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Characteristics and Significance of DNA Binding Activity of Plasmid Stabilization Protein ParD from the Broad Host-range Plasmid RK2*

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A region of the plasmid RK2 has been shown to stabilize plasmid replicons in a broad host-range manner. This region encodes two divergently transcribed operons: parCBA and parDE. The parCBA operon specifies a multimer resolution system, while the parDE operon alone is capable of stabilizing an RK2-derived minireplicon under defined growth conditions in several different Gram-negative bacteria. The observed autoregulation of the parDE operon is most likely the result of ParD protein binding within the PparDE region. The characteristics of ParD binding to this region and the role of such binding in plasmid stabilization were examined with purified ParD protein. The results indicate that the binding of a single dimer of ParD protein to the promoter region most likely blocks interaction of RNA polymerase holoenzyme with the promoter. DNase I protection experiments indicate that ParD binds to a discrete sequence of 48 base pairs in length. While the binding of ParD to PparDE is essential for proper regulation of expression of the ParD and ParE proteins in vivo, the analyses of binding properties of mutant ParD proteins suggest that binding to this region does not play a direct role in plasmid stabilization.

Naturally occurring plasmids are extrachromosomal elements that replicate independently from the bacterial host chromosome. They are maintained in a controlled manner at characteristic copy numbers, ranging from very low (one per host genome) to quite high (hundreds per genome) (reviewed in Refs. 1 and 2). If these copies were segregated to the daughter cells upon division in a random manner, lower copy number plasmids would be predicted to be lost at a rate increasing with decreasing copy number, approaching 25% loss per generation for single copy elements. However, naturally occurring plasmids are generally very stable, with loss rates as low as once in every 10⁹ cell divisions. This high degree of stability has been shown to be due to plasmid-encoded stabilization elements (reviewed in Refs. 3 and 4).

The elements responsible for plasmid stabilization can be classified in several groups, based on their mechanism of action. Systems that resolve plasmid multimers to monomers (and thus increase the number of units able to be segregated) have been described for several plasmids. Other systems encode a stable toxic component whose function is repressed by the presence of a less stable inhibitor. Upon plasmid loss from the cell, the inhibitor is inactivated to allow toxin-mediated killing of the cell, thus ensuring stable plasmid maintenance within the population. Two distinct classes of such genetic systems have been found: one represented by the F ccd locus (5) and the other by the R1 plasmid hok/sok locus (reviewed in Ref. 6). Alternatively, several plasmids appear to encode elements, known as partitioning elements, that assure stability by physically directing segregation of the plasmids into each daughter cell. Such elements have been described for several plasmids, including F, the plasmid form of the phages P1 and P7, the R1 family, and the Agrobacterium plasmid pTAR (reviewed in Refs. 3 and 4). A locus with similar function but dissimilar genetic structure has been characterized for pSC101 (7).

A better understanding of how these plasmid stabilization systems function has been attained through genetic and biochemical analyses. One of the common features noted is the autoregulation of many of the operons encoding the stabilization proteins. Since proteins involved in plasmid stabilization may also possibly interact with the host chromosomal segregation machinery, such autoregulation may be important to prevent disruption of the chromosomal or plasmid segregation systems (as appears to be the case for the P1 prophage (8)). In some systems, it is likely that the DNA binding responsible for autoregulation also plays a direct role in the stabilization of the plasmid. This has been suggested for plasmids R46 (9) and pIP404 (10), whose multimer resolution systems encode resolvases that have been shown to bind and carry out DNA recombination at the site of promoter autoregulation. The cis-acting partitioning sites for the R1 group of plasmids (11, 12) and pTAR (13) overlap the autoregulatory region in the promoter, suggesting duality of function. Therefore, the examination of the autoregulatory properties of plasmid stabilization elements may yield important information on plasmid stabilization in general.

Plasmid RK2 is a broad host-range, 60 kb1 plasmid of the Inc P1 family (reviewed in Ref. 14). A 3.2-kb region of this plasmid capable of stabilizing heterologous replicons in a broad host-range manner has been described (15-17). This region encodes two operons transcribed in a divergent manner: parCBA and parDE. A multimer resolution system plus at least one additional stabilizing function has been identified to date within this region (16-18). Recently, it was observed that, under several conditions, the parDE operon encoded within a 780-bp sequence was adequate to promote plasmid

1 The abbreviations used are: kb, kilobase pair; bp, base pair.
plasmid for overexpression of two of the mutated parD genes. Before achieved from plasmids in which a T7 promoter was used to express cation of the ParD protein and analysis of its characteristics of ParD binding and the role of this binding in plasmid stabilization functions were examined by purification of the ParD protein and analysis of its DNA binding properties at the PparDE region.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmid Construction—The Escherichia coli strain used for the overexpression of the wild-type and mutant ParD proteins was the protease-deficient strain BL21 (22). This strain was grown in LB medium (23) (Life Technologies, Inc.) with antibiotic selection at 250 μg/ml for penicillin and 40 μg/ml for kanamycin. The plasmids used in this study are listed in Table I; manipulations of these plasmids were performed using described cloning techniques (23).

