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Mapping the Topography of DNA Wrapped around Gyrase by Nucleolytic and Chemical Probing of Complexes of Unique DNA Sequences

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Summary

Complexes between DNA gyrase and DNA fragments of unique sequences were used to probe the topography of the DNA with nucleases and dimethyl sulfate. The results indicate that the flanking regions, each 50 bp in size, of a 145–155 bp DNA segment resistant to staphylococcal nuclease contain groups of pancreatic DNAase I-susceptible sites that are spaced 10–11 nucleotides apart. Pairs of adjacent DNAase I-sensitive sites on complementary strands are typically staggered by 2–4 bp. The binding of DNA to gyrase confers no protection against alkylation of the DNA by dimethyl sulfate. These properties of the gyrase–DNA complex are reminiscent of those of the nucleosome, and the common underlying structural feature appears to be the wrapping of the DNA around a protein core. The gyrase–DNA complex differs from the nucleosome, however, in that it must possess features necessary for the catalysis of DNA chain breakage and the modulation of the DNA–enzyme interaction by ATP. We present evidence that the breakage and rejoining of the DNA by gyrase occur within a central region of the staphylococcal nuclease-resistant DNA segment. The relation of this observation to the mechanism of DNA supercoiling by gyrase is discussed. Addition of ATP or its β,γ-imido analog has essentially no effect on the patterns of susceptibilities to DNAase I, implying that the DNA–enzyme contacts mapped by the nuclease are little affected by ATP-induced conformational changes.

Introduction

The enzyme DNA gyrase was originally discovered as an activity in Escherichia coli that catalyzes the ATP hydrolysis-coupled negative supercoiling of DNA (Gellert et al., 1976). Probably as a result of this supercoiling function in vivo, gyrase has been shown to affect a number of vital processes, including replication and transcription (for review see Cozzarelli, 1980). More recently, it has been found that gyrase, as well as a number of other type II DNA topoisomerases, can also convert topologically knotted double-stranded DNA rings to the unknotted form (Liu et al., 1980; Mizuuchi et al., 1980), and can catenate and decatenate duplex DNA rings (Kreuzer and Cozzarelli, 1980; Baldi et al., 1980; Hsieh and Brutlag, 1980). From elementary topological considerations of the unknotting, catenation and decatenation reactions with duplex DNA rings, it is apparent that the enzyme must be capable of introducing transient breaks into both strands of a DNA molecule. The unlinking of two duplex rings, for example, cannot be accomplished without a sequence of events involving the transient breakage of both strands of at least one of the duplex rings, the passage of the other duplex ring through the break and the resealing of the broken strands. The negative supercoiling of a DNA, on the other hand, could be achieved with either transient single-stranded breaks or transient double-stranded breaks. An examination of the way the linking numbers of the DNA molecules change in the course of such a reaction, however, suggests that here, too, double-stranded breakage and rejoining events are involved. The linking number of a DNA is found to reduce by units of 2 in this reaction (Brown and Cozzarelli, 1979; Mizuuchi et al., 1980). This dyadic change is entirely consistent with the double-stranded transient breakage and passage mechanism, provided that the DNA segments straddling the transient break are not allowed to rotate relative to each other around the helical axis (Fuller, 1978).

There is little information, at the molecular level, on how negative supercoiling of a DNA is achieved by the passage of a DNA segment through a transient double chain scission. In order to gain some insight, we have initiated experiments on the topography of the gyrase–DNA complex. We present the mapping of gyrase–DNA contacts at the nucleotide level. A model describing a possible mechanism for the negative supercoiling of DNA by gyrase is presented elsewhere (Wang et al., 1980).

Results

Selection of Gyrase Binding Sites

We have selected three gyrase binding sites for probing contacts between the enzyme and DNA at the nucleotide level. Sites 1 and 2 were selected by the preferential retention of restriction fragments containing these sequences on glass fiber filters (Coombs and Pearson, 1978; Thomas et al., 1979), and site 3 was selected as one of the sites where gyrase-effected double-stranded cleavage occurs preferentially when alkali is added. A detailed description of the selection procedures for these sites is presented in the Experimental Procedures.

Probing the Unique DNA Gyrase–DNA Complexes with Pancreatic DNAase I Indicates That the DNA Is Wrapped outside Gyrase

Details of the interaction between gyrase and DNA were obtained by probing the complex with pancreatic DNAase I in the manner described by Galas and Schmitz (1978), using appropriate restriction fragments containing the selected gyrase-binding sites.

The results of a representative experiment are depicted in Figure 1. A 199 bp restriction fragment

