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A switch in the formation of alternative DNA loops modulates λ site-specific recombination

(higher-order structures/DNA bending/bacteriophage)

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ABSTRACT **The virally encoded Xis protein is one of the components in the site-specific recombination reactions of bacteriophage λ. It is required for excisive recombination and inhibits integrative recombination. The mechanism of Xis inhibition of the integration reaction was investigated by methylation protection assays (footprinting analyses) in conjunction with recombination assays. Xis is shown to mediate the formation of a specific *att*P looped structure involving cooperative and competitive long-range interactions among integrase, integration host factor, and Xis proteins. This higher-order structure precludes supercoiled *att*P from engaging in the productive partner interactions that lead to execution of the first strand exchange in integrative recombination. In addition to its previously characterized role in excision, Xis-induced DNA bending is postulated to act as a regulatory switch (in an alternative loop mechanism) that converts the *att*P intasome from an integrative-competent complex to a nonreactive one.**

Two site-specific recombination reactions of bacteriophage λ direct the integration and excision of phage DNA into and out of the *Escherichia coli* chromosome. Each one is characterized by a distinct set of long-range interactions that are required for the formation of specific higher-order structures. In this paper, we describe a set of long-range interactions that are responsible for Xis inhibition of integrative recombination.

λ integrative recombination between the 240-(bp) base-pair phage attachment site (*att*P) and the 25-bp bacterial attachment site (*att*B) requires supercoiling of the *att*P partner, the virally encoded integrase protein (Int), and the bacterially encoded integration host factor (IHF) (for reviews, see refs. 1 and 2. Two additional proteins, the phage-encoded Xis and the host-encoded factor for inversion stimulation (FIS), are involved in excisive recombination between the two prophage *att* sites (*att*L and *att*R). In both recombination reactions, the exchange of the top strands precedes that of the bottom strands (3, 4). Strand exchange is carried out by Int, a type I topoisomerase with two DNA binding domains (5–9). It binds, by way of its carboxyl-terminal domain, to "core-type" sites flanking the region of strand exchange, and, by way of its amino-terminal domain, to "arm-type" sites that are distal to the crossover region (see Fig. 1). The major role of the sequence-specific accessory factors IHF, Xis, and FIS is to induce the sharp DNA bends that are required for the formation of recombinogenic structures (10–13). Although FIS is not an essential protein and cannot replace Xis, it stimulates the Xis-dependent reactions when Xis is limiting (ref. 14; this work).

The Xis and FIS proteins, in addition to their role in excisive recombination, also inhibit integrative recombination (14–16). This paper addresses the mechanism(s) by which they exert their inhibitory effect. Previous analyses of three and four recombination proteins binding to *att*P were carried out with linear molecules, which are not competent substrates for integrative recombination (17–19). We show here that binding of Xis to supercoiled *att*P prevents Int and IHF from binding to those sites that are occupied only during integrative recombination. The binding alterations are not due to simple occlusion of sites by bound Xis. Rather, they result from long-range cooperative and competitive interactions that require the core sites, the arm sites involved in excision, and the correct DNA helical phase. Formation of the Xis-mediated *att*P structure correlates with Xis inhibition of the first strand exchange in integration. The results presented here are consistent with a model in which Xis-induced DNA bending acts as a switch that converts the *att*P intasome from an integrative-competent complex to a nonreactive one.

