Molecular Basis of the Supercoil Deficit Induced by the Mini-F Plasmid Partition Complex

Received for publication, April 9, 2008, and in revised form, October 28, 2008. Published, JBC Papers in Press, November 10, 2008, DOI 10.1074/jbc.M802752200

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Formation of a partition complex on plasmid F by binding of SopB protein to the sopC centromere is the first step in the partition process that ensures stability of F in dividing cells. Establishment of the complex enables nonspecific binding of SopB to neighboring DNA, which extends the partition complex and provokes reduction of negative supercoiling of the plasmid. This reduction is believed to reflect winding of DNA into positive supercoils about SopB to create a nucleoprotein structure of probable importance to partition. We have searched for evidence that SopB alters plasmid topology. Permutation analysis indicated only modest bending of linear DNA fragments, and in vivo DNase I footprinting revealed no enhanced cleavages indicating curvature. In vitro, SopB binding left no topological trace in relaxed-circular DNA treated with topoisomerase I or in nicked circles closed by ligase. In vivo, novobiocin-mediated inhibition of DNA gyrase relaxed a plasmid carrying the partition complex but left no residue of positive supercoils. Hence, SopB does not reduce plasmid supercoiling directly. We did observe that SopB partly prevented removal of negative supercoils from plasmid DNA by topoisomerase I and partly prevented ligation of nicked circles, indicating that it acts as a physical obstacle. The supercoil deficit is thus better explained as SopB recoating of just-replicated DNA, which shelters it from gyrase and from topological changes in SopB-free DNA. This topological simplicity distinguishes the Sop partition complex from other complexes described.

The partition mechanisms that ensure stability of low copy number bacterial replicons resemble eukaryotic mitosis stripped to its simplest elements. This is most clearly seen in the case of plasmids. Each carries a characteristic set of sequence repeats that serve as a centromere. A specific protein, generally termed ParB, binds to the centromere to form a partition complex, functionally equivalent to a kinetochore. A cytoskeleton-like ATPase or GTase, ParA, then acts on the partition complexes to transport their attached plasmids to daughter cells. The proteins are encoded in an autoregulated two-gene operon, parAB, to which the centromere is usually adjacent. Most bacterial chromosomes specify a similar set of functions but how these contribute to chromosome partition is at present less well defined. Our focus here is plasmid partition complexes, whose formation following replication of the centromere is the essential first step of the partition mechanism.

Recent studies have indicated that partition complexes serve not only to link plasmid molecules to their cognate ParAs but also to activate the dynamic properties of these proteins that enable them to segregate plasmid copies (1, 2). The dynamics of ParA proteins stem from their ability to form metastable polymers. In vivo, dynamic behavior is seen only if the cognate ParB and centromere are both present (2); ParB proteins, though able to interact with ParA (3, 4) and to stimulate ATP hydrolysis (5, 6), do not alone promote ParA dynamics. Some aspect of partition complex structure appears to be needed to explain this key event in partition.

One factor could be size. Centromeres typically consist of a cluster of ParB binding sites, and formation of a partition complex might raise local ParB concentration to the point where it is a strong enough signal to promote ParA polymerization. Reduction of binding site number has been seen to diminish centromere efficiency (7). The effective size of certain partition complexes may be increased by spreading, the recruitment by the ParB component of further ParB molecules which bind nonspecifically to neighboring DNA for up to several kilobases (8–10). It is also possible that ParA senses not size itself but partition complexes which have paired (“metaphase”), and that larger partition complexes pair more readily.

Alternatively, the feature of partition complexes essential for ParA stimulation could be the specific nature of their superstructure. Recent studies of P1 partition complex structure have led to the proposal that the combination of two types of binding site in the parS centromere, two distinct motifs in ParB that recognize them and a flexible peptide that links the motifs could confer the versatility that enables partition complex assembly, pairing of plasmid copies and condensation of neighboring DNA via spreading (11, 12). A spring washer structure deduced for the ParR-parC complex of pB171 suggested a mechanism for trapping and feeding ParM-ATP molecules onto the growing tip of the ParM filament (13). While we do not yet know to what extent the structural features so far identified are exploited in vivo, it is clear that defining partition complex structure will be necessary for understanding events at the ParA-partition complex interface.

