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The partition locus of plasmid pSC101 is a specific binding site for DNA gyrase

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A protein in extracts of *Escherichia coli* that specifically binds the stabilizing *par* sequence of pSC101 was identified as DNA gyrase. The purified enzyme protects *par* against digestion by DNase I and exonuclease III. Competition assays demonstrate that gyrase has a 40-fold higher affinity for the 100-bp *par* sequence than for non-specific DNA and that *par* is the major gyrase-binding site in pSC101 derivatives used in this and other studies. Within *par*, AT-rich sequences occur with a pronounced 10-bp periodicity that is shifted by 5 bp from a similar periodicity of GC-rich sequences. As judged by DNase I digestion, the GC sequences are exposed on the outside of the DNA wrapped around gyrase. The data suggest that the site-specificity of DNA gyrase may be partly determined by the bendability of the DNA. A 4-bp deletion that interferes with Par function *in vivo* also reduces the affinity for gyrase *in vitro*. However, a deletion of *par* causes little reduction in superhelical density *in vivo*. We conclude that DNA gyrase, while involved in the Par function, may not affect plasmid stability through its supercoiling activity or by an influence on DNA replication.

Key words: plasmid pSC101/*par* sequence/DNA gyrase

Introduction

The mechanism of segregation of bacterial replicons at cell division is unknown. From the high degree of stability of low copy number plasmids (see below) and of the chromosome itself (Howe and Mount, 1975; Jaffé *et al.*, 1986), segregation cannot be random. For investigations of the segregation mechanism, plasmids offer two advantages: they can be readily manipulated genetically and biochemically and, since they are dispensable for cell growth, their stability can be observed over the course of many generations. Stability functions believed to promote partitioning (i.e. regulated, non-random distribution of plasmids between daughter cells) have been identified in plasmids R1 (Nordstrom *et al.*, 1980), F (Ogura and Hiraga, 1983), P1 (Austin and Abeles, 1983) and pSC101 (Meacock and Cohen, 1980).

The plasmid pSC101 is not lost from the host cells during 1000 generations of growth under non-selective conditions (Cohen *et al.*, 1985). Deletion of a 400-bp *par* fragment (Figure 1) makes the plasmid unstable; it is completely lost in <100 generations, despite the maintenance of its copy number and the lack of accumulation of oligomers (Meacock and Cohen, 1980). There is no ORF in the essential sequence of the *par* locus (Miller *et al.*, 1983; Tucker *et al.*, 1984). Rather, two direct repeats and one inverted repeat of a 13-bp sequence (Figure 1) give it the appearance of a protein-binding site. Inasmuch as the *par* sequence can stabilize plasmids unrelated to pSC101 (Meacock and Cohen, 1980), it would seem that the sequence provides a binding site for a host protein (Cohen *et al.*, 1985).

Partial deletions of the *par* site have intermediate phenotypes: deletion of two of the three repeats makes the plasmid unstable, but less so than a complete deletion. Plasmids lacking only one repeat appear to be as stable as the wild-type, but are unable to co-exist in the same cell with a wild-type plasmid, even under selective conditions. Tucker *et al.* (1984) suggested that a failure to compete in replication with the par^+ plasmid might be the basis for this Cmp^- phenotype.

For lack of an *in vitro* assay for partitioning, we looked instead for a host protein that would bind specifically to the *par* sequence and might be involved in its function. Here we provide evidence that DNA gyrase binds specifically to the sequence and may contribute to its role in maintaining the plasmid.

Results

A par-specific binding protein in the crude extract

An exonuclease III protection assay (Wu, 1985) was used in a search for proteins that bound the *par* sequence of pSC101. A 392-bp fragment containing the *par* sequence (Figure 1) was 5′-labeled at the *Eco*RI end and digested with exonuclease III in the presence of a 400-fold weight excess of DNA that lacked the sequence. The predominant products, as analyzed by electrophoresis on a denaturing gel, were fragments of ~200 nt (Figure 2a), as expected from the action of exonuclease III. [This enzyme digests double-stranded DNA from both 3′ ends, thereby releasing two half-length single strands which are resistant to further degradation (Kornberg, 1980)]. Digestion of the *par* fragment in the presence of a crude extract of *Escherichia coli* produced, in addition, two fragments of ~285 and 275 nt (Figure 2a). This indicates the presence in the crude extract of a protein which binds the labeled fragment and protects it from the nuclease. When the limits of exonuclease digestion were mapped with a fragment labeled at the other 5′ end, the crude extract was observed to protect a region of 120–140 bp, approximately from nucleotides 170 and 180 to 300 and 310 (Figure 1). This region contains the 60–100 bp sequence shown by deletion analysis to be essential for the Par function. When, in addition to the labeled fragment (2.6 ng) and the non-specific competitor DNA (1 μg) present in the assay, 50 or 125 ng of unlabeled *par* fragment was added, the protection of the labeled DNA decreased from 35% to 14% and 9%, respectively, presumably due to competition of the unlabeled fragment for the binding protein. This distribu-

