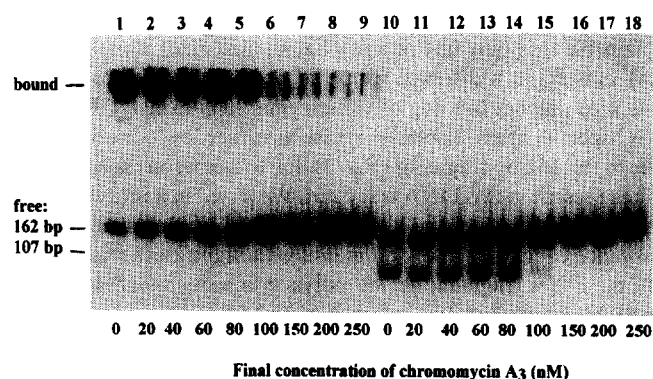


Fig. 3. Inhibition of the DNA gyrase mediated binding and cleavage of the 162 base pair fragment with CHR. Band shift assay (lanes 1–9) was done with 1 ng  $^{32}$ P-labelled fragment; DNA gyrase (reconstituted from subunits) from *Streptomyces noursei* 300 ng. Cleavage assay (lanes 10–18) contained = 1 ng  $^{32}$ P-labelled fragment, subunits of DNA gyrase from *S. noursei*. Concentration of CHR are nanomolar (nM). Electrophoresis: see section 2.

minor groove binders are effective inhibitors of the supercoiling reaction. From the present results it is evident that they affect especially the ciprofloxacin-induced cleavage to different extent. The gyrase cleavage reaction is completely blocked by CHR at 10  $\mu$ M which corresponds to a total molar ratio, ligand per basepair  $r' = 0.36$  (Table 1). Nt inhibits the ciprofloxacin-induced linearization of the plasmid above 50  $\mu$ M corresponding to a much higher ratio of  $r' = 1.8$ . This indicates that the GC-specific CHR is virtually more potent to inhibit this reaction step than the AT-specific Nt. Dst-3 is also more effective than Nt, but it is somewhat less potent than CHR.

Results of studies on the influence of these three minor groove binders on the binding and cleavage of the 162 bp fragment containing a single gyrase site are demonstrated in Figs. 3 and 4 and are summarized in Table 1. There is a large difference in the inhibitory effects on gyrase binding between Nt, Dst-3 on one hand and CHR on the other side. The displacement of gyrase from the DNA fragment induced by CHR occurs already at a final concentration of 30 nM of the ligand ( $I_i$ , Table 1) which corresponds to  $r'$  of 1 (total ratio) and is complete at  $r'$  of 2.6 (100 nM), whereas Nt and Dst-3 show an initial inhibitory effect at extremely high ligand concentration only (Fig. 4A, Table 1); i.e. CHR is about 10,000-fold more effective than Nt.

Fig. 4B shows that in contrast to these binding data the gyrase mediated cleavage of the 162 bp fragment is effectively inhibited by all three ligands. The concentrations for initial,

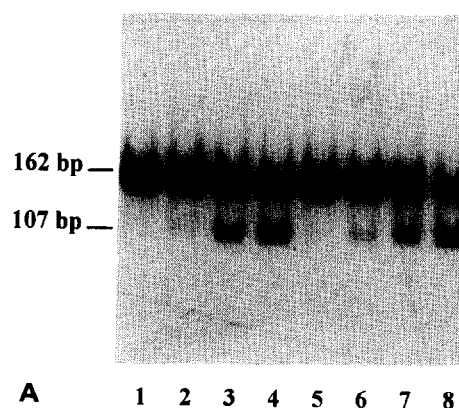


Fig. 3. (A) Cleavage of the 162 base pair fragment mediated by gyrase from *S. noursei* (lanes 1–4 = 10, 25, 40, 80 ng enzyme, respectively) and *E. coli* (lanes 5–8 = 7.5, 15, 30, 50 ng enzyme, respectively). These results allow us to assume that the cleavage site of *Streptomyces* gyrase is located at or very near to the *E. coli* gyrase site at base pair 990 (see Fig. 5).

