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IHF-binding sites inhibit DNA loop formation and transcription initiation

Yi-Xin Huo[1,2], Yuan-Tao Zhang[1], Yan Xiao[1], Xiaodong Zhang[3], Martin Buck[3], Annie Kolb[2] and Yi-Ping Wang[1,*]

[1]National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, P. R. China, [2]Institut Pasteur, Molecular Genetics Unit and CNRS URA-2172, 25 rue du Dr Roux, 75724 Paris Cedex 15, France and [3]Department of Life Sciences, Imperial College London, London SW72AZ, UK

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ABSTRACT

Transcriptional activation of enhancer and σ^{54}-dependent promoters requires efficient interactions between enhancer-binding proteins (EBP) and promoter bound σ^{54}-RNA polymerase ($E\sigma^{54}$) achieved by DNA looping, which is usually facilitated by the integration host factor (IHF). Since the lengths of the intervening region supporting DNA-loop formation are similar among IHF-dependent and IHF-independent promoters, the precise reason(s) why IHF is selectively important for the frequency of transcription initiation remain unclear. Here, using kinetic cyclization and *in vitro* transcription assays we show that, in the absence of IHF protein, the DNA fragments containing an IHF-binding site have much less looping-formation ability than those that lack an IHF-binding site. Furthermore, when an IHF consensus-binding site was introduced into the intervening region between promoter and enhancer of the target DNA fragments, loop formation and DNA-loop-dependent transcriptional activation are significantly reduced in a position-independent manner. DNA-looping-independent transcriptional activation was unaffected. The binding of IHF to its consensus site in the target promoters clearly restored efficient DNA looping formation and looping-dependent transcriptional activation. Our data provide evidence that one function for the IHF protein is to release a communication block set by intrinsic properties of the IHF DNA-binding site.

INTRODUCTION

The expression of σ^{54}-dependent promoters requires efficient communication between enhancer and promoter *via* DNA loop formation, and in most cases the distance between enhancer and –12 promoter region is around 100–140 bp (1–3). It is generally believed that protein induced DNA bending should be used to lower the energetic cost of DNA looping and to facilitate the enhancer–promoter communication (4–6). For example, the regulatory protein NifA from *Klebsiella pneumoniae* binds to enhancer and activates σ^{54}-dependent transcription from the *nifH* promoter; in addition, the integration host factor, IHF, binds between the *nifH* promoter and enhancer (7). IHF bends the DNA in the *nifH* promoter regulatory region and greatly stimulates NifA-mediated activation of *nifH* transcription *in vitro* and *in vivo*. NifA and IHF are functionally synergistic. IHF induces DNA bending up to 160° (6) and facilitates DNA looping formation that leads to productive interactions between NifA and RNAP-σ^{54} holoenzyme which results in transcription initiation (7). An IHF-induced bend is also needed for the DNA looping at *glnH*p2, *Pu* and *pspA* (8–11). However some σ^{54} promoters such as *glnA*p2 and *nifLA* have no obvious IHF-binding site and can transcribe well in the absence of IHF *in vitro* (9,12), indicating that the DNA looping can occur without any DNA bender *in vitro*. The distance between enhancer and promoter is similar for IHF-dependent and IHF-independent promoters, and why promoters lacking an IHF-binding site can achieve DNA looping in the absence of IHF *in vitro* is not fully understood. It was proposed that IHF-independent promoters such as *glnA*p2 and *nifLA* contain an intrinsic DNA bend (9). However, most IHF-dependent promoters also contain several sequences AAAA, AATT and TTTT (Table 1), each of which can contribute to an 18–19°

*To whom correspondence should be addressed. Tel: +86 10 6275 8490; Fax: +86 10 6275 6325; Email: wangyp@pku.edu.cn
Present address:
Yi-Xin Huo, Department of Chemical and Biomedical Engineering, University of California, Los Angeles, CA 90095, USA

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

bend (13). Therefore, the following two issues remain: (i) whether an intrinsic bend between enhancer and promoter is sufficient for DNA loop formation; (ii) what is the exact role of IHF in DNA looping.

