P1 ParA interacts with the P1 partition complex at *parS* and an ATP–ADP switch controls ParA activities

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The partition system of P1 plasmids is composed of two proteins, ParA and ParB, and a *cis*-acting site *parS*. *parS* is wrapped around ParB and *Escherichia coli* IHF protein in a higher order nucleoprotein complex called the partition complex. ParA is an ATPase that autoregulates the expression of the *par* operon and has an essential but unknown function in the partition process. In this study we demonstrate a direct interaction between ParA and the P1 partition complex. The interaction was strictly dependent on ParB and ATP. The consequence of this interaction depended on the ParB concentration. At high ParB levels, ParA was recruited to the partition complex via a ParA–ParB interaction, but at low ParB levels, ParA removed or disassembled ParB from the partition complex. ADP could not support these interactions, but could promote the site-specific DNA binding activity of ParA to *parOP*, the operator of the *par* operon. Conversely, ATP could not support a stable interaction of ParA with *parOP* in this assay. Our data suggest that ParA-ADP is the repressor of the *par* operon, and ParA-ATP, by interacting with the partition complex, plays a direct role in partition. Therefore, one role of adenine nucleotide binding and hydrolysis by ParA is that of a molecular switch controlling entry into two separate pathways in which ParA plays different roles.

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Introduction

Partition of bacterial and plasmid chromosomes is an active positioning process that ensures proper segregation of daughter chromosomes at cell division. Recent fluorescence studies have illustrated that these chromosomes are specifically oriented within cells (Gordon *et al.*, 1997; Niki and Hiraga, 1997, 1998; Webb *et al.*, 1997). Genes and proteins responsible for partition have been identified in plasmids and more recently in several bacterial genomes, although the mechanism of this process remains elusive.

The partition of the *Escherichia coli* prophage/plasmid P1 is effected by two plasmid-encoded proteins, ParA and ParB. For most of the cell cycle, P1 plasmids are specifically localized at the $\frac{1}{4}$ and $\frac{3}{4}$ positions of cell length. These positions become the midpoint of a newborn cell following cell division, but plasmids move rapidly (presumably following replication) to the new $\frac{1}{4}$ and $\frac{3}{4}$

positions (Gordon *et al.*, 1997). This behaviour is similar to that of F, the sex factor plasmid of *E.coli*, and in F has been shown to be dependent on its partition system (Niki and Hiraga, 1997). Homologues of P1 ParA and ParB have been identified that are encoded by other plasmids such as F, as well as by various bacterial chromosomes (Motalebi-Veshareh *et al.*, 1990; Williams and Thomas, 1992; Koonin, 1993; Ireton *et al.*, 1994; Mohl and Gober, 1997). It is therefore likely that some and perhaps many of the steps performed by these proteins are shared among the different bacterial and plasmid partition systems.

The P1 *par* operon contains the *parA* and *parB* genes upstream of a *cis*-acting site, called *parS* (Abeles *et al.*, 1985) (Figure 1A and B). All three elements are absolutely essential for plasmid stability. P1 ParB and *E.coli* integration host factor (IHF) bind to *parS* to form a higher order nucleoprotein complex called the partition complex (Davis and Austin, 1988; Funnell, 1988b, 1991). IHF binds to a sequence that is flanked by several ParB recognition sequences and induces a large bend in *parS*, thereby facilitating ParB's binding to its sequences and greatly increasing the affinity of ParB for *parS* (Funnell, 1991; Funnell and Gagnier, 1993). ParB recognises two different sequence motifs, a heptamer called BoxA and a hexamer called BoxB, which are asymmetrically arranged in *parS* (Figure 1B). ParB is a dimer in solution (Funnell, 1991), but the stoichiometry of ParB bound to *parS* is not known. Other ParB homologues, such as F plasmid SopB and *Bacillus subtilis* SpoOJ, have also been shown to be site-specific DNA binding proteins and to be required for proper partition of their respective chromosomes (Ogura and Hiraga, 1983; Mori *et al.*, 1989; Watanabe *et al.*, 1989; Ireton *et al.*, 1994; Lin and Grossman, 1998).

ParA plays at least two roles in the P1 partition cycle. The first, which is better understood, is as a repressor of *par* gene expression (Friedman and Austin, 1988). ParA's repressor role is mediated via its site-specific DNA-binding activity to a large inverted repeat in the promoter region of the *par* operon, *parOP* (Hayes *et al.*, 1994). The autoregulation is particularly important since both the absolute and relative levels of ParA and ParB are critical for the partition process (Abeles *et al.*, 1985; Funnell, 1988a; Hayes *et al.*, 1994). The operon repression by ParA is increased by ParB as shown by transcriptional studies (Friedman and Austin, 1988). Consistent with this, DNase I footprinting experiments have shown that ParB stimulates ParA's binding activity to *parOP* *in vitro* (Davey and Funnell, 1997). However, ParA's repressor role can be bypassed genetically and ParA is still essential for P1 plasmid stability (Friedman and Austin, 1988; Davis *et al.*, 1996), from which it has been inferred that ParA has a second, direct role in the partition process.

