The P1 Plasmid Partition Complex at parS

II. ANALYSIS OF ParB PROTEIN BINDING ACTIVITY AND SPECIFICITY*

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The P1 plasmid prophage is partitioned by a very high affinity protein complex at its partition site, parS, that contains the P1 ParB protein and Escherichia coli integration host factor (IHF). ParB binds to regions of parS that flank the IHF binding site. In this report, we have examined the sequences to which ParB binds, the spatial relationship between them, and the effect of IHF on ParB binding patterns. Methylation protection and interference experiments were performed on supercoiled plasmids. Mutations that interfered with the action of both proteins in vivo were identified following random mutagenesis of parS. These studies revealed that ParB binds to a complicated, nonsymmetrical region in the right side of parS. ParB recognizes a partial copy of this sequence, TCGCA, in the left side of parS with much lower affinity. The presence of IHF greatly facilitates the interaction of ParB with parS such that both sides bind with an equal affinity that is much greater than to either side alone. The stimulation by IHF is strongly influenced by helical phasing. These observations support the proposal that ParB is directed, by the bend created by IHF, to bind simultaneously to properly placed sequences flanking the IHF site.

Stable maintenance of the P1 plasmid prophage in Escherichia coli requires proper segregation, or partition, of daughter plasmid copies at cell division. The plasmid components of the P1 partition system are the ParA and ParB proteins, and the centromere-like site, parS (1). The E. coli integration host factor, IHF, is the only known host protein involved in the P1 partition system (2), although it is generally assumed that other, as yet unidentified, components from E. coli also participate in this process (3, 4).

ParB and IHF bind with high affinity to parS, forming a multiprotein complex which is the normal substrate for subsequent steps in partition (5, 6). In vitro, ParB and IHF bind specifically and cooperatively to parS; binding of each protein increases the affinity of the other by several orders of magnitude (5). In addition, this high affinity binding requires that the parS substrate is supercoiled (5). Measured by DNase I protection experiments, IHF binds to an approximately 30-bp region in the center of parS (5, 6), which includes a reasonable copy of its consensus sequence. IHF binding creates a large bend, probably at least 140°, in the binding site (5). DNase I protection studies showed that ParB interacts with sequences on the left and right sides of the IHF binding site (6, 7).

In vivo, ParB is essential for partition (1). The lack of IHF has only a small effect on plasmid stability, but competition experiments with other versions of parS have established that the wild-type partition complex contains IHF (2, 5). The Par activity of parS is its ability to stabilize a low copy number plasmid in cis. In trans, parS will destabilize a second low copy number plasmid that is also partitioned by P1 par. This incompatibility, or Inc activity, is assumed to be a result of competition between these plasmids for the partition process (8). The minimal site required for wild-type incompatibility (also called incE) (1) has been narrowed down to an 84-bp region, which corresponds to the region protected from DNase I attack by ParB and IHF (6), and represents the wild-type partition site or parS*. A smaller region in the right side of parS, which we call parS-small, is sufficient for Par activity in vivo but has lost the ability to destabilize plasmids partitioned by parS* (9). This is because of a much reduced ParB binding affinity for parS-small, which cannot be stimulated by IHF (2, 5). Therefore the minimal ParB binding site is in parS-small, but the left side of parS is required for high affinity protein binding and wild-type Par and Inc activities in vivo (2, 5, 6, 9).

IHF is involved in many DNA-protein interactions in E. coli (for review see Ref. 10). It was originally identified as a host protein required for phage λ site-specific recombination (11), and this system continues to provide most of the detailed knowledge about IHF. IHF binds to and bends sequences in the λ attP site, which allows the λ Int protein to bind to distant, multiple sites in a specific, three-dimensional complex (12–17). IHF helps Int contact weak binding sites (the core sites) by affecting their proximity to strong binding sites (the arm-type sites). It seems likely that IHF plays a similar role at parS; that the IHF promoted bend allows ParB to contact distant sites, in the left (weak) and right (strong) sides of parS, in a specific three-dimensional conformation (5, 6). ParB is a dimer in solution (5), and it is reasonable to suspect that at least one dimer ParB binds at parS. Thus a core of ParB could expose two or more DNA binding domains on its outer surface. High affinity binding of ParB and IHF would be a consequence of the formation of a structure in which parS DNA is wrapped, or looped, around ParB and IHF.

We have tested two predictions of this model. First, ParB binding sites should be positioned so as to face the DNA binding domains of a central ParB core. Therefore proper spacing, or helical phasing, between the left and right sides of parS should be critical for complex formation. Second, simultaneous occupation of the left and right sides of parS by ParB should accompany complex formation.
We were particularly interested in the way that ParB recognizes the wild-type parS site and how IHF affected the ParB binding patterns. Previous DNease I footprinting experiments with ParBs were not sensitive enough to determine the specificity of individual bases (6, 7). However, the patterns that we observed and colleagues to propose that the sequence ATTTCA(C/A), which is repeated four times in parS, was an important element. Site-directed mutagenesis showed that at least one of these sites was dispensable (6).