MATERIALS AND METHODS

The construction of plasmids pWR1, pPH201 (20), pJT17 (21), pJT27 (18), pJT29 (22), pJT115 (23), pSN8, and pSN108 (3) has been described. pLV8 is an *att*P plasmid derived from pLV1 (24), in which the *Eco*RI site at position +13 in the *att* DNA was replaced by an *Sph* I site at +12, and the pBR327 sequences between 401 and 1283 as well as the phage DNA from +94 to +241 were deleted. Plasmids pLV9, pLV10, and pLV11 were constructed by cleavage of pLV8 with *Bst*BI (+48) and *Nco* I (+94) and ligation of a 46-bp synthetic oligonucleotide carrying the mutations in the P'1, P'2, and P'3 sites, respectively (24). pVL14 was made by cleaving pLV8 with *Xho* I (−13) and *Sph* I (+12) and replacing the intervening 27 bp with a double-stranded *Xho* I–*Sph* I synthetic oligonucleotide containing 4 additional bp (see Fig. 3). pLV13 was constructed by cleavage of pLV8 with *Sph* I and insertion of a single *Sph* I–*Sma* I adaptor (Genetics Design, Houston) (Fig. 3). pLV24 was obtained by cleaving pLV8 with *Xho* I, filling in the 3' ends with the Klenow fragment of DNA polymerase, and ligating to recircularize. pLV26 was constructed by cleaving pLV8 with *Xho* I and inserting a single *Sal* I–*Sma* I adaptor (New England Biolabs). pLV25 was made by ligating a gel-purified 1380-bp *Xho* I–*Pst* I fragment from pLV9 with a gel-purified 1012-bp *Pst* I–*Xho* I fragment from pSK8 (a P2$^-$ *att*P-containing plasmid) (S. Kim and A.L., unpublished). Plasmids pBF8-12 and pBF9-12 are *att*P-containing plasmids that carry the previously described mutations (25) at the C' and C core sites, respectively (B. Franz and A.L., unpublished). pSN66 is an *att*R-containing plasmid in which an *Xho* I site was introduced at −13 (S. Nunes-Duby and A.L., unpublished).

Plasmid DNA was prepared either as described (26) or with the Quiagen kit as recommended by Quiagen (Studio City, CA). Preparation of *att*B suicide substrate prenicked at the right exchange site was carried out as described (3). ^{32}P labeling of DNA was accomplished with [γ-^{32}P]ATP and

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

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polynucleotide kinase (5′ ends) or with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase (3′ ends). Digestion with an appropriate restriction enzyme was used to obtain fragments with a single end labeled.

Methylation protection experiments with supercoiled plasmid DNA (1–2 nM) and the proteins at the concentrations indicated in Fig. 2 (expressed as recombination units per 20 μl ref. 27) were carried out in 0.2 ml of 3.25 mM Tris·HCl, pH 7.4/50 mM NaCl/50 mM sodium cacodylate, pH 8.0/5 mM spermidine/1 mM 2-mercaptoethanol/1 mM EDTA/1 mg of bovine serum albumin per ml/1% (vol/vol) glycerol. When present, IHF was incubated with the DNA for 10 min prior to addition of Xis and Int. After modification with dimethyl sulfate, the DNA was digested with an appropriate restriction enzyme and 3′ end-labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase. Cleavage of the DNA at the sites of modification was accomplished with the adenine guanine reaction of Maxam and Gilbert (28) and gel electrophoresis was carried out as described (9).

Standard recombination assays were carried out in 20 μl of 75 mM NaCl/25 mM Tris·HCl, pH 7.9/6 mM spermidine/5 mM EDTA/2 mM dithioerythritol/1 mg of bovine serum albumin per ml/1–10 nM DNA. For certain recombination assays the reactions were carried out in the buffer specified for dimethyl sulfate modification experiments as indicated. Purification of proteins, electrophoresis, and quantitation of products were as described (27).

RESULTS

Xis-Mediated *attP* Structure. There are a total of 15 binding sites for the recombination proteins on *att* DNA; however, different combinations of sites are used for integration and excision (see Fig. 1 *A* and B). Some protein binding sites are required for both reactions, some (P1, P′3, and H1) are used exclusively for integration, and others (P2 and P′1) are used only for excision (14, 18, 21, 24, 29, 30). To study those sites involved in Xis inhibition of integration, methylation protection experiments were carried out on supercoiled *attP* DNA, which is the topological state required for the integration reaction (17) (see *Materials and Methods*). Binding by Int, IHF, and Xis alone can be seen as diminution and/or enhancement of the methylation pattern obtained in the absence of protein (Fig. 2*A*, lanes 1–6). In the absence of Xis, the previously proposed Int and IHF binding site occupancies for the integrative mode are observed (18, 31): occupancy of all IHF binding sites, lack of P2 protection, and occupancy of the remaining Int binding sites with the core and P1 sites exhibiting enhanced protection (Fig. 2*A*, lanes 7–9). However, addition of Xis alters this binding pattern: binding of Int to P1 is decreased, Int binding to P2 is stimulated, and binding of Int to P′3 and of IHF to H1 is inhibited (Fig. 2*A*, lanes 10–12).

