Probing the structure of complex macromolecular interactions by homolog specificity scanning: the P1 and P7 plasmid partition systems

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The P1 plasmid partition locus, *P1 par*, actively distributes plasmid copies to *Escherichia coli* daughter cells. It encodes two DNA sites and two proteins, ParA and ParB. Plasmid P7 uses a similar system, but the key macromolecular interactions are species specific. Homolog specificity scanning (HSS) exploits such specificities to map critical contact points between component macromolecules. The ParA protein contacts the *par* operon operator for operon autoregulation, and the ParB contacts the *parS* partition site during partition. Here, we refine the mapping of these contacts and extend the use of HSS to map protein–protein contacts. We found that ParB participates in autoregulation at the operator site by making a specific contact with ParA. Similarly, ParA acts in partition by making a specific contact with ParB bound at *parS*. Both these interactions involve contacts between a C-terminal region of ParA and the extreme N-terminus of ParB. As a single type of ParA–ParB complex appears to be involved in recognizing both DNA sites, the operator and the *parS* sites may both be occupied by a single protein complex during partition. The general HSS strategy may aid in solving the three-dimensional structures of large complexes of macromolecules.

Keywords: homolog specificity scanning/macromolecular interactions/plasmid partition systems/protein–DNA/protein–protein interactions

Introduction

Genetic techniques can give detailed information about contact points in macromolecular interactions. One powerful approach available involves isolation of a mutant form of one molecule which disrupts the interaction, and a subsequent second-site reversion which adjusts the second molecule in such a way as to restore the interaction. This method has been used successfully to analyze a protein–DNA complex (Yoderian *et al.*, 1983). The chief limitation of this approach is the difficulty in predicting which starting mutations will give the appropriate revertants.

This requires either massive screening or a detailed knowledge of the contact region of one of the interacting partners. These requirements are particularly difficult to satisfy for most protein–protein interactions.

Evolution can, in principle, supply a source of suitable mutations and their appropriate compensatory revertants. As protein complexes evolve, they develop species specificities. The components are no longer interchangeable with their progenitors because mutations altering a contact point on one component arise, along with the necessary compensatory change in the other component. By identifying such correlated differences between one species and another, it should be possible to define pairs of residues involved in protein–protein as well as protein–DNA interactions. Unfortunately, the correlated differences must be identified from among many other differences that are silent, or which remodel the internal architecture of the proteins.

Mapping of the relevant differences can be achieved by constructing hybrid proteins in which a region from one species is substituted for that of another. One strategy involves looking for regions from a homolog which, when incorporated into the hybrid protein, block the protein–protein interaction of interest. This is known as homolog-scanning mutagenesis (Cunningham *et al.*, 1989) and has been successfully used to identify epitopes for binding of human growth hormone to its receptor and to monoclonal antibodies raised against it. This approach has limitations for its general application because changes that affect the internal architecture of the protein often give rise to completely non-functional hybrids which are indistinguishable from those with the desired properties in a loss-of-function screen. Here, we take the approach of scoring hybrids that switch their specificity so that they now interact with the partner from the other species (homolog specificity scanning, HSS). We have applied this principle to interactions between the plasmid partition proteins ParA and ParB from plasmids P1 and P7 and their DNA sites. By applying the method to both protein partners we were able to generate information on critical protein–DNA and protein–protein contacts involved in partition.

The plasmid prophages of bacteriophage P1 and P7 encode partition (*par*) systems which direct the proper segregation of daughter plasmids to *Escherichia coli* daughter cells. The plasmids encode two proteins, ParA and ParB, and a downstream *cis*-acting site, *parS* (Figure 1; Abeles *et al.*, 1985; Ludtke *et al.*, 1989). Homologs of these plasmid Par proteins have recently been shown to be important for the segregation of chromosomes in bacterial species (Lin *et al.*, 1997; Mohl and Gober, 1997; Webb *et al.*, 1997). The P1 and P7 *par* systems have similar characteristics. The ParB protein binds specifically to *parS*. It acts cooper-

actively with the host protein integration host factor (IHF) to form a wrapped *parS* complex (Davis and Austin, 1988; Funnell, 1988). ParA is essential for partition (Davis *et al.*, 1996). It is an ATPase whose activity is stimulated by ParB and double-stranded DNA (Davis *et al.*, 1992). It does not appear to form a stable complex at *parS*, although there is evidence that it acts via the site for some essential step in partition (Youngren and Austin, 1997).

In addition to their central roles in partition via *parS*, both proteins play an additional role: that of co-regulators of the *par* operon. ParA binds to an operator site in the vicinity of the operon promoter and represses transcription (Davis *et al.*, 1992; Davey and Funnell, 1994). ParB stimulates the repressor activity of ParA, thus establishing autoregulation of the operon which is sensitive to the level of both gene products (Friedman and Austin, 1988).

Although the P1 and P7 plasmid partition systems are closely related, the individual components are not interchangeable. The *parS* sites only function with their cognate ParB proteins. This is due to a secondary contact with the DNA (the discriminator contact) which involves a short peptide sequence near the ParB C-terminus (the discriminator recognition sequence, DRS) and a pair of hexamer sequences that lie at each boundary of the *parS* site (Radnedge *et al.*, 1996). This contact does not appear to provide binding energy, but requires a proper 'fit' for the protein to orient itself to the site. A species-specific interaction between ParA and ParB has been shown *in vitro*: ParA ATPase activity is only stimulated by ParB from the same species (Davis *et al.*, 1992). Lastly, the autoregulatory interaction between ParA and the *par* operon operator sequence is also species specific, with the P1 and P7 proteins recognizing dissimilar operator sites (Hayes *et al.*, 1994). Here we apply HSS to localize the determinants of the ParA-ParB interaction, to better define the location of the ParA-operator contact and to probe the secondary structure of the proteins.

