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Helical-repeat dependence of integrative recombination of bacteriophage λ: Role of the *P1* and *H1* protein binding sites

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ABSTRACT **The efficiency of site-specific recombination of bacteriophage λ was found to depend on the spacing between distant protein binding sites. Insertions and deletions of up to 30 base pairs were made in the nonessential regions between the *H1* and *H2* protein binding sites. Recombination was found to occur in substrates with changes of integral multiples of a DNA helical repeat, whereas recombination was defective in substrates with nonintegral changes. The λ recombinogenic complex is especially interesting because two different proteins are involved: integration host factor (IHF), which has been shown to bend DNA, and the phage-encoded integrase protein (Int), which has been shown to have two distinct DNA-binding domains. The importance of angular displacement of protein binding sites was confirmed by addition of ethidium bromide to defective substrates. Significant stimulation of recombination was observed when sufficient drug intercalated and unwound the DNA to allow improved orientation of sites. The orientation effects are dependent on supercoiling, as spacing is less important in conditions where supercoiling and the *P1–H1* sites are not required for recombination.**

Higher-order structures have been postulated for generating specific complexes capable of stimulating DNA replication, recombination, transcription, and other cellular events that require specificity and precision (1). One example of such a system is that responsible for executing site-specific recombination in bacteriophage λ (reviewed in refs. 2 and 3). Integrative and excisive recombinations involve two DNA substrates and up to four proteins binding to multiple sites (4–8). These components interact in a way that allows the reactions to be tightly regulated as a function of cellular physiology (8–10).

Site-specific recombination is employed by λ to stably maintain the lysogenic state (11, 12). This developmental pathway is a means of preserving its host population and is initiated when the phage DNA (*attP*) integrates into a specific location on the *Escherichia coli* genome (*attB*). When the appropriate physiological signals are present, lysogeny is terminated by excision of the phage DNA and the lytic pathway is followed. Both the integration and the excision reactions require the virus-encoded integrase protein (Int) and the bacterium-encoded integration host factor (IHF) (13, 14). The virus-encoded excisionase (Xis) is required for excisive recombination and inhibits integrative recombination (15), whereas the bacterium-encoded factor for inversion stimulation (FIS) modulates the excision reaction (8). Each of these proteins has been purified and can be used in *in vitro* recombination reactions.

Int is a type I topoisomerase that carries out the cutting and ligation during strand exchange (16–18). It binds to two distinct DNA consensus sequences: the core-type sites that border the points of strand exchange in each DNA substrate and the arm-type sites that are located distal to this region in the phage DNA (4, 5). IHF, which bends the DNA (refs. 19–21; J.F.T. and A.L., unpublished results), binds to three sites on the phage DNA and is an accessory factor in this reaction, being required for the formation of a recombinogenic structure but not for either strand cleavage or ligation (6, 18).

Studies employing nuclease protection, chemical modification, and electron microscopy (22–26) have indicated that the recombination proteins bind to their DNA substrates and interact to form a higher-order complex. Evidence for cooperative and competitive interactions among proteins in this complex has been obtained. In particular, long-range interactions involving an Int (*P1*) and IHF (*H1*) binding site with proteins bound at least 100 base pairs (bp) distant have been observed (23, 24), with one of these interactions requiring supercoiled DNA (24). Supercoiling of *attP* has also been found to be necessary for efficient integrative recombination *in vivo* or *in vitro*, with a monovalent salt concentration above 40 mM (27). At less than 40 mM salt, supercoiling is not required (28).

In certain other systems in which long-range protein–protein and protein–DNA interactions play a role, the face of the helix on which the binding sites are located is more important than the distance between sites. Varying the normal spacing by integral numbers of helical turns allows retention of activity, whereas nonintegral numbers of turns results in a reduction or loss of activity. Examples of this include repression of transcription by AraC, LacI, and λ repressors; enhancement of transcription in simian virus 40; and stimulation of *hin* inversion by FIS (29–36). To see whether long-range interactions present in the more complicated structures involved in integrative recombination behave in a similar fashion, a series of *attP* substrates with different spacings between the *P1–H1* sites and the core-*P'* arm was examined in a range of recombination conditions.

