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Genetics

Specificity determinants of the P1 and P7 plasmid centromere analogs

(partition/segregation/protein–DNA interactions/*parS*/integration host factor)

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ABSTRACT **The cis-acting *parS* sites of P1 and P7 are similar in sequence and promote active partition of their respective plasmid prophages to daughter cells when the cognate Par proteins are supplied. Forty of the 94 relevant bases differ between the P1 and P7 *parS* sites, and the protein-site interactions show complete species specificity. A method was developed to predict which subset of the differing *parS* bases is responsible. When the four P1 bases thus identified were substituted into the P7 *parS* site, a complete switch to P1 specificity was observed. The P1-specific bases constitute two CG dinucleotide elements situated 66 bp apart. They lie within repeats of the TCGCCA sequence implicated in secondary contacts with the P1 ParB protein. The equivalent TC dinucleotides in the P7 site were found to be involved in P7 specificity. However, three other P7 bases can also contribute, including two in the heptamer repeats primarily responsible for ParB binding, and the P7-specific information shows some redundancy. The motifs containing the specificity dinucleotides and the primary ParB binding (heptamer) sites bear no obvious relationship of spacing or orientation to each other. For the ParB protein to contact both types of motif at the same time, the topology of the interaction must be complex.**

Bacterial plasmids maintained at low copy numbers have acquired mechanisms to guarantee their faithful inheritance in growing cells (1). Critical among these are active partition systems expressly implicated in promoting segregational stability of a number of low-copy-number plasmids (1–3).

Bacteriophage P1 lysogenizes *Escherichia coli* as a highly stable plasmid with a copy number that can be as low as two per dividing cell (4, 5). This stability is dependent on the presence of an active partition cassette consisting of two essential genes, *parA* and *parB*, and an adjacent cis-acting site, *parS* (6–8). The *parS* site is sufficient to promote partition of a test plasmid if the protein products of *parA* and *parB* (ParA and ParB, respectively) are both supplied in trans (9). ParB binds specifically to a series of heptamer motifs in *parS* (10), where it interacts cooperatively with the host-encoded integration host factor (IHF) (10–12). IHF bends the *parS* site (13), probably wrapping it around a ParB–IHF protein core (11). ParA is an ATPase that is stimulated by ParB and double-stranded DNA but has not been shown to bind directly to *parS* (14).

The active partition system of the P7 prophage plasmid is organized similarly and is homologous to that of P1, indicating that these cassettes apparently are derived from a shared progenitor (15). However, the systems have diverged sufficiently that they demonstrate species specificity with respect to complementation, partition-mediated incompatibility, and partition-site activities (15, 16). Although the relative positions of the protein binding motifs in the P1 and P7 *parS* sites are conserved perfectly (refs. 15 and 16; see Fig. 1) and the sites are 57% identical over the 94-bp sequences illustrated in Fig. 1, the P1 *parS* site fails to work with the P7 proteins, and vice versa (ref. 16; see Fig. 1). Presumably, this specificity is due to key macromolecular interactions in which the individual components of the P1 and P7 systems are not interchangeable. The specific P1 nucleotides that determine site specificity must either be required for the cognate proteins to interact correctly with the site, or alternatively, they specifically prevent the proteins of noncognate type from doing so. In either case, by pinpointing the elements that determine this specificity, it should be possible to define crucial steps in the active partition pathway.

MATERIALS AND METHODS

Medium, Enzymes, and Materials. The following antibiotics were added to L medium (Sigma): chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 25 μg/ml. Sources of materials and enzymes have been described (16).

Bacterial Strains and Bacteriophage. *E. coli* HB101 (17) was used as a host for plasmid propagation and in cloning procedures. Partition-site assays used strains CC1531 (16), CC1532 (16), and CC1654. These strains are derivatives of CC1572 [*recA13 galK pro* Str^r (λ*xis6 ind*$^-$)] (16) that harbor pGB2, pALA480, and pALA283, respectively. Bacteriophage λ*c*I857-P1:5RΔ1005 (18) was maintained on strain YMC (19).

Plasmid DNA Procedures. Protocols for plasmid DNA manipulation and sequencing have been described (16, 20).

