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DNA Bending and Twisting Properties of Integration Host Factor Determined by DNA Cyclization

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The binding of many proteins to DNA is profoundly affected by DNA bending, twisting, and supercoiling. When protein binding alters DNA conformation, interaction between inherent and induced DNA conformation can affect protein binding affinity and specificity. Integration host factor (IHF), a sequence-specific, DNA-binding protein of *Escherichia coli,* strongly bends the DNA upon binding. To assess the influence of inherent DNA bending on IHF binding, we took advantage of the high degree of natural static curvature associated with an IHF site on a 163-bp minicircle and measured the binding affinity of IHF for its recognition site contained on this DNA in both circular and linear form. IHF showed a higher affinity for the circular form of the DNA when compared to the linear form. In addition, the presence of IHF during DNA cyclization changed the topology of cyclization products and their ability to bind IHF, consistent with IHF untwisting DNA. These results show that inherent DNA conformation anisotropy is an important determinant of IHF binding affinity and suggests a mechanism for modulation of IHF activity by local DNA conformation.

Integration host factor (IHF) is a heterodimeric protein that binds specifically and deforms its DNA target into a mostly planar bend (Prentki *et al.,* 1987a; Nash, 1996) with an estimated magnitude of 140° to 180° (Thompson and Landy, 1988; Rice *et al.,* 1996). IHF is involved in a diverse set of systems representing nearly all of the major functions and activities of DNA including transcription, replication, recombination, transfer, partitioning, and packaging (Nash, 1996). A common theme in many of these systems is the requirement for higher ordered multiprotein/DNA complexes. IHF was originally discovered as a host factor required for the integration of phage λ into the *Escherichia coli* chromosome (reviewed in Nash, 1981). Its function in the assembly of the λ "intasome" (Echols, 1986; Richet *et al.,* 1986) is that of DNA deformation (Goodman and Nash, 1989; Moitoso de Vargas *et al.,* 1989; Segall *et al.,* 1994).

While there is an apparent consensus binding sequence for IHF, it is a relatively poor predictor of binding affinity and sequence information is delocalized over 30 to 40 bp (Goodrich *et al.,* 1990). Regions beyond the consensus sequence may play an important role in IHF binding through the influence of specific bases on DNA structure and its local anisotropy (Huisman *et al.,* 1989; Kur *et al.,* 1989; Prentki *et al.,* 1987b; Gamas *et al.,* 1987; Kosturko *et al.,* 1989; Tsai *et al.,* 1990; Goodman *et al.,* 1999).

To assess the role of DNA conformation in IHF binding, the binding affinity of IHF for a small, 163-bp DNA molecule with one IHF binding site was measured. Circularization of this short DNA, which induces inherent DNA bending, increased IHF's affinity for binding. Topological analysis of ligation–circularization products made in the presence of IHF revealed that IHF induces a change in linking number which can most easily be explained by DNA untwisting at the binding site.

MATERIALS AND METHODS

Construction and verification of monomer circle. The 163-bp *Nla*III fragment was derived

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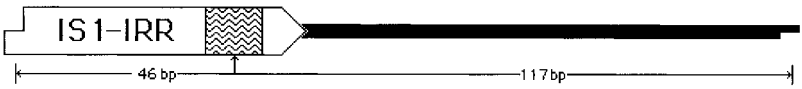

FIG. 1. DNA fragment used in small circle construction. The linear 163-bp DNA is derived from the right end of IS*1* which contains the IHF binding site (hatched box) and part of pBR322 from coordinates 3199 to 3299 (heavy line). The IHF binding site is 46 and 117 bp from the ends of the fragment which are *Nla*III sites with four single-stranded 3′ overhanging ends.

from pMP29 (Prentki *et al.*, 1987b). The IHF binding site present on this fragment occurs in the inverted right repeat of IS*1* (Prentki *et al.*, 1987b) and is centered 46 and 118 bp from the IS*1* and pBR322 *Nla*III sites, respectively (Fig. 1). To generate the labeled reporter DNA, the region encompassing this fragment was amplified by a standard polymerase chain reaction using [^{32}P]dATP (100 mCi, the total [dATP] was 20 mM (the ratio of ^{32}P:^{31}P was 1:30); the other dNTPs were 200 μM). The amplified product was cut with *Nla*III (which leaves 4 bp CATG 3′-overhang, Fig. 1) and the 163-bp fragment was isolated from an 8% polyacrylamide gel and eluted by the crush and soak method (Maniatis *et al.* 1982). The DNA concentration was calculated from the DNA radioactivity and the [^{32}P]dATP specific activity. This labeling procedure produced high specific activity DNA so that very small amounts of DNA were used in IHF-binding gel-shift reactions; this allowed the use of simplifying assumptions in the estimation of binding affinity.

