Recognition of the P1 plasmid centromere analog involves binding of the ParB protein and is modified by a specific host factor

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The P1 plasmid partition system is responsible for segregation of daughter plasmids during division of the *Escherichia coli* host cell. The P1-encoded elements consist of two essential proteins, ParA and ParB, and the *cis*-acting *incB* region. The *incB* region determines partition-mediated incompatibility and contains the centromere-like site *parS*. We have isolated and purified the two proteins. ParB binds specifically to the *incB* region *in vitro*. DNase I footprinting assays place a strong binding site over the 35-bp *parS* sequence previously shown to be sufficient for partition when the Par proteins are supplied *in trans*. A weaker site lies within the *incB* region in sequences that are important for specifying incompatibility, but are not essential for partition. Gel band retardation assays show that a host factor binds specifically to the *incB* sequence. The factor strongly stimulates binding of ParB. Cutting the region at a site between the two ParB binding sites yields two fragments that can bind ParB but not host factor. Thus, information for host-factor binding lies in the region determining the specificity of plasmid incompatibility. The roles of parB and the host factor in partition and the specificity of plasmid incompatibility are discussed.

Key words: *E.coli*/plasmid/centromere/partition/DNA binding

Introduction

The prophage form of bacteriophage P1 is an autonomous unit copy plasmid. All the information required for stable plasmid maintenance is present in a 4-kb section of the P1 DNA. This consists of two blocks of information. One (*rep*) contains the replication functions. The other, the *par* region, contains information that is required for the accurate partition of daughter plasmids during cell division (Austin *et al.*, 1982; Austin and Abeles, 1983b). The *par* region appears to be sufficient to promote proper partition of any low copy number replicon into which it is inserted (Austin and Abeles, 1983a; Austin *et al.*, 1986). It encodes two proteins, ParA and ParB, both of which are essential for the process (Austin and Abeles, 1983b; Friedman and Austin, 1988) and contains a *cis*-acting site *parS* (Martin *et al.*, 1987).

A simple experimental system for partition has been constructed (Martin *et al.*, 1987). It consists of a small unit copy target plasmid containing a 49-bp fragment encompassing the *parS* site. The plasmid is inherently unstable due to random distribution of the copies to daughter cells. However, when the two P1 Par proteins are supplied *in trans*, the target

plasmid is stably maintained (Martin *et al.*, 1987). Thus, the 49-bp *parS* site contains all the *cis*-acting information necessary for active partition. It is probable that the site is acting as an analog of the centromere of a eukaryotic chromosome. It is proposed that, in concert with the Par proteins, the site is involved in the recognition of the plasmid copies by some sort of mitotic apparatus that actively distributes the copies to daughter cells (Austin and Abeles, 1983b). A notable feature of the *parS* sequence is the presence of a 13-bp perfect palindrome (Figure 1). A sequence containing just 35 bp across this palindrome is functional and a single base change within the palindrome blocks *parS* activity (Martin *et al.*, 1987).

As a first step in probing the molecular events involved in partition, we present data on the ability of Par proteins to interact with the *parS* site.

The properties of the minimal system differ from those of the intact *par* region in one important respect. The intact *par* region exerts a strong P1-specific incompatibility effect; plasmids containing it can efficiently displace a P1 miniplasmid (or any plasmid that relies on P1 *par* for its maintenance) from the cell. This is presumed to be due to competition between the plasmids in the partition process (Austin and Abeles, 1983b). The sequences that exert this incompatibility effect have been termed *incB*. The *incB* sequence consists of some 109 bp and includes the *parS* site (Abeles *et al.*, 1985; Martin *et al.*, 1987). However, the minimal *parS* site, though functional for partition, is not sufficient to exert the *incB* effect. This is not due to a loss of all ability to compete with other plasmids but rather to an altered specificity that no longer allows competition with plasmids bearing the full wild-type site. Thus, plasmids partitioned by the minimal site compete strongly with each other but not with plasmids that have the large (*incB*⁺) site (Martin *et al.*, 1987). By deleting a few base pairs from the *incB* sequence, a novel incompatibility group has been created.