In addition, they noted the presence of the sequence TOGGCA in both the left and right sides of parS. Here, we present biochemical and genetic evidence that ParB recognizes a complicated sequence combining both motifs in the right half of parS. This recognition is not altered by IHF. The presence of IHF and its associated degradation of DNA allow ParB also to contact a partial copy of this sequence in the left side of parS; these ParB sites must be on the same face of the DNA helix.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Phage, and Plasmids—E. coli strains (and relevant genotypes) DH5α (recA) and BR4301(DH5hip) (2) were used for plasmid experiments. E. coli LE30(mutD5) (18) was the mutator strain. akn-miniP1 is a kanamycin-resistant derivative of AP15R3 (19, 20). The immα clear plaque phage (Mc2 and Aβ-M2027) used to select immα lysogens are described by Sternberg (21). The plasmid pALA207 contains wild-type parS in a 748-bp Sau3A1 fragment inserted in the BamHI site of pBR322 (1). pLG23 has this parS Sau3A1 fragment cloned into pBluescript SK+/− (Stratagene). pBEF179 was constructed by subcloning an EcoRI-XhoI parS fragment from pBEF165 (5) into the EcoRI and SalI sites of pBR322. pBEF182 was derived directly from pBR322; the region between BamHI and StyI was deleted, and the BamHI site was destroyed.

**Reagents and Media—Dimethyl sulfoxide (DMS) was obtained from Aldrich, and DTT was purchased from Sigma-Aldrich. LB medium and plates were prepared as described by Sambrook et al. (18). Where indicated, the media contained 100 μg of ampicillin/ml and/or 25 μg of kanamycin/ml. EMBO agar plates (22) were prepared from Bectin Dickinson EMB (Levine) agar base without lactose. Restriction enzymes and enzymes used for cloning were purchased from New England Biolabs, Pharmacia LKB Biotechnolog Inc., and Boehringer Mannheim and were used according to their directions.

**DNA and Proteins—Plasmid DNA used in vitro was prepared by the alkaline lysis method and banded twice in CsCl-ethidium bromide gradients. Purified plasmid DNA (freshly diluted in ethanol) was purified as described (5). E. coli IHF was generously provided by Howard Nash (NIMH).

**DMS Methylation Protection—pBEF179 (1 μg, approximately 2 nm) was incubated with ParB and/or IFH in a 200-μl reaction containing 50 mM sodium cacodylate (pH 8), 150 mM KCl, 1 mM Na2EDTA, and 100 μg of bovine serum albumin/ml for 20 min at 30 °C. Five microliters of 1 M DMS (freshly diluted in ethanol) was added for 1 min at 30 °C. The reaction was quenched by the addition of 200 μl of a solution containing 2 mM NaHCO3, 1 M 2-mercaptoethanol, 20 mM adenine hemisulfate, and 60 μg of yeast tRNA/ml at 0 °C. The DNA was precipitated with 70% ethanol, dried, and resuspended in 100 μl of 0.3 M NaOAc, 10 μg of bovine serum albumin/ml and proteins as indicated in the text. Following a 20-min incubation at 30 °C, 10 μl of the mix was filtered through a nitrocellulose filter (2), washed with binding buffer, dried, and counted by liquid scintillation to determine binding efficiencies. The remaining 190 μl was filtered through another nitrocellulose filter and washed four times with 0.3 ml of binding buffer. The filter was incubated in 0.5 ml of 50 mM Tris-HCl (pH 8), 5 mM Na2EDTA, 0.3 M NaOAc, 50% SDS on a rocker for 45 min. After cooling, the eluate was mixed with 5 μg of yeast tRNA and extracted three times with phenol/CHCl3 and with CHCl3.

**End Labeling and Analysis of DNA—pBEF179 DNA (treated as above) was digested with Sall and end labeled with [α-32P]dCTP and DNA polymerase I large fragment (23). Following phenol extraction and ethanol precipitation, the DNA was redigested with BgIII and again phenol extracted and ethanol precipitated. This treatment labeled the “lower” strand of parS (Fig. 1). The “upper” strand was labeled with [α-32P]dATP and DNA polymerase I large fragment after the plasmid was digested with BglII. This was followed by a second digest with Rsal, and the DNA was processed as above. For interference experiments, a control sample of DMS modified [3H]DNA was also labeled. The DNA was cleaved in a Maxam-Gilbert G > A reaction (as modified by Craig and Nash (12)) and analyzed on 6% sequencing gels (24). The gels were dried on Whatman DE81 paper and exposed to film. Quantitation was performed using a Molecular Dynamics Phosphoimager.

**Plasmid Stability Tests—** The EMBO test is a quick and simple color test (22, 24). α-MiniP1 lysogens were streaked on EMBO plates selected for miniA X kan-P1 (clear plaque phage that are sensitive to λ repressor). Stable λ-miniP1 lysogens were immune and grew up as light colored cells. However, cell populations that lost λ-miniP1 at higher frequencies contain many plasmidless cells that were killed and lysed by the selector phage, producing a much darker appearance on EMBO plates.