The identification of an ATP-binding motif in the ParA protein sequence (Motalebi-Veshareh *et al.*, 1990) led to

Fig. 1. Schematic representation of the P1 *par* operon, *parS*, and the DNA fragments containing *parS* used as substrates in this study. (A) The *par* operon containing the *parA* and *parB* genes, the *cis*-acting *parS* site, and the operator/promoter region *parOP* are drawn approximately to scale. (B) The P1 DNA sequence between *TaqI* and *StyI* restriction sites: The BoxA and BoxB *ParB* recognition motifs are indicated by boxes; the 84 bp minimal *parS* site defined by Davis *et al.* (1990) is between (and includes) Box B1 and Box A4. The IHF binding site, determined by DNase I footprinting studies (Funnell, 1991), is underlined. Twenty-one nucleotides are omitted between the *TaqI* site and the left end of *parS*. (C) DNA fragments used as *parS* substrates: black shading represents *parS* as the P1 sequence between the *TaqI* and *StyI* restriction sites [as in (B)]. Gray shading corresponds to P1 sequence adjacent to *parS*. White shading represents vector (pBR322) sequence. *parS*Δ-390 contains only the left part of *parS*, between the *TaqI* and *DraI* sites [as in (B)]. The construction of all fragments is described in the Materials and methods. Each fragment is designated by its length in bp.

the discovery of a weak ATPase activity in ParA that was stimulated by ParB (Davis *et al.*, 1992). In fact, much of the homology among the members of the ParA-like family of proteins is due to their putative Walker A- and B-type ATP binding regions (Motallabi-Veshareh *et al.*, 1990; Koonin, 1993). *In vivo*, the ATP-A motif is essential for both the repressor and partition activities of P1 ParA (Davis *et al.*, 1996). *In vitro*, all of ParA's activities are strongly influenced by binding to adenine nucleotides. They affect ParA's dimerization state, its site-specific DNA binding activity to *parOP*, its conformation as measured by circular dichroism and its interaction with ParB at *parOP* (Davis *et al.*, 1992; Davey and Funnell, 1994, 1997). However, different adenine nucleotides (ATP, ADP and nonhydrolyzable analogues) influence each activity in different ways. For example, both ATP and ADP promote dimerization of ParA (Davey and Funnell, 1994). ATP stimulates ParA's DNA binding activity to *parOP* ~10-fold, but ADP and non-hydrolyzable ATP analogues stimulate 5- to 10-fold better than ATP. ParB stimulates ParA's *parOP* binding activity, but only in the presence of ATP; no stimulation is observed with ADP or ATPγS (Davey and Funnell, 1997). Measured by circular dichroism, ATP-bound ParA adopts a more helical conformation than that of ADP-bound ParA. These observations led to the suggestion that different forms of ParA exist, depending on the bound nucleotide, and have different roles in repression and partition.

The role of ParA in the partition mechanism is not understood. The ParA protein is not required for the formation of the partition complex. Models for ParA action include both assembly and disassembly activities during a plasmid pairing step or during attachment of plasmids to a fixed cellular structure such as a site on the bacterial membrane. Since the partition complex at *parS* is essential for the plasmid positioning reaction, ParA must interact with this complex, directly or indirectly, to

perform its roles. There is some evidence that ParA or ParA-like proteins can influence ParB's binding to the partition site (Youngren and Austin, 1997; Jensen *et al.*, 1998); however, a complex of ParA bound to the partition complex has not yet been observed. For example, in the *parA* partition system of plasmid R1, which is analogous but not homologous to that of P1, the ParM ATPase stimulates an intrinsic pairing activity of ParR, the protein that binds to the partition site *parC* (Jensen and Gerdes, 1997; Jensen *et al.*, 1998).

In this study, we have examined ParA's interactions with the partition complex at *parS*, and the influence of adenine nucleotides on this activity. We found that ParA does assemble onto the partition complex at *parS*, and that this interaction is completely dependent on ATP. These results provide biochemical evidence that ParA does participate directly in partition via incorporation into a higher-order structure at *parS*. In addition, we demonstrate that ParA-*parOP* complex formation prefers a different form of ParA than that of the ParA-ParB-*parS* complex. These observations suggest that ATP- and ADP-binding contribute to the discrimination between the repressor and partition forms of ParA *in vivo*.