Ligation reactions. The radioactively labeled, 163-bp linear DNA (~50 pM for low concentration reactions; 500 pM for high concentration reactions) was preincubated with or without 100 nM IHF (a generous gift of Howard Nash and George Chaconas) in 20 μl IHF binding buffer (50 mM Tris HCl, pH 7.4, 70 mM KCl, 7 mM $MgCl_2$, 3 mM $CaCl_2$, 1 mM EDTA, 1 mM mercaptoethanol, 200 μg/ml BSA, 10% glycerol) with 1 μM ATP at 25°C for 15 min; 1 unit of T4 DNA ligase (New England Biolabs) was added and incubated at 25°C for 60 min (high DNA concentration with or without IHF; low DNA concentration with IHF) or 8 h (low DNA concentration without IHF); the reaction was extracted with phenol:chloroform (1:1), ether extracted, ethanol precipitated, and resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8). Ligation in the presence of IHF was carried out similarly at 50 pM DNA.

Chloroquine gel electrophoresis. Topoisomers open circle (OC) and covalently closed circle (CCC) of 163-bp circles were resolved on 6% polyacrylamide/TBE gels with 30 μg/ml chloroquine, loaded under 150 V, and run at 300 V (10 V/cm) for 3 to 5 h at 25°C. The gel was dried and exposed to film.

IHF binding assay. Circular and linear 163-bp DNA at ~50 pM each were incubated with purified IHF in 20 μl of IHF binding buffer at 25°C for 15 min. Two microliters of loading dye (xylene cyanol 0.01%, BSA 1 mg/ml, 50% glycerol) was added and gently mixed. The reaction was loaded onto an 8% polyacrylamide gel (19:1 acrylamide:bis-acrylamide, pre-run for 3 h at 4°C and 250 V) at 4°C under 50 V (2.5 V/cm) and then run at 250 V for 4 h. The gel was dried and exposed to film. Gel bands were excised and Cerenkov counted in a scintillation counter.

RESULTS

DNA Minicircles Made in the Presence and Absence of IHF

A 163-bp DNA substrate containing a single binding site for IHF derived from the IRR end of IS*1* was used as the DNA substrate for the ligation–circularization reaction (Fig. 1). This IHF site has previously been shown to bind and be bent by IHF (Prentki *et al.*, 1987a). The linear form contains a 4-bp 3′ overhanging end derived from *Nla*III cleavage. The products of ligation at high linear DNA concentration (~500 pM) were linear concatamers and monomeric and concatemeric circles (Fig. 2, lane 1). Their relative abundances conform to the predicted relationship between intra- and intermolecular cyclization kinetics based on DNA

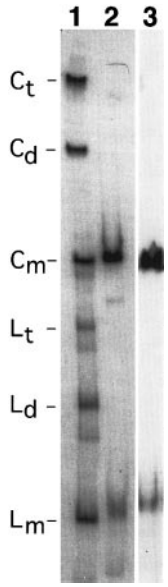

FIG. 2. Ligation products of the 163-bp DNA. Products of ligation in the absence of IHF at high DNA concentration (lane 1) or at low DNA concentration (lane 2) or ligation at high DNA concentration in the presence of IHF (lane 3) were resolved on 6% polyacrylamide gel. The identities of the bands are indicated as C, circular; L, linear; m, d, t: monomer, dimer, and trimer, respectively.

length and concentration (J/I ratio; Maniatis *et al.*, 1982). Ligation at low linear DNA concentration (~50 pM) produced only monomeric circles (Fig. 2, lane 2), as expected for an increased J:I ratio. The identity of the monomeric circle was shown by heat denaturation and gel mobility (below) as well as restriction digestion and electron microscopy (data not shown).

Ligation of the 163-bp DNA at high linear DNA concentration in the presence of IHF (100 nM) produced exclusively monomeric circles (Fig. 2, lane 3). In addition, the rate of monomer circularization at low linear DNA concentration was about 50-fold faster in the presence of IHF (data not shown). Similar effects have been shown for CAP on DNA cyclization products and kinetics (Kahn *et al.*, 1992). Because of these enhanced kinetics, monomeric circles for further studies were prepared by ligation in the presence of IHF, except where indicated.

Binding Affinity of IHF for Minicircles

Monomeric circles (163-bp) produced by ligation in the presence of IHF were subsequently purified and used to study IHF binding. The relative binding affinities of IHF for the circular and linear forms of the 163-bp DNA were distinguished using a gel-shift assay in which equal amounts of 163-bp circular and linear DNA were mixed and titrated with IHF (Fig. 3). The identities of the IHF–DNA complexes with linear and with circular DNA were determined by the mobilities of their complexes in independent binding reactions (data not shown). The IHF–

