the sensitivity in a simple experiment to approximately one in 10^{13} for a 1-s integration time.

Numerous improvements in the sensitivity of this very simple scheme can be envisioned. (i) Overall 100-s counting times are reasonable. (ii) The material in the sample can be enriched in heavy isotopes, which could readily improve the sensitivity by several more orders (at least three) of magnitude. (iii) Gated detection of the ions, following a simple time-of-flight scheme that rejects all ions with masses less than 30 GeV (mass HF + 10 GeV), would greatly reduce wing noise. Rejection ratios for HF should be at least 10^3. Thus a realistic ultimate sensitivity that might be achieved for REMPI searches for HNe^x in HF is roughly 1×10^{-20}.

Conclusions

Our considerations of galactic chemical evolution have provided new insight into the optimum chemical environments in which to search for X^- particles, with B (corresponding to C^x) being the best candidate of the stable nuclides. Other good candidates include F (corresponding to Ne^x) and Tc (corresponding to Ru^x).

Although a variety of experiments have been conducted to search for stable X^- particles, none has successfully located them as yet. The least ambiguous abundance limit appears to be that obtained for a B sample, 1×10^{-12} to 1×10^{-14} for X^- masses from 100 to 10,000 GeV, although the limit obtained from the H sample also infers some interesting information. Laser spectroscopic techniques can be used to search for stable X^- particles. Further searches of the types already performed for such particles should be undertaken, in view of the importance that their discovery would have on particle physics and possibly on astrophysics.

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Research Articles

DNA Looping Generated by DNA Bending Protein IHF and the Two Domains of Lambda Integrase

LINA MOITOSO DE VARGAS, SUNGHOON KIM, ARTHUR LANDY

The multiprotein-DNA complexes that participate in bacteriophage lambda site-specific recombination were used to study the combined effect of protein-induced bending and protein-mediated looping of DNA. The protein integrase (Int) is a monomer with two autonomous DNA binding domains of different sequence specificity. Stimulation of Int binding and cleavage at the low affinity core-type DNA sites required interactions with the high affinity arm-type sites and depended on simultaneous binding of the sequence-specific DNA bending protein IHF (integration host factor). The bivalent DNA binding protein is positioned at high affinity sites and directed, by a DNA bending protein, to interactions with distant lower affinity sites. Assembly of this complex is independent of protein-protein interactions.

PROTEIN-INDUCED DNA BENDING AND PROTEIN-MEDIATED DNA looping contribute to the overall structure of higher-order complexes in several transcription, replication, and

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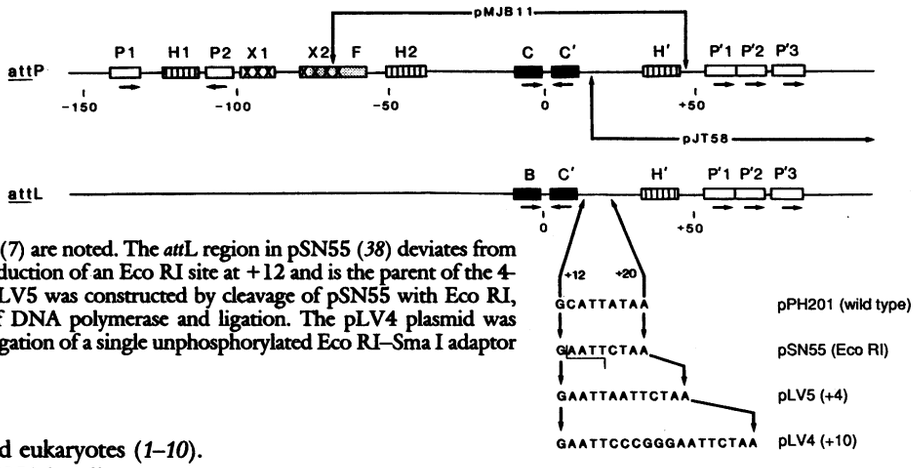

