Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 11910–11914, December 1992
Biochemistry

Deformation of DNA during site-specific recombination of bacteriophage λ: Replacement of IHF protein by HU protein or sequence-directed bends

(DNA bend/nucleoprotein complex/curved DNA)

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Contributed by Howard A. Nash, September 4, 1992

ABSTRACT *Escherichia coli* **IHF protein is a prominent component of bacteriophage λ integration and excision that binds specifically to DNA. We find that the homologous protein HU, a nonspecific DNA binding protein, can substitute for IHF during excisive recombination of a plasmid containing the prophage attachment sites *attL* and *attR* but not during integrative recombination between *attP* and *attB*. We have examined whether IHF and HU function in excisive recombination is mediated through DNA bending. Our strategy has been to construct chimeric attachment sites in which IHF binding sites are replaced by an alternative source of DNA deformation. Previously, we demonstrated that properly phased bends can substitute for the binding of IHF at one site in *attP*. Although this result is highly suggestive of a critical role of IHF-promoted bending in λ integration, its interpretation is obscured by the continued need for IHF binding to the remaining IHF sites of these constructs. In the present work, we engineered a population of sequence-directed bends in the vicinity of the two essential IHF sites found in *attR* and *attL*. Even in the absence of IHF or HU, pairs of these attachment sites with properly phased bends are active for both *in vitro* and *in vivo* excision. This success, although tempered by the limited efficiency of these systems, reinforces our interpretation that IHF functions primarily as an architectural element.**

A variety of biochemical and genetic experiments indicate that *Escherichia coli* IHF protein plays an important role during integration of bacteriophage λ DNA into the host chromosome and excision of integrated λ prophage DNA from the chromosome (1). IHF is a heterodimeric protein that binds to sequences that contain a well defined consensus sequence (2). Three such sequences, called H1, H2, and H′, are found at attachment sites, the loci of λ integration and excision (3). At these and other sites IHF sharply bends the DNA to which it binds (4–10). Our laboratory has put forward and has been testing the hypothesis that deformation of DNA is the critical function for IHF in recombination. HU protein is a homolog of IHF that also binds and compacts DNA (11), but with little or no sequence specificity (12). In this paper, we address the degree to which HU protein can replace IHF and the extent to which such a replacement can be mimicked by another mode of deforming DNA.

MATERIALS AND METHODS

Plasmids. Plasmids used in Table 1 are as follows: pHN894 (*attP*, phage attachment site; constructed identically to pWR1; ref. 13), pHN861 (*attB*, bacterial attachment site; ref. 14), pHN868 (*attR*, right prophage attachment site), pHN872 (*attL*, left prophage attachment site), pHN864 (*attR–attL*), and pHN1533 (*attP–attB*; recombinant product of *in vitro* excisive recombination between pHN868 and pHN872; this work). pHN868, pHN872, and pHN864 were derived by *in vitro* integrative recombination (14).

All chimeric *att* sites were derived from plasmids pHN1160 and pHN1278, which contain functional *attR* and *attL* sites, respectively. pHN1160 was derived from pHN986 (15), which carries *attP*. The 0.4-kilobase (kb) *Sph* I/*Hpa* I fragment of an *attR* derived from *in vitro* recombination of pHN986 with wild-type *attB* (pBB105) was ligated to the 2.9-kb *Nco* I/*Sph* I fragment of pHN1158 (the *Nco* I end was treated with T4 DNA polymerase prior to restriction with *Sph* I to generate a blunt end). pHN1158 is identical to pHN986 except for a *Sna*BI site engineered at −60 (−59T and −61C) by site-directed mutagenesis (16). pHN1278 was constructed as follows: the *attP*-containing plasmid, pHN1099 [equivalent to pHN986 except, instead of an engineered *Sph* I site at −23, there is an *Sph* I site engineered at +29 (+29G and +30C)] was recombined *in vitro* with an *attB*-containing plasmid, pHN861. The recombinant product was cleaved with *Cla* I and the 2.8-kb fragment containing *attL* was isolated and recircularized to yield pHN1278. pHN1302 is equivalent to pHN1278 except for an engineered *Xmn* I site at +40 (+35G, +36G, +45C). Chimeric *attL*s are equivalent to pHN1302 except that each contains a single insert at the *Xmn* I site; the inserts were derived from a population of DNA fragments containing sequence-directed bends (ref. 15; see also Fig. 2). Chimeric *attR* sites were made by a similar cloning strategy by ligating the same population of DNA segments into the unique *Sna*BI site of pHN1160 (see Fig. 2).

