DNA binding and antigenic specifications of DNA gyrase

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Received 9 November 1983; Accepted 28 November 1983

ABSTRACT

Complexes of DNA gyrase and minichromosomal DNA containing the origin of replication of *Escherichia coli* (*oriC*) can be formed without metabolic energy and visualised by electron microscopy. The A subunit, part of the A₂B₂-DNA gyrase complex is the binding protein. Various binding sites are scattered around the minichromosomal DNA including *oriC*. The minimal origin contains the only prominent and reproducible binding site. Binding to this site is suppressed by oxolinic acid and the ATP analogue β - γ -imido ATP. If gyrase isolated from the gram-positive bacterium *Bacillus subtilis* is used no binding to *oriC* is seen. This observation is consistent with antigenic differences between the A subunits of the two microorganisms. The binding to *oriC* might reflect a requirement for DNA gyrase during the initiation of DNA replication.

INTRODUCTION

The *E.coli* chromosome is present within the cell as a negatively supercoiled covalently closed circle comprising about 4×10^6 bp. This topological status is actively maintained by topoisomerases. In *E.coli*, DNA gyrase type II topoisomerase catalyzes the supercoiling of relaxed closed circular DNA (for reviews, see 1,2). The holoenzyme is a tetramer containing two each of the *gyrA* and the *gyrB* gene products (3). The activity of subunit A is inhibited by nalidixic or oxolinic acids, while that of the B subunit is inhibited by coumarin antibiotics like novobiocin. On the basis of inhibition studies employing these drugs, DNA gyrase has been implicated in almost every transaction of bacterial DNA. The use of these inhibitors, however, has not provided clear evidence as to whether the enzyme is directly involved in cellular processes which require the coupled breakage and rejoining of DNA strands, or indirectly by controlling the superhelical state of DNA. Moreover, thermal inactivation of gyrase in conditional lethal mutants defective in either the *gyrB* or *gyrA* gene products, does not affect processes such as DNA replication, gene expression or phage propagation in the same way as gyrase inhibitors (4, 5; E. Wahle, K. Muller

and E. Orr, in preparation). The use of gyrase B mutants suggested that the inactivation of DNA gyrase is inhibitory mainly to the initiation of chromosome replication rather than to ongoing replication (4; Orr, unpublished). This suggestion is further reinforced by the requirement for a specific superhelical density for the *in vitro* initiation of replication of ColE1 (6, 7, 8) or minichromosomes containing *oriC* (9).

Here we present our study of DNA gyrase complexes visualized by electron microscopy. In our experiments we have employed purified gyrase from both *E.coli* and *B.subtilis*, two genetically unrelated bacteria, and minichromosomal DNAs containing *oriC*, differing in their topological structure. This study enabled us to determine the binding specificity of DNA gyrase to a sequence within *oriC* and the effect of gyrase inhibitors on this binding. Our data are consistent with a wrapping of DNA around the enzyme (10).

MATERIALS AND METHODS

DNA and Enzyme Preparations

pOC51 was prepared by CsCl-EtBr gradients (11). Subunits A and B of DNA gyrase from either *E.coli* or *B.subtilis* were purified by affinity chromatography on heparin-sepharose followed by novobiocin-sepharose (12). *E.coli* gyrase was assayed by its ability to stimulate ColE1 replication in an extract prepared from temperature sensitive strains defective in gyrase activity (7). The activity of *B.subtilis* gyrase was tested by supercoiling relaxed covalently closed circular ColE1 DNA prepared with calf thymus topoisomerase I.

Cleavage of pOC51 by Gyrase

Cleavage reaction contained in 250 μ l : 25 mM Hepes pH 8.0, 5 mM Mg-acetate 50 mM KCl, 5 mM spermidine-HCl, 5 mM dithiothreitol, 50 μ g oxolinic acid, 25 μ g supercoiled pOC51 DNA and 5 μ g of each subunit A and B of *E.coli* gyrase. After 30 min at 30°C, SDS at final concentration of 0.5% and proteinase K at 100 μ g/ml were added. The mixture was further incubated at 37°C for 20 min. DNA was deproteinised by phenol extraction and was applied to a 0.8% agarose gel.

