The P1 ParB protein is required for active partition and thus stable inheritance of the plasmid prophage. ParB and the Escherichia coli protein integration host factor (IHF) participate in the assembly of a partition complex at the centromere-like site parS. In this report the role of IHF in the formation of the partition complex has been explored. Next, ParB protein was purified for these studies, which revealed that ParB forms a dimer in solution. Next, the IHF binding site was mapped to a 29-base pair region within parS, including the sequence TAACTGACTGTTT (which differs from the IHF consensus in two positions). IHF induced a strong bend in the DNA at its binding site. Versions of parS which have lost or damaged the IHF binding site bound ParB with greatly reduced affinity in vitro and in vivo. Measurements of binding constants showed that IHF increased ParB affinity for the wild-type parS site by about 10,000-fold. Finally, DNA supercoiling improved ParB binding in the presence of IHF but not in its absence. These observations led to the proposal that IHF and superhelicity assist ParB by promoting its precise positioning at parS, a spatial arrangement that results in a high affinity of ParB for parS.

The prophage of bacteriophage P1 exists as an autonomously replicating plasmid in Escherichia coli (for review see Ref. 1). The plasmid copy number is very low (one to two/Escherichia coli, for review see Ref. 12). IHF participates in a variety of processes in E. coli, including recombination, replication, and transcription, as well as P1 plasmid partition (for review see Ref. 12).

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of plasmids at cell division (3). Plasmids containing parS-small were unable to destabilize wild-type λ-miniP1 plasmids (with parS-large, the natural context) in wild-type cells (14). However, in cells without IHF, parS-small was an effective competitor of parS-large (6). These experiments led to several important conclusions. First, they showed that IHF allows the partition system to discriminate between the two versions of parS. Second, the sequences upstream of the DraI site were essential for ParB binding. Finally, wild-type λ-miniP1 partitions via the intact large site. Thus the wild-type parS site is parS-large and must contain all information required for both proteins to bind.

This study addresses key questions regarding the mechanism of IHF action within the partition complex and presents the first quantitative analysis of the IHF effect on ParB binding. Is the role of IHF to increase ParB affinity for parS? Where and how does IHF exert its effect? The influence of substrate topology on the formation of the partition complex as well as several physical properties of ParB protein have also been examined. The experiments presented here lead to the hypothesis that the precise geometry of the partition complex, promoted by IHF and superhelicity, is crucial for high affinity binding of ParB to DNA.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Phage—**E. coli K12 strains (and relevant genotypes) were DH5 (recA1; Bethesda Research Laboratories) and BR4501 (DH5; 6). λKan-miniPi1 is a kanamycin-resistant derivative of λ-P1:5R-5 (15). The λ-miniPi1parS-[Dra] insertion mutant derivatives were constructed by cloning the insertions onto a pBR322 (16) plasmid containing about 2 kb of P1 DNA and then cross-link the mutations onto λ-miniPi1. The cloning procedures and genetic crosses have been described in detail previously (6).

Briefly, an 8-bp Sall restriction enzyme linker was inserted into the DraI site in parS (Fig. 1), producing pBPE140. Next, the 1.2-kb kanamycin resistance gene from Tn903 (from pUC4-K; Pharmacia Biotechnology Inc.) was cloned into the new Sall site, producing pBPE150. pBPE150 was crossed with λ-P1:5R, selecting for recombinant phage that had picked up the entire plasmid (ampicillin resistant). Lysogens of these progeny were induced, and plaque purified, and phage DNA was restriction mapped. About 50% of the progeny phage had picked up the Sall insert, yielding λ-miniPi1parS::Sal.

A variety of parS-containing plasmids were used in this study. The pBR322 derivatives pALA207 (parS-large, or parS+) and pBPE127 (parS-small) have been described previously (2, 6). pBPE146 (parS::Sal) contains an 802-bp Sall fragment (the parS fragment analogous to that in pALA207) in the pBR322 BamHI site. pBPE143 (DNase footprinting substrate) contains the P1 TaqI-EcoRV parS fragment (Fig. 1), converted to an EcoRI fragment with synthetic linkers. The vector for pBPE143 was pBPE141, a deletion derivative of pBR322 which lacks all sequences between PucI and EcoRI but retains the EcoRI site.

Low copy number parS derivatives were in pFM3, a miniF derivative (17). pBPE161 contains the Sall1 parS-large fragment from pALA207, and pBPE162 the BamHI parS-small fragment from pBPE127. For DNA binding studies, the vector plasmid was pBend5 (a gift from Sankar Adhya, NCI), a derivative of pBend2 (18). pBend5 contains a direct repeat of 17 duplicated restriction sites (from the ends were aligned in modulating cloning sites, HincII and HinfI) and unique sites that are all unique within this region.

**Spectroscopic Analysis of ParB**—This procedure was modified from that in Ref. 20. All absorption spectra were measured on a Cary 118 spectrophotometer, and the data were analyzed on a VAX computer. A model amino acid mixture of N-acetyl-L-tyrosinamide, N-acetyl-L-tyrosinamide, and N-acetyl-L-phenylalaninamide was prepared at a ratio of 1:4:14. This corresponds to the ratio of tryptophan to tyrosine to phenylalanine in ParB, determined. After a four-fold dilution with 8 M guanidine HCl, the absorption spectrum of native ParB, from 245 to 340 nm, was measured and compared to tyrosine to phenylalanine in ParB, predicted from the DNA sequence (2). FFLC-purified ParB (Fraction IV) was concentrated by centrifugation in Centricon-30 filters (Amicon) and run over a 2-ml Sephadex G-25 column in freshly prepared 25 mM Tris-HCl, pH 7.5; 200 mM Na2SO4; 0.1 mM EDTA; 1 mM dithiothreitol (DTT); 10% glycerol. TBE is 89 mM Tris, 89 mM borate, 1 mM EDTA.

