Assembly of P1 plasmid partition complexes at the partition site, parS, is nucleated by a dimer of P1 ParB and Escherichia coli integration host factor (IHF), which promotes loading of more ParB dimers and the pairing of plasmids during the cell cycle. ParB binds several copies of two distinct recognition motifs, known as A- and B-boxes, which flank a bend in parS created by IHF binding. The recent crystal structure of ParB bound to a partial parS site revealed two relatively independent DNA-binding domains and raised the question of how a dimer of ParB recognizes its complicated arrangement of recognition motifs when it loads onto the full parS site in the presence of IHF. In this study, we addressed this question by examining ParB binding activities to parS mutants containing different combinations of the A- and B-box motifs in parS. Binding was measured to linear and supercoiled DNA in electrophoretic and filter binding assays, respectively. ParB showed preferences for certain motifs that are dependent on position and on plasmid topology. In the simplest arrangement, one motif on either side of the bend was sufficient to form a complex, although affinity differed depending on the motifs. Therefore, a ParB dimer can load onto parS in different ways, so that the initial ParB-IHF-parS complex consists of a mixture of different orientations of ParB. This arrangement supports a model in which parS motifs are available to inter- as well as intramolecular parS recognition.

In bacterial cells, the dynamic localization of low copy number plasmids is directed by their partition systems, which ensure the proper segregation and thus stable inheritance of these plasmids in growing cell populations. The P1 plasmid exists as a stable, autonomously replicating, low copy number plasmid in Escherichia coli, and its partition system has served as a paradigm for homologous systems found in many naturally occurring bacterial plasmids as well as several bacterial chromosomes (reviewed in Refs. 1 and 2). These partition systems typically consist of two proteins, ParA and ParB, that act on a plasmid partition site, parS (P1 nomenclature). The partition reaction occurs through a series of DNA-protein and protein-protein interactions that lead to P1 plasmid localization within an E. coli cell. ParB acts as the key partition site binding protein (3, 4). ParB initially loads onto parS as a dimer and then recruits multiple dimers to form a large nucleoprotein complex, which is proposed to mediate P1 plasmid pairing through ParB-ParB interactions (5–7). ParA is an ATPase that interacts with this complex and through an unknown process, mediates the specific localization of P1 plasmids (6, 8–10). The only host factor known to participate in P1 partition is E. coli integration host factor (IHF), which assists ParB in the initial DNA binding step by greatly increasing the affinity of ParB for parS (4).

ParB is an unusual site-specific DNA-binding protein in that it recognizes two distinct sequence motifs, called A-boxes (ATTTCAA/C) and B-boxes (TCGCCA), in parS (11, 12). There are multiple copies of these boxes in parS, and they are asymmetrically arranged around an IHF binding site (Fig. 1). The spacing and orientation of these binding motifs are critical for complex formation in vitro as well as parS activity in vivo (12, 13). IHF bends parS, which allows ParB to contact its specific binding motifs flanking the bend, resulting in a high-affinity protein-DNA complex. In the absence of IHF, ParB binds specifically but more weakly to parS and requires only the right half of parS for activity. In vivo, a 22-bp sequence on the right side of parS (boxes A2-A3-B2, called parS-small in Fig. 1) is sufficient but not optimal for partition (14). The optimal parS site extends from box B1 to box B2 and requires IHF. All motifs except boxes A1 and A4 are necessary for wild-type parS activity in vivo (11–13). Interestingly, however, the position and orientation of boxes A1 and A4 have been conserved in a variety of related parS sites (2).

Biochemical assays have shown that the C-terminal half of ParB (residues 142–333) contains all of the information necessary to form the dimeric, high-affinity (IHF-stimulated) ParB complex at parS (15). Recently, the crystal structure of ParB-(142–333) bound to a 25-bp DNA duplex containing the parS-small sequence has been determined (16). ParB contains two essentially independent DNA-binding domains (a helix-turn-helix (HTH) domain between residues 147–270 and a dimer domain between residues 275 and 333) separated by a short (4-residue) flexible linker. This arrangement permits ParB to contact a variety of A- and B-box combinations within parS.
functional studies predicted, the crystal structure of ParB-(142–333) confirms that the HTH motif binds the A-boxes within parS and the dimerization domain binds the B-boxes (15–18). The dimerization interaction creates a novel DNA binding motif that requires contributions from each monomer to bind a box B sequence. In fact, one ParB dimer contacts its different motifs on separate copies of the same 25-mer parS-small oligonucleotide, effectively bridging different DNA molecules. The bridging activity illustrates how ParB might contact separate motifs intramolecularly across an IHF-directed bend but also suggests that it may participate in pairing plasmids intermolecularly across parS sites (16).