Plasmids. Plasmids pALA283 (8) and pALA480 (16) contain the P1 and P7 *parA* and *parB* genes in the pSC101-based vectors pLG338 (21) and pGB2 (22), respectively. Plasmid pALA1626 (16) is a P1 miniplasmid vector lacking the *par* genes and the *incA* copy-number-control locus. It replicates at a moderate copy number via the P1 origin (16). A set of pALA1626-based framework plasmids was constructed containing portions of the P1 or P7 *parS* loci flanked by restriction enzyme sites. These allowed insertion of synthetic double-stranded oligonucleotides to regenerate altered *parS* sites while maintaining the continuity of the junction sequences. For example, framework plasmid pALA1709 contains P1 *parS* regions II, III, and IV (see Fig. 1) into which altered region I sequences were cloned as synthetic oligonucleotides with *Hpa* I and *Bam*HI compatible ends to give plasmids pALA1735 and pALA1775. All plasmids containing altered or hybrid *parS* regions were made by similar strategies, and their resulting *parS* sequences are shown in the relevant figures.

Partition-Site Assays. Details of the pickup partition assay have been described (16, 23). Briefly, the *parS* site to be tested was inserted into the elevated-copy-number P1 mini-

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Abbreviation: IHF, integration host factor.

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plasmid pALA1626, and the resulting plasmid was incorporated into bacteriophage λcI857-P1:5RΔ1005 by homologous recombination. The phage provides the copy-number-control locus, *incA*, which is absent from pALA1626, and the resultant composite replicates at a low copy number (S.J.A., unpublished data). The composite containing the cloned *parS* site was introduced into a strain supplying the relevant Par proteins in trans. Its stability was estimated by measuring loss of the composite plasmid after ≈25 generations of unselected growth.

RESULTS

A Systematic Approach for Determining the Basis of Specificity in the Related P1 and P7 *parS* Sites. A protein-binding site can be regarded as a linear array of modular elements (the bases), which are recognized by specific features on the surface of the cognate proteins. The P1 and P7 *parS* sites, although similar, must differ in one or more of these critical contacts. The P1 nucleotides that differ from those in the P7 *parS* site must include all of the information responsible for P1 specificity. However, some of the bases unique to the P1 *parS* site may contain no relevant information and could therefore be altered without effect. Other bases may be critical for function, but not for specificity, such that either the P1 or P7 base is acceptable at that position. The key information for P1 *parS* site specificity must reside in the remaining nucleotides, which cannot be changed, even to the P7 type, without loss of function. Thus, it should be possible to identify the bases that determine P1 specificity by systematic substitution of P7 bases into the P1 *parS* site and assaying for loss of P1 site function. The identity of these bases can be confirmed by substituting them simultaneously into an otherwise intact P7 *parS* site, which should now switch to P1 specificity. The key bases responsible for P7 *parS* site specificity, which need not necessarily correspond to their P1 counterparts, could be determined by a reciprocal approach.