Results

ParA interacts with the P1 partition complex

To investigate a possible interaction between ParA and the partition complex, we modified our standard gel mobility shift assay to include ATP and Mg²⁺ in the electrophoresis gel and buffer. In their absence (with typical electrophoresis buffers), stable interactions between ParA and the partition complex at *parS* have never been obtained. We re-examined these conditions in light of several observations. First, ParA is an ATPase that requires magnesium for ATP hydrolysis (Davis *et al.*, 1992). Secondly, all of ParA's activities and its conform-

Fig. 2. ParA interacts with the partition complex. 32 P-labeled DNA fragments (a *Bam*HI digest of the plasmid pBEF166; Materials and methods) were incubated in the presence (+) or absence (-) of IHF (200 nM) and of ParB (500 nM), and with increasing concentrations of ParA (250 nM, lanes d and g; 500 nM, lanes e and h; 1000 nM, lanes f and i). All reaction mixtures contained 2 mM ATP. They were analyzed by electrophoresis in a 5% polyacrylamide gel in TBM buffer containing 0.1 mM ATP. The positions of various protein-DNA complexes (I, I+B and I+B+A), free *parS*-252 fragment, and the 120 bp and 3040 bp vector DNA fragments are indicated on the left. Note that the 3040 bp fragment carries an IHF binding site.

ation are differentially affected by bound ATP, ADP or ATP analogues (Davey and Funnell, 1994, 1997). Finally, although ParA's site-specific DNA binding activity to *parOP* was readily detected by DNase I footprinting studies, it was not detectable in a standard gel-shift assay. We therefore considered the possibility that nucleotide and magnesium must be present continuously during electrophoresis, rather than just in the reaction buffer. Indeed, with the addition of magnesium and ATP to the gel and electrophoresis buffer, we have been able to visualize an interaction between ParA and the partition complex (Figure 2).

A typical experiment is shown in Figure 2. The 32 P-labeled DNA substrates were a mixture of three fragments of 3040, 252 and 120 bp, but *parS* was contained only within the 252 bp fragment (hence *parS*-252). The *parS* fragment was specifically bound by IHF and ParB, as shown by the formation of two distinct complexes: complex 'I' (Figure 2, lane b) and complex 'I+B' (lane c), corresponding to the partition complex. In the presence of ParA, a third complex was observed as a supershift of the partition complex (complex 'I+B+A'; Figure 2, lanes d-f), revealing an interaction between ParA and the partition complex. A stable interaction was observed only at high ParA and ParB concentrations; at lower concentrations more smearing in the complexes was apparent (Figure 2; and see below).

The ParA complex I+B+A was specific for *parS*-DNA and was dependent on the presence of ParB. Control fragments, radioactively labeled but without a *parS* site, were not bound by ParA in this assay. This is most easily seen for the small 120 bp fragment in Figure 2. (Note that the large DNA fragment, which contains an IHF binding site from pBR322 sequences, binds only IHF.) In

Fig. 3. The ParA interaction is competed by excess DNA containing full *parS* sites. Protein binding to 32 P-labeled DNA fragments (1.5 nM each) was measured in the presence (+) or absence (-) of IHF (400 nM), ParB (500 nM) and ParA (500 nM). All assays contained 2 mM ATP. Unlabeled DNA fragments with either full *parS* sequences (*parS*-434; lanes e-h) or partial *parS* sequences (*parS*Δ-390; lanes i-l) were included at the following concentrations: lanes e and i, 1.5 nM; lanes f and j, 3 nM; lanes g and k, 6 nM; and lanes h and l, 12 nM. As in all experiments in this study, the reactions also contained unlabeled salmon sperm DNA as non-specific competitor DNA (100 µg/ml). They were analyzed by gel mobility shift assay as described in Figure 2. Positions of protein complexes at *parS* and free DNAs (as in Figure 2) are indicated on the left.

the absence of ParB, ParA could not bind to the IHF/*parS* complexes (Figure 2, lanes g-i) or to *parS* alone (Davis *et al.*, 1988; data not shown). Therefore the interaction of ParA with *parS*-252 is not due to non-specific DNA binding activity of ParA and these results indicate that ParA interacts specifically with the partition complex assembled on *parS*.

The specificity of the ParA-partition complex interaction was also tested by competition experiments. Increasing concentrations of unlabeled *parS*-434 or *parS*Δ-390 (Figure 1C), carrying a full or partial *parS* site, respectively, were included in the reaction mixture with 32 P-labeled *parS*-252. The *parS*-434 DNA fragment carried an intact *parS* site, and it competed very efficiently with *parS*-252 for formation of the ParA complex at limiting ParA concentration (500 nM). *parS*Δ-390 carries the left end of *parS*, but the IHF binding site is disrupted and the stronger ParB recognition sequences of the right half of *parS* are missing (Funnell and Gagnier, 1993, 1994). This fragment exhibits a very low binding affinity for ParB (Davis and Austin, 1988), and it was unable to compete with *parS*-252 for complex I+B+A formation (Figure 3). Therefore, we conclude that a full *parS* site and thus an intact partition complex formed on this *parS* site (the *parS*-434 fragment) are necessary for this competition to occur. As ParA has no affinity for *parS* without ParB, we infer that this competition occurs through ParB. At higher concentrations of *parS*-434 competitor (4- and 8-fold excess versus *parS*-252), ParB binding to *parS*-252 was

also competed (Figure 3, lanes g and h). This observation is expected as the concentration of ParB becomes limiting with increasing concentrations of *parS* substrate.