Overexpression of the ParD wild-type or mutant proteins was achieved from plasmids in which a T7 promoter was used to express the gene. The original insertion of the wild-type parD gene downstream of the T7 promoter present on pBluescript SK(+) (pRR136) (19) yielded a mutant with greater protein expression capacity (described under "Results"). This plasmid, pRR136-6, was the parental plasmid for overexpression of two of the mutated parD genes. Before these genes were introduced, the ApIII site destroyed in the generation of pRR136-6 was restored by site-directed mutagenesis to generate pCE-2.1 The sources of the mutated parD genes parDδ and parDδ were plasmids derived from pRR120 (19), in which the 0.5-kb stabilization region had been mutagenized by insertion of nine BglII linkers (5' - CAGATCTG-3') at the centrally located parD BglII site (pRR120-Dδ net insertion of 69 bp) or by insertion of one BglII linker at the parD AvaII site (near the 5' end of the gene) followed by Klenow-mediated filling in of this AvaII site (pRR120-Dδ net insertion of 15 bp). These mutations were then introduced into the parD gene of pCE-2 by replacing the ApIII to EcoRV region of pCE-2 with the AplI to anti-tet sequences fused to the 3' end of parD. ParD-containing fractions from this chromatography were identified both by electrophoresis on a 12% polyacrylamide gel prepared as described by Schagger and von Jagow (25) and by analysis for specific ParD-binding activity using gel retardation assays as described below. These fractions were then pooled and dialyzed against buffer A plus 25 mM KCl overnight. The ParD wild-type or mutant protein was purified by first binding the (NH4)2SO4 precipitated proteins to a heparin-Sepharose CL-6B column (bed volume of 6 ml; Pharmacia) in buffer A plus 25 mM KCl. The bound protein was eluted with a linear gradient of 25 to 400 mM KCl in buffer A; all ParD proteins with the exception of ParD10 eluted at approximately 100 mM KCl (the ParD10 protein failed to bind to this column material). ParD-containing fractions from this chromatography were identified both by electrophoresis on a 12% polyacrylamide gel prepared as described by Schagger and von Jagow (25) and by analysis for specific ParD-binding activity using gel retardation assays as described below. These fractions were then pooled and dialyzed against buffer A plus 25 mM KCl overnight. The ParD protein was then bound to a DEAE-Sephacl column (5-m bed volume) in this buffer and eluted with a linear gradient of 25–200 mM KCl in buffer A. Wild-type ParD and ParDδ were over 95% pure after this step, while the ParD10 protein was 5% pure (as judged by Coomassie staining in polyacrylamide gel electrophoresis). These protein preparations were used in most analyses without further manipulation.

For ParD protein analysis by fluorescence spectroscopy, ParD protein was further enriched by chromatography using carboxymethyl-Sepharose resin (Pharmacia). ParD protein did not bind to this resin and was recovered in the column flow through. Peak ParD-containing fractions were pooled and dialyzed against 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 25 mM KCl in preparation for spectroscopy.

The DNA binding activity of ParD and ParD proteins was determined using crude cell extracts. Cultures (200 ml) were induced as described above, harvested, resuspended in buffer A plus 25 mM KCl to a volume of 1.0 ml, and lysed by sonication. The crude cell extracts were prepared by removal of debris by centrifugation at 12,000 × g for 30 min. These extracts were then tested without further purification.