Fig. 1. Protein binding sites in *attP* and *attL* and their relation to relevant plasmids. The coordinates for *attP* and *attL* show 0 within the 7-bp overlap, with positive numbers to the right (P' arm) and negative numbers to the left (P arm or B arm) (*14*). The relative orientations of Int binding sites are indicated by arrows for both arm-type (P1, P2, P'1, P'2, P'3) and core-type (C, C', B) sites. Also indicated are the binding sites for IHF (H1, H2, H'), Xis (X1, X2), and FIS (F). The *attP* sequences contained in pMJB11 (*16*) and pJT58 (*7*) are noted. The *attL* region in pSN55 (*38*) deviates from the canonical sequences in pPH201 (*34*) by the introduction of an Eco RI site at +12 and is the parent of the 4-bp (pLV5) and 10-bp (pLV4) insertion mutants. pLV5 was constructed by cleavage of pSN55 with Eco RI, filling in the 3' ends with the Klenow fragment of DNA polymerase and ligation. The pLV4 plasmid was constructed by cleavage of pSN55 with Eco RI and ligation of a single unphosphorylated Eco RI–Sma I adaptor (New England Biolabs).

recombination systems in both prokaryotes and eukaryotes (*1–10*). Although the mechanisms of protein-induced DNA bending are not fully understood, much has been learned from the detailed analysis of CAP (catabolite gene activator) binding (*11*). In most documented cases of DNA looping, two distant but similar DNA sites are linked by a multimeric protein (a homodimer or a homotetramer). Formation of DNA loops is considered an efficient means of incorporating several proteins and distant DNA regions into complex regulatory systems requiring specificity, precision, and flexibility (*2*).

We describe a system in which both protein-induced bending and protein-mediated looping cooperate in a highly organized and specific manner to form the functional higher order complexes that comprise the site-specific recombination system of bacteriophage lambda (λ). In this structure, the binding of two different and distant DNA sequences by a protein with two autonomous DNA binding domains, the integrase (Int) protein of λ, is facilitated by DNA bending induced by IHF (integration host factor).

The site-specific recombination reactions of the λ virus include four proteins and 15 protein binding sites that comprise the four DNA substrates (each containing an attachment, *att*, site). The virally encoded Int and Xis proteins and the bacterially encoded IHF and FIS (factor for inversion stimulation) are participants in excisive recombination between the prophage *att*L and *att*R sites. Only Int and IHF are required for integrative recombination between the phage and bacterial *att* sites (*att*P and *att*B) (*12*).

Int, a type I topoisomerase, is directly responsible for the cleavage and rejoining reactions that take place during strand exchange at the borders of the homologous 7-bp "overlap" regions of two recombining partners (*13–15*). The Int protein binds to two classes of DNA sequences (Fig. 1). The "core-type" sites overlay each of the four points of strand cleavage, and the five "arm-type" sites are distal to the region of strand exchange (*16*). Recognition of these two classes of sequences is achieved via the two autonomous DNA binding domains of Int—arm-type sites by the amino-terminal domain and core-type sites via the carboxyl-terminal region (*17*).

IHF is a sequence-specific DNA binding protein that affects many cellular processes (*18*). It induces sharp DNA bends in various physiological situations that may or may not involve the binding of other proteins (*4–7*). Similarly, HU, a nonspecific DNA binding