IHF can recognize its binding sites by both direct and indirect readout. It has been proposed that IHF-binding sites (although some of them are not consensus) have common DNA structural properties, allowing them to be recognized by IHF through indirect readout (5). Therefore, it is reasonable to explore whether the DNA structural properties of IHF-binding sites can themselves affect looping. In this study, we measured the looping-formation ability of the DNA fragments from eight native promoters (six of them contain IHF-binding sites, and two of them not) and one generic control DNA, using the ligase-mediated cyclization method (14–17). Results showed that the DNA fragments containing an IHF binding site have much less looping-formation ability than those having no IHF-binding site. When an IHF consensus-binding site (IC site, the H′ site of phage λ) was introduced into *glnA*p2 and generic DNA, it strongly inhibited loop formation. Furthermore, *in vitro* transcription assays were carried out using wild-type *glnA*p2 and its IC insertion derivatives. Results show that IHF-binding site can generally inhibit the enhancer-dependent expression of *glnA*p2 in a position-independent manner in the absence of IHF protein, and the inhibitory role is manifest through reducing DNA looping between enhancer and core promoter. In agreement with previous studies, IHF-binding to the IC sites leads to a general restoration of the transcription initiation of all *glnA*p2 derivatives. Clearly, the IHF target site and by inference its flanking sequences are not passive but have a negative regulatory role in DNA loop formation, and one role of IHF is to restore DNA loop formation.

MATERIALS AND METHODS

DNA

The 216-bp DNA fragments containing entirely the enhancer and promoter regions were amplified by PCR reactions from genomic DNA. *PnifB*, *PnifE*, *PnifH*, *PnifJ*, *PnifLA* and *Pu* were from *Klebsiella pneumoniae*; *PnifH* was from *Rhizobium meliloti*, *glnA*p2 was from *Escherichia coli* while the tandem *glnA*p1 promoter was silenced as in ref. 18 by removing the cAMP receptor protein (CRP)-binding site. All fragments contain *EagI* at both ends (introduced by PCR primers). The fragments were then cloned into pBR322 individually. The IC-constructs were constructed by inserting IHF-binding site at precise positions of target DNA by site-directed mutagenesis PCR reactions. The sequences are shown in Table 1 and Supplementary Table 1. All plasmids were sequenced and maintained in *E. coli* DH-5α cells. When these pBR322-derived plasmids were digested by *EagI*, the small fragments could form 210-bp circular monomers if they were further treated with T4 DNA ligase. The *glnA*p2 derived base pair insertion or deletion mutants were also constructed by site-mutagenesis PCR reactions. They can form circular monomers from 207 to 213 bp in ligation assays.

The templates for *in vitro* transcription assays were constructed by inserting the promoters in front of the two strong transcriptional terminators T1 and T2 of the transcriptional vector pJCD01 (19). All promoters were PCR amplified from the corresponding PBR322 derived plasmids. The upstream primer contains a *BamHI* site and the downstream primer contains a *SalI* site. Each fragment was cut by *BamHI* and *SalI* and the *BamHI-SalI* fragment was ligated to *BamHI-SalI* digested pJCD01.

Radioactive labeling

The mixture of two *EagI*-digested fragments, about 210 bp and the rest of the plasmids, was labeled by incubation with [γ^{-32}P]-ATP and T4 DNA polynucleotide kinase (New England Biolabs). Unincorporated ATP was removed by using Sephadex G-50 spin column and the samples were then used for the ligation experiments.