Fig. 1. Physical map and protein sequences of the P1 and P7 *par* regions. A physical map of the features common to the P1 and P7 *par* loci is shown above with a scale in kbp. Similar promoter-operator (PO), helix-turn-helix (HTH), Walker A and B ATPase boxes, motif 4 and the partition sites (*parS*) are present in both species (Davis *et al.*, 1996). They are shown on this map and boxed in the protein sequences shown below. The ParA and ParB protein sequences of P1 (above) and P7 (below) were aligned using the GAP program (Wisconsin package, version 8.0, Genetics Computer Group, Madison, WI). The junctions of the hybrids used in this study are shown as linked arrows pointing in the direction of the included sequences. The junctions are shown at the position of the splice in the DNA sequence: when the junction occurs in a stretch of conserved amino acids, its position within the protein can be regarded as being anywhere within that stretch. HTH, the best fit HTH motifs for each protein (Hayes *et al.*, 1994; Radnedge *et al.*, 1996). ATPase A and B; the putative Walker A and B boxes for the ATPase active site (Davis *et al.*, 1996). DRS, the ParA DRS that imposes species specificity on recognition of the *parS* site (Radnedge *et al.*, 1996). Hybrids A0.2, A3, A6 and A6.3 show no partition or repression activity and are considered as uninformative for this study.

	<i>parA</i>	<i>parB</i>	Partition test (% retention)		Repression test (Miller units)	
			P1 <i>parS</i>	P7 <i>parS</i>	pP1	pP7
pALA1413			95	<2	68	4097
pALA1414			>2	81	2635	87
pALA1416			>2	NC	334	3811
pALA1462			NC	<2	2951	557
pALA1126			<2	<2	342	4348
pALA1115			<2	<2	2668	321
pBR322			<2	<2	2953	4307

Fig. 2. The partition and repressor activities of hybrid *par* operons. Maps of *par* operons with ParA and ParB ORFs (arrows), and the *par* promoter-operator sequences (small boxes on left end) derived from P1 (open regions) or P7 (closed regions) are shown. The operons were inserted into vector pBR322 (horizontal lines) and introduced into test strains where the partition activity and the operon repression activity of the proteins was tested. The partition test assays the ability of the proteins to promote retention of a mini-P1 plasmid carrying the P1 or P7 *parS* partition site during ~25 generations of unselected growth (Materials and methods). The repression test measures the β -galactosidase expression from plasmids carrying *lacZ* under the transcriptional control of the P1 or P7 *par* promoter-operator regions (Materials and methods). NC, no colonies formed in the partition tests, i.e. the test plasmid containing the relevant *parS* site could not be introduced into the strain.

Results

Tests for species specificity of *par* operon components

We have produced a series of P1/P7 hybrid *par* operons using a modified strand overlap extension (SOE) polymerase chain reaction (PCR) technique (Horton *et al.*, 1990; Radnedge *et al.*, 1996). These were inserted into a pBR322 vector and introduced into the test cells. Each hybrid was tested for species specificity in separate assays for operon promoter repression and for plasmid partition. For repression assays, a pGB2-based reporter plasmid was introduced which has the *lacZ* gene under control of either the P1 or P7 promoter-operator. For partition assays, cells containing the hybrid operon plasmids were transduced by a low-copy number mini-P1 plasmid containing either the P1 or P7 *parS* site. The maintenance stability of the *parS* plasmid was followed during ~25 generations of unselected growth (see Materials and methods).

The majority of the hybrid operons constructed showed repressor activity of one specificity or the other and some of these also promoted partition. In no case was species specificity compromised; none of the hybrids showed significant repression of both operator types or partition of both types of *parS* site. A few of the hybrids with hybrid junctions in ParA were completely uninformative as they showed no repressor activity of either specificity and had no effect in partition tests. They are listed in the legend to Figure 1.

Species specificity for repression

As previously shown (Hayes *et al.*, 1994), the P1 Par proteins only repress the P1 promoter and the P7 proteins only repress the P7 promoter (Figure 2). Hybrids were tested that have the complete ParA protein from one species and the ParB protein from the other (ParA/B hybrids). The hybrid with P1 ParA and P7 ParB (pALA1416) repressed the P1 operator-promoter and that with P7 ParA and P1 ParB (pALA1462), the P7 operator-promoter (Figure 2). This is consistent with the finding that ParA is essential for repression and binds to the operator (Hayes *et al.*, 1994).

ParB enhances repression in the wild-type operons (Hayes *et al.*, 1994). Deletion of *parB* from either the P1 or the P7 operon increased expression several fold (Figure 2). In contrast, the ParA/B hybrid operons showed no ParB component to repression: repression of the appropriate reporters was several fold less effective than that given by the wild-type operons and deletion of ParB from the hybrid constructs had no significant effect on repression (Figure 2). Thus, the ParA/B hybrid operons use the ParA protein for repression, but the ParB protein derived from the other species does not enhance it. We conclude that some functional interaction between ParB and ParA or ParB and the operator is species specific.