50% and complete inhibition for CHR are the same for the cleavage as well as for the binding step and suggest that the ligand directly interferes with the enzyme binding and subsequent cleavage reaction by interaction with the GC-preferred regions of the DNA. Dst-3 inhibits the cleavage of the fragment at 20-fold higher concentrations and Nt inhibits at 200–250-fold higher concentrations than CHR. Nevertheless, the inhibitory concentrations of Nt on the cleavage of this specific gyrase site correlate with concentrations of this ligand which inhibit the overall supercoiling reaction of pBR 322. Dst-3 inhibits this specific site about 5-fold more effective than the overall supercoiling of the plasmid but CHR inhibits 100-fold stronger. The most striking differences in the behaviour of the three ligands we found in their influence on the binding of gyrase to the DNA fragment.

#### 4. Discussion

In our present work we used the novobiocin-Sepharose purified gyrase from *Streptomyces noursei*, its subunits and the reconstituted enzyme to investigate the interference of three minor groove binders with an active gyrase site contained in a DNA fragment. From these studies three aspects can be visualized: (i) *Streptomyces* gyrase binds selectively to the 162 bp fragment; (ii) the enzyme binding to its cleavage site is specifically abolished by CHR but less by Dst-3 and not by Nt; and (iii) the ciprofloxacin induced cleavage of the fragment mediated by *Streptomyces* gyrase is preferentially inhibited by CHR,

Table 1  
Influence of netropsin, distamycin A and chromomycin A<sub>3</sub> on the activity of DNA gyrase from *Streptomyces*

Ligand	Supercoiling of pBR 322			Linearization of pBR 322			Binding to the 162 bp-fragment			Cleavage of the 162 bp-fragment		
	$I_C$ ( $\mu$ M)	$I_{50}$ ( $\mu$ M)	$I_i$ ( $\mu$ M)	$I_C$ ( $\mu$ M)	$I_{50}$ ( $\mu$ M)	$I_i$ ( $\mu$ M)	$I_C$ ( $\mu$ M)	$I_{50}$ ( $\mu$ M)	$I_i$ ( $\mu$ M)	$I_C$ ( $\mu$ M)	$I_{50}$ ( $\mu$ M)	$I_i$ ( $\mu$ M)
Nt	30*	8*	2.5*	100	50	10	–	–	300	25	12	8
Dst-3	10*	5*	1.5*	20	10	3	–	300	50	2	1	0.8
CHR	10*	5*	1*	10	5	1	0.1	0.06	0.03	0.1	0.06	0.03

$I_C$ ,  $I_{50}$  and  $I_i$  = complete, 50% and initial inhibition;  $\mu$ M = final concentration of ligands, micromolar.

\*Compare data with [12].

– = inhibition could not be found up to 1 mM concentration of ligands.