Footprinting analyses with *att*Ps carrying mutations in single protein binding sites indicated that the arm sites involved in excision, P2, P′1, P′2, H2, H′, and X1X2/F, as well as the core sites were necessary for the Xis-dependent interactions (Table 1). Similar results were obtained with nuclease protection experiments on linear *attP* DNA (data not shown). Thus, Xis mediates the formation of a unique *attP* structure. This structure depends upon those protein binding sites that are required for excision and it excludes the binding of Int and IHF to those sites that are used only during integrative recombination (see Fig. 1*C*).

Helical Phase Dependence. An *attL* recombinogenic complex was previously shown to depend upon the bending protein IHF and the two-domain Int protein (12). To determine whether the Xis-mediated *attP* structure also involves DNA looping, its dependence on the helical phase of the P and P′ arms was ascertained. Four *attP* mutants were constructed. In two of them, the helical phase of the P (pLV24) or P′ (pLV14) arm was changed approximately a half-helical turn by insertion of 4 bp. In the other two (pLV26 and pLV13), the helical phases (of P and P′, respectively) were changed approximately one helical turn by insertion of 10 bp (Fig. 3).

The protein binding patterns of the supercoiled *attP* spacing mutants were examined by methylation protection and compared to those of the *attP* parent (pLV8). The 10-bp insertion mutants preserve the binding characteristics of the wild type for the integrative and the Xis-mediated complexes (Table 1). In contrast, the 4-bp insertion mutants fail to exhibit the IHF-dependent stimulation of Int binding to the

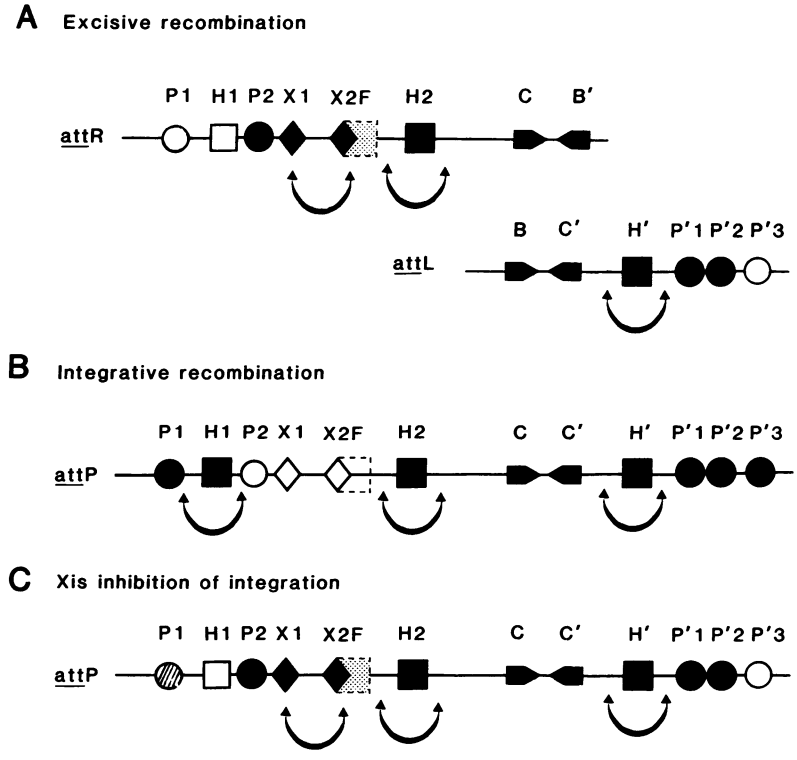

FIG. 1. Patterns of protein binding and DNA bending in excisive, integrative, and Xis-mediated complexes. Protein binding in *att* complexes for each of the three conditions (*A–C*) is indicated by filled symbols at the binding sites for IHF (□) (H1, H2, H′), Xis (◇) (X1, X2), FIS (▨), Int arm-type (○) (P1, P2, P′1, P′2, P′3), and Int core-type (■) (C, C′, B, B′). (Occupancy of C and B′ in *attR* is inferred from the requirements of the recombination reaction.) Protein-induced bending in each complex is indicated (↻). In all of these interactions, Xis at X2 can be replaced by FIS at F (▨) (ref. 3; this work). Protein binding and DNA bending patterns are shown for *attR* and *attL* complexes in excisive recombination (*A*), for the supercoiled *attP* complex in integrative recombination (*B*), and for the supercoiled Xis-mediated *attP* complex that is recombinationally inert (*C*).