**Proteins—**Purified E. coli IHF was generously provided by Howard Nash (NIMH). ParB Fraction III is Bio-Rex 70 purified ParB described previously (6). Protein molecular mass standards were from Sigma and Bio-Rad.

**Spectroscopic Analysis of ParB**—This procedure was modified from that in Ref. 20. All absorption spectra were measured on a Cary 118 spectrophotometer, and the data were analyzed on a VAX computer. A model amino acid mixture of N-acetyl-L-tryptophanamide, N-acetyl-L-tyrosinamide, and N-acetyl-L-phenylalaninamide was prepared at a ratio of 1:4:14. This corresponds to the ratio of tryptophan to tyrosine to phenylalanine in ParB, determined. After a four-fold dilution with 8 M guanidine HCl, the absorption spectrum of this denatured ParB was measured and compared to tyrosine to phenylalanine in ParB, predicted from the DNA sequence (2). FFLC-purified ParB (Fraction IV) was concentrated by centrifugation in Centricon-30 filters (Amicon) and run over a 2-ml Sephadex G-25 column in freshly prepared 25 mM Tris-HCl, pH 7.5; 200 mM Na2SO4; 0.1 mM EDTA; 1 mM dithiothreitol (DTT); 10% glycerol. The UV absorption spectrum of native ParB, from 245 to 340 nm, was determined. After a four-fold dilution with 8 M guanidine HCl, the absorption spectrum of this denatured ParB was measured and compared to tyrosine to phenylalanine in ParB, predicted from the DNA sequence (2). FFLC-purified ParB (Fraction IV) was concentrated by centrifugation in Centricon-30 filters (Amicon) and run over a 2-ml Sephadex G-25 column in freshly prepared 25 mM Tris-HCl, pH 7.5; 200 mM Na2SO4; 0.1 mM EDTA; 1 mM dithiothreitol (DTT); 10% glycerol. The UV absorption spectrum of native ParB, from 245 to 340 nm, was determined. After a four-fold dilution with 8 M guanidine HCl, the absorption spectrum of this denatured ParB was measured and compared to tyrosine to phenylalanine in ParB, predicted from the DNA sequence (2). FFLC-purified ParB (Fraction IV) was concentrated by centrifugation in Centricon-30 filters (Amicon) and run over a 2-ml Sephadex G-25 column in freshly prepared 25 mM Tris-HCl, pH 7.5; 200 mM Na2SO4; 0.1 mM EDTA; 1 mM dithiothreitol (DTT); 10% glycerol. The UV absorption spectrum of native ParB, from 245 to 340 nm, was determined. After a four-fold dilution with 8 M guanidine HCl, the absorption spectrum of this denatured ParB was measured and compared to tyrosine to phenylalanine in ParB, predicted from the DNA sequence (2). FFLC-purified ParB (Fraction IV) was concentrated by centrifugation in Centricon-30 filters (Amicon) and run over a 2-ml Sephadex G-25 column in freshly prepared 25 mM Tris-HCl, pH 7.5; 200 mM Na2SO4; 0.1 mM EDTA; 1 mM dithiothreitol (DTT); 10% glycerol. The UV absorption spectrum of native ParB, from 245 to 340 nm, was determined. After a four-fold dilution with 8 M guanidine HCl, the absorption spectrum of this denatured ParB was measured and compared to tyrosine to phenylalanine in ParB, predicted from the DNA sequence (2). FFLC-purified ParB (Fraction IV) was concentrated by centrifugation in Centricon-30 filters (Amicon) and run over a 2-ml Sephadex G-25 column in freshly prepared 25 mM Tris-HCl, pH 7.5; 200 mM Na2SO4; 0.1 mM EDTA; 1 mM dithiothreitol (DTT); 10% glycerol. The UV absorption spectrum of native ParB, from 245 to 340 nm, was determined.
endomucinases (which cut within the bla gene of pBR322). The double digestion ensured complete cutting and linearization but did not change the specific activity of the linear DNA. Nicked plasmid was prepared as described previously (21). All DNAs were extracted twice as described previously (21). All DNAs were extracted twice. Nicked plasmid was confirmed by agarose gel electrophoresis.

Binding Assays—Filter binding reaction mixtures contained \(^{[32P]}\)DNA, 0.1 mg of sonicated salmon sperm DNA/ml, and 0.1 mg of BSA/ml in binding buffer. After an incubation for 20 min at 30 °C, they were filtered through nitrocellulose filters (6) and quantitated by liquid scintillation counting. HIF binding reaction mixtures (10 \(\mu\)l) contained 5 fmol of \(^{[32P]}\)DNA (each fragment) and 0.1 mg of BSA/ml in binding buffer with 10% glycerol. They were incubated for 20 min at 30 °C, bromphenol blue was added to 0.001%, and the samples were loaded onto a 5% or 6% polyacrylamide gel (see legends to Figs. 6 and 7) in TBE buffer. The gel was run for 4 h at 4 °C, dried, and exposed to film.

RESULTS

Properties of ParB—ParB protein has been purified to greater than 99% homogeneity using only two column chromatography steps. The final step, gel filtration (Fig. 2), removed essentially all the small contaminants present after Bio-Rex 70 ion exchange chromatography (6). The purification was monitored by parS binding activity and yielded high levels of pure protein (about 20 mg from 2 liters of E. coli culture). Using the purified ParB (Fraction IV), the UV absorption spectrum and extinction coefficient were determined (see “Experimental Procedures”) to measure protein concentration accurately in subsequent assays. At the absorption maximum of 280 nm the extinction coefficient of ParB was 0.34 (mg/ml)\(^{-1}\) cm\(^{-1}\), or 1.31 \(\times\) 10\(^{4}\) M\(^{-1}\) cm\(^{-1}\) (monomers).