Binding of Gyrase to pOC51 DNA

The binding mixture contained 25 mM Hepes pH 8.0 80 mM KCl, 4 mM Mg-acetate, 2 mM ATP, 5 mM dithiothreitol, 30 μ g/ml of each subunit A and subunit B, and 20 μ g/ml pOC51 DNA. After 10 min of incubation at 30°C glutaraldehyde was added at a concentration of 0.2% and incubated for another 15 min at 30°C.

Glutaraldehyde and unbound enzyme were separated from the complexes by chromatography on Sepharose 4B. Half the circular DNA was linearized with restriction endonuclease *XhoI* and half with *SmaI* and repurified by a second Sepharose 4B step.

Electron Microscopy

Samples containing gyrase complexes were adsorbed to mica, washed on distilled water for 2 hours, stained with 2% uranylacetate solution for 5 min dehydrated with ethanol and rotary shadowed with Pt/IR (13). Gyrase complexes were photographed at primary magnification of 6000x.

Localisation of Gyrase Complexes

Positions of the DNA gyrase molecules were determined on 16-fold enlarged negatives using a digitizer. In each experiment about 300 molecules with 1.0-2.0 DNA gyrase molecules bound on each were measured. Orientation of measured molecules and determination of peak positions were obtained by a computer program (14), comparing DNA molecules from a given experiment cut independently with *XhoI* and *SmaI* after the binding of gyrase. All these parameters were checked manually as well.

"Western Blotting" Analysis

Antiserum against *E.coli* subunit B of DNA gyrase was raised in rabbits. 1 µg of *E.coli* and *B.subtilis* purified subunits were applied to a 10% SDS-polyacrylamide gel, blotted on to a nitrocellulose membrane by electrophoresis and visualized by using Fluorescein-conjugated goat anti rabbit IgG (15). Antiserum raised in rabbit against *E.coli* gyrase B subunit was applied in a 1:150 dilution. Fluorescein-conjugated goat anti rabbit IgG were diluted 1:250.

Solid Phase Immunoassay

Purified subunits A or B (2 µg/50 µl) from either *E.coli* or *B.subtilis* were incubated in polyvinyl chloride wells (16). After 2 h at 37°C the wells were washed several times with phosphate-saline buffer containing 1 mg/ml bovine serum albumin (buffer A). Mouse anti gyrase (A or B) serum was added at a dilution of 1:1000 in buffer A and incubated at 37°C for 1 h. After washing several times with buffer A the wells were incubated with ¹²⁵I-rabbit anti mouse IgG serum (kindly donated by Miss S. Griffiths and Dr T. Harrison) at 37°C for 1 h. After several washes with buffer A, the wells were air dried and the radioactivity measured in a Gamma Counter.

RESULTS

Gyrase Cleavage Sites

The *in vitro* interaction of isolated gyrase with DNA has mainly been studied

Figure 1. Cleavage of pOC51 DNA by gyrase. The analysis of pOC51 DNA either cleaved by DNA gyrase or uncleaved is shown in Figure 1a tracks 1 and 2, respectively. The linear fragment was electrophoresed out of the agarose and phenol extracted several times. The purified gyrase cleaved (Figure 1b, track 1; track 2 = uncleaved control). DNA was digested by either *XhoI* (Figure 1c, track 1) or *SmaI* (Figure 1c, track 2) and analysed by 1.5% agarose gel electrophoresis.

by following its double strand breakage activity in the presence of oxolinic acid and subsequent treatment with sodium dodecyl sulfate. Although the DNA in this reaction is broken into defined fragments, no extensive homology of nucleotide sequences around the cleavage sites could be demonstrated (17, 18, 19). Nevertheless, the presence of preferred cleavage sites suggested that the enzyme might recognize a specific sequence or structure on a given template.

Since initiation of replication at *oriC* is inhibited by the loss of gyrase activity (4, 9), we hypothesized that the enzyme might interact specifically with this region. We, therefore, first attempted to locate gyrase binding sites on the minichromosome pOC51 (11). We examined the cleavage sites induced by DNA gyrase in the presence of oxolinic acid. The reaction yielded only one linear fragment (Figure 1a) which has subsequently been purified from the agarose gel. In order to map this site on the linearized minichromosome the DNA was cleaved with the restriction enzymes *SmaI* or *XhoI*. Both enzymes have a single recognition site on this DNA. Analysis of the digested products by gel electrophoresis demonstrated that

the restriction digests contained far too many, yet still defined fragments, with different molecular weights (Figure 1c). This result suggests that gyrase might bind to several sites on pOC51 DNA but under our assay conditions double strand cleavage is induced only once per DNA molecule. The experiment, however, failed to elicit a major binding site of DNA gyrase on the minichromosome.