Pairing of plasmids following DNA replication has been proposed to occur during plasmid partition, and in the R1 plasmid system, pairs have been detected by electron microscopy (Jensen *et al.*, 1998). One possible explanation for the P1 I+B+A complex is that this gel band represents pairs of *parS*-252 partition complexes. In this case, we would expect that the competition experiment in Figure 3 would yield a novel band that migrated more slowly than the *parS*-252 I+B+A band and represented *parS*-252/*parS*-434 pairs. (*parS*-434 I+B+A complexes migrate more slowly than *parS*-252 I+B+A complexes when both substrates are labeled; data not shown). However, bands migrating more slowly than *parS*-252 I+B+A complexes were not detected when *parS* fragments of larger size were included in the binding reaction (Figure 3). Either such complexes are not formed or they do not survive during the electrophoresis. Therefore, we conclude that an I+B+A complex represents ParA assembled onto a partition complex at a single *parS* DNA molecule.

The ParA effect depends on the concentration of ParB

We have analyzed the ParA-partition complex interaction over a broad range of ParB concentrations. Interestingly, the nature of ParA's effect on the complex depended on whether ParB was present at high (500–1000 nM) or low (30–125 nM) levels (Figure 4A and B). Using high ParB concentrations that completely shifted the IHF complex to a ParB-IHF partition complex, ParA produced the more slowly migrating or 'supershifted' I+B+A complex (Figure 4A, lanes i and j). However, at lower ParB concentrations, ParA specifically destabilized the partition complex, and in particular removed ParB from *parS* (Figure 4A, compare lanes c and g). In effect, ParA appears to titrate ParB from, or prevent ParB binding to *parS* under these conditions. At intermediate concentration, a combination of both effects was seen (Figure 4A, lane h). These interactions are further illustrated in Figure 4B. At low ParB concentration, and thus at high ParA:ParB ratios, the destabilization effect predominated, whereas at higher ParB concentrations the partition complex became less sensitive to the destabilizing effects of ParA. The stable supershifted I+B+A complex required high ParB concentrations. We do not yet know whether both of these effects reflect activities of ParA *in vivo* (see Discussion), and for the purposes of this study we have focussed on the supershifted complex and the evidence that ParA assembles onto the partition complex via an interaction with ParB.

Chemical footprinting of ParA on the partition complex

The interactions between ParA and *parS* were observed only when ParB was assembled on *parS*. From this we conclude that ParA assembles via a ParA-ParB protein-protein interaction. However, this does not rule out the possibility that ParA interacts specifically with *parS* DNA following its recruitment by ParB. Therefore, we examined the protein interactions with *parS* by footprinting experiments. We first performed DNase I footprinting experi-

Fig. 4. ParA interactions with the partition complex at high and low levels of ParB. 32 P-labeled DNA fragments were incubated in the presence (+) or absence (-) of the indicated proteins and analyzed as described in the legend to Figure 2. (A) ParA-partition-complex interactions at different concentrations of ParB. IHF and ParA concentrations were 400 and 1000 nM, respectively. ParB was included at the following concentrations: lanes c and g, 50 nM; lanes d and h, 200 nM; lanes e and i, 500 nM; lanes f and j, 1000 nM. Reaction mixtures contained 2 mM ATP, and the electrophoresis gel and buffer contained 0.1 mM ATP. Positions of protein complexes at *parS* and free DNAs (as in Figure 2) are indicated on the left. (B) Analysis of ParA-partition complex interactions at lower concentrations of ParB. ParB concentration, in nM, is indicated above each lane. IHF concentration was 400 nM. ParA concentrations were: lanes c, g, k and o, none; lanes d, h, l and p, 62.5 nM; lanes e, i, m and q, 250 nM; lanes f, j, n and r, 1000 nM. Binding reactions were analyzed as in (A). Positions of protein complexes at *parS* and free DNAs (as in Figure 2) are indicated on the left.

ments. The patterns of IHF and ParB binding, measured as protection of the DNA from DNase I cleavage, were the same as those described previously (Davis *et al.*, 1990; Funnell, 1991). ParA did not extend the observed footprint, and we saw no significant difference between *parS* footprints with and without ParA (data not shown). This result suggested that ParA does not bind directly to *parS*. We then used 1,10-phenanthroline-copper (OP-Cu) to footprint the complexes directly in the electrophoresis gel. OP-Cu is a chemical nuclease which interacts with DNA through the minor groove, where it initiates an oxidative attack on the C1-hydrogen of deoxyribose leading to cleavage of the DNA backbone (Spassky *et al.*, 1988; Sigman *et al.*, 1991). Resistance to cleavage is caused by blocking access

