Replacement of Integration Host Factor Protein-induced DNA Bending by Flexible Regions of DNA*

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The Escherichia coli integration host factor (IHF) protein is required for site-specific recombination of bacteriophage λ DNA. Previously, we had shown that alternative modules of static DNA curvature could partially replace IHF in recombination. Now we use regions of single-stranded DNA as a flexible tether to address whether the function of IHF in recombination is simply to reduce persistence length. Although we find that these modules clearly enhance recombination in the absence of IHF, they are not perfect replacements. In addition, evidence is presented that the efficacy of a flexibility swap is specific to a particular IHF site. This may indicate that additional functions beyond simple deformation of DNA are required of IHF. During the course of these experiments we discovered that these flexible sequences are still specific sites for IHF binding and function.

Deformation of DNA is a common feature of nucleoprotein complexes. In some cases, DNA structure has been shown to have a specific function and not simply be a consequence of protein binding or DNA sequence (1–4). DNA bending is known to be associated with virtually all types of nucleoprotein systems including DNA replication, transcription, and recombination (5, 6). Interestingly, DNA deformation is often relegated to an accessory function that simply modulates the function of other essential components. In the few cases where DNA deformation has been proven to be functional, it is unclear how the structure affects function. Although there is reason to believe that the deformation in and of itself induces effects on the structure of neighboring DNA (7), a parsimonious theory is that DNA bending could simply reduce the native persistence length of DNA to allow adjacent regions of DNA and/or proteins bound there to interact.

The Escherichia coli bacteriophage λ site-specific recombinase system was the first system where DNA deformation was proved to be functional (1). In this system, a 250-bp site attP on the phage is required for conservative integration of the phage genome into a unique site attB on the E. coli chromosome. attP and attB share 15 bp of perfect sequence identity within which the cleavage and ligation steps of recombination occur. The integrated prophage product has phage bacterial junctions called attL and attR for left and right attachment sites, respectively. The phage-encoded integrase (Int) protein performs all of the recombination steps. The prophage can be excised from the bacterial chromosome by Int but only in conjunction with another phage-encoded protein, excisionase (Xis). In addition to the phage-encoded proteins, one host protein, integration host factor (IHF), is absolutely required for efficient recombination. IHF is a site-specific DNA-binding protein that, upon binding, invariably bends its DNA target into a virtual U-turn (8, 9). Three such high affinity IHF binding sites exist on attP whereas none are found on attB. Consequently, recombination distributes one of these sites to attL and two to attR, although the most distal site on attR is not required for excisive recombination.

Previously, we had demonstrated that the requirement for IHF binding for both integrative and excisive recombination could be functionally replaced through a bend swap, an exchange of bending modules. Specifically, IHF-induced bends were substituted with sequence-directed DNA deformation that approximates native IHF-induced curvature (1, 4). Recombination substrates, in which all of the required IHF sites were replaced with properly phased DNA bends, were competent for recombination in the absence of IHF (4). The results of these experiments strongly suggest that the role of IHF in phage λ site-specific recombination is to act as an architectural element.

Studies on excisive substrates, which, unlike attP, each require a single IHF site, have demonstrated that one role of IHF is to assist Int binding to the DNA (10, 11). Int possesses two DNA binding domains. The amino-terminal domain binds with high affinity to arm-type sites found at the periphery of attP, attR, and attL, whereas the carboxyl-terminal domain, which possesses recombination functions, binds with lower affinity to core-type sites that flank the region of strand exchange. One effect of IHF binding is to stabilize the interaction of individual Int promoters simultaneously bound to both high and low affinity sites. Because such bivalent binding would require the intervening DNA to bend and because that segment of DNA is well below the persistence length of B-form DNA, it is possible that the sole function of IHF is to reduce the persistence length of the DNA between these two types of sites.

We have tested whether a module of flexibility can replace IHF for function in bacteriophage λ recombination. It has been demonstrated that regions of single-stranded DNA possess much higher capacity for isotropic flexibility than native duplex DNA (12–14). Heteroduplex regions of DNA possess single-stranded character with reduced persistence length (13). By substituting the essential IHF binding sites with heteroduplex DNA we were able to examine whether flexible DNA could substitute for IHF in excisive recombination. We found that, although these flexible regions could adequately replace IHF in

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‡The abbreviations used are: bp, base pair; Int, integrase protein; Xis, excisionase; IHF, integration host factor; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay.
the formation of at least one type of nucleoprotein complex, they failed to completely support IHF-independent excisive recombination. Moreover, despite making dramatic changes in the IHF recognition sites, we observed that the heteroduplex DNA was still a specific site for functional IHF binding.