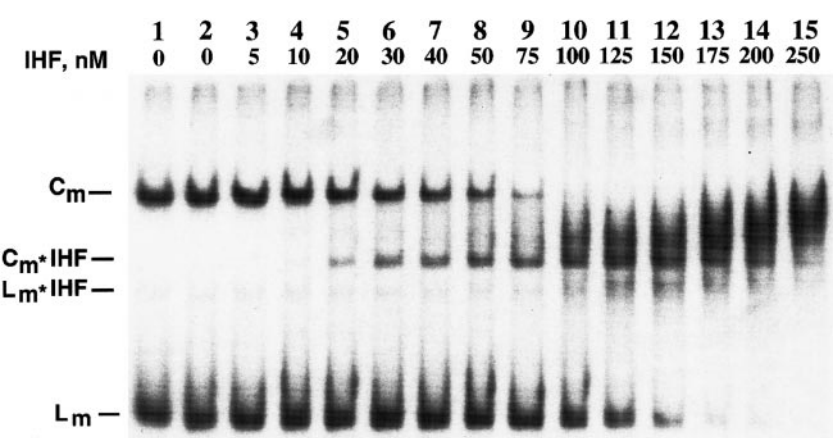

FIG. 3. IHF binding to 163-bp circular and linear DNA. Monomer circular (C_m) and linear (L_m) 163-bp DNA were mixed in equal amounts and incubated with IHF at the indicated concentrations. Complexes with IHF with C_m and L_m are indicated.

linear DNA complexes showed the classic "retarded" mobility relative to unbound linear DNA. In contrast, the migration of the IHF–circular DNA complex was accelerated relative to unbound circle. This "gifted" mobility of a protein–DNA complex probably involves both compaction of the circular DNA by IHF-induced bending, reducing its hydrodynamic radius, and the lack of a linear DNA-end reptation effect. Similar results and interpretations were reported for IHF (Sun *et al.,* 1996) as well as other DNA bending proteins (Douc-Rasy *et al.,* 1989; Teyssier *et al.,* 1996).

The intensities of the unbound linear and unbound circular DNA bands (Fig. 3) show that IHF complexes form at lower IHF concentrations on the circular DNA than on the linear DNA, indicating that IHF has a higher affinity for the circular form. Relative binding affinities were estimated from the half-maximal binding equilibrium relationship: K_d = [IHF] when the fraction of DNA bound = the fraction of DNA unbound; e.g., in Fig. 3, these IHF concentrations were 25 nM for circular and 125 nM for linear. From Fig.3 and additional determinations, IHF showed about 5-fold higher affinity for circular compared to linear form DNA. This probably primarily reflects enhancement of specific binding since the nonspecific binding affinity of IHF is estimated to be ~100- to 1000-fold lower than specific binding affinity (Shindo *et al.,* 1995; Yang and Nash, 1995; Wang *et al.,* 1995; Mengeritsky *et al.,* 1993) and since the nonspecific target in the 163-bp DNA is relatively small.

Topologies of Circles Made in the Presence and Absence of IHF

The 163-bp linear DNA has, at 10.6 bp per turn (Peck and Wang, 1981), approximately 15.5 turns of the double helix. The product of the ligation of this molecule will be either covalently closed on both strands (covalently closed circle with 15 turns or 16 turns) or covalently closed on only one strand (a topologically relaxed, open circle). These forms are separable by gel electrophoresis in the presence of chloroquine. Chloroquine intercalation into DNA causes unwinding of the double helix; this causes positive twisting in the rest of a CCC which can be converted to positive writhe (buckling and figure-8 formation). Therefore, chloroquine intercalation causes a CCC to become more positively supercoiled and its mobility in PAGE will be altered (as discussed below). In contrast, the topology of an OC will not be significantly distorted by chloroquine because the free ends rotate about the closed strand as chloroquine unwinds the DNA; therefore, its mobility is relatively unaltered.

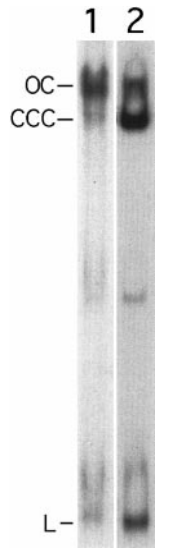

FIG. 4. Topoisomer resolution of circles made in the absence of IHF or presence of IHF (lane 2). Products of ligation at low DNA concentration in the absence of IHF (lane 1) or presence of IHF (lane 2) were run on a 6% gel with 30 μg/ml chloroquine. The positions of open circular (OC), covalently closed circular (CCC), and unligated linear (L) species are indicated.