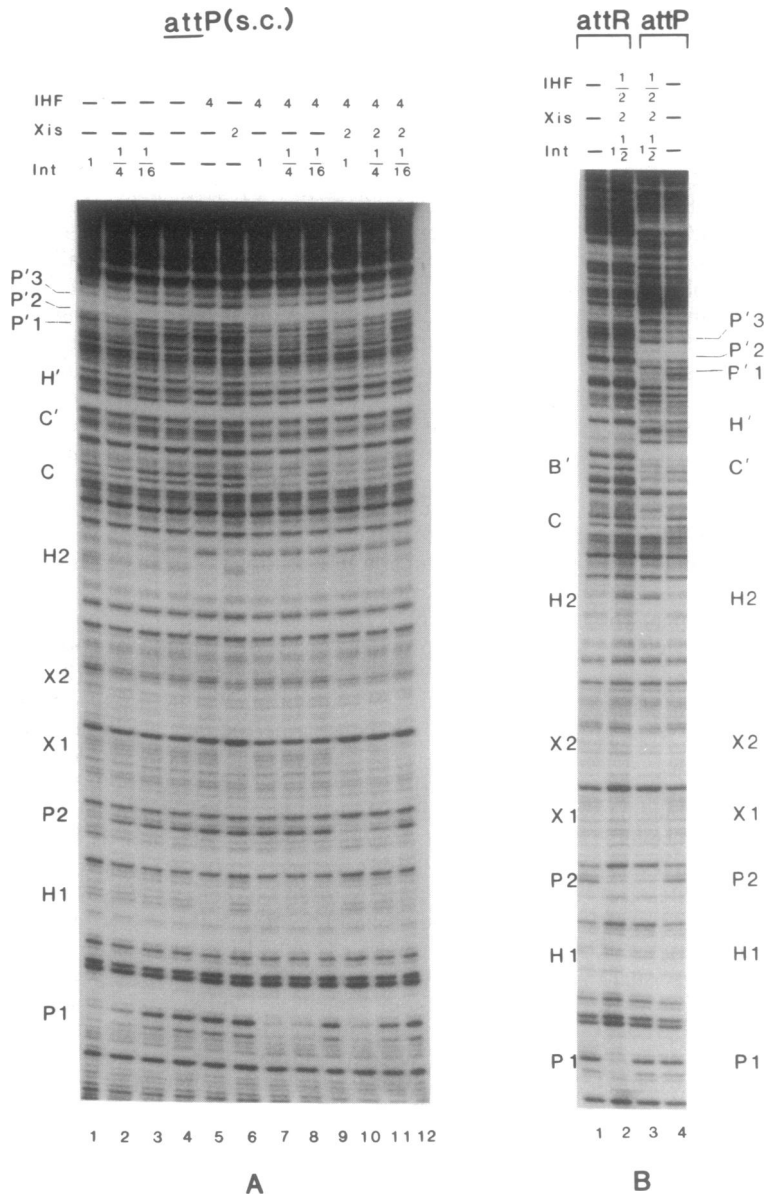

FIG. 2. Footprinting analyses of Xis-dependent interactions. Methylation protection assays with the proteins in the unit amounts (recombination units per 20 μl) indicated above each lane were carried out on supercoiled DNA that was subsequently digested with restriction enzyme and end-labeled with ^{32}P. The protein binding sites are noted to the left or right of the gel ladder using the symbols shown in Fig. 1. (*A*) The supercoiled (s.c.) *att*P plasmid pLV8 was analyzed by 3′ end-labeling the bottom strand at the *Hin*dIII site. (*B*) The supercoiled *att*R plasmid pSN66 in an excisive reaction mixture and the resulting supercoiled *att*P product were analyzed by 3′ end-labeling the bottom strands at the *Hin*dIII site.

core and P1 sites. Furthermore, they behave differently in response to Xis: the inhibition of Int binding to P′3 and IHF binding to H1 and the stimulation of Int binding to P2 are not observed (Table 1).