The additional sequences required to act as a large (*incB*⁺) site lie directly to the left of the minimal *parS* sequence and include the 3' end of the ORF for ParB (Figure 1; Martin *et al.*, 1987). We present studies showing specific protein–DNA interactions in the region of the additional sequences. These studies suggest a model for how partition sites are recognized.

Results

Isolation and purification of the ParA and ParB proteins

Plasmids were constructed that place the strong λ pL promoter upstream of the *parA* ORF. Plasmid pALA269 contains both the *parA* and *parB* ORFs, whereas pALA406 is a similar construct containing *parA* only. These were introduced into strains containing the temperature-inducible cI857 repressor of λ pL. On induction, these strains greatly over-

Fig. 1. The sequence of the *PlincB/parS* region. The upper strand sequence is shown above and is mapped below. The amino acid sequence is the end of the *parB* ORF. The sequence coordinates are those of the P1 plasmid-maintenance region of Abeles *et al.* (1985). The half-arrows mark the 13-bp perfect inverted repeat. The small open boxes show the position and relative orientations of the repeated sequence 5'-ATTTCAC-3'. The *parS* and *incB* boxes show the extent of the minimum known sequences that have the small site *parS* activity (35 bp) and the large site *incB* activity (109 bp). The restriction sites on the map show the positions of the center of each recognition sequence.

produce the respective Par proteins (Figure 2). In agreement with our previous observations (Abeles *et al.*, 1985), the mobilities of the two proteins are very similar so that when both are present, they form a single band on SDS-PAGE. The proteins were substantially purified by the methods described below. The results of analyzing the ParB product by gel electrophoresis are shown in Figure 2. The products were positively identified as ParA and ParB, respectively, by Western blotting using antisera prepared against synthetic ParA and ParB carboxy-terminal peptides (Materials and methods; data not shown). The ParB preparation does not contain detectable amounts of ParA (or vice versa) by the same criterion. The apparent mol. wts of the purified products match those previously determined for the Par proteins (Abeles *et al.*, 1985) and are too similar to be readily separated on gels. Thus the gel band formed at the earliest step of the ParB purification contains some ParA as the plasmid used also contains the *parA* ORF. Most of the ParA protein is removed at the first column step as it does not bind to Affigel blue. After the second step (phosphocellulose chromatography) no ParA contamination of the *parB* product is detectable. Reconstruction experiments show that if any residual ParA was still present it would have been removed very efficiently at the third column step (heparin-agarose chromatography).

The ParB protein forms a specific complex with *incB* region DNA

We have studied the DNA binding properties of the purified Par proteins by the gel band retardation method (Garner and Revzin, 1980; Fried and Crothers, 1980). In this assay, the

Fig. 2. Purification of ParB protein. SDS-PAGE gels were stained with Coomassie blue. **A,B** 12.5% gel with crude lysates of induced cells containing: **A**, the vector control plasmid and **B**, the plasmid carrying the *parA* and *parB* ORFs under the control of the λ pL promoter. The ParA and ParB co-migrate in these gels with an apparent mol. wt of 44 kd. The ParA content of the material run in **lane B** is eliminated by subsequent purification steps (see Materials and methods) and is already undetectable in the material eluted from the phosphocellulose column (**lane E**). **C-F**, 10–15% gradient gel with peak ParB fractions from: **C**, Mono S column step (final product); **D**, heparin-agarose column step; **E**, phosphocellulose column step; **F**, Affigel blue column step. The prominent band of ~32 kd in **lane F** also reacts with the ParB-specific antibody; it is assumed to be a degradation product of ParB and is removed in subsequent chromatography. This degradation product does not affect the DNA binding, since the specific activity of the products in lanes **C-F** is essentially unchanged (data not shown). The numbered positions are those of bands formed by mol. wt standards and indicate their mol. wts in kd.

proteins are incubated with various 32 P-labelled linear DNA fragments and the mixtures subjected to polyacrylamide gel electrophoresis. Under these conditions, protein-DNA complexes remain together and migrate more slowly through the gel than the free DNA fragments. The positions of the free DNA and the protein-DNA complexes can be visualized by autoradiography. A large excess of unlabelled salmon sperm (SS) DNA was mixed with the fragments to compete out any non-specific DNA binding activities present in the protein preparations.