Species specificity for partition

Cells containing the P1 *par* operon only support the partition of the plasmid carrying the P1 *parS* site, and the P7 operon only supports function of the P7 *parS* site (Hayes and Austin, 1993; Figure 2). This is due to a species-specific contact between the N-termini of the ParB proteins and their cognate recognition sequences in *parS* (Hayes and Austin, 1993). The ParA/B hybrid operons expressed from their natural promoters were uninformative in partition tests because they blocked the introduction of the cognate *parS* test plasmids (Figure 2). This problem may be due to partial derepression of these operons: modified operons driven from a low-level constitutive promoter did not show this block (Figure 3). Comparison of these modified ParA/B hybrid operons to their non-hybrid P1 and P7 equivalents showed them to be completely inactive for partition (Figure 3). We conclude that the ParA protein must come from the same species as ParB and *parS* for partition to work. ParB recognizes *parS* in a species-specific manner for partition (Hayes and Austin, 1993) and ParA is essential for partition (Davis *et al.*, 1996). Thus some essential interaction between ParA and ParB or between ParA and *parS* is species specific.

Further localization of the specificity determinants for *par* promoter repression

Figure 4 describes a series of hybrid operons in which the P7-P1 hybrid junction was moved progressively down-

Plasmid	Hybrid	<i>parA</i>	<i>parB</i>	Partition test (% retention)	
				P1 <i>parS</i>	P7 <i>parS</i>
pALA1570	—			90	<1
pALA1571	—			<1	92
pALA2147	A/B			<1	<1
pALA2143	A/B			<1	<1
pALA2144	A5			91	<1
pALA2141	A5.1			13	<1
pALA2142	A5.4			<1	<1
pALA2145	A6.1			<1	<1
pALA2146	A6.2			<1	<1

Fig. 3. The partition activity of operons driven by a low-level constitutive promoter. The assays and map symbols are as used in Figure 2. *p_c*, constitutive promoter. Constitutive synthesis did not compromise the partition activity of P1 wild-type or hybrid A5 operons (c.f. Figure 2). The block to the establishment of the P1 *parS* test plasmid seen with the A/B hybrid (Figure 2) and with hybrids A5.1–A6.2 (Figure 5) was partially alleviated when the constitutive promoter was used: the test plasmids could be introduced into cells containing the constitutive constructs although the resulting colonies grew slowly under selection.

Plasmid	X-over	<i>parA</i>	<i>parB</i>	Partition test (% retention)		Repression test (Miller units)	
				P1 <i>parS</i>	P7 <i>parS</i>	pP1	pP7
pALA1413				88	<1	117	3865
pALA1543	A0.1			<1	<1	1	2030
pALA1547	A0.3			<1	<1	2664	9
pALA1491	A1			<1	<1	3187	20
pALA1495	A2			5	<1	3126	57
pALA1503	A4			<2	<1	2885	65
pALA1507	A5			90	<1	3091	107

Fig. 4. The properties of operons with hybrid ParA proteins. The map symbols and tests are the same as in Figure 2. The hybrid constructs are driven from the natural P7 promoter-operator region. The shaded result confirms a result previously published using the same hybrid operon in a different vector and using a different reporter construct (Hayes *et al.*, 1994). The strong repression of the P1 operator seen with hybrid A0.1 reflects overexpression of the operon because the hybrid ParA protein has P1 repressor specificity but the operon producing it has a P7 operator.

stream through the ParA open reading frame (ORF) from left to right. The data allowed refinement of the mapping of the P7 ParA operator specificity determinant previously shown to lie somewhere within the N-terminal 87 amino acids (Hayes *et al.*, 1994). Substitution of the promoter-operator region and the first 22 P1 ParA amino acids by their P7 equivalents (hybrid A0.1) had no effect on operator specificity: the products continued to specify P1 operator-promoter repression (Figure 4). However, substitution of 66 amino acids (A0.3) caused a complete switch to P7 operator specificity. Thus, all the information required to discriminate between the P1 and P7 operator sequences lies in the 44 amino acid region from residues 22–66 of P7 ParA. This region includes a putative DNA binding helix–turn–helix (HTH) motif (Figure 1). It is likely that the P7 ParA HTH region binds the P7 operator but cannot effectively bind to the P1 operator sequence. The P1 ParA sequence contains a somewhat different HTH motif in this position (Figure 1) which presumably

specifies binding to the P1 operator whose sequence and position relative to the transcription start point differ from that of the P7 operator (Hayes *et al.*, 1994).

A ParA–ParB protein–protein interaction determines the ability of ParB to enhance repression

Hybrids A0.3–A5 contain the P7 ParA HTH sequence and repress the P7 operator (Figure 4). The C-terminal end of ParA and all of the ParB protein are derived from P1 in these hybrids. The repression efficiency varied with these hybrids (a range of 30- to 300-fold repression was observed) but it was roughly comparable to that achieved by the wild-type P1 operon (40-fold; Figure 2). This suggests that these hybrid ParA proteins cooperate with the P1 ParB protein to achieve full repression as P1 ParA does in the wild-type operon. This was confirmed for the hybrid operon A5: P7 operator repression was reduced 3-fold on deleting the *parB* gene (Figure 5).

Plasmid	Hybrid	<i>parA</i>	<i>parB</i>	Partition test (% retention)		Repression test (Miller units)	
				P1 <i>parS</i>	P7 <i>parS</i>	pP1	pP7
pALA1507	A5			90	<2	2991	100
pALA2133	A5			<2	<1	3060	332
pALA2112	A5.1			NC	<1	2290	591
pALA2134	A5.1			<2	<1	2567	1216
pALA2115	A5.4			NC	<1	2986	846
pALA2135	A5.4			<2	<1	2022	999
pALA2116	A6.1			NC	<1	2618	506
pALA2136	A6.1			>2	<1	2050	729
pALA2117	A6.2			NC	<1	2376	663
pALA2137	A6.2			>2	<1	2438	732
pALA1462	A/B			NC	<1	3811	334
pALA1115				<2	<2	2668	321
pBR322				<2	<2	2732	3143

Fig. 5. The properties of operons with ParA proteins having a hybrid junction close to the C-terminus. The map symbols and tests are the same as in Figure 2. The properties of each hybrid operon are compared with those of the corresponding operon (immediately below it) from which the *parB* gene has been deleted. The difference between the P7-specific repression seen with hybrid A5.1 and its *parB*-deleted equivalent is highly significant but the apparent differences between hybrids A5.4, 6.1 and 6.2 and their *parB*-deleted derivatives are not. NC, no colonies formed in the partition tests, i.e. the test plasmid containing the relevant *parS* site could not be introduced into the strain containing the hybrid operon.