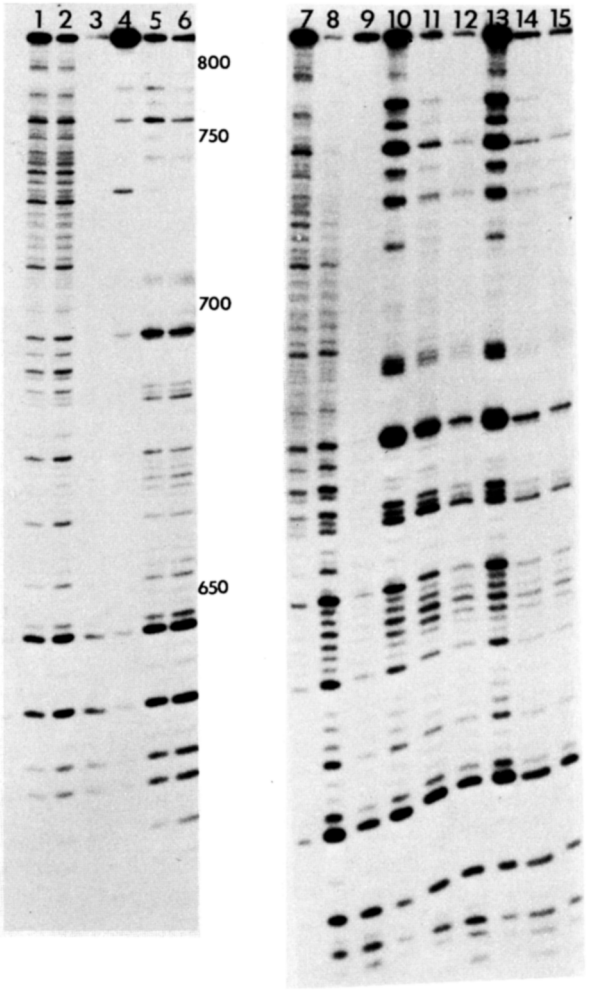

Figure 1. Pancreatic DNAase I Cleavage Patterns of a Singly End-Labeled DNA Restriction Fragment in the Presence and Absence of Gyrase

The Hinf I restriction fragment 7a of pBR322 DNA was labeled at the 5′ ends with ^{32}P and then cleaved with restriction endonuclease Hind III. The 199 bp fragment spanning from positions 631 to 830 on the Sutcliffe (1978) map was isolated by preparative gel electrophoresis as described by Maxam and Gilbert (1980). This fragment was probed with pancreatic DNAase I in the presence and absence of gyrase, as described in the Experimental Procedures.

Two separate experiments are shown. Samples were run in groups of three, with DNAase concentrations of 0.002 μg/ml (lanes 1, 4, 7, 10 and 13), 0.006 μg/ml (lanes 2, 5, 8, 11 and 14) and 0.02 μg/ml (lanes 3, 6, 9, 12 and 15). (Lanes 1–3 and 7–9) DNAase I cleavage patterns of the end-labeled DNA in the absence of gyrase; (lanes 4–6 and 10–12) corresponding patterns in the presence of excess gyrase. The numbers to the right of lane 6 give the sequence location (Sutcliffe, 1978). Samples run in lanes 13–15 correspond to those run in lanes 10–12, except 1 mM ATP was present during digestion with DNAase I, and the gel was electrophoresed for a slightly longer time. Only those lanes showing less than one cleavage per DNA fragment, such as lanes 4 and 7, were scanned in sections with a densitometer and quantitated as described in the text.

labeled at one of the 5′ ends was digested lightly with pancreatic DNAase I in the absence or presence of gyrase, and the digests were analyzed on a DNA sequencing gel and autoradiographed. Lanes 1–3 show the patterns of cleavage of the fragment in the absence of gyrase and lanes 4–6 show the patterns of cleavage in the presence of gyrase. The three samples within each set were digested with increasing amounts of the nuclease, to facilitate the comparison between patterns obtained in the presence and absence of the bound protein (Galas and Schmitz, 1978).

An examination of these patterns indicates that the binding of DNA gyrase to this 199 bp fragment yields a unique pattern of enhanced and diminished suspectibilities to cleavage by pancreatic DNAase I along the DNA strand. The total absence in the digests with gyrase of some bands present in the controls without gyrase demonstrates that the gyrase-binding site is saturated.

The regions where the pancreatic DNAase I cleavage patterns differ in the presence and absence of bound gyrase coincide with the regions mapped by protection against staphylococcal nuclease. It has been shown previously that staphylococcal nuclease digestion of the gyrase complex of calf thymus or phage T7 DNA yields protected DNA fragments averaging 143 bp in length (Liu and Wang, 1978a; Klevan and Wang, 1980). For the selected sites of unique sequences, a length of 145–155 bp of each site resistant to this nuclease has been mapped, as indicated in Figure 2. Outside this region, the DNAase I cleavage patterns in the presence and absence of gyrase are essentially identical. Upon comparison of samples that have been lightly digested such that there is less than one DNAase I scission per DNA fragment, the ratios of peak heights on the densitometer tracing of the bands in the presence and absence of gyrase in the affected region can be normalized to the average value of the ratios of bands in the unaffected region, where the intensities differ primarily by the amount of radioactivity in the samples. This provides a rough quantitation of the extent of enhancement and protection of each DNAase I-produced band. The patterns obtained from the autoradiogram shown in Figure 1 and similar experiments are schematized in Figure 2. Aside from the sites of enhanced suspectibility to DNAase I attack (simple arrows), essentially all other phosphodiester bonds within the staphylococcal nuclease-resistant region are protected to varying extents against DNAase I by the binding of gyrase. The hollow arrows shown in Figure 2 will be described in a later section.