A striking structural idiosyncrasy appears to characterize the SopB-sopC partition complex of plasmid F. SopC is a tandem...
array of twelve 43-bp units (Fig. 1), each containing a short inverted repeat to which SopB binds as a dimer (14–16). It was found by Biek and Shi (8) and Lynch and Wang (17) that mini-F and other plasmids carrying sopC extracted from cells producing SopB are less negatively supercoiled than plasmids without sopC. These authors proposed, on the basis of various prokaryotic precedents (18–20), that upon binding to sopC, SopB protein wraps it in a right-handed sense, creating a zone of positive supercoiling and leaving negative supercoiling of the remaining plasmid DNA to be adjusted by topoisomerase action. An overall increase in linking number results, which is seen following deproteinization. Notably, the supercoil deficit induced by a single 43-bp unit was comparable to that seen with the entire sopC, implying that the initial partition complex could nucleate the addition of further SopB molecules and so extend the zone of local positive supercoiling over flanking DNA (8). This suggestion received strong support from demonstrations that SopB could inhibit methylase and gyrase action, as well as promoter activity, on DNA flanking sopC (9, 16). Hence the F plasmid partition complex has generally been viewed as an extended nucleoprotein structure whose formation involves introduction of local positive supercoiling.

However, direct evidence that the increased linking number of mini-F plasmids results from creation of a zone of positive supercoiling, by wrapping of DNA on SopB or by other means, has not been reported. We report here an exploration of the topological consequences of SopB binding to DNA in vitro and in vivo. Our findings indicate that rather than introducing positive supercoils, SopB raises mini-F linking number by sheltering it from DNA gyrase.

**EXPERIMENTAL PROCEDURES**

**Proteins, Enzymes, and Reagents**—SopB was purified as described previously (21). HU was a gift from J. Rouvière-Yaniv. Restriction and nicking endonucleases, T4 DNA ligase, T4 polynucleotide kinase (PNK), Klenow fragment of DNA polymerase I, and Taq polymerase were purchased from New England Biolabs, calf thymus topoisomerase I (Topo I)2 from Promega, and Taq polymerase were purchased from New England Biolabs, calf thymus topoisomerase I (Topo I)2 from Promega, and Taq polymerase were purchased from New England Biolabs, calf thymus topoisomerase I (Topo I)2 from Promega, and Taq polymerase were purchased from New England Biolabs, calf thymus topoisomerase I (Topo I)2 from Promega, and Taq polymerase were purchased from New England Biolabs. Bovine serum albumin (Fraction V), chloroquine and Sybr green from Sigma.

**Filter Binding Assay**—SopB DNA binding activity was assayed by double filter binding as previously described (21). The specific and nonspecific DNA species were, respectively, a 144-bp sopC fragment produced by PCR amplification from pJYB55 (22) in the presence of [α-32P]dATP, using oligonucleotides sopC22 (5’-CTAGTGATCCCCCGGCTCGAG-3’) and sopC23 (5’-CCCTCACTAAAGGAACAAGGC-3’), and a 127-bp repE fragment from pDAG114 (23) using repE1 (5’-AGTTACCAGCGTATGCTGATTTTT-3’) and repE2 (5’-ACGATATGAGTCGTGAGCGT-3’).

**Electrophoretic Mobility Shift Assay**—Binding reactions were started by adding purified SopB protein to 50 mM HEPES-KOH (pH 7.5), 50 mM KCl, 10 mM MgCl2, 10% glycerol, 100 μg·ml−1 bovine serum albumin, 50 μg·ml−1 sonicated salmon sperm DNA, and 1 mM dithiothreitol containing 0.5 mM 32P-labeled restriction fragments with (106 bp) and without (83 bp) a SopB binding site in a final volume of 10 μl. After incubation at 30 °C for 10 min, the mixtures were analyzed by electrophoresis in 6% polyacrylamide gels in TBE (90 mM Tris borate, 1 mM EDTA) and phosphorimaging as previously described (22). For DNA binding analysis, a 43-bp sopC unit generated by oligonucleotide annealing (sequence shown in Fig. 1) was inserted into the SalI restriction site of pBend2 (24), giving pJYB69. Fragments of 169 bp were excised from pJYB69 to yield a circularly permuted set (see Fig. 2C), and radioabeled using PNK and [γ-32P]ATP. SopB binding reactions were assembled and processed as above.

**Preparation of Plasmid DNA Substrates**—A p15A-based plasmid carrying sopC, pZC204 (25), was purified according to the Qiagen miniprep procedure. When necessary, pZC204 monomers were further purified by agarose gel electrophoresis and extraction using the Qiagen kit.

Relaxed CCC DNA for Topo I assays was prepared by incubating pZC204 DNA (600 ng) with Topo I (30 units) in T buffer (25 mM HEPES-KOH, pH 7.8, 100 mM KCl, 5 mM MgCl2, 100 μM EDTA, 10% glycerol, 1 mM dithiothreitol) at 30 °C for 15 min. Removal of supercoils was verified by agarose gel electrophoresis, and the relaxed DNA was used directly in subsequent reactions.