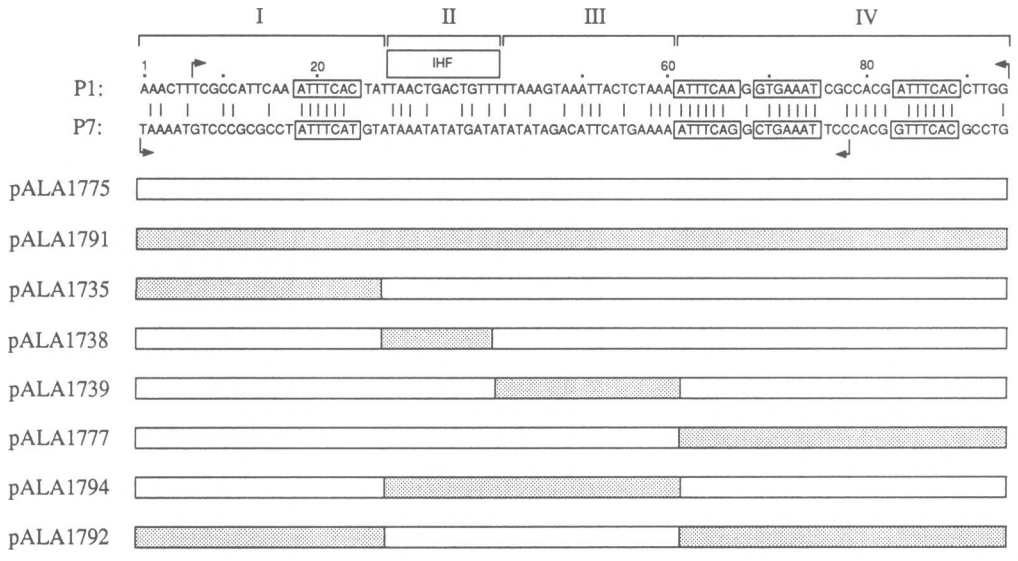
Approximate Localization of the Determinants for P1 *parS* Specificity. Partition-site activity was measured by using the pickup partition assay (16). This assay detects both P1 and P7 partition-site activities efficiently and defines homologous minimal P1 and P7 *parS* sites of similar lengths as illustrated in Fig. 1. It does not detect any activity for the truncated 22-bp P1 *parS* core sequence (bp 61–82, Fig. 1; unpublished data), which shows modest partition site activity in an alternate assay system (24). The P1 *parS* site was arbitrarily divided into four regions, and a series of P7 segment replacement experiments was conducted (Fig. 1). Regions II and III are fully interchangeable between the sites, either individually (pALA1738 and pALA1739) or simultaneously (pALA1792 and pALA1794) and therefore do not contain sequences that contribute to the specificity of the systems. These regions both appear to be involved in IHF protein binding (11, 16), with region II corresponding roughly to the IHF recognition consensus sequence (11, 16, 25). Region I of the P7 *parS* site can only partially substitute for the corresponding region in P1 *parS* (pALA1735), and region IV of the P7 site cannot replace the equivalent sequence in the P1 site (pALA1777). Substitution of P7 region IV into the P1 *parS* site (pALA1777) not only eliminated P1 *parS* site activity but also conferred partial function as a P7 site (Fig. 1). Therefore, the specificities of the P1 and P7 *parS* sites are determined by sequences in both regions I and IV.

Nucleotide Substitutions in Region I. The P1 and P7 *parS* sites differ at 15 of 27 positions in region I (Fig. 2). The crucial bases in this region that contribute to the specificity of the P1 site were identified by substituting subsets of P1-specific nucleotides with the appropriate P7 bases (Fig. 2). The three leftmost (pALA1750) and four rightmost (pALA1752) P1-specific bases in region I could be altered to the corresponding P7 bases without an appreciable effect on P1 *parS* site activity. However, all or some of the central eight nucleotides are necessary for full P1 *parS* site activity (pALA1751). An examination of the effect of replacing these eight bases individually with P7-specific bases revealed that only the C → T and G → C substitutions in pALA1763 and pALA1764, respectively, had any appreciable effect on P1 *parS* site activity. When these two alterations were combined (pALA1774), a marked reduction in P1 *parS* site activity was demonstrable. The simultaneous substitution of the remaining six nucleotides of the central region had no effect (pALA1773). Therefore, the modest requirement for P1 specificity in region I appears to be due just to the CG dinucleotide at the eighth and ninth positions (Fig. 2).

Nucleotide Substitutions in Region IV. The P1 and P7 *parS* sites differ at 9 of 34 positions in region IV (Fig. 3). A similar approach to that described in the preceding section was adopted to determine which of these bases contribute to P1 *parS* site specificity (Fig. 3). The five rightmost (pALA1779) and two leftmost (pALA1793) P1-specific nucleotides could be altered to the appropriate P7-specific bases without impairing P1 *parS* site ability. However, when the central CG dinucleotide sequence was altered to the corresponding P7 sequence, all activity as a P1 site was eliminated (pALA1789). Lesser effects were observed when these bases were changed separately (pALA1783 and pALA1784). Therefore, the CG dinucleotide at bp 76 and 77 should be the region IV determinant for P1 *parS* site specificity.