Purified DNA samples from circles made in the absence of IHF [circles(−)IHF] and circles made in the presence of IHF [circles(+)IHF] were separated by gel electrophoresis (6% polyacrylamide) in the presence of 30 μg/ml chloroquine (Fig. 4). The monomer circle band that migrated as a single band on a native (no chloroquine) gel (Fig. 2, lanes 2 and 3) was resolved into two bands on the chloroquine gel (Fig. 4). Both circles(−)IHF (lane 1) and circles(+)IHF (lane 2) preparations showed these two species on chloroquine gels. The identities of these two

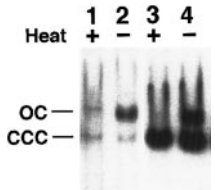

FIG. 5. Heat denaturation of circle topoisomers. Circles made in the absence of IHF (lanes 1 and 2) or in the presence of IHF (lanes 3 and 4) were heated to 95° (lanes 1 and 3) or not (lanes 2 and 4) and run on a 6% polyacrylamide, 30 μg/ml chloroquine gel. The positions of open circular (OC) and covalently closed circular (CCC) are indicated.

monomeric species as OC and CCC (predicted by the differential effect of chloroquine on the mobility of OC and CCC species, discussed below) were shown by the following denaturation experiment: Circles(+)IHF and circles(−)IHF were heated to 95°, quickly cooled to −70°, and separated on a chloroquine gel (Fig. 5). For both circle preparations, heating caused the upper band to disappear, consistent with it being single stranded, while the lower band was quantitatively retained; this suggests that the upper band is nicked (OC) and the lower band is covalently closed (CCC). Similar results were reported by Sun *et al.* (1996) using denaturing gels. The effects of ethidium bromide on ligation product topology (below) are also consistent with these interpretations.

The nick in the OC species can be attributed to either lack of ligation on one strand or postligation nicking of a CCC species. The latter mechanism could be caused by (1) nuclease activity, (2) mechanical breakage, or (3) ^{32}P decay. These are unlikely because of, respectively, (1) the numerous phenol/chloroform treatments, (2) the small size of the DNA, and (3) the failure of the ligation products' CCC:OC ratio to vary over 3 weeks (1.5 half-lives) when compared to the CCC:OC ratio from freshly ligated products (data not shown); this indicates that most molecules had only one [^{32}P]dATP incorporated, consistent with the prediction of incorporation during PCR (see Materials and Methods).

The CCC and OC bands of the circles(−)IHF and circles(+)IHF preparations were quantitated from three experiments. For circles(−)IHF, 83% of the total monomer circle products were OC and 17% were CCC. The abundance of OC is consistent with torsional misalignment of the 163-bp DNA ends, as predicted by the torsional misalignment (~0.5 turns) and the large energy cost to torsionally align the ends in short DNA molecules. This can be approximated at 0.5 kcal/mol assuming a torsional rigidity constant of 10^{-19} erg cm and the twist distributed equally over the entire 163-bp (Law *et al.*, 1993). In contrast, circles(+)IHF were composed of 25% OC and 75% CCC. This indicates that IHF binding substantially counteracts the thermodynamic disfavor for CCC formation inherent in the structure of the 163-bp DNA. Such a linking deficit can be accommodated by either altering twist or writhe of the DNA.

Circles Made in the Presence of Ethidium Bromide

The enhanced production of CCC over OC in the presence of IHF suggests that DNA twisting may be involved. This hypothesis was investigated by performing the ligation reaction in the presence of ethidium bromide (EtBr) which unwinds DNA by 26° per each intercalated EtBr molecule (Clendenning and Schurr, 1994). Ligation products made at low linear DNA concentration in the presence of increasing amounts of EtBr, then purified from the EtBr before separating by gel electrophoresis in the presence of chloroquine, are shown in Fig. 6. Ligation products in the absence of EtBr showed a OC: CCC_1 ratio of 4:1 (Fig. 6A, lane 1). The addition of increasing EtBr resulted in a progressive change in the ratio of topoisomers and the appearance of two new species, CCC_2 (Fig. 6A, lanes 7 to 9, and Fig. 6B, lanes 2 to 9) and CCC_3 (Fig. 6B, lanes 8 to 13), with only one of these species being prevalent at any given EtBr concentration.

The identities of these CCC species were deduced from the combined effects of EtBr on unwinding the DNA, the topological fixation by ligase, and the relative migrations of these three