Gel Mobility Shift Analysis—The capacity of the ParD proteins to bind specifically to the PparDE sequence was tested by gel retardation, using a modification of the procedures described by Hope and Struhl (26) and Perri et al. (27). The DNA fragment used to test for binding contained RK2 sequences from the Sau3AI site in parC to the Sau3AI site in parD (see Fig. 1A), encompassing the parCBA and ParD operon divergent promoters (20). This 548-bp fragment was recovered from the plasmid pTD3 with EcoRI and HindIII digestion, then end-labeled by incubation with Klenow enzyme for 10 minutes in the presence of α-[^32]PdATP (6000 Ci/ml; DuPont NEN) as described elsewhere (23). In many cases, this DNA fragment was subsequently digested with 22U in a 1 M salt buffer (50 mM Tris-HCl, pH 8.0, and 10 mM MgCl2) to generate two fragments, one (316 bp) carrying ParCBA and the other (232 bp) carrying ParD. The digested DNA was used in gel retardation assays without further purification. Protein fractions with potential binding activity (diluted 10-fold) were incubated with the DNA for 30 min at 30°C, then run on a 5% polyacrylamide gel prepared as described by Schagger and von Jagow (25) and stained with 0.5% Coomassie blue.
and incubated at 9 °C for 10 min to allow adsorption of nonspecific DNA binding activity. In cases where crude protein preparations were to be tested, MgCl₂ was eliminated from the binding buffer. This reduced the ParD binding affinity in gel retardation only slightly, but significantly lowered nuclease activity in the crude protein preparations. ³²P-labeled DNA in 5 μl of binding buffer was then added and the mixture was incubated at room temperature for 20 min. The temperature of incubation was not carefully controlled because no difference in binding characteristics were seen with incubation at 0 °C, 15 °C, room temperature, or 37 °C (results not shown). Finally, 2 μl of a 25% stock of Ficoll 400 (Pharmacia) was added, and 16 μl of each reaction were loaded for electrophoresis on a 5% polyacrylamide gel prepared with Tris-borate-EDTA buffer as described elsewhere (23). The gel was pre-electrophoresed for 1 h to remove compounds potentially inhibitory to binding, and the gel was changed immediately prior to loading and electrophoresing the samples. The samples were electrophoresed for 5 h at 10 V/cm, until xylene cyanol dye loaded in a separate lane had migrated 10 cm. The gel was then dried and autoradiographed to visualize the migration of the labeled DNA fragments. Analysis of the effect of MgCl₂ during electrophoresis was performed by its addition to a concentration of 12 mM to both the gel and the running buffer.

Cross-linking of ParD Protein.—Wild-type or mutant ParD proteins were cross-linked in buffer containing 50 mM HEPES, pH 7.5, 10 mM MgCl₂, and 100 mM KCl (cross-linking buffer). Proteins to be tested were first dialyzed from buffer A into buffer B (HEPES, pH 7.5, 10 mM MgCl₂, and 100 mM KCl) prior to cross-linking. The cross-linking buffer was composed of buffer B and the addition of glycogen (5 mg/ml) followed by rapid electrophoresation and dialysis against a cross-linking buffer (23). The concentration of ParD protein was determined by its absorbance at 280 nm, using a calculated molar extinction coefficient of 13,520 M⁻¹ cm⁻¹ (32). The concentration of the DNA was determined by absorbance at 260 nm, using a sequence-based, calculated molar extinction coefficient of 1,072,600 M⁻¹ cm⁻¹.

Fluorescence emission was measured at room temperature using a fluorometer. Excitation and emission filters were used to excite the intrinsic tryptophan (excitation 295 nm; emission 348 nm). The DNA was excited at 295 nm to decrease inner filter effects. The protein concentration was quenched by its addition to a concentration of 12 mM to both the gel and the running buffer. The gel was pre-electrophoresed for 1 h to remove compounds potentially inhibitory to binding, and the gel was changed immediately prior to loading and electrophoresing the samples. The samples were electrophoresed for 5 h at 10 V/cm, until xylene cyanol dye loaded in a separate lane had migrated 10 cm. The gel was then dried and autoradiographed to visualize the migration of the labeled DNA fragments. Analysis of the effect of MgCl₂ during electrophoresis was performed by its addition to a concentration of 12 mM to both the gel and the running buffer.