Nicked circular DNA for ligation assays was prepared by incubating monomeric pZC204 with N.BstNBI endonuclease in NEBuffer 3 for 1 h at 55 °C, then purifying the DNA by phenol/chloroform extraction and ethanol precipitation.

**Topo I Assay**—Negatively supercoiled or relaxed pZC204 (at 2 nm) was incubated with SopB (0.014–4.0 μM) in 10 μl of T buffer at 30 °C for 10 min. Three units of Topo I (0.5 μl) were then added, and incubation continued for 10 min. Reactions were stopped by addition of 10 μl of 2% SDS, 50 mM EDTA, and incubated at 55 °C for 15 min, and the DNA was purified by phenol/chloroform extraction and ethanol precipitation prior to analysis by agarose gel electrophoresis and Sybr green staining.

**Ligase Assay**—Nicked pZC204 (1 nm) was incubated with SopB (0.025–2.5 μM) in 10 μl of B buffer (20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 10 mM MgCl2, 10% glycerol) at 30 °C for 15 min. 1 μl of ligase mix (40U T4 DNA ligase in B buffer containing 10 mM ATP) was then added and incubation continued for 20 min. Reactions were stopped by addition of 5 μl of Stop mix (3.3% SDS, 100 mM EDTA, 25% glycerol, bromphenol blue) and incubated at 55 °C for 15 min, and the DNA was purified and analyzed as for the Topo I assay.

**Electrophoresis**—The electrophoresis medium for all experiments was 1% agarose in 40 mM Tris acetate, 1 mM EDTA. Topo

2 The abbreviations used are: Topo I, topoisomerase I; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
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RESULTS

Several measurements of the linking number difference (ΔLk) between wild type mini-F and its Δ sopC equivalent have shown it to be $-12 \pm 1$ (8, 25, 26, 28, 29). The correspondence of this number with that of specific binding sites in sopC is purely fortuitous; mini-F with a single sopC unit (43 bp) centers more a ΔLk about 7 higher than that of the Δ sopC mutant, implying that much of the supercoil deficit in mini-F plasmids (about half on the basis of these figures) arises from nonspecific binding of SopB to the DNA that flanks sopC. In any case, only ten of the binding sites in sopC are likely to be effective; footprinting data (30) indicate that the degenerate terminal repeats do not bind SopB strongly. Anticipating that both specific and nonspecific binding would contribute to plasmid topology, we began by characterizing both.

DNA Binding by SopB—To establish the conditions needed for analyzing topological effects of SopB binding to sopC, we purified SopB as the native protein, and used a filter retention assay to measure its binding to DNA in the presence of nonspecific competitor DNA. Fig. 2A shows binding of radioactively labeled 144-bp fragments with or without a 43-bp sopC unit as a function of SopB concentration. Binding to the sopC fragment was by far the stronger under these conditions, as expected. A double-reciprocal plot of the binding data (not shown) yielded an apparent $K_d$ of 0.015 μM and 2.9 μM for the sopC and nonspecific fragments respectively. The $K_d$ for sopC binding is 15-fold higher than the DNA concentration (1 μM) used, not a vast excess but sufficient for confidence that the $K_d$ is accurate within a factor of 2. The nonspecific binding constant is similar to that reported previously (21). The substrates used in previous determinations of specific SopB-sopC affinity (30, 31) differ too much from the single-binding site, linear fragments used here to allow meaningful comparison.

The electrophoretic mobility shift analysis shown in Fig. 2B confirmed the filter retention results. SopB showed no significant binding to the control 83-bp fragment but formed a distinct band (“B”) by retarding a radioactively labeled 106-bp fragment containing one 43-bp unit of sopC. In addition, a smear of DNA appeared behind the sopC fragment and migrated more slowly as SopB concentration rose. Only the sopC fragment contributes to this; the 83-bp control band is not diminished even at the highest concentration of SopB used. The smearing suggests that some of the SopB-sopC complexes are not fully stable in the gel buffer and disintegrate during electrophoresis.

Bending of sopC DNA by SopB Protein—As a first test of the likelihood that SopB wraps DNA, we examined a necessary consequence of wrapping, SopB-induced curvature. Curvature of linear DNA decreases the distance between its ends and increases its effective cross-sectional area, thus retarding its reptational migration in gel matrices (32, 33). This phenomenon has served as the basis of a widely used method for measuring bend angles. Retardation of a fragment bent in the middle results not only from the added mass of the bound protein but also from the maximum reduction in the end-to-end distance, and is therefore greater than retardation of a fragment bent near the end which is caused mainly by protein bulk. The bend angle is proportional to the ratio between these two retardations and can be calculated from it (34).