MATERIALS AND METHODS

The construction of plasmids pWR101 (22); pJT17 (9); pJT29 (10); pJT3, pJT27, and pJT38 (23); and pJT111 (37) has been described. Plasmids with altered spacing were constructed by using three related strategies: (*i*) cleavage of the parent(s) with a restriction enzyme and ligation; (*ii*) cleavage of the parent(s) with a restriction enzyme, filling in the 3′ protruding end with DNA polymerase (large fragment), and ligation; and (*iii*) cleavage of the parent with a restriction enzyme, filling in the 3′ protruding end, ligation of a single, unphosphorylated linker to each 3′ end, and annealing as described (23, 37). The strategy and parent(s) of each *attP* are shown in Table 1. All plasmids were sequenced with a kit from New England Biolabs by using recommended procedures and had the sequences expected except for pJT59, in which one base from the inserted linker was lost during construction (shown in parentheses in the pJT60 sequence in Fig. 1).

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Abbreviations: EtdBr, ethidium bromide; IHF, integration host factor; FIS, factor for inversion stimulation.

Table 1. Generation of spacing mutations

Spacing	Plasmid	*P1–H1*	Core-*P'*	Filled in	Linker
−31	129**	38N	111N	−	
−29	128**	38N	111N	+	
−20	115*	111N	111N	−	
−18	114*	111N	111N	+	
−15	131**	60B	115N	+	
−11	38	27N	27N	−	Ref. 23
−9	88	27N	27N	+	
−8	133**	73B	111N	+	
−7	132**	71B	111N	+	
−2	65	27S	38N	+	
−1	39*	see legend		−	
0	27	3H	3N	−	Ref. 23
1	176**	39N	39N	+	
2	8	3N	3N	+	
4	51	27S	27S	+	
7	59	27S	3N	+	B10
8	60	27S	3N	+	B10
9	75	59N	59N	+	
10	98	60N	60N	+	
11	126	59B	59B	+	
12	127	60B	60B	+	
15	64	60B	3N	+	B10
17	92	64N	64N	+	
19	93	64B	64B	+	
21	73	60B	27S	+	B10
23	71	60B	27S	+	B12
27	94	71B	71B	+	
29	99	94N	94N	+	
31	106	99N	99N	+	

All of the plasmids with spacing mutations are listed with their change in spacing relative to wild-type (column 1) and their plasmid number (column 2). All plasmids additionally have the prefix pJT. Most of the changes in spacing were made in the *P2* binding site (Fig. 1). Plasmids marked * have changes in the *X2* site, whereas those marked ** have changes in both *P2* and *X2*. pJT39 was constructed from a plasmid that had a spontaneous deletion of 1 bp (−81). Columns 3–6 describe the construction of each plasmid. The parent plasmids donating the *P1–H1* region and the core-*P'* region are listed in columns 3 and 4, respectively, with the plasmid number of the DNA and the restriction enzymes used to cleave each abbreviated as N (*Nde* I), B (*Bgl* II), H (*Hin*fI), or S (*Sal* I). Column 5 signifies whether or not the single-stranded ends generated by the restriction enzymes were filled in with DNA polymerase prior to ligation, and column 6 lists the linkers inserted between ends, where applicable (B10, GAAGATCTTC; B12, GGAAGATCTTCC). These data can be used to generate the sequence of each plasmid with the exception of pJT59, which lost a single base during construction (noted in parentheses in the pJT60 sequence in Fig. 1).

Recombinations were carried out overnight in 25 mM Tris·HCl, pH 7.9/6 mM spermidine/5 mM EDTA/5 mM dithiothreitol containing bovine serum albumin at 1 mg/ml. The NaCl, protein, and DNA concentrations are listed in each figure legend. Purification of proteins, labeling of *attB*, and electrophoresis of products were as described (23). The agarose gels were autoradiographed after drying, and the amount of recombination was quantitated with an LKB Ultroscan 2220 laser densitometer.