A second assay measured the frequency of plasmid loss. Cells containing αkan-miniP1 derivatives were initially grown for several generations in LB with kanamycin. Portions were diluted at least 5,000-fold into LB and grown for 15–20 generations at 30 °C. The cell population was sampled at the beginning and at the end of growth in this medium by plating onto LB plates. Subsequent colonies were transferred with toothpicks to LB plates with kanamycin to check for the presence of λ-miniP1 in the original population. At least 200 colonies were tested at each time point. The percent retention represents the ratio of the frequency of kanamycin-resistant colonies at the end of growth in LB, to the frequency at the start, expressed as a percentage. To measure incompatibility exerted by a pBR322-derived plasmid in the same cell, the experiment was similar except that ampicillin was always present in the LB medium and plates.

**Random Mutagenesis—** Three-ml cultures of E. coli LE30(mutD) containing pALA207 were grown overnight in LB medium and used to isolate plasmid DNA, which was then introduced into DH5αkan-miniP1 lysogens by transformation. Transformants were grown overnight in LB with ampicillin and then plated on LB plates with both ampicillin and kanamycin. Cells resistant to both ampicillin and kanamycin were streaked in patches onto EMBO plates, and light (Inc−) derivatives were isolated and sequenced. Independent minipreparations from LE30 were used to ensure independent hits of all mutations isolated. More than 90% (42/51 independent mutations) contained mutations in parS. We routinely sequenced at least 50 bp on either side of parS and found no Inc− mutations in P1 DNA outside the 84-bp region defined by Davis et al. (6) as the minimal wild-type Inc− site. We therefore assumed that the remaining Inc− plasmids (5/51) represented mutations in the vector sequences, and these were discarded. The parS mutations were recloned into fresh

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**FIG. 1.** Map of the region of pBEF179 containing parS. P1 DNA containing parS is shown as the filled rectangle, from the TraI to StyI sites (1) (note that the TraI was destroyed in the construction), in a modified pBR322 vector (double lines). The restriction sites that are relevant for protection and interference studies are indicated.
pBR322, and all retained their Inc* phenotypes. As a control, a similar pBR322 derivative containing the lacZ gene was mutagenized under identical conditions and transformed into DH5α lac cells. Approxi-
mately 0.7% (12/1609) of these transformants were Lac- on Lac-
MacConkey plates, whereas no transformants (0/800) from unmuta-
genized DNA were.

In vitro, pALA207 DNA was treated with 1 M NH₂OH, 5 mM Na₂EDTA (pH 6) for 30 min at 75 °C, used to transform DH5α, and screened for Inc* phenotypes as above. This protocol yielded only one single mutation in parS (HA2) and nine multiple mutations (three to six hits/parS site). To obtain a higher frequency of single
mutations, plasmid DNA was linearized with EcoRI, treated with
hydroxylamine for 6-24 h at 37 °C, religated, and used to transform
DH5α. This protocol produced one double and 11 single mutations in
parS but also gave a higher background of vector mutations (51). Mutagenesis frequency was checked by mutagenizing pBR322 (am-
picillin- and tetracyclin-resistant) under identical conditions. Ap-
proximately 1-2% of ampicillin-resistant transformants were sensi-
tive to tetracyclin.

Site-directed and Insertion Mutagenesis—Site-directed mutagene-
sis was performed using the Kunkel method described in Ref. 23. The
substrates were the single-stranded DNA form of pLG23 and syn-
thetic oligonucleotides containing the altered bases. The Sau3A1 linker to measure the fragments from the resulting mutants were cloned into the BamHI site of pBR322, producing pLG30 (2-bp insertion, "parS::Xba") and pLG31 (2-bp substitution, "parS::GAGA"). Additional insertion mutations were created following digestion of pLG30 with XbaI. First, the ends of the DNA were filled in with DNA polymerase I large fragment and dNTPs, and religated, yielding the "6-bp" insert. Next, the DNA was treated with mung bean nuclease, religated, and transformed. Because this nuclease was not completely proficient at digesting the 4-base overhang from the restriction cut (a net -2 bp), three deletion derivatives (see text) were obtained. Mung bean nuclease digestion (XbaI and
NheI) was partially proficient at digesting the sticky ends that had escaped nuclease
attack by IHF (Fig. 2B) (5, 6). The pattern in this region was consistent with patterns observed at other footprinted IHF binding sites. For example, the protection at positions −4, 2, and 3 and the enhancement at position −5 (Fig. 2B) are also seen in the H1 and H2 binding sites in attP (12). It has been observed that IHF preferentially protects A residues from methylation (12, 25). This was also evident at parS (Fig. 2B). In addition, one A residue in the lower strand becomes especially susceptible to DMS attack in the presence of IHF. Interestingly, the position of this en-
hancement coincides with the predicted center of the bend that IHF produces at parS, as measured by gel mobility shift assays (5).