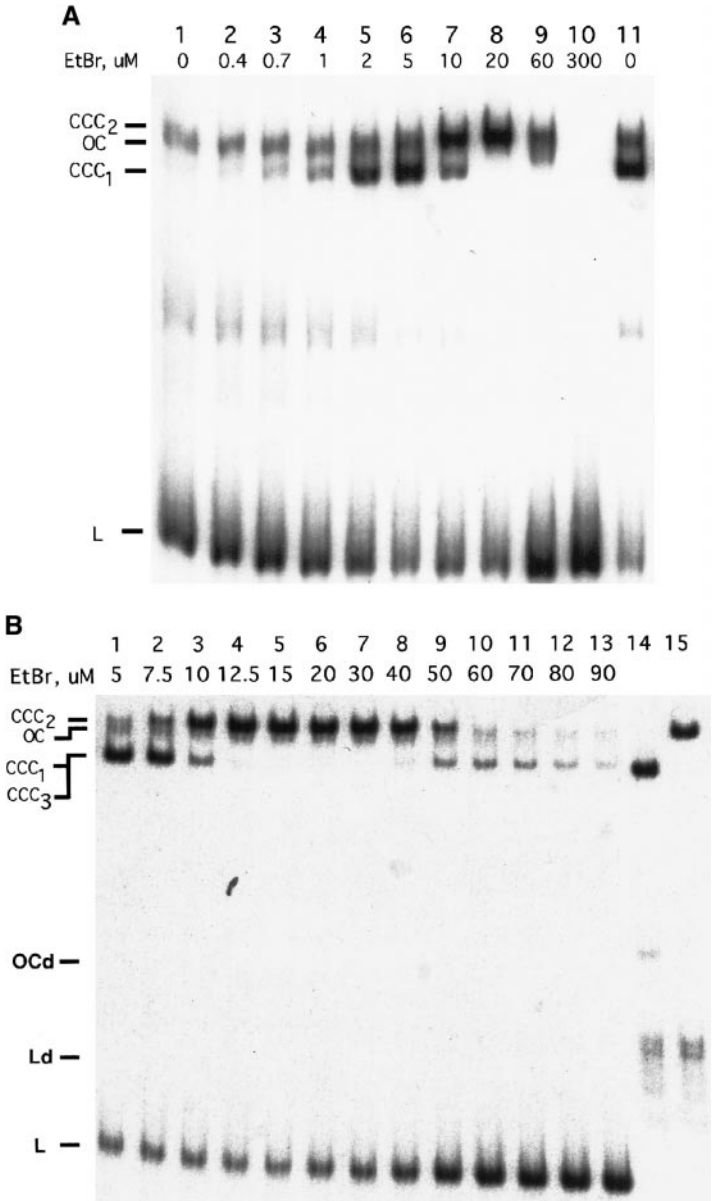

FIG. 6. Ligation in the presence of ethidium bromide. Products of ligation were separated by electrophoresis as described in Fig. 5. A. The 163-bp DNA was ligated for 90 min in the presence of ethidium bromide (EtBr) at the indicated concentrations; a parallel reaction without EtBr but with IHF (100 nM) is shown in lane 11. The positions of linear (L), open circular (OC), and the two covalently closed circular topoisomers, CCC_1 and CCC_2, are indicated. B. Ligation in the presence of EtBr (at indicated concentrations) without IHF (lanes 1 to 13) showing resolution of CCC1 and OC (lane 3) and production of CCC3 (lanes 10 to 14). The positions of linear (L), open circular (OC), and the three covalently closed circular topoisomers, CCC_1, CCC_2, and CCC_3, are indicated. Reaction products at 5 and 20 μM EtBr (as in lanes 1 and 6) were heated to 95° and cooled to −70° before loading (lanes 14 and 15, respectively). The positions of denatured linear (Ld) and open circular (OCd) are indicated.

CCC species in the presence of chloroquine. That CCC_1 and CCC_2 are covalently closed circles is shown by their resistance to heat denaturation (Fig. 6B, lanes 14 and 15; see also Fig. 5; CCC_3 was not tested). Since CCC_1 is the first species formed at low EtBr levels, it follows that it has a Lk = 15 and therefore a "linking deficit," $\Delta Lk = -0.5$. At higher EtBr levels, it follows that CCC_2 and CCC_3 have Lk = 14 and 13 and $\Delta Lk = -1.5$ and -2.5, respectively. Their migration reflects their topological states after chloroquine intercalation. By analogy to the previous discussion of OC and CCC_1, the topologies of CCC_1, CCC_2, and CCC_3 in 30 μg/ml chloroquine can be deduced: CCC_2 is completely relaxed (nearly comigrates with OC; Fig. 6B, see lane 2 for resolution of the OC and CCC_2 species) while the CCC_1 and CCC_3 species are equally positively and negatively supercoiled, respectively, since they comigrate (Shure and Vinograd, 1976).

To compare the effects of the presence of IHF and EtBr, a parallel ligation was carried out in the presence of 100 nM IHF without EtBr; this produced the expected OC:CCC_1 ratio of 1:3 (Fig. 6A, lane 11). The amount of ligated circle product formed and the ratio of OC:CCC_1 produced in this reaction with IHF alone is nearly identical to the amount and ratio produced in the presence of 5 μM EtBr without IHF (Fig. 6A, lane 6). Depending on the direction of twist imparted by IHF, the CCC_1 toposiomer formed in the presence of IHF could be Lk = 15 or 16, since the twist potential is symmetric about $\Delta Lk = 0.5$ (i.e., twisting and untwisting energies are equal), at least for 247-bp DNA cyclization (Shore and Baldwin, 1983b). Its comigration with the CCC_1 product formed in the presence of EtBr suggests it is most likely Lk = 15 since these gel conditions will resolve Lk = 16 from Lk = 15 topoisomers (data not shown; in the presence of chloroquine, Lk = 16 is more positively supercoiled and therefore migrates faster than Lk = 15). These results suggest that IHF, like EtBr, untwists DNA.