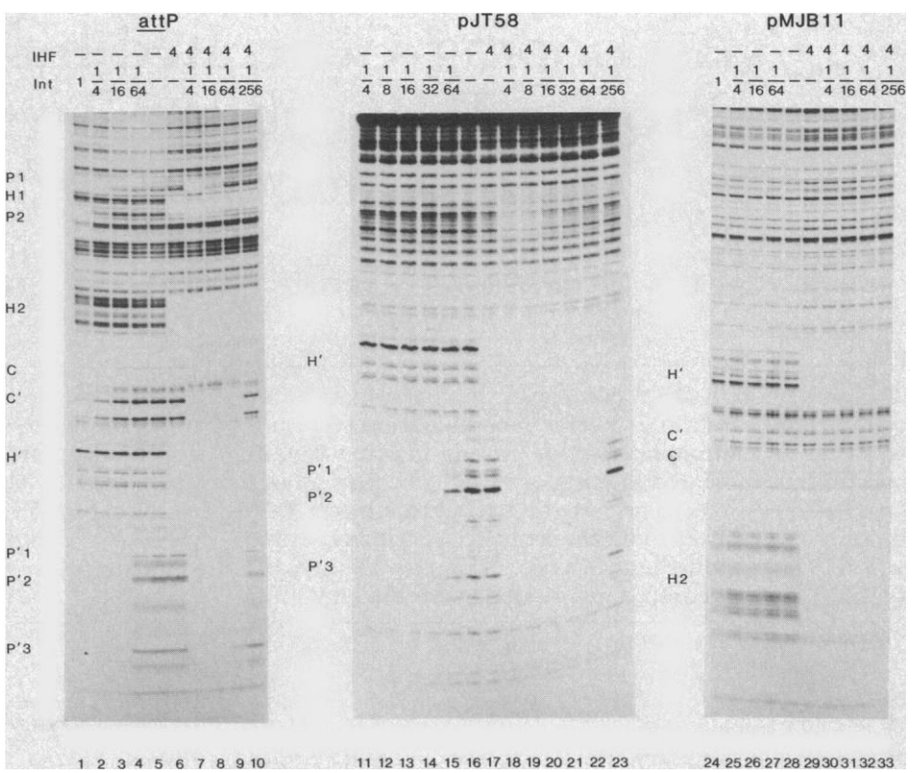

Fig. 2. Int and IHF promoted nuclease protection of *att* DNA's containing different combinations of protein binding sites. Nuclease protection assays were performed with NCS (neocarzinostatin) in the presence of the indicated amounts of Int and IHF (given as recombination units per 20 μl) (*24*). The plasmid pJT58 does not contain core sites and pMJB11 does not contain arm sites (Fig. 1). The protein binding sites (Fig. 1) protected by Int and IHF are shown on the left. The *att*P DNA is an Nco I–Aat II fragment from plasmid pWR1 (*34*) that was 5' end–labeled on the bottom strand at the Nco I site. DNA from pJT58 is a Nco I–Ssp I fragment 5' end–labeled on the bottom strand at the Nco I site. DNA from pMJB11 is an Eco RI–Sal I fragment 5' end–labeled on the top strand at the Eco RI site. The reaction mixture (100 μl volume) consisted of 120 m*M* NaCl, 10 m*M* $MgCl_2$, 50 m*M* tris-HCl (*p*H 7.4), 10 m*M* 2-mercaptoethanol, 10 percent (volume by volume) glycerol, and bovine serum albumin (BSA) at 2 mg/ml as described (*17*). The ^{32}P-labeled fragments (1×10^{-11} to 5×10^{-11} *M*) and proteins were purified as described (*17, 29*).

protein closely related to IHF, has been implicated (as a multimeric array) in conferring conformational DNA changes that promote specific protein-DNA interactions (*9, 19, 20*). IHF binds to three sites in *att* DNA, inducing a bend at each site estimated to be more than 140° (*6, 7, 21*).

There are several lines of evidence that the lambda recombination proteins may participate in the formation of a higher order complex in which long-range protein-protein and protein-DNA interactions take place (*22–25*). The dual binding capacity of Int was proposed to provide a means for generating looped DNA structures by bridging two different and distant DNA sites (an arm-type site and a core-type site), possibly with the assistance of other interactions involving IHF, Xis, and FIS (*17, 25*). In earlier experiments, IHF stabilization of Int binding to core-type sites was reported, but no mechanisms were deduced (*21*), and there were conflicting results on the role of arm-type sites in enhancing core binding (*26, 27*).