RESULTS

A series of plasmids was constructed with altered spacing between the region of strand exchange and the distal *P1* and *H1* protein binding sites required for integrative recombination. All the changes were made in regions not required for integrative recombination with most being in the *P2* Int binding site. This protein binding site is involved in regulating recombination but is not required for integration (23, 38). The construction of these 29 DNAs with insertions or deletions of up to 31 bp is described in *Materials and Methods* and outlined in Table 1 and Fig. 1 along with data that allow reconstruction of the sequences.

Because IHF binding to the *H1* site is sensitive to some mutations outside the consensus sequence, recombination conditions of high Int and IHF concentrations were chosen to minimize any such effects (23). In these conditions, no significant difference in recombination efficiency between wild-type *attP* and an *attP* mutated in the *P2* site with no spacing change was observed. The recombination efficiency of *attP* DNAs with spacing mutations is shown in Fig. 2. Mutants with approximately one helical turn of DNA either inserted or deleted are nearly as efficient as wild type. Mutants with differences of two helical turns are far less efficient but still noticeably better than substrates with changes of half-helical-turn increments.

To show that these defects are caused by protein binding sites being on the wrong face of the DNA helix, conditions were altered to allow twisting of the helix to restore the proper orientation of sites. Ethidium bromide (EtdBr) is known to intercalate into DNA and unwind it by 26° per molecule bound (40). If sites are out of phase with respect to wild-type orientation, they should be correctable by the intercalation and unwinding caused by the binding of drugs in the region between the interacting protein binding sites.

The effect of added EtdBr on four different *attP* DNAs is shown in Fig. 3. Wild-type *attP* is unaffected by up to 12.5 μM EtdBr but is then progressively inhibited by increasing amounts of the drug. Concomitant with this inhibition is a reduction in the relaxation of unreacted supercoiled *attP* by Int topoisomerase (data not shown). This is caused by a reduction in the effective superhelical density rather than by a direct enzymatic inhibition of Int. Int topoisomerase activity is present at high EtdBr concentrations, based on the increased supercoiling of DNA evident after removal of EtdBr [as also observed with topoisomerase I (41)].

In contrast to wild-type *attP*, the partially defective *attP* with a 2-bp insertion is stimulated by 3–12.5 μM EtdBr, with

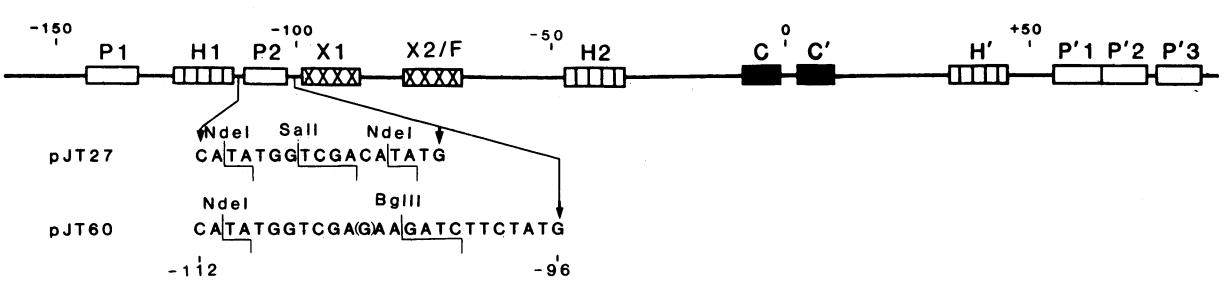

FIG. 1. Protein binding sites in wild-type *attP*. The consensus binding sequences for Int (arm-type, □; core-type, ■), IHF (□), and Xis (□) present on *attP* are drawn to scale. Also, FIS can bind to the *X2/F* site instead of Xis. The center of the core region is designated nucleotide 0, and positions to the left and right are given negative and positive numbers, respectively (39). The sequences for two representative *attP* DNAs with mutations are shown below the *P2* region. Relevant restriction sites are noted on both. The nominal numbering of the first and last bases shown is −112 and −96. The base deleted in pJT59 (G) is indicated in the pJT60 sequence.