The molecular weight of a monomer of ParB, predicted from the DNA sequence (2), is 38,519 (although it runs with 44-kDa proteins on denaturing gels (Fig. 2)). The elution profile from the sizing column (Fig. 2) suggested a native molecular mass of about 140 kDa. However, sedimentation analyses implied a smaller size: ParB sedimented with, or slightly more slowly than BSA (67 kDa) (Fig. 3). Because gel filtration and sedimentation are sensitive to shape as well as to size, the simple interpretation is that ParB forms an asymmetric dimer. The Stokes radius, estimated from the gel filtration data, is 47 Å (Fig. 2), and the sedimentation coefficient from glycerol gradients is approximately 4 S. From these values, the native molecular mass of ParB was calculated to be approximately 80 kDa (23), consistent with its existence as a dimer.

To control for differences caused by buffers and running conditions sedimentation was repeated several times. The results were similar under all conditions tested: (i) in 200 mM Na\(_2\)SO\(_4\) (the FPLC buffer) and in 150 mM KCl (the binding assay buffer); (ii) at 4 and 20 °C; (iii) in sucrose and in glycerol gradients; and (iv) with partially purified Fraction III (from the Bio-Rex column).

Chemical cross-linking experiments confirmed that ParB forms a dimer (Fig. 4). DSP is a thiol-cleavable cross-linking reagent that reacts with primary amines (mainly lysine residues) (25). ParB (10 \(\mu\)g/ml) was treated with DSP, and the products were separated on SDS-polyacrylamide gels in the absence of 2-mercaptoethanol. ParB was converted rapidly to a product that ran as a dimer-sized smear (Fig. 4A). This species is heterogeneous because of intra- as well as intermolecular cross-links. When the samples were electrophoresed in the presence of 2-mercaptoethanol, all cross-links were cleaved (Fig. 4B), converting ParB to a monomer species (now slightly larger because DSP adds molecular weight).
ParB behaved identically at the two salt concentrations used routinely in the analyses of ParB; that is, the FPLC buffer and binding assay buffer (Fig. 4). The experiment was repeated at 10-fold higher (100 μg/ml) and 50-fold lower (0.2 μg/ml) concentrations with similar results (in the latter case, protein was detected by immunoblots rather than Coomassie staining; data not shown). Therefore, cross-linking and thus dimer formation were not functions of protein concentration (between 0.2 and 100 μg/ml). It is possible that higher oligomers form but are not cross-linkable in this experiment. To be consistent with the gel filtration and sedimentation data, however, the simple interpretation that ParB exists as a dimer in solution is preferable.

**IHIF Acts by Binding to and Bending parS—**IHIF is an accessory protein in P1 partition (6). It is a site-specific binding protein that recognizes the sequence 5′' T/C AAmnTTGAT A/T (12). The closest match within parS is 5′' TAACTGACTGT'TT, immediately to the left of the DraI site (Fig. 5). DNase I footprinting of parS showed that IHIF protected 29 bp including the predicted recognition sequence (Fig. 5). The binding site resembles other footprinted IHIF binding sites; it includes the consensus adjacent to an A + T rich region. Goodrich et al. (26) have recently developed a computer program to assess the similarity among published IHIF binding sites and generated an extended consensus recognition sequence. Using this program, the IHIF site within parS shows a similarity score of 54.9, which is within the range of scores of other footprinted binding sites. Deletion of the sequences between TaqI and DraI yields a version of parS, parS-small (Fig. 1), to which ParB still binds, but this activity is no longer stimulated by IHIF (6). Thus parS-small is insensitive to IHIF because the binding site has been removed.

IHIF has been shown to bind DNA (27-29). In the λattP site, for example, IHIF creates bends that help to juxtapose λ Int protein binding sites properly (30, 31). Goodman and Nash have shown recently (32) that an IHIF binding site can be replaced by an exogenous bend, arguing that IHIF acts through the bend and not by IHIF-Int protein-protein contact. The organization of parS, ParB binding sites flanking the IHIF binding site (Fig. 5B), strongly suggested that IHIF served to bend parS and bring distant ParB sites closer together. Because this bend is a key element in any three-dimensional model of the structure of the partition complex, the action of

**Fig. 4. Cross-linking ParB with DSP.** DSP-treated ParB protein electrophoresed in the absence (A) or presence (B) of 2-mercaptoethanol. ParB Fraction IV was diluted to 10 μg/ml in 50 mM Hepes, pH 7.5; 150 mM NaCl; 0.1 mM EDTA (NatCl lanes); or 25 mM Hepes, pH 7.5; 200 mM Na2SO4; 0.1 mM EDTA (Na2SO4 lanes) at 23 °C. DSP (20 μg/ml in dimethylformamide) was added to 0.1 mg/ml at t = 0. One-mL portions were removed at the indicated times, the reaction was quenched with glycine (25 mM), and the samples were sonicated salmon sperm DNA/ml. After phenol extraction and ethanol precipitation the samples were resuspended in 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% Molecular mass standards from Fig. 2; and S, ParB protein (undiluted and untreated).