We were interested in how ParB contacts the parS site, particularly across the IHF-directed bend and potentially across adjacent DNA molecules. Chemical footprinting experiments have shown that all A- and B-boxes within parS are protected by a dimer of ParB (5), but the crystal structure implies that one dimer cannot contact all motifs simultaneously (16). In this study we examined how a dimer of ParB recognizes the parS site. Do the different A- and B-boxes work together in providing a high-affinity binding site for ParB, or is there a distinct set of ParB-IHF-parS complexes that uses specific A- and B-box combinations? Does ParB have a binding preference for specific A- or B-boxes within parS? To address these questions, variants of parS were created with A- and B-boxes systematically mutated individually or in combination with each other. We found that ParB prefers different subsets of motifs when it initially binds to parS. Our results support the model that the arrangement of recognition motifs in parS is necessary to build the proper architecture of partition complexes at and across partition sites.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—E. coli strain DH5 (F<sup>−</sup> endA1 hsdR17 (rK<sup>−</sup> mK<sup>−</sup>) supE44 thi-1 gyrA96 recA1) was used to construct and maintain plasmids. The plasmid pBend5, a derivative of pBend2 (19), was used as the vector to create plasmids containing the parS variants, which were the DNA substrates for biochemical experiments. All parS variants used in this study were constructed by cloning complementary synthetic oligonucleotides (80–88-mers, depending on the variant; see supplemental Table 1) into the HpaI site of pBend5. The plasmid parS sequences were confirmed by DNA sequencing at York University (Toronto) and/or Macrogen Inc. (Seoul, South Korea).

**Reagents**—Synthetic oligodeoxynucleotides were purchased from Invitrogen. Sources for other reagents were as follows: [α-<sup>32</sup>P]dCTP and S-[<sup>3</sup>H]adenosyl-l-methionine were from PerkinElmer; bovine serum albumin and salmon sperm DNA, from Sigma; restriction enzymes, Klenow DNA polymerase, and HaeIII methylase, from New England Biolabs.

**DNA and Proteins**—For gel mobility shift assays, the DNA substrates were total Xho I restriction digests of plasmid DNA. DNA fragments were labeled at their 3′-ends with [α-<sup>32</sup>P]dCTP and DNA polymerase I (Klenow fragment), and then purified by phenol-chloroform extraction and ethanol precipitation steps. For nitrocellulose filter binding assays, supercoiled plasmid DNAs were purified in cesium chloride gradients, and labeled with S-[<sup>3</sup>H]adenosyl-l-methionine and HaeIII methylase as described (4).

ParB (fraction V) was purified as described previously (9). IHF was purified essentially as described (20), except that the phosphocellulose step was followed by Mono-S chromatography in 15 mM phosphate buffer, pH 6.4, 5% glycerol, and protein was eluted with a 50 mM–1 M NaCl gradient in the same buffer.

**Gel Mobility Shift Assays**—The standard reaction mixture (10 μl) contained 1 nM 32P-labeled DNA in 50 mM Hepes-KOH buffer, pH 7.5, 150 mM KCl, 10% glycerol, 80 μg/ml of bovine serum albumin/ml, and 250 μg/ml of salmon sperm DNA/ml. IHF, when present, was included at 500 ng/ml (so its binding would not be limiting). The mixtures were assembled on ice, incubated for 30 min at 30°C, and then analyzed by electrophoresis in 5% polyacrylamide gels in 90 mM Tris borate, 2 mM EDTA. Electrophoresis was performed at 150 V for 3.5 h at 4°C. The gels were dried onto Whatman DE81 paper and exposed to a phosphor screen for imaging by a PhosphorImager. Data were quantitated using ImageQuant software (GE Healthcare). For each lane in the gels, the radioactivity (as phosphorimaging counts) in the area corresponding to a dimeric ParB-IHF-parS complex was measured and expressed as a fraction of the value corresponding to the parS DNA fragment in the absence of ParB.