IHF enhancement of core protection and of core cleavage. Nuclease protection experiments (*28*) were used to assess possible interactions between Int bound at both arm- and core-type sites (Fig. 1). In the presence of Int alone, protection of core-type sites was observed in fragments obtained from *att*P and *att*L, but not in a fragment lacking the arm-type sites (plasmid pMJB11 contains the region from −70 to +46 of *att*P) (Fig. 2). In the presence of IHF, Int binding to the core sites was enhanced by a factor of approximately 64 in the *att*P and *att*L fragments (Figs. 2 and 3). However, no IHF stimulation of Int binding was observed in the fragment lacking arm-type sites (Fig. 2). Thus, under these experimental conditions, core binding and its enhancement by IHF requires the P′ arm Int sites.

In contrast to the strong IHF stimulation of Int binding to core, there was only approximately a fourfold stimulation of Int binding to the P′1 and P′2 sites (Figs. 2 and 3). In a similar analysis, with a fragment lacking the C and C′ core sites (no DNA to the left of +4) IHF binding had no effect on Int protection of the P′ arm-type sites. However, replacement of the core sites with non-*att* DNA (pJT58) doubled the enhancement of Int binding to all three P′ arm sites (Fig. 2). Thus, the increase in protection of the three P′ arm Int sites depends on the presence of any DNA sequences to the left of the C′ core site, although for the specific interactions with P′1 and P′2 the correct core-type Int recognition sequences are required.

To demonstrate that the P′-dependent enhancement of core protection is functionally relevant, we present data that it also promotes specific cutting of these sites by Int. The DNA cutting and resealing activity of Int proceeds by a covalent Int-DNA intermediate that is difficult to detect since it is rapidly converted to religated DNA product (*15, 29–31*). In addition, when linear DNA fragments are used, the predominant religated product of Int nicking is indistinguishable from the initial substrate. Consequently, we used a cleavage assay that depends on the ability of an *att* site DNA substrate to trap covalently linked Int-DNA complexes (*29*).

Although the substrates used in these experiments contain only a single core site, they act by a similar mechanism-based inactivation as already described (*29, 30, 32*). Specific cleavage by Int at the site of strand exchange generates a rapidly diffusible tetranucleotide (Fig. 4, top). Loss of this oligomer deprives the covalently bound Int of the 5′-hydroxyl acceptor it requires to reseal the nick, thus converting a transient covalent Int-DNA complex into a stable one. This assay provides a measure of protein-DNA interactions that is very different from that of nuclease protection. While the latter offers a short-term view of the protein-DNA complexes in equilibrium, the cleavage assay gives a long-term cumulative account of dynamic interactions.

Two substrates (with and without the P′ arm Int sites) were prepared from a plasmid that contains *att*L (Fig. 4). In the absence of IHF, incubation of Int with either substrate led to the formation of covalent Int-DNA complexes with comparable efficiencies. However, in the presence of IHF the behaviors of the two substrates differed. A 16-fold increase in cutting by Int was observed with the substrate containing the P′ arm Int sites, whereas no stimulation of cleavage was seen with the substrate lacking these sites (Fig. 4). Thus, the IHF-mediated delivery of Int from the P′ arm site to the core site leads to efficient cleavage at the normal site of strand