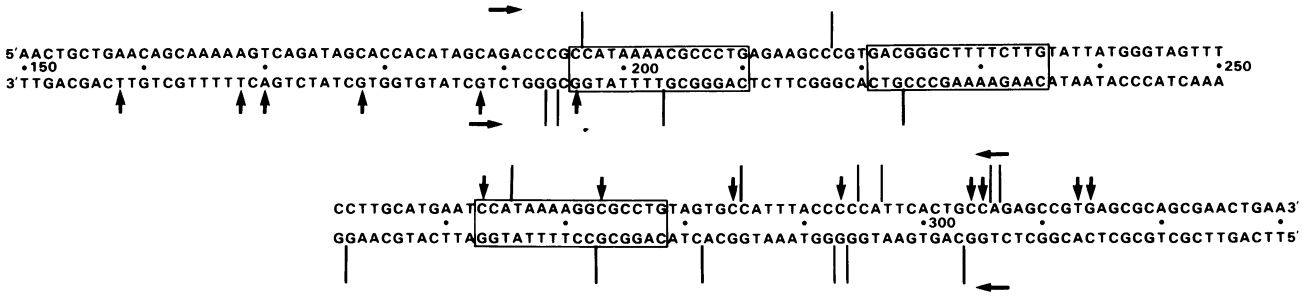

Fig. 1. The *par* sequence and summary of nuclease protection data. Boxes enclose the three repeat sequences (see text). Short vertical arrows mark the limits of exonuclease III digestion in the presence of purified gyrase; with spermidine present, only the stopsites at the periphery were observed. The main stopsites in the presence of crude extract, mapped at lower resolution, were at positions 170 and 180 on one side and 300 and 310 on the other side of the sequence. Horizontal arrows mark the approximate boundaries of the DNase I footprint. Vertical bars indicate the phosphodiester bonds at which cleavage is enhanced by DNA gyrase. The sequence and its numbering are from Armstrong *et al.* (1984). Cuts by *Afl*II, outside the sequence shown here, are between nucleotides 418 and 419 on the top strand and by *Ava*I are between 397 and 398; cutting of an *Eco*RI site created by cloning (Meacock and Cohen, 1980) occurs between nucleotides 26 and 27 on the top strand.

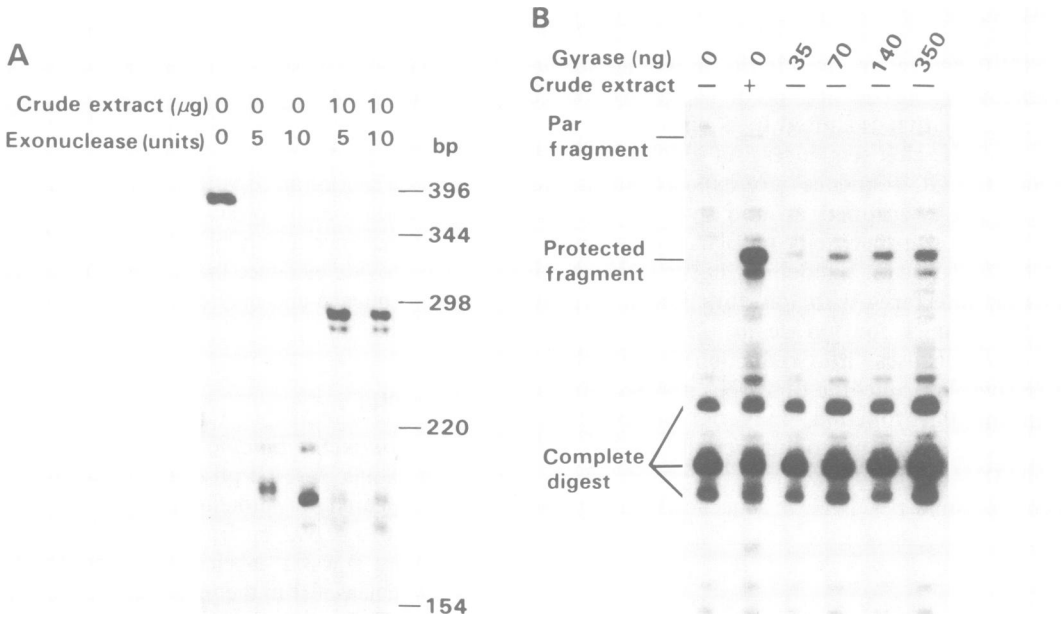

Fig. 2. Protection of *par* against exonuclease III digestion. The *Eco*RI–*Afl*II *par* fragment, 5'-labeled at the *Eco*RI end, was digested (**A**) with exonuclease III in the absence or presence of crude extract, or (**B**) with 10 units of exonuclease III in the absence or presence of crude extract (12 μg protein) or of purified gyrase.

tion of the binding protein between the labeled fragment and the specific and non-specific competitor is consistent with the degree of specificity determined later (see below). No competition was observed with *Hin*fI-cut pBR322; addition of 28 or 72 ng resulted in 31% and 36% protection, respectively. Based on the mapping data and the competition experiments, we conclude that the protection is specific for the *par* sequence.

The par-binding protein is DNA gyrase

The characteristics of the active protein: elution behavior on heparin–agarose, mol. wt in gel filtration (>250 000), site-specific binding to DNA and the size of the region it protected against exonuclease, were reminiscent of DNA gyrase. Like gyrase, the *par*-binding protein appeared to have a lower affinity for supercoiled DNA: in the exonuclease protection assay, a 10-fold molar excess of supercoiled DNA containing *par* failed to compete with the labeled *par* fragment (data not shown). We therefore determined directly whether pure DNA gyrase might replace the *par*-binding protein.

Purified gyrase did protect the *par* fragment from exonuclease III digestion. The major digestion products obtained in the presence of gyrase were indistinguishable from those produced in the presence of crude extract (Figure 2b). On the heparin–agarose column, the peak of supercoiling activity coincided with that of the *par*-binding activity. The ratio of specific activities in the supercoiling and in the exonuclease protection assays was identical for the heparin–agarose peak fraction and purified gyrase (data not shown). Thus, the *par*-binding protein which we isolated is DNA gyrase.