To construct plasmids containing both an *attL* and an *attR*, each *attL* plasmid was cleaved at a unique *Pvu* II site and was ligated to an *Apa*LI/*Hin*dIII fragment containing *attR* (≈1.2 kb; treated with T4 polymerase to fill in the ends). In each case, plasmids were isolated that had the *att* sites separated by ≈2 kb and oriented head to head. pHN1394 was made from pHN1278 and pHN1160. pHN1396 was made from pHN1319 and pHN1385 (see Fig. 2). pHN1460 was made from pHN1414 and pHN1399 (see Fig. 2). Derivatives of these plasmids that were phenotypically similar as substrates for recombination were generated by replacing the 1240-base-pair (bp) *Pst* I/*Pst* I KnR cassette with a 1113-bp *Bsp*HI partial digestion fragment from pBR322 (coordinates 3193–4307), which contains the gene for AmpR. Both vector and insert were treated with T4 polymerase prior to ligation to generate blunt ends. The AmpR versions of pHN1394, pHN1396, and pHN1460 are designated pHN1485 (wt R-L in Table 2), pHN1486 [Chimeric R-L (bent) in Table 2], and pHN1487 [Chimeric R-L (straight) in Table 2], respectively.

Restriction digests and DNA sequencing (17, 18) were used to confirm the above constructions.

***In Vivo* Excisive Recombination.** Excisive recombination was assessed in the following *E. coli* strains: N6377 (wild type), HN1563 ($hupA^-$, $hupB^-$), HN1436 (hip^-), and

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HN1519 (*himA*$^-$, *hupA*$^-$, *hupB*$^-$). Each of these strains carried the deleted prophage λ *int*$^+$ *xis*$^+$ Δ(*Sal* I/*Xho* I) *cIts857* (*cro-chl(A)*)ΔH1. Strain N6377, C600 *thr*$^-$ *leu*$^-$ *thi*$^-$ *pro*$^-$ *gal*$^+$(*chlD–pgl*)Δ8, was provided by M. E. Gottesman (Columbia University); strain HN1436 was derived from N6377 by P1 transduction of the ∇3[*hip*]::*cat* allele (19). Strain HN1563 was derived from N6377 by sequential P1 transduction of *hupA16*::*kan* and *hupB11*::*cat* alleles from K4389 (N99 *hupA16*::*kan*) and K4388 (N99 *hupB11*::*cat*), respectively (P1 lysates were the generous gift of Dhruba Chattoraj, National Cancer Institute). K4832, (N99 *himA*Δ*Sma* I *hupB11*::*cat*) was a gift from A. E. Granston (National Institute of Mental Health); strain HN1519 was derived from K4832 by P1 transduction of the prophage from N6377 and the *hupA16*::*kan* allele was from K4389. After checking the HU/IHF phenotype of N6377 (wild type), HN1436 (IHF$^-$), HN1563 (HU$^-$), and HN1519 (IHF$^-$, HU$^-$) by Western blotting and by bacteriophage plating tests (20), the strains were transformed with the *attL–attR* plasmids pHN1485, pHN1486, and pHN1487 or an *attP–attB* plasmid pBP86 (21).

These plasmid-bearing strains were used to study excisive recombination *in vivo*. Each of the following steps in the protocol was done in LB medium with vigorous shaking. Cells were grown to midlogarithmic phase at 30°C and induced to produce Int and Xis by adding an equal volume of prewarmed medium at 60°C. The culture was then incubated at 42°C for 30 min. Cultures were then returned to 30°C for 30 min. Plasmid DNA was then isolated (22), digested to distinguish substrates from products, and resolved by agarose gel electrophoresis.