The results in Figure 3 show that a binding activity in the ParB preparation specifically recognizes DNA fragments containing the *incB* sequences, forming a distinct retarded complex. Labelled control fragments present in the same mixtures were not bound. An identical binding activity could be seen using partially purified ParB eluted by a salt gradient from an Affigel blue column. When extracts from cells not containing ParB were fractionated in an identical fashion, none of the fractions had binding activity (data not shown), indicating that ParB protein is required for the binding. No binding was evident when purified ParA protein was used (data not shown). We conclude that ParB, or some material dependent on the presence of ParB binds specifically to the 175-bp P1 fragment containing the *incB/parS* sequences.

Fig. 3. Gel band retardation assay for protein binding. Protein binding and electrophoresis were carried out as described in Materials and methods. The lanes show the effect of protein binding to end-labeled fragments containing the P1 *incB* region, or the smaller pBR322 control fragment, or a mixture of the two at $\sim 10^{-9}$ M. **Lanes a, d, g, j and m:** 211-bp fragment including the 174-bp *TaqI*–*HincII* region. **Lanes b, e, h, k and n:** the 185-bp *EcoRI*–*EcoRV* control fragment from pBR322. **Lanes c, f, i, l and o:** equal mixtures of the two fragments. Lanes a, b: no protein. Lanes c, d, e: ParB protein, 50 μ g/ml. Lanes f, g, h: crude host factor, 10 μ g/ml. Lanes i, j, k: ParB, 10 μ g/ml plus host factor, 10 μ g/ml. Lanes l, m, n: ParB 50 μ g/ml plus host factor 10 μ g/ml. Lane o: ParB 50 μ g/ml plus host factor, 10 μ g/ml plus $10\times$ more SS DNA (to 400 μ g/ml). The numbered arrows show the positions of complexes 1–4. The mobility of complex 4 varies somewhat with the concentration of SS DNA present (see Results).

ParB binds to the minimal *parS* sequence

We have further characterized the ParB binding to the 174-bp *incB* sequence by the DNase I protection (footprinting) technique (Galas and Schmitz, 1978; Materials and methods). The results are shown in Figure 4. Increasing concentrations of ParB reveal a clear area of protection corresponding to the symmetrical sequence in *parS*. Thus, ParB binds directly to the minimal *cis*-acting site required for partition. Figure 5 shows that this binding requires only the minimal *parS* sequence; the same *parS* bases were protected when a synthetic 35-bp *parS* site surrounded by pBR322 sequences was used as a substrate for binding. Although the DNase I cutting pattern of the region does not allow the protected region to be mapped to a precision of one base, it is clear that most of the 13-bp palindrome is protected and that the protection could extend several bases to the left of the symmetry (Figures 4 and 5).

Inspection of Figures 4 and 5 suggests that the protection of the upper strand (Figure 1) is strong in the left element and inner portion of the right element of the symmetrical sequence but weaker in the outer portion of the right element and in the 3-bp spacer between the inverted repeats. The pattern seen with the lower strand seems weaker, with protection evident mainly in the left repeat element and the spacer region. Thus, the binding appears to be asymmetric with respect to the 2-fold symmetry of the site. This implies that P1 bases outside the symmetrical elements (i.e. the 3-bp core or the four bases to the left of the symmetry) are involved in binding.

A second region within the *incB* sequence binds ParB

Figure 4 shows that a second region of protection lies ~ 50 bp to the left of the symmetrical sequence. This lies outside the minimal *parS* sequences but is within the region necessary for the *incB* activity (Figure 1). This sequence shows little or no strand bias in the protection pattern. The sequence 5'-ATTTCAC-3' appears both in this site and in each of the two symmetrical elements of *parS*. We propose that this sequence is an important element for ParB recognition.