Stimulatory factors for gyrase action

After phosphocellulose chromatography of crude extracts active in the protection assay, gyrase was found in the

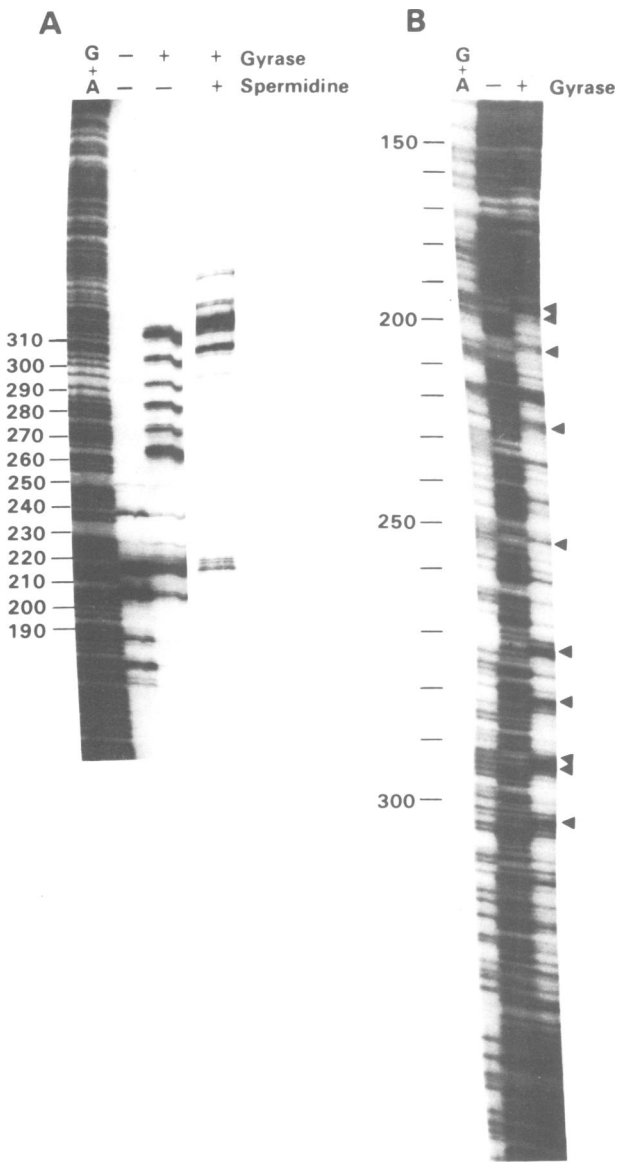

Fig. 3. Exonuclease III and DNase I footprints. (**A**) The *Eco*RI−*Afl*II *par* fragment was 5′-labeled at the *Eco*RI end and digested with exonuclease III in the absence or presence of 140 ng of gyrase and 5 mM spermidine; **lane 1** is a G+A sequencing ladder (Maxam and Gilbert, 1977). (**B**) The *Eco*RI−*Afl*II *par* fragment was 5′-labeled at the *Afl*II end and digested with DNase I in the absence or presence of 140 ng of gyrase; arrows indicate sites of enhanced cleavage; **lane 1** is a G+A sequencing ladder. A footprint made in the presence of 5 mM spermidine was identical.

flowthrough fraction but the protection activity was very feeble. The activity could be restored by the addition of a fraction eluted at high ionic strength. This fraction stimulated the activity of the flowthrough and of purified gyrase up to 10-fold but contained no gyrase subunit. It could be replaced by spermidine at 0.5−5 mM (see below) or by concentrations of H-protein sufficient to coat all the DNA in the assay (Hübscher *et al.*, 1980). Proteins HU and IHF were inactive. However, the most abundant and effective protein factor with stimulatory activity was an apparently novel 15.5-kd protein with histone-like properties which we purified to homogeneity. Upon sequences analysis, the 12 N-terminal residues of this isolated protein were found to be identical to amino acids 11−22 of the ribosomal protein L16 (Brosius and Chen, 1976). The amino acid compositions of the two proteins (omitting the first 10 of L16) were very similar (average deviation in the molar content of individual amino acids was 4.2%; data not shown).

It seems likely that the 15.5-kd protein is released from dissociated ribosomes during our standard procedure for the preparation of cell lysates, owing to the presence of lysozyme, EDTA and high concentrations of salt (see Materials and methods). When cell extracts were made instead by mechanical disruption in the presence of 10 mM Mg^{2+} and 300 mM NaCl and the intact ribosomes (Van Holde and Hill, 1979) removed by high speed centrifugation, the stimulatory protein could not be found. It was not present in the supernatant before or after the Biorex 70 chromatography that had been used previously for purification from the standard lysate.

In the absence of stimulatory factors, multiple pause sites for exonuclease III were often observed on each side of the gyrase-binding site (Figure 3a). The spacing between these sites was 10 bp. When spermidine was present, these clustered pause sites were no longer observed. Instead, the digestion of the majority of DNA fragments stopped at the extreme right or left boundary of the complex and the amount of protected DNA was increased.

Specificity of the gyrase−par interaction

The exonuclease protection assay, performed in the presence of an excess of competitor DNA, demonstrates that the affinity of DNA gyrase for the *par* sequence is substantially higher than for random DNA. In filter-binding experiments, a 40-fold weight excess of non-specific DNA was required to reduce the binding of the labeled *par* fragment by 50% (data not shown).

Upon DNase I digestion of *par* under conditions similar to those used for the exonuclease protection assay, DNA gyrase left its characteristic footprint (Fisher *et al.*, 1981; Morrison and Cozzarelli, 1981; Kirkegaard and Wang, 1981) on the *par* sequence (Figure 3b). This footprint extended over the same region as did the exonuclease footprint, the strongest protection being in the middle. This central region of ~30 bp was flanked on both sides by several sites of enhanced cutting, separated from each other by 10 or 20 bp. The predominant site of SDS-induced cleavage in the presence of oxolinic acid (Sugino *et al.*, 1977; Gellert *et al.*, 1977) was in the middle of the central region between nucleotides 247 and 248 on the top strand and between nucleotides 251 and 252 on the bottom strand (data not shown). The nuclease protection data are summarized in Figure 1.