***In Vitro* Recombination.** Purified proteins (Int, IHF, Xis, and HU) were used in all experiments. Purification of Int, IHF, and Xis has been described (23–25). HU, purified from an IHF-deficient strain of *E. coli* (RW1912 = LE392 with the following relevant genotype: ∇3[*hip*]::*cat himA*Δ*82*), was the gift of R. McMacken and L. Huang (Johns Hoplins University). All reaction mixtures (20 μl) included plasmids containing *att* sites at 2–4 nM and Int at 60 nM in buffer containing 30 mM Tris·HCl (pH 7.8), 70 mM KCl, 5 mM spermidine, 0.1 μg of EDTA per ml, 50 μg of bovine serum albumin per ml, and 1% glycerol. Xis (20 nM) was added to all excision reaction mixtures. IHF and HU were added where indicated. Specific reaction conditions are described in the figure legends.

Quantitation of Recombination. Quantitation of DNA substrates and products (see Tables 1 and 2) was determined by using the NIH densitometry program IMAGE written by W. Rasband. Agarose gels containing resolved DNA species were stained with ethidium bromide (0.1 μg/ml for 3 hr). Gels were then photographed with Polaroid type 665 positive/negative film under UV light (310 nm) with red and yellow filters. Negatives were scanned and band intensities were quantitated with IMAGE against a set of DNA standards of known concentration and length.

RESULTS

HU Can Replace IHF During *in Vitro* Excisive Recombination. We carried out *in vitro* λ site-specific recombination in the presence of either IHF or HU (Table 1). We examined both integration and excision reactions and we positioned the appropriate *att* sites either on the same plasmid substrate or on different substrate plasmids.* With IHF, all recombination reactions were efficient (≥25% of the substrates were converted to products in 45 min), but only prophage *att* sites recombined detectably when HU replaced IHF. The efficiency of excisive recombination under these conditions is never very high but is clearly better when *attR* and *attL* are in cis. It should be noted that reactions without either IHF or HU failed to yield recombinant product (data not shown); this confirms that a host factor is an essential ingredient for site-specific recombination.

Table 1. Integrative and excisive recombination *in vitro*

	% recombination (IHF/HU)	
	attR–attL	*attP–attB*
Intramolecular	73/9	49/<0.2
Intermolecular	34/1	25/<0.2

Supercoiled plasmids with attachment sites on different plasmids (intermolecular) or on the same plasmid (intramolecular) were used as substrates for intermolecular and intramolecular recombination, respectively. All reaction mixtures contained the designated attachment sites at 2 nM and Int at 60 nM. Excision reaction mixtures also contained 20 nM Xis. IHF or HU (30 nM) was added where indicated. Incubations were at 25°C for 45 min. DNA in each reaction mixture was then digested to distinguish substrates and products and resolved by agarose gel electrophoresis. Values for % recombination are the average of two trials.

The following attributes were comparable in HU-stimulated and IHF-stimulated excisive recombination: the structure of the recombinant products, the requirement for particular Int binding sites in *attL* and *attR*, the dependence on Xis protein and ionic conditions, and the response to supercoiling (data not shown). As yet the only difference observed occurs during recombination in the presence of competitor DNA. Fig. 1 shows that, as increasing amounts of pBR322 (competitor) are added, reactions with HU yield less and less recombinant product while those that contain IHF are unaffected. The ability of competitor DNA to inhibit recombination with HU is consistent with the apparent