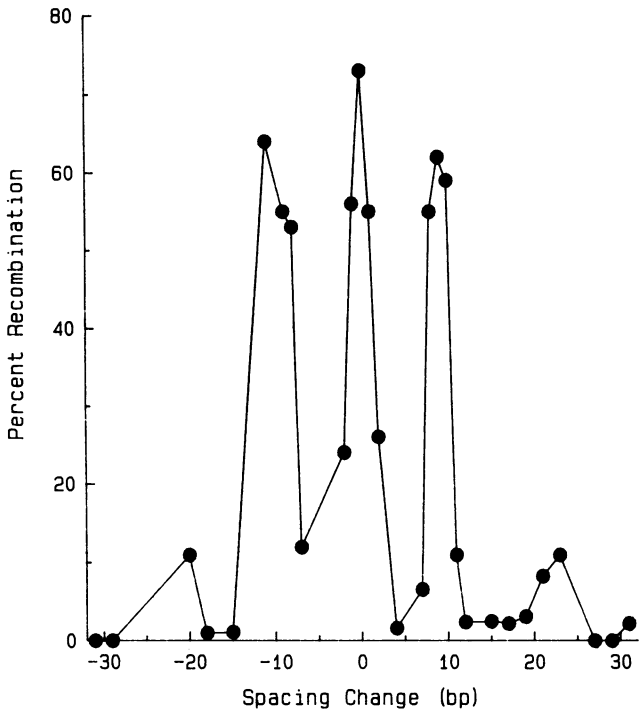

FIG. 2. Spacing dependence of integrative recombination. The efficiency of integrative recombination is plotted versus the change in spacing between wild-type *attP* and the *attP* being tested. The final level of recombination is shown so that both competent and defective *attP* DNAs could be compared at a single time point; however, for the *attP* DNAs for which data could be obtained, the initial rate of reaction was directly related to the final extent (data not shown). Recombinations were performed with 1 nM supercoiled *attP*, 1 nM linear *attB*, 2 units of Int, and 1 unit of IHF at 75 mM NaCl in 20 μl.

recombination going from 30% to 45%. At higher concentrations, it is also inhibited by the loss of superhelical density caused by the drug. With no EtdBr, an *attP* with a 4-bp insertion is completely defective (<0.1% recombination). However, addition of 6 μM EtdBr results in detectable recombination with maximal recombination at 25 μM EtdBr (at least 30-fold stimulation relative to no EtdBr). An *attP* with a 7-bp insertion is inhibited at a lower EtdBr concentration than any of the other *attP* DNAs tested, with a complete loss of function at 25 μM. An *attP* whose defect is in the *P1* site rather than spacing remains completely defective at all EtdBr concentrations (data not shown).

To see whether the spacing effect is dependent on supercoiling, conditions in which supercoiling is not required for recombination (low salt) were tested (27). No linearized *attP* examined (including wild-type) is competent for recombination at salt concentrations >40 mM, but efficient reaction can be obtained at low salt (Fig. 4). Reaction of wild-type and −11 *attP* DNAs are relatively unaffected by linearization, yielding >50% recombinants, whereas *attP* DNAs that are highly defective when supercoiled are enhanced 2- to 9-fold, yielding 14–18% product when linearized. Cleavage adjacent to the mutated sites with *Nde* I restriction endonuclease results in the separation of the *P1–H1* region from the remainder of the *attP* region by 3000 bp and renders all of the tested *attP* DNAs identical in function. This suggests that the *P1–H1* sites can still play a role in stimulating integration in low salt but are not absolutely required. *attP* DNAs with deletions of the *P'2 P'3* or the *P'1 P'2 P'3* Int sites are completely defective in all conditions tested (data not shown).