MATERIALS AND METHODS

**Bacterial Strains and Plasmids—**E. coli DH5α was host for plasmid propagation. pHN64 (15) contains both a wild type attL and an attR locus.

**DNA Substrates—**For quantitation, some DNA substrates were end-labeled with 32P using the RTS T4 kinase labeling system (Life Technologies, Inc.). Labeled oligonucleotides were purified using a nucleotide removal kit (Qiagen) and eluted with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. All DNA substrates were purified by gel electrophoresis through 2% agarose followed by extraction using a gel extraction kit (Qiagen); DNA was eluted with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Double-stranded attL and attR DNA fragments were generated by the PCR using pHN864 as a template and the following oligonucleotides (Table 1): sG1117 and sG1212 for attR, sG646 and sG449 for attL, and sG153 and sG449 for saf attL. The PCR was performed with Taq polymerase (Promega) with the following temperature incubation profile: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and a final extension at 72 °C for 2 min. The heteroduplex (T-bubble) DNA substrates were generated by annealing and extending partially complementary oligonucleotides (Table 1; sG145 and sG146 for FattR, sG147 and sG148 for FattL, and sG148 and sG152 for saf FattL). For each particular substrate, the appropriate oligonucleotides were mixed together to a final concentration of 0.1 μM in 1.5× Sequenase® buffer (U. S. Biochemical Corp.) in a 10-μl volume, placed in a 65 °C heat block, and allowed to cool down to room temperature over approximately 45 min. The 3′ ends of these annealed oligonucleotide pairs were then extended by addition of dithiothreitol to 6.7 mM, dNTP to 0.2 mM, and 7 units of Sequenase® (U. S. Biochemical Corp.) in a final volume of 15 μl. The concentration of each DNA substrate was determined by the Q-test. The Int heteroduplex substrates (2) were sensitive to mung bean single-stranded nuclease treatment. On the other hand, Int and IntE174K were added to 70 nM. Xis and IHF were either PCR amplicons or restricted plasmid DNA and present at a concentration of 0.1 μM. Reactions were incubated for 3 min at 95 °C, chilled on ice, and placed in a 65 °C heat block that was allowed to cool down to room temperature. The DNA was then dialyzed against 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. This results in three isotopically labeled species, namely the attL saf-mutant homoduplex and two attL saf-heteroduplexes. Of these three species only the two attL saf-heteroduplex species were substrate for Int cleavage; the homoduplex was intact (data not shown).

**Mung Bean Endonuclease Digestion—**Isotopically labeled DNA substrates (2 μl) were incubated with 20 units of mung bean endonuclease (Stratagene) in 1× mung bean nuclease buffer (Stratagene) at 30 °C for 30 min.

In **Vitro Recombination Assay—**All recombination reactions were performed in 25 mM Tris-HCl, pH 7.8, 60 mM KCl, 250 μg/ml bovine serum albumin, 5 mM spermidine, 0.5 mM EDTA in 1% glycerol. Reactions were incubated at 25 °C at the indicated times. DNA substrates were either PCR amplicons or restricted plasmid DNA and present at a final concentration of 2 nM. In cases where PCR amplicons were used exclusively as the recombination substrates, 0.1 μg of carrier DNA (salmon sperm or cleaved pBR322) was added. Pure IntE174K, Xis, and IHF were gifts from Howard Nash. Pure Int was the gift of Life Technologies, Inc. Int and IntE174K were added to 70 nM. Xis and IHF were added to 30 nM and 50 nM, respectively. attL × attL reactions were performed as described above except Xis protein was omitted. The statistical significance of values used to calculate average recombination efficiencies was determined by the Q-test. The Int heteroduplex cleavage assay was assembled in the same manner as the excisive recombination reactions except the attL and FattL saf-heteroduplexes, described above, were used as substrates.

**Electrophoretic Mobility Shift Assay (EMSA)—**Each binding reaction consisted of purified proteins at the same concentrations used in the recombination reactions but were executed in the following buffer: 50 mM Tris-HCl, pH 7.8, 60 mM KCl, 50 mg/ml bovine serum albumin, 10% glycerol in a final volume of 20 μl. The concentration of each DNA substrate was 5 nM. Reactions were incubated at the designated temperature for 30 min to achieve equilibrium. The entire reaction volume was immediately loaded onto an 8% native polyacrylamide gel. Electrophoresis was performed at 5 V/cm for 3 h at 22 or 4 °C. Gels were dried and exposed to a PhosphorImager screen (Molecular Dynamics). Bands were annotated with ImageQuant software. Competition experiments to determine equilibrium dissociation constants were performed as described previously (16). Statistical significance of values used to calculate averages was determined by the Q-test.