Fig. 5. *In situ* footprinting of protein–DNA complexes with 1,10-phenanthroline-copper. The substrates for these experiments were *parS*-211 DNA fragments (Figure 1C) that were ^{32}P -labeled at the 3' end of either the upper or lower strand. The upper strand is defined as the strand containing the sequence shown in Figure 1B, and the lower strand is its complement. (A) Protein–DNA complexes were formed, resolved by electrophoresis and treated with OP-Cu as described in Materials and methods. DNA from each complex (same nomenclature as in Figure 2) was recovered and analyzed on 6% sequencing gels. On each gel, Maxam-Gilbert G>A sequencing reactions were included as markers. The ParB BoxA and BoxB motifs, and the IHF binding site (as in Figure 1B) are drawn beside each gel. Squares and circles mark bands that show differences between I+B+A complexes and I+B complexes [see (B)]. (B) Quantification of OP-Cu cleavage patterns. The cleavage pattern of each complex from (A) were quantified by PhosphorImager analysis. The data are represented as differential scans: a scan of each protein complex (thick line) is superimposed on that of the nearest lower complexity complex (thin line). The ParB BoxA and BoxB motifs, and the IHF binding site (as in Figure 1B) are drawn below the scan of the G>A lane of each gel. Bands which showed differences in protection due to the addition of ParA (I+B+A complexes versus I+B complexes) are marked with squares (stronger protection) or circles (weaker but reproducible changes).

to the DNA either as a result of protein steric hindrance or altered geometry that narrows the minor groove. Enhanced cleavage by OP-Cu results from altered DNA geometry that leads to a widened minor groove. Since OP-Cu is a much smaller probe than DNase I, we asked whether we could detect subtle effects of ParA on the footprint at *parS*.

Protein–DNA complexes were formed and resolved by electrophoresis in the presence of ATP as before (e.g. Figure 2), and then treated with OP-Cu directly in the gel (see Materials and methods). After recovery of the treated DNA from the gel, the footprint patterns were resolved in a denaturing polyacrylamide gel (Figure 5A). The radioactive bands in these footprints therefore represent residues that were protected, enhanced or not affected by protein binding, and were quantified by PhosphorImager analysis (Figure 5B). While the patterns of protection from OP-Cu cleavage were not as dramatic as typical DNase I protection patterns, quantitative analyses showed specific protection by IHF and ParB at *parS*. Both strands

of *parS* DNA exhibited protection by IHF (in complex I) between boxes A1 and A2, in agreement with previous DNase I footprints (Funnell, 1991) and with IHF contacting the DNA through the minor groove (Rice *et al.*, 1996). ParB protected sequences on both strands on boxes B1, A1, A2 and A3 (complex I+B).

Addition of ParA to the partition complex (complex I+B+A) resulted in no major new regions of protection, consistent with the DNase I footprinting results. However, several small but reproducible changes in the region protected by ParB and IHF were observed. The main changes observed on both strands were protection at one edge of the IHF binding site, next to the A1 box. One enhanced cleavage was always present at the left edge of the protected region on the lower strand, which is outside the minimal P1 *parS* sequence. Nevertheless, these changes are within or very close to the residues already protected by ParB and IHF, and it is unlikely that ParA also contacts them directly. These footprinting results suggest that when

Fig. 7. ADP is preferred by the site-specific DNA binding activity of ParA. 32 P-labeled *parOP* DNA fragments (1 nM) were incubated in the presence (+) or absence (-) of ATP or ADP, and with increasing amounts of ParA. The (+) and (-) above each lane indicate components added to the reaction mixtures. Nucleotide, when included, was present at 2 mM. ParA concentrations were: lanes a, e and i, none; lanes b and f, 250 nM; lanes c, g, j, k and l, 500 nM; lanes d and h, 1000 nM. Each panel shows the results of a single gel, although not all lanes were contiguous in each panel. The gels were run in TBM buffer containing either 0.1 mM ADP (left panel), 0.1 mM ATP (central panel) or no nucleotide (right panel). The positions of free and ParA-bound *parOP* ('A' complex) are indicated on the left.

ent on ParB and ATP. In contrast, ParA's site-specific DNA binding activity to the *par* promoter, *parOP*, prefers the ADP-bound form of ParA over the ATP-bound form.

ParB is a central element for the ParA-partition-complex interaction. The observations that support a direct ParA-ParB interaction at *parS* are (i) ParB was absolutely essential for this interaction (Figures 2 and 3), (ii) no new protein-DNA contacts were detected at *parS* by chemical footprinting experiments when ParA interacted with the partition complex (Figure 5), and (iii) the nature of the ParA effect (assembly or dissociation) was dependent on ParB concentration (Figure 4). ParA and ParB have previously been shown to interact directly *in vitro* as ParB stimulates ParA's ATPase activity (Davis *et al.*, 1992) and ParA's DNA binding activity to *parOP* (Davey and Funnell, 1997). This study provides the first biochemical evidence that ParA interacts with the partition complex at *parS*, mediated via a ParA-ParB interaction.