DISCUSSION

These data show that the long-range interactions between the *P1–H1* sites and the core and/or *P'* regions that are required

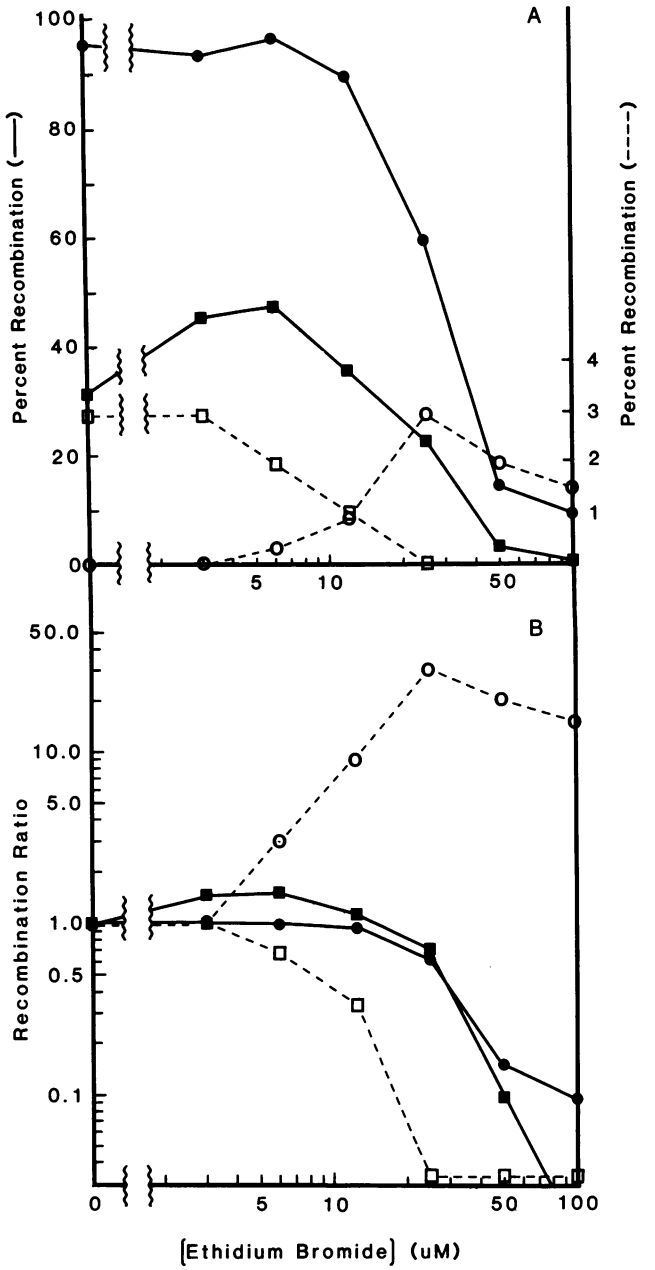

FIG. 3. EtdBr dependence of recombination. The efficiency of recombination is plotted versus EtdBr concentration for *attP* DNAs with spacing changes of 0 (●), +2 (■), +4 (○), and +7 (□). The solid symbols correspond to the left scale and the open symbols correspond to the right scale. Reactions were carried out with 1 nM supercoiled *attP*, 1 nM linear *attB*, 4 units of Int, and 1 unit of IHF at 75 mM NaCl. EtdBr was added 10 min after the start of the reaction to allow formation of the recombinogenic complex without competition from bound drug. No significant recombination had taken place at that time.

for proper function are dependent on the correct angular displacement of these sites. Variation of the length of DNA between these interacting regions results in periodic changes in recombination efficiencies that are most easily explained by postulating that the interacting sites must be on the same face of the DNA helix. Substrates with the wrong phasing of sites are unable to properly align even with random, thermal fluctuations of DNA torsion angles. However, the alignment of distant sites can be altered by untwisting the DNA with the aid of EtdBr. Intercalation of a small number of drug molecules allows the sites to be reoriented so that *attP* DNAs with insertions of 2 or 4 bp attain a better alignment of sites