A host factor binds specifically to the *incB* region and facilitates ParB binding

Using the gel band retardation assay described below, we found that crude extracts of cells contain a novel binding activity that is specific for *incB* region DNA (Figure 3). This activity must be a host factor as it was detected in cells without any P1 *par* genes. Like ParB, the factor bound only to those fragments that contain the *incB* region and formed a single retarded band in the gels. However, the complex formed (complex 2, Figure 3) has a very different mobility from the ParB-promoted complex (complex 1). When purified ParB protein was added with the crude extract, neither of these specific complexes was seen and two new slower migrating complexes were evident (complexes 3 and 4). Moreover, in contrast to the reactions with the individual components at the same concentration, the binding reaction was almost complete with respect to the DNA. By varying the concentrations of the components, we could show that combinations of ParB and host factor could form complexes 3 and 4 at protein concentrations well below those required with the individual components for efficient formation of complex 1 or 2, respectively. This synergistic effect is unlikely to be due to some trivial effect of ionic conditions as the ParB and host extracts were both extensively dialyzed against the same buffer prior to the binding reactions. We conclude that either the formation or the stability of bound complexes is favored by an interaction involving both ParB and host factor. Note that if the concentration of one component is severely limited, both factor-specific and complex 3 and 4 bands can be seen (Figure 3). Moreover, at low ParB concentrations, complex 3 predominates. Thus, a progression from individual complexes (1 or 2) to complex 3 and subsequently to a mature complex 4 can be achieved by increasing one of the components from a low concentration with a fixed concentration of the other. The mobility of the complex 4 is rather variable (Figure 3). It appears to decrease with increasing SS DNA concentration. In similar experiments, binding was studied using crude extracts of cells containing overproduced Par proteins. As expected, extracts of cells containing both ParB and ParA proteins gave complex 3 and 4 bands with high efficiency. Extracts of cells containing only ParA behaved exactly like control extracts with no Par proteins; they show only the host-factor effect and give complex 2. Purified ParA has no activity in the assay (data not shown). Thus, no ParA binding activity was detectable in either crude extracts or purified preparations.

ParB and host-factor binding sites are separable

The gel studies described above use target DNA containing the 174-bp (large site, *TaqI*–*HincII*) *incB/parS* region. A similar fragment containing only a *parS* small site (*AhaIII*–*HincII*) was prepared. Assays using this fragment

Fig. 4. DNase I protection by ParB protein bound to the *incB* region. DNase I protection patterns are shown on DNA containing the 174-bp *incB* region excised and end-labeled as described in Materials and methods. **Panel A:** upper strand labeled (see Figure 1). **Panel B:** lower strand labeled. **Panel C:** control fragment from pBR322 DNA. **Lanes 1, 2:** sequencing lanes. **Lane 3:** no ParB protein added. **Lanes 4, 5, 6, 7:** 0.005, 0.05, 0.5, 5 μ g of ParB protein used per 100 μ l assay. The half-arrows indicate the position of the inverted repeat sequence in *parS* (Figure 1). The open boxes indicate the position of the sequence ATTCAC or its opposite strand complement. The sequences are oriented such that the end of the *parB* ORF occupies the lower part of panel A and the upper part of panel B.

showed that it contains a ParB binding site but no host-factor site (Figure 6). Thus, a complex 1 analog was formed with ParB but host factor alone gave no complex at all. We also used a fragment containing only the remaining *TaqI*–*AhaIII* region (Figure 7). This fragment also showed no sign of host-factor binding. It did, however, give a small amount of retarded complex with ParB, but only at high ParB concentrations. This binding site was much more evident when the concentration of non-specific competitor DNA was reduced (Figure 7), suggesting that it has a low affinity for ParB in comparison with *parS*. This activity presumably reflects binding of ParB to the secondary site seen in the footprints

of this region.

We conclude that two ParB binding sequences exist in the *incB/parS* region and that these can be separated by cutting at the central *AhaIII* site. However, continuity across the *AhaIII* site is essential for host factor to bind. Presumably, a specific site for host-factor binding includes the sequence disrupted by *AhaIII* cutting. Thus, host-factor and ParB binding sites may overlap, but are separable.

Partial purification of host factor

We have partially purified the host factor from crude extracts using chromatography on heparin–agarose columns.