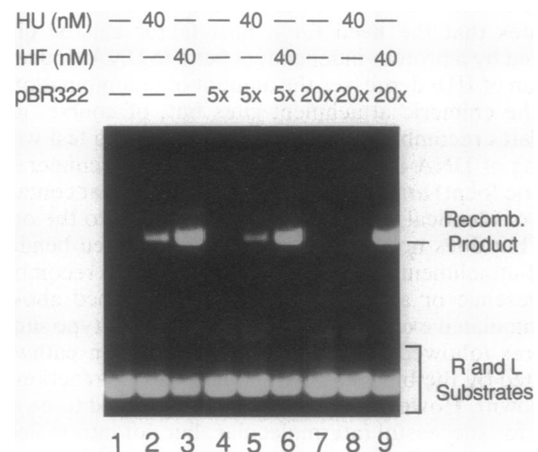

FIG. 1. Excisive recombination *in vitro* with HU or IHF protein. A supercoiled plasmid (pHN1394) containing wild-type *attR* and *attL* was used as substrate. All reaction mixtures contained 60 nM Int and 20 nM Xis and were incubated for 40 min at 25°C. HU and IHF (40 nM) were added where indicated. pBR322 predigested with *Msp* I was added as nonspecific competitor at 0×, 5×, or 20× (wt/wt) the concentration of the substrate plasmid (12 μg/ml). Reactions were stopped by incubating at 70°C for 15 min. $MgCl_2$ was then added to 12 mM followed by digestion with *Mlu* I and *Kpn* I (10 units of each enzyme per reaction). Upon agarose gel electrophoresis, the substrate DNA fragments (1974 and 1999 bp) are not resolved from each other but they are well separated from one recombinant product (3514 bp); the other recombinant product (459 bp) and the pBR322 fragments were run off the end of the gel.

*Integrative and excisive recombination are defined as site-specific recombination events that occur between *attP* and *attB* and between *attR* and *attL*, respectively. Although these reactions normally join and uncouple segments of DNA, respectively, each can function efficiently in intramolecular inversion, intramolecular resolution, and intermolecular fusion.

inability of HU to recognize a specific DNA sequence. This result also rules out the possibility that the HU preparations used in these experiments are contaminated with significant levels of IHF.

Excision in the Absence of IHF or HU with Modified *att* Sites. The ability of HU to eliminate the need for IHF during excisive recombination, while consistent with the idea that deformation of DNA is a crucial function for IHF, fails to prove this hypothesis. Since IHF and HU are homologous proteins, they should have many features in common, any of which could account for our data. For example, if a critical contact between a protomer of IHF and another protomer of the same protein or between IHF and Int (or Xis) were essential for recombination, such contacts may also be made by HU.

We have used bend swap experiments to distinguish between these alternatives. Such studies have showed that sequence-directed bends, properly phased, can substitute for binding of IHF at the H2 site of *attP* during integration (15). Although this result strongly implicated bending as an important function of IHF, we could not test whether IHF was performing additional roles since the protein was still demanded at the remaining sites (H1 and H′). To determine whether bending is the exclusive role of IHF, and therefore the basis of its successful replacement by HU, we engineered bend swaps on the essential IHF sites of *attL* and *attR*. This approach is facilitated by the need of only two IHF sites (H2 and H′) during excisive recombination (14, 26).

A population of substrates containing sequence-directed bends in the vicinity of H2 in *attR* or within the H′ consensus of *attL* was constructed (Fig. 2). Each chimeric attachment site was then tested for *in vitro* intermolecular recombination with a wild-type partner (i.e., chimeric *attR*s were paired with wild-type *attL*s and vice versa) in the presence of IHF, Int, and Xis (data not shown). The chimeric *attL* and *attR* sites that were most proficient in excisive recombination were then tested for their ability to recombine with one another in the presence or absence of IHF (Fig. 3). In the absence of IHF, wild-type *att* sites fail to recombine but the chimeric (bent) sites yield recombinant product. This demonstrates that the need for a host factor can be entirely replaced by a protein-independent bend in DNA. Indeed, the addition of IHF depresses the amount of recombination seen with the chimeric attachment sites but, of course, greatly stimulates recombination of wild-type sites. To test whether bending of DNA is the critical feature of the chimeras, the chimeric (bent) *attL* was paired with an *attR* that contains an insert of identical size and base composition to the original insert but does not contain a sequence-directed bend. This pair of attachment sites (lanes 3 and 6) fails to recombine in the presence or absence of IHF. As described above for HU-stimulated excisive recombination of wild-type sites, the chimeras followed the standard recombination pathway as indicated by the biochemical attributes of the reaction (data not shown). However, the *attR* chimera needed to be supercoiled for successful intermolecular, but not intramolecular, excisive recombination (data not shown).