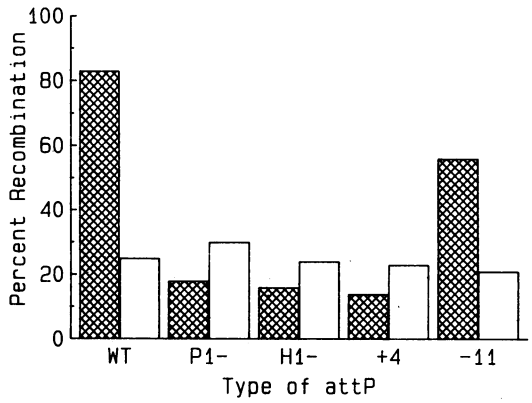

FIG. 4. Linear, low-salt recombination. The efficiency of a typical set of recombination reactions is plotted for each of the *attP* DNAs examined. Reactions were carried out with 1 nM linear *attP*, 0.2 nM linear *attB*, 8 units of Int, and 3 units of IHF at 27 mM NaCl. The *attP* DNAs were linearized with either *Nco* I (which cuts outside of the minimal *attP* on the *P'* side) (crosshatched bars) or with *Nde* I (which cuts between the *P2* and *X1* binding sites) (open bars). Reaction efficiency is measured by the amount of *attB* that recombined. Qualitatively similar results were obtained with other protein and DNA concentrations.

and recombination is stimulated as a function of EtdBr concentration. As predicted, higher concentrations of EtdBr are required to maximally stimulate an *attP* with a 4-bp insertion than one with a 2-bp insertion. Similarly, an *attP* with a 7-bp insertion is inhibited at lower EtdBr concentration than any other *attP* because its suboptimal orientation is made even worse by EtdBr. Presumably, this *attP* would regain function at higher concentrations were it not for the loss of superhelicity.

The stimulation of recombination of an incorrectly oriented *attP* is especially dramatic because it is superimposed on the inhibitory effects of EtdBr. Binding of EtdBr results in the loss of negative supercoiling, and the efficiency of integration is directly related to superhelical density (24). Five or six intercalated molecules should be required to restore the proper alignment of an *attP* with a 4-bp insertion. Since there are about 70 bp between *H1* and *H2*, not more than one EtdBr molecule per 12 bp should be required. This is approximately the amount necessary to completely relax the plasmid, assuming all binding sites have equal affinity. When the EtdBr concentration is increased above that necessary for maximal stimulation, no relaxation of the supercoiled substrate by Int topoisomerase is seen, indicating that the plasmid is fully relaxed at that drug concentration. This suggests that even greater stimulation could be achieved if supercoiling were not a factor. Furthermore, such a high loading of drug molecules on the DNA could also lead to direct competition between proteins and drugs for binding sites. Thus, the inhibition of wild-type recombination by added EtdBr can be explained in several ways in addition to the loss of proper phasing between distant sites, but the stimulation of a normally inefficient *attP* can best be explained by reorientation of distant sites.

Systems thought to include a DNA loop closed by a protein–protein linkage can be divided into two general classes: those with large loops in which the interacting points can encounter one another during thermal motions in the DNA and those with small loops in which protein–protein interactions or protein-induced DNA bending must play a role. With large loops the DNA acts as a tether to locally increase the concentration of bound protein (42), the DNA being sufficiently flexible that binding sites can freely rotate for optimal interactions. Examples of this type of interaction include transcriptional enhancer proteins and suitably placed *lac* repressor sites (42–44).