RESULTS

Purification of the ParD Protein.—To obtain ParD protein sufficiently pure to analyze some of its biochemical properties, an overexpressing plasmid was constructed. The parDE operon on plasmid pRR120 (a derivative of pBluescript SK(+)) (19) was first modified by removing the parE sequences downstream of the first parE DdeI site (located 9 codons from the translational start of the 103-codon parE gene), to eliminate expression of ParE. The native promoter for this operon was then deleted, bringing the T7 promoter of the vector adjacent to the parD gene to generate pRR136 (19). In the process of this manipulation, a construct was identified that contained an extra 47 bp of AT-rich sequence (76% AT) of unknown origin between the T7 promoter and the start of the parD sequence (see Fig. 1B for sequence). When this construct was tested for T7-promoted expression of the parD gene in the strain BL21, the level of inducible expression was estimated...
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Specific DNA Binding by the ParD Protein as Detected by Gel Retardation—It has been shown that the ParD protein is responsible for the regulation of the parDE operon by auto-repression of its own promoter, PparDE (19–21), and this repression appears to be due to binding of the protein at the promoter sequence (20, 21). Using purified ParD protein, a more detailed analysis of its DNA binding properties was carried out by gel retardation assays. It was determined, using other labeled DNA probes and either crude cell extract or purified ParD, that under the binding conditions described, no additional binding sites exist within the DNA sequence from the parC Sau3A1 site (Fig. 1A) to the end of the 0.8-kb stabilization region, 94 bp downstream from the end of the parE stop codon (results not shown). The binding of the ParD protein to the PparDE region generated a surprisingly large gel shift at protein concentrations above 0.55 μM, as seen in Fig. 2A. This shift was dependent on the presence of protein, since it was sensitive to digestion with proteinase K and extraction with phenol. The DNA binding activity of ParD was heat stable (heating the protein to 100 °C for 10 min did not reduce activity as determined by gel shift) and also resistant to incubation with heparin at 300 μg/ml for 10 min prior to electrophoresis (not shown).

The specificity of the interaction of the ParD protein with the PparDE region was evident from several observations. In all gel retardation assays, 0.5–1.0 μg of AluI-digested pUC18 DNA was included as a nonspecific competitor; its presence did not diminish the binding to the PparDE region. In addition, many of the gel retardation assays utilized a 548-bp endlabeled DNA probe that was digested with the restriction endonuclease Dral to generate two single-ended labeled fragments, a 232-bp fragment carrying PparDE and a 316-bp fragment carrying PparCBA (20). In these cases, gel retardation by ParD protein was restricted to the PparDE-containing fragment (Fig. 2) unless very high quantities of protein were added; in excess of 100 times that needed to bind to all of the PparDE fragments present. Finally, the addition of unlabeled DNA eliminated binding to the labeled fragment only when the unlabeled DNA contained the PparDE sequence, with no difference seen for linear or supercoiled cold competitor (not shown).

Upon closer examination of the ParD-dependent gel retardation of PparDE DNA, several unusual characteristics were noted. Varying the quantity of labeled probe 100-fold (from 0.05 to 5 ng) did not change the concentration of protein required for maximal DNA binding (not shown), suggesting that the binding was dependent on the concentration of protein rather than on the molar ratio of protein to PparDE binding sites. This hypothesis was further supported by the observation that dilution of the reaction mix immediately prior to electrophoresis caused dissociation of the complexes. Furthermore, the addition of 12 mM MgCl₂ in both the gel and the running buffer changed the characteristics of ParD binding to the PparDE sequence as analyzed by gel retardation (compare Panels A and B, Fig. 2). The added MgCl₂ seemed to affect the binding to the 232- and 548-bp fragments differently, reducing the gel shift for the 548-bp fragment substantially while only slightly affecting the gel shift for the 232-bp fragment. In the presence of MgCl₂ a new retarded complex was observed at lower protein concentrations (0.55–1.1 μM), perhaps representing a Mg⁺⁺-dependent interaction with the PparDE sequence. It was also observed that the [³⁵S] methionine-labeled ParD protein itself migrated in the Trisborate gels used in analysis of gel retardation at approximately the position of the maximally shifted complex of the ParD protein.

by Coomassie staining of polyacrylamide gels to be 10 times that observed without the extra AT-rich sequence (not shown). The reason for this enhanced level of protein expression is unknown; perhaps the AT-rich sequence allows more efficient use of the Shine-Delgarno sequence for translational initiation. This clone, named pRR136-6, was chosen for overexpression of the ParD protein for larger scale purification. The ParD protein was purified as described under "Experimental Procedures." Throughout the purification protocol, the capacity of the ParD protein to bind to its own promoter sequence in gel retardation assays (20) was used to monitor the location of the ParD protein in column eluates.