To correlate the protein binding patterns with recombination function, assays were carried out with each of the *att*P phasing mutants. The recombination efficiencies for the 10-bp insertion mutants (64% and 71% for the P and P′ arm mutants, respectively) were similar to the wild type (72%), and recombination with the 4-bp insertion mutants was undetectable (data not shown). The observed phase dependence does not by itself establish the existence of DNA looping. However, in conjunction with other available data (10–13), these results provide further support for a higher-order looped complex in *att*P during integrative recombination. Furthermore, they suggest that a loop structure is responsible for the Xis-mediated interactions described here.

Correlation Between the Xis-Mediated Structure and Xis Inhibition of Integration. If the Xis-dependent interactions in the *att*P complex are responsible for Xis inhibition of integration, the two phenomena should occur under the same reaction conditions and also have the same protein binding site requirements. To satisfy the first test, the supercoiled *att*P substrate used in the methylation protection assay was recombined with

Table 1. Behavior of *att*P plasmids in complex formation

Plasmid	Characteristics of *att*P sequence	Integrative complex	Xis-mediated complex
pWR1	Canonical sequence	+	+
pLV8	*Xho* I, *Sph* I	+	+
pJT17	P1⁻	−	+
pJT27	P2⁻	+	−
pJT29	H1⁻	−	+
pJT115	X2∇	+	−
pBF8-12	CO∆′8	−	−
pBF9-12	∆9OC′	−	−
pLV9	P′1⁻	+	−
pLV10	P′2⁻	−	−
pLV11	P′3⁻	−	+
pLV25	P2⁻, P′1⁻	+	−
pLV13	+10 bp in P′ arm	+	+
pLV14	+4 bp in P′ arm	−	−
pLV24	+4 bp in P arm	−	−
pLV26	+10 bp in P arm	+	+

The ability (+) or the inability (−) to undergo the formation of an integrative (Fig. 1B) or Xis-mediated (Fig. 1*C*) complex is shown for the *att*P plasmids whose *att* sequence characteristics are noted.

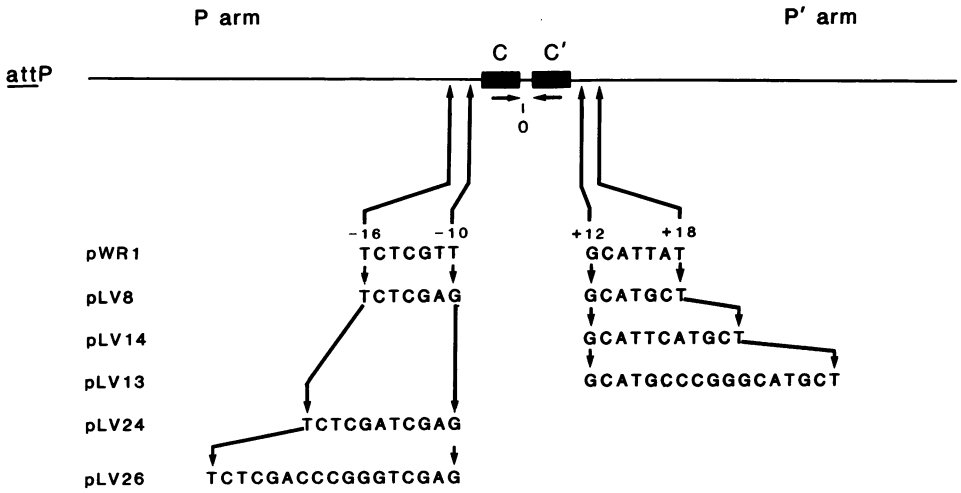

FIG. 3. DNA sequence changes in plasmids with mutant *att*P sites. The 0 position of the coordinates for *att*P is flanked on the right by positive numbers, the C' core-type Int site (■), and the P' arm protein binding sites. On the left it is flanked by negative numbers, the C core-type Int site, and the P arm protein binding sites (see Fig. 1 for more detail). The *att*P DNA in pLV8 deviates from the canonical sequences in pWR1 by the introduction of an *Xho* I site at −13 and an *Sph* I site at +12 and is the parent of the +4 and +10 insertion mutants shown below it.

a labeled linear *att*B partner under the conditions shown in Fig. 2*A*. Consistent with the proposed mechanism, recombination proceeds well under conditions corresponding to lanes 7–9 (Fig. 2*A*), and it is inhibited by the addition of Xis, corresponding to lanes 10–12 (data not shown).