Fig. 5. DNase I protection of the isolated 35-bp *parS* sequence. The sequence is derived from a plasmid containing a synthetic 35-bp *parS* sequence inserted between the *EcoRI* and *SphI* sites of pBR322 DNA (Martin *et al.*, 1987). The half-arrows indicate the position of the *parS* palindrome. The upper strand is represented (Figure 1). The sequence is oriented such that the sequence of Figure 1 runs from the bottom to the top of the gel. **Lanes 1, 2:** sequencing lanes. **Lane 3:** no protein. **Lanes 4, 5, 6, 7:** 0.05, 0.15, 0.5, 1.5 µg ParB per 100 µl reaction. The strongly enhanced bands are due to some non-specific activity present in this preparation of ParB. Other preparations such as that used in Figure 4 do not give this effect.

An active fraction can readily be separated from the bulk of the cellular proteins as it elutes at high salt concentrations. SDS-PAGE analysis shows that the bulk of this material forms a complex of bands with mol. wts in the range of 10–15 kd.

Discussion

We have purified the ParB protein to ~90% homogeneity

Fig. 6. ParB binds to the isolated *parS* site but host factor does not. Protein binding to the 174-bp *TaqI*–*HincII* region (**lanes a–f**) is compared with binding to the 114-bp *AhaIII*–*HincII* region (**lanes g–l**). The binding seen with the 114-bp region is presumably in the *AhaIII*–*StyI* (*parS*) portion of this fragment as the remaining *StyI*–*HincII* portion gave no detectable binding activity (data not shown). **Lanes a–d and g–h** have decreasing concentrations of ParB (50, 20, 5, 2 µg/ml). **Lanes e, f and k, l:** decreasing concentrations of host factor (100 and 10 µg/ml crude host factor). In each case, the faint band immediately above the unretarded band is a contaminant of these particular fragment preparations; it is also seen in controls that have no protein added.

Fig. 7. The isolated *TaqI*–*AhaIII* region has a weak ParB binding site but does not bind host factor. The labeled fragment contained the 62-bp *TaqI*–*AhaIII* region. The reactions in **lane d** (ParB protein 50 µg/ml) and **lane e** (ParB, 50 µg/ml plus host factor, 10 µg/ml) run under standard conditions for ParB binding (400 µg/ml unlabeled SS DNA competitor). **Lanes a** (ParB, 50 µg/ml), **b** (ParB, 50 µg/ml plus host-factor, 10 µg/ml) and **c** (host factor, 10 µg/ml) result from binding carried out with a 10-fold lower SS DNA concentration.

using only its electrophoretic mobility as an assay. This material binds specifically to the minimal *parS* site. The activity is not present in control extracts from cells without

Fig. 8. A selective pairing model for small and large partition sites. Protein-protein interaction specifically pairs like plasmids via the *parS* site (marked by half-arrows). Plasmids using the minimal *parS* site can pair as shown (A + B). The existence of an additional ParB binding site and host-factor binding sequences on plasmids with the large (*incB*⁺) site causes a distortion which allows pairing with plasmids in the same state (C + D) but not with plasmids using the minimal *parS* site (B + C). Squares represent ParB protein and circles represent host factor. The exact positioning of proteins and the stoichiometry of the components are speculative.

the *parB* gene. Thus, it is very likely that ParB binds directly to the DNA site. We have not been able to detect a ParA binding activity to the region either with purified ParA or with crude extracts containing it. Thus, a direct binding role for ParA seems unlikely although it cannot be entirely ruled out.

The smallest functional *parS* site described to date is one consisting of only 35 bp of P1 sequence spanning the region of symmetry (Figure 1; Martin *et al.*, 1987). Within the limits of the footprinting technique, this sequence is the same as that bound by the ParB protein. Thus, it seems likely that the ParB-*parS* interaction is the key mechanism for recognition of the plasmid partition site and, in all probability, represents the first step in partition.

We have suggested a model for partition in which partition begins with the specific pairing of copies withdrawn from a free pool (Austin and Abeles, 1983b). Pairs of plasmids are then able to bind to one of a number of equivalent sites on some sort of mitotic apparatus, perhaps the nascent septum of the dividing cell, that directs one member of each pair to each daughter cell. The model helps to explain the incompatibility of like plasmids and the compatibility of unlike ones as it assumes that only like plasmids can pair. If the P1 system does work this way, an obvious role emerges for ParB. ParB may bind specifically to the *parS* sequences of the available plasmid copies and facilitate their pairing by a specific protein-protein interaction (Figure 8). It seems probable that ParA plays a role at some later stage, perhaps in the recognition of the pre-paired complexes by the mitotic machinery.