The most prominent feature of the DNAase I-mapped "footprints" of DNA gyrase on the three sites examined is the unique spacing between adjacent sites of enhanced pancreatic DNAase I cleavage. In site 3 (Figure 2), for example, a group of six DNAase I sites on the left side of the top strand can be identified (a–f), with a spacing of 10 or 11 nucleotides between adjacent pairs within this group. A similar group of four sites can also be found on the right side (g–j). On

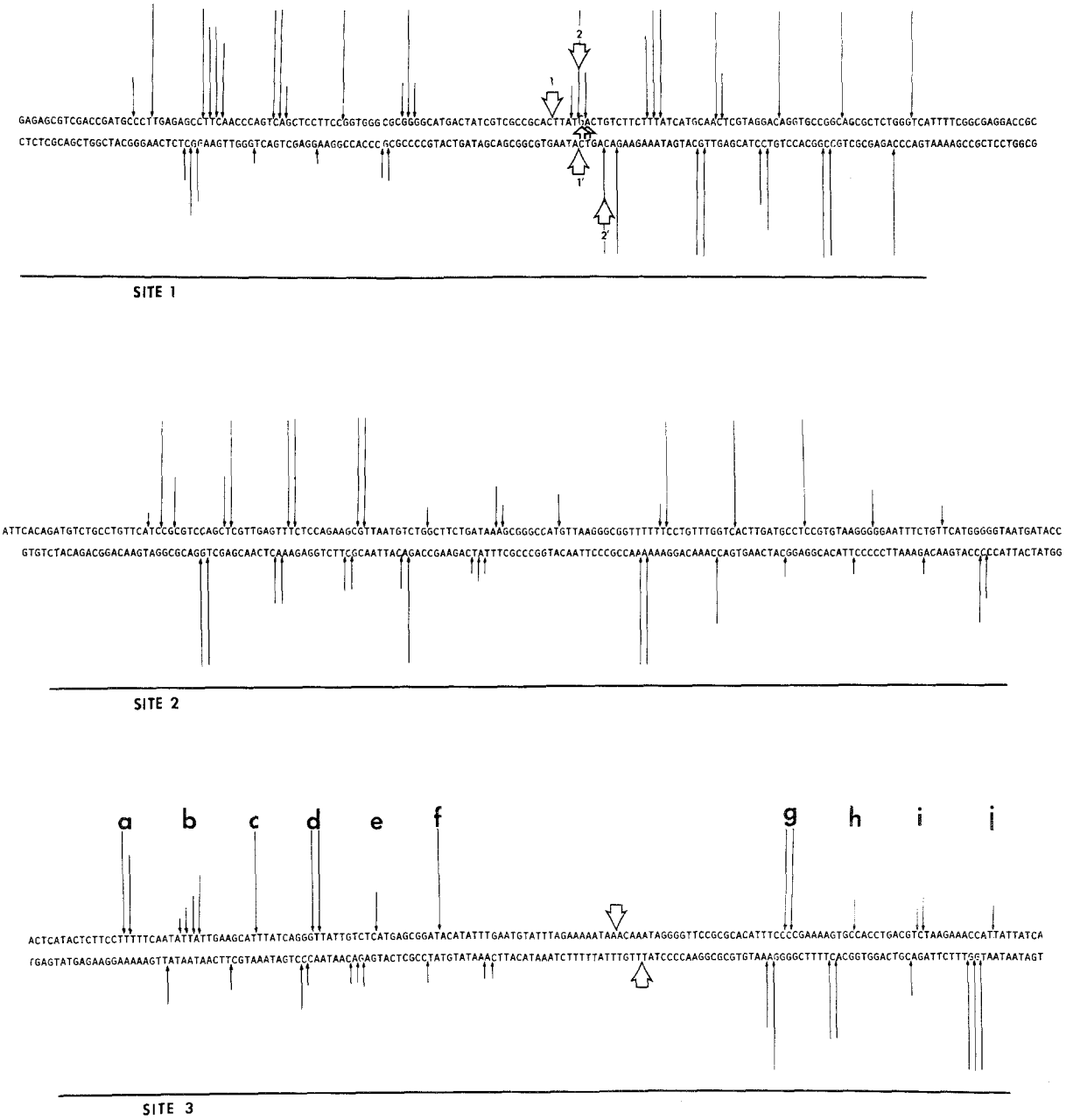

Figure 2. Topography of Gyrase–DNA Interaction at Three Sites

Simple arrows: locations of the phosphodiester bonds with apparently enhanced susceptibility to pancreatic DNAase I. The length of each arrow is proportional to the magnitude of the effect, except that no distinction is made if the apparent enhancement is 8 fold or greater.

Large hollow arrows: positions of gyrase-effected cleavage upon alkali treatment under appropriate conditions, as described in Results and Experimental Procedures.

Smaller hollow arrows between the DNA strands of site 1: two purines where enhanced reactivity to dimethyl sulfate is observed in the presence of gyrase.

The sequences given are from Sutcliffe (1978): from left to right they span base pairs 644–804 for site 1, base pairs 2033–1866 for site 2 and base pairs 4148–4308 for site 3. The underlined region for each site corresponds to the 145–155 bp fragment protected by gyrase against staphylococcal nuclease. Similar to the case with nucleosomes, there are internal staphylococcal nuclease cleavages within this protected region (Sollner-Webb et al., 1978). The general pattern of these internal cleavages resembles that of the DNAase I cleavage sites, and we have not attempted their mapping.