Fig. 1. Purification of the ParD protein. Panel A shows the genetic structure of the parDE operon and the divergent promoter region controlling the RK2 par genes. Panel B shows the nucleotide sequence of the fortuitous DNA inserted 5' to the start of translation of the parD gene in plasmid pRR136-6. This sequence (not underlined) enhances expression of ParD protein approximately 10-fold. The sequence of the non-transcribed DNA strand is shown, beginning with the start of transcription of the pBluescript SK (+) T7 promoter and continuing to the start codon for the parD gene. Panel C shows the proteins present at each step of purification of the wild-type ParD protein, as analyzed using a 12% low molecular weight polyacrylamide gel (25) and Coomassie staining. Lane 1, 0.3 A₂₆₀ units of E. coli BL21 (pGP1-2, pRR136-6) cells prior to induction; lane 2, 0.3 A₂₆₀ units of these cells following induction; lane 3, 100 μg of the S-100 fraction; lane 4, 100 μg of proteins recovered in the high salt DEAE column flow-through fractions; lane 5, 100 μg of protein recovered in the 0–60% (NH₄)₂SO₄ precipitation step; lane 6, 30 μg of protein eluting from the Heparin-Sepharose CL-6B column at about 100 mM KCl; and lanes 7 and 8, 2.5 μg and 10 μg, respectively, of protein eluting from the low salt DEAE column at about 0 mM KCl. The positions of migration of molecular mass markers are indicated (using bovine serum albumin, 66,000 daltons; ovalbumin, 45,000 daltons; glyceraldehyde-3-phosphate dehydrogenase, 36,000 daltons; carbonic anhydrase, 29,000 daltons; trypsinogen, 24,000 daltons; trypsin inhibitor, 20,000 daltons; and α-lactalbumin, 14,000 daltons). The position of the ParD protein is also indicated.
Protein and the 232-bp PparDE DNA (see Fig. 7, Panel B, PparDE. Corrected for dilution and inner filter effects. EDTA, and 25 mM KCl was titrated against successively added 1-2-p1 oligonucleotide encoding the PparDE site protected in DNase I footprinting, yielding fragments of 316 bp (carrying PparCBA) and 232 bp (carrying PparDE). The sizes of these fragments are indicated. These DNAs were incubated with 0.138, 0.275, 0.55, 1.1, 2.2, 4.4, and 8.8 μM ParD (final concentration), respectively, to produce the results seen in lanes 1-7 of both panels. Both gels were electrophoresed for 3 h at 10 V/cm and autoradiographed to yield the results shown.

Characterization of the ParD Binding Site by DNase I Footprinting—Due to the complications observed in the gel retardation assays, DNase I footprinting was carried out in an effort to determine the precise binding site and concentration of protein required for binding. As shown in Fig. 4, the maximum footprint extended for 48 bp on both strands with no evidence of exposed regions or sites of enhanced DNase I cleavage within this sequence. The position of the footprint completely covered both the PparDE -10 box and start of transcription. The complete footprint was seen at ParD concentrations of 4.4 μM and above; at concentrations of 2.2 and 1.1 μM, the footprint did not extend sufficiently to cover the terminal nucleotides nearest the parD gene. At 0.55 μM, only partial protection was seen throughout the region, and no protection was seen below 0.275 μM ParD. This would suggest that the dissociation constant for the ParD protein is between 0.55 and 1.1 μM, based on this concentration representing partial binding at this sequence. The footprinting data for both transcribed (Fig. 4A) and nontranscribed (Fig. 4B) DNA strands show almost identical footprints, with no significant differences.

Since it is proposed that the binding of the ParD protein to this region blocks transcription of the parDE operon by blocking initiation of transcription (20, 21), the binding site of E. coli RNA polymerase to the PparDE region was also determined by DNase I footprinting. The binding observed extended past that seen for the ParD protein upstream of the parDE operon, but the downstream border for both proteins was within 3 bp (see Fig. 4, C and D). The close identity of the regions bound by ParD and RNA polymerase suggest further that autoregulation is due to ParD-mediated interference of RNA polymerase binding to the PparDE sequence.

Multimeric Form of the ParD Protein in Solution—The first approach taken to determine the native form of the ParD protein in solution was through the use of glutaraldehyde cross-linking, particularly since ParD (predicted M, of 9,103) has 6 lysine residues out of 83 amino acids (16, 19). When a
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**Fig. 4. DNase I protection of ParD-bound PparDE DNA.** Panel A shows the ParD-mediated DNase I protection of the transcribed strand of the PparDE region following the described protocol (see "Experimental Procedures"). The DNA was incubated in reactions containing no ParD protein (lanes 1 and 9) or concentrations of 0.138, 0.275, 0.55, 1.1, 2.2, 4.4, or 8.8 μM ParD (lanes 2-8, respectively). The samples were then electrophoresed in an 8% sequencing gel, along with G and G+A sequencing ladders (not shown). The solid bar indicates the region of protection by ParD protein at concentrations of 1.1 μM and above, with the hatched region protected at 2.2 μM and above, and the stippled region protected at 4.4 μM and above. Numbers represent nucleotides from the major start of transcription from the PparDE promoter, the first G residue in the AflIII site (20, 21).