**Analysis of the Data**—All variants were tested typically three to six times for binding by ParB over a range of ParB concentrations, and the results from these titrations were averaged. The error in individual values was typically less than 10% of substrate, ranging from 2 to 15% of substrate. When a dimer of ParB binds parS, the interaction occurs in an equimolar fash-
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ion. Therefore, the averaged data set from a particular variant was then fit to the Langmuir binding equation (21–23) using SigmaPlot 2000 software.

\[ I = I_0 + \frac{(I_a - [B] + K_d)}{(1 + [B]/K_d)} \]  
(Eq. 1)

The band intensity, \( I \), is a function of the free ParB concentration \([B]\) and the dissociation constant \((K_d)\), and \([B]\) is defined by the following equation,

\[ [B] = -(\frac{[S] - [B] + K_d}{S}) + \sqrt{[S] - [B] + K_d^2 + 4K_d^2[B]}/2 \]  
(Eq. 2)

where \([S]\) is the total concentration of parS substrate, \([B]_\text{app}\) is the total concentration of ParB, and SQRT is the square root of the values in parentheses.

RESULTS

Not all A- and B-boxes Are Required for a Dimer of ParB to Recognize and Bind to parS—The first step in partition complex assembly is the binding of one ParB dimer to parS across the IHF-directed bend (5). We were interested in the nature of this protein-DNA interaction as well as the role of different motifs in partition complex function. Our recent crystal structure of ParB-(142–333) complexed with parS-small had raised questions about how ParB recognizes a full parS site (16). We typically study the interaction by gel mobility shift assays in order to examine specific complexes and monitor the stoichiometry of assembly (5). In situ OP-Cu footprinting of the dimeric ParB-IHF-parS complex showed protection of all A- and B-boxes within parS, suggesting two possible mechanisms of ParB binding to parS: 1) a single dimer of ParB binds all or most of its recognition sequences on both the left and right arms of parS simultaneously; or 2) a dimer of ParB can bind various combinations of motifs across an IHF-directed bend, so that the initial complex actually represents a mixture of complexes with different modes of ParB binding to parS. To examine these possibilities, we created a spectrum of mutant sites in which A- or B-boxes were removed by substitution with a restriction site (Fig. 2). In our nomenclature, mutations are designated by the box name in lowercase (for example, parS[a2] lacks the box A2-specific sequence; Fig. 2). Substitution allowed for the removal of specific base pair contacts with ParB while maintaining the sequence, spacing, and orientation of the remaining boxes. The use of different restriction sites also marked each parS variant differently. We constructed a negative control, called parS[Δab], which lacks all A- and B-box sequences but retains the IHF binding site (Fig. 2). We defined parS\(^+\) as all sequences between and including box B1 to B2 because box A4 is dispensable for partition in vivo (13, 24), and its omission allowed us to reduce the number and complexity of variants that we created. We did subsequently confirm that box A4 was dispensable in our assays; we observed no quantitative differences in ParB binding affinity between parS\(^+\) and the parS\(^+\)-A4 site (see below and Table 1).

We first tested the effect of mutating individual motifs on the formation of the partition complex. The parS substrates were incubated with ParB and IHF, and the resulting complexes were separated by gel electrophoresis (Fig. 3). An apparent dissociation constant \((K_{d\text{app}})\) was then determined through quantitative analysis of ParB-IHF-parS complex formation (Table 1). The data were evaluated using the Langmuir formula (see “Experimental Procedures”), a common way of describing specific, high-affinity binding of two molecules (21–23). The half-interval method was then used on the fitted curves to obtain the \(K_{d\text{app}}\) value, where \(K_{d\text{app}}\) is defined as the amount of ParB
necessary for 50% maximal binding. The theoretical Langmuir curves and experimental binding curves produced $K_{\text{d,app}}$ values that were very similar.