To estimate the degree of bending that SopB produces upon binding to a single 16-bp sopC site, we inserted the site into the vector pBend2, then re-excised it on fragments of ~164 bp (Fig. 2C) and measured SopB-induced retardation of each fragment on polycrylamide gels. The distance migrated by the complex relative to that migrated by the bare fragment, Rf, is plotted against the distance from the fragment end to the binding site center in Fig. 2D. SopB retards the fragments according to the binding site position and most strongly for the more central positions, implying that the apex of the bends is at or within a few base pairs of the center of the sopC site.

The relative mobilities of fragments with centrally located and terminally located bends was shown by Thompson and Landy (34) to conform fairly closely to the relation: ratio of
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FIGURE 2. DNA binding properties of SopB. A, site-specific and nonspecific binding. Radioactively labeled sopC (circle) and repE (square) DNA fragments were incubated with increasing concentrations of SopB, and the amounts retained by filtration were measured. The data are from three independent experiments. B, gel retardation of SopB-sopC complexes. Mixed radioactively labeled sopC (106 bp) and control (83 bp) fragments were incubated with increasing concentrations of SopB and complex formation revealed by PAGE and phosphorimaging. B denotes a specific retarded complex. C, circularly permuted set (in open rectangle) fragments (164 bp), generated from pJYB69 with restriction endonucleases indicated at the top. Top scale, distance in bp; bottom scale, distance relative to center of Smal fragment binding site (vertical line). D, permutation analysis of SopB-induced DNA bending at sopC. Radioactively labeled fragments of the permuted set (in C) were incubated with SopB (60 nM) and complexes separated from naked DNA as in B. The distance moved by each complex divided by that of the corresponding unbound fragment yields the relative migration (µ(μu), closed circles), which is plotted against the distance between the nearer end and the binding site center (arrowhead in Fig. 1). Non-linear regression analysis fitted the data to a parabolic curve whose equation enabled extrapolation to the fragment ends (dotted lines; open circles) and calculation of µu values for the theoretically most terminal complex realizable and for the terminus itself (vertical dotted line). Bend angles were calculated from µu (middle)/µu (end) = cos(α/2).

We also attempted to measure the cumulative bend produced by binding at two naturally spaced sites, reasoning that the 43-bp interval would correspond closely enough to four complete helical turns (of 10.5 bp) for the individual bends to be in phase and thus measurable in the same way. The value obtained, ~70° (data not shown) was considerably less than twice the solo angle, suggesting that in sopC successive SopB binding sites are not bent in the same plane. It also suggests that, at least under our electrophoresis conditions, two bound SopB dimers do not interact to loop the intervening DNA into a tight bend.

Permutation analysis allows curvature estimations only for specifically bound protein. Multiplicity of binding sites, potential for rapid exchange of SopB between them and site-to-site variation prevents the use of this method for estimation of bending induced by nonspecifically bound protein. In general the degree of induced curvature is correlated with binding affinity (35–38), and if SopB follows the pattern we expect that even in an extended, relatively long-lived complex, nonspecifically bound SopB dimers would bend DNA to a variable degree and on average by less than 50°.

The ~50° bend induced in sopC scarcely qualifies as wrapping, and an in-phase 10-fold repetition in sopC would introduce at most only ~1.5 turns. If the contributions of specific and nonspecific binding to the mini-F supercoil deficit are about equal (see above), explaining the ΔLk of +12 on the basis of directly introduced positive supercoils seems problematic.

Effect of SopB Protein on Plasmid Topology—If SopB directly increases plasmid linking number, this should be manifested as a residue of positive supercoils after removal of supercoiling from the SopB-free portion of plasmid DNA. A binding mixture consisting of SopB protein and a negatively supercoiled plasmid carrying sopC, pZC204, was treated with calf thymus topoisomerase I, which removes both negative and positive supercoils. After deproteinization the DNA was subjected to agarose gel electrophoresis, with the result shown in Fig. 3A. In the absence of SopB, topoisomerase I relaxed pZC204 to a mixture of topoisomers which two-dimensional gel electrophoresis (not shown) showed to consist largely of species −1 to +4 (lane 2). We assume that the topoprofile is not centered at zero because of the difference in temperatures at which Topo I treatment and gel electrophoresis were carried out. The profile was not detectably altered at SopB concentrations ≤ 0.05 μM. At SopB concentrations ≥ 0.1 μM the same topoisomers were the major products but in addition a series of higher order topoisomers appeared (lanes 3–5). Their sign was determined by subjecting the 0.9 μM SopB sample to two-dimensional electrophoresis (Fig. 3B), in which the chloroquine present in the second electrophoresis buffer causes helix unwinding and a
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compensatory reduction of negative superhelicity. The faster migrating, more highly wound topoisomers from the first dimension are slowed in the second, and the slower ones accelerated, showing that the SopB-related topoisomers were of negative sign. (Distinct migration of positive and negative topoisomers of a given density is also seen in the standard gel conditions of Fig. 3A, as observed previously (39)). The essential result of this experiment is that SopB does not generate new positively supercoiled species or increase the amounts of those made in its absence, and so does not affect plasmid DNA in vitro as it does in vivo. It suggests that the increased in vivo linking number of sopC plasmids is an indirect consequence of SopB binding.