The role of ParA in the partition reaction

The essential function of ParA in the partition process is certainly related to its interaction with the partition complex. *In vitro*, the outcome of ParA's interaction with the partition complex depended on the concentration of ParB. At high ParB concentrations, the supershifted I+B+A complex was formed. At high ParA:ParB ratios and lower ParB concentrations however, ParA removed or titrated ParB from the partition complex. Both of these effects illustrate a ParA-ParB interaction and that ParA can interact with ParB assembled in the partition complex. The amount of ParB in the cell is relatively high (~7000 dimers/cell; Funnell and Gagnier, 1994), and preliminary estimates suggest it is ~10-fold higher than the amount of ParA (M.J.Davey and B.E.Funnell, unpublished results). Thus, it is likely that the stable I+B+A complex observed here forms for at least part of the cell cycle. Two steps of the partition process where ParA has been proposed

to act are during a plasmid-pairing step and/or during attachment to a fixed cellular structure. Our experiments did not detect a pairing step mediated by ParA and ParB; however, it is entirely possible that such pairs do not survive electrophoresis. ParA might also act as a scaffold that joins the plasmid to, and/or dissociates the plasmid from fixed host factors at the cell membrane (a localization step). This possibility is supported by data on subcellular fractionation of ParA, SopA and QsopA, the A partition proteins of P1, F and QpH1 plasmids, respectively. These studies have shown that these proteins are partly membrane associated (Lin and Mallavia, 1998; our unpublished observations). Moreover, MinD, an *E.coli* cell division protein which is homologous to the ParA proteins (Motallebi-Veshareh *et al.*, 1990), is associated with the inner membrane (deBoer *et al.*, 1991) and is essential for membrane localization of the MinE cell division protein (Raskin and deBoer, 1997). However, host factors required for P1 and ParA localization have yet to be identified.

It is unclear whether the destabilizing action of ParA represents an activity of ParA that occurs *in vivo*, or simply is an *in vitro* consequence of adding too much ParA with respect to ParB. In the latter case for example, too much ParA could aggregate and sequester ParB so it can no longer bind to DNA. However, this destabilization activity is intriguing in the light of proposals that ParA could be responsible for separating plasmid pairs or dissociating the plasmid from its membrane anchor in response to some cellular signal. In such a scenario, formation of the I+B+A complex should be favored when ParA is present at its normal low ratio with respect to ParB *in vivo*. At the proper cell cycle signal, perhaps accompanied by ATP hydrolysis, ParA would dissociate plasmids from their anchor. In addition, ParA-mediated dissociation potentially provides an attractive explanation for the 'incompatibility' caused by raising the ratio of ParA to ParB *in vivo*. Excess ParA destabilizes P1 plasmids, implying that a correct ParA:ParB ratio is important for partition (Abeles *et al.*, 1985). While excess ParA repressor could lower protein levels below those needed for optimal partition, another possibility arising from our observations is that excess ParA would promote destabilization of partition complexes by bypassing the cellular signal needed for proper dissociation.

Many of the active partition systems described so far for plasmids and chromosomes are composed of two proteins and, where it has been investigated, of a *cis*-acting site (reviewed in Hiraga, 1992; Williams and Thomas, 1992). The partition proteins of the B family interact with *cis*-acting sites to highly and specifically organize their respective partition complexes. The ParA-like proteins share a specific version of the ATP-Walker A motifs and are probably all ATPases (Motallebi-Veshareh *et al.*, 1990; Koonin, 1993). By analogy with P1, we speculate that the conformational changes upon ATP binding and hydrolysis should regulate the activities of these proteins during their partition reactions.

An ATP-ADP switch distinguishes ParA's repressor and partition activities

Adenine nucleotide binding modulates ParA's activities and conformation as has been shown previously (Davey and Funnell, 1994, 1997), and these observations are

Fig. 8. Model describing the control of ParA's activities by adenine nucleotide binding and hydrolysis. Both ATP and ADP promote formation of ParA dimers. In the right pathway, ATP binding by ParA promotes a conformation that preferentially allows its interaction with the partition complex formed by ParB and IHF bound to *parS*. Once recruited by ParB, ParA participates in later as yet undefined steps in the partition mechanism, although we think it likely that at least one of these steps requires ATP hydrolysis. In the left pathway, ATP hydrolysis by ParA, which is stimulated by DNA and by ParB, converts ParA to its repressor form, ADP-ParA. These two pathways may not be completely independent; for example, ADP-ParA could be produced following partition in the right pathway. More details of this model are presented in the Discussion.

extended by our present study. This study reveals the preference for involvement of two different forms of ParA in its two separate pathways (repression versus partition). A ParA-*parOP* complex was detected in presence of ADP, whereas no stable interaction was detected in the presence of ATP (Figure 7). ParA-*parOP* interactions measured by DNase I footprinting showed that ADP was a better cofactor than ATP for DNA binding, although both stimulated binding over that seen with no nucleotide (Davey and Funnell, 1994). We interpret these observations as follows: ADP or ATP mediated dimerization of ParA is important for its DNA binding activity, but ADP-ParA is the better repressor form of ParA. In contrast, the ParA-partition-complex interactions required ATP absolutely. Therefore, the repressor activity of ParA prefers the ADP bound form, and the partition activity prefers the ATP bound form.