Evaluation of the binding site requirements is complicated by the fact that Xis inhibition of integration can only be studied with complexes that are competent for integration. This limits the analysis to those sites that are dispensable for the integration reaction and are also required for the Xis-dependent methylation protection patterns. Two protein binding sites fulfill these criteria, P2 and P'1 (Table 1). The double mutant $P2^-$, $P'1^-$ was constructed and shown to be competent for integrative recombination, as expected (Fig. 4). When analyzed by methylation protection assays this mutant failed to exhibit the Xis-dependent methylation protection patterns characteristic of wild-type *att*P (Table 1). Concomitantly, the mutant has become at least four times more resistant to Xis inhibition of integration than wild-type *att*P, at 0.5 unit of Xis (Fig. 4). It should be noted that the Xis concentrations conferring inhibition of integration in the wild-type *att*P are equivalent to those required to promote excisive recombination. The inhibition of $P2^-$, $P'1^-$ that is observed at higher Xis concentrations may be due to a transient occupancy of the mutant Int binding sites that is too unstable to be detected in footprinting experiments (see Table 1), or it may result from nonspecific (aberrant) interactions.

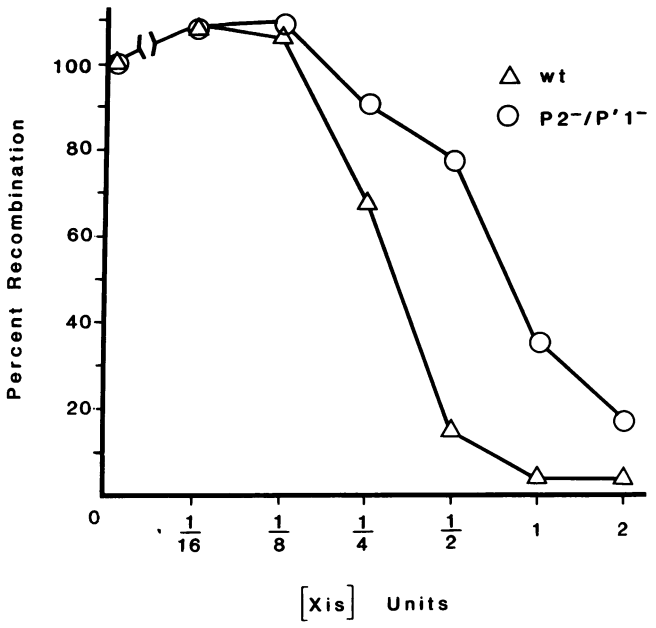

FIG. 4. Xis inhibition of integrative recombination. Integrative recombination assays were carried out with supercoiled *att*P and linear *att*B. The recombination efficiencies for the wild-type (△) and $P2^-$, $P'1^-$ (○) *att*Ps were normalized to 100% in the absence of Xis (32% and 26%, respectively).

Reaction Steps Affected by Xis Inhibition. To determine which step of integrative recombination is inhibited by Xis, a suicide substrate with a nick at the bottom strand cleavage site was constructed as described (3) (see diagram at top of Fig. 5). Recombination of this substrate with a supercoiled *att*P allows the first strand exchange of recombination to occur but inhibits the second strand exchange. This leads to the accumulation of reaction intermediates, predominantly in the form of α structures, and to trace amounts of a nicked linear recombinant (3). As shown in Fig. 5, addition of Xis to the reaction abolishes formation of the reaction intermediate as well as the nicked recombination product. Thus, Xis-mediated interactions lead to the formation of an *att*P complex that is incapable of carrying out the first strand exchange of integrative recombination.