How to account for the negatively supercoiled species? It is not simple to exclude direct inhibition of Topo I by SopB. Non-specific DNA binding by SopB subverts attempts to control for inhibition by inclusion of a second, non-sopC plasmid; replacement of pZC204 by the vector pACYC184 gave essentially the same result as that shown in Fig. 3A. Although near maximum relaxation of most substrate molecules throughout the SopB concentration range (Fig. 3A) is not the action expected of a directly inhibited enzyme, Topo I is reported to be processive in the buffer conditions used (40), and this, coupled with occasional dissociation provoked by SopB, could account for the heterogeneity of the reaction products.

An alternative to inhibition of Topo I is prevention of its action through competition for the DNA substrate. Negatively supercoiled molecules could arise from a plasmid subclass of distinct tertiary structures particularly receptive to SopB, or simply be the remainder of SopB-DNA complexes that are inherently unstable, as suggested above (Fig. 2B), or readily destabilized by Topo I. The heterogeneity of the plasmid population could reflect an all-or-none response to SopB, implying strong co-operativity and suggesting that protein-protein contacts facilitate nonspecific binding of SopB. These uncertainties led us to examine alternative approaches.

Lack of Supercoiling of Relaxed Plasmid DNA by SopB—We next determined whether SopB could induce supercoiling in plasmid DNA that had already been relaxed by Topo I action. As a control we used the “histone-like” protein HU, which is known to wrap DNA into negative supercoils (41). Relaxed pZC204 was incubated with SopB or HU then treated again with Topo I to remove supercoils from protein-free DNA, and the products were fractionated by agarose gel electrophoresis (Fig. 4A). Comparison of lanes 2 and 3 shows that the initial Topo I relaxation had not gone to completion but did so during the second incubation. Supercoiling introduced by HU was readily seen (lanes 4 and 5), and its negative sense was verified by two-dimensional gel electrophoresis (Fig. 4C). In contrast, the barely discernible reduction of topoisomers +2→+4 at the highest concentration of SopB (compare lane 8 to lanes 6 and 7) represents a shift in topoisomer distribution of much less than Δlk = 1 (compare Fig. 4C to Fig. 3B).

Because under the conditions of this experiment SopB induced no significant change in the topology of the target molecule, it was important to verify that SopB was actually binding DNA. Fig. 4B shows the result of incubating both CCC and OC forms of pZC204 and a second, non-sopC plasmid (pCN) with SopB in T (Topo I reaction) buffer prior to electrophoresis in an agarose gel. Depletion of naked pZC204 DNA (first CCC then OC) was followed by that of pCN (lane 3), and at 2 μM SopB all species were strongly retarded (lane 4), showing that within the range of concentrations used in the experiment of Fig. 4A SopB was binding to both sopC and nonspecific DNA.

To verify the apparent inability of SopB to introduce supercoils, we used a ligation assay, independent of
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A pZC204 substrate, showing the sites of N.BstNBI-mediated nicking (N). B, nicked circular pZC204 DNA was incubated with SopB (at the concentrations shown) and T4 DNA ligase, and the products were separated by agarose gel-electrophoresis. Lane 1, linear DNA standards (length in kb is indicated on the left); lane 2, N.BstNBI-nicked pZC204 substrate; lanes 3–9, nicked pZC204 after incubation with proteins as indicated. C, T4 DNA ligase is not inhibited at high SopB concentrations. A linear 575-bp sopC fragment (lanes 2 and 3), N.BstNBI-nicked pZC204 substrate (lanes 4 and 5) or both (lanes 6 and 7) were incubated with T4 DNA ligase in the absence (lanes 2, 4, and 6) or presence of 2.5 μM SopB (lanes 3, 5, and 7). Lane 1, linear DNA standards (length in kb is indicated on the left). Note that to ensure the resolution of the ligation products, the gel was such that the original purified linear DNA fragment runs off the gel. The position of the pZC204 open circle (OC) band is indicated on the right.