Although our data clearly show that bent DNA can replace IHF function, the level of recombination we observe is not high. The difference in efficiency is even greater when the initial rate of recombination, rather than the extent of the reaction, is measured (data not shown). Although the efficiency of the chimeras could be improved by placing the *att* sites in cis, their efficiency relative to IHF-promoted recombination of wild-type sites was not enhanced (data not shown). In addition, HU failed to significantly affect excisive recombination between chimeric *att* sites (data not shown). If one assumes that deformation of DNA is the sole function of IHF, the limited efficiency of the chimeras could be ascribed to inadequacies in our replacement—e.g., the bend is of the wrong magnitude, is included on too large of a piece of DNA, or is phased inappropriately. We doubt that the latter explanation is correct. Other chimeras, whose inserts differ in size from those used in Fig. 3 by nearly an integral number of helical turns (8, 9, 11, and 12 bp), show similar efficiency while those that differ in size by nearly a half-integral number of turns (4, 6, and 16 bp) are significantly worse (data not shown). Thus, our best chimera appears to be adequately phased. We have not tested to see whether much smaller inserts that have a similar sequence-directed bend or inserts with a different bend magnitude would be more effective replacements for IHF.

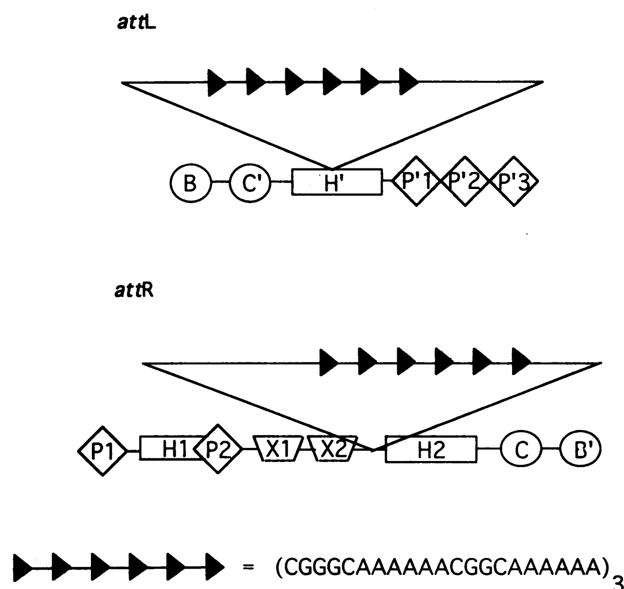

FIG. 2. Construction of chimeric attachment sites. (*Top* and *Middle*) Attachment sites *attL* and *attR* and position of the sequence-directed bend inserts (drawn approximately to scale). All *attR* plasmids were derived from pHN1160 (3.1 kb), which contained an engineered *Sna*BI site between X2 and H2; it is proficient for recombination. The *Sna*BI site was used to introduce a population of bent DNA fragments as described (15). (*Bottom*) Each insert included a 77-bp *Xba* I cassette, which contains six repeats (solid arrowheads) of a tract of six adenines phased to form a stable bend (A tract DNA; 63 bp). This A tract was flanked by spacer DNA of variable length; thus, the size of the insert (126–159 bp) and the position of the bend were distinct in individual clones. Similarly, a population of *attL* derivatives was constructed with A tract inserts (103–145 bp) at an engineered *Xmn* I site within the IHF consensus of the H′ locus. The *attR* (pHN1385) and *attL* (pHN1319) that were most efficient in intermolecular recombination with a wild-type *att* partner are referred to in subsequent figures as bent or chimeric (bent) *attR* and *attL*, respectively. The insertions in pHN1385 and in pHN1319 have 46 and 16 bp to the left and 16 and 33 bp to the right of the A tract cassette, respectively. In addition, chimeric (straight) derivatives of pHN1319 (pHN1414) and pHN1385 (pHN1399) were also constructed. For these plasmids, the 77-bp region, which includes the A tract, was replaced with an unbent segment of DNA of identical base composition and size (pBR322 coordinates 1311–1387 with an A to C change at position 1327 and a G to A change at position 1373).