**Fig. 5. DNase I footprinting of IHIF at parS.** A, the lower strand is a 180-bp fragment (see "Experimental Procedures") analyzed on a 6% gel; the upper strand a 452-bp fragment analyzed on a 5% gel. Lower and upper refer to the strands as drawn in B. IHIF binding reactions (20 μl) contained 20 fmol of DNA in 50 mM Hepes-KOH, pH 7.5; 150 mM KCl; 1 mM EDTA; 2.5 mM CaCl2; 10% glycerol; 0.1 mg BSA/ml, and the indicated amount of IHIF. They were incubated for 15 min at 30 °C, MgCl2 (to 10 mM) and DNase I (to 50 μg/ml) were added for 90 s, and the reaction was stopped by the addition of 60 μl of 0.6 M ammonium acetate, 0.1 M EDTA, and 20 μg of sonicated salmon sperm DNA/ml. After phenol extraction and ethanol precipitation the samples were resuspended in 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue and electrophoresed on sequencing gels in TBE buffer. On each gel, the Maxam-Gilbert C+T and A+G sequencing reactions of the same DNAs are the left two lanes. The position of the 13-bp IHIF consensus sequence is shown as a filled box, and the heptamers proposed as ParB recognition sites (7) are unfilled boxes. A control fragment (the pBR322 EcoRI-EcoRV fragment) showed no protection by IHIF (not shown). B, sequence of parS (2); GenBank Accession number X029544) indicating the region protected from DNase I cleavage by IHIF as thick black lines. The protection on the lower strand can be defined unambiguously because the flanking bases were clearly unaffected by the presence of IHIF (see A). The corresponding protection on the upper strand is correct ±1 bp on each side. The IHIF consensus is shown within the shaded box and the proposed ParB heptamers within the dashed boxes.
IHF was explored further. parS was cloned into the bending vector pBend5 (see “Experimental Procedures”). Digestion of the resulting plasmids (parS was cloned in both orientations) with a variety of restriction endonucleases produced a series of fragments of identical length, in which parS was present in circularly permuted positions along the DNA. DNA-IHF complexes were analyzed by polyacrylamide gel electrophoresis; because the fragments were the same length, differences in mobility should reflect differences in shape. As predicted, IHF bends parS (Fig. 6). Fragments cut with three different enzymes (with bound IHF) showed differential mobility, and these differences were also dependent on the orientation of parS with respect to pBend5 sequences (Fig. 6A). Theoretically, fragments with the bend in the center will migrate more slowly than those with the bend at the end (33). The average center of the bend is thus extrapolated to be the center of the most slowly migrating fragment. The experiment was repeated with a larger number of restriction enzymes, and the relative mobilities of IHF-bound versus -free fragments were plotted as a function of the center of each fragment. When plotted in this fashion, both orientations of parS gave the same result: the center of the band mapped within the IHF binding site (Fig. 6B).

parS fragments electrophoresed anomalously even in the absence of bound protein. First, they consistently migrated more slowly than would be predicted from their size. In this experiment, the 255-bp parS fragments migrated with 310-bp DNA marker fragments (not shown). Second, the permuted set of unbound parS fragments did not comigrate with each other (Fig. 6A). Both observations indicated the presence of an intrinsic bend or distortion. The parS site, although very A + T rich, does not contain any obvious blocks of adenine residues in phase with the helix, the “A-tract bend” sequences (34). The shift was insufficient to map this distortion precisely, and it is unknown whether this intrinsic structural feature of parS is important for ParB or IHF binding.

Construction of IHF Binding Site Mutations—The parS-small site described above is missing all sequences upstream of the DraI restriction site (Fig. 1) and thus lacks more than just the IHF binding site. IHF binding site mutations were constructed by inserting sequences into the DraI site (Figs. 1 and 5B). An 8-bp SalI restriction enzyme linker insertion created parS::Sal, and a 1.2-kb kanamycin resistance gene insertion produced parS::kan. These “parS-[Dra]” mutations should leave all ParB sites intact but change their spacing by 8 bp and 1.2 kb, respectively.

The parS::Sal site was characterized biochemically (Fig. 7). Two different techniques have been used to measure protein binding by IHF binding without ParB was measured by gel mobility shift assays and was about 10–20-fold weaker to parS::Sal than to parS (Fig. 7A). Some specificity was apparently retained, as the parS::Sal fragment was a slightly better substrate than a control fragment (included in the same reaction mixture), measured as disappearance of the unbound fragments with increasing IHF concentration. The bound DNA appeared as a shifted smear, presumably caused by some dissociation of the complexes within the gel. Thus the insertion mutation quantitatively and qualitatively damaged IHF binding. In filter binding assays, ParB could bind to parS::Sal (Fig. 7B), but this binding was insensitive to IHF (Fig. 7C). Since DNA-IHF complexes do not stick to nitrocellulose filters (6, 9) this technique measures ParB binding directly (Fig. 7B) and IHF binding by its ability to stimulate ParB binding activity (Fig. 7C). In the absence of IHF, ParB bound equally to parS-large, parS::Sal, and parS-small (Fig. 7B). Therefore, parS::Sal and parS-small were equivalent ParB substrates in vitro.

IHF Increases ParB Affinity in Vitro—The filter retention assay provides a quick and quantitative measure of ParB activity. Using this assay, IHF increases the affinity of ParB for parS. However, because some protein-DNA complexes do stick poorly to nitrocellulose, it was important to show that IHF increases ParB affinity for parS rather than simply ParB affinity for the filters. Therefore, unlabeled plasmids containing parS-large, parS-small, and parS::Sal were asked to compete with 3H-labeled parS-large plasmids in the presence of limiting ParB (and excess IHF). The rationale for the competition was as follows. In the absence of IHF, ParB binding to all three parS versions was identical (Fig. 7B). Only parS-large was stimulated by IHF (Fig. 7C; 6). If this stimulation represents increased ParB affinity for parS, then only unlabeled parS-large should compete with 3H-labeled parS-large.
Conversely, competition by all unlabeled parS versions would signify that IHF influences the affinity of ParB bound to different versions of parS to stick to nitrocellulose. The results demonstrated that parS-large was a better ParB substrate than parS-small or parS::Sal because only parS-large was an effective competitor of 3H-labeled parS-large (Table I). Therefore, IHF increases ParB affinity for the wild-type site, parS-large.