The results showed that not all motifs are necessary for the initial recognition of $\text{parS}$ by ParB, and that some motifs are more important than others (Figs. 3 and 4; Table 1). Mutation of box B1 or box A2 had no significant effect on complex formation compared with that with $\text{parS}^+$. However, removal of the other three motifs did reduce ParB affinity for $\text{parS}$. Mutation of box A3 or box B2 each reduced complex formation by ~3–4-fold. The removal of box A1 produced the greatest reduction in complex formation. The $K_{\text{d,app}}$ was 30-fold greater than when using $\text{parS}^+$. Therefore in this assay box A1 is a stronger recognition sequence than box B1 for ParB to bridge over the IHF directed bend. Comparison of $\text{parS}^+[a2]$ to $\text{parS}[a2]$ indicates that box A3 is the preferred A-box on the right arm of $\text{parS}$. Finally, the data indicate that this initial ParB-IHF-$\text{parS}$ complex is only a precursor of the functional partition complex, because not all motifs necessary for ParB function are necessary to form this first complex. We also noted that ParB bound to all $\text{parS}$ variants with the same stoichiometry as to $\text{parS}^+$, as the mobility of the ParB-IHF-$\text{parS}$ complexes were identical in all gels that we examined (Fig. 3).

**Motif Use Is Influenced by Substrate Topology**—The effect of mutation of box A1 on complex formation was unexpected because genetic assays had shown that it is dispensable for $\text{parS}$ activity in vivo (11). We confirmed that the single motif mutations we used in this study behaved identically in vivo to mutations published previously (12, 13, 24) by using incompatibility assays, which measure the ability of $\text{parS}$ to destabilize a miniP1 partitioned by $\text{parS}^+$. The $\text{parS}[b1]$, $\text{parS}[a2]$, $\text{parS}[a3]$, and $\text{parS}[b2]$ substitutions destroyed $\text{parS}$ activity (were Inc−) compared with $\text{parS}[a1]$, which behaved as $\text{parS}^+$ (Inc+); data

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**Table 1**

Summary of ParB binding activity to $\text{parS}$ site variants in the presence of IHF

| $\text{parS}$ variant | Boxes remaining* | $K_{\text{d,app}}$b |
|-----------------------|------------------|---------------------|
| $\text{parS}^+$       | B1 A2 A3 B2      | 17 ± 4              |
| $\Delta ab$           | – – – –          | NB                  |
| b1                    | – A1 A2 A3 B2    | 7 ± 2               |
| a1                    | B1 – A2 A3 B2    | 580 ± 40            |
| a2                    | B1 A1 – A3 B2    | 10 ± 2              |
| a3                    | B1 A1 A2 – B2    | 61 ± 25             |
| b2                    | B1 A1 A2 A3 –    | 58 ± 23             |
| b1,a1                 | – – A2 A3 B2    | 690 ± 210           |
| b1,a2                 | – A1 – A3 B2    | 49 ± 20             |
| b1,a3                 | – A1 A2 – B2    | 119 ± 59            |
| b1,b2                 | – A1 A2 A3 –    | 56 ± 28             |
| a1,a2                 | B1 – – A3 B2    | >1 μM               |
| a1,a3                 | B1 – A2 – B2    | NB                  |
| a1,b2                 | B1 – A2 A3 –    | NB                  |
| a3,b2                 | B1 A1 A2 – –    | 59 ± 35             |
| a2,b2                 | B1 A1 – – A3 –  | 400 ± 160           |
| a2,a3                 | B1 A1 – B2 –    | 450 ± 150           |
| b1,a3,b2              | – A1 A2 – –     | 28 ± 6              |
| b1,a2,b2              | – A1 – A3 –     | 550 ± 180           |
| b1,a1,a3              | – A1 – – B2    | 500 ± 220           |
| RevA[A2]              | 1A 2A – –      | NB                  |
| $\text{parS}^+[A4]$   | B1 A2 A3 B2 A4  | 14 ± 7              |
| b1,a2,a3,b2–A4        | – A1 – – A4    | 760 ± 210           |
| a1,a2,a3,b2–A4        | B1 – – – A4    | NB                  |

*a Dashes indicate the position of boxes that have been mutated (with respect to $\text{parS}^+$).

*b $K_{\text{d,app}}$ was calculated as described under “Experimental Procedures.” NB, no measurable binding activity. >1 μM, substrate binding below 50% at 1 μM ParB.