Electron Microscopic Visualization of Gyrase-DNA Complexes

Since it is possible that oxolinic acid might have altered the enzyme such that its specificity is masked by freezing a "walking" molecule at secondary (non specific) sites on the DNA, we looked for other techniques enabling us to monitor binding with and without the drug. The most direct one seemed to be visualization by electron microscopy. This approach was stimulated by recent findings that nalidixic and oxolinic acids inhibit cellular DNA replication at concentrations which neither alter the superhelical density of cellular DNA (20, 21) nor inhibit the supercoiling activity of DNA gyrase *in vitro* (22; Orr and Staudenbauer, unpublished). Moreover, experiments which examined the effect of thermal inactivation of DNA gyrase on gene expression strongly indicated that both nalidixic and oxolinic acids might affect transcription via a mechanism different from the inactivation of DNA gyrase (E. Wahle, K. Muller and E. Orr, in preparation).

DNA gyrase was incubated with minichromosomal DNA, subsequently fixed with glutaraldehyde and analysed by electron microscopy. The reaction conditions for the binding assay were identical to the optimal conditions for supercoiling *in vitro* of ColE1 (22) or minichromosomal DNA and for ATPase activity of gyrase (22; Orr, unpublished) except that spermidine was omitted. We first used supercoiled DNA in order to examine effects of ATP and K^+ -concentrations. After fixation of bound gyrase, the DNA was linearized with restriction enzymes.

As can be seen from Figure 2a, gyrase complexes formed on plasmid DNA are spherical particles with a diameter of 22 ± 2 nm, a size similar to that of RNA polymerase prepared under the same conditions. This size is a good correlation to 400 000 daltons, the proposed molecular weight of the holo-enzyme. The number of gyrases bound per DNA molecule was neither altered by the omission of ATP, nor by the use of the ATP analogue β - γ -imido ATP. KCl at concentrations higher than 150 mM abolished the binding, but had no effect at lower concentrations.

We then investigated the effect of different templates on gyrase binding activity. The ratio of gyrase complexes to DNA was not altered

Figure 2. Visualization of *E. coli* gyrase on pOC51 DNA. Supercoiled or linear pOC51 DNA was used in the binding assay and prepared for electron microscopy. Circular DNA was linearized by either *XhoI* or *SmaI* after binding. (a) Holoenzyme complexes formed on linear *XhoI* cut pOC51 DNA. Same preparation as used in Figure 3c. (b) Complexes formed on ccc DNA with subunit A alone and cut with *XhoI*. Arrows indicate a big and a small complex of subunit A with DNA. Magnification: 35 000x.

when either linearized, relaxed or supercoiled DNA was used. The number of gyrase molecules bound to the DNA followed a Poisson distribution.

Binding of Subunit A

The A subunit is believed to contain the DNA cleavage and rejoining site of the holoenzyme. This is supported by the finding that if gyrase is allowed to induce cleavage of duplex DNA, the A subunit is covalently linked to each of the 5'-phosphoryl ends of the cleaved DNA (23, 24). We therefore examined the binding of either the A or the B subunits of gyrase to pOC51 DNA. While no binding could be observed in the presence of the B subunit alone, complexes of the DNA with A subunit could be visualized. We observed two kinds of complexes: small particles with a diameter of 16 ± 1 nm and larger ones with a diameter of 21 ± 2 nm (Figure 2b). Since the A subunit of gyrase can exist in solution as dimers (3, 25), it can probably bind to DNA in multimeric forms. Although filter binding experiments failed to detect stable complexes between DNA and gyrase A subunit (26), the formation of such complexes between *Micrococcus luteus* subunit A and pBR322 DNA has recently been observed by electron microscopy (27).