ParB protected sequences on both sides of the IHF site. The extent but not the pattern of protection was influenced by the presence of IHF. The strongest protection was consist-
tently observed at the G residues in the G-rich strand of Box B, and the complementary strand G was moderately protected. The Gs in the GAAA sequence of Box A were somewhat protected, and this protection was more variable from experi-
ment to experiment. Without IHF, high concentrations of ParB were required to protect, and the left side of parS required much more ParB than the right side (Fig. 2A). In the presence of IHF, both sides of parS were protected equi-
formally with increasing ParB concentrations, and less ParB was necessary for maximal protection. We repeated the ParB titration (with IHF) at lower concentrations to measure the concentration dependence more accurately. ParB binding to parS under these conditions was essentially stoichiometric. To illustrate the relative protection in the left and right sides of parS, the extent of DMS modification at the Box B motifs is shown quantitatively in Fig. 3. The values for the three G residues in the G-rich strand (which were protected similarly by ParB) were averaged and plotted as a function of ParB concentration (Fig. 3). These results are consistent with previous DNase I footprinting experiments and protein binding assays (2, 5, 6), which show that the minimal ParB binding site is contained within the right side of parS. The left sequences interact with ParB in the presence of IHF or at very high ParB concentrations. The increased affinity that

RESULTS

Methylation Protection of parS on Supercoiled DNA—ParB and IHF bind very tightly and cooperatively to parS (2, 5, 6). Although the interaction of IHF with its binding sites has been well characterized (12, 16, 25, 26), the exact specificity of ParB binding is unclear. We have used methylation of G and A residues by DMS (27) to probe the intimate contacts of ParB and IHF with parS*. We were interested in (a) the specific bases that these proteins contact and (b) how the ParB contacts and/or specificity is altered by IHF. Since the tightest and most specific binding of ParB and IHF requires supercoiled DNA contacts on supercoiled plasmids. Methylation by DMS was chosen because this modification does not break the DNA backbone. In addition, we created a parS plasmid, pBEF179, that allowed unique end labeling of each strand of DNA after the binding reactions were complete and without any gel isolation steps (Fig. 1; see “Experimental Procedures”). pBEF179 DNA was incubated with ParB and/or IHF and treated briefly with DMS. After removal of protein and DMS, portions of the samples were labeled and analyzed on sequenc-
gels. These gels were scanned in a Phosphorimager to quantitate the extent of modification and thus protection at G and A residues. Variability in the amount of radioactivity loaded on each lane was corrected by normalizing each pattern to the values of G residues that were outside of parS and unaf-
fected by protein.

We examined the effects of each protein separately and the influence of IHF on ParB methylation patterns. Methylation patterns for both strands from one such experiment are shown in Fig. 2A. Fig. 2B summarizes the protection data from several experiments on the sequence of parS. For the purposes of this discussion, we have defined a numbering system to differentiate the two halves or "subsites" of parS from the IHF binding site. Since the precise ends and center of parS are unknown, we used the center bp of the IHF consensus (which is well defined (10)) as position 0. Residues in the right half or subsite of parS are positive; residues in the left subsite are negative (Fig. 2B). We designated the two different repeated motifs in parS as Box A (ATTTCGCA) and Box B (TCGCCA).

It was apparent that IHF and ParB affect distinct nonoverlapping regions of parS, even when both proteins were present (Fig. 2B). The bases altered by exposure to IHF fall entirely within the region shown previously to be protected from nuclease attack by IHF (Fig. 2B) (5, 6). The pattern in this region was consistent with patterns observed at other footprinted IHF binding sites. For example, the protection at positions −4, 2, and 3 and the enhancement at position −5 (Fig. 2B) are also seen in the H1 and H2 binding sites in attP (12). It has been observed that IHF preferentially protects A residues from methylation (12, 25). This was also evident at parS (Fig. 2B). In addition, one A residue in the lower strand becomes especially susceptible to DMS attack in the presence of IHF. Interestingly, the position of this en-
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FIG. 2. DMS protection at parS. Panel A, DMS methylation protection patterns of the lower (left) and upper (right) strands of parS at various concentrations of ParB (nm dimer). Where indicated, IHF was also present at 22 nM. For reference, the rectangle shows the position of the TCGCCA box, or Box B, in the right and left sides of parS (see "Results"). The thick vertical lines next to the sequence indicate the IHF binding site, as defined by DNase I protection studies (5). The positions of the TaqI (T), DraI (D), and StyI (S) sites are shown. Panel B, summary of the DMS protection data from several experiments on the sequence of parS (GenBank accession no. X02864). Our numbering system defines the center A of the IHF consensus (TAACTGACTGTTTT) as position 0. Sequences to the left are negative and to the right are positive. The IHF binding site is shown as the thick black lines above and below the sequence. Box A sequences are shown as the thin rectangles, and Box B as the thick rectangles. Strong protection is indicated by triangles pointed toward the affected base; weaker or more variable protection is shown as circles. Filled symbols (V, □) represent protection by ParB and IHF, and unfilled symbols (V, ○) represent protection by ParB alone. Arrows show the positions of enhancements (by IHF). DMS modification at each position was quantitated with a Phosphoimager and may be a result of overloading the sample or incomplete digestion by BglII ("Experimental Procedures"). Otherwise, and in all other experiments (data not shown), the pattern of protection in the IHF binding site was identical with and without ParB.