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**FIGURE 3.** DNA binding activity of ParB to $\text{parS}$ variants lacking one motif. The DNA substrates were $^{32}$P-labeled restriction digests of plasmid DNA. DNA fragments were incubated with increasing amounts of ParB in the presence of IHF and analyzed by electrophoresis. The positions of free DNA (parS, 213 bp), the parS-IHF complexes, and the parS-IHF-ParB complexes are indicated. Note that the large vector fragment (3 kb) contains an IHF binding site. ParB dimer concentration (in nM) is shown above each lane. A, $\text{parS}^+[A4]$. B, $\text{parS}^+$. C, $\text{parS}[^{Δab}]$. D, $\text{parS}[b1]$. E, $\text{parS}[a1]$. F, $\text{parS}[a2]$. G, $\text{parS}[a3]$. H, $\text{parS}[b2]$. APRIL 13, 2007 • VOLUME 282 • NUMBER 15 JOURNAL OF BIOLOGICAL CHEMISTRY 10947
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not shown). We considered the possibility that DNA topology was important because plasmid DNA would normally be negatively supercoiled in vivo. We used nitrocellulose filter binding assays with 3H-labeled plasmid DNA in vitro to test the influence of mutations in each motif on ParB binding to supercoiled substrates (Fig. 5). This assay is effective in examining ParB binding directly because under these conditions IHF binds DNA to nitrocellulose poorly (4). In contrast to the assays on linear DNA, ParB bound parS sites with single right-side mutations (parS[A2], parS[a1], and parS[b1]) to similar extents in this assay (Fig. 5A). The mutations in the right side of parS, however, had effects on supercoiled DNA similar to those they manifested on linear DNA. Mutation of box A2 did not affect ParB binding activity, whereas mutation of box A3 or box B2 reduced ParB affinity for these parS variants.

Because removing either one of the left-side boxes did not significantly affect binding, we removed both boxes B1 and A1 (Fig. 5A). The ParB binding activity to parS[b1,a1] was greatly reduced; therefore either box B1 or box A1 on the left arm of parS is necessary for this complex, but either one will suffice in this assay. This result confirms that the complexes measured in these filter-binding assays are using recognition motifs on both the left and right arms of parS over an IHF-directed bend, and thus the use of box B1 by ParB is strongly influenced by the topological context of the parS site. Therefore the ability to use box B1 is dependent on negative supercoiling, but in plasmids with this topology, either box A1 or B1 is sufficient to bridge across the IHF-directed bend.

The role of Right-side Motifs in ParB Recognition—We next examined parS variants lacking two motifs (Fig. 2). First, each right-side motif was mutated in combination with either box A1 or box B1 (Fig. 6A and Table 1). All double mutants lacking A1 were poor substrates for ParB, parS sites lacking box B1 and one right-side motif were similar to or slightly weaker than the corresponding parS sites with single right-side mutations. Therefore two motifs on the right side are sufficient to mediate a relatively strong interaction with ParB as long as there is a specific left-side interaction. We concluded that box B1 could not contribute significantly because the substrates were linear. We confirmed this conclusion by testing two of these right-side mutations (a2 or a3) in the presence of either a1 or b1 substitutions in parS sites on supercoiled plasmids in nitrocellulose filter binding assays. In this experiment, parS[a1,a2] behaved as parS[b1,a2], and parS[a1,a3] behaved as parS[b1,a3] (Fig. 5B). Therefore, as seen with the single mutants, either box B1 or A1 is sufficient for a specific interaction of ParB with the left side of parS on supercoiled substrates.

Because, with the exception of box A1, the gel-shift and filter-binding assays showed similar motif preferences for ParB, we
FIGURE 6. DNA binding activity of ParB to parS variants lacking multiple motifs. ParB binding was examined by electrophoretic mobility shift assays as in Fig. 3. The data points are an average of at least three independent determinations, and the binding curves were calculated as described under "Experimental Procedures." A, DNA binding activity of ParB to parS site variants with a single motif mutated from either side of parS: +, parS*; ▲, Δab; ■, b1,a2; ●, b1,a3; ○, b1,b2; □, a1,a2; △, a1,a3; ○, a1,b2. B, DNA binding activity of ParB to parS site variants with two motifs mutated from the right side of parS: [a1,a2], [a1,a3], [a1,b2], [a2,a3]; [a1,a2,a3]; [a2,b2]. C, DNA binding activity of ParB to parS site variants with a single motif remaining on either side of parS (empty symbols): +, parS*; ▲, Δab; ■, a2,a3; ●, a2,b2; △, a3,b2; □, a1,a2,a3; ○, a1,a2,b2; △, a1,a3,b2; △, a1,b1,a3,b2; △, a1,b2,a3,b2; △, a1,b3,a2,b2; △, a1,b3,a2,b2. D, DNA binding activity of ParB to parS site variants, shown to high concentrations of ParB: +, parS*; ▲, Δab; ■, b1,a2; △, b1,a3,b2; ○, b1,a2,b2; △, b1,b2,a3; ●, a1,b2; ○, a2,b2; ○, a2,a3; ○, RevA1a2.
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![Graph](image)