Figure 3. Localization of DNA gyrase on pOC51 DNA. Binding of DNA gyrase to pOC51 DNA and the electron microscopical preparation were done as described in experimental procedures. Samples containing circular DNA were split after the removal of glutaraldehyde. Half the DNA was linearized with the restriction enzyme *XhoI* and half with *SmaI*. In each experiment about 300 molecules with 1.0–2.0 DNA gyrase molecules bound on each were measured. Orientation of measured molecules and determination of peak positions were primarily obtained by both computer program and manually, comparing (to best fit) DNA molecules cut independently with *XhoI* and *SmaI*. All histograms (1 interval = 41 bp) shown are from DNAs which were cut with *XhoI* for electron microscopical preparations. DNA gyrase was bound to supercoiled DNA (a) to covalently closed relaxed (b) and to linear DNA (c). In histogram (d) DNA was cut with *XhoI* and *SmaI* into two fragments for the assay. Histograms of both fragments are shown joined together. For evaluation of the small fragment only 130 molecules were used. Histogram 3e shows an identical experiment as in (a), but without ATP and in (f) ATP was replaced by β - γ -imido ATP. Oxolinic acid was added to the binding assay at a concentration of 200 μ g/ml in (g). *B. subtilis* subunits A and B instead of *E. coli* enzyme were bound to supercoiled DNA in histogram (h).

Binding Specificity of Gyrase to Minichromosomal pOC51 DNA

In order to localize bound gyrase molecules relative to a given restriction endonuclease cleavage site by electron microscopy, we used *SmaI* and *XhoI*, which cleave the minichromosome once. Electron micrographs were done at a primary magnification of 6000x. Contour lengths of DNA molecules and positions of gyrase molecules were measured on negatives enlarged 16 times

using a digitizer. Orientation of measured molecules and determination of peak positions were obtained by comparing (to best fit) gyrase complexes on DNA molecules cut independently (after the binding) by *XhoI* and *SmaI*. Peak positions on linear DNA were deduced by comparing DNA molecules linearised by *XhoI* and *SmaI* (independently) before and after the binding of gyrase. Orientation of peaks as well as plotting of histograms have all been performed by a computer program (14) and checked manually. The results obtained confirm the conclusion from the cleavage experiment that the enzyme can bind to any of many sites on the DNA (Figure 3). The number of sites outside *oriC* is too high to be resolved by the electron microscopic method employed. However, all experiments carried out under optimal conditions produced a peak centered around 235^{+23} bp, which is located within the minimal origin sequence comprising 245 bp (28). These data have been confirmed by 17 individual experiments analysing about 300 DNA molecules in each. While the peak in the origin region was always the most prominent one, none of the other peaks were reproducible when histograms of different experiments were compared. Figure 3 demonstrates that the *oriC* gyrase site has not been altered whether supercoiled (a), relaxed (b) or linear (c) DNA was used in the binding assay. The *oriC* peak could also be detected when only subunit A was present in the binding assay (not shown). Omission of ATP (Figure 3e) did not diminish this binding site, but it was no longer noticeable when the ATP analogue β - γ -imido ATP was used (Figure 3f). It has been speculated that the ATP analogue induces changes in the enzyme structure (17). One might, therefore, assume that such changes inhibit the binding of the holoenzyme to its specific site. Alternatively, gyrase binding to *oriC* might induce structural changes in the neighbouring DNA region which cannot be established in the presence of the ATP analogue. A possible freezing of the enzyme by the ATP analogue on a secondary binding site cannot, as yet, be ruled out.

To analyse the *oriC*-site more rigorously, we examined gyrase complexes bound to a mixture of a 463 bp fragment containing *oriC* and the remaining larger fragment making up the rest of pOC51. The two fragments were produced by a double digestion using *SmaI* and *XhoI*. As shown in Figure 3d, one major gyrase binding site is located on the small fragment at a position identical to the *oriC* site on the whole minichromosome. On the small fragment the probability of a gyrase being bound to one kb is 42% higher than on the remaining larger fragment. These results, along with the lack of a prominent gyrase binding site on the larger fragment, further indicate that the *oriC*

Figure 4. Immunological analysis of *E.coli* and *B.subtilis* subunit B. *E.coli* and *B.subtilis* purified subunits were blotted on to a nitrocellulose membrane by electrophoresis and visualized by using Fluorescein-conjugated goat anti rabbit IgG. Lane (a) *E.coli* subunit B, (b) *B.subtilis* subunit B.

site contains a specific gyrase binding site.

The average length of the small *oriC* fragment measured from the electron micrographs is 167^{+14} nm, while the length of fragments complexed with one gyrase molecule is 145^{+19} nm. This difference in length corresponds to about 70 bp. The decrease in length of the *oriC* fragment complexed with gyrase can be explained by the wrapping of a DNA segment around the enzyme molecule. Such interaction has previously been proposed following the presence of nuclease protected areas on the complex DNA-gyrase (10).