IHF confers on ParB correlates with ParB's ability to recognize both sides of parS with equal affinity.

Methylation Interference at parS—The protection experiments indicated that ParB was close to sequences in both Box A and Box B. We used methylation interference experiments (28) to determine which bases were important for ParB recognition of parS by asking how chemical modification interfered with protein binding. The plasmid pBEF179 was lightly modified with DMS such that each parS site should contain no more than 1 modified base. The methylated DNA was incubated with ParB, with and without IHF, and the protein-DNA complexes were separated from free DNA by filtration through nitrocellulose filters. The bound DNA was eluted from the filters, and ^32P labeled, cleaved, and analyzed on sequencing gels. The bound DNA was compared with control DNA (DMS-modified substrate) that was similarly chemically cleaved and run on the same gel; bands that were missing or underrepresented in the former sample correspond to bases at which methylation interferes with protein binding.

Again, the patterns of modification and interference were scanned, quantitated, and normalized for variable sample loading.

Several details of the experiment are noteworthy. First, this strategy allows us to examine only complexes containing ParB, since IHF-DNA complexes alone do not stick well to nitrocellulose filters (2, 29). We assumed that the IHF binding site was defined as the region protected from DNase I digestion by IHF (6, 6) and that interference within this region was caused by IHF. Second, we used subsaturating concentrations of ParB to ensure that the assay would be sensitive to
small changes in affinity caused by the methylation of a single residue. The plasmid was ³H-labeled to measure binding efficiencies; under the particular conditions used, 10–20% of the DNA was bound by protein and to the filters. Finally, ParB binding in the absence of IHF was weak, and significant nonspecific competitor was required to observe specific complexes on nitrocellulose filters (5).

Typical interference patterns are shown in Fig. 4A. The positions of strong and weak interference are summarized (shown as triangles and circles, respectively) in Fig. 4B. Strong interference was defined as substantial and consistent underrepresentation of a band in the bound sample. Weak interference effects were smaller and/or more variable. In the right side of parS, ParB interference patterns were observed in a 15-bp region, from coordinates 31 to 45 (Fig. 4). The strongest interference was observed in the G residues in the Box B sequences, especially in the lower strand. The pattern was identical with and without IHF, although more ParB and nonspecific competitor were required to isolate specific complexes without IHF. In the left side of parS, only the Box B G residues exhibited interference, which was only seen when IHF was present. This result further confirms that the left side of parS is required only for high affinity ParB binding. These methylation and protection experiments suggest that ParB recognizes a complicated and/or composite sequence as its minimal ParB binding site in the right half of parS and that it uses a subset of these sequences, namely Box B, to recognize the left half of parS. They also suggest that IHF does not alter the recognition specificity of parS and support the idea that IHF stimulates ParB affinity by increasing the proximity of the left (weak) and right (strong) sites through the bend that it creates at its binding site.

Finally, the interference patterns observed in the IHF binding site were quite modest. Interference patterns have been reported to be weak at saturating IHF concentrations (25), which were used here so that complex formation would be most sensitive to ParB. We did not analyze IHF patterns further, since both nuclease and DMS protection experiments indicated that IHF binding at parS was very similar to its mode of binding at other sites (12, 16, 25, 30).

**Mutagenesis of parS**—We next used a genetic approach to determine which features of the DNA sequence were important for partition complex formation. The genetic screen was based on the incompatibility properties of parS. When parS is present on a second plasmid, it destabilizes low copy number plasmids, such as λ-miniP1 chimeras, that are partitioned by P1 par (1). Previous experiments have established that wild-type incompatibility (Inc+) requires both ParB and IHF (2, 6). The substrate for mutagenesis was pALA207, a pBR322 derivative containing parS⁺ (1). We screened a pool of randomly mutagenized pALA207 plasmids for mutants that were no longer or less able to destabilize λ-miniP1parS⁺ (Inc⁻). We used a simple color test, the EMBO test, for λ-miniP1 stability (see "Experimental Procedures"). Briefly, on EMBO agar plates seeded with immA selector phase, cells containing stable λ-miniP1 plasmids were light, and cells containing unstable plasmids were dark.

This approach had several advantages. First, mutagenesis was targeted specifically to parS. Davis et al. (6) have narrowed down the region essential for Inc⁺ to approximately 84 bp between the TaqI and StI sites, which corresponds to the region protected from DNase I digestion by ParB and IHF. Second, the Inc phenotypes of the high copy number plasmid pALA207 are more severe than low copy number derivatives containing parS (2, 5). Therefore we anticipated that this screen might be more sensitive to partial Inc⁻ mutations. Finally, the amount of P1 DNA in pALA207 provided enough homology to allow us to cross the resulting mutations into λ-miniP1.