The two strands of site 1 were examined with samples of the 199 bp fragment, ^{32}P-labeled at either the 5′ or the 3′ termnus of the Hinf I end, as described in the legend to Figure 1. Site 2 was mapped with a 219 bp fragment, which is bounded by a Hpa II and a Hinf I site and spans positions from 1811 to 2030 on the map (Sutcliffe, 1978). Samples of the fragment singly labeled at the 5′ end of either the Hinf I terminus or the Hpa II terminus, or at the 3′ end of the Hinf I terminus were used. Site 3 was mapped with a 253 bp fragment from the unique Eco RI site to the Fnu 4HI site at position 4108. Samples of the fragment labeled at the 5′ terminus of the Eco RI end or the Fnu 4HI end were used in the mapping.

Lower-case letters: groups of DNAase I cleavage sites discussed in the text.

the lower strand, two groups of sites, again with a spacing of 10 or 11 nucleotides, are seen in the flanking regions. The 10–11 bp spacing of these sites coincides with the helical repeat of DNA, and is reminiscent of the DNAase I cleavage pattern seen in the nucleosome (Noll, 1974; Felsenfeld, 1978; Prunell et al., 1979). The presence of these groups of DNAase I-sensitive sites in the flanking regions of gyrase-covered sites 1 and 2 is again evident (Figure 2).

Within these flanking regions, the DNAase I-sensitive sites on one strand and those on the complementary strand are staggered. Typically, a site of enhanced cleavage on one strand is separated from the proximal site of enhanced cleavage on the complementary strand by 2–4 bp. This again resembles the case of the nucleosome. It has been deduced by less direct methods than the one employed here that approximately 2–3 bp separates the proximal sites on the complementary strands where cleavage by DNAase I occurs readily (Sollner-Webb and Felsenfeld, 1977; Lutter, 1977, 1979).

The conclusion that can be drawn from the DNAase I "footprinting" results is that each gyrase-binding site spans about 145–155 bp of DNA, and that within such sites are two flanking regions, each about 50 bp in length, that are wrapped around the enzyme. We will argue below that the central region of about 40 bp is located in the gyrase topoisomerization site—the site at which breaking and rejoining of the DNA strands occur.

The DNAase I Cleavage Pattern of Gyrase–DNA Complex Is Unaffected by ATP or the β,γ-Imido Analog of ATP

We have attempted to detect conformational changes of the gyrase–DNA complex, upon addition of ATP or its β,γ-imido analog pNHppA, by carrying out the DNAase I probing in the presence of these compounds. One such experiment is shown in Figure 1, lanes 7–12; lanes 7–9 show the cleavage patterns of the DNA–gyrase complex in the absence of ATP, and lanes 10–12 show the patterns in the presence of 1 mM ATP. No difference is observed. Analysis of the solutions at the end of digestion with DNAase I showed that ATP was still present in excess; the lack of a difference is therefore not due to the exhaustion of ATP. Similar experiments with pNHppA instead of ATP also show no detectable difference.

DNA Is not Protected by Gyrase against Methylation by Dimethyl Sulfate

Probing of the gyrase–DNA complex with dimethyl sulfate, according to the method of Gilbert et al. (1975), shows that there is no protection along the entire length of DNA in the complex. Among the three sites examined, the only difference observed is that the binding of gyrase to site 1 causes a 2 fold enhancement of methylation at two purines, a G and an A, demarcated by the two unnumbered hollow arrows in Figure 2. The addition of ATP or pNHppA caused no change in these patterns.

To ensure that the lack of protection by bound gyrase against the alkylating agent is not due to dimethyl sulfate-induced dissociation of the complex, in a separate experiment the complex was first treated with the alkylating agent and then probed with DNAase I *before* the alkali treatment and heating step that is used to effect DNA cleavage at the methylated purine sites. The same pattern is observed with DNAase I alone or with exposure of the DNA–gyrase complex to both dimethyl sulfate and DNAase I (data not shown). Thus the protein–DNA interactions remain unperturbed during treatment with the alkylating agent, and the conclusion is valid that there is no protection of the DNA by gyrase against methylation by dimethyl sulfate.

A similar situation has been observed in dimethyl sulfate treatment of nucleosomes obtained by staphylococcal nuclease digestion of chromatin. Differential reactivity of the random-sequence 140 bp DNA with and without the associated histones occurs at only one guanine in the central region, where methylation is enhanced in the presence of the histones (McGhee and Felsenfeld, 1979). The implication in both cases is that the DNA in the complex is freely accessible to solvent along its entire length.

The Sites of DNA Breakage and Rejoining by Gyrase Appear to Be Located in the Central Region of the Gyrase-Bound Sequence

It is known that under certain conditions, such as treatment with dodecyl sulfate in the presence of the gyrase inhibitor oxolinic acid, a bound gyrase can cleave DNA (Sugino et al., 1977, 1978; Gellert et al., 1977). The sites of cleavage on the complementary strands are staggered by 4 bp, and a protein moiety is found to be linked to each of the 5′ ends when such a cleavage occurs (Morrison and Cozzarelli, 1979). The protein–DNA link has been identified to be a phosphodiester bond formed between a tyrosine hydroxyl group on a gyrase α protomer and a DNA 5′ phosphoryl group (Tse et al., 1980).

It is likely that the positions on DNA where gyrase-induced cleavages occur under these conditions represent positions where transient breaks are introduced during the normal course of gyrase-catalyzed topoisomerization. Mechanistically, it is important to know the disposition of these gyrase cleavage sites relative to the pattern of gyrase–DNA contacts revealed by the nuclease probing experiments.