Topo I SopB was added to pZC204 DNA into which three single-strand breaks had been introduced by digestion with nicking endonuclease N.BstNBI (Fig. 5A). If binding of SopB distorts the DNA sufficiently to create a supercoil this will be trapped by ligation of the breaks and seen as a change in the topoisomer profile. Fig. 5B shows the topoisomers resulting from ligation in the presence of increasing concentrations of SopB. Ligation in the absence of SopB was >90% complete and produced topoisomers which had 1–4 supercoils in our electrophoresis conditions (lanes 3 and 9). This pattern was little changed by inclusion of SopB protein. Densitometric scanning showed that the topoisomer distribution was not affected at any concentration of SopB. However, at 0.25 μM SopB and above (lanes 6–8) a fraction of the substrate remained incompletely ligated (OC band), about one-third at the highest SopB concentration used, raising the suspicion that SopB might have been inhibiting ligase. The experiment depicted in Fig. 5C shows that this was not the case. A linear 575-bp DNA fragment consisting largely of sopC was incubated with T4 DNA ligase. In the absence of SopB (lane 2), approximately equal amounts of circular (lower band) and linear (upper band) dimers were formed together with lesser amounts of higher order linear and circular multimers. In the presence of SopB (lane 3) most of the sopC fragment was ligated to linear multimers at the expense of circular forms, presumably because SopB binding reduces the flexure needed for the ends of a single fragment to meet. The same pattern of ligation products was seen when the OC pZC204 closure reaction (lanes 3 and 4) was carried out together with the sopC fragment ligation (lanes 5 and 6). These results show that T4 DNA ligase is just as active in the presence of SopB as in its absence, and hence that the accumulation of OC pZC204 DNA in the experiment of Fig. 5B is not due to direct inhibition of ligase by SopB. The simplest explanation for failure to fully ligate nicked DNA is that SopB renders the nicks inaccessible to ligase.

In Situ Footprinting of sopC—If sopC is bent significantly in vivo we would expect to be able to detect this as enhanced cleavage in or around the SopB binding sites. None was seen in the in vitro DNase I protection analysis reported by Mori et al. (30), but the potential for assisted bending mediated by HU, exemplified by the Gal repressor complex (42), or by other histone-like proteins had not been explored. DLT1472, which carries sopB on the chromosome under lac promoter control, was transformed with pZC204. Exponentially growing cells of the resulting strain were induced with IPTG or left uninduced, and then treated briefly with cold ethanol and incubated with DNase I (the “drunken cell” technique (27)). Plasmid DNA was then extracted for use as a template for polymerization from primers specific for the degenerate terminal sopC repeats. The footprint shown in Fig. 6A corresponds to the expected SopB binding sites. Topoisomer analysis of separate samples from same cultures confirmed increased pZC204 linking number in the induced cells (Fig. 6B). Densitometric scanning (Fig. 6C) revealed no enhanced cleavage, only protection. This in vivo result is consistent with the results of our in vitro experiments which indicate that SopB-induced wrapping of a magnitude sufficient to account for the natural mini-F supercoil deficit does not occur.

Absence of Positive Supercoils from SopB-bound Plasmid in Vivo—Although to this point all tests of positive sense supercoiling by SopB had proved negative, it remained possible that in growing cells other factors enable SopB to change topology in this way. We therefore carried out an in vivo version of the search for SopB-dependent positive supercoiling described above (Fig. 3), using novobiocin inhibition of DNA gyrase to allow depletion of negative supercoiling by Topo I. Retention of sufficient novobiocin for topology studies requires use of an active efflux mutant; we chose the E. coli B strain AS19 used by several workers (e.g. Ref. 43), and transduced placc:sopB to it, forming DLT2503, to allow inducible SopB synthesis. In DLT2503 cells carrying pZC204 (sopC+), growth with 100 μM IPTG results in production of SopB at 11 times the level in cells harboring mini-F (data not shown); since this corresponds to the ratio of pZC204 and mini-F copy numbers (26, 44), the SopB:sopC ratio and the size of partition complexes are expected to be normal in this strain. Strains AS19 and DLT2503, each carrying pZC204, were grown in the presence of IPTG, and novobiocin was added to or withheld from cultures prior to extraction of plasmid DNA. Topoisomer profiles of plasmids extracted during novobiocin treatment are shown in Fig. 7. In AS19, without SopB, negatively supercoiled pZC204 molecules (lane 1) were progressively relaxed (lanes 2 and 3), so that by 60 min about a third of the topoisomers contained fewer than 10 negative supercoils; a small fraction of these were positive sign topoisomers (up to +4), which might result at least partly from changes in temperature and buffer, as in the experiment of Fig. 3. In DLT2503, SopB itself provoked the deficit in negative supercoiling characteristic of partition complex formation (compare lanes 1 and 4). The combined effects of novo-
biocin and SopB were additive (lanes 5 and 6), so that by 60 min (lane 6) none of the initial population of negatively supercoiled plasmid remained and nearly all topoisomers had fewer than 10 supercoils. This redistribution did not however extend into the zone of positive sign topoisomers. Because in the conditions of this experiment novobiocin treatment by itself can convert a substantial fraction of the plasmids to the linking number range −10 to −1 (lane 3), doing so on molecules already harboring −12 SopB-induced positive supercoils should have resulted in transformation of this fraction into molecules with a positive net linking number. The absence of any increase in the minor population of positive sign topoisomers (lanes 5 and 6) implies that binding of SopB to sopC-carrying plasmids in vivo does not introduce a significant degree of positive supercoiling.