The CG Dinucleotide Pairs Determine P1 *parS* Site Specificity. To show that the P1-specific information does indeed reside in the two CG pairs in regions I and IV of the P1 *parS* site, a hybrid site was constructed consisting of the P7 *parS* sequence with just the four relevant bases changed to CG pairs. The resulting construct (pALA1799) was fully active when the P1 proteins were supplied, despite the fact that 90 of the 94 bp of the site are derived from the P7 sequence (Fig. 4*B*). The hybrid site displayed only marginal activity as a P7 site (Fig. 4*B*). The introduction of additional P1-specific material in region I did not appreciably increase P1 *parS* site activity but did eliminate the residual P7 activity (data not shown). Thus, one or more of these additional P1-specific bases play some minor role in restricting the specificity of the system to the P1 type. However, introduction of just the P1-specific CG dinucleotide elements in regions I and IV almost achieves a complete shift from P7 to P1 *parS* site specificity.

Specificity Determinants in the P7 *parS* Site. A systematic search for the P7 *parS* specificity determinants was not carried out using the strategy outlined above. However, the segment swap experiments described previously revealed that P7 *parS* specificity, like P1 *parS* specificity, was determined by elements in both regions I and IV of the site (Fig. 1). The critical nucleotides were identified directly by simultaneously replacing sequences in both regions I and IV of the P1 *parS* site with P7-specific sequences and screening the resulting constructs for a switch to P7 *parS* site specificity (Fig. 4*C*). In the simplest case, the critical determinants for P7 *parS* site specificity would consist of the P7 bases that occupy the same positions as the four critical P1 *parS* bases. This four-base exchange, consisting of a pair of TC dinucleotide elements, was insufficient to confer P7 *parS* site activity (pALA1917). Thus, the P1 and P7 *parS* site specificity determinants do not show a simple reciprocal relationship. One possible explanation might be that the TC dinucleotides are required for P7 *parS* site specificity but that some additional information is also needed. This information should reside within region I or IV (pALA1792; Fig. 1). In fact, such additional information was identified in region I (pALA1921; Fig. 4*C*) and was further narrowed down to the

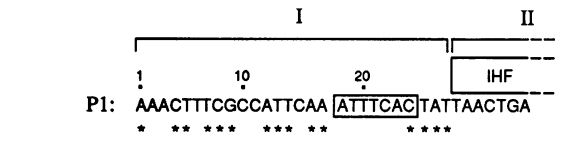


	% Retention	
	pALA283 (P1 *parAB*)	pALA480 (P7 *parAB*)
pALA1775	83 ± 6	<2
pALA1791	<2	75 ± 10
pALA1735	36 ± 4	4 ± 1
pALA1738	82 ± 7	<2
pALA1739	81 ± 12	<2
pALA1777	<2	35 ± 7
pALA1794	77 ± 1	<2
pALA1792	<2	74 ± 8

FIG. 1. Segment substitution experiments in the P1 and P7 *parS* sites. The sequence alignment of the two sites is shown with a common nucleotide numbering system applying only to this study. Identical nucleotides are linked by the vertical lines. Heptamer repeat motifs recognized by the ParB proteins (10, 16) and the consensus sequences for IHF binding (11, 16) are boxed. The bent arrows denote the approximate limits of the P1 *parS* site (unpublished data) and the precise limits of the P7 *parS* site (16) in the pickup assay system. Reassortment of the four regions (I–IV) shown above the sequences gave the hybrid *parS* sites illustrated below. Open and stippled areas indicate P1- and P7-derived sequences, respectively. Retention frequencies in pickup partition-site assays (see *Materials and Methods*) were determined in the presence of pALA283 and pALA480, which supply the P1 and P7 partition proteins, respectively. Averages of at least three individual experiments ± 1 SD are given. No retention was detected when the vector plasmid lacking Par protein production (pGB2) was present in trans.

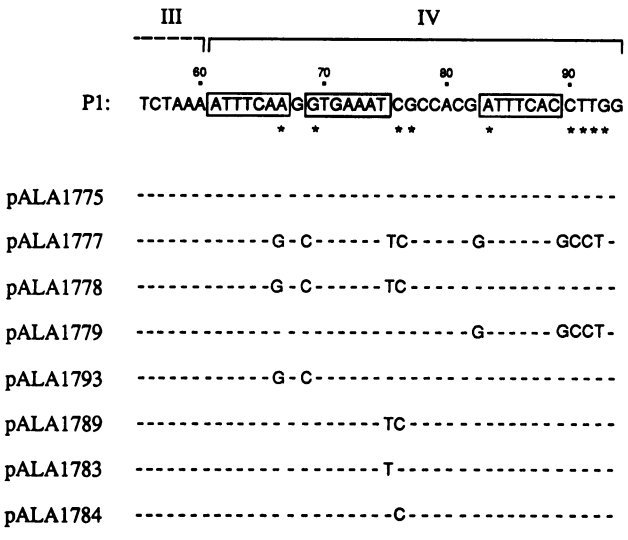
single G nucleotide at position 7 (pALA1931; Fig. 4*C*). The specificity of the P7 *parS* site can therefore be determined by five nucleotides (bp 7–9, 76, and 77), four of which constitute TC dinucleotide repeat sequences that map at corresponding positions to the CG dinucleotide sequences responsible for P1 *parS* site specificity.