For site 3, which was selected for the presence of a preferred gyrase-induced cleavage site, the locations of gyrase-induced breaks are readily determined. A restriction fragment bearing this site and ^{32}P-labeled at one unique end was first incubated with gyrase in the presence or absence of oxolinic acid

and then exposed to alkali. Sizing of the products by electrophoresis in a sequencing gel reveals a single cleavage site in the labeled strand when both ATP and oxolinic acid were present (Figure 3a upper tracing). By comparing with samples of the same end-labeled restriction fragment that had been subjected to DNA sequencing reaction treatment and had been run in adjacent lanes on the same gel, the position of cleavage was determined. In the absence of either ATP or oxolinic acid, no cleavage is detectable. The enhancement of gyrase-effected cleavage by ATP has been observed previously by Morrison et al. (1980). Similar experiments led to the identification of a single cleavage site on the complementary strand, where gyrase-effected cleavage occurs in the presence of ATP and oxolinic acid. These sites of cleavage are indicated by the hollow arrows in Figure 2 (site 3).

For sites 1 and 2, which were selected for their preferential binding of M. luteus gyrase, it turns out that the topoisomerase-induced DNA cleavage does not occur efficiently. Alkali treatment of gyrase bound to site 2 results in no cleavage whether ATP and

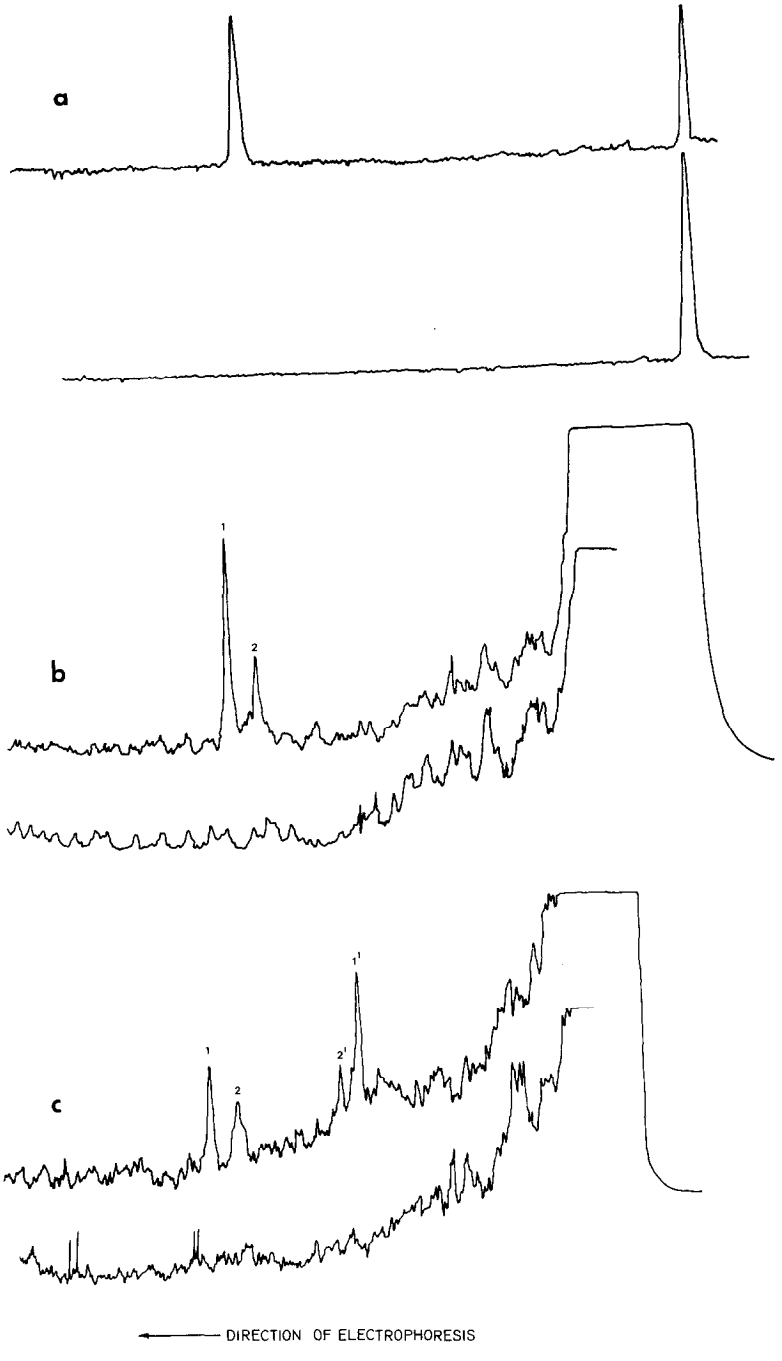

Figure 3. Gyrase-Effected Cleavage of DNA at Sites 1 and 3

(a) The 253 bp fragment bearing site 3, labeled at the 5′ terminus of the RI end, was incubated in the presence (top tracing) and absence (bottom tracing) of gyrase, as described in the Experimental Procedures. Oxolinic acid and ATP were present in the incubation mixture at concentrations of 250 μg/ml and 2 mM, respectively. The induction of cleavage by alkali and the analysis of the products by electrophoresis on a sequencing gel were done as described in the Experimental Procedures. Omission of either oxolinic acid or ATP during incubation with gyrase abolishes the gyrase-effected cleavage at this site (tracings not shown).