**FIGURE 7. Comparison of ParB DNA binding activity with and without IHF.** Electrophoretic mobility shift assays were performed as described under "Experimental Procedures." A, DNA binding activity of ParB in the absence of IHF to parS site variants with a single motif mutated (empty symbols) or parS site variants missing the left arm (filled symbols). The data points are an average of at least three independent determinations, and measurement errors were typically less than 2% of substrate. +, parS; ▲, Δab; □, b1; ○, a1; △, a2; ▼, a3; ◇, b2; ■, b1,a1; ●, b1,a1,a2. B–E, increasing amounts of ParB were incubated with each of the parS substrates in the presence and absence of IHF and analyzed by electrophoresis. The positions of free DNA (parS), parS-IHF complexes, parS-ParB complexes, and parS-IHF-ParB complexes are indicated. ParB dimer concentration is shown in nm above each lane. B, parS. C, parS(b1,a1). D, parS(a2). E, parS(a3).

With parS sites lacking one or more motifs in the absence of IHF (Fig. 7A). Consistent with previous observations that the right side of parS, or parS-small, is the minimal partition site, removal of motifs on the left side of parS did not affect ParB binding in the absence of IHF. In other words, without the bend, ParB does not interact productively with the left side of parS. Examination of the right-side mutants showed that box A2 mutations did not affect minimal binding activity, but mutations in box A3 or box B2 essentially destroyed specific ParB binding activity as measured in this assay.

We directly compared ParB binding with and without IHF for several of these parS derivatives (Fig. 7, B–E). As seen previously, ParB binding to parS− is strongly stimulated by IHF (>50-fold; Fig. 7B). IHF also strongly stimulated ParB binding to parS variants with one right-side mutation (Fig. 7, D and E). In fact, seeing as the binding of ParB was very weak to parS[a3] or parS[b2] without IHF, ParB binding to these sites was essentially IHF-dependent (Fig. 7E and data not shown). ParB binding to sites lacking both left-side motifs (parS[b1,a1]; Fig. 7C) was similar with and without IHF. These data further confirm that a dimer of ParB normally sits on parS so that it bridges motifs across the IHF-directed bend. We constructed one other parS variant to emphasize the importance of the direction of the IHF-directed bend. We took advantage of the observation that boxes A1 and A2 were sufficient to form a high-affinity complex and constructed an A1-A2 parS site in which the orientation of A1 and A2 were reversed but their positions in parS were maintained (parS[RevA1A2]; Fig. 2). This arrangement changed the orientation of these box A motifs with respect to the bend but not with respect to each other. This parS variant showed no specific DNA binding activity by ParB in this assay (Fig. 6C; Table 1), further confirming that the direction of the IHF bend is important for ParB-DNA contacts.

**DISCUSSION**

ParB Recognizes Different Subsets of Its Motifs When It Binds to parS—A central question that arose from the crystal structure of P1 ParB protein bound to parS-small was how ParB would bind and assemble on the complete parS site across an IHF-directed bend. In this study, we have addressed the initial recognition step by ParB using a large number of parS sites with different combinations of its box A and box B motifs. Our results reveal several modes of ParB loading onto parS and that ParB has a motif preference that is dependent on motif position and on supercoiling of the plasmid substrate. High-affinity interactions do not require all box A and box B motifs. In the simplest arrangement, only one motif is necessary on either side of the bend. On linear DNA molecules, the main ParB contact on the left side of parS is with box A1, whereas on supercoiled substrates, ParB can use either box A1 or box B1. On the right side of parS, the data indicate that the highest affinity interactions are either with box A2 or a combination of box A3 and box B2. Because previous chemical footprinting of the dimeric ParB complex at parS (done in the gel) showed protection of all motifs in parS (5), we conclude that a dimer of ParB first loads onto parS− in several different ways, leading to a mixture of different complexes of ParB making contacts with specific subsets of motifs.