Partial Redundancy of the P7 *parS* Specificity Determinants. In the course of searching for the postulated additional information required for P7 *parS* site activity, constructs were also made with additional P7-derived bases in region IV (Fig. 4*C*). When combined with the TC dinucleotide elements, the introduction of two P7-specific bases at positions



		% Retention	
		pALA283 (P1 *parAB*)	pALA480 (P7 *parAB*)
pALA1775	----------	83 ± 6	<2
pALA1735	T--AA-GTC--GCG-CT------TGTA-------	36 ± 4	4 ± 1
pALA1750	T--AA----------	84 ± 2	<2
pALA1751	------GTC--GCG-CT----------	22 ± 7	4 ± 3
pALA1752	----------TGTA-------	83 ± 7	<2
pALA1762	------G----------	80 ± 2	<2
pALA1763	-------T----------	63 ± 7	<2
pALA1764	--------C----------	57 ± 13	<2
pALA1765	----------G----------	85 ± 7	<2
pALA1766	----------C----------	87 ± 3	<2
pALA1767	----------G----------	85 ± 5	<2
pALA1768	----------C----------	89 ± 7	<2
pALA1769	----------T----------	81 ± 8	<2
pALA1773	------G----GCG-CT----------	80 ± 5	<2
pALA1774	-------TC----------	16 ± 9	<2

FIG. 2. Nucleotide substitution experiments in region I of the P1 *parS* site. Symbols correspond to those described in the legend to Fig. 1, except that nucleotides that differ between the P1 and P7 *parS* sites are denoted by stars beneath the P1 sequence. The set of plasmids used to define the key bases contributing to the specificity of the P1 *parS* site is shown. Alterations toward the P7 sequence are indicated by the appropriate bases. Dashes denote the presence of a P1 nucleotide. All constructs also contained regions II, III, and IV of the P1 site. Partition-site tests were performed as outlined in the legend to Fig. 1.



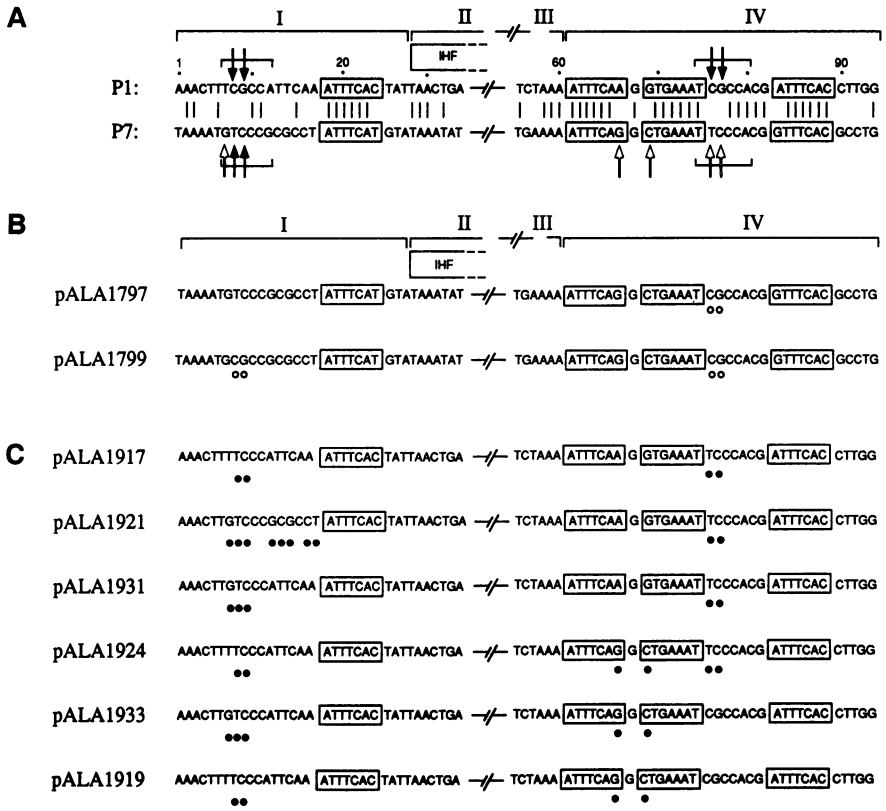
% Retention	
pALA283 (P1 *parAB*)	pALA480 (P7 *parAB*)
83 ± 6	<2
<2	35 ± 7
<2	32 ± 5
85 ± 6	<2
85 ± 5	<2
<2	<2
61 ± 7	<2
38 ± 12	<2