These results can be used to estimate the degree of twist imparted by IHF by equating it with the twist imparted by EtBr at 5 μM. To estimate the number of EtBr molecules bound to the 163-bp DNA, the three CCC species formed at increasing EtBr concentrations were quantitated and plotted as a function of EtBr concentration (data not shown). This analysis showed that the CCC_1, CCC_2, and CCC_3 species were most abundant at EtBr concentrations of 5, 30, and 65 μM, respectively (the CCC_3 peak at 65 μM was partially obscured by reduced ligation rates at higher EtBr levels). Since the CCC species differ by 360° twist and each EtBr molecule imparts 26° twist, then an additional 14 EtBr molecules are bound per DNA molecule at 30 μM EtBr for CCC_2 and 28 EtBr molecules at 65 μM EtBr for CCC_3. The number of EtBr molecules bound per 163-bp DNA molecule at 5 μM EtBr was estimated by equating the equilibrium binding constant equations of the CCC_1- and CCC_2-peak EtBr concentration (assuming the concentration of unbound EtBr and unbound DNA base pairs are not significantly different from their respective total concentrations).

Solving for the unknown EtBr molecules per 163-bp DNA at 5 μM EtBr, (X),

$$\frac{(X)}{(5\ \mu\text{M})\ (163\ \text{bp})} = \frac{(X + 14)}{(30\ \mu\text{M})\ (163\ \text{bp})},$$

indicates that about 3 EtBr molecules were bound per 163-bp DNA molecule at 5 μM EtBr; qualitatively similar results were obtained when the equilibrium binding constant equation of the CCC_3-peak EtBr concentration was evaluated, however, the reduced ligation rates at higher EtBr levels limited accurate estimation of the peak EtBr concentration. Three bound EtBr molecules produce a total twist of about 78°. By this analysis, the equivalent ratios of topoisomers formed by IHF and by 5 μM EtBr suggest that IHF imparts approximately 78° of (negative) twist to the DNA.

IHF Binding to Circles Made in the Presence and Absence of IHF

The binding affinity of IHF for circles(+)IHF and circles(−)IHF was investigated by gel-shift (Fig. 7). Note that the unbound topoisomers in circles(+)IHF and circles(−)IHF comigrate in

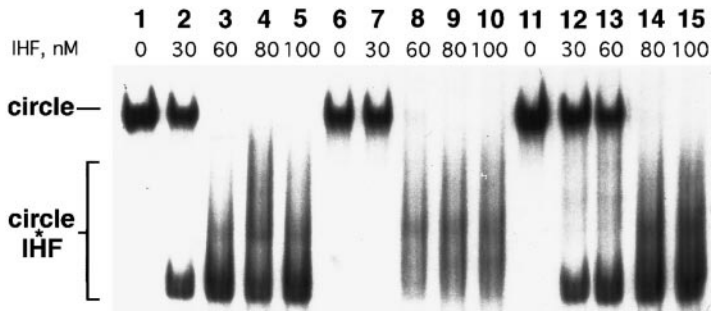

FIG. 7. IHF binding to circles made in the presence or absence of IHF. The 163-bp circles made in the presence of IHF (lanes 1 to 5), circles made in the absence of IHF (lanes 6 to 10), or a mixture of these two circle preparations (lanes 11 to 15) were incubated with IHF at the indicated concentrations. The positions of unbound circular DNA and IHF–circle complexes are indicated.

this gel without chloroquine. Circles(+)IHF usually showed slightly higher affinity, as indicated by quantitation of the unbound DNA bands from several experiment (compare lanes 2 and 7 in Fig. 7); however, the trend was not statistically significant (data not shown). Despite the similarity in their binding affinities for IHF, it is clear that IHF forms different complexes with these species. Circles(+)IHF forms one predominant fast-migrating complex with IHF (Fig. 7, lanes 2–5) while circles(−)IHF complexes are smeared with slower mobility compared to circles(+)IHF complex (Fig. 7, lanes 8–10). Mixing the two DNA preparations did not inhibit the formation of stable complexes characteristic of circles(+)IHF DNA (Fig. 7, lanes 11–15).