Par is the major gyrase-binding site in the plasmid

The par^+ plasmid pCM128 was cut with *Eco*RI and *Ava*I yielding a *par* fragment of 400 bp and a second fragment of 3600 bp. A mixture of the end-labeled fragments was incubated with increasing amounts of DNA gyrase and, after nitrocellulose filtration, the bound fragments were eluted, separated by gel electrophoresis and quantitated by scintillation counting. Gyrase interacts with ~150 bp of DNA. On a 400-bp fragment, the enzyme can, therefore, select its binding site from a region of 250 bp without extending over the end of the DNA. Similarly, on a 3600-bp fragment, 3450 bp are available for binding. Random binding would

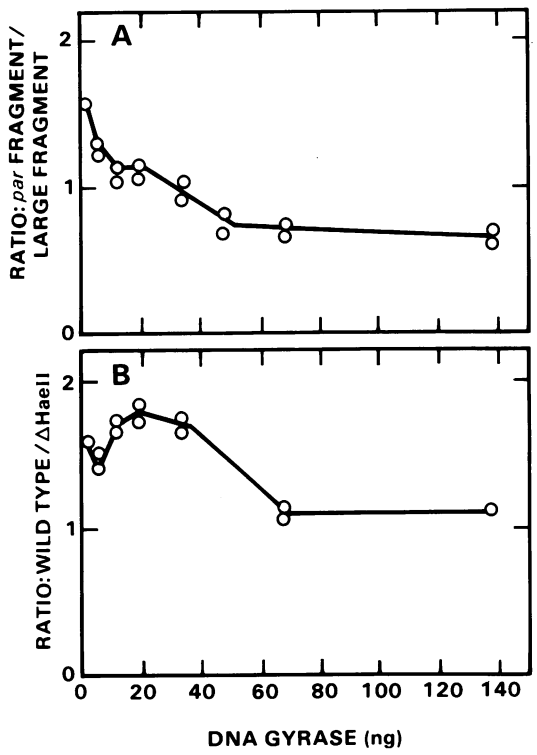

Fig. 4. Filter-binding competition experiments. (**A**) Competition between the *par* fragment and the remaining plasmid DNA. Approximately 40 fmol of pCM128, cut with *Eco*RI and *Ava*I and labeled at the 3′ ends, was incubated with the amount of DNA gyrase indicated and the fragments bound to the enzyme were quantitated (see Materials and methods). After subtraction of background, the ratio of radioactivity in the two fragments was corrected for the ratio in the input DNA; the background ratio of the DNAs trapped on the filter in the absence of gyrase was 1.06. (**B**) Competition between wild-type *par* and ΔHaeII *par*. The 3′-end-labeled wild-type *Eco*RI−*Ava*I *par* fragment (20 fmol) was mixed with 20 fmol of an otherwise identical ΔHaeII *par* fragment (from pCM31) and incubated with the amounts of DNA gyrase indicated. The ratio of *Hae*II-sensitive and -resistant fragments was determined and corrected for the ratio in the input DNA; the background ratio of the DNAs trapped on the filter was 1.2.

thus be expected to result in a 15-fold preference for the large fragment. However, in a number of experiments, DNA gyrase at low concentrations showed a 1.5- to 2-fold preference for the small fragment (Figure 4a). The affinity of gyrase for the *par* fragment is, therefore, 20- to 30-fold higher than for the rest of the plasmid.

A qualitatively similar result was obtained in experiments in which equimolar amounts of relaxed pCM128 (par^+) and pCM328 (Δpar) were mixed and supercoiled by DNA gyrase. At low enzyme concentrations, the par^+ plasmid was supercoiled with a preference of ~1.3 (data not shown).

Interaction of gyrase with a mutant par site

A 4-bp deletion (ΔHae; 272−275) abolishes a *Hae*II site in *par* and interferes only marginally with the Par function, rendering the plasmid Cmp^- (Tucker *et al.*, 1984). An equimolar mixture of end-labeled ΔHaeII and wild-type *par* fragments was used for a filter-binding competition experiment. After elution from the filters, the *Hae*II-sensitive and -resistant fragments were quantitated. At low concentrations of DNA gyrase, the wild-type fragment was bound with an ~1.5-fold higher affinity (Figure 4b).

Influence of par on supercoiling in vivo

Samples from exponentially growing cultures were lysed rapidly with hot SDS and the plasmids were analysed by electrophoresis in chloroquine gels and by Southern blotting (Lockshon and Morris, 1983). A direct comparison of a par^+ plasmid with one that differed only by the 4-bp *Hae*II deletion (see above) revealed no reproducible differences in superhelicity. Due to their different size, plasmids lacking the entire *par* sequence could not be directly compared to par^+ plasmids. From a comparison with plasmids of known superhelical density [measured by band-counting (Keller, 1975) and by dye titration] we estimate that the wild-type plasmid pPM30 in an exponentially growing culture had a superhelical density of −0.068 (26 negative superhelical turns) and that the deletion of *par* caused a loss of superhelicity of only 5−10%, the limit of detection in our experiments (data not shown).

Discussion

The *par* sequence, responsible for the stability of pSC101, is a specific binding site for DNA gyrase. Whereas specific binding of gyrase to other DNA sequences has been observed (for review, see Maxwell and Gellert, 1986), our findings together with genetic studies (Meacock and Cohen, 1980; Tucker *et al.*, 1984) document for the first time a biological role for such an interaction.