FIG. 3. Nucleotide substitution experiments in region IV of the P1 *parS* site. Symbols correspond to those described in the legend to Fig. 1, except that nucleotides that differ between the P1 and P7 *parS* sites are denoted by stars beneath the P1 sequence. The set of plasmids used to define the key bases contributing to the specificity of the P1 *parS* site is shown. Alterations toward the P7 sequence are indicated by the appropriate bases. Dashes denote the presence of a P1 nucleotide. All constructs also contained regions I, II, and III of the P1 site. Partition-site tests were performed as outlined in the legend to Fig. 1.

67 and 69 in region IV resulted in a functional P7 *parS* site (pALA1924). These two bases are symmetrically disposed in the P7 *parS* heptamer repeats. Further analysis (pALA1933) showed that an alternative set of five P7-derived bases also suffices for P7 *parS* site specificity (P7 residues 7–9, 67 and 69). Thus, different combinations of P7 bases (compare pALA1924, pALA1931, and pALA1933) can confer P7 *parS* site specificity and the relevant information is contained within seven nucleotides that show partial redundancy. However, as only some of the possible subsets of these seven bases (e.g., pALA1919) have been tested, it cannot be stated conclusively that the two sets of five P7-specific bases in pALA1931 and pALA1933 represent the simplest functional combinations.

DISCUSSION

The differing bases that determine specificity in two related cis-acting DNA sites (sites A and B) should define points at which key macromolecular interactions occur. If these critical differences are scattered among many trivial ones, it is not a simple task to find them. The systematic approach used



% Retention	
pALA283 (P1 *parAB*)	pALA480 (P7 *parAB*)
29 ± 1	66 ± 11
83 ± 4	14 ± 1
<2	7 ± 1
<2	61 ± 7
<2	65 ± 7
<2	73 ± 10
20 ± 6	72 ± 2
8 ± 4	15 ± 1

FIG. 4. Specificity shifting of the P1 and P7 *parS* sites. (*A*) Alignment of regions I and IV of the P1 and P7 *parS* sites. See Fig. 1 legend for symbols. The hexamer repeats are indicated by brackets above and below the sequences. Vertical arrows mark the bases involved in P1 and P7 species specificity; open arrows indicate P7 bases involved in redundancy. (*B*) Specificity shifting of the P7 *parS* site. The indicated constructs consist of P7 sequences except for P1-specific bases, which are denoted by open circles beneath the sequences. (*C*) Specificity shifting of the P1 *parS* site. The indicated constructs consist of P1 sequences except for P7-specific bases, which are denoted by filled circles beneath the sequences. Partition-site tests were performed as outlined in the legend to Fig. 1.

here may have some general application. The relevant site A bases are identified as those that cannot be changed to the site B type without loss of site function. The choice of bases is refined by using block substitutions prior to testing the remaining individual bases. The critical site A bases, thus identified, are substituted together into an otherwise normal site B. If the base identification is correct, the specificity then switches from the site B to the site A type, as was found with the four bases identified in the P1 *parS* site. The approach assumes independence of information conferred by each relevant base and a lack of redundancy. Redundancy would have prevented the approach from working for the P7 *parS* site. For example, although the P7 TC dinucleotide (bp 76–77) can contribute to P7 specificity (pALA1931; Fig. 4), its replacement by P1 bases does not lead to a significant loss of function as a P7 site (pALA1797; Fig. 4), due to redundancy. Fortunately, useful information about the relevant P7 bases could be determined directly, based on the assumption that the critical P1 and P7 bases might be similarly placed.