**RESULTS**

DNA flexibility swaps were studied in a excisive recombination, taking advantage of the simple substrate requirements. Unlike integrative recombination, the excisive recombination substrates, attL and attR, do not have strict topology requirements and can be reduced to short PCR amplicons (Fig. 1). In addition to DNA substrates, native excisive recombination absolutely requires the phage-encoded proteins Int and Xis and the host protein IHF. Int must bind to both the P2 and P1 arm-type sites in addition to the core elements (C, C′, B, and B′) whereas Xis binds to the X1 and X2 sites. IHF binding and deformation are required at the H2 and H′ sites.

**Design of Fatt Substrates—**Previously, we had shown that sequence-directed bends facilitate moderate recombination efficiency in place of IHF (1, 4). Initial nucleoprotein intermediates, such as the one speculated at attL (10, 11), require IHF to bend DNA for proper Int binding, e.g. Int must simultaneously bind the C′ and the P1′ sites of attL. To construct excisive recombination sites with flexible regions or Fatt sites (flexible attachment sites) we made use of heteroduplex DNA. Regions of heteroduplex are known to be more flexible than native B-form duplex DNA (13). The solved cocrystal structure of IHF bound to DNA (8, 9) was used as a guide to design our heteroduplex replacement substrates. We substituted the nine base pairs between the two positions on the DNA where IHF induces kinks, with nine thymidine residues on each strand (T-bubble, Fig. 2). Thymidines were chosen for their lack of self base pairing and their poor stacking capacity. The same strategy was used for both attL and attR.

**Fatt sites** were first characterized to ensure the existence of the desired T-bubble. Sources of flexure are known to retard the rate of DNA migration during native polyacrylamide gel electrophoresis (17). Fig. 3 shows the wild type versions of attL and attR side by side with their Fatt counterparts. Because each type of att site is otherwise identical, decreased mobility can be ascribed to the T-bubble within the IHF locus of each Fatt site; a denaturing gel of the same substrates shows no mobility differences (data not shown). In addition, Fatt sites were sensitive to mung bean single-stranded endonuclease whereas the wild type att sites were resistant to digestion. On FattL, mung bean endonuclease treatment was predicted to yield two duplex DNA fragments of similar length (about 60 bp), because cleavage(s) could, in principle, occur anywhere within the single-stranded region of the T-bubble. The products of cleavage of FattR were predicted to yield two fragments of approximately 60 and 80 bp. As shown in Fig. 3, cutting was restricted to the region of the T-bubble as judged by the size of the DNA cleavage products.

In **Vitro Excisive Recombination—**We began by testing Fatt sites as substrates in various forms of in vitro recombination. Fatt sites were first used in excisive recombination mediated by a hyperactive mutant of the Int protein that possesses a single missense mutation that changes the glutamate at amino acid position 174 to a lysine (IntE174K). Bruist and co-workers (18) have shown that IntE174K binds with higher affinity to core-type sites, which they conclude facilitates the bivalent binding of the IntE174K promoter in the absence of IHF. This single amino acid change creates an integrase with higher specific
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Table I

| Oligonucleotides used in this study | Sequence (5' to 3') |
|-----------------------------------|---------------------|
| oSG46 (attL)                      | GGAATTCCGTTGAAGCCTGCTTTTTTATACTAAGTTGGC |
| oSG49 (attL)                      | GGAATTCAAATAATGATTTATTTTGACTGATAGTGACCTGTTC |
| oSG117 (attR)                     | GGAATTCCCGTTTCGCTCAAGTTAGTATAAAAAAGC |
| oSG145 (attR)                     | GGAATTCATAGTGACTGCATATGTTGTGTTTTAC |
| oSG146 (attR)                     | GGAATTCCCGTTTCGCTCAAGTTAGTATAAAAAAGCTGAACGAGAAACGTAAAATGATATTTTTTTTTATATATTAAATTAG |
| oSG147 (attL)                     | GGAATTCCGTTGAAGCCTGCTTTTTTGTACTAAGTTGGC |
| oSG148 (attL)                     | GGAATTCAAATATGATTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTTTTTTTTTATGCTTTTTTATAATGCC |
| oSG153 (attL) saf1G1)             | GGAATTCCGTTGAAGCCTGCTTTTTTGTACTAAGTTGGC |

Underlined bases have been altered from the wild type sequence.