A protein that we isolated on the basis of its stabilizing influence on the gyrase−*par* complex is probably an artifact of ribosomal dissolution and is probably without any physiological significance as a DNA-binding protein. A commonly used procedure for cell lysis leads to a massive release of ribosomal proteins which may masquerade as DNA-binding proteins. Indeed, H-protein initially described as an *E.coli* histone analog (Hübscher *et al.*, 1980) has recently been identified as the ribosomal protein S3 (M.Cox, personal communication). Protein n″, which stimulates the synthesis of the complementary strand on single-stranded φX174DNA (Kornberg, 1980), is heavily contaminated with ribosomal protein, L13 (E.H.Lee and A.Kornberg, unpublished observation). However, other histone-like proteins might play a comparable role *in vivo*, enhancing or modulating the activity of gyrase.

The 10-bp phasing of sites of enhanced DNase cutting in gyrase−DNA complexes together with topological evidence suggests that DNA is wound around gyrase in a nucleosome-like fashion (Liu and Wang, 1978a,b). Our finding that the invasion of the gyrase−DNA complex by exonuclease III, in the absence of spermidine, proceeds through multiple pause sites which are 10 bp apart is consistent with this model; spermidine stabilizes the protein−DNA complex against this invasion.

The reasons for the site specificity of gyrase have been obscure. The sequence immediately surrounding the major site of oxolinic acid-induced cleavage in *par* on the bottom strand (AG↓GA) agrees with a portion (PuT/G↓GPu) of a proposed consensus for gyrase-binding sites (Lockshon and Morris, 1985); however, the rest of the sequence fits the consensus poorly. Histone octamers not only bind to DNA in a fashion similar to gyrase, they also share with gyrase the ability to bind specifically to various DNA sequences bearing no apparent similarity. The site specificity of the histone octamer depends on the periodic arrangement of

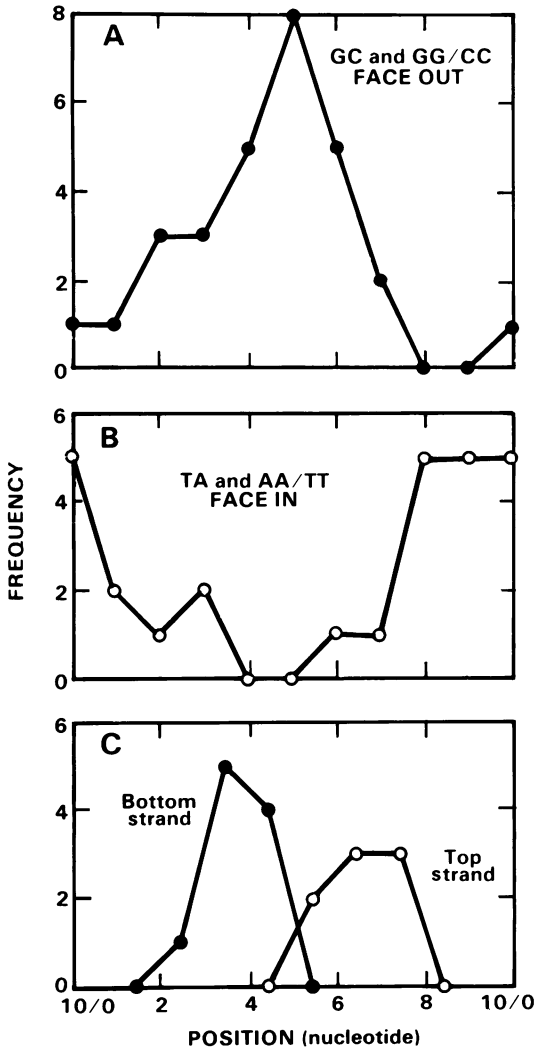

Fig.5. Sequence periodicity in *par*. (**A**) The *par* fragment was analyzed as a sequence of overlapping dinucleotides. All GC and GG/CC dinucleotides were scored (complementary sequences are equivalent). Nucleotides 191–240 (Figure 1) were represented as repeats of a unit of 10 nt: 191, 201, 211, etc., being counted as position 1; 192, 202, 212, etc., as position 2; and so forth. The analysis was repeated for nucleotides 261–310. The two half-sites were superimposed with a 1-bp shift between them, 261, 271, etc., being counted as position 2. This shift is justified because (i) the two half-sites showed similar periodicities, displaced from each other by 1 nt and (ii) the overall periodicity of DNase I cuts in gyrase–DNA complexes is 9.9 (Morrison and Cozzarelli, 1981), corresponding to a 1-nt deviation from a 10-bp period over a sequence of 100 nt. The frequency of occurrence of the dinucleotides in each position is shown. (**B**) The same analysis was accomplished for the dinucleotides TA and AA/TT. (**C**) The locations of enhanced cutting by DNase I in the gyrase–*par* complex are shown in the same manner as the dinucleotide distributions.

certain di-, tri- and tetranucleotides which are more easily bent around the protein (Drew and Travers, 1985; Satchwell *et al.*, 1986): in nucleosomes, short sequences containing As and Ts tend to be 'inside,' arranged with a 10-bp periodicity so that their minor grooves face the protein core, while sequences containing Cs and Gs are predominantly found 'outside' with their minor grooves exposed to the solution. In *par*, a closely corresponding sequence pattern is found: 'inside' and 'outside' dinucleotides occur with a periodicity of 10 bp. The two peaks are shifted one half of a helical turn with respect to each other (Figure 5). The sites of enhanced DNase I cutting straddle the GC dinucleotides, confirming that in the gyrase–DNA complex these nucleotides face toward the solution just as in a nucleosome. Twenty base pairs in the centre of gyrase-binding sites are usually more strongly protected against DNase I (Maxwell and Gellert, 1986) and, in *par*, lack any apparent sequence periodicity of the type described above. A good binding site for gyrase may thus consist of two bendable arms flanking a core of 20 bp which contains some moderately conserved nucleotides.