The plasmid pALA207 was mutagenized in vivo by growth on the E. coli mutator strain LE30(mutD) and in vitro with hydroxylamine (see "Experimental Procedures") prior to transformation into cells containing λkan-miniP1. Next, independent pools of transformants were grown for several generations without kanamycin so that λ-miniP1 would be lost from the Inc⁺ population faster than from the Inc⁻ population. Cells were subsequently plated on LB plates with kanamycin; this lysogen population should contain a higher proportion of Inc⁺ pALA207 derivatives. Kanamycin-, ampicillin-resistant cells were tested on EMBO plates for λ-miniP1 stability and thus for Inc phenotypes.

From the mutD mutagenized plasmids, we isolated 42 independent mutants in parS which conferred a lighter color than wild-type pALA207 to cells containing λ-miniP1parS⁺. In fact, there was a range of colors from light to dark, and we picked everything that looked lighter than wild-type parS controls. The mutations were in 11 different positions in parS (Fig. 5). They included 10 transitions (C to T, and T to C),

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**Figure 4**. DMS interference at parS. Panel A, interference patterns for the lower (L) and upper (U) strands of parS. pBEF179 DNA was treated with ParB (2 nM dimer) and IHF (10 nM) (left) or with ParB only (135 nM) (right). Protein-DNA complexes (+ protein) were isolated as described under “Experimental Procedures” and compared with premethylated, untreated DNA (− protein). The radioactive gels were scanned, quantitated, and normalized for variable loading. The numbering system is described in Fig. 2. Panel B, summary of interference patterns from several experiments. Strong interference (♀, ♂) and weak interference (●, ○) (see "Results") in the presence (filled symbols) and absence (unfilled symbols) of IHF are indicated.
and one single-bp deletion. From the hydroxylamine-mutagenized DNA, 12 more independent mutants were identified in seven different positions (Fig. 5). These included two single-bp deletions, four different single transitions (C to T), and one double transition in which adjacent C residues were changed to adjacent Ts. The four substitution mutations had also been identified in the mutD population. Only one mutation, MD12, was in the IHF binding site (see below). The positions of all others coincided well with the regions identified by chemical interference as important ParB-DNA contacts (Fig. 4).

We reasoned that mutations affecting the IHF-stimulated component of ParB binding would be masked and thus would be Inc- in the absence of IHF (because parS+ also lacks stimulation by IHF). Conversely, mutations affecting basal ParB recognition, or the minimal site, should be Inc- regardless of the presence or absence of IHF. We tested Inc phenotypes in the E. coli strain DH5hip, which contains a mutation in the β subunit of IHF (2, 31), using the EMBO test. As predicted, DH5hip(λ-miniP1parS+) cells containing plasmids with parS mutations in the left half of parS (MD25, HA2, HA13) and in the IHF site (MD12) were now as dark as parS+ controls; that is, the mutants were Inc-. All mutations in the right side of the site (MD10, MD3, MD9, MD33, MD17, HA10, MD1, MD5, HA11, and MD2) were lighter than parS+ and were still Inc-.

The EMBO test was convenient to analyze a large number of mutants but was not very quantitative. We chose several representative mutations and measured the actual loss frequency of λ-miniP1parS+ caused by plasmids carrying these versions of parS (Table I). The strongest Inc- phenotype was the deletion HA10. In the right side of parS, MD10, MD33, and MD2 showed good Inc- phenotypes, whereas MD3 and MD9 were less severe. The HA2 (left) and MD12 (IHF site) mutations were also partially Inc- The numbers correlated very well with the classification determined using the EMBO test. The lightest colors were the most Inc-, and intermediate shades were partially Inc-. Therefore we continued to rely on this test for quick qualitative characterization of all mutants.

![Fig. 5. Mutations in parS. The positions and base changes for mutations in parS are shown below the sequence. The nomenclature represents the first independent isolate of each mutation (HA, hydroxylamine; MD, mutD). The number of independently identified mutations at a given position (by both mutagens) is also indicated (# hits). Note that the deletions MD12 and HA11 remove 1 base from a run of identical bases; their positions are arbitrarily displayed as the last base. The thick boxes represent Box B sequences, and the thin boxes show Box A. The thick horizontal line indicates the IHF binding site, and the dashed box shows the IHF consensus.](image-url)
TABLE II

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<th>parS mutation in λ-miniP1</th>
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<tr>
<td>None (parS+)</td>
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The mutant parS sites were crossed into λ-miniP1, and the stability of resulting λ-miniP1 plasmids was measured after overnight growth in LB medium (about 16 generations) at 30 °C.