**DISCUSSION**

Previous work had shown that in vivo SopB can block the action of several enzymes on DNA and reduce the supercoil density of plasmids carrying its specific binding site, sopC (8, 9, 17, 29). Both enzyme occlusion and supercoil reduction were attributed to an ability of the SopB-sopC complex to nucleate formation of a larger complex which through SopB-SopB interaction and nonspecific DNA-SopB binding extends over neighboring DNA. While spreading of the partition complex has been deemed a sufficient basis for enzyme occlusion, reduction of negative supercoiling has been assumed to require in addition the wrapping of DNA on SopB to form local positive supercoils (45–47). Our present results do not support this explanation for the supercoil deficit in mini-F plasmids. SopB introduced no positive supercoiling in either CCC or relaxed plasmid DNA that was detectable after treatment with Topo I, introduced no positive supercoiling in either CCC or relaxed plasmid DNA that was detectable after treatment with Topo I, whether in vitro (Fig. 4) or in vivo (Fig. 7), and left no topological trace after closure of nicked plasmid circles with ligase (Fig. 5).

The possible objection that Topo I action might dislodge SopB from DNA, and with it any DNA deformation, is not tenable in the case of ligase which acts only at nicks. We did find however that SopB bends a single specific site by about 50°, meaning that the cumulative bend over the ten effective sites of sopC potentially induces writhe that in closed circular plasmid DNA would require a compensating introduction of supercoiling. The fact that this was not seen in topoisomer analyses easily sensitive enough to detect them implies that the bends were too planar or were not sufficiently in phase to be additive, despite a binding site interval expected to correspond closely to four integral helices of 10.5 bp. Indeed, the two-binding site bend analysis (not presented) indicated a cumulative curve much less than that expected for phased bends. In addition, nonspecific binding is expected to induce various degrees of bending at various intervals and may well nullify bending at sopC. The absence of a net topological effect of SopB on a sopC-carrying plasmid is thus not necessarily incompatible with moderate bending at individual binding sites.

Because both specific and nonspecific binding contribute to the linking number change in vivo, it was important to allow both to occur on the target DNA in our assays, and to this end we omitted excess nonspecific competitor DNA from the Topo I relaxation and ligase closure experiments. Under these conditions, nonspecific DNA of the assay molecule was as effective a

**FIGURE 6. In situ DNase I footprint and topoisomer analysis.** A, footprints on sopC. pZC204-carrying cells with sopB induced (+) or uninduced (−) were subjected to in situ DNase I treatment, and their DNAs then extracted and used as templates for extension of primers sopC8 (top strand) and sopC9 (bottom strand). Vertical blocks indicate SopB protected regions; numbered from the sopB-proximal end. B, chloroquine-agarose gel electrophoresis of pZC204 topoisomers from the cultures sampled for footprinting in A. C, scan of the in situ DNase I footprints on the bottom strand around binding site 11. Gray line, sopB induced; dark line, uninduced. To correct for differences in loading, the scans were normalized for band intensities outside the SopB-protected area. * indicates a blemish in the gel which causes artifactual peaks in the scan. The sequence of the 43-bp sopC repeat unit is shown beside the corresponding scan, with the footprint shown as bold type and the 16-bp SopB binding site indicated by arrows.