We propose the following model, illustrated in Figure 8, to describe how adenine nucleotide binding and hydrolysis orient ParA in one or the other pathway. ParA's affinity for ATP is slightly higher than that for ADP (K_d s of 33 and 50 μ M, respectively; Davey and Funnell, 1997). In normal exponentially growing *E. coli* cells, when ATP levels are ~8-fold higher than ADP levels (Franzen and Binkley, 1961), most ParA is in its ATP-bound form. This conformation preferentially allows its recruitment to the

partition complex, mediated by ParA's interaction with ParB. It is in this conformation that ParA performs its partition roles. We assume that ATP hydrolysis plays a role here, as discussed above, but the steps at which it acts have yet to be defined.

The ADP form of ParA will be produced primarily by ATP hydrolysis, although some fraction of ParA may bind ADP directly. ParA is a relatively weak ATPase, and ATP hydrolysis is stimulated by DNA (of no specific sequence) and by ParB (Davis *et al.*, 1992). Therefore, either (or both of) ParA's interactions with DNA or ParB will stimulate formation of the repressor form. Importantly, this stimulation by ParB explains or at least contributes to the observation that ParB increases repression by ParA. Since ADP-ParA is preferentially used as the repressor, it cannot interact (and perhaps interfere) with the partition complex at *parS*. In this way, the repressor activity is kept separate from the partition activity.

Finally, while partition and repression can be separated and can act independently (Friedman and Austin, 1988; Davis *et al.*, 1996), they may influence each other under normal circumstances. Our model suggests that ATP hydrolysis during the act of partition could make more repressor available and thus reduce Par protein production after the positioning reaction has occurred. In this respect, we have observed that *in vivo*, plasmids containing *parS* lower the level of Par proteins whose genes are transcribed from *parOP* (unpublished results). This decrease in expression occurs when *parS* is present either on the same or on a different plasmid than the plasmid containing *parOP* and the *par* operon.

There are a variety of examples of molecular switches that are controlled by nucleotide binding and hydrolysis. These include GTP-regulated events in signal transduction (reviewed in Sprang, 1997) as well as ATP-regulated switches in DNA mismatch repair (Gradia *et al.*, 1997), replication (Sekimizu *et al.*, 1987; Crooke *et al.*, 1992; Klemm *et al.*, 1997) and transposition (Yamauchi and Baker, 1998). These switches are believed to act as gates controlling progression through their respective pathways. For example, the human mismatch repair protein hMSH2-hMSH6 binds to DNA mismatches in its ADP-bound form but not in its ATP-bound form (Gradia *et al.*, 1997). The release of ADP from hMSH2-hMSH6 is thought to be stimulated by other components of the repair machinery and thus signal dissociation of this protein from the mismatch so that the excision repair pathway can proceed. Here we have described a switch that allows discrimination between two alternative pathways rather than progression through one pathway only. This may be in addition to the involvement of an ATP-regulated switch in progression through the partition pathway, and the model in Figure 8 does invoke a role for ATP hydrolysis that is later than the association of ParA with ParB. P1 may have taken advantage of the 'off' state for partition (the ADP form) and recruited it to perform another role, repression. The challenge now is to identify the partition steps at which ParA participates, and the host components with which ParA and ParB interact.

Materials and methods

Reagents and enzymes

Sources for reagents were as follows: ADP, ATP, bovine serum albumin (BSA; Fr V), OP-Cu, mercaptopropionic acid (MPA), 2,9-dimethyl-

1,10-phenanthroline (neocuproine) and CuSO₄ were from Sigma; ATPγS [adenosine-5'-O-(3-thiotriphosphate)] and bovine pancreatic DNase I, Boehringer Mannheim; [α-³²P]dATP and [α-³²P]dCTP, Dupont NEN; and dithiothreitol (DTT), Bio-Rad. Restriction enzymes and DNA polymerases were purchased from New England Biolabs.