In vivo, the effect of parSLG31 was not very dramatic (Table III). Since ParB also increases IHF affinity for parS (5), we expect that IHF can bind to this damaged site only in the presence of ParB (see “Discussion”). This may explain why no substitution mutations in the IHF site were identified in our genetic screen; small defects in IHF binding would always be hidden by ParB binding and would appear Inc+ in vivo.

![Protein binding to various parS mutants](image_url)

**Fig. 6.** Protein binding to various parS mutants. Nitrocellulose filter retention assays on 1H-labeled plasmid DNA were performed as described previously (5). The substrates were the pALA207 derivatives containing the indicated mutations: O, parS; pALA207; V, parSLG31; pLG33; ●, parS; Xba; pLG30; and ●, parS; Nhe; pLG33.

**Panel A.** ParB binding activity. The DNA concentration was 1 nM, and the assays included 2 μg of unlabeled salmon sperm DNA as nonspecific carrier. ParB concentration is reported as the concentration of the dimer. Panel B, IHF binding activity in the presence of 30 nM ParB (dimer). The DNA concentration was 1 nM.
The P1 Plasmid Partition Complex

A Higher Order Complex at parS—The P1 plasmid partition complex consists of ParB and IHF bound to parS. Previous evidence (5, 6) has suggested that parS is specifically wrapped around a core of ParB and IHF. (a) The right and left sides of parS, divided by the IHF binding site, are both required for formation of the high affinity complex. The right side contains the stronger ParB binding site. In the presence of IHF, ParB binds to the left or weaker side as well, resulting in an affinity much greater than to either side individually (or in the absence of IHF). (b) IHF binds to and bends parS. The complex with IHF and ParB is strongly stimulated by DNA supercoiling, which should favor wrapping. The experiments described here provide further confirmation of this model. First, when ParB binding was stimulated by IHF, both left and right parS Box B sequences were occupied equivalently by ParB (Figs. 2 and 3). Second, changing the spacing within parS showed that stimulation of ParB by IHF required that binding sites in the left and right sides of parS were on the same face of the helix (Table III and Fig. 6).

The spacing changes that we introduced into parS were more severe than the IHF site mutation, parSLG31 (Table III and Fig. 6). Although this mutation damaged IHF binding in the absence of ParB, IHF was still able to stimulate ParB binding in vitro, and parSLG31 was only slightly Inc in vivo. This is very likely because ParB increases IHF affinity to parS (5), and thus IHF would bind well to parSLG31 only in the presence of ParB. Preliminary experiments using gel mobility shift assays support this conclusion (data not shown). Presumably at very low IHF concentrations in vitro and in vivo we would see a more dramatic effect of this mutation. A severe IHF binding site mutation constructed by Davis et al. (6), in which 4 bases in the IHF consensus were changed, was Inc in their assays. Therefore, IHF must bind to parS to produce an Inc phenotype in wild-type cells.

Interaction of ParB with parS—We used DMS methylation protection and interference experiments to probe protein-DNA contacts. The patterns for IHF suggested that this protein binds to parS in much the same way as to other well characterized sites (12–14, 25), by binding in the minor groove and creating a large bend as the site is wrapped around it. We were primarily interested in defining the intimate ParB contacts at the wild-type parS site and how this binding was affected by IHF. Previous experiments reduced parS to an 84-bp sequence and led to the suggestion that two types of repeated motifs may be important for ParB recognition (6, 7). The Box A sequence (ATTTCA(C/A)) is repeated four times, and Box B (TCGCCA) is found twice in parS (Fig. 2B). (The limits of the motifs are defined by their similarity to each other and are thus somewhat arbitrary.) The DMS methylation protection and interference experiments exhibited a complex pattern of ParB-DNA contacts and suggested that ParB uses at least portions of both motifs when it binds to parS. Methylation interference experiments indicated that the subsite in the right side of parS is a combination of Box B and the center two inverted Box A repeats, whereas the left subsite appears to be only Box B. Interference experiments argue that two of the four Box A repeats are dispensable (Fig. 4), even though all four were partially protected from DMS by ParB (Fig. 2). IHF affected the affinity of ParB for parS but not the pattern of bases affected by ParB.

We used the incompatibility properties of parS to search for mutants in parS that interfered with protein binding. Since the entire 84-bp parS sequence is protected from DNase I attack by ParB and IHF, we assumed that no other protein binds directly to parS. Fifty-four independent muta-

tions mapped to 12 different positions in parS (Fig. 5). The mutations outside the IHF binding site were very close or identical to the important bases identified by chemical interference experiments (Fig. 4). They were in both Box B sequences and the two central Box A sequences (Fig. 5). The similarity with biochemical interference patterns makes it unlikely that the mutations directly interfere with the binding of a third protein and not ParB. The Inc− phenotypes of mutations in the right half of parS were seen in the presence and absence of IHF, whereas those in the left were noted only when IHF was present. In vitro, interference caused by methylation of bases in the left of parS was observed only in the presence of IHF. Therefore the genetics and biochemistry reveal a similar pattern of ParB interactions at parS.