(b) The 199 bp fragment bearing site 1, singly labeled at the Hinf I site, was used. (Top tracing) incubation mixture contained gyrase, 0.3 mM pNHppA, but no oxolinic acid. The effects of ATP and oxolinic acid on gyrase-effected cleavage within this site are described in the text. (Lower tracing) result of the control experiment, in which gyrase was omitted in the incubation mixture. Because of the low amounts of cleaved products, longer exposure of the autoradiogram and higher sensitivity of the densitometer setting than those used (a) were necessary, and result in a noisy base line.

(c) The experiment was the same as that depicted in (b), except that a 200 bp Hinf I restriction fragment labeled at both 5′ ends was used. This fragment spans positions 631 to 851 on the Sutcliffe (1978) map, and contains the 199 bp fragment used in (b).

oxolinic acid are present or absent. For site 1, alkali treament of complexes formed between M. luteus gyrase and the fragments bearing site 1, under conditions specified in Figure 3, results in a low amount of DNA cleavage at two specific pairs of sites. The locations of the cleavage sites were determined as depicted in Figures 3b and 3c. Two samples of site 1-bearing DNA, one uniquely end-labeled (Figure 3b) and one labeled at both 5′ ends (Figure 3c), were subjected to gyrase-induced cleavage, and the products were sized by electrophoresis as described for site 3. The densitometric scans in Figure 3b show cleavage positions 1 and 2 on one strand; and the scans in Figure 3c display, in addition, the breakage sites 1′ and 2′ on the other strand. The sequence locations of these scissions are indicated in Figure 2 by the two pairs of hollow arrows labeled 1, 1′ and 2, 2′. The gyrase-effected double-stranded breakage at the pair of phosphodiester bonds 1, 1′, in contrast to the situation within site 3, is not affected significantly by the presence or absence of ATP and oxolinic acid during alkali treatment. The breakage at the pair 2, 2′ is enhanced by the presence of ATP and oxolinic acid, and the relative intensities of the labeled bands resulting from cleavages at 2, 2′ are higher than those resulting from cleavages at 1, 1′ if ATP and oxolinic acid are both present (results not shown).

Discussion

There is a striking parallel between the gyrase–DNA complex and the nucleosome, both in their patterns of cleavage by pancreatic DNAase and staphylococcal nuclease, and in their reactivities toward dimethyl sulfate. The most significant structural feature underlying these similarities is probably the wrapping of the DNA duplex outside a protein core.

The details of the spatial coiling of the DNA around the protein core appear to be different in the two cases. Aside from a possible difference in the handness of coiling (Liu and Wang, 1978a), the central 40 or so bp of the DNA segment in contact with gyrase are much less accessible to cleavage by pancreatic DNAase I. As we will discuss below, the DNA in this central region is likely to reside in a catalytic site where DNA breakage and rejoining occur. It has been shown previously that when staphylococcal nuclease-digested gyrase complexes of an essentially random collection of DNA sequences are digested with pancreatic DNAase I, there exists a region where DNAase I cleavage occurs rarely; this region appears to be bounded by DNAase I cleavage sites that are separated by a nonintegral multiple of the DNA helical repeat (Liu and Wang, 1978b). These earlier results, obtained with DNA of random sequence, are in complete accord with the more direct results reported here for gyrase-binding sites of unique sequence.

The gyrase–DNA complex must also possess two features that are absent in the nucleosome case: the catalysis of DNA chain breakage and the modulation of the enzyme–DNA interaction by ATP. We have previously reported that the addition of ATP has no effect on either the length of the DNA protected by gyrase against staphylococcal nuclease, or the DNAase I digestion pattern of the gyrase–DNA core complex obtained by staphylococcal nuclease digestion (Liu and Wang, 1978b). By using gyrase complexes of DNA of unique sequence, a much more detailed picture is now emerging on how the enzyme interacts with the DNA. Even at this level of probing, however, no ATP-mediated effects are detected. Any reasonable mechanism of ATP hydrolysis-coupled DNA supercoiling by gyrase must involve ATP-mediated conformational changes of the complex. It thus appears that either these ATP-mediated conformational changes involve little change in the DNA–enzyme contacts that we have probed with the nucleases, or that such conformational states are sufficiently transient that, for a population of molecules in a steady state in the presence of ATP or its nonhydrolyzable analog, those molecules in such conformational states form only a minor subpopulation and are therefore not detectable. We have presented elsewhere a model on how DNA supercoiling might be achieved by the enzyme without noticeably affecting the enzyme–DNA contacts mapped with nucleases (Wang et al., 1980). The gyrase-effected DNA cleavage sites appear to be located within the central region of the 145–155 bp DNA segment in contact with gyrase. We are uncertain about the rules that determine the disposition of such sites relative to the DNAase I-sensitive sites that mark the wrapping of the flanking regions of the DNA around gyrase. There does not appear to be a common dyad or pseudodyad axis for the gyrase-effected sites of breakage and the DNAase I-sensitive sites in the flanking regions.