Previous experiments showed that IHF substantially increased the affinity of ParB for parS (6). These binding experiments were performed at a DNA concentration (3 nM) too high to estimate directly binding constants of the "high-affinity" complex. ParB binding in the presence of IHF was repeated at very low DNA concentration to measure ParB affinity. The dissociation constant, \( K_d \), equals the free protein concentration at 50% maximal binding; this is approximately equal to the total protein concentration if the DNA concentration (and thus bound protein) is a negligible proportion of the total. Thus when [DNA] \( \ll K_d \), dissociation constants can be estimated from the protein concentration at 50% maximum binding. DNA concentration was reduced by including less DNA in a greater reaction volume (2 ml). At 0.5 pm plasmid, 50% was bound at 10 pm ParB (dimer) (Fig. 8A). The apparent \( K_d \) (apparent because one does not know whether all the protein was active, or the stoichiometry of ParB binding) was estimated from the protein concentration at 50% maximum binding activity without IHF. Nitrocellulose filter retention assay measuring ParB binding to \(^{3}H\)-labeled pALA207 in the presence of a 2-fold excess of unlabeled competing plasmid. Binding reaction mixtures (20 \( \mu \)l) contained 110 fmol of \(^{3}H\)-labeled pALA207, 220 fmol of unlabeled plasmid containing the indicated parS site, 75 nM IHF, and 6 nM ParB (120 fmol of dimer). The mixtures were assembled on ice, and the proteins were added last. ParB was the limiting component; DNA and IHF were in excess.

![Fig. 7. Protein binding to various parS sites. A, IHF binding to parS and parS::Sal. The 794-bp Sau3A1 parS restriction fragment from pALA207 and the 802-bp Sau3A1 parS::Sal fragment from pBEF146 were \(^{32}P\) labeled and cut with RsaI, producing two fragments: a 460-bp parS fragment (or 468 parS::Sal fragment) and a 334-bp fragment used as an internal control. Binding reactions were analyzed on 5% polyacrylamide gels. B, ParB binding activity without IHF. Nitrocellulose filter binding assays were performed on \(^{3}H\)-labeled pALA207 (parS-large) (○), pBEF146 (parS::Sal) (■), pBEF127 (parS-small) (▽), and pBR322 (vector) (▲). C, IHF stimulation of ParB binding. The substrates for filter retention assays were pALA207 (parS-large) (○) and pBEF146 (parS::Sal) (■), and the ParB concentration was 10 nM. In B and C the DNA concentration was 3 nM, and ParB concentrations are reported as the concentration of dimers (M, 77,000).

**TABLE I**

| Unlabeled plasmid (parS site) | Bound \(^{3}H\)-labeled pALA207 (parS-large) |
|-------------------------------|---------------------------------------------|
| None                          | 62 (1.0)                                    |
| pALA207(parS-large)           | 23 (0.37)                                   |
| pBEF127(parS-small)           | 63 (1.0)                                    |
| pBEF146(parS::Sal)            | 59 (0.95)                                   |
| pBR322 (none)                 | 62 (1.0)                                    |

![Fig. 8. Measurements of ParB binding affinity (A) with and (B) without IHF. The \(^{3}H\)DNA substrates were pALA207 (parS) and the vector pBR322. A, binding reactions (2 ml) contained 0.5 pm DNA and 0.5 nm IHF where indicated. Data points are ○, pALA207 with IHF; O, pALA207 without IHF; and ▲, pBR322 with IHF. B, binding reactions (20 ml) contained 3 nM pALA207 (○, O) or pBR322 (▼, ▲) in the presence (filled symbols) or absence (empty symbols) of 2 \( \mu \)g of unlabeled salmon sperm DNA (nsDNA). The concentration of ParB dimers is reported.](image-url)
The P1 Plasmid Partition Complex

μl), 50% of the substrate was bound at approximately 0.1 μM ParB (Fig. 8B). Nevertheless, the apparent K_0 is still much greater than the substrate concentration. Thus by this rationale, IHF stimulates ParB binding by about 10,000-fold.

Nonspecific DNA carrier was omitted to assess the contributions of both specific (to pALA207) and nonspecific (to pBR322) DNA binding to total binding activity. In the presence of IHF and at low ParB concentration, binding was very specific (Fig. 8A). Without IHF, ParB discriminates very poorly between specific and nonspecific DNA although specific binding activity can be analyzed by including unlabeled nonspecific DNA in the reaction (Fig. 8B). Simplistically then, IHF increases specific recognition of parS by ParB. In vivo, of course, recognition of a specific sequence always occurs in the presence of a vast excess of nonspecific competitor.

DNA:IFH complexes are detected on filters by their ability to stimulate ParB binding to parS (Fig. 7C,6). At 0.5 pmol DNA, the DNA concentration was still too high to measure the K_0 for IHF binding to parS in the presence of ParB; 50% of the parS plasmid was bound at 2 pmol IHF (data not shown). It was technically difficult to lower the DNA concentration further. On linear fragments, the K_0 for IHF was about 10 nM (data not shown). Therefore IHF affinity for parS was greater than the parS penetration complex. It is likely that both DNA supercoiling and ParB contribute to this increase in IHF affinity (>50,000-fold; see “Discussion”).

IHF Affects ParB Affinity in Vivo—Competition, or incompatibility, assays were used to examine the effect of IHF on partition in vivo. Experimentally, one tests the ability of a plasmid containing a given version of parS to destabilize a second resident plasmid partitioned by P1 par. The resident plasmid used in all experiments was λ-miniP1, partitioned by different versions of parS. Thus the resident parS sites were tested within a par system that was as close to the natural context as possible, with the Par proteins supplied in cis from their natural promoters. The parS-[Dra] insertion mutations were crossed into λ-miniP1, producing λ-miniP1parS::Sal and λ-miniP1parS::kan. These particular IHF-insensitive mutations were used because the original parS small site cannot be tested directly in λ-miniP1; the TaqI-DraI deletion removes the parS::Sal were not identical in the absence of IHF; the latter was always slightly less stable than the former, regardless of the competing plasmid. Although the differences were small, they imply that the spacing changes created by the parS-[Dra] insertions have affected something in addition to IHF binding. This may reflect subtle effects on ParB binding, on subsequent partition steps, or on binding of other (as yet undefined) proteins.