The stability phenotypes of our parS mutations (Table II) can also be explained by ParB binding defects, although this must be confirmed biochemically. For example, strong mutations in the right side of parS (MD2, MD10, MD33) require IHF for Par activity. They presumably reduce basal ParB binding activity so that stable ParB binding and thus partition are dependent on IHF stimulation. This requirement for IHF emphasizes that the 84-bp parS site is the normal substrate for partition in wild-type cells; and we expect that plasmids partitioned by a 22-bp parS-small site containing MD2, MD10, or MD33 would be Par− with and without IHF. The Par−, Inc− phenotype of parSHA10 suggests that ParB cannot bind at all to this site. Perhaps single-base substitutions in the various motifs in the right side of parS are tolerated because no single base is absolutely required but spacing changes between them are not. Further characterization of our mutants in vivo and in vitro should help clarify the relationship among ParB binding, stability, and incompatibility.

Austin and co-workers (6, 32) have examined the limits of the right half of parS, the stronger ParB binding site. A 35-bp sequence (coordinates 26–60) contained enough information for ParB to bind in vitro and to exert incompatibility in the absence of IHF in vivo (6). More recently, they have narrowed it down in vivo to about 22 bp (coordinates 26–47), although it was slightly less efficient that the 35-bp version (32). All of our right side mutations fell within this 22-bp region (Fig. 5). They identified mutations in the 35-bp sequence, which were in positions similar to the mutations that we isolated. We have extended this analysis to cover the complete, wild-type site. We have established that the sequence TCGCCA, which coincides very well with the left boundary of the 84-bp wild-type site (6), contains an important recognition element for ParB.

How Does ParB Recognize Its Site—parS is an unusual specific DNA binding site. The stronger right subsite is asymmetric, and it is unknown whether ParB views the DNA sequence as distinct motifs, for example as two symmetrical Box A repeats superimposed on Box B, or in some more complicated fashion. The arrangement of parS, and the binding properties of ParB, lead us to consider and speculate on several possibilities. For example, two different recognition sequences implies two different DNA binding domains per monomer of ParB. Since the Box A and Box B sequences in the right subsite overlap (or are very close), the DNA binding domains on ParB must be correspondingly close. Alternatively, one larger binding domain of ParB may fit the entire right region, but the subunit that contacts the left subsite has its binding domain only partially filled. Perhaps if a copy of the right subsite replaced the left one (and was appropriately positioned), the requirement for IHF would be eliminated.

If ParB recognizes Box A and Box B independently, then
one dimer at parS could accommodate both Box B sequences and perhaps both required Box A sequences, depending on the geometry of the DNA binding domains in the protein complex. We do not know the stoichiometry of ParB at parS. Previous experiments suggested that this number is not greater than four monomers, but isolated ParB-DNA complexes were not analyzed (5). Although DMS interference assays (Fig. 4) argue that two of the Box A motifs are not important, protection from DMS by ParB of all four Box A sequences (Fig. 2) may suggest the presence of a tetramer or two dimers at parS. In this case, two of the Box B binding domains in ParB would be empty. Are these filled and unfilled sites involved in pairing plasmids prior to cell division? A plasmid pairing step is proposed in several models of partition (3, 4). For example, one ParB tetramer may bind to two plasmids simultaneously, filling four Box B and four Box A domains (two each per plasmid). We have no evidence for pairing in vitro, but a factor or structure that mediates pairing may be missing. This could also explain the differences between ParB interference and protection patterns (Figs. 3 and 4). In the absence of this factor in vitro, a tetramer would contain two extra Box A domains that could contact the extra but unnecessary Box A sequences on the same plasmid.

Another possibility is that ParB may be sensing something other than or in addition to the linear DNA sequence. For example, the two required Box A sequences form a short inverted repeat. Perhaps ParB binding to Box B facilitates the formation of a short hairpin or cruciform from the adjacent Box A palindromes, and both binding properties are required for a stable ParB-DNA complex. A hairpin or cruciform could also provide a structure that the partition complex of a sister plasmid must recognize to form a partitionable pair.

The geometry of the ParB complex with IHF (ParB bound to the left and right Box B sequences) would further stabilize ParB binding at parS and increase the ability to pair with sister plasmids. This idea could explain why such a complicated site is necessary as a specific DNA binding site.

Incompatibility and Stability—With the exception of parS-HA10, our mutations damaged incompatibility more than stability (Tables I and II). It is possible that the stability and incompatibility properties of the partition site evolved with different requirements. Perhaps a strong site on a P1 plasmid was more important to protect against competing plasmids than was necessary to stabilize itself. Alternatively, our stability assays in the laboratory may not be sensitive enough to detect small deficiencies that would be dangerous to long term stability in a natural environment.

Nevertheless, it is clear that P1 uses a strong, multiprotein complex as its normal substrate for partition. This structure must also contain domains that recognize other components of the partition pathway, such as ParA or host proteins. The nature of the partition events after formation of this complex remains an important question in the bacterial cell cycle.

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REFERENCES