IntE174K promotes recombination on both att over the wild type resulting in a significant increase in recombination efficiency. The protein could produce a synergistic effect with the T-bubble in independent recombination, we reasoned that this mutant Int activity that can even perform a small amount of IHF-independent recombination (18). If Fatt sites contribute to IHF-independent recombination, we reasoned that this mutant Int protein could produce a synergistic effect with the T-bubble resulting in a significant increase in recombination efficiency over the wild type att sites. As shown in Table II, although IntE174K promotes recombination on both att and Fatt substrates in the absence of IHF, the recombination of Fatt sites was almost 5-fold more efficient than wild type sites. In the presence of IHF, recombination of Fatt sites and att sites was of similar efficiency. Hence, IHF more than doubled the efficiency of Fatt site recombination but increased recombination between wild type att sites by 12-fold. This demonstrates that the introduced T-bubble is providing a significant benefit to the Fatt sites in favor of recombination competent nucleoprotein complexes.

We repeated these experiments with wild type Int. In the presence of Int and Xis alone we failed to detect any recombination product with the wild type att sites. Similarly, Fatt sites also failed to produce detectable recombination in the absence of IHF. In contrast, wild type att sites recombined efficiently when IHF was added to reaction mixtures. Surprisingly, the Fatt sites also recombined with added IHF, achieving about half the efficiency of the wild type sites. This suggests that IHF still binds to these sites. This was not expected, because we had both altered the sequence of the IHF consensus at some of the most conserved residues and disrupted base pairing within this region.

EMSA with Fatt Sites—It had been shown previously (10, 11) that excisive recombination substrates give very specific mobility shifts during native electrophoresis when bound to particular recombination proteins. Thus we tested Fatt sites for their capacity to generate nucleoprotein complexes with combinations of Int, Xis, and IHF. In the presence of IHF alone, both attL and attR shifted specifically into a single nucleoprotein complex. Moreover, the Fatt sites were also shifted into complexes with similar mobility (Fig. 4, A and B). They differed, however, in that both FattR and FattL failed to form complexes as discrete as the wild type sites. The shifted Fatt complexes also migrated slightly faster than the complexed att sites. Taken together, this may indicate that IHF has a more distributive mode of binding on the Fatt sites. To further assess IHF binding to Fatt sites we quantitatively measured the dissociation constants of IHF to each of the Fatt sites. Dissociation constants were measured by a competition of IHF binding for the 119-bp wild type attL, under limiting IHF concentrations (16). Nonspecific DNA has 100 to 1000-fold lower affinity for IHF than specific sites (19). As shown in Table III, these T-bubble-containing Fatt sites bound IHF within a factor of 2 of their respective wild type site. Hence, part of the IHF site containing some of the most conserved residues can be replaced with a T-bubble with only a small loss of affinity. Our results suggest that the T-bubbles, when embedded in native DNA, are preferred sites for IHF binding. Future experiments will be required to determine whether enhanced flexibility is the feature that allows retention of IHF binding affinity.

For Int to yield a functional complex on attL, it must bind simultaneously to both the P'1 and C' sites. In the native system, IHF is required to sufficiently facilitate this structure. Int bound to attL yields two weak complexes. Upon addition of IHF, both the substrate and the faster migrating complex get chased into the slower shifted complex. This latter species has been previously shown to consist of bivalent Int binding to P'1 and C' and bound IHF (10, 11). Henceforth, we will refer to these complete nucleoprotein single att species as unimolecular complexes. In contrast, FattL makes, almost exclusively, one...
complex with Int that comigrates indistinguishably with the att-Int-IHF unimolecular complex. Thus in the absence of IHF, Int and P2 Int site overlap. Little change is observed when IHF is added to reactions that contain FattL. Because we have already shown that IHF binds to FattL and because IHF binding is required for wild type recombination, we assume that IHF is still binding but fails to significantly affect the migration of this complex. We

**FIG. 1.** Bacteriophage λ excisive recombination sites. Each line represents a specific functional locus for excisive recombination. Symbols represent protein binding sites. Conservative recombination is represented by the large X. The H1 IHF site and P2 Int site overlap.