A different type of experiment confirmed that IHF increases ParB affinity for parS in vivo. In previous studies it was observed that very high levels of ParB specifically destabilize both high and low copy number plasmids containing parS (5). The effect was shown to be a segregation defect, explained by the aggregation of ParB-bound plasmids. The destabilization of λ-miniP1 was so severe that lysogens could not form colonies. If IHF influences ParB affinity for parS, then removal of IHF should reduce the lysogenization defect. The plasmid pBEF104 contains the parB gene in the trp promoter vector, pRPG48 (5). The ratio of colony-forming lysogens in DH5(pBEF104) to DH5(pRPG48) was ≤10 (5). The experiment was repeated in cells lacking IHF; the lysogenization ratio in DH5(pBEF104) to DH5(pRPG48) was 0.21. The DH5(pBEF104) λ-miniP1 lysogens were unstable (data not shown), but they could form small colonies. β-Galactosidase measurements (∼36 of parB-lacZ protein fusions

| Strain | Competing plasmid(parS) | % Retention of resident λ-miniP1 containing |
|--------|-------------------------|-------------------------------------------|
|        | parS:large | parS:Sal | parS:kan |
| DH5    | pMF3 (none) | >99 | 96 | 86 |
|        | pBEF161(parS:large) | 30 | 2 | <1 |
|        | pBEF162(parS:small) | >99 | 20 | 21 |
| DH5hip | pMF3 (none) | 96 | 89 | 84 |
|        | pBEF161(parS:large) | 34 | 18 | 12 |
|        | pBEF162(parS:small) | 44 | 16 | 10 |
The P1 Plasmid Partition Complex

(transcribed from the trp promoter) showed that ParB expression was not lower in DH5α/pip cells (data not shown). Therefore, the lack of IHF diminished but did not eliminate the inhibitory effects of excess ParB, evidence that ParB binding to parS was reduced in the absence of IHF.

Topology Strongly Influences ParB Binding—During the development of the ParB binding assay, it was observed that ParB preferred supercoiled plasmid DNA; linear DNA fragments carrying parS were poor substrates for ParB. Subsequently, supercoiled, relaxed and nicked circular, and linear forms of the parS-containing plasmid, pALA207, were examined (Fig. 9A). Supercoiled DNA was clearly the best substrate. In addition, however, ParB could distinguish between relaxed circular and linear DNA. Plasmids linearized with different restriction enzymes, and short DNA fragments, gave identical results (data not shown). Competition experiments confirmed that supercoiled DNA was a higher affinity substrate. 3H-Labeled supercoiled pALA207 (parS-large) plasmids were mixed with an excess of unlabeled supercoiled, relaxed circular, or linear forms (Table III). Both relaxed circular and linear forms were poor competitors compared with supercoiled plasmid although relaxed and linear pALA207 competed slightly better than nonspecific supercoiled pBR322 DNA at high competitor concentration.

It appeared that both superhelicity and plasmid shape (i.e. circular versus linear shape) influenced the binding activity of 3H-labeled substrates (Fig. 9A). Subsequent experiments indicated that the supercoiling effect required ParB and IHF and that ParB was responsible for the shape discrimination. In the absence of IHF, all circular molecules behaved similarly, and all were better substrates than linear DNA (Fig. 9B). Thus without IHF, ParB binding was insensitive to supercoiling but sensitive to shape. IHF stimulated ParB binding to all forms (compare the ParB concentrations in Fig. 9, A and B) although the stimulation on supercoiled circles was greater than on relaxed ones. At higher IHF concentrations, supercoiled circles were still better substrates than relaxed ones (Fig. 9C), implying that superhelicity does more than just improve IHF affinity. At the concentration of IHF used in Fig. 9A (25 nM), all IHF binding sites should be occupied fully (Figs. 9A and 9C). Therefore the free energy of supercoiling was used only in the complete partition complex.

The effect of plasmid shape on ParB binding in Fig. 9 seems puzzling since ParB is a site-specific binding protein, and the ends of the linear forms are at least 1.5 kb from parS. The competition experiment in Table III provides a preliminary explanation. Although poor competitors of 3H-supercoiled plasmid, linear forms behaved similarly to relaxed circular forms. This observation suggests that ParB affinity for both forms may be similar, but ParB-relaxed circular DNA complexes are trapped more efficiently than ParB-linear DNA complexes on nitrocellulose. This interpretation needs to be confirmed, but it seems the simplest way to rationalize why 3H-linear molecules appear to be such poor substrates. Nevertheless, the important conclusion here is that superhelicity is required for high affinity protein binding to parS.

DISCUSSION

One of the earliest steps in P1 partition is the assembly of a protein complex at parS. This study describes several biochemical and biological characteristics of this partition complex and its components: ParB, IHF, and the parS DNA substrate. In particular, the role of IHF and DNA supercoiling has been examined. (i) IHF greatly increases ParB affinity for parS (Fig. 8 and Table I). (ii) IHF binds to and bends parS (Figs. 5 and 6). (iii) The IHF binding site is between ParB binding sites (Fig. 5; 7). Only the sequences downstream of the IHF site (between the DraI and StyI sites; Fig. 5) are necessary for low affinity ParB binding in the absence of IHF (Fig. 7B; 5, 7) whereas the intact parS-large site is required for high affinity (IHF-stimulated) ParB binding. (iv) The complex containing IHF and ParB strongly prefers a superhelical substrate (Fig. 9). The IHF-induced bend in parS will change the proximity and alignment of ParB binding sites with respect to each other. These data suggest that tight ParB binding requires that ParB contact recognition sequences on each side of the IHF site simultaneously (Fig. 5) and that this contact requires the bend provided by IHF and the free energy of supercoiling. In this model, the wild-type partition complex contains parS-large DNA specifically wrapped around ParB and IHF. The precise geometry of the partition complex, promoted by IHF and superhelicity, is required for high affinity ParB binding. ParB binding increases IHF affinity as well; thus ParB and IHF bind cooperatively with respect to each other.