As the DNA length is reduced, there is a sharp drop in the local concentration of tethered protein due to the stiffness of the DNA. Superimposed on this is a dependence on helical phasing caused by the lack of free rotation, as observed with DNA cyclization kinetics (45). When small loops are closed, the DNA cannot rotate to allow alignment of sites, generating the pronounced periodic pattern of interaction seen here and in other systems (46). Evidence for these small loops has been obtained by DNase I digestion, chemical reactivity, gel mobility shifts, and electron microscopy (22–26, 29–35). In some, but not all, of these loops, supercoiling is required for interaction. Additional evidence for a looped structure in *att* DNA has been found from topological analysis of recombinant products (47, 48).

The λ *att* complex is especially interesting because it differs from most of the other systems examined in that it has two different proteins involved in generating the loop. One of these, IHF, is likely to assist in this process by bending the DNA (refs. 19–21, J.F.T. and A.L., unpublished results) and facilitating close approach of distant regions. However, binding of a bend-inducing protein within the loop structure will not necessarily promote loop formation, as a bend in the wrong direction can prevent proper interactions. This has been observed with binding of catabolite gene activator protein (CAP) between two AraC sites (30) and may also be one reason that Xis (which bends DNA) is able to inhibit integrative recombination. Closing of the loop may be due to Int–Int interactions or, because each Int molecule has two DNA-binding domains (4, 50), DNA–Int–DNA interactions.

The capacity of *attP* to accept a DNA arm that varies by 40 bp has implications for the structure of the complex involved. Although the nucleoprotein complex observed by electron microscopy was described as "nucleosome-like" (25), it seems unlikely that the *P*-arm DNA is smoothly wrapped around a protein core. The entire *attP* DNA must wrap around at least once to yield products with the observed topological properties (47, 48), but much of this DNA cannot be required for interaction with protein because of the large insertions and deletions that are allowable.

While *attP* DNAs with an integral number of helical turns inserted or deleted recombine better than their half-helical-turn counterparts, there is a large damping of recombination efficiency superimposed on this periodicity. This has also been observed to varying extents with other systems in which distant sites require proper angular orientation. The relatively symmetric effect on recombination efficiency of insertions and deletions of the same size is not necessarily expected because of the energetic costs associated with making the smaller loop. The slight differences observed (e.g., recombination maxima at −11 and +9) may be caused by the specific sequences inserted and deleted or may be providing some structural information about the complex. Flexibility within the Int protein between the different DNA-binding domains (L. Moitoso de Vargas, C. Pargellis, and A.L., unpublished results) may be important in permitting these large length variations.

The contrasting behavior of the various *attP* DNAs indicates that different recombination pathways are followed at high and low salt concentrations. At high salt, both supercoiling and proper spacing of sites are required for the proper assembly of a recombinogenic complex. At low salt, these are dispensable for reaction (although both can still affect it). This suggests that their role in normal (high salt) recombination can be assumed by an alteration in the properties of some other component in the reaction. One difference between high- and low-salt conditions is the affinity of Int for core-type sites. Core binding to *attB* is observed at low salt and is undetectable at high salt. Richet *et al.* (24) have

proposed that *attB* comes in "naked" (of Int) to the *attP* complex at high salt and is stabilized by interactions with Int molecules bound to the arm sites. Extending this model, the Int bound at *P1* would participate in the interaction between *attB* and *attP* to promote recombination in a supercoiling-dependent manner. At low salt, Int could bind to *attB* independently of *P1* stimulation. *attB* could then synapse with *attP* without supercoiling or correctly positioned *P1–H1* sites. The role of the *P'* sites in the recombination reaction is likely more complicated, as they are required even at low salt. The excision reaction may also have altered requirements in low salt (49).

The role of helical phasing in promoting λ recombination provides insight into the mechanism of the reaction and the structure of the protein–DNA complexes that mediate it. The utilization of small, constrained loops provides a means of incorporating multiple proteins, long stretches of DNA, and supercoiling into the regulation of a variety of cellular processes. These looped structures are likely to be present in other systems with regulatory regions encompassing hundreds of base pairs and provide a general mechanism for integrating many inputs (such as the IHF, FIS, and Xis modulation of recombination) into a given regulatory event.

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