Both the minimal site and the larger (*incB*⁺) site are proficient for partition and any differences in their efficacy are modest (Martin *et al.*, 1987). What, then, is the difference between the two? We propose that they differ in an essential self-recognition event during partition.

Partition-mediated incompatibility can be readily explained only if available plasmids are withdrawn at random from a pool for partition (Novick and Hoppensteadt, 1978). Plasmids must be recognized as daughters because they have partition sites of identical specificity. An incoming plasmid will compete in this process if it too has the same site. A random assortment of the two plasmids will result in the phenomenon described as incompatibility. A useful way of modelling this self-recognition event emerges from the pre-pairing model described above. Differentially marked plasmids with identical partition sites will pair with each other, irrespective of whether they are daughters or not, resulting in random assortment.

We have shown that deletion of a small region to the left of the minimal P1 partition site results in a site that is still effective for partition, but is no longer recognized as being identical to the wild-type. Thus, the mechanism that recognizes partition sites as 'self' has been altered. If the pre-pairing model applies, minimal P1 partition sites only pair with other minimal sites and have lost the ability to pair with large sites (Martin *et al.*, 1987). Our current results may provide an important clue as to the biochemical basis for this change in specificity. The additional base pairs encode a specific binding site for a host protein and an additional ParB binding site. We propose that binding of one or both of these factors to the additional bases alters the topology of the whole partition site (by bending, for example) in such a way that the site can only pair with sites in the same state (Figure 8C and D). The minimal site can only pair with itself because it cannot achieve this state and remains extended (Figure 8A and B). After binding, the events that drive the partition itself must work on either type of paired complex. The model predicts that ParB protein may be an essential component for binding to the putative 'mitotic apparatus', but the host factor is not, as it is only involved in the selectivity of the pairing process. We are currently attempting to test this hypothesis using an *in vitro* pairing assay.

Materials and methods

Materials

Media, reagents, enzymes, buffers and chemicals were as previously described (Abeles, 1986) unless otherwise stated. Enzymes were used under conditions recommended by the manufacturers.

Bacterial strains and plasmids

Strain MZ1 contains a defective λ prophage encoding the temperature-sensitive λ cI857 repressor (Zuber *et al.*, 1987). Strain N100 is described elsewhere (Sternberg and Weisberg, 1975).

Plasmid pALA118 was constructed as follows: the *Hind*III end of the *Eco*RI-*Hind*III fragment containing P1 *par* was converted to a *Sal*I end using a synthetic linker. The resulting fragment was inserted between the *Eco*RI and *Xho*I sites of the λ pL vector pRK12 (Abremski and Hoess, 1983).

Plasmid pALA269 was derived from pALA118 by deletion from the unique *Nru*I site in the P1 insert (Abeles *et al.*, 1985) to the *Pvu*II site of the vector. Plasmid pALA406 consists of the *Clal*-*Ssp*I fragment of pALA269 that includes λ pL through the end of the *parA* gene inserted into the *bla* gene of pBR322 which was cut with *Sca*I and *Clal*. The insertion event involved an undefined rearrangement of the vector sequences such that the *parA* gene of an intact pALA269 fragment reads clockwise with respect to the pBR322 physical map. The plasmid confers tetracycline resistance. Plasmid pALA161 consists of the 174-bp *Taq*I to *Hinc*II P1 fragment carrying *incB* inserted between the *Acc*I and *Hinc*II sites of the polylinker in M13mp10 (Messing and Vieira, 1982), excised as a *Eco*RI-*Hind*III fragment, and inserted between the homologous sites of pBR322. The conventional map sense of the *par* region runs clockwise with respect to the pBR322 map.