Ligation-mediated cyclization assays

Ligation experiments were carried out with 0.2 pmol 210-bp DNA fragment at 22°C in ligation buffer [50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 100 μg/ml BSA]. The concentration of T4 DNA ligase (New England Biolabs) was 0.25 U/μl. At specific time intervals, the same amount of reaction mixture was withdrawn from the reaction solution and quenched with 1 μl 0.5 M EDTA and heated (65°C, 10 min). The ligation products were separated and analyzed as described previously (13). When *E. coli* DNA ligase (New England Biolabs) was used as in Supplementary Figure 4B, the ligation experiments were carried out at 22°C in its suggested ligation buffer.

Protein purification

These were conducted as we have described previously (18).

In vitro single-round transcription assays

Assays were done in buffer A (18) at 30°C using 40 nM Eσ^{54}, 50 nM IHF, 50 nM NtrC-P, 2 nM DNA and 2 mM ATP. Elongation was started by adding a nucleotides-heparin mixture (final concentrations 200 μM CTP and GTP, 20 μM UTP and 2.5 μCi [α-^{32}P]UTP and 120 μg/ml heparin). Relaxed templates were obtained by digesting supercoiled plasmids with *AseI* for 2 h. When PspFΔHTH was used as the activator, STA buffer with PEG (25 mM Tris-acetate pH 8.0, 8 mM Mg-acetate, 100 mM KCl and 3.5% W/V PEG-6000) was used as the reaction buffer. The transcript of *glnA*p2 has a length of 121 nt which contain 20 UTPs. The absolute intensities for each of the transcripts from the above assays were quantified on a Molecular Dynamics Storage Phosphorimager using ImageQuant software.

RESULTS

Ligation-mediated cyclization assay for DNA Cyclizability

DNA elasticity as well as the efficiency of DNA loop formation of a particular fragment can be determined by measuring its *j*-factor, a measure of the efficiency of the DNA cyclization. DNA conformational changes that bring the two self-complementary ends into transient base-paired contact can occur spontaneously (17,20). The *j*-factor equals the molar concentration of one end of a linear DNA molecule in the vicinity of its own other end in the appropriate angular and torsional orientation. Higher *j*-factors therefore indicate a higher probability of loop formation, or increased flexibility in DNA conformation while lower *j*-factors indicate a reduced ability in loop formation, or increased rigidity in DNA conformation.

To obtain *j*-factors experimentally, we measured the ratio of the covalently formed closed monomer circles, $C(t)$, to the covalently joined dimeric species, $D(t)$, during the early stage of fragment ligation (17). The *j*-factor value is obtained by extrapolating $2M_0C(t)/D(t)$ to zero ligation time, where M_0 is the initial concentration of the fragments.

Two factors need to be considered when choosing the length of DNA fragments for the experiments (13). On one hand, shortening the DNA fragment length increases the sensitivity of the *j*-factor to the DNA flexibility. On the other hand, the initial DNA concentration should be about the value of *j*-factor to obtain comparable amounts of $C(t)$ and $D(t)$. One has to deal with a very low amount of DNA if the fragments are too short because the *j*-factor drops very fast with shortening DNA length. In many cases, the length of DNA fragments was chosen to be around 200 bp (13). To satisfy all the parameters mentioned above, we chose 210 bp as the DNA length, which is sufficient to cover all the enhancer and core promoter regions of the native promoters and contains around 20 DNA helical repeats assuming the helical pitch of DNA is about 10.5 bp.

In this study, eight σ^{54} promoters and one generic DNA were studied first. The DNA fragments were prepared as described in 'Materials and Methods' section and the sequences are given in Table 1. The generic DNA is from λ phage and does not contain any known curved DNA sequences such as AAAA, AATT and TTTT as investigated previously (13). Two of the promoters (*glnA*p2 from *E. coli*., *nifLA*p from *K. pneumoniae*) as well as the generic DNA do not contain IHF-binding sites while the others contain their native IHF-binding sites.