IHF-Independent Excisive Recombination *in Vivo*. To test the physiologic relevance of our observations on replacements of IHF, we examined excisive recombination *in vivo* (Table 2). Plasmids containing chimeric or wild-type *attR* and *attL* sites were propagated in a set of four strains that could be induced to express Int and Xis; the members of the set provided all different combinations of IHF and HU protein. Plasmid DNA isolated after induction was examined for the appearance of recombinant product. As predicted by our observations *in vitro*, the $IHF^{+}HU^{-}$ and $IHF^{-}HU^{+}$ strains

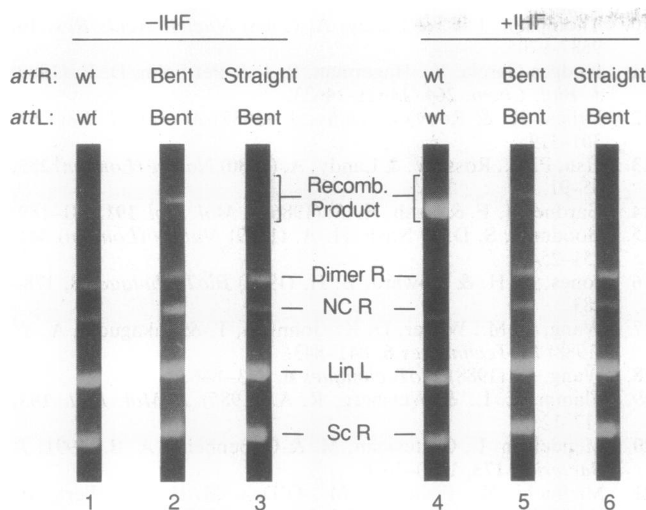

FIG. 3. *In vitro* excisive recombination in the absence and presence of IHF. Supercoiled plasmids containing wild-type (wt) or chimeric *attR* and *attL* were used as substrates for excisive recombination. All reaction mixtures were incubated for 3 hr at 25°C. IHF (30 nM) was added where indicated. Reaction mixtures were then treated with *Kpn* I, which linearizes recombinant product and *attL* substrate. Substrates and products were then resolved by agarose gel electrophoresis and stained with ethidium bromide. Recombinant product appears as linear form molecules with the combined size of the two substrates (≈6 kb). Sc R, supercoiled form of *attR*; Lin L, linear form of *attL*; NC R, relaxed circular form of *attR*; Dimer R, supercoiled form of *attR* dimer. Chimeric *att* sites, bent or straight, are described in Fig. 2.

still sponsor recombination of wild-type *att* sites, but cells lacking both IHF and HU fail to support any detectable excisive recombination. In contrast, recombination of chimeric (bent) *att* sites recombines at a similar (albeit lower) efficiency regardless of the status of the HU and IHF genes. This result not only supports our *in vitro* observations, but it also serves as a control to indicate that the strain lacking both IHF and HU is able to synthesize Int and Xis. Chimeric (straight) *att* sites fail to recombine in any of the tested strains. Also consistent with our *in vitro* results is the observation that *in vivo* integrative recombination is efficient in the strains that provide IHF but fails to yield detectable products in strains defective in IHF irrespective of the HU genotype.

DISCUSSION

In this work, we have shown *in vivo* and *in vitro* that HU protein is a moderately effective replacement for IHF protein during excisive recombination. It has been reported that HU and IHF have similar effects *in vitro* on the transposition of bacteriophage mu (27) and the replication of the origins of *E. coli* (28) and bacteriophage λ (29). In all these cases, despite the absence of a well-defined target consensus sequence, HU is effective in amounts that are insufficient to cover a substantial fraction of the substrate DNA. Unless HU is used catalytically, it must have a significant element of specificity in its binding. This could reflect some undiscovered sequence specificity. Alternatively, HU may preferentially bind to the active locus as a result of interactions with other proteins bound to the region or as a result of a preference for DNA structures that are induced by these proteins. The λ prophage attachment sites offer an excellent opportunity to distinguish between these possibilities.

Table 2. Intramolecular recombination *in vivo*

	% wild-type recombination			
	IHF^+HU^+	IHF^+HU^-	IHF^-HU^+	IHF^-HU^-
wt R-L	100	81	16	<1
ch R-L (bent)	7	5	7	5
ch R-L (straight)	<1	<1	<1	<1
wt P-B	100	86	<1	<1

All values are relative to the result for wild-type attachment sites in a wild-type host (41% and 72% conversion into recombinant products for excisive and integrative recombination, respectively). All values are the average of duplicate experiments. Strains with the IHF and HU phenotypes indicated were derived from the following parent strains: IHF^+HU^+, N6377; IHF^+HU^-, HN1563; IHF^-HU^+, HN1436; IHF^-HU^-, HN1519. Wild-type (wt) and chimeric (ch) attachment sites are described in Fig. 2.