Plasmid pALA288 contains the 49-bp *HincII*–*AhaIII* fragment inserted in the *HincII* polylinker site of M13mp10 and excised as a *EcoRI*–*HindIII* fragment and inserted into the homologous sites of pBR322. The conventional orientation of the *parS* site runs counterclockwise relative to the pBR322 map. Plasmid pALA408 was derived from pALA288 by deletion of the *SylI*–*SylI* fragment leaving the *SylI*–*HincII* fragment as the sole P1 sequence. Plasmid pALA271 consists of the 2.5-kb *HindIII*–*AhaIII* P1 *par* region inserted between the *HincII* and *HindIII* sites of the M13mp10 polylinker, excised as a *BamHI*–*HindIII* fragment and inserted between the homologous sites of pBR322. The *par* genes run clockwise relative to the pBR322 map.

End-labeled DNA fragments

End-labeled DNA fragments were prepared as previously described (Abeles, 1986). The fragment containing the 174-bp *TaqI*–*HincII* P1 sequence has short polylinker sequences at each end resulting in a total length of 211 bp. The 211-bp fragment was excised from the M13mp10 polylinker site with *EcoRI* and *PstI* and was labeled at the *EcoRI* end. The 185-bp *EcoRI*–*EcoRV* control fragment from plasmid pBR322 was labeled at the *EcoRI* end. The 62-bp *TaqI*–*AhaIII* region was isolated as a *DdeI*–*BamHI* fragment from plasmid pALA271 (Abeles *et al.*, 1985). The labeled *DdeI* end is linked by 55 bp of pBR322 and polylinker sequence to the *TaqI* end of the P1 sequence. The *SylI* end of the P1 sequence is modified by a short polylinker sequence, giving a *BamHI* terminus. The fragment containing the 114-bp *AhaIII*–*HincII* region that includes *parS* was excised from the M13mp10 polylinker site with *HindIII* and *BamH* and labeled at the *BamHI* end.

DNA footprinting

DNA footprinting was carried out essentially by the DNase I protection method of Galas and Schmitz (1978), except that Par protein was allowed to bind to DNA in 20 mM Tris–HCl pH 8, 40 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA and 50 µg/ml bovine serum albumin. Labeled DNA was used at 0.1 µg/ml.

Gel band retardation experiments

Labeling of fragments, binding of protein and gel electrophoresis were carried out essentially as described by Abeles (1986). The binding step was carried out at 30°C for 20 min. In all cases, a large excess of unlabeled SS DNA was added to compete for non-specific binding activities. ParB binding alone was carried out at 400 µg/ml of SS DNA. A 10-fold reduction of the concentration led to a reduced efficiency of binding to the labeled fragments. For host-factor binding and host-factor plus ParB binding, 40 µg/ml of SS DNA was used because, although the binding 400 µg/ml was equally efficient, the retarded complexes tended to smear over a wider range of mobility. Labeled DNA was used at 0.1 µg/ml. The protein concentrations used for the experiments recorded in Figure 3 were chosen as they best illustrate the synergistic effect of the two factors on binding. When the proteins were added alone at significantly higher concentrations the proportion of DNA in the specific retarded band could be increased but some retardation or degradation of the control DNA was observed.

Isolation and purification of ParB

The strain used was MZ1 (Zuber *et al.*, 1987). Plasmid pALA269 was introduced by transformation. This plasmid consists of the *HindIII*–*NruI* region of P1 *par* (Abeles *et al.*, 1985) that includes the *parA* and *parB* genes. The *par* operon transcription is under control of the strong *pL* promoter of phage λ. 300-ml cultures were grown in L broth supplemented with 20 µg/ml of tetracycline at 30°C to an OD₆₀₀ of 0.5. The cultures were then shifted to 42°C to induce transcription from *λpL*, and incubated for 3 h with aeration. The cells were pelleted by centrifugation and resuspended in 3 ml of cold P buffer plus 150 mM NaCl (P buffer was 50 mM Tris–HCl pH 7.6, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol).