Another level at which the Xis-mediated interactions might be important is preventing reversal of the excision reaction. An excisive reaction was carried out in which the *att*R (pSN66) and *att*L (pPH201) partners were both supercoiled. The protein binding patterns of the supercoiled *att*P product and the initial *att*R partner were then examined by methylation protection (Fig. 2B). The protection patterns of the *att*P

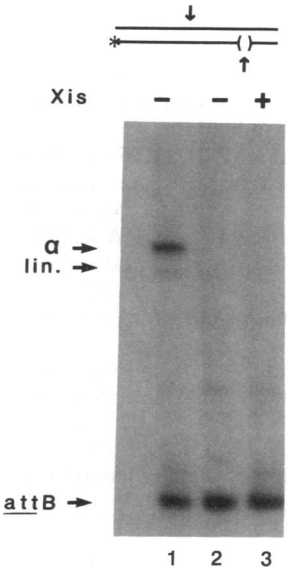

FIG. 5. Integrative recombination between a supercoiled *att*P and a linear *att*B suicide substrate nicked at the right (bottom) exchange site. A linear *att*B fragment from pSN108, containing a nick at the right exchange site, was prepared and 3' end-labeled on the bottom strand at the *Bam*HI site (see diagram above) as described (3). It was recombined with the supercoiled *att*P plasmid pSN8 under normal reaction conditions. In the absence of Xis (lane 1), the major product is the expected α structure recombination intermediate (α) and a small amount of nicked linear recombinant (lin.). No products are seen in the presence of Xis (lane 3) or the minus protein control (lane 2).

recombination product were identical to those obtained with the Xis-mediated *att*P complex, except for binding to the P1 site (compare lanes 10–12 in Fig. 2*A* to lane 3 in Fig. 2B). Although Int binding to P1 is reduced in the Xis-induced *att*P complex, this binding is totally abolished in the *att*P resulting from recombination. This more prominent effect in the latter case is expected since these molecules were selected by excisive recombination and P1 is not required for this reaction (21, 29). These data indicate that the Xis-dependent interactions correlating with inhibition of integration are also present in the *att*P that results from excisive recombination. Therefore, another role of Xis could be to prevent reintegration after excision.

DISCUSSION

The data presented here provide insight into the mechanism and structure responsible for Xis inhibition of integrative recombination. Xis is shown to mediate a number of cooperative and competitive interactions that secure the *att*P–protein complex in a specific, recombinationally inert structure. Xis-induced DNA bending promotes interactions in the P and P′ arms that (*i*) decrease Int binding to P1, (*ii*) stimulate Int binding to P2, (*iii*) inhibit IHF binding to H1, and (*iv*) preclude Int binding to P′3 (Fig. 2). The multiple Xis-dependent interactions are difficult to understand in terms of protein binding sites arrayed on a linear DNA since many of the observed effects are quite far from the Xis binding site (P′3 is 140 bp from X1X2). The simplest model of these long-range interactions postulates DNA loops, generated by the bending proteins IHF and Xis/FIS and tethered by the bivalent Int protein. This view is consistent with the dependence upon the core-type Int sites and the correct helical phase of the P and P′ arms.

The integrative supercoiled *att*P complex probably consists of a P arm loop, promoted by the IHF-induced bends at H1 and H2, and a P′ arm loop, promoted by IHF at H′. These two loops would allow interactions between those arm-type Int binding sites necessary for integration and the core-type Int sites of *att*P and *att*B (Fig. 1B). In the Xis-mediated *att*P complex (Fig. 1*C*) a bend shift in the P arm loop from H1 to X1X2/F leads to the recombinationally inert structure defined by the interactions described above.

These models are consistent with several lines of evidence for a looped structure in the *att*P recombinogenic complex (23, 32–35), with the previously established protein binding site requirements for integration (18, 21, 22, 29), and with the recent finding that Xis decreases the cleavage efficiency of an *att*P heteroduplex (19). Xis has previously been shown to promote the initial steps of excisive recombination (36, 37). The results presented here suggest that it may favor the lytic pathway in two additional ways: by inhibiting integrative recombination after infection and by making the excisive reaction irreversible after prophage induction.

The action of Xis in switching the *att*P–Int–IHF complex into a nonreactive mode is consistent with the recently proposed role for DNA bending proteins as regulatory switches (12). Regulatory schemes involving DNA looping have been described in a number of prokaryotic and eukaryotic systems (for reviews, see refs. 38 and 39). The additional feature of two DNA bending proteins acting as the switch for alternative DNA looping is unlikely to be unique. Specific binding sites for DNA bending proteins are being found in an increasing number of replication, transcription, and recombination systems. Utilizing DNA bending proteins as the switch in an alternative loop mechanism would allow the incorporation of several control signals and the coordinate regulation of complex systems involving multiple proteins and long regions of DNA.

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