Panel B shows the DNase I protection pattern of ParD binding to the nontranscribed strand carrying PparDE. Lane 1 shows the G + A Maxim-Gilbert sequencing reaction. Lane 2 shows the result of DNase I digestion without added ParD, and lanes 3-9 the results of digestion in the presence of 0.138, 0.275, 0.55, 1.1, 2.2, 4.4, or 8.8 μM ParD protein, respectively. Indication of the region protected at the different ParD concentrations is as described for Panel A, as is the basis for the nucleotide numbering.

Panel C demonstrates the difference in DNase I protection patterns using E. coli RNA polymerase holoenzyme (8 pg added in a 20-μl volume), lane 1; no protein, lane 2; or the ParD protein (at 4.4 μM concentration), lane 3. The solid bars indicate regions of complete protection by ParD protein, and the striped region indicates the sequence protected by RNA polymerase. The thin line from bp -1 to +3 indicates reduced protection by the RNA polymerase. The asterisk at bp -27 of the RNA polymerase footprint indicates a site of enhanced DNase I cleavage. Numbering is as described in Panel A. Panel D represents a summary of the DNase I footprinting data for both ParD protein and RNA polymerase. The regions of protection with ParD protein at concentrations of 1.1 μM or higher are shown as solid boxes, protected sequences at 2.2 μM ParD or higher are shown as hatched boxes, and protected areas at 4.4 μM ParD or higher are shown as stippled boxes. The RNA polymerase region of complete protection is indicated as striped boxes. Numbering of the sequence is based on the major start of PparDE transcription as +1; the -35 and -10 boxes for this promoter are indicated as well as the restriction sites NdeI and AflIII and the start of the parD coding region. Possible repeated sequences to which ParD may bind, based on genetic and biochemical data (20, 21), are indicated as arrows with the repeated sequence included.

solution of ParD at 11.0 μM (100 ng/μl) was treated with increasing concentrations of glutaraldehyde, a form with an apparent \( M \), of 19,000 was observed on polyacrylamide gel electrophoresis (Fig. 5). Increasing the concentration of ParD protein to 110 μM (1.0 μg/μl) did not yield appreciable quantities of forms higher than this putative dimer species (not shown), suggesting that ParD is a dimer in solution at these concentrations.

Confirmation of this result was obtained using time-resolved fluorescence anisotropy. As shown in Fig. 6, at concentrations above 10 mM, the rate of tumbling of the protein is consistent with it being a dimer, in agreement with the glutaraldehyde cross-linking results. No indication of substantial quantities of multimers larger than dimer was seen even at 100 μM. Below 10 μM, the average molecular weight diminishes, suggesting that dissociation into monomer occurs
in a concentration-dependent manner.

Requirement of PparDE DNA Binding by ParD for Plasmid Stabilization—The parD gene of plasmid RK2 has been shown to be the first of two genes expressed in an operon that constitutes a minimal plasmid stabilization region, with both genes required for plasmid stabilization activity (19). However, the mechanism by which this operon achieves plasmid stabilization is not yet clear. It is possible that the binding of the ParD protein to the PparDE site acts not only in auto-repression of PparDE but also as the cis-acting site in partitioning. This possibility was addressed by examining mutated parD genes, to analyze whether such a correlation between DNA binding and plasmid stabilization exists.

Mutations in the parDE operon have been generated by insertion of DNA linker oligonucleotides and then tested for their effect on both plasmid stabilization and repression of the PparDE promoter in trans (19). It was found that insertion of either 23, 7, or 3 extra codons at the BglI site at approximately the center of the parD gene inactivated the ParD protein for PparDE repression in vivo but did not adversely affect plasmid stabilization capacity (19). One additional mutation also yielded this phenotype: the insertion of 5 codons at the 5' end of parD (results not shown). These observations preliminarily indicate that ParD binding at PparDE is not required for stabilization. Alternatively, the degree of binding at this site that is required for stabilization may either be lower than that required for PparDE repression or of a different nature or sequence specificity. To resolve these possibilities, two mutant ParD proteins were chosen for purification and testing for DNA binding by gel retardation: the ParD# mutant and the ParD1O mutant (Fig. 7). The ParD# protein behaved much like the wild-type ParD in purification, resulting in a preparation comparable to ParD in purity (not shown). This protein was tested by the gel retardation assay using PparDE and found to bind to this region specifically, albeit with an affinity 2–4-fold lower than seen for wild-type protein (Fig. 7B). These results indicate that a mutation that reduces ParD affinity for PparDE binding as determined by gel retardation also reduces the capacity of this protein in vivo to repress its promoter in trans.