What is the nature of this cooperation? As the hybrid ParA proteins such as A5 repress the P7 operator but cooperate with the wild-type P1 ParB protein to enhance repression (Figure 5), it can be concluded that species-specific enhancement of repression by ParB is due to a protein-protein interaction with ParA rather than direct recognition of the operator by ParB.

Mapping the ParA determinant that interacts with ParB to enhance repression

If ParB binds to ParA in a species-specific fashion to enhance repression, ParA should contain specific information for recognition of ParB. As hybrid A5 interacts productively with P1 ParB (Figure 5), this ParA information must lie within the P1 portion of the hybrid, i.e. within a 117 amino acid region of the P1 ParA C-terminus (Figure 6).

Additional hybrids were constructed with hybrid junctions in the vicinity of the ParA C-terminus. Hybrids A5.1–A6.2 all exhibited a somewhat weakened repression (Figure 5). It is likely that the internal architecture of these hybrid ParA proteins is disturbed by conflicts between residues of P1 and P7 origin, causing the hybrid proteins to be somewhat unstable or prone to misfolding. However, it is clear that hybrid A5.1 retains the capability of cooperating with P1 ParB for repression because, on deleting ParB from the respective operon, repression was reduced 2-fold (Figure 5). Deletion of *parB* from hybrids A5.4–A6.2 had little or no effect on repression showing that these hybrids have lost most or all of the capacity to interact with ParB for repression (Figure 5). We conclude that a species-specific determinant for interaction with P1 ParB lies within the P1 ParA sequences to the right of

the hybrid junction A5.1 i.e. within the 110 C-terminal amino acids of P1 ParA (Figure 6). At least part of this determinant lies in the 18 amino acid interval between the junctions in hybrids A5.1 and A5.4 (P1 ParA residues 289–307; Figure 1).

A ParB determinant for cooperation with ParA in promoter repression

As shown above (Figure 2), the P7 ParB protein is unable to cooperate with the P1 ParA protein to enhance repression. However, ParB hybrids containing as little as 13 amino acids of the P1 N-terminus enhance the repressor activity of P1 ParA (hybrid B0.1; Figure 7). Thus, information for a productive interaction with P1 ParA for repression lies within this short region of the P1 ParB N-terminus (Figure 6).

All the hybrid ParB proteins in Figure 7 contain this N-terminal sequence. However, hybrids with junctions toward the middle of the ParB ORF (B0.4–B4), although retaining a ParB component to repression (c.f. pALA1416, Figure 7), show somewhat reduced repression levels. Presumably the ParB proteins of these hybrids are capable of enhancing repression by interacting with P1 ParA but they are partially inactive due to messenger instability or to internal conflicts which compromise folding.

A ParA–ParB interaction determines the ability of ParA to promote partition

Partition activity requires that the ParA and ParB–*parS* components come from the same species. As the hybrid ParB proteins B0.2, B0.3, B0.4 and B5 promote partition via the P7 *parS* site but use the wild-type P1 ParA protein for function (Figure 7), it can be concluded that species-

Fig. 6. Features mapped to the P1 and P7 *par* regions. The P1 (above) and P7 (below) *par* physical maps are shown with their common features marked as in Figure 1. Narrow boxes below the maps show the positions and maximum extents of the species-specificity determinants as mapped in this work, and the DRS (Radnedge *et al.*, 1996). The ParA–ParB interactions for partition, operon repression and ParA ATPase stimulation appear to be the same. However, the P1 regions in ParA and ParB required for proper repression (open boxes) are slightly smaller than those required for efficient partition (open boxes plus shaded extensions), and the ParB determinant for interaction with ParA for ATPase stimulation was less precisely mapped (open box plus shaded and hatched extensions). The ‘folding/dimerization’ specificity determinants represent pairs of determinants (shaded boxes) which must be from the same species for partition to proceed. They probably reflect protein regions which must match for function, although they could theoretically be messenger RNA stability determinants. As the analysis used here can only detect the outermost members of a nested or overlapping series of such determinants, other such determinants may exist in the region between these determinants (hatched connectors).

Plasmid	X-over		Partition test (% retention)		Repression test (Miller units)	
			P1 <i>parS</i>	P7 <i>parS</i>	pP1	pP7
		<i>parA</i> → <i>parB</i>				
pALA1416	A/B	→	<1	NC	551	3811
pALA2107	B0.1	→	<1	4	32	3862
pALA2108	B0.2	→	<1	95	66	3629
pALA2109	B0.3	→	<1	97	75	3866
pALA2110	B0.4	→	<1	32	177	3982
pALA2111	B0.5	→	<1	<1	237	3845
pALA1465	B1	→		<2	177	3503
pALA1469	B2	→	<2	<2	161	3987
pALA1473	B3	→	<2	<2	161	3447
pALA1477	B4	→	<2	<2	172	4323
pALA1481	B5	→	<2	85	20	3641
pALA1485	B6	→	<2	<2	56	3811
pALA1413		→	95	<1	68	4097
pALA2139	A5/B0.2	→	<1	87	2310	60

Fig. 7. The properties of operons with hybrid ParB proteins. The map symbols and tests are the same as in Figure 2. Values in the shaded area duplicate and resemble those previously published (Radnedge *et al.*, 1996). They are included to facilitate analysis of the data set as a whole. The last construct was made by combining the hybrid genes from hybrid A5 (Figure 3) and hybrid B0.2 (Figure 7).

specific promotion of partition by ParA is due to a protein–protein interaction with ParB rather than direct recognition of the *parS* site by ParA.