**FIGURE 7. In vivo topoisomer analysis.** Plasmids extracted from exponentially growing cultures of pZC204-carrying cells synthesizing SopB (+) or not (−) and incubated for 0, 20, and 60 min with novobiocin were subjected to two-dimensional gel electrophoresis with 0 and 1 μg·ml⁻¹ chloroquine in the first and second dimensions, respectively. SopB had no detectable effect on the topology of pACYC184 in either the presence or absence of novobiocin (results not shown).
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binding target as sopC. This apparent lack of specificity is not unexpected; there are only 10 specific binding sites but \( \sim 3500 \) potential nonspecific sites in pZC204 of which we estimate about 220 could be simultaneously occupied by SopB. Moreover it corresponds to the behavior of its P1 ParB homologue which, in the absence of IHF protein to mold the parS centromere, was seen to bind equally well to DNA with and without parS when nonspecific DNA was not added (48). ParB was also reported to bind equally well (in the absence of IHF) to the parS of circular forms and, in competition experiments, of linear fragments. If SopB behaves essentially like ParB, its affinity for linear sopC as measured here (Fig. 2) is probably close to its affinity for the circular substrates used in subsequent experiments.

Because SopB-sopC complexes do not restrain supercoils in vitro (Fig. 4) or in vivo (Fig. 7) and their footprints give no sign of significant DNA distortion in vivo (Fig. 6), the origin of the supercoil deficit in mini-F plasmids remains an open question. Certain of our observations may point to the answer. Coating of DNA by SopB could prevent access of single-strand interruptions to ligase, explaining the progressive failure of open circle closure as a function of SopB concentration (Fig. 5). The increase in negatively supercoiled plasmid DNA in proportion to the SopB added to the Topo I relaxation assay (Fig. 3) suggests that bound SopB shields DNA from topoisomerase action. Such shielding can also explain SopB-mediated suppression of DNA cleavage by the gyrase inhibitor, oxolinic acid, reported by Lynch and Wang (9). These authors also suggested that SopB could increase linking number by supplanting more general DNA-binding proteins whose wrapping action contributes to negative superhelicity, but preventing access to gyrase is the only mechanism supported by existing evidence. We propose that the in vivo SopB-induced supercoil deficit has the same origin as enzyme occlusion, the masking of both sopC and adjacent nonspecific DNA by SopB.

The environment of newly replicated plasmid DNA should initially favor binding of SopB over supercoiling. Replication and resolution of progeny plasmid molecules, and resealing to CCC form, must be completed before gyrase introduces supercoils (49). Because much of cell’s SopB is concentrated at extended partition complexes (3, 50), reassembly of new complexes after replication of \( \text{parC} \) complexes could occur quickly and allow extension of the complexes before the onset of supercoiling, resulting in a zone impervious to gyrase. We assume that the SopB-SopB interactions responsible for attracting SopB to the sopC flanks generate a structure compact enough to resist transmission of topological changes occurring in the SopB-free region of the plasmid. Restriction of the normal negative supercoiling reaction to this region would result in the higher linking numbers observed. An alternative explanation, that some particularity of the nucleoprotein structure stimulates host topoisomerase I activity relative to that of gyrase, cannot be ruled out, but we know of no evidence for it.

The view of the SopB-DNA complex as an extensive region with simple DNA topology is in marked contrast to several recent descriptions of partition complexes. P1 ParB is generally similar to SopB; it spreads from its centromere and creates a supercoil deficit, but binding to the complex array of sites in its centromere involves a degree of topological versatility (11) unlikely to be required of SopB. The host IHF protein is enlisted to help form the ParB-parS complex, which is thus strongly bent. The ParR-parC complexes of pB171 (13) and pSK41 (51) form spiral structures in crystals, which were suggested to interact with their ParM actin filaments by encircling them. ParR-parC complexes of plasmid R1 were seen to bind to parC via ribbon-helix-helix motifs rather than a helix-turn-helix as with SopB, also bind to nonspecific DNA in a centromere-dependent manner (i.e. spread). Another partition complex, PrgO-cenA of pGENT, has an intrinsically curved centromere composed of closely spaced repeats, like ParR-parC, but appeared by AFM to have a relatively open configuration (53), like SopB-sopC.

Clearly a wide variety of partition complex structure is compatible with the single function of plasmid segregation. However, the relative simplicity of the Sop complex apparent from our work raises the question of what purpose is served by the complexity of the others. Experiments aimed at defining more precisely the interactions of SopB with DNA may reveal further levels of Sop complex organization.

Acknowledgments—We thank M. Chandler’s laboratory for pBend2 and pCN148, J. Rech and M. Bouvier for technical assistance, F. Pasta, F. Cornet, P. Polard, and H. Krisch for comments on the manuscript, and members of the group Dynamique des réplicons bactériens for discussions.

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