The ParD1O protein, however, could not be purified using the protocol developed for the wild-type ParD. Therefore, DNA binding activity was initially tested for a ParD1O preparation that was approximately 5% pure: no specific binding to the PparDE sequence could be detected (not shown). To confirm this result, crude cell extracts from E. coli BL21 carrying either ParD wild-type or ParD1O were prepared and tested for gel retardation activity (in the absence of MgCl2, in order to minimize the activity of nucleases present in the extracts). While the wild-type protein bound as expected (Fig. 7D, lanes 3–6), no binding activity could be detected for ParD1O (Fig. 7D, lanes 11–14), even though the protein could be detected by Coomassie staining of crude extract proteins separated by polyacrylamide gel electrophoresis (not shown). This lack of binding, furthermore, was not the result of an inhibitory factor in the ParD1O extract, since the wild-type ParD protein bound well even in the presence of this extract when the two were mixed (Fig. 7D, lanes 7–10). The observation that ParD1O protein functions effectively in plasmid stabilization but yet binds to PparDE at much reduced affinities, if at all, suggests that binding to this site does not play a direct role in plasmid stabilization.

DISCUSSION

This work has centered around the characterization of the interaction between the ParD protein and its binding region.
**DNA Binding Activity of ParD Protein of Plasmid RK2**

**Fig. 7. DNA binding properties of PparDE repression-defective ParD mutants.** Two mutated ParD proteins that displayed the phenotype of being functional in plasmid stabilization yet defective in repression of PparDE in *vivo* were chosen for examination of their DNA binding activities. Panel A shows the predicted central amino acid sequence of the wild-type ParD and ParD# proteins, indicating the amino acids inserted (the two native amino acids D43 and Q44 were altered to A43 and E67 as part of the insertion). The gel retardation properties of this protein are shown in Panel B. Purified (1.0 μg) L-[35S]Met-labeled wild-type ParD was electrophoresed in lane 1, with the majority of the protein migrating as indicated. Lane 2 shows the migration of the PparCBA (316 bp) and PparDE (232 bp) fragments in the absence of protein. Lanes 3–9 show gel retardation with the wild type ParD protein and lanes 10–16 gel retardation using the ParD# protein. The same final concentrations of each protein were used as follows: 8.8 μM, lanes 3 and 10; 4.4 μM, lanes 4 and 11; 2.2 μM, lanes 5 and 12; 1.1 μM, lanes 6 and 13; 0.55 μM, lanes 7 and 14; 0.275 μM, lanes 8 and 15; and 0.138 μM, lanes 9 and 16. Panel C shows the predicted N-terminal amino acid sequences of wild-type ParD and the other potential PparDE binding mutant, ParD10. The analysis of the gel retardation properties of these proteins in crude cell lysates is shown in Panel D. Lanes 1 shows the migration of the PparCBA (316 bp) and PparDE (232 bp) fragments in the absence of protein. Lane 2 shows the gel retardation pattern generated with 4.4 μM purified ParD protein. Lanes 3–6 show the gel shift properties of 0.5, 0.2, 0.05, and 0.02 μl, respectively, of the wild-type ParD-containing extract prepared as described under “Experimental Procedures.” The complexes between ParD and the 232-bp PparDE fragment are indicated. Lanes 7–10 show the retardation properties of 0.5, 0.2, 0.05, and 0.02 μl, respectively, of the wild-type ParD-containing extract in the presence of 0.2 μl of the ParD10-containing extract. Lanes 11–14 show gel retardation using 0.5, 0.2, 0.05, and 0.02 μl, respectively, of the ParD10-containing extract.

at the PparDE promoter sequence. At a ParD concentration of 1.1 μM, the protein binds to a 33-bp sequence beginning within the promoter –35 box and extending 2 bp past the transcriptional start site, protecting this sequence from DNase I digestion. The location of this binding site fits well with the sites of other prokaryotic transcriptional repressors, suggesting interference with transcription by steric hindrance of RNA polymerase binding (33). Approximately centered within this region lie the two inversely repeated sequence motifs proposed as possible DNA binding sites (Fig. 4D), based on genetic and biochemical evidence (20, 21). The region protected from DNase I digestion is extended an additional 16–17 bp downstream of the PparDE transcriptional start site when the ParD concentration is increased to 4.4 μM, suggesting weaker binding of ParD to a site in this region. Sequences with partial homology to either of the proposed repeat motifs may be found in this downstream region; one or more of these may be responsible for the weaker binding. Exact identification of the sequence to which the ParD protein binds will require further genetic and biochemical dissection of this region.