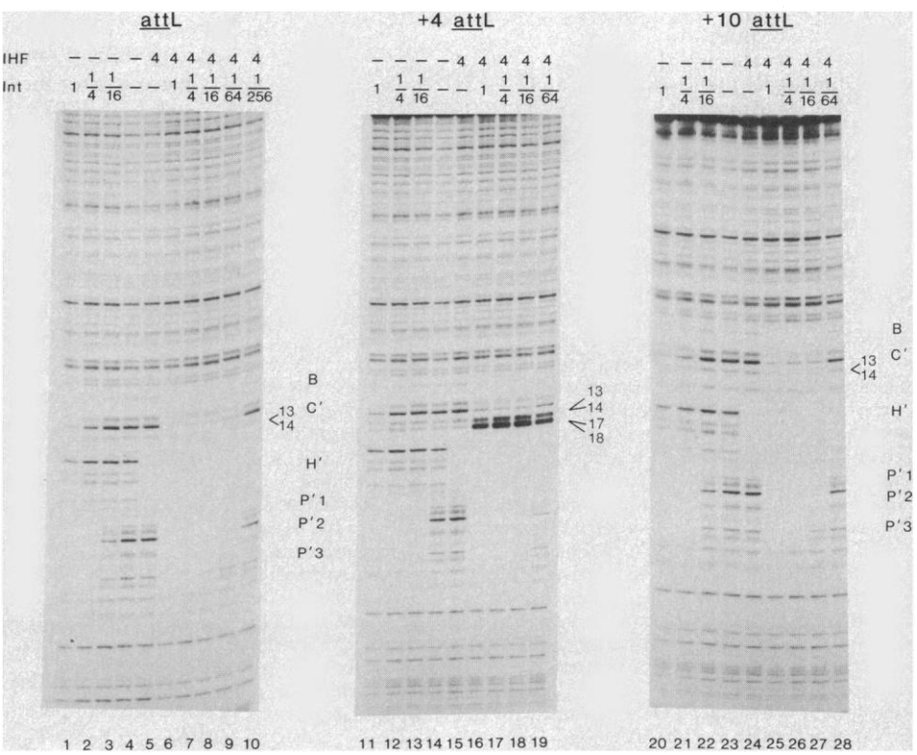

Fig. 3. Nuclease protection of *att*L and *att*L spacing mutants. Nuclease protection assays in the presence of the indicated concentrations of Int and IHF were performed as in Fig. 2. Protein binding sites are to the right of the protected regions for *att*L and +10 spacing mutant and to the left of protected regions for +4 mutant. Sequences of *att* site DNA's are shown in Fig. 1. The *att*L DNA is a Bam HI–Ban II fragment from pSN55 that was 5′ end–labeled on the bottom strand at the Bam HI site. The +4 *att*L spacing mutant is a Bam HI–Ban II fragment from pLV5 that was 5′ end–labeled on the bottom strand at the Bam HI site. The +10 *att*L spacing mutant is a Bam HI–Dra III fragment from pLV4 that was 5′ end–labeled on the bottom strand at the Bam HI site.

exchange.

The effect of helical phase. Interactions, via DNA looping, between proteins bound at two closely located DNA sites are favored when the proteins bind to the same face of the DNA helix (*33*). Therefore, if the IHF-mediated enhancement of core binding is a consequence of protein-induced looping, it should be influenced by the relative helical phase of the arm and core sites.

Two *att*L mutants were constructed in which the relative positions of the core and P′ sites were changed by approximately a half-helical turn (a 4-bp insertion, pLV5) or a full helical turn (a 10-bp insertion, pLV4) as shown in Fig. 1. The binding behavior of the two spacing mutants was examined by nuclease protection assays and compared to that of the wild-type *att*L parent (pSN55). In the absence of IHF, the pattern and degree of Int protection in both mutants were similar to those of wild type (Fig. 3). However, the addition of IHF led to different protection patterns in the two mutants. With the +10 spacing mutant the IHF-induced enhancement of Int binding was identical in pattern to that of the wild-type parent at both the core and arm sites, although the IHF stimulation was two to four times lower (Fig. 3). In contrast, the protection patterns in the +4 spacing mutant showed (i) an increased intensity of the bands at +13 and +14 and at +17 and +18 and (ii) a twofold enhancement of Int binding to all three P′ arm sites, a result similar to those obtained when the core sites were replaced by heterologous DNA (Fig. 3). The increased cutting by neocarzinostatin at those positions, as well as the anomalous enhancement of P′3 protection, could reflect distortion of the *att* DNA by aberrant Int interactions with the core and arm sites. Thus, preservation of the correct helical phase is required for the proper IHF-mediated enhancement of Int binding to core. Furthermore, with regard to the IHF enhancement of Int binding to the P′ arm sites, rotating the core sites by a half-helical turn is equivalent to replacing the core with heterologous DNA.