**FIG. 2.** T-bubble heteroduplexes in FattL and FattR. A, comparison of the region of heteroduplex DNA in FattL with the same region in the wild type attL. The arrowheads indicate the position of two kinks observed in the cocrystal structure of IHF bound to the H9 IHF site of attL. B, comparison of the region of heteroduplex DNA in FattR with the same region in the wild type attR. The most conserved portion of the IHF consensus sequence is aligned at the bottom (W = A or T, Y = T or C, and N is any base).

**FIG. 3.** Mung bean nuclease cleavage of att sites. Each of the designated att sites was isotopically labeled, incubated with or without mung bean nuclease (MN) (see “Materials and Methods” for conditions), and separated by native gel electrophoresis.
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**Table II**

| att site substrates | Integrate | IHF | Percent recombinationa |
|---------------------|-----------|-----|------------------------|
| attL × attR         | Int       | −   | 100%                   |
| attL × attR         | Int       | −   | <1                     |
| attL × attR         | IntE174K  | +   | 150                    |
| FattL × FattR       | Int       | +   | 12                     |
| FattL × FattR       | Int       | <1  | 82                     |
| FattL × FattR       | IntE174K  | +   | 160                    |
| FattL × FattR       | IntE174K  | −   | 57                     |

a Percent recombination is defined as the fraction of substrate counts chased into product and is normalized to the percent of attL × attR converted to recombinant product with Int, Xis, and IHF. Each percent recombination value is the average of two trials, each of which was incubated at 25 °C for 3 h.

b 100% is equivalent to 17% of the substrate converted to product.

and P′1 sites of attL, thus creating a sharp bend as is required in exusive recombination, then attL × attL recombination is inhibited. Recombination efficiency is even depressed if only one attL partner is bound to IHF. This allows us to distinguish whether FattL behaves like unbent attL or more like IHF-bound bent attL. Table IV shows that IHF inhibited Int-mediated attL × attL recombination. In contrast, FattL × attL recombination was equally efficient with or without added IHF but was only as efficient as attL × attL recombination in the presence of IHF. Hence, recombination with Fatt sites mimicked the wild type attL sites in the presence of IHF. This result further confirms that at least FattL is both competent for IHF-independent recombination with wild type Int and possesses qualities consistent with functional DNA deformability.

**Heteroduplex Cleavage Assays**—The staggered cleavages and subsequent ligations mediated by Int take place in the overlap region (bp −2 to +4) of each att site. Nash and co-workers (21, 22) showed that if one recombination partner possesses a heteroduplex in the overlap region, cleavage of that att site becomes uncoupled from the subsequent steps in recombination, i.e. strand exchange and ligation. Subsequent to cleavage, the Int promoter, still covalently attached to the 3′ end of the DNA, efficiently catalyzes the formation of a hairpin by reacting with the adjacent 5′ hydroxyl. For this assay to function, the complete repertoire of recombination proteins specific to that att site must be present. For example, cleavage fails to occur in the absence of IHF. Implicit to the cleavage step is the requirement that the nucleoprotein complexes must synapse productively (capture). Thus the heteroduplex cleavage assay tests the competency of a pair of att sites for capture and cleavage.

We assessed the ability of FattR, Int, and Xis to induce cleavage on saf+/+ 1G FattL heteroduplex in the presence and absence of IHF. As shown in Fig. 5, cleavage products for heteroduplex saf-attL sites are observed only in the presence of Int, Xis, and IHF. In contrast, heteroduplex saf-FattL is cleaved in the absence or presence of IHF, although IHF does seem to stimulate cleavage. No cleavage was observed in the absence of an attR species (data not shown). Hence, the Fatt sites are capable of efficient capture and cleavage without IHF. Thus the recombination defect that we observe likely occurs after the first-strand cleavage but prior to formation of the completed recombinant product.

**DISCUSSION**

Previously, it has been shown that segments of statically bent DNA could modestly replace IHF for function in excusive recombination (4). This strongly suggested that DNA deformation is likely to be at least one of the roles of IHF in this system. Here we have asked a more specific question. Is the role of IHF merely to reduce DNA persistence length? Implicit in this question is that the other recombination components i.e. att sites, Int and Xis, possess all the necessary features to specify all other aspects of active complexes. According to this model, IHF would act to facilitate the contortion of the DNA but only inasmuch as is required to overcome the natural stiffness of the DNA. If true, then IHF-induced deformation should become dispensable with the introduction of a sufficiently flexible tether. For this investigation, we used heteroduplex DNA. Our results show that, although the introduced T-bubbles facilitate the formation of some intermediates and some forms of recombination, they fail to fully replace IHF in all steps of excusive recombination. The cause of the failure of recombination appears to reside mostly in attR where a role of persistence length reduction seems inadequate to explain the function of IHF. Interestingly, we found that the heteroduplex T-bubbles were still specific targets for IHF binding and function.