The trinucleotides GGC/GCC and AAA/TTT show the most pronounced periodicity in nucleosome core DNA (Satchwell *et al.*, 1986). Each pair would, statistically, be expected to occur 3.1 times in a random sequence of 100 bp (= 98 overlapping trinucleotides). In fact, the GGC/GCC pair occurs nine times and the AAA/TTT pair occurs seven times in the two arms of *par*. Of these 16 trinucleotide pairs, 10 are completely and one is partly contained within the three 13-bp repeats that are believed to be essential for the *par* function. Among these three repeats only seven out of 13 nucleotides are conserved. Four of the seven nucleotides represent the runs of As in the middle of each sequence. Since runs of As are the predominant feature of bent DNA (Wu and Crothers, 1984), the significance of this homology is questionable. The repeats are, therefore, most readily interpreted as easily bent segments of the *par* locus rather than as the reiteration of one specific binding sequence.

From the known involvement of DNA gyrase in several aspects of DNA replication, one might suspect that the deletion of *par* destabilizes the plasmid by interfering with its replication. However, the level of single cell resistance to ampicillin suggests that the copy number of various *par* mutants is the same as that of the wild-type (Tucker *et al.*, 1984). This suggestion concerning copy number is supported by the analysis of plasmid DNA in Southern blots (our unpublished data; C.Miller and S.Cohen, personal communication). Also, significant amounts of catenated plasmids were not detected (Meacock and Cohen, 1980; our unpublished observations). Thus the stabilizing effect of the gyrase–*par* interaction is most likely not due to a stimulation of plasmid replication at any stage. Alternatively, a higher degree of superhelicity of the plasmid brought about by a strong gyrase-binding site might facilitate the interaction with a different protein (possibly host-encoded) that is important for stability. However, this would not explain the Cmp^- phenotype which appears not to be correlated with any loss of superhelicity. Furthermore, the loss of superhelicity in par^- plasmids is small and apparently within the physiological range (Balke and Gralla, 1987). The possibility remains that the Par effect may not be related to the known catalytic activities of gyrase in supercoiling and decatenation. Rather, the tight binding of DNA gyrase to one particular site (alone or in conjunction with other proteins) might serve in the intracellular organization of the DNA, a role which cannot be fulfilled by the association of the enzyme with many weaker binding sites.

Such a structural function has been suggested for the eukaryotic topoisomerase II, which is homologous to gyrase (Lynn *et al.*, 19865; Uemura *et al.*, 1986): the enzyme is found associated with the so-called 'chromosome scaffold' (Earnshaw *et al.*, 1985; Berrios *et al.*, 1985) and is required throughout the mitotic cycle for structural changes of the

chromosome (Uemura *et al.*, 1987). With regard to possible structural functions of DNA gyrase, a few other observations should be mentioned: membrane-binding of pSC101 has been reported to depend on *par* (Gustafson *et al.*, 1983) and the T4-encoded topoisomerase, also homologous to gyrase (Huang, 1986a,b), has been described as a membrane-associated protein (Huang, 1975; Takacs and Rosenbusch, 1975). A temperature-sensitive *gyrB* mutant not only fails to segregate its nucleoids, presumably due to lack of decatentation, but also is grossly defective in the placement of its septa (Orr *et al.*, 1979), a phenotype clearly distinct from the arrest of cell division associated with other mutations affecting DNA replication (Donachie *et al.*, 1984). The Par function of pSC101 might thus reflect a role of DNA gyrase in the process of host chromosome segregation, septum placement and cell division.

A Cmp^- mutation with properties similar to the pSC101 mutants has been described for the *Staphylococcus* plasmid pT181 (Gennaro and Novick, 1986). The authors propose that this mutation interferes with plasmid replication and note that the mutant plasmid has a lowered superhelical density.

Materials and methods

Proteins

Sources were as follows: *Afl*III from Amersham; all other restriction enzymes and exonuclease III from New England Biolabs. DNase I from Worthington. DNA gyrase A and B subunits were each purified from overproducing strains (Mizuuchi *et al.*, 1984; Kaguni and Kornberg, 1984); the two subunits were mixed in an equimolar ratio at a total concentration of 0.7 mg/ml.

DNA

Plasmid pPM30, a 4-kb par^+ derivative of pSC101 (Meacock and Cohen, 1980), or pCM128 [identical to pPM30 except for a copy number mutation (Tucker *et al.*, 1984)] were used as sources of the *par* fragment; pCM328 is a *par* deletion derivative of pCM128 (Tucker *et al.*, 1984). pCM31 is pACYC184 (Chang and Cohen, 1978) containing the ΔHaeII *par* sequence of pWTT316 (Tucker *et al.*, 1984). Plasmids were isolated by the cleared lysate procedure (Clewell and Helinski, 1969) or the alkaline-SDS method (Maniatis *et al.*, 1982), banded at least twice in CsCl–EtBr gradients and further purified by gel filtration or chromatography on hydroxylapatite. Some DNA preparations with an unusually high background in filter-binding experiments were improved by slow filtration through nitrocellulose filters. DNA fragments were isolated from low-gelling- temperature agarose gels by phenol extraction, further purified on DEAE–cellulose and labeled according to Maniatis *et al.* (1982). Non-denaturing agarose and polyacrylamide gels were run in 1 × TBE (Maniatis *et al.*, 1982). Very small quantities of DNA were determined in a TK0100 fluorometer (Hoefer Scientific, San Francisco, CA).