Our *in vivo* experiments confirm previous reports (30–32) that residual levels of excisive recombination can be seen in IHF^- strains and our work provides the first evidence that this residual recombination depends strongly on HU function. The inability of an IHF^-HU^- strain to support excisive recombination implies that other small basic proteins of *E. coli* (12) are not functionally equivalent to HU. We note that the levels of recombination we observe in IHF^-HU^+ strains are substantially higher than those reported previously. This could reflect a strong dependence of HU-stimulated recombination on the effective concentration of attachment site pairs, since in previous studies (30–32) the attachment sites were on separate DNA species or, when located on the same species, were more than 6 times farther apart than in our studies. It must be emphasized that both previous and present studies agree that there is little or no residual integrative recombination in IHF^- strains. This is consistent with our *in vitro* data, which show that HU cannot replace IHF for integration. Integration and excision differ most notably in their requirement for the H1 site; perhaps HU cannot bind to this region or cannot function there.

Our experiments have also shown that adding sequence-directed bends to both *attL* and *attR* leads to a significant level of Int- and Xis-promoted recombination in the absence of any host factors. This result is a significant extension of our previous experience, in which we replaced one IHF site in *attP* with a sequence-directed bend. In that case, efficient recombination was still dependent on IHF, presumably because of the unaltered H1 and H′ IHF binding sites. The new constructs permit us to eliminate the possibility that the artificial bend merely served as an alternative binding site for IHF. They also extend our previous conclusion that functional attachment sites do not demand a structure that is highly constrained; our most successful constructs expand the size of *attL* and *attR* by 126 and 139 bp, respectively. We conclude that, although certain parts of the attachment sites may need to come together to generate a functional locus (33, 34), the pathway of DNA between these parts appears to accommodate great variation. Indeed, we are struck by the observation that, both *in vivo* and *in vitro*, addition of IHF causes at best a modest inhibition of function of our chimeras. It is possible that IHF fails to bind to these sites (although no alteration of the IHF consensus sequences was made in *attR*, an adjacent sequence-directed bend might cause interference), but it is also possible that the attachment site structure accommodates an additional protein-induced DNA deformation.

IHF is a particularly striking example of a protein that deforms DNA. Electron microscopic (4–6) and gel-mobility experiments (7–10) indicate that IHF introduces a sharp turn at its binding site. In this paper, we have seen that two different agents that deform DNA, HU and A tracts, can at least partially replace IHF protein during excisive recombination. To what extent does this replacement reflect the fundamental mechanism by which IHF activates recombination? It is tempting to imagine that the replacements are relatively direct—i.e., one agent that deforms DNA replaces

another. However, it is hard to rule out the possibility that the replacements are not direct but produce a bypass for an essential function of IHF. According to this hypothesis, IHF does something that is essential for recombination but this action is independent of, or in addition to, bending of DNA. Replacements of IHF with elements that lack this action must activate recombination by doing something that IHF does not do—e.g., producing a deformation in DNA dissimilar to the bend caused by IHF. One would imagine that such a pathway would have unusual properties. The attributes of *in vitro* excisive recombination under conditions in which IHF is replaced by HU or by sequence-directed bends are quite similar to those of the standard reaction. The most notable exception, an unusual dependence on supercoiling for bend swap chimeras of *attR*, is plausibly explained by a difference in the nature of compaction of DNA caused by IHF versus the A tracts. Thus, although we cannot eliminate the possibility of a bypass mechanism for the IHF experiments described in this paper, we think it is unlikely.

We are indebted to Drs. Roger McMacken and Li Huang for generously providing us antisera to HU and purified HU protein without which we could not have performed these experiments. We thank the members of our laboratory for critical reading of this manuscript. We also thank Drs. Anca Segall and Andrew E. Granston for helpful discussions throughout the course of this work. S.C.N. is a Howard Hughes Medical Institute–National Institutes of Health Research Scholar.

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