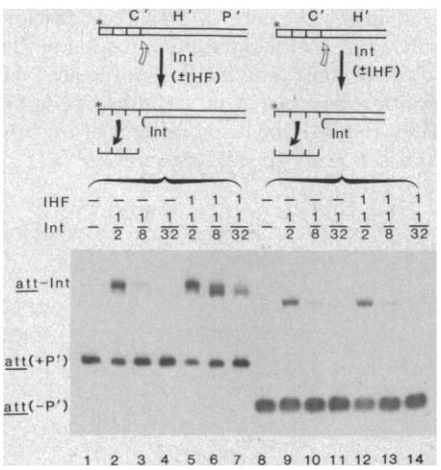

Fig. 4. Int cleavage of DNA substrates with and without P′ arm Int sites. (Top) Representation of Int cleavage (open arrow) of substrates that contain (left) or lack (right) the P′ arm sites and are labeled with ^{32}P at the 5′ end (*). (Bottom) Gel electrophoresis of reactions with the indicated substrates. The amount of Int and IHF in each reaction is given as the number of recombination units per 20 μl. The left margin shows the relative mobility of covalent complexes (*att*-Int) (*29*), *att* DNA with the P′ binding sites [*att* (+P′)], and *att* DNA lacking the P′ binding sites [*att* (−P′)]. Reaction mixtures (20 μl) consisted of 10 m*M* tris-HCl (*p*H 7.4), 80 m*M* NaCl, 5 m*M* EDTA, BSA at 2 mg/ml, 4 × 10^{-10}*M* DNA; the reactions were stopped by the addition of SDS to a concentration of 0.1 percent. Samples were analyzed by electrophoresis on a 5 percent acrylamide, 0.1 percent SDS gel that was subsequently dried and autoradiographed. The substrate containing P′ arm Int sites was an Xba I–Nco I fragment from pSK3 5′ end–labeled on the top strand at the Xba I site. The substrate lacking P′ arm Int sites is an Xba I–Bst BI fragment from pSK3 5′ end–labeled on the top strand at the Xba I site. Plasmid pSK3 contains *att*L and has an Xba I site at +1 from pSN84 (*30*) and a Bst B1 site at position +48 from pLV1 (*39*), and a Hind III site that was introduced at +23. Restriction fragments used to generate these substrates were prepared by cleavage of pSK3 with Xba I, ^{32}P-labeling of the 5′ ends (*17*), filling in the 3′ ends with unlabeled nucleotides and the Klenow fragment of DNA polymerase, and cleaving with either Nco I or Bst BI. The ^{32}P-labeled fragments were purified by electrophoresis on a 5 percent polyacrylamide gel with subsequent elution on NA-45 DEAE membranes (Schleicher & Schuell, Inc.) as described (*30*) and incubated with the appropriate proteins at room temperature for 2 hours.

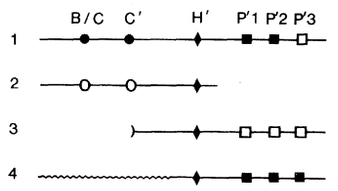

Fig. 5. Patterns of IHF-mediated enhancement of Int binding (filled symbols). IHF binding at the H′ site (◆) mediates enhanced binding (filled symbols) of Int at core-type (●) and arm-type (■) sites. *att* DNA (⎯), non-*att* DNA (〰) and the end of a DNA fragment (◗) are indicated. The pattern of enhanced binding for both the P′ arm and the core Int binding sites depends upon the presence or absence of adjacent DNA and other Int binding sites.

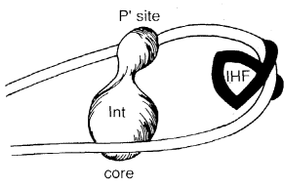

Fig. 6. Representation of potential interactions in the *att*L loop structure. The IHF-induced bending of *att*L DNA (double solid line) is shown promoting interactions between an arm-type binding site and a core-type binding site via the two autonomous DNA binding domains of one Int monomer.