Mapping P1 ParA residues that interact with the P1 ParB for partition

Hybrid A5 is fully competent for partition using the P1 *parS* site (Figure 4). This contains 115 amino acids of the P1 ParA C-terminus. Thus, a P1 sequence sufficient to allow an otherwise P7 ParA to interact properly with P1 ParB for partition exists in the 115 N-terminal amino acids of P1 ParA. The data in Figure 5 could not be used

to localize the interacting determinant more precisely because hybrid operons with junctions in ParA farther to the right than A5 are uninformative for partition. Cells containing them could not be transformed with the test plasmids containing the P1 *parS* site for which the ParB protein is cognate (Figure 5). This problem is due, at least in part, to the partial derepression of the respective operons (Figure 5) and was overcome by replacing the operon promoter–operator region with a suitable constitutive promoter (Figure 3). Like the wild-type P1 operon, hybrid A5 is fully active for partition when driven from the constitutive promoter. Hybrid A5.1 showed a low but

significant level of partition activity. All hybrids with junctions farther to the right were inactive (Figure 3). Thus the ParA determinant for species-specific interaction with ParB lies within the 115 amino acids of the ParA C-terminus (Figure 6), and sufficient information for partial activity lies within the 109 P1 ParA amino acids to the right of the block of identical amino acids bordering the junction in hybrid 5.1 (Figures 1 and 6).

ParA hybrids A0.3–A4 act as efficient repressors but do not promote partition (Figure 4; Radnedge *et al.*, 1996). These may have internal conflicts that prevent them participating in some critical step in partition without interfering with their interaction with the cognate operator site for repression. Note that hybrid A0.1 is uninformative for partition because it is derepressed, having the promoter-operator of P7, but the repression specificity of P1 (Figure 4).

Mapping P1 ParB residues that contact P1 ParA for partition

Hybrid B0.2 is fully competent for partition using the P7 *parS* site (Figure 7). Thus the N-terminal 28 amino acids of P1 ParB are sufficient to promote a productive interaction with P1 ParA for partition (Figure 6). The P1 and P7 ParB sequences are quite divergent in this region (Figure 1). One or more of the many differing residues appear to be crucial for a proper interaction with the cognate ParA.

All the hybrid ParB proteins in Figure 7 contain this N-terminal P1 sequence. However, hybrids with junctions toward the middle of the ParB ORF (B0.5–B4) are inactive for partition. We speculate that these hybrids have internal conflicts which compromise folding or dimerization.

A functional P7 partition complex that uses the P1 ParA–ParB interaction determinants

Figure 4 shows that the mostly P7 ParA protein in hybrid A5 is capable of function and contains the P1 C-terminal residues necessary for interaction with P1 ParB. Similarly, the mostly P7 ParB protein in hybrid B0.2 (Figure 7) is capable of function and contains the P1 N-terminal residues necessary to interact with P1 ParA protein. If these conclusions are correct, an operon combining these two hybrid genes should function as a P7 Par system for partition and repression but make use of the P1 determinants for the necessary ParA–ParB interaction. This operon was constructed and is fully functional for P7-specific repression and partition as predicted (pALA2139; Figure 7).

A ParA activity which can block plasmid propagation

Hybrid operons with natural promoters and ParA and ParB from different species not only fail to support partition but cause a block to the establishment or propagation of plasmids with the cognate *parS* site (Figure 2). For example, test plasmids containing P1 *parS* cannot be introduced into cells containing pALA1462 (P7 ParA plus P1 ParB; Figure 2). This block to plasmid establishment is dependent on the presence of P1 ParB and P1 *parS* (Figure 2). This property resides in the P7 ParA C-terminus because hybrids A5.1–A6.2 show the same effect (Figure 5). The phenotype is caused, in part, by the partial

GST-ParB	ParA (pg. ADP per assay)		
	None	P1 ParA	P7 ParA
None	<5	17	20
P1	<5	118	20
P7	<9	25	117
B1	<5	90	24

Fig. 8. ParA ATPase stimulation by ParB. Purified P1 or P7 ParA protein was assayed for ATPase activity in the presence of the various ParB–GST fusion proteins as described in Materials and methods. Arrows represent the composition of the proteins with P1 portions open and P7 portions filled. Each assay was carried out in a 20 µl reaction.

operon derepression to which these hybrids are subject (Figure 5). It was substantially alleviated when the operons were placed under the control of a low-level constitutive promoter (Figure 3). As P1 ParB can be overproduced without causing a block of this sort (Davis *et al.*, 1996; hybrid A0.1, Figure 4), it appears that it is the presence of elevated levels of the ParA protein C-terminal sequence from the wrong species that causes the block. The sequence resides between the hybrid junctions of A5 and A5.1 (Figure 5). We propose that, when present at an elevated level, this sequence interacts with the ParB–*parS* complex in an inappropriate fashion which blocks the ability of the *parS* plasmid to be established in the cell.