**IHF Binding to Flexible DNA**—The recently solved cocrystal

interpret this to mean that the primary reduction in electrophoretic migration is the result of DNA distortion by the T-bubble and Int acting in concert and that IHF binding fails to add significantly to both that distortion and the electrophoretic mobility. Hence by EMSA, IHF’s role appears to be mimicked by the T-bubble.

We repeated this analysis with attR and FattR (Fig. 4B). In this case, we examined shifted complexes with Int, IHF, Int/Xis, and Int/Xis/IHF. Distinct complexes with attR were observed with each of these protein combinations. We observed that FattR and attR yielded similar but not identical complexes with each protein combination with one important exception, namely the Int/Xis/FattR complex nearly comigrated with the unimolecular complex of attR bound to Int, Xis, and IHF. However, the addition of IHF to the Int/Xis/FattR further enhanced the migration rate of the complex, making the two FattR complexes distinguishable. This differed from Int/FattL complexes that were indistinguishable by EMSA in the presence and absence of IHF. This is the first indication that the T-bubble, at least within attR, may not functionally replace the IHF-bound complex. Nevertheless, although this IHF-independent complex on FattR is merely similar and not identical to the unimolecular complex on wild type attR, it is still dependent on both Int and Xis for its formation.

To determine whether the T-bubble flexibility accounted for this drastic change in IHF-independent EMSA complexes, we repeated the above EMSA experiments but performed all of the steps, including polyacrylamide gel electrophoresis, at 4 °C. At lower temperatures, others have shown that base-stacking interactions are stronger for single-stranded regions of DNA, yielding a greater persistence length (14). Thus as the temperature is reduced we hypothesize that the T-bubble will become stiffer. As shown in Fig. 4, C and D, there are nominal differences in the extent and relative migration of nucleoprotein complexes on the native att sites at this lower temperature; however, the number of major complexes remains the same.

The most dramatic change is that the Fatt sites are now dependent on IHF for efficient production of the unimolecular complex. Hence, this temperature-induced change, which is consistent with reduced flexibility, provides further evidence that under nonpermissive conditions these Fatt sites can behave like wild type att sites; they are competent for unimolecular complex formation but only in the presence of IHF.

**attL × attL Recombination**—To further characterize FattL as a substrate for recombination, we took advantage of the attL × attL recombination pathway. This form of recombination is a naturally occurring secondary pathway that has recently been characterized in vitro (20). Via this route, attL solely requires Int to perform recombination; addition of IHF is strongly inhibitory. If Int is able to simultaneously bind the C′
structure and biochemical studies have shown that, although IHF makes several contacts with its DNA site and in particular with certain bases in the consensus sequence, its binding is mostly degenerate, meaning that the contacts made are not completely specific. Thus IHF binds not through individual base-mediated specific contacts but through the contextual structure that the sequence creates. The complex that is formed during EMSA with IHF and Fatt sites fails to migrate with the corresponding wild type att sites and is somewhat smeared. Although we have measured the affinity via the dissociation constants and they are within a factor of 2 of the wild type, we cannot say to what degree the kinetics, contacts, or final structure match the wild type site interactions; those experiments are in progress.

Recombination using Fatt sites in the presence of IHF is almost as efficient as the wild type att sites under the same conditions. Because IHF function is predicated on precise binding (even one base out of register is detrimental to recombination) (23), our results suggest that IHF must be binding to T-bubbles with virtual wild type positioning. This binding is, of course, within the context of each Fatt unimolecular complex with Int or Int-Xis. One possible model would have IHF binding to the T-bubble with relaxed positioning (e.g., a small amount of one-dimensional sliding) with specificity increasing in the context of each unimolecular complex.

Fig. 4. EMSA of att sites. Native electrophoresis was used to separate att containing nucleoprotein complexes at 25°C (A and B) and 4°C (C and D). Each reaction contained 0.1 pmol of the isotopically labeled DNA substrate and 0.1 µg of carrier DNA. Brackets indicate the position of the wild type att complexes only.