Two CG dinucleotide elements disposed at opposite ends of the P1 *parS* site are necessary and sufficient to confer complete P1 *parS* site activity to an otherwise P7 *parS* site. Similarly, two TC dinucleotide elements at identical positions play an important role in P7 specificity (Fig. 4). What is the function of these sequences? First, the roles of the dinucleotides at each end of P1 *parS* are probably the same because they are nested within direct hexamer repeats: TCGCCA at bp 7–12 and bp 75–80 (Fig. 4). The integrity of the rightmost copy of this repeat was previously shown to be important for partition (24, 26). The equivalent P7 6-bp sequences that contain the TC dinucleotides can also be regarded as direct hexamer repeats ($^{G}_{T}$TCCC$^{G}_{A}$). In this instance, the significance of the rightmost repeat is less clear, because this motif is involved in redundancy with region I and the last two bases of this repeat can be deleted without effect (16). Second, the dinucleotides are probably recognized by ParB. They are included in regions protected by the appropriate ParB proteins in both systems (10, 16) and are in close contact with ParB, at least in the P1 *parS* site (26). However, the heptamer repeat sequences are also required for ParB binding (10, 16), and a single heptamer unit acts as an individual ParB binding site (16). By comparing the ends of each *parS* site, it can be seen that the heptamer repeats and the dinucleotide-containing hexamer repeats show no simple relationship of spacing or orientation to each other (Fig. 4). Thus, they are not likely to be parts of an extended ParB binding consensus sequence. Funnell and Gagnier (26) recently confirmed the importance of both types of repeat in the P1 *parS* site and provided evidence that both P1 motifs are recognized by ParB. This probably occurs via separate sites on the ParB protein. Presumably, ParB protein contacts the two recognition elements in some topologically complex fashion. This can be envisioned most easily if each ParB protein joins two separate plasmids by a pairing reaction or bridges the ends of a single *parS* site in a looped structure. The latter seems plausible, because the ends of the *parS* site are thought to be aligned through IHF-mediated looping (11, 13). IHF protein bridging of the *parS* site ends might explain the partial redundancy observed with the P7 specificity elements in which the absence of P7-specific nucleotides in region IV can be compensated for by the presence of additional P7 information in region I (Fig. 4*C*).

The specificity determinants of the P1 and P7 *parS* sites are not reciprocal, as the P7 determinants include extra bases and a degree of redundancy. An extra P7 base in region I (bp 7), adjacent to the leftmost P7 dinucleotide (bp 8–9), can compensate for the lack of the rightmost P7 dinucleotide (bp 76–77) in region IV as long as P7 bases 67 and 69 are also present. These latter bases conserve the perfect inverted heptamer repeats of the primary ParB binding sequence, converting it to its P7 form (Fig. 4). Presumably, the P7 versions of the heptamer repeats are more favorable for P7 ParB binding than the P1 equivalents, thereby contributing to specificity. Preliminary *in vitro* binding studies appear to confirm that the P7 ParB protein preferentially recognizes the heptamer repeats in P7 *parS* but that the P1 ParB protein interacts equally well with either the P1 or P7 heptamer boxes (M. A. Davis and S.J.A., unpublished data). The presence of redundant P7 information may explain why some of the hybrid *parS* sites confer significant partition activity with both sets of Par proteins (e.g., pALA1797 and pALA1933; Fig. 4). Presumably this is due to the presence of both the critical P1 region IV determinant at bp 76–77 and the P7 heptamer box information at bp 67 and 69.

Our results with the P7 determinants indicate that heptamer box information can compensate for suboptimal dinucleotide-containing hexamer repeats and that region I information can compensate for suboptimal region IV information. The available assay is not precise enough to rule out some minor advantage of having all these elements present together as in the original P7 site. However, the elements clearly show considerable redundancy. These effects are most easily explained if the regions involved contact each other in a cooperative complex, consistent with our suggestion that the two ends of such *parS* sites contact each other by looping around a compact protein core (11).

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