The resuspended pellets were stored at –70°C. After thawing on ice, the cell suspension was sonicated with six 20-s bursts separated by 45 s of chilling on ice (Branson sonicator microprobe, setting 6). The suspension was centrifuged at 27 000 g for 30 min. The supernatant was loaded onto a 3-ml Affigel blue column (Sigma Corp.) equilibrated in P buffer (150 mM NaCl). The column was washed with 10 column volumes of the same buffer and eluted with a 20-ml 150–1000 mM NaCl gradient in P buffer. Fractions were assayed by SDS–PAGE, looking for the prominent band of ~42 kd that elutes near the middle of the gradient. The peak fractions were pooled, dialyzed against P buffer (150 mM NaCl) and then loaded onto a 3-ml phosphocellulose column equilibrated in P buffer (150 mM NaCl). This column was washed with 10 vol of the same buffer and then eluted with a 20-ml 150–1000 mM NaCl gradient in P buffer. Peak fractions were located by gel electrophoresis, pooled and dialyzed ex-

tensively against P buffer (150 mM NaCl). This material was loaded onto a 3-ml heparin–agarose column equilibrated with the loading buffer, washed with 20 column vol of buffer and eluted with a 20-ml 150–1000 mM NaCl gradient in P buffer. Peak fractions were determined by SDS–PAGE analysis and Western blotting as described below. At this stage, the material is substantially pure ParB material but contains a minor ParB species of ~30 kd which may be a degradation product with an intact carboxy terminus. Most of this material was removed by a final column step. Pooled fractions from heparin–agarose were loaded onto a Mono-S column (Pharmacia) equilibrated with H buffer plus 100 mM NaCl. (H buffer was P buffer but with 50 mM Hepes pH 7.6 instead of Tris–HCl.) The column was run on a Pharmacia FPLC apparatus, washed with 35 ml of H buffer (100 mM NaCl) and eluted with a 50-ml H buffer gradient of 100–500 mM NaCl. ParB protein eluted at ~300 mM. The peak fractions were pooled and dialyzed against P buffer with 150 mM NaCl and 50% glycerol, and stored at –20°C. The binding activity of this material was stable for at least 6 months. The final product yielded ~0.5 mg of protein and was judged to be >90% homogeneous.

ParA isolation and purification

MZ1 cells containing pALA406, a plasmid in which an isolated *parA* open reading frame is under control of *λpL*, were grown and disrupted in the same way as those for ParB purification. After centrifugation at 27 000 g for 30 min, a 20% streptomycin sulfate solution was added slowly with stirring to the supernatant to give a final concentration of 4%. After removal of the precipitate by centrifugation at 27 000 g for 15 min, the supernatant was raised to 50% saturation with ammonium sulfate and the precipitate removed by centrifugation. The procedure was repeated to 60% saturation with ammonium sulfate and the insoluble pellet recovered by centrifugation. The pellet was resuspended in 2.0 ml of cold P buffer plus 150 mM NaCl and extensively dialyzed against P buffer plus 50 mM NaCl.

The solution was applied to a heparin–agarose column equilibrated with the same buffer, washed with 20 column vol of buffer and eluted with a 50–1000 mM NaCl gradient in P buffer. Peak fractions containing ParA were located by SDS–PAGE and Western blotting with ParA antibody (see below). The peak fractions were pooled and dialyzed against P buffer with 150 mM NaCl and 50% glycerol. The protein was stored at –20°C. Total yield of the protein was ~0.5 mg and it is >90% homogeneous. SDS–PAGE analysis showed only one visible band of mobility equivalent to a mol. wt of 44 kd (data not shown). This band is ParA as judged by Western blotting against antisera specific for the carboxy-terminal residues of ParA (see below).

Host-factor extracts

Strain N100 was grown in 1 l of L broth to OD₆₀₀ of 0.5, and the cells harvested by centrifugation and resuspended in 10 ml P buffer (150 mM NaCl) and frozen at –70°C. After thawing, the suspension was lysed and centrifuged to remove insoluble material as described above for ParB preparation. The supernatant was raised slowly to 4% streptomycin sulfate and the precipitate removed by centrifugation for 30 min at 27 000 g. The completed extract was stored at 4°C and remained active for several weeks.

Western blotting for detection of ParA and ParB

Western transfer was carried out as originally described (Towbin *et al.*, 1979). The antisera used were prepared by standard techniques against synthetic peptides corresponding to the last 12 amino acids encoded by the *parA* and *parB* ORFs, respectively. Within the concentration ranges used, the antisera were specific for their cognate proteins and did not give any signal with control extracts or with the other Par protein.

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