If the arm-core interactions are critical in constructing functional complexes, recombination should also depend on helical phase. The labeled fragments used in the helical-phase nuclease protection experiments (from pSN55, pLV4, and pLV5) were recombined with an excess of supercoiled *att*R (pPH202) (*34*). Similar recombination efficiencies were obtained for the wild type and +10 mutant, 100 and 84 percent, respectively; but recombination was only 1 percent with the +4 mutant. Identical results were obtained when this reaction was carried out under nuclease protection conditions (Fig. 2), or under standard recombination conditions (*24*) with linear *att*R and supercoiled *att*L's.

Structure of the recombinogenic complex. Our data show that IHF binding to the H′ site, previously shown to induce a DNA bend >140° (*7*), brings the core and P′ arm sites into close proximity, thereby promoting Int-mediated interactions between core and the P′ arm. It is unlikely that a direct IHF-Int interaction can account for the large (64-fold) effect seen on core binding. This conclusion and the requirement for arm sites in the stimulation of core binding are based on the experimental data in Figs. 2 and 4: a fragment containing two IHF binding sites (H2 and H′) but lacking arm-type Int sites fails to exhibit any core protection in the presence of IHF binding. Similarly, the IHF-enhanced cleavage of *att* site DNA also requires the presence of P′ arm sites. That these effects were not observed in earlier experiments (*21, 26*) is probably due to the high degree of cooperativity that can mask the consequences of single site mutations in the Int binding sites (*24, 35*).

The asymmetry in IHF-induced enhancement of core binding relative to arm binding (64-fold and 4-fold, respectively) is consistent with the much higher affinity of Int for arm-type sites than for core-type sites (*16*). Int binding to the strong arm-type sites is the primary event, which promotes Int binding to the low affinity core-type sites. We, and others (*27*), had previously observed that the P′ arm can influence core binding even in the absence of IHF, as seen for the core protection in *att*P compared to that of PMJB11 (Fig. 2). Evidently Int, or the intrinsically curved *att* DNA (*36*), is sufficiently flexible to permit slight, but unstable, Int-mediated interaction between core and the P′ arm.

Although the IHF enhancement of Int binding to P′ arm sites is not large, the patterns are revealing (Fig. 5). Replacement of core sites with heterologous DNA leads to a less constrained loop as P′

bound Int can now only establish loose and nonspecific DNA contacts in its core-binding domain. Deletion of the DNA to the left of the C′ core site abolishes all IHF effects on Int binding to the P′ arm sites. These results do not support a direct IHF-Int interaction between H′ and the P′ arm sites. Rather, they suggest a model in which IHF binding at the H′ site is required to induce a bend and stabilize a DNA loop whose size and stability is determined by the spacing and specific sequences recognized by the two DNA binding domains of Int. In the normal *att* site these interactions most likely involve an arm-Int-core configuration (Fig. 6). The loop structure identified here as the *att*L recombinogenic complex is consistent with the analysis of mutant *att* sites (*26, 37*) and provides insight into the role of the P′ arm during excision.

Extrapolations. DNA loop structures have been previously shown to be generated by one sequence-specific binding protein, in the form of a homodimer or homotetramer, that tethers two similar DNA binding sites (*8–10*). In contrast, the *att*L loop involves two different classes of sequence-specific DNA binding proteins: a bend-inducing protein, IHF, and a bivalent binding protein, Int. This general structure of a bending protein and one or more bridging proteins has been implicated in several other systems (*4, 9, 19, 20*) and has several interesting features.

As described above, DNA binding proteins "poised" at high affinity sites can be directed by the appropriate DNA bending protein to interactions with lower affinity sites. This design provides flexibility for constructing singular but overlapping regulatory circuits; the presence or absence of particular sequence-specific bending proteins would determine which pairs of binding sites are favorably juxtaposed for tethering. In this way, a single sequence-specific bending protein could coordinately activate a large number of unrelated tethering proteins at many different loci. In addition (since the bending and bridging proteins do not have to interact), this strategy affords a mechanism of regulation that is completely independent of any requirements for protein-protein recognition.

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