The N-terminal region of ParB specifies ParA ATPase stimulation *in vitro*

We have previously described the purification of P1 and P7 Par proteins and shown that ParB stimulates the ATPase activity of ParA in a species-specific manner (Davis *et al.*, 1992). Figure 8 shows the results of modified experiments in which ParB proteins were isolated as ParB–glutathione *S*-transferase (GST) fusions (see Materials and methods). In agreement with the original results with the native ParB protein (Davis *et al.*, 1992), the ATPase activity of P1 ParA protein was stimulated only by P1 ParB–GST, and P7 ParA ATPase only by P7 ParB–GST (Figure 8). A further ParB–GST fusion construct was made that produces a B1 hybrid ParB–GST fusion. The ParB–GST protein from this construct stimulated the ATPase activity of P1 ParA but not that of P7 ParA (Figure 8). Thus, the determinant for specific stimulation of ParA ATPase activity resides within the first 68 amino acid residues of P1 ParB (Figure 6). This is a maximum estimate of the region as GST fusions of hybrids B0.1–B0.5 were not made. The region includes the residues shown above to be necessary for the ParA–ParB interaction for partition (Figure 6).

Discussion

We have mapped determinants for species-specific interactions of Par components (Figure 6). Operon repression involves a specific interaction between ParA and the

operator DNA. The specific determinant includes a HTH motif in the N-terminal region of ParA and probably contacts the operator directly. Direct binding of ParA to the operator sequence has been shown previously (Davis *et al.*, 1992).

Specific enhancement of repression by ParB was shown to be due to a protein–protein interaction with ParA rather than direct recognition of the operator by ParB. This is consistent with the observation that ParB does not appear to bind to the operator by itself, and does not cause any qualitative modification of the footprint of ParA bound to operator DNA (Davis *et al.*, 1992). The ParA–ParB interaction involves determinants in the C-terminal region of ParA and the N-terminal end of ParB (Figure 6). ParA and ParB interact directly *in vitro* (Davis *et al.*, 1992). Thus it is likely that the mapped determinants contact each other directly to enhance repression.

Partition requires a species-specific contact between the DRS motif in the ParB C-terminus and four or five base pairs in the *parS* DNA (Hayes and Austin, 1993; Radnedge *et al.*, 1996). Species-specific promotion of partition by ParA was found to be due to a protein–protein interaction with ParB rather than a direct recognition of *parS* by ParA. This is consistent with the observation that ParA does not bind to the *parS* site by itself, and does not cause any qualitative modification of the footprint of ParB bound to *parS* DNA (Davis *et al.*, 1992).

The same ParA–ParB interaction is probably involved in repression and partition. The determinants for the two activities are coincident, although the regions required for a fully productive interaction for partition are somewhat extended (Figure 6). We suggest that the same core ParA–ParB contacts are required for both activities but some nearby residues must also match properly if the complex is to be fully competent for partition.

The ParA–ParB interaction for *in vitro* stimulation of ParA ATPase activity by ParB probably involves the same core residues (Figure 6), although only the ParB determinant was mapped and the mapping was less precise (Figure 6). It is likely that repression, partition and the stimulation of ATPase activity all make use of the same ParA–ParB interface, and that ATPase stimulation is an integral part of repression or partition. Both activities are dependent on the integrity of the putative ParA ATP binding site (Davis *et al.*, 1996). ParA operator binding requires nucleotide binding but not hydrolysis (Davey and Funnell, 1994). Thus, the ParA ATPase and its specific stimulation by ParB are likely to be involved directly in the partition event.

We have shown that the ParA N-terminus and a second ParA region to the left of hybrid A5 must be from the same species for the protein to function in partition tests (Figure 4). Similarly, ParB regions to the right of the hybrid junction in B0.3 and to the left of the junction in hybrid B5 have to come from the same species for ParB to function fully (Figure 7). We suggest that these regions represent pairs of points within the proteins which form critical bonds for folding a portion of the protein or for forming a homodimer interface (Figure 6). Other such specificities might exist in the region between these determinants (Figure 6, hatched regions); the analysis used here can only detect the outermost members of a nested

Fig. 9. A structural interpretation of the data. The protein and DNA sequences of P1 are open and those of P7 filled in black. Upper panel: the wild-type P1 ParA (A) ParB (B) complex recognizes the P1 operator site (O_{P1}) and the P1 partition site (S_{P1}) via species-specific contacts (open circles). Other species-specific contacts specify the proper ParA–ParB interaction and allow the proteins to fold properly. Lower panel: when the ParA protein from hybrid A5 and the ParB protein from hybrid B0.2 are combined, the resulting operon is fully functional (Figure 7), recognizing the P7 operator and P7 *parS* sites, but making use of the P1 determinants for the required ParA–ParB interaction.

or interdigitated series of contacting residues within a single protein.

Assuming that the mapped determinants (Figure 6) correspond to direct contact points between the components, a complex is indicated similar to that shown in Figure 9. For simplicity, a single heterodimer is shown and the proteins are shown attached simultaneously to the operator and the partition site; a situation which is not necessary for function, but which can presumably occur *in vivo*. Figure 9 also serves to illustrate how this structural interpretation can be applied to hybrid A5/B0.2 (Figure 7) which uses P7 determinants for operator and partition site recognition, but P1 determinants for the ParA–ParB interaction.

The structural interpretation used in Figure 9 is doubtless greatly oversimplified. Multiple ParA and ParB subunits are probably present (Hayes and Austin, 1993; Davey and Funnell, 1994; Lobocka and Yarmolinsky, 1996) and other contact points that do not impose species specificity are likely to be involved in the structure. Nevertheless, the overall topology of the complex or complexes formed are probably constrained in the way shown. A stable complex resembling this structure may carry out the functions of repression and partition simultaneously. However, it is also possible that the contacts mapped are not all formed at the same time or with the same individual molecules, i.e. they may occur during a series of sequential events which constitute the function of the Par system.