DNA and plasmids

The plasmids pBEF165 and pBEF166 contain the P1 *parS* sequence between the P1 *TaqI* and *SlyI* restriction sites, cloned in opposite orientations into a modified pBlueScript vector (Funnell, 1991). Plasmid DNA was prepared by the alkaline lysis method followed by two CsCl-ethidium bromide gradients (Sambrook *et al.*, 1989). *parS*-252 and *parS*-211 DNA fragments were used as substrates for DNA binding and complex formation assays (the numbers after *parS* correspond to the length of the DNA in bp). *parS*-252 was generated by digestion of pBEF166 with *Bam*HI. *parS*-211 was made from either pBEF165 or pBEF166 by digestion with *Bam*HI and *Sma*I. *parS*-434 and *parS*Δ-390 DNA fragments were generated by PCR using pLG44 (Funnell and Gagnier, 1995) and pBEF118 (Funnell and Gagnier, 1994), respectively, as templates, and purified using a Quiaex II kit (Qiagen) following electrophoresis in 1.2% agarose gels. The DNA substrate containing the P1 operator/promoter sequence, *parOP*, was a 171 bp *Bam*HI-*Eco*RI fragment prepared from the plasmid pMD9 (Davey and Funnell, 1994). *parS* and *parOP* DNA fragments were labeled at their 3' ends with [α-³²P]dATP or [α-³²P]dCTP and DNA polymerase I large fragment, and purified by phenol-chloroform extraction and ethanol precipitation steps (Sambrook *et al.*, 1989).

Proteins

ParA (Fr IV) was prepared as described previously (Davey and Funnell, 1994), except that fraction IV was adjusted to 50% glycerol (v/v) with 10 mM DTT and stored at -80°C until use. ParB (Fr V) was prepared as described (Davey and Funnell, 1997), except that protein from the MonoS column (Pharmacia) was eluted with a 100 mM to 1 M KCl gradient. IHF was prepared as described previously (Nash *et al.*, 1987) except that the polymin P precipitation step was omitted. ParA, ParB and IHF molar concentrations are expressed as the concentration of dimers.

Gel mobility shift assays

The standard binding reaction mixture (10 μl) contained 1 nM ³²P-labeled *parS* DNA in a buffer containing 50 mM HEPES-KOH pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 10% glycerol, 100 μg BSA/ml, 100 μg sonicated salmon sperm DNA/ml and 1 mM DTT. Where noted, reaction mixtures contained 2 mM nucleotide (ATP, ATPγS or ADP). They were assembled on ice and the proteins were added in the following order: IHF, ParB and ParA. The mixtures were incubated for 15 min at 30°C and analyzed by electrophoresis in 5% polyacrylamide gels in TBM buffer (90 mM Tris-borate, 10 mM MgCl₂). Where indicated, 0.1 mM of ATP or ADP was included in the polyacrylamide gel and the top electrophoresis buffer (the buffer at the cathode side of the gel). Electrophoresis was performed at 150 V for 4–6 h at 4°C; under these conditions, ~25% of the nucleotide was depleted from the top buffer. The gels were dried onto Whatman DE81 paper and exposed to a phosphor screen for imaging by a PhosphorImager (Molecular Dynamics).

In situ OP-Cu footprinting

In situ footprinting assays were performed as described by Sigman *et al.* (1991). Binding reaction assays were scaled up 3-fold using *parS*-211 DNA fragments as the substrates. Since the *parS* inserts in pBEF165 and pBEF166 are in opposite orientations, ³²P-labeling at the 3' ends of the *Bam*HI sites of *parS*-211 DNA fragments made from these two plasmids labeled the upper and lower DNA strands, respectively. Protein-DNA complexes were separated from each other and from free DNA by electrophoresis in TBM buffer with ATP as described above. The wet gels were immersed in 200 ml of 10 mM Tris-HCl pH 8, followed by the addition of 20 ml of solution A (0.45 mM CuSO₄, 2 mM OP-Cu). *In situ* digestion of the protein-DNA complexes was initiated by the addition of 20 ml of solution B (1:200 dilution of MPA in H₂O). The reactions were stopped after 15 min at room temperature by the addition of 20 ml of solution C (5.8 mg neocuproine/ml in ethanol). Free DNA and protein-DNA complexes were localized by autoradiography of the wet gel and excised from the gel. The DNA was recovered by elution overnight at room temperature in 0.5 M ammonium acetate, 1 mM EDTA followed by phenol/chloroform extraction and ethanol precipitation. The purified DNA samples were resuspended in 5 μl of 98% formamide, 10 mM Na₂EDTA pH 8, 0.025% xylene cyanol, 0.025% Bromophenol

Blue, heated at 95°C for 5 min and separated by electrophoresis in 6% denaturing polyacrylamide gels. Sequencing standards for footprinting reactions were prepared by dimethyl sulfate (Aldrich) modification and NaOH cleavage to produce a G>A ladder (Sambrook *et al.*, 1989). The gels were dried on Whatman DE81 paper and exposed to a phosphor screen for quantification on a PhosphorImager (Molecular Dynamics). The cleavage pattern for each complex was scanned and normalized, in the area outside of *parS*, to the cleavage pattern of unbound *parS* DNA that was isolated from the same gel.

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