The requirements for IHF and supercoiling and the organization of multiple binding sites are seen in the AttP·Int-IHF complex, the intasome. Many elegant studies have established that Int, IHF, and DNA supercoiling cooperate to allow Int protein to contact distant, multiple binding sites in a specific three-dimensional way (27-33, 37). The important function of IHF is probably to provide bends in the DNA substrate (32). Richet et al. (30) have shown that the major role of superhelicity is proper positioning of recombination proteins on their sites to favor formation of the intasome. The observations described in this study and summarized above lead to the proposal that IHF and supercoiling play similar architectural roles at parS and that this is a general feature of IHF-assisted complex formation.

A recent recurring theme in the study of protein-nucleic acid complexes is “higher order nucleoprotein structures,” formed by multiple protein-DNA and protein-protein interactions (38). Such structures allow protein-mediated communication of distant DNA sites, or “action at a distance” (38, 39). This study suggests that P1 has borrowed IHF from its host to assemble such a structure at parS. This parallels

![Figure 9](attachment:image.png)
DNA structures such as cruciforms (41). However, there is no prior reason to invoke helix-opening events in partition. The model of the partition complex should eventually incorporate some additional information. First, important protein-protein contacts and ParB stoichiometry are still speculative. The bend-swap experiments in attP (32) imply that IHF supplies the bend, not specific IHF-ParB contacts. Simultaneous ParB binding to sequences on both sides of the IHF site requires ParB-ParB contact, but the number of ParB molecules involved is unknown. Binding experiments at high DNA concentration (when the equilibrium is pushed toward complex formation) indicated that two dimers are sufficient to bind a parS plasmid to nitrocellulose (42) (Table I). However, stoichiometry must be determined on isolated partition complexes.

In addition, the contribution of intrinsically bent or kinked sequences (the DNA secondary structure) has not been assessed. Aberrant electrophoretic mobility has been reported for a variety of DNA sites involved in higher order nucleoprotein structures, such as the attP site (43), the λ origin of replication (44), and the Fis binding site (45). It has been suggested that such secondary structure may be important for complex assembly.

**Partition without IHF**—The fact that partition works without IHF indicates that ParB must bind to parS in vivo in its absence. The intracellular concentration of ParB may be high enough to promote some ParB binding. In fact, preliminary experiments with antibodies directed against ParB indicated about 2,000–4,000 dimers/cell. In a cylinder the size of an E. coli cell (3 μm long by 1 μm in diameter), this corresponds to micromolar ParB concentrations. Alternatively, it is possible that some other host factor or factors substitute for IHF in vivo.

**Affinity Versus Specificity**—The in vivo competition suggested that ParB has similar affinities for parS::Sal, parS::kan, and parS-small because parS::small destabilized both λ-miniP1parS-[Dral] insertion mutants (Table II). In vitro, parS-small and parS::Sal behaved identically (Fig. 7B and Table I). One experiment by Martin et al. (14) does suggest that parS-small is not exactly identical to parS-[Dral] insertion mutants. They reported that a miniF derivative partitioned by parS-small was not destabilized by coreisose λ-miniP1parS-[Dral] and proposed two alternative competition or incompatibility states for parS, incB* (parS-large) and incB (parS-small), determined by IHF (14, 35). They also showed recently that the incB specificity difference disappeared when the competing plasmid was of high copy number, using a λ-miniP1 with a parS-[Dral] insertion mutation (35). The experiments presented here show that λ-miniP1 partitioned by the parS-[Dral] insertion mutants was always competed by plasmids carrying parS-large, regardless of the presence of IHF and of the copy number of the competing plasmid (Table II). Thus it is very unlikely that IHF is the determinant of the observed specificity difference with the miniF experiment (14). The lack of upstream ParB sequences in parS-small differences in context (i.e. the vector sequences), or differences in the amount of partition proteins when supplied in trans, may be responsible for the insensitivity of this miniF derivative to λ-miniP1. Nevertheless, all experiments with the parS-[Dral] mutants support the idea that the in vivo action of IHF is the same as its role in vitro; it increases ParB affinity for wild-type parS.

**Partition with IHF**—The observation that wild-type λ-miniP1 is insensitive to competition by parS-small (Table II; 6) shows that wild-type partition complexes contain IHF. Therefore, the nucleoprotein structure that IHF promotes is the substrate for subsequent steps in partition. However, ParB binding, rather than this structure, is sufficient (although not optimal) for partition since both IHF and the upstream ParB site are dispensable. It is unclear why P1 has chosen to use IHF to assist ParB and has conserved the complexity of the parS site. It has been suggested that IHF is a link to the physiological state of the cell (12). For example, IHF makes ParB binding sensitive to supercoiling (Fig. 9). There is evidence that intracellular supercoiling varies during transcription (46) and in response to certain environmental stimuli such as osmolarity (47) and anaerobiosis (48). Alternatively, higher order nucleoprotein structures may increase the fidelity of a reaction (38). Because IHF improves ParB affinity for specific sites more than its nonspecific DNA binding activity (Fig. 8) P1 plasmids may be less likely to pair with heterologous plasmids when the ParB-IHF-parS complex is used. Finally, one can argue that a strong system is always better than a weak one. In the laboratory, time scales are brief relative to those that exert selection pressure during evolution. It has been observed consistently in this laboratory that the par system is slightly less efficient without IHF (Table II; 6). In nature, after thousands of generations of growth, this difference in efficiency may yield cell populations practically devoid of the plasmid. Thus a weaker par system may confer little more advantage than no par system at all.