The presence of multiple gyrase-effected cleavage sites in site 1 and the absence of cleavage site in site 2 are puzzling. The former could be due to the existence of two subpopulations of gyrase molecules that are bound differently to the particular DNA fragment. To reconcile this with the observation that the DNAase I cleavage pattern appears to be a unique one, one would have to invoke the possibility that one subpopulation is small but has a much higher yield of the cleaved products when alkali is added. A second possibility is that there might be flexibility in the interaction between the central DNA segment and the enzyme cleft where the topoisomerization catalytic site is located. Such flexibility might promote the breakage and rejoining of DNA at a favorable sequence with the central region. A third possibility is that the rather harsh alkali treatment for the induction of cleavage might cause artifacts, and the cleavage site so mapped might be only rough locations.

The lack of detectable DNA cleavage by bound

gyrase is not limited to site 2. With a DNA fragment (4108–161 in the sequence given by Sutcliffe [1978]) that contains site 3 and spans a longer stretch of sequence than the fragment used to give the results depicted in Figure 2 (4044–4361), binding sites that overlap with site 3 are detectable by the presence of additional groups of DNAase I-sensitive sites with the characteristic spacing, and are interlaced with the sets of such sites shown in Figure 2. Nevertheless, no additional gyrase-effected cleavage sites have been detected with the longer fragment. The detection of such "silent" gyrase-binding sites where gyrase-effected DNA breakage rarely occurs indicates that DNA sequences where gyrase-effected breakage occurs preferentially should not in general be identified as preferred gyrase-binding sites. The gyrase-effected DNA breakage may also have DNA sequence or structural requirements that are not required in the normal reactions catalyzed by the enzyme. Thus the lack of such cleavage sites within a binding site does not imply that the binding site is not a catalytically productive one.

In spite of the uncertainties discussed above concerning the details of the gyrase-induced DNA cleavage, it nevertheless appears certain that the positions of these cleavages are located within the 40 bp central region bounded by two 50 bp DNA segments that are wrapped around the enzyme. As we have reviewed in the introduction, the gyrase-catalyzed even-number change in linking number is consistent with a double-stranded transient breakage and passage mechanism (Brown and Cozzarelli, 1979; Liu et al., 1980; Mizuuchi et al., 1980), provided that the DNA segments straddling the transient break are not allowed to rotate relative to each other around the helical axis (Fuller, 1978). The structural features of the gyrase–DNA complex can now provide a clear molecular picture of why there is no relative rotation between the DNA segments straddling the break. A basic postulate for topoisomerses is that the transient breakage of a DNA strand by a topoisomerase is accompanied by the covalent linking of one end of the broken DNA strand to the protein (for review see Wang and Liu, 1979). Under certain conditions, double-stranded breakage of DNA by gyrase (Sugino et al., 1977, 1978; Gellert et al., 1977), accompanied by the covalent linkage (Morrison and Cozzarelli, 1979) of each of the 5′ ends resulting from the breakage to an α protomer of the enzyme (Sugino et al., 1980; Tse et al., 1980), has been observed. Furthermore, it has been established recently that the gyrase–DNA complex consists of a nominal 140 bp segment of DNA resistant to staphylococcal nuclease and an $\alpha_2\beta_2$ tetrameric gyrase molecule (Klevan and Wang, 1980). The picture that evolves from these results is that, when a transient break occurs in the central region of the DNA segment, the DNA segment remains tightly bound to the enzyme. Furthermore, each of the two 5′ ends is presumably anchored covalently to an α protomer of the tetrameric protein. Thus relative rotation between the segments on the two sides of the break cannot occur. Since all other type II DNA topoisomerases from procaryotic and eucaryotic organisms also change the linking number of DNA in units of 2 (Liu et al., 1980; Baldi et al., 1980; Hsieh and Brutlag, 1980), it is most likely that these enzymes each have a multisubunit structure to facilitate the anchoring of the free DNA ends that are transiently generated during catalysis.

Experimental Procedures

Enzyme and DNA Preparations

The M. luteus DNA gyrase was prepared as described by Klevan and Wang (1980). Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories, staphylococcal nuclease and pancreatic DNAase I from Worthington and pBR322 DNA was prepared by standard methods.

Filter Retention of Gyrase–DNA Complexes

Labeled digests of pBR322 DNA were incubated for 30 min at 30°C with gyrase at a ratio of 1 tetrameric gyrase molecule per pBR322 genome in 50 mM Tris–HCl, pH 7.6, 20 mM $MgCl_2$, 20 mM KCl, 1 mM $CaCl_2$ and 100 μ/ml bovine plasma albumin. The presence of Ca(II) in the binding buffer fulfilled the staphylococcal nuclease digestion requirement for some of the later experiments, and was otherwise nonessential. Retention of the protein–DNA complex on Whatman GF/C filters was carried out as described by Thomas et al. (1979) and the retained DNA was eluted with the binding buffer plus 1% sodium dodecyl sulfate. The filtrates and eluates were loaded directly on polyacrylamide gels for comparison with the untreated restriction digests; the preferential retention of specific fragments by gyrase was quantitated by scanning of the autoradiogram of the gel with a densitometer (Helena).

Experiments with restriction endonuclease Hinf I digests showed that fragments 7a and 3 (numbered according to Sutcliffe, 1978) were preferentially retained. DNA derived from these two regions was also selected by gyrase binding from restriction endonucleases Taq I and Ava I, or Alu I and Bam HI, double digests; it therefore appears that these regions contain the preferential gyrase-binding sites. The Hinf I fragments 7a and 3 were employed for most of the experiments; the gyrase-binding sites they bear are designated site 1 and site 2, respectively.