Typical distributions of ligation products for different ligation times are shown in Figure 1A. The *j*-factor is

Table 1. Sequences of the native σ^{54}-dependent promoters and their derivatives

*K.p nif*B:	5′-GGACGCGGGTTGCCGGTTAAAAAGTCTACTTTTCATGCGGTTGCGAAATTAACCTCTGG-3′
*K.p nif*E:	5′-GTTGGCTTTGTCGCAAAGCCAACAACCTCTTTTCTTTAAAAATCAAGGCTCCGCTCTGG-3′
*K.p nif*H:	5′-GCGGCGACAAATAACTAACTTCATAAAAATCATAAGAATACATAAACAGGCACGGCTGG-3′
*K.p nif*J:	5′-GGCGACTCTCTTCATAACGCGTTGAATTCGCGCTAACTCTTCTGTCATCCGCGAGCTGG-3′
*K.p nif*U:	5′-GACGAACCTTGTCAGGACTAATACACAACCATTTGAAAAATATTAATTTTATTCTCTGG-3′
K.p nifLA:	5′-AAAAAGCGCCTGCTTTTCCCCTACCGGATCAATGTTTCTGCACATCACGCCGATAAGGG-3′
*R.m nif*H:	5′-TCAATTTCCAGATCTAACTATCTGAAAGAAAGCCGAGTAGTTTTATTTCAGACGGCTGG-3′
*E.c glnH*p2:	5′-TATAAATCGTGCATCACGTTTTTGCCGCATCTCGAAAAATCAAGGAGTTGCAAAACTGG-3′
E.c g*lnA*p2:	5′-CAGCCCTTTTGCACGATGGTGCGCATGATAACGCCTTTTAGGGGCAATTTAAAAGTTGG-3′
E.c IC-44:	5′-CAGCCCTTTTGCACGATGGTGCGCATGATAACGTTGCTTATCAATTTGTTGCACCTTGG-3′
E.c IC-50:	5′-CAGCCCTTTTGCACGATGGTGCGCATGTTGCTTATCAATTTGTTGCACCTAAAAGTTGG-3′
E.c IC-53:	5′-CAGCCCTTTTGCACGATGGTGCGCTTGCTTATCAATTTGTTGCACCATTTAAAAGTTGG-3′
E.c IC-54:	5′-CAGCCCTTTTGCACGATGGTGCGTTGCTTATCAATTTGTTGCACCAATTTAAAAGTTGG-3′
E.c IC-55:	5′-CAGCCCTTTTGCACGATGGTGCTTGCTTATCAATTTGTTGCACCCAATTTAAAAGTTGG-3′
E.c IC-57:	5′-CAGCCCTTTTGCACGATGGTTTGCTTATCAATTTGTTGCACCGGCAATTTAAAAGTTGG-3′
E.c IC-59:	5′-CAGCCCTTTTGCACGATGTTGCTTATCAATTTGTTGCACCGGGGCAATTTAAAAGTTGG-3′
E.c IC-61:	5′-CAGCCCTTTTGCACGATTGCTTATCAATTTGTTGCACCTAGGGGCAATTTAAAAGTTGG-3′
E.c IC-64:	5′-CAGCCCTTTTGCATTGCTTATCAATTTGTTGCACCTTTTAGGGGCAATTTAAAAGTTGG-3′
E.c IC-69:	5′-CAGCCCTTTTGCTTATCAATTTGTTGCACCACGCCTTTTAGGGGCAATTTAAAAGTTGG-3′

The native (as previously reported) or introduced IHF-binding sites (if present) were boxed and the consensus sequences were shadowed. The centre of the IHF binding site is the first nucleotide of the consensus sequence. The IHF site of the *nif*J promoter is located in the opposite orientation. The –24 regions of σ^{54}-dependent promoters were double underlined. The IC-constructs in this table were constructed by inserting IHF-binding site at precise positions of *E. coli glnA*p2 promoter. The whole sequences of these fragments and other sequences used in this study were shown in Supplementary Table 1. If not specifically indicated, each fragment used in this study is 216 bp in length and contains the entire enhancer and core promoter. After digestion with *Eag*I and ligation, these fragments can form 210-bp circular monomers in the ligation reactions.