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**Table III**

| Unlabeled plasmid   | H-Labeled PAlA207 at 'H-labeled to unlabeled DNA ratios of |
|---------------------|----------------------------------------------------------|
| None                | 1:2                                                      |
| pBR322 supercoiled  | 1:0                                                      |
| pAlA207 supercoiled | 0.86                                                     |
| pAlA207 relaxed     | 0.99                                                     |
| pAlA207 linear      | 0.86                                                     |

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B. E. Funnell, unpublished results.
explaining the evolutionary pressure to conserve the strong ParB binding site.

Finally, it remains to be determined how the partition complex at parS interacts with ParA and functions in subsequent events in partition. The identification of other host components and the characterization of the interactions involved are the next steps in defining the process of plasmid partition.

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REFERENCES

1. Yarmolinsky, M. B., and Sternberg, N. (1988) in The Bacteriophages (Calendar, R., ed) pp. 291–438, Plenum Press, New York
2. Abeles, A. L., Friedman, S. A., and Austin, S. J. (1985) J. Mol. Biol. 185, 291–272
3. Austin, S. J., and Abeles, A. L. (1983) J. Mol. Biol. 169, 373–387
4. Ogura, T., and Hiraga, S. (1983) Cell 32, 351–360
5. Funnell, B. E. (1988) J. Bacteriol. 170, 954–960
6. Funnell, B. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6657–6661
7. Davis, M. A., and Austin, S. J. (1988) EMBO J. 7, 1881–1888
8. Kikuchi, Y., and Nash, H. A. (1978) J. Biol. Chem. 253, 7149–7157
9. Nash, H. A., and Robertson, C. A. (1981) J. Biol. Chem. 256, 9240–9253
10. Miller, H. A. (1985) Cold Spring Harbor Symp. Quant. Biol. 49, 691–698
11. Flamm, E. L., and Weinberg, R. A. (1985) J. Mol. Biol. 183, 117–128
12. Friedman, D. I. (1988) Cell 55, 545–554
13. Sternberg, N., and Austin, S. I. (1983) J. Bacteriol. 153, 800–812
14. Martin, K., Friedman, S. A., and Austin, S. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8544–8547
15. Funnell, B. E. (1989) J. Cell Biol. 13D, 86
16. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H., and Falkow, S. (1977) Gene (Amst.) 2, 95–113
17. Manis, J. L., and Kline, B. C. (1977) Mol. Gen. Genet. 152, 175–182
18. Zweib, C., Kim, J., and Adhya, S. (1989) Genes & Dev. 3, 606–611
19. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
20. Edelhoch, H. (1967) Biochemistry 6, 1948–1954
21. Funnell, B. E., Baker, T. A., and Kornberg, A. (1986) J. Biol. Chem. 261, 5616–5624
22. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
23. Siegel, L. M., and Monty, K. J. (1987) Biochim. Biophys. Acta 112, 346–362
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Lomant, A. J., and Fairbanks, G. (1976) J. Mol. Biol. 104, 243–261
26. Goodrich, J. A., Schwartz, M. L., and McClure, W. R. (1990) Nucleic Acids Res. 18, 4993–5000
27. Przentki, P., Chandler, M., and Galas, D. J. (1987) EMBO J. 6, 2479–2487
28. Robertson, C. A., and Nash, H. A. (1988) J. Biol. Chem. 263, 3554–3557
29. Thompson, J. F., and Landy, A. (1988) Nucleic Acids Res. 16, 9687–9705
30. Richet, E., Abcarian, P., and Nash, H. A. (1986) Cell 46, 1011–1021
31. Moitoso de Vargas, L., Kim, S., and Landy, A. (1989) Science 244, 1457–1469
32. Goodman, S. D., and Nash, H. A. (1989) Nature 341, 251–254
33. Wu, H. M., and Crothers, D. M. (1984) Nature 308, 509–513
34. Kuo, H., Wu, H., and Crothers, D. (1986) Nature 320, 501–506
35. Davis, M. A., Martin, K. A., and Austin, S. J. (1990) EMBO J. 9, 991–998
36. Miller, J. H. (1972) in Experiments in Molecular Genetics, pp. 352–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Yang, C., and Nash, H. A. (1989) Cell 57, 869–880
38. Echols, H. (1990) J. Biol. Chem. 265, 1497–14700
39. Wang, J. C., and Giaever, G. N. (1988) Science 240, 300–304
40. Gellert, M. (1981) Annu. Rev. Biochem. 50, 879–910
41. Frank-Kamenetski, M. D. (1990) in DNA Topology and Its Biological Effects (Cozzarelli, N. R., and Wang, J. C., eds) pp. 185–215, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
42. Drlica, K., Pruss, G. J., Burger, R. M., Franco, R. J., Hsieh, L.-S., and Berger, B. A. (1990) in The Bacterial Chromosome (Drlica, K., and Riley, M., eds) pp. 195–204, ASM Press, Wash., D. C.
43. Ross, W., and Landy, A. (1982) J. Mol. Biol. 156, 523–529
44. Zahn, K., and Blattner, F. R. (1987) Science 236, 416–422
45. Johnson, R. C., and Simon, M. I. (1987) Trends Genet. 3, 262–267
46. Liu, L. F., and Wang, J. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7024–7028
47. Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G., and Bremer, E. (1988) Cell 52, 569–584