DISCUSSION

DNA structure is clearly a fundamental aspect of the DNA binding activity of IHF. One consistent theme among the pleiotropic effects of IHF is its bending of DNA, as epitomized by its function in λ phage recombination. As shown in this current work, DNA conformation, in the form of inherent DNA curvature, enhanced IHF–DNA interactions; IHF binding affinity was demonstrated to be fivefold higher for a 163-bp circular DNA compared to its linear form. If IHF binding affinity is dependent on the "bendability" of the DNA, as proposed by Snyder *et al.* (1989), that is, if it is dependent on the flanking-sequence protein–DNA contacts that are made only when the DNA is bent around IHF, then stabilization of the bent state would preserve these contacts and increase binding affinity (Hales *et al.*, 1996; Shindo *et al.*, 1995). This model has also been proposed for other DNA-bending proteins (Kim *et al.*, 1997; Teyssier *et al.*, 1996; Kahn and Crothers, 1992; Dripps and Wartell, 1987). In this case, circularization of the 163-bp DNA favors binding by reducing the energetic cost of IHF-induced bending. We assume that the bend energy imparted by DNA circularization represents only *part* of the total bend energy of IHF, since IHF bends DNA 140° to 180° over the ~30-bp footprint, while circularization of 163-bp DNA would provide only 66° of curvature over 30 bp. In our analysis, we have made four additional assumptions (see below), which in part may limit the enhancement of IHF binding affinity for circular substrates. While the free energy of IHF-induced DNA bending probably represents a significant cost to the total binding energy of IHF, estimation of this free energy by comparing the binding affinities of IHF to circular and linear substrates is precluded if any of the following assumptions prove incorrect:

(1) IHF bends DNA equally whether DNA is in linear or circular form. The structural response of a small circle to IHF-induced bending, i.e., the formation of a quasi-ellipse, may place structural limitations on the degree of IHF-induced bending, compared to linear DNA with free ends. Mutations in an IHF binding site

do not affect the degree of bending by IHF binding, even when binding affinity is reduced (Shindo *et al.,* 1995). However, others (Hales *et al.* 1996; Goodman *et al.* 1999) reported that the flanking DNA adjacent to the IHF binding site can alter the degree or kind of induced bend.

(2) IHF-induced DNA bending is facilitated by circularization-induced DNA curvature. It is clear that the IHF-induced bend is more severe than, and perhaps qualitatively different from, the "smooth" bending of DNA by circularization, e.g., IHF makes two kinks within its recognition site which contribute most of the DNA bending (Rice *et al.,* 1996).

(3) Circularization-induced curvature does not alter binding site presentation, that is, DNA orientational (rotational) anisotropy, through interaction with other sequence-induced DNA anisotropy (Kahn and Crothers, 1992).

(4) Circularization-induced curvature does not significantly alter DNA twist. Since bending derived from the natural curvature of these minicircles is likely uniformly distributed in the absence of IHF, any associated twist component would be expected to be nominal over the length of the IHF binding site. This ignores the twist component inherent in CCC. However, the effect of CCC twist on IHF–DNA complex stability was weak in the gel-shift experiments, with no strong effect on binding affinity; bending seems to be a dominant effect in this case.

Nevertheless, the topological constraints imposed by circularization limit the degrees of freedom of motion of the DNA into a subset of conformations in which the thermodynamic cost of bending by IHF is reduced, perhaps by stabilizing the protein–DNA contacts that IHF uses to induce the DNA to bend. The stability of a specific IHF/DNA complex has been shown to be dependent on bending, per se, where bending was either sequence-induced (inherently bent) or circularization- or loop-induced ("exogenously"-bent). On an IHF binding site (H′) required for λ excisive recombination, Segall *et al.* (1994) have shown that the K_d of IHF is decreased 10-fold in the presence of another DNA binding protein, Int, which binds simultaneously to two sites flanking H', creating a loop of bent DNA at the IHF site. Similarly, circularization-induced bending not only increases the affinity of IHF (this work; Sun *et al.,* 1996) but also may be a general phenomenon of DNA bending proteins (Pil *et al.,* 1993; Kahn and Crothers, 1992).

While there are limited data, it is unclear whether all types of DNA target deformation would enhance IHF binding. There are numerous cases in which IHF binds specifically to inherently bent DNA (poly-A/T elements, Hales *et al.,* 1996; Sun *et al.,* 1996) as well as nonspecifically, like that of HU (which binds nonspecifically), to inherently curved DNA (Shimuzu *et al.,* 1995). In the structure derived from the cocrystal of IHF bound to the H′ site, the internal DNA bending elements within the IHF binding site (described above) also possess narrow minor grooves which Rice *et al.* (1996) contend may be critical in their interaction with IHF's two antiparallel β strands. Thus the DNA target itself may possess a conformational "blueprint" to introduce anisotropic flexibility at critical points that are compatible with IHF-induced distortions. Base-induced deformations that are degenerate with respect to DNA sequence may explain the degeneracy of the IHF consensus (Nash 1996; Goodman *et al.,* 1999).

DNA Cyclization and Circle Topology

A widely used assay for the natural fluctuations in DNA twist is the capture of topological states by ligase joining the DNA ends. The rate of intramolecular cycling is dependent on the contributions of the energies of DNA bending and twisting which become dominant for DNA $<$500 bp and increase exponentially as DNA length decreases (Shore and Baldwin, 1983a,b). The energy to attain an integral twist value (where both strands are aligned) increases exponentially from a linking deficit (ΔLk) ΔLk = 0 to ΔLk = 0.5.