Motif Use Depends on Plasmid Topology—The ability to form a stable ParB complex using only the box B1 motif on the left side of parS required negative superhelicity in the plasmid substrate. At low concentrations of ParB, ParB bound well to parS[a1] only on supercoiled plasmid DNA (Figs. 4 and 5A). On supercoiled DNA, either box A1 or box B1 was necessary and sufficient for ParB to interact with the left arm of parS. We note that differences in affinity with \( K_d \) values below 5 nM would not...
have been detected in the filter binding assay because the DNA concentration was ~5 nM. It is therefore possible that differences in affinity between box B1 and A1 were below our limit of sensitivity. The motif preference on the right side of parS (either A2 or A3+B2) was similar on both linear and supercoiled DNA (Figs. 4 and 5A). These findings suggest that supercoiling is most important in stimulating tighter interactions with the left arm of parS, specifically box B1, although it may improve affinity for all motifs. How does supercoiling make box B1 a strong binding site for ParB? Negatively supercoiled plasmid DNA exists primarily in branched plectonemic conformations, and strong bends in the DNA, such as the one in mid DNA exists primarily in branched plectonemic conformation. The idea that a dimer of ParB can bind the right arm of parS presented by IHF, are typically positioned at the superhelix ends (reviewed in Refs. 25 and 26). The diameter of the superhelix axis is inversely dependent on the superhelical density, and negative supercoiling should favor tighter wrapping of parS around ParB. Therefore, we presume that negative superhelicity affects the orientation and position of box B1 so that it is closer to the protein than when the DNA is relaxed.

ParB Recognition of the Right Side of parS—The data indicate that a dimer of ParB can bind the right arm of parS in several ways in the presence of IHF, but two combinations yield the strongest complexes. The general picture is that ParB interacts best with either box A2 or a combination of boxes A3 and B2. On linear DNA, the best substrates with the fewest motifs are parS sites with only boxes A1 and A2 (parS[A1,A2]) or with boxes A1, A3, and B2 (parS[A1,A3,B2]). The presence of either only box A3 or only box B2 on the right side of parS (in combination with box A1) supported weaker but specific complex assembly. The ability of ParB to assemble a complex using only one motif on the right side of parS was absolutely dependent on the IHF bend and on a specific interaction with the left side of parS.

ParB Geometry on parS with IHF—Taken together, the results show that a dimer of ParB spans the IHF-directed bend in a variety of combinations, particularly on a supercoiled substrate. A dimer can span the IHF-directed bend by binding to one motif on each side of parS. What does this mean for the geometry of the protein in these complexes? The crystal structure of ParB-(142–333) shows two essentially independent DNA-binding domains that can rotate with respect to each other (16), so the flexibility of ParB, and also potentially the DNA, allows ParB to contact parS across the bend in a variety of conformations. The biochemical data here support this prediction. The arrangement of HTH motifs on opposite sides of the protein is consistent with each HTH motif binding an A-box spanning the bend, and the simplest arrangement would be with boxes A1 and A2. Both contacts are necessary because box A2 is not sufficient to bind ParB in the absence of IHF (Fig. 7). The observation that the affinity of ParB for the A1+A3+B2 combination is significantly higher than either A1+A3 or A1+B2 alone indicates cooperativity between boxes A3 and B2 (Fig. 6C; Table 1). However the flexible linker between the HTH domain and the dimerization DNA binding interface is too short for the protein to fold back and contact two adjacent motifs (16). We think that the best explanation for these observations is that the ParB complexes are dynamic; that box A3 and box B2 motifs in close proximity increase the affinity compared with each alone, because the protein comes on and off between these motifs rapidly and during migration of the complexes in the gel. Indeed, we think that the data support the idea that ParB interactions may always be dynamic in the presence of multiple motifs, especially in the wild-type site. For example, although the two best subsites (on linear DNA) are the A1+A2 or A1+A3+B2 combinations, they are still weaker sites than parS+ (Table 1). The motifs help each other, even though the HTH domains cannot contact more than two box A motifs simultaneously, consistent with rapid shuttling among these different combinations of complexes in solution. The reason that box B2 helps A3 and not A2 is likely that some rotations of ParB are more favorable, such as the one that contacts boxes A1 and A2. Because boxes A2 and A3 are inverted repeats, binding of ParB to boxes A1 and A3 would require one HTH domain to rotate 180° with respect to the protein conformation when ParB contacts boxes A1 and A2. We speculate that this rotation is also stabilized by the box B2 dimer domain interaction. Finally, the DNA surfaces contacted by ParB should be positioned facing inwards (between the bend), or there is enough flexibility in the DNA that they are anchored in this orientation by the protein. Consistent with this idea, previous in vivo and in vitro studies have shown that insertions in parS are tolerated only if they are integral turns of the helix and are between the left and right sides of parS (12, 13).