Table III
Equilibrium dissociation constants (K_d) of IHF bound to att sites

| att site | K_d [M] |
|----------|---------|
| attL     | 0.8     |
| attR     | 0.5     |
| FattL    | 2.0     |
| FattR    | 0.5     |

Each value is the average of three trials, each of which was within 10% of the average.

Table IV
In Vitro recombination with attL

| attL Substrates | IHF | Percent recombination |
|-----------------|-----|-----------------------|
| attL × attL     | −   | 100%                  |
| attR × attL     | +   | 41%                   |
| FattL × attL    | −   | 36%                   |
| FattL × attL    | +   | 32%                   |

Each reaction contains a 9-kb attL derived from pHN864 and a PCR amplicon containing either attL or FattL.

Percent recombination is defined as the fraction of substrate counts chased into product and normalized to the percent of attL × attL recombinant product with Int and is the average of two trials. All individual values were within 10% of their respective averages. Reactions were incubated at 25°C for 12 h.

100% is equivalent to 7% of the substrate converted to product.
Perhaps it is not surprising that IHF does bind these T-bubbles with specificity. HU (a homolog of IHF that also binds and bends DNA but lacks target sequence specificity) typically has a \( \mu \)M dissociation constant with native duplex DNA. It has been shown, however, that HU is highly specific for gapped DNA, decreasing its dissociation constant 1000-fold (24). Castaing et al. (24) proposed that this substrate specificity may indicate a role for HU in DNA repair where DNA heteroduplexes are substrates. We suggest that so much of the free energy of binding is sacrificed to bend the DNA that HU shows site specificity when the target sequence is significantly more flexible. In a related study of the TATA-binding protein that also bends a specific DNA site, Geiduschek and co-workers (25) were able to show that properly placed regions of flexure, in the form of heteroduplex bubbles, could replace residues from within its recognition sequence and retain specific binding and bending capacity. Because Yang and Nash (19) have shown that single-stranded DNA is not a preferred substrate for IHF over nonspecific double-stranded DNA, it is unlikely that the single-strandedness of the T-bubble is conferring specificity. Although our results reflect that IHF is similar to HU in binding preference for flexible DNA, it is remarkable that the central 9 bases of a 32-base site can be functionally replaced with 9 residues of non-base-paired thymidines. We suggest that whereas native DNA targets have evolved through changes in their sequence to enhance their ability to be bent by a particular protein, it is possible to artificially modify the DNA structure to obviate those features without altering the protein–target affinity and structure.

The Role of IHF in Recombination—We used our Fatt sites in two forms of recombination that require excisive substrates. The attL \( \times \) attL pathway gave straightforward results. Fatt \( \times \) attL in the absence of IHF gave similar recombination efficiencies when compared with attL \( \times \) attL in the presence of IHF. According to the model put forth by Segall and Nash (20), attL \( \times \) attL recombination is an alternative pathway of recombination where IHF acts as a noncompetitive inhibitor by creating a complex that is recalcitrant to recombination. The ability of the T-bubble to impede this form of recombination mimics IHF binding. In this pathway, IHF appears to play only one role, to bend DNA.

Interpretation of excisive recombination is more complicated. We found that Fatt sites were superior to wild type att sites as substrates in IntE174K-mediated IHF-independent recombination. However, excisive recombination in the presence of wild type Int was undetectable for both wild type att sites and Fatt sites when IHF was omitted from the reaction. Because recombination relies on a specific IHF site on each att substrate, the failure of efficient recombination could rest with either or both T-bubble replacements. Having shown by EMSA that unimolecular complexes readily form on Fatt sites in the absence of IHF but fail to proceed to recombinant product, we explored an assay that is indicative of a downstream intermediate in the recombination pathway. The heteroduplex cleavage assay requires a sufficiently competent nucleoprotein complex on att sites such that the subsequent steps of productive synapsis and cleavage can occur. Fatt sites were competent for cleavage both in the presence and absence of IHF, hence, the T-bubble can replace IHF in this assay. Hsu and Landy (26) have shown that Int alone can resolve topologically relaxed Holliday junctions without IHF, suggesting that the role(s) of IHF precedes the resolution step. Taken together, the T-bubble deficiency can be staged after synapsis and first-strand cleavage but before resolution.