Mapping of DNA Sequences That Are Protected from Staphylococcal Nuclease Attack by Gyrase

The staphylococcal nuclease-resistant regions of DNA restriction fragments in the presence of bound gyrase were mapped by incubating a particular DNA fragment with gyrase at molar ratios varying from 1 to 5 gyrase tetramers per DNA fragment. A typical reaction mixture (25 μl) contained, in addition to gyrase: 2 μg DNA, 35 mM Tris–HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 1 mM spermidine, 1 mM $CaCl_2$, 20 mM KCl and 20% glycerol. Following incubation at 30°C for 30 min, 5 U of staphylococcal nuclease were added and incubation was continued for 10 min. The mixture was then extracted once with an equal volume of buffer-saturated phenol and 3 times with ether. The DNA was recovered by 2 cycles of ethanol precipitation. Labeling of the DNA with ^{32}P at the 5′ ends by polynucleotide kinase was then carried out, aliquots of the labeled fragments were digested with appropriate restriction enzymes and the products were sized by polyacrylamide gel electrophoresis. Since the nucleotide sequence of the fragment is known, sizing of the products of two restriction enzymes that cleave inside the region protected by gyrase against staphylococcal nuclease allows the demarcation of the boundaries of this protected region.

Probing Gyrase–DNA Interactions with Pancreatic DNAase I and Dimethyl Sulfate

The "footprinting" (Galas and Schmitz, 1978) of gyrase bound to unique sequences of DNA was carried out with restriction fragments singly labeled with ^{32}P at one of the 5′ or 3′ ends. Labeling at the 3′ end by E. coli DNA polymerase and an appropriate α-^{32}P-labeled deoxynucleoside triphosphate was carried out with restriction fragments with 5′ overhangs, as described by Maxam and Gilbert (1980). A typical DNAase I reaction mixture (45 μl) contained less than 0.1 μg of an end-labeled DNA fragment and more than a 10 fold molar excess of gyrase in the same buffer used in staphylococcal nuclease digestion. The concentration of ATP and its β,γ-imido analog, if present, were 2 mM and 0.3 mM, respectively. DNAase I incubations were for 1 min at 30°C, with DNAase I concentrations varying from 0.001 to 0.02 μg/ml. Digestions were stopped by the addition of 50 μl of a solution containing 0.1 M Na_3EDTA, 0.6 M sodium acetate and 25 μg/ml tRNA. The solutions were brought to a concentration of 0.2% in sodium dodecyl sulfate, heated for 5 min at 90°C, treated with proteinase K for 2 h at 50°C and ethanol-precipitated twice before redissolving in a gel loading solution containing 95% formamide and tracking dyes. Upon electrophoresis of the samples in a 10% polyacrylamide gel containing 7 M urea, the results were quantitated by densitometric scanning of the autoradiogram of the gel. Sequence positions were assigned by electrophoresis of the DNAase I-cleaved DNA adjacent to sequencing reactions (Maxam and Gilbert, 1980) of the same fragment; a DNA piece bearing the 3′ OH resulting from DNAase I cleavage migrates a half-rung more slowly than a corresponding fragment on the sequencing ladder that terminates in a 3′ phosphate (McConnell et al., 1978). Dimethyl sulfate reactions were similarly performed with the concentrations of the alkylating agent ranging from 20 to 200 mM, and incubations were carried out for 1.5 min at 30°C. Further treatment of the samples was performed as described in Maxam and Gilbert (1980).

Mapping the Sites of Cleavage by Gyrase

The sites of cleavage by gyrase on restriction fragments that had been selected for their preferential retention on filters were mapped by the same method described previously for E. coli and M. luteus DNA topoisomerase I-effected DNA cleavage (Tse et al., 1980). The gyrase–DNA mixtures were identical to the ones described for the pancreatic DNAase I reaction, except that 2 mM ATP was also included to enhance DNA cleavage by gyrase (Morrison et al., 1980). In some samples, oxolinic acid was also present at 100–250 μg/ml. After incubation for 30 min at 30°C, 20 μl of 1 M KOH was added to each mixture, and incubation was continued for 1 min. The reaction mixture was neutralized by the addition of 100 μl of a solution containing 0.25 M Tris–HCl, pH 5.5, and 0.6 M sodium acetate. Treatments with sodium dodecyl sulfate and proteinase K were the same as described for the DNAase I reaction. For each restriction fragment, two sets of cleavage reactions were carried out to obtain the location of the cleavage site on each strand.

We have also mapped the prominent sites of cleavage by gyrase on pBR322 DNA. The plasmid DNA was linearized by treatment with Eco RI restriction endonuclease, 5′ end-labeled with ^{32}P and then digested with Hind III restriction enzyme, which cuts off one of the labeled ends by cleaving 31 bp from it. After inactivation of the endonucleases by heating at 65°C for 10 min and cooling, gyrase was added. The gyrase-effected cleavage and the recovery of the DNA by ethanol precipitation were carried out as described above. The ethanol precipitate was resuspended in 20% glycerol containing tracking dyes and was analyzed by electrophoresis on both an alkaline agarose gel (McDonnell et al., 1977) and a 6% polyacrylamide gel containing 7 M urea (Maniatas et al., 1975). The sizes of the end-labeled fragments and the intensities of the corresponding bands on the autoradiograms of the gels give the distances of the prominent gyrase cleavage sites counterclockwise from the RI site on the Sutcliffe (1978) map.

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