Other reagents

5′-Phosphoryl pentadeoxynucleotides of mixed sequence [$p(dN)_5$] were from Pharmacia, spermidine trihydrochloride from Sigma, labeled deoxyribonucleotides from Amersham, and [γ-^{32}P]ATP (6000 Ci/mmol) from NEN. Hydroxylapatite was obtained from Biorad, low-gelling-temperature agarose from FMC, and nitrocellulose filters from Millipore (HA) or Schleicher and Schuell (BA85). The pH of all buffers was adjusted at 50 mM at room temperature.

Crude extracts

E.coli H560 (F^+ *polA end tsx rpsL*) was grown in a 200 l fermentor in L broth to late log phase, suspended in 10% sucrose, 50 mM Tris–HCl, pH 7.5 and frozen in liquid nitrogen. The cells were thawed, adjusted to OD_{600} = 200 in 10% sucrose, 50 mM Tris–HCl (pH 7.5), 0.2 M or 1 M NaCl, 20 mM EDTA, 20 mM spermidine (neutralized with HCl) and 1 mM dithiothreitol (DTT). Lysozyme (200 μg/ml) was added, the suspension was stirred in a cold room for 45 min and 1/100 volume of 10% Brij58 was added. The suspension was spun for 45 min at 0°C and 13 000 r.p.m. in a Beckman JA 14 rotor. The supernatant (Fr. I) was used for assays and for protein purification. The specific protection of the *par* sequence was observed in extracts made at either 0.2 or 1 M NaCl but the specific activity of the crude fraction was 2-fold higher at the higher level. This result may reflect an increased yield of DNA gyrase or of one or more stimulatory factors.

Alternatively, cells were adjusted to OD_{600} = 200 in 50 mM Tris–HCl (pH 7.5), 0.3 M NaCl, 10 mM $MgCl_2$, 1 mM DTT and disrupted by two passages through a French pressure cell at 11 000 p.s.i. cell pressure. The lysate was spun for 20 min at 15 000 r.p.m. and 2°C in a Beckman JA 20 rotor and the supernatant was spun again for 3.5 h at 50 000 r.p.m. and 4°C in a Beckman 60 Ti rotor. The fate of the ribosomes was followed by a determination of the RNA content of each fraction. The second supernatant was taken to be ribosome free.

Assays

The exonuclease III protection assay (Wu, 1985) contained in 20 μl: 25 mM Hepes-KOH (pH 7.6), 10% glycerol, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 2 mM K phosphate (pH 7.5), 100 μg of BSA/ml, 1 μg of $p(dN)_5$, 1 μg of salmon sperm DNA, 10 fmol of 5′-end-labeled *par* fragment, 10 units of exonuclease III and other proteins as indicated. The mixture was assembled on ice. The digestion was started by addition of $MgCl_2$ to 10 mM and incubated at 37°C for 5 min. It was stopped by addition of 2.5 M NH_4 acetate and transfer to ice. The DNA was precipitated with ethanol and analyzed on denaturing 4% acrylamide gels of 0.75 × 105 × 240 mm (Maniatis *et al.*, 1982). For high resolution mapping, 8% acrylamide buffer gradient gels (Biggin *et al.*, 1983) of 0.4 × 200 × 500 mm were used. In some experiments, gyrase and DNA were incubated for 10 min at 37°C in the presence of 10 mM $MgCl_2$ before exonuclease III was added. The results were essentially the same as with the standard procedure. When purified gyrase was used, K phosphate and $p(dN)_5$ could be omitted from the assay.

DNase I footprinting (Galas and Schmitz, 1978) was done under the same conditions as the exonuclease digestion with the following modifications: phosphate, $p(dN)_5$ and EDTA were omitted, 1 mM $CaCl_2$ was included and the mixture was incubated for 10 min at 37°C in the presence of 10 mM $MgCl_2$ before 1 or 2 ng DNase I was added. The reaction was stopped after 30 s as described above and analyzed on buffer gradient sequencing gels.

For filter-binding experiments, gyrase and DNA were incubated (20 μl volume) for 40 min at room temperature in 25 mM Hepes-KOH (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM spermidine trihydrochloride, 1 mM DTT and 100 μg of BSA/ml. The mixture was diluted with 1 ml of wash buffer (binding buffer lacking DTT and BSA) and filtered through 13 mm diameter nitrocellulose filters at 2–3 ml/min. The filters had been previously boiled in four changes of distilled water and soaked in wash buffer containing 10 μg of salmon sperm DNA/ml. For elution of DNA, each filter was immediately placed into a microcentrifuge tube containing 300 μl of 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 500 mM Na acetate and left at room temperature for several minutes; SDS was added to 0.5% and the filters were incubated at 65°C for 30 min. The capped tubes were inverted, pierced at the bottom with a hot needle, inserted into plastic tubes and spun for several seconds in a bench-top swinging-bucket centrifuge. The filters were re-eluted in new microcentrifuge tubes containing 200 μl of 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5% SDS and 5 μg of tRNA each. The buffer was spun out after a few minutes as before and the nucleic acids were ethanol precipitated from the combined eluates.

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References

Armstrong,K.A., Acosta,R., Ledner,E., Machida,Y., Pancotto,M., McCormick,M., Ohtsubo,H. and Ohtsubo,E. (1984) *J. Mol. Biol.*, **175**, 331–347.

Austin,S. and Abeles,A. (1983) *J. Mol. Biol.*, **169**, 353–372.

Balke,V.L. and Gralla,J.D. (1987) *J. Bacteriol*, **169**, 4499–4506.

Berrios,M., Osheroff,N. and Fisher,P.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4142–4146.