Using tryptophan fluorescence quenching to monitor ParD binding to DNA, it was estimated that two ParD monomers bound to the 48-bp double-stranded oligonucleotide sequence at a protein concentration of 4.7 μM (Fig. 3). Most likely, this represents binding to the two inversely repeated motifs to generate the 33-bp region of DNase I protection.

Interaction between ParD and the PparDE site was also analyzed by monitoring specific retardation of a PparDE sequence-containing DNA fragment. This assay demonstrated a large decrease in mobility upon addition of sufficient quantities of ParD protein. This large shift was shown not to be due to DNA bending or the binding of a large number of ParD monomers. It is most likely that, while the specificity of binding is valid (as supported by the DNase I footprinting), the degree of gel retardation is an artefact of the assay. Indeed, when free ParD protein was electrophoresed along with ParD-bound PparDE DNA, the free protein migrated to roughly the same place as the bound complex (Fig. 3). While such migration could be coincidental, it suggests that the position of

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migration of the PparDE DNA is largely influenced by the electrophoretic properties of ParD and as such, its position does not reflect information about the nature of specific ParD-DNA complexes.

The gel mobility shift experiments also suggest that the complex formed between the ParD protein and its specific binding site is not stable, based on sensitivity of the binding to dilution of the mixture or increased times of electrophoresis. Since the ParD protein migrates at approximately the same position as the protein:DNA complex, such instability could result in a dynamic equilibrium between ParD and its binding site, with DNA dissociating from one protein to the other. This interpretation is supported by the failure to obtain distinct intermediate-sized DNA:protein complexes using heteromultimeric mixtures of ParD and Par*. If the ParD proteins and PparDE DNA are in dynamic equilibrium, then approximately 44,000 fmol of monomeric ParD was required to bind to the PparDE DNA in gel shift assays; however, it was shown that, at this ParD concentration (2 × 10^6 M), DNA binding could be detected. Time resolved fluorescence anisotropy also indicated that at similar ParD concentrations (1–5 × 10^4 M), the average molecular weight of ParD in solution was decreasing from that of a dimer to a monomer. Time resolved fluorescence anisotropy of ParD in solution was decreasing from that of a dimer to a monomer. Time resolved fluorescence anisotropy of ParD in solution was decreasing from that of a dimer to a monomer.

It has been shown that the ParD protein is essential for plasmid stabilization in the context of either the 3.2-kb or the 2.7-kb regions of RK2 (16, 17). The function of the ParD protein in stabilization, however, is not known. Strong genetic (20, 21) and biochemical evidence (this study) support a role of ParD in the autoregulation of the parDE operon. Regulation of this operon is significant, since many plasmid partitioning genes inhibit partitioning function when overexpressed, including the P1 prophage proteins ParA and ParB (5, 6), the F protein SopB (35), and the NRI protein StbB (11). If indeed a classical partitioning function is represented within the parDE operon, a characteristic feature would be the presence of a cis-acting site by which the region can be linked to a putative cellular segregation machinery. Such a cis-acting site has been shown to be located either in the promoter region of the partitioning operon, or as the case for the R1 group of plasmids (11, 12) and pTar (13), or downstream from the partitioning genes, as is found for the incD site of the F plasmid (36, 37), the parS site of the P1 prophage (38), and the STB or REP3 site of the yeast 2 μM circle (39). Within the RK2 par region, evidence for a determinant which might correspond to such a cis-acting site is lacking (16, 17). The possibility that ParD binding to its promoter region may serve as the cis-acting site for partitioning is supported. Results presented here however, show that ParD binding to the PparDE region is dispensable for plasmid stabilization; the mutant ParD10 lacks detectable PparDE DNA binding activity while retaining its role in plasmid stabilization. Thus the ParD-PparDE interaction is not likely to represent a cis-acting partitioning site, raising the possibility that ParD plays some alternate role in plasmid stabilization in addition to its autoregulatory capacity. Since the ParE protein is also essential for plasmid stabilization (19), additional studies involving the ParD and ParE proteins and their interactions are being pursued to examine further the mechanisms employed by the parDE operon to achieve plasmid stabilization.

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REFERENCES

* R. C. Roberts, A. Ström, and D. R. Helinski, unpublished results.