In the previous bend swap experiments IHF could be functionally replaced with either sequence-directed DNA deformation or bending induced by a heterologous protein (1, 4). This was strong evidence in favor of a role for induced DNA structure in recombination. Assuming that DNA deformation plays an essential role in recombination, the failure of Fatt sites to recombine in the absence of IHF can be explained in two ways. 1) Our persistence length-reduction model is correct, but our T-bubble is an insufficient replacement. 2) Our model is inadequate, and IHF is required for an additional role(s) that enhanced flexibility cannot accommodate. In the first case, perhaps the simplest explanation for the failure of T-bubbles to replace IHF binding is that they are simply not flexible enough to effectively reduce the persistence length of the DNA. This seems inconsistent with the EMSA experiments. Unimolecular complexes on Fatt sites in the absence of IHF yielded complexes similar to wild type att sites with IHF. It is possible, however, that the results of the EMSA have been overinterpreted to conclude that a band of similar migration necessarily suggests similar structure. It is also possible that the complexes are additionally stabilized in situ by the gel matrix via the cage effect (27) and that T-bubbles simply lack sufficient flexibility in solution.

Whereas Int and FattL yielded a complex indistinguishable from Int-IHF-FattL by EMSA, the unimolecular complex on FattR was merely similar to the same complex in the absence of IHF. Could the complex on FattR be deficient in the absence of IHF? Consistent with this notion, we had previously identified HU as a modest replacement for IHF but only in excisive recombination. A further analysis (10) by EMSA showed that, although HU could indistinguishably replace IHF on attL unimolecular complexes, it was only able to create a similar complex on attR. Interestingly, the unimolecular complexes on attR containing HU migrated slightly slower than the wild type complexes. This same phenomenon was observed in the FattR unimolecular complexes when IHF was omitted. In addition, in experiments in which sequence-directed bend swaps could facilitate IHF-independent recombination, it was found that the reactions were dependent on the supercoiling of attR (4), and attL topology was unimportant. Thus there is reason to believe that IHF may perform additional roles on at least attR that are beyond a simple reduction of DNA persistence length.

What additional features of IHF might contribute to recombination? We consider three possibilities. First, we can view the role of IHF as the consequence of adding structure in conjunction with reducing persistence length. In this case the shape of the deformation would add to and be necessary for the configuration of the final nucleoprotein complex. Hence, structural information would be required from Int, Xis, att sites, and IHF to create the proper nucleoprotein complex. This would also account for the success of the bend swap as functional replacements (1, 4).

Second, the failure of IHF-independent Fatt site recombination may be the result of additional IHF functions that cannot be accommodated by these alternative structures. For example, we cannot rule out protein-protein interactions between Int-IHF, IHF-Xis, and/or IHF-IHF (i.e. attR-bound and attL-bound). However, because IHF interacts with a plethora of nucleoprotein systems, it seems unlikely that there is a fortuitous interaction specific to any one protein, although there is evidence that this may occur in an isolated case (28). In addition, we also cannot eliminate charge shielding through simple DNA binding. If charge neutralization of the polyamion backbone of DNA was required for function, then the T-bubble would be deficient and thus recombination abrogated. The ability of IntE174K to perform marginal recombination could be explained if it, too, shielded charge at a site otherwise protected by IHF. The change from acidic amino acid to basic amino acid...
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obviously has pronounced effects on recombination. It is possible that IntE174K is creating a new contact with the DNA. This would obviate one of the functions of IHF permitting the small amount of observed recombination.

Third, IHF may possess properties that in subsequent steps makes it recalcitrant to recombination. According to this model, IHF would need to bend DNA to create the initial DNA structures. However, these structures and/or some other additional feature would interfere with subsequent steps, and thus the intermediate would need to be altered to complete the reaction. In a parsimonious version of this model, IHF would need to bend DNA to create the initial DNA intermediate would need to be altered to complete the reaction. In that work, the authors liken the role of IHF to a compressed spring that in later steps needs to be sprung for completion of transposition. Moreover, a similar scheme has been proposed for Flp-induced architecture during Flp-mediated recombination (30). In a recombination with Fatt sites, deformation of DNA could initiate the formation of the appropriate complexes; however, with the inability to undo these structures, the intermediates would be reversibly trapped and would likely revert to substrates. This would readily account for the Fatt sites ability to function in capture/cleavage without IHF and still explain their inability to yield sufficient recombinant products.

In conclusion, the results of experiments performed with Fatt sites are consistent with a role for DNA deformation in the native IHF-dependent recombination pathway. Although Fatt sites fail to achieve conditions that can fully replace IHF function, highlighting the limitations of alternative structures, these results have focused our attention on additional contributions of IHF to the recombination pathway. Our discovery of the apparent binding specificity of IHF to flexible DNA substrates may indicate previously unknown functions in native DNA metabolism.

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