Biggin,M.D., Gibson,T.J. and Hong,G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3963–3965.

Brosius,J. and Chen,R. (1976) *FEBS Lett.*, **68**, 105–109.

Chang,A.C.Y. and Cohen,S.N. (1978) *J. Bacteriol.*, **134**, 1141–1156.

Clewell,D.R. and Helinski,D.R. (1969) *Proc. Natl. Acad. Sci. USA*, **62**,

1159–1166.
Cohen,S.N., Miller,C.A., Tucker,W.T., Meacock,P.A. and Gustafson,P. (1985) In Helsinki,D.R., Cohen,S.N., Clewell,D.B., Jackson,D.A. and Hollaender,B. (eds), *Plasmids in Bacteria*. Plenum Press, New York, pp. 383–395.
Donachie,W.D., Begg,K.J. and Sullivan,N.F. (1984) In Losick,R. and Shapiro,L. (eds), *Microbial Development*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 27–72.
Drew,H.R. and Travers,A.A. (1985) *J. Mol. Biol.*, **186**, 773–790.
Earnshaw,W.C., Halligan,B., Cooke,C.A., Heck,R.R.S. and Liu,L.F. (1985) *J. Cell Biol.*, **100**, 1706–1715.
Fisher,L.M., Mizuuchi,K., O'Dea,M.H., Ohmori,M. and Gellert,M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4165–4169.
Galas,A. and Schmitz,O.J. (1978) *Nucleic Acids Res.*, **5**, 3157–3170.
Gellert,M., Mizuuchi,K., O'Dea,M.H., Itoh,T. and Tomizawa,J.-I. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4772–4776.
Gennaro,U.L. and Novick,R.P. (1986) *J. Bacteriol.*, **168**, 160–166.
Gustafson,P., Wolf-Watz,H., Lind,L., Johansson,K.-E. and Nordstrom,K. (1983) *EMBO J.*, **2**, 27–32.
Howe,W.E. and Mount,D.W. (1975) *J. Bacteriol.*, **124**, 1113–1121.
Huang,W.R. (1975) *Virology*, **66**, 508–521.
Huang,W.R. (1986a) *Nucleic Acids Res.*, **14**, 7379–7390.
Huang,W.R. (1986b) *Nucleic Acids Res.*, **14**, 7751–7765.
Hübscher,U., Lutz,H. and Kornberg,A. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5097–5101.
Jaffé,A., D'Ari,R. and Norris,V. (1986) *J. Bacteriol.*, **165**, 66–71.
Kaguni,J.M. and Kornberg,A. (1984) *Cell*, **38**, 183–190.
Keller,W. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4876–4880.
Kirkegaard,K. and Wang,J.C. (1981) *Cell*, **23**, 721–729.
Kornberg,A. (1980) *DNA Replication*. W.H.Freeman and Company, San Francisco.
Liu,L.F. and Wang,J.C. (1978a) *Proc. Natl. Acad. Sci. USA*, **75**, 2098–2102.
Liu,L.F. and Wang,J.C. (1978b) *Cell*, **15**, 979–984.
Lockshon,D. and Morris,D.R. (1983) *Nucleic Acids Res.*, **11**, 2999–3017.
Lockshon,D. and Morris,D.R. (1985) *J. Mol. Biol.*, **181**, 63–74.
Lynn,R., Giaever,G., Swanberg,S.L. and Wang,J.C. (1986) *Science*, **233**, 647–649.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Maxam,A.M. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560–564.
Maxwell,A. and Gellert,M. (1986) In Anfinsen,C.B., Edsall,J.T. and Richards,F.M. (eds) *Advances in Protein Chemistry*. Academic Press, Orlando, FL, pp. 69–107.
Meacock,P.A. and Cohen,S.N. (1980) *Cell*, **20**, 529–542.
Miller,C.A., Tucker,W.T., Meacock,P.A., Gustafson,P. and Cohen,S.N. (1983) *Gene*, **24**, 309–315.
Mizuuchi,K., Mizuuchi,M., O'Dea,M.M. and Gellert,M. (1984) *J. Biol. Chem.*, **259**, 9199–9201.
Morrison,A. and Cozzarelli,N.R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1416–1420.
Nordstrom,K., Molin,S. and Aagard-Hansen,M. (1980) *Plasmid*, **4**, 215–227.
Ogura,T. and Hiraga,S. (1983) *Cell*, **32**, 351–360.
Orr,E., Fairweather,N.F., Holland,I.B. and Pritchard,R.H. (1979) *Mol. Gen. Genet.*, **177**, 103–112.
Satchwell,S.C., Drew,M.R. and Travers,A.A. (1986) *J. Mol. Biol.*, **191**, 659–675.
Sugino,A., Peebles,C.L., Kreuzer,K.N. and Cozzarelli,N.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4767–4771.
Takacs,B.J. and Rosenbusch,J.P. (1975) *J. Biol. Chem.*, **250**, 2339–2350.
Tucker,W.T., Miller,C.A. and Cohen,S.N. (1984) *Cell*, **38**, 191–201.
Uemura,T., Morikawa,K. and Yanagida,M. (1986) *EMBO J.*, **5**, 2355–2361.
Uemura,T., Ohkura,M., Aduchi,Y., Morino,K., Shiozaki,K. and Yanagida,M. (1987) *Cell*, **50**, 917–925.
Van Holde,K.E. and Hill,W.E. (1979) In Nomura,M., Tissieres,A. and Lengyel,P. (eds), *Ribosomes*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 53–91.
Wu,C. (1985) *Nature*, **317**, 84–87.
Wu,H.-M. and Crothers,D.R. (1984) *Nature*, **308**, 509–513.

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