Effect of Novobiocin and Oxolinic Acid

The addition of novobiocin and oxolinic acid to the binding assay enhanced complex formation by a factor of 1.5-2. Although complexes bound to the *oriC* site were found when novobiocin was added to the assay mixture (not shown), no specific complex formation of gyrase at this site could be visualized in the presence of oxolinic acid at concentrations used for the cleavage experiment (Figure 3g). Formation of non-specific complexes might prevent the enzyme from "searching" the specific site at *oriC* (see above).

Bacillus subtilis Gyrase

In order to further substantiate the specific interaction of *E.coli* DNA gyrase with *oriC* DNA, we used *Bacillus subtilis* DNA gyrase in the binding

Table 1. Solid-phase Immunoassay

Antigen	Serum	% activity
<i>E.coli</i> subunit B	anti B	100
<i>B.subtilis</i> subunit B	anti B	100
<i>E.coli</i> subunit A	anti A	100
<i>B.subtilis</i> subunit A	anti A	15

Purified subunits A or B (2 μ g) from either *E.coli* or *B.subtilis* were incubated with mouse anti gyrase (A or B) serum followed by incubation with 125 I-rabbit anti mouse IgG serum. 100% activity represents 32 000 cpm for subunit A and 34 000 cpm for subunit B.

assay instead of the *E.coli* enzyme. Although gross complex formation was not affected, the histogram shown in Figure 3h demonstrates that this enzyme does not recognize the *oriC* site. The failure of *B.subtilis* gyrase in binding to the *oriC* site might reflect both binding specificity and other differences between the A subunits of *E.coli* and *B.subtilis*. Earlier experiments indicated that an enzyme made up of *B.subtilis* A subunit and *E.coli* B subunit was inactive in the supercoiling of ColE1 DNA while the reciprocal combination was fully active (29). One interpretation of these results was that the A subunits of these microorganisms did not recognise the template employed similarly.

To further elucidate the differences between the two proteins we used antiserum raised against *E.coli* gyrase A or gyrase B subunits. Figure 4 and Table 1 show that *B.subtilis* gyrase B subunit reacts with the antiserum raised against the *E.coli* gyrase B subunit. However, by employing the "Western blotting" technique (15) we could not detect any cross reactivity of the *B.subtilis* gyrase A subunit with antiserum raised against the *E.coli* subunit A. Since the "Western blotting" technique implies the transfer of denatured protein and subsequent immunoassay, we tried to avoid the denaturation step by using a solid phase radioimmunoassay (16). Table 1 demonstrates that a limited cross reactivity between the *B.subtilis* subunit A and the *E.coli* antiserum can be detected.

DISCUSSION

The data presented in this paper demonstrate that the A subunit of *E.coli* DNA gyrase is a DNA binding protein. This result deviates considerably from

Figure 5. Location of gyrase binding site within *oriC* as identified by electron microscopy. The DNA sequence of the estimated gyrase binding centre around bp 235 is shown. The most prominent repeats thought to be involved in gyrase recognition are indicated by arrows. Its location within *oriC* is indicated by arrows connected by a bracket. The extent of the mini-chromosomal *oriC* region (28), the location of the two *oriC* promoters (filled bars, 35), and the binding sites for the B' protein (hatched bars, 36) are given for orientation of gyrase binding site within *oriC*. Relevant endonuclease restriction cut sites of *oriC* are given below.

previous reports suggesting that both the A and the B subunits of *E. coli* are necessary for the binding activity (for review see 2). The physiological significance of this binding however, is not yet clear. Two different techniques, the electron microscopy and the induction of gyrase mediated DNA cleavage in the presence of oxolinic acid, confirm that the enzyme can bind to many sites on the minichromosome DNA containing *oriC*. The electron microscopy technique enabled us to monitor the distribution pattern of gyrase complexes along the DNA easily. It has thus become evident that *oriC* contains a sequence which is recognised preferentially by the enzyme. This site, centred around 235 bp of *oriC* (30), was the only prominent binding site, repeatedly detected in all the experiments carried out under optimal conditions. The binding to this site only was abolished in the presence of β - γ -imido ATP, oxolinic acid, or when *B. subtilis* gyrase replaced the *E. coli* enzyme. Based on these results, we would like to propose that the strong binding of *E. coli* gyrase to *oriC* is different from its binding to the other sites and reflects the need for gyrase during the initiation of DNA replication (see below).