When the P1 ParB–*parS* complex encounters elevated levels of the P7 ParA region from residues 284–289, plasmid establishment is completely blocked. This phenomenon resembles that seen with mutant P1 ParA protein ParAM314I in the P1 *par* system. This protein interacts inappropriately with P1 ParB to block plasmid propagation (Youngren and Austin, 1997). It was suggested that such interactions might prevent the partition complex from dissociating from the plasmid DNA and hence block

subsequent replication (Youngren and Austin, 1997). Note that the M314I mutation and the P7 284–289 determinants are relatively close to each other in the aligned ParA sequences (Figure 1).

The HSS method employed here constitutes a genetic approach to understanding aspects of the tertiary (and possibly secondary) structure of complex interactions between macromolecules. The general method may be applicable to other systems where suitable homologs exist and suitable functional assays are available. The information gained, together with physical information as to the shape of the individual components, might provide unique solutions for the structure of complexes presently too large to be solved by physical methods. Partition is a dynamic process and it is quite likely that some of the interactions mapped here occur only transiently during the cell cycle. The general approach used here should permit the mapping of functionally important but transient interactions that might be difficult to map by most physical methods.

Materials and methods

Media, enzymes and materials

Bacteria were grown at 37°C in L-medium (Sambrook *et al.*, 1989) unless otherwise stated, with or without ampicillin at 100 µg/ml. Solid L-medium contained 1.5% agar with chloramphenicol at 10 or 2.5 µg/ml when necessary. MacConkey plates contained 1% lactose and 100 µg/ml ampicillin. Enzymes were obtained and used as previously described (Radnedge *et al.*, 1996). [γ -³²P]ATP was obtained from Amersham Corporation (Arlington Heights, IL). Other biochemical reagents were obtained from New England Biolabs (Beverly, MA).

Bacterial strains and bacteriophage

Strain CC2056 [λ xis6ind⁺ recA56 trp^{am} thi lac^{am} (Radnedge *et al.*, 1996)] was used for the partition tests, DH5 α (Sambrook *et al.*, 1989) for isolation of plasmid DNA and JM105 [F' traD36 lacI⁺ Δ (lacZ)M15 proA⁺B⁺/thi rpsL (Str^r) endA sbcB15 hsdR4 (r_K⁺ m_K⁺) Δ (lac-proAB) (Radnedge *et al.*, 1996)] for extracts used in ATPase assays.

DNA procedures

Plasmid DNA purification, DNA sequencing, oligonucleotide preparation and other DNA techniques were carried out as previously described (Radnedge *et al.*, 1996).

Synthesis of fragments with hybrid junctions

Fragments containing the junction regions of hybrid operons were constructed by a modified SOE PCR technique (Horton *et al.*, 1990) as previously described (Radnedge *et al.*, 1996). Primary overlapping products were produced using pALA1413 (P1 *par*) and pALA1414 (P7 *par*) DNA as templates. For some of the constructions, VENT polymerase (New England Biolabs) was used for the PCRs.

Vector and basic *par* operon plasmids

Plasmids pBR322 and pGB2 were as described (Bolivar *et al.*, 1977; Churchward *et al.*, 1984). Plasmid pGEX-2T was as described (Pharmacia Biotech Products catalog, 1998). Plasmids pALA1413, pALA1414, pALA1416 and pALA1462 consist of a *parA parB* operon, flanked upstream by an *EcoRI* site and downstream by an *XbaI* site, inserted into the *EcoRI*–*HindIII* sites of pBR322, using a *HindIII*–*XbaI* oligonucleotide linker (Radnedge *et al.*, 1996). The natural *par* promoters are present, but not the downstream *parS* sites. Plasmids pALA1413 and pALA1414 (Figure 2) have wild-type P1 and P7 *par* operons, respectively. Plasmid pALA1416 has a P1 *parA* gene followed by P7 *parB* and pALA1462 has a P7 *parA* gene followed by P1 *parB*. The *ParA*–*ParB* intergenic regions differ between the P1 and P7 operons but are functionally interchangeable (data not shown). The junctions of the hybrid operons are such that pALA1416 has the P1 intergenic sequence and pALA1462 the P7 sequence. The nucleotide sequences of the PCR-generated portions of all hybrid constructs were confirmed by automated DNA sequencing.

Plasmids with hybrid junctions in *parA* and *parB*

The hybrid plasmids used in Figures 4 and 5 have P1 *par* operons with the P1 *par* regulatory region and a portion of the P1 *parA* gene replaced by the equivalent P7 sequences. In each case, the P7 portion of the sequence was amplified by PCR using a primer with the leftward restriction site (see below) and a hybrid oligonucleotide (primer 1) designed to contain the desired crossover, and using pALA1414 DNA as a template. A P1 portion of the sequence was amplified using a primer with the rightward restriction site and a hybrid oligonucleotide (primer 2) complementary to primer 1, and pALA1413 as template. The products of these two PCRs were annealed and used to generate the appropriate hybrid fragment with leftward and rightward restriction site ends (Horton *et al.*, 1990). The ends of the fragment were trimmed at the leftward and rightward restriction sites and the fragment used to replace the corresponding fragment of the P1 *par* operon in pALA1413 or the hybrid operon construct pALA1462 to produce the hybrid protein constructs. Hybrid A0.1 through A3 used *EcoRI* and *SacII* as the leftward and rightward restriction sites and the fragment was inserted into the equivalent sites in pALA1413. Hybrids A4, A5 and A6 used *Bsu36I*–*MluI* and hybrids A5.1, A5.4 and A6.1–6.3 used *AgeI*–*MluI* inserted into the equivalent sites in pALA1462. The plasmid numbers of the final products are given in Figures 4 and 5. Variants of these plasmids that have hybrid junctions in *ParA* but have the *parB* gene deleted (Figure 5) were made by cutting the parental hybrid plasmid with *BglII* and *XbaI* and replacing the sequence between these sites with a double-stranded oligonucleotide (5'GATCTAACTGATAT plus 5'CTAGATATCAGTTA) which restores the end of *parA* and its stop codon, but results in the deletion of *parB*.