The linear 163-bp has a predicted Lk = 15.5 ± 0.1 (at 10.6 ± 0.1 bp/turn). The predominance of OC in the ligation products (CCC:OC ratio 1:5) probably reflects the nonintegral ΔLk of the 163-bp DNA and the large energy cost necessary to distribute ~180° of

twist (to bring $\Delta Lk = 0$ for CCC formation) over only 163-bp. The presence of IHF during ligation increased the ratio of CCC:OC products to 3:1. Qualitatively similar results were reported by Sun *et al.* (1996). The 3:1 ratio was also achieved by ligation in the presence of 5 μM EtBr. These results show that the thermodynamic disfavor for CCC formation inherent in the 163-bp DNA can be overcome either by IHF binding or by EtBr intercalation, most likely by directly altering the net twist which would better align the DNA ends. Our data do not allow a direct determination of the magnitude of untwisting induced by IHF, which would require kinetic data on CCC formation induced by IHF and determination of binding stoichiometry. However, an estimate of 78° of untwisting was made by equating the twist induced by EtBr with that of IHF binding. The derived cocrystal structure showed a 15° dihedral angle out of the plane, which represents twist; however, the linear DNA substrate contained a nick (Rice *et al.,* 1996) and may not be quantitatively representative of a native solution state or our end-ligated minicircles. The relative contributions of IHF-induced bending and twisting to the increase in production of and binding to CCC is more complicated to interpret since there can be an inherent coupling between DNA bending and twisting (Marko and Siggia, 1994). In addition, by bending DNA 180°, IHF could introduce writhe, which can be translated vibrationally into twist, and this is, in principle, reversible (reviewed in Calladine and Drew, 1997).

IHF Binding to Circles(+)IHF and Circles(−)IHF

To further evaluate the role of DNA twisting, the binding affinity of IHF for the 163-bp circles made in the presence or absence of IHF [circles(+)IHF or circles(−)IHF] were compared. The two circle preparations showed similar binding affinity for IHF under our reaction conditions, but differed in their relative stabilities during electrophoresis: complexes with circle(+)IHF showed distinct bands, while complexes with circle(−)IHF were smeared. Smearing between the positions of migration of stably bound complex and unbound DNA may represent marginally stable complexes that dissociate stochastically during electrophoresis, perhaps due to the suboptimal binding conditions in the gel compared to the *in vitro* IHF binding conditions (e.g., differences in ionic environment or temperature). This observation was also reported for IHF binding to mutated sites with reduced binding affinity and caused a similar smearing of the IHF/DNA complex (Thompson *et al.,* 1986; Lee *et al.,* 1991; Shindo *et al.,* 1995). Alternatively, the smearing may represent a dynamic equilibrium between a series of metastable structural states, each of which migrates differently during electrophoresis. The correspondence between the ability of IHF to form stable complexes and the higher fraction of CCC in the preparation is consistent with the formation of stable complexes with CCC and unstable complexes with OC. Sun *et al.* (1996), however, reported a lack of binding of IHF to the CCC species of a 156-bp DNA substrate containing *two* IHF binding sites that were circularized in the presence of saturating IHF. Ligation of DNA when both IHF sites are occupied could create a CCC conformation (when subsequently purified from IHF) that precludes rebinding of one IHF molecule at the low IHF concentrations that were tested. This contrasts our system where only *one* IHF molecule is likely binding during both facilitated ligation and subsequent rebinding reactions.

The importance of torsional alignment of IHF–DNA contacts in IHF binding affinity is suggested by deletions or insertions of one base pair between core consensus elements or flanking bend elements that severely disrupted IHF binding (Lee *et al.,* 1991; Shindo *et al.,* 1995). Although such manipulations do not only change twist alignment, this model may be extended to explain the differential binding of IHF to OC and CCC species. Since the two topoisomers probably have similar inherent bending, the IHF/DNA complex stability differences during electrophoresis may be due to differences in DNA twisting (although the four assumptions outlined above cannot be ruled out). The instability of complexes of IHF with the OC species

in circles(−)IHF reflects the IHF-induced twist energy required to align the optimal protein/DNA contacts, while stable complexes with the inherently untwisted substrate, the CCC species in circles(+)IHF, reflect at least partial alignment that reduces IHF-induced twist energy and therefore stabilizes binding. These arguments parallel those of the increased binding affinity for prebent (circularized) DNA because of the reduced energy necessary for IHF-induced bending.

In summary, the influences of DNA structure that are shown to modulate the affinity of IHF binding, bending, and twisting have significant implications for the function of IHF as a regulatory factor in many DNA reactions. Since IHF binding is modulated by DNA structure, it has adapted to binding to a wide range of sequences, since conformation specification is degenerate. This may reduce the number of mutational steps required to create an IHF binding site and the sequestration of IHF as a functional member in nucleoprotein complexes.

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