The reason for the relative small difference between the binding of

gyrase to *oriC* and to the other sites is as yet not understood. It is nevertheless likely to result from the strong affinity of gyrase to DNA and from a possible requirement for other cellular proteins e.g. DNA binding proteins, while binding to *oriC*.

DNA gyrase binds to *oriC* when linear as well as supercoiled DNA is used. Thus, it probably recognises a DNA sequence rather than a structure. Sequence analysis of *oriC* around position 235 bp reveals an extended inverted repeat (Figure 5). This repeat, comprised of 9 bp, is conserved in several gram-negative bacteria (31). Since the two A subunits of gyrase holoenzyme might be expected to recognise identical sequences, it is tempting to speculate that they bind symmetrically to both sequences. Although the initial binding of gyrase to *oriC* does not require ATP, an energy requiring step could follow, analogous to the proposed interaction of the phage T4 topoisomerase II during initiation of T4 replication (32). The observation that the non-metabolised ATP analogue β - γ -imido ATP suppresses gyrase binding at *oriC* favours this idea.

Binding of gyrase, to *oriC* only, could not be detected in the presence of oxolinic acid, a drug used to map putative bindings sites on DNA by examining gyrase cleavage sites. The reason for this phenomenon is not entirely understood. It has however been pointed out recently, that oxolinic acid affects cellular processes not via its interference with the supercoiling activity of the enzyme, but by other, not entirely known, mechanisms (20, 21, Wahle, Muller and Orr, submitted). If the binding of gyrase to *oriC* reflects a true physiological activity of the enzyme, the effect of oxolinic acid on the binding to this site might add to the uncertainty concerning its mode of action *in vivo*.

The enhancement of gyrase complex formation by oxolinic acid could explain previous reports that gyrase binds at some, not all, oxolinic cleavage sites in the absence of the inhibitor (see ref. 2).

DNA supercoiling is maintained *in vivo* and *in vitro* by the activities of DNA gyrase and relaxing enzyme(s) (7, 33). Changes in this balance towards DNA relaxation lead to inhibition of DNA replication in *E.coli* (4,5,7). The failure of *B.subtilis* gyrase to bind at *oriC* could reflect its inefficiency in supercoiling the minichromosome (Orr, unpublished) as well as a specific interaction of the *E.coli* enzyme with this sequence. Furthermore, the A subunits (the DNA binding proteins) of these microorganisms differ considerably in their antigenic properties. These observations imply that supercoiling may not only be a critical step in initiation of replication but also a selecting mechanism in the replication of different extrachromosomal replicons in a given host.

This idea is consistent with the observation that ColE1 type plasmids are rapidly relaxed in extracts prepared from gram-positive bacteria, e.g. *B.subtilis* and fail to initiate DNA replication (Orr and Lother, unpublished).

It is generally believed that the ATPase site of gyrase is located on the B subunit of the enzyme (1, 2). The conservation of this subunit in *B.subtilis*, as well as in other microorganisms (Orr and Lother, in preparation), probably reflects its indispensable role as the energy binding protein in the enzyme complex. The limited cross reactivity between the *B.subtilis* subunit A and the *E.coli* anti gyrase A suggests a limited conservation of binding regions on the gyrase A subunit for either the gyrase B subunit or the nalidixic acid-like drugs or for both.

As mentioned above, gyrase might play an "active" role during *oriC* replication. To date, however, we have not sufficient data to assign any gyrase activity to the *oriC* binding site, nor to any others. It therefore cannot be excluded that the binding at *oriC* reflects a requirement for negative supercoils only, prior to the initiation step. It has been proposed that several proteins, e.g. the *dnaA* gene product (34), RNA polymerase (35), and membrane proteins (36, 37) are capable of binding to *oriC*. The interaction of these proteins with *oriC* might depend on a negative supercoiled template. It is possible that the preferential binding of gyrase at *oriC* reflects the essential role of this enzyme in preparing this template for DNA replication.

ACKNOWLEDGEMENTS

This work was supported by a grant No. G80/0084/OCB from the Medical Research Council (U.K.) and by a short term EMBO award (E.O.). We are grateful to Dr W. Messer and Dr T.A. Trautner for their valuable discussions regarding the manuscript. We thank Beate Rueckert for making the electron microscopic preparations and measuring the molecules. Last, but not least, we would like to thank Dr T. Harrison and Miss Susanne Griffiths for their assistance with the antisera experiments.

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