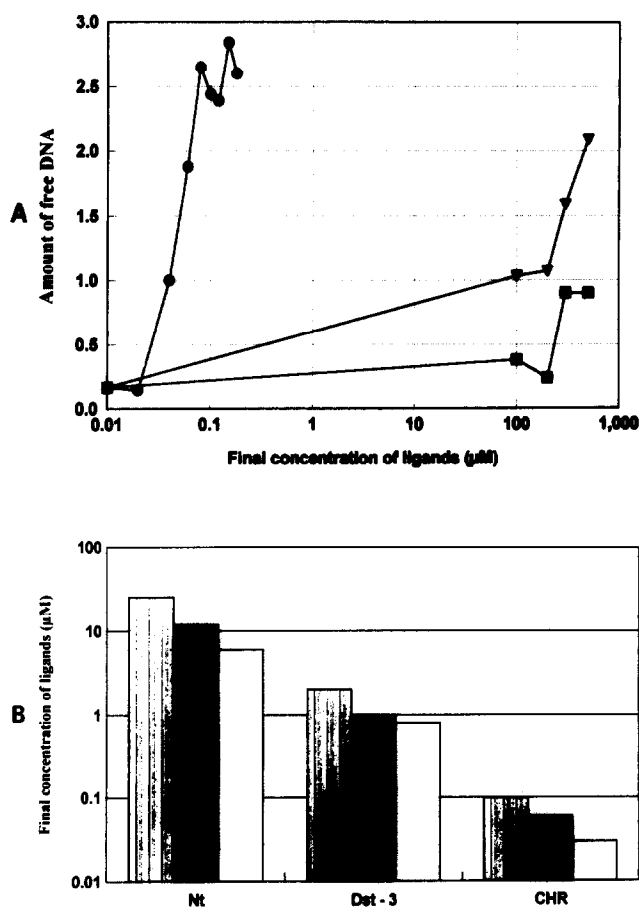


Fig. 4. (A) Inhibition of the binding of gyrase from *Streptomyces noursei* to the 162 bp fragment by ligands. Final concentration of ligands means micromolar concentration in the assay (circles); CHR, squares; Nt, triangles; Dst-3, amount of free DNA derived from densitometrically integrated peak areas. DNA = 1 ng  $^{32}$ P-labelled fragment. (B) Inhibition of the gyrase mediated cleavage of the 162 bp fragment by ligands. Final concentration of ligands means micromolar at which ligands show complete (dark grey), 50% (black) or initial (light grey) inhibition of the ciprofloxacin-mediated cleavage. DNA = 1 ng  $^{32}$ P-labelled fragment.

20-fold less by Dst-3 and 200-fold less by Nt. It should be mentioned that we assigned the cleavage site of *Streptomyces noursei* gyrase to the 990 base pair position as known for the *E. coli* enzyme, since the size of the ciprofloxacin-induced cleavage fragment justifies this assumption.

Dst-3 and Nt can quite effectively inhibit the supercoiling and cleavage reaction of gyrase when the plasmid is used as a substrate, but (Table 1) they are nearly ineffective to prevent binding of the enzyme to the DNA fragment (Fig. 4A). The pronounced inhibitory effect of the minor groove binder CHR on the binding of gyrase and cleavage of the fragment by gyrase suggests that these reaction steps, mediated by the Gyr A protein, are directly blocked by interaction of the drug with GC-sequences (Fig. 5) in the minor groove. The lower effectiveness of Dst-3 and the inability of Nt to compete with gyrase binding on the 162 bp fragment is most probably related to the small number of AT affinity sites at and around the gyrase cleavage site (Fig. 5). Strong binding of Dst-3 and Nt will occur preferentially on AT clusters [13]. Since about 120 base pairs of DNA

are wound around the native enzyme tetramer  $A_2B_2$  [25], it is plausible that only one or a few ligand molecules competing on AT clusters at a distance from the cleavage center are insufficient to prevent binding of the enzyme. In contrast, CHR as a GC-specific agent displaces gyrase at rather low concentration from the 162 bp fragment (Fig. 3) due to numerous specific GC sites present in this DNA fragment. We tested in a mobility shift assay the affinity of the ligands to the 162 bp fragment (in the absence of the enzyme) and measured a significant retardation of the DNA fragment (in 8% polyacrylamide, 5% cross-linked with bis-acrylamide) in presence of 2  $\mu$ M CHR whereas Nt and Dst-3 even at 1 mM showed no or only a weak retardation effect (data not shown). CHR binding to GC sequences in the minor groove requires  $Mg^{2+}$  ions [18–21].