Plasmids containing the P1 regulatory region, P1 *ParA* and hybrid junctions in *ParB* (Figure 7) were generated as previously described (Radnedge *et al.*, 1996). Plasmids with junctions B0.1–B0.5 were made by constructing a fragment spanning the hybrid junction with *XhoI* and *HpaI* ends and using it to replace the equivalent fragment in the hybrid operon construct pALA1416. Plasmids with junctions B1–B6 were as previously described (Radnedge *et al.*, 1996).

Plasmids that have *par* operons under the control of a low-level constitutive promoter

Plasmids pALA1570 and pALA1571 are pBR322 derivatives in which the promoter–operator regions of the P1 and P7 *par* operons, respectively, have been substituted by a low-level constitutive promoter (Davis *et al.*, 1996). These operons produce the *Par* proteins constitutively at approximately normal physiological levels and are fully functional for partition. The *XhoI*–*BamHI* fragment of pALA1570 was excised and replaced by the equivalent fragment of pALA1416 to give pALA2147, thus putting the hybrid operon under control of the constitutive promoter. The *AgeI*–*NheI* fragment of pALA1571 was excised and replaced by an equivalent fragment of pALA1462, pALA1507, pALA2112, pALA2115, pALA2116 or pALA2117 to give plasmids pALA2143, pALA2144, pALA2141, pALA2142, pALA2145 and pALA2146, respectively, placing their respective hybrid operons under constitutive promoter control.

Plasmids that produce *ParB*–GST fusion proteins

Plasmid pALA1119 was made by cutting pGEX-2T with *BamHI* and ligating two fragments into it. The first was the *MunI*–*BamHI* fragment of pALA1413 containing most of P1 *parB*. The second was a double-stranded oligonucleotide consisting of the N-terminus of P1 *ParB* with *BamHI* and *MunI* ends (5'AATTGTTGGTCTGTTTTCTTTGAC-ATG plus 5'GATCCATGTCAAAGAAAAACAGACCAAC). Plasmid pALA1174 was produced as follows: pGEX-2T was cut with *BamHI* and a double-stranded oligonucleotide with *SphI*, *NsiI* and *XbaI* sites ligated to it (5'GATCGGCATGCATCTAGA plus 5'ATTTCTAGATG-CATGCC) to form pALA1163. This plasmid was cut with *SphI* and the 61 bp *NlaIV* N-terminal fragment from P7 *parB* ligated to it to form pALA1164. This plasmid was cut with *SphI* and *XbaI*, and the *SphI*–*XbaI* fragment of pALA1414 containing the rest of the P7 *ParB* ORF was inserted to form pALA1174. Plasmid pALA1166 was made by replacing the *MunI*–*XbaI* fragment of pALA1119 with the corresponding fragment from pALA1465.

Assays for repression of *par* transcription by *par* operon products

The pGB2-based reporter plasmids pALA1435 and pALA1463 are derivatives of plasmid pGB2 that have the P1 and P7 *par* operator–promoter regions, respectively, upstream of a *lacZ* transcription reporter gene (Hayes *et al.*, 1994). They were separately introduced into strain DH5 α by transformation selecting for spectinomycin resistance. The

resulting reporter strains were transformed by the *par* operon plasmid to be tested, and β -galactosidase assays carried out as previously described (Hayes *et al.*, 1994).

The colony color partition assay

Partition tests were performed by measuring the stability of mini-P1 *parS* plasmids when the Par proteins are supplied *in trans* from the operon to be tested. The *parS* plasmids contain a *supF* suppressor locus which renders the *lacZ* (amber) mutant host cells Lac⁺. The proportion of cells retaining the plasmid was assayed by plating the cells on MacConkey lactose indicator plates and counting the proportion of colonies that contain red pigment (Radnedge *et al.*, 1996). Mini-P1 plasmids pALA1952 (P1 *parS*) and pALA1993 (P7 *parS*) carrying the host *supF* gene were described previously (Radnedge *et al.*, 1996). These plasmids were incorporated into a λ phage vector by homologous recombination. The chimeric phage was then introduced by infection into the test strain containing the *par* operon plasmid to be tested. The λ -mini-P1 chimera forms a low copy number plasmid under P1 replication control which is very unstably maintained unless the strain supplies the appropriate Par proteins (Radnedge *et al.*, 1996).

ATPase assays

ATPase assays were carried out as previously described (Davis *et al.*, 1992, 1996) with modifications. Purified P1 and P7 ParA proteins were made as previously described (Davis *et al.*, 1996). The ParB proteins were produced as N-terminal fusions of the ParB protein to GST using the pGEX-2T cloning system. The proteins were purified using the Pharmacia GST purification module (Pharmacia Biotech Products). P1 ParB-GST was produced from pALA1119, P7 ParB-GST from pALA1174, and a ParB-GST with hybrid junction B1 from pALA1166.

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