Why Does parS Contain Multiple Box A and Box B Motifs?—On a supercoiled plasmid in our assays, either box A1 or B1 was sufficient to bind ParB, but in vivo, box B1 is the required motif for activity of the full parS+ site. Why is box A1 not sufficient for parS+ activity in vivo, and are there conditions in which it is necessary? One explanation for the former question is that the architecture of a complex that loads solely onto box A1 may be different from one loaded via box B1, and that architecture as well as protein affinity is important for the next steps in the partition reaction. For example, perhaps the pairing reaction requires that the HTH interactions are intermolecular across A-boxes on the right side of parS. The conservation of box A1 among a number of related plasmid partition sites (2) implies that box A1 is utilized or required under certain lifestyles of the plasmid, for example ones that involve altered plasmid topology. Box A1 may expedite partition complex assembly under conditions in which plasmid DNA is not negatively supercoiled (or less negatively supercoiled), such as immediately following passage of a replication fork or during initial transfer of the plasmid into a bacterial cell (by infection of phage such as P1 or by conjugation of other plasmids, for example).

The idea that multiple binding sites can modulate protein complex architecture is observed for the phage A Int recombinase, another bivalent DNA-binding protein for which activity is also influenced by IHF and by Xis, a phage-encoded protein required for excise but not integrative recombination. The N-terminal domain of Int binds to “arm” DNA sites, which increases affinity of its “core”-binding and catalytic domains for core DNA sites. IHF binding at several sites is required to arrange arm and core sites into the proper architecture for recombination. The organization of the multiple copies of arm sites differs for integrative versus excise recombination (in attP versus attL and attR, respectively), and the use of different
P1 ParB Recognition of Partition Site parS

arm sites and thus the architecture of Int-DNA complexes are modulated by binding of Xis to influence the directionality of the recombination reaction (27, 28). The presence of multiple copies of recognition sequences for partition site-binding proteins is a general feature of many different partition systems, such as in the F plasmid sopC site, even though all copies may not be necessary for plasmid stability in the laboratory (29).

parS, mediated by ParB protein, is the loading site for the segregation machinery. Following recognition and binding of parS by a dimer of ParB, multiple dimers load onto parS to form large complexes visualized in vivo as large foci using immunofluorescence or fluorescent forms of ParB (6, 10). In vivo, ParB has been observed to spread along the DNA away from parS (30) and to pair parS sites (7). Pairing between P1 parS sites has not been seen in vitro however (5, 9) in the type of gel shift experiments performed here, from which we predict that stable pairing requires many ParB molecules. The evidence also suggests that ParB must spread onto several hundred base pairs around parS for efficient partition (31), consistent with the idea that many ParBs must assemble and act together during the partition reaction. The specific interactions of ParB require the parS+ site, even though a dimer can load relatively efficiently on sites with subsets of its recognition motifs. We propose that the requirement for multiple motifs reflects specific higher order interactions with ParB. These could reflect the sequence requirements to load additional dimers of ParB onto parS or to bridge adjacent parS sites in a pairing reaction. The latter is particularly attractive, because the crystal structure of ParB-(142–333) shows a three-way bridging activity of the protein, that is, the potential to bridge across parS sites as well as across the IHF-directed bend is supported by structural data. The interactions of ParB with all four essential motifs (B1, A2, A3, and B2) would supply the specificity for parS recognition both intra- and intermolecularly, whereas other protein-protein interactions, likely mediated by the oligomerization domains in the N-terminal half of ParB (32), would supply the strength to keep pairs together.

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