NMR [19] and footprinting data [18,20] showed that in presence of  $Mg^{2+}$  CHR binds as a dimer to the central sequence 5'TGGCC of an octanucleotide and of an 18-mer with a binding constant of  $2.7 \times 10^7 M^{-1}$  [18]. This central sequence reported for a strong site of CHR is identical with the recognition and cleavage site of gyrase in the 162 bp fragment (Fig. 5). Thus we propose that CHR strongly competes at the cleavage site of gyrase and in the surrounding GC-rich regions. NMR measurements further revealed that binding of the CHR dimer to the sequence GGCC causes a modification of the groove width resulting in an A-like conformation [19]. This conformational change can drastically diminish the enzyme affinity constant which additionally prevents gyrase binding. In contrast to that Nt and Dst-3 are inserted into the minor groove of B-DNA with a minimal change in groove width [22]. It has been argued that intrinsic curvature of DNA surrounding the regions of the 990 site of pBR 322 could be an essential factor responsible for the preference of gyrase binding and cleavage of this site [23]. On the other hand, it was shown by X-ray crystal structure analysis of the decamer CATGGCCATG that a curvature occurs over the GGCC center [24]. It seems possible that this intrinsic bend compressing the major groove is a local structural element which could promote the enzyme mediated cleavage. Minor groove binding of CHR to the GGCC center abolishes DNA curvature which may be another feature of the inhibitory effect. The CHR-induced inhibition is also a reflection of the drug binding in surrounding sequences, since in the gyrase DNA complex a DNA segment of about 120 base pairs is occupied by the protein [25]. This may be the reason that Nt and Dst-3, which have a strong binding site at a 10 bp distance from the cleavage site (Fig. 5, AT-cluster 1001–1005) do not prevent the binding of gyrase to the fragment, but both drugs influence the

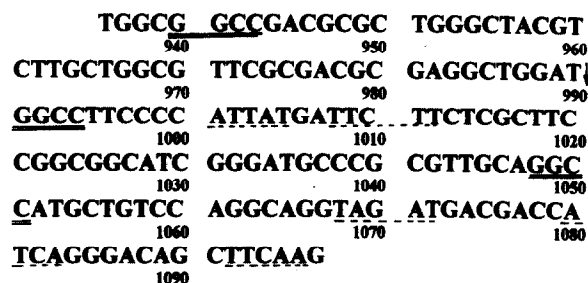


Fig. 5. Sequence of the 162 bp fragment from pBR 322 (see section 2), GC content = 64 mol%. Suggested strong binding sites for CHR (==), possible strong and weak binding sites for Nt and Dst-3 (-----), specific gyrase cleavage site at AT<sub>990</sub> (see arrow).

cleavage step of the enzymatic reaction. Dst-3 inhibits the cleavage 10 times more effective than Nt. We explain this fact with possible binding of Dst-3 molecules e.g. at the nucleotides 1007–1012 and 1068–1072. At regions consisting of AT- and GC-basepairs Dst-3 will bind with a higher probability [13,26] than Nt does resulting in a higher inhibitory efficiency of Dst-3 (Fig. 4B). This implies that the enzyme is still bound to its substrate DNA but is prevented to cleave when a few minor groove binders block sequences in surrounding regions of the cleavage center. Therefore it is quite possible that a higher number of AT-clusters in the vicinity of a gyrase site can lead to a strong competitive binding effect of Nt and Dst-3. This could be anticipated since the inhibitory effect on the supercoiling activity of *Streptomyces* gyrase differs not significantly between these AT-selective ligands and CHR (Table 1, [12]), which awaits, however, further investigation.

Our results show that sequence specific minor groove binders, such as CHR can probe the recognition site of gyrase as also demonstrated for restriction endonucleases [13,27,28]. This could reflect the role of the minor groove for the gyrase–DNA interaction. Although DNA sequence recognition by proteins preferably occurs in the major groove [29], in some cases the minor groove is involved in DNA protein interaction; e.g. minor groove contacts have been shown to exist for DNase I binding to DNA [30] and for a binding domain of Hin recombinase [31]. Whether in the process of gyrase–DNA interaction and cleavage the minor or major groove or both play a role for contacts with domains of the Gyr A protein cannot be concluded from the present data.

**Acknowledgements:** We thank Mrs. U. Amthor for technical assistance and Dr. M. Roth for cultivation of the *Streptomyces* strain by applying a continuous culture procedure. This work was supported by the Deutsche Volkswagen-Stiftung (Grant AZI/65733).

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