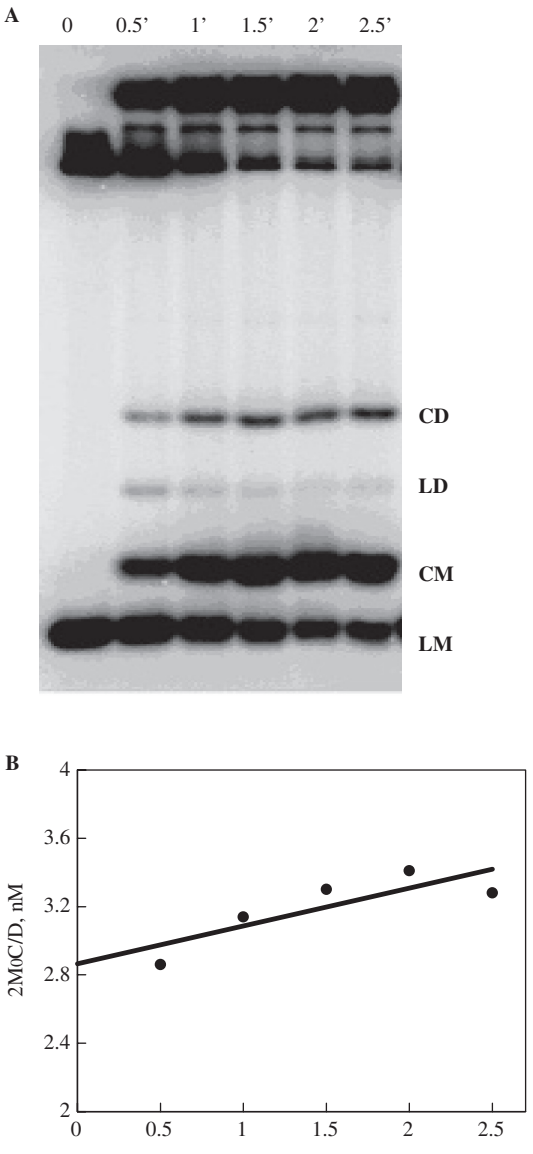

Figure 1. Determination of *j*-factor from ligation time course. (**A**) A typical gel shows the LM (linear monomers), LD (linear dimers), CM (circular monomers) and CD (circular dimers) obtained by ligation of the 210-bp DNA fragments. PhosphorImaging was used to quantify the intensities of the bands. The bands at the top of the gel correspond to the ligation products of the large fragment of the plasmid DNA. (**B**) The *j*-factor is obtained by extrapolating the ratio $2M_0C(t)/D(t)$ to zero reaction time. Both LD and CD were included in $D(t)$.

determined by quantitative analysis of the gel as shown in Figure 1B. As explained previously (13), the rate of the cyclization is much higher for the dimers than for the original fragments because the *j*-factor for 420-bp fragments is much higher than that for 210-bp fragments. As a result, the majority of the dimers are present in circular form except at the very beginning of the ligation reaction. When $D(t)$ is determined, it included both linear and circular dimers to make more reliable the extrapolation to zero reaction time. As shown in Supplementary Figure 4A and in agreement with previous observations (21), the *j*-factor value does not depend on the concentrations of DNA ligase within the appropriate range. Moreover it does not vary using either T4 or *E. coli* DNA ligases, suggesting that it is independent of the type of DNA ligase.

Table 2. Measurements of *j*-factor (nM) of native promoters and generic DNA

Generic DNA	6.9 (0.3)
Generic-IC	1.9 (0.4)
*K.p nif*LA	7 (0.5)
*K.p nif*B	0.8 (0.2)
*K.p nif*E	1.1 (0.1)
*K.p nif*H	1.15 (0.2)
*K.p nif*J	1.2 (0.1)
*K.p nif*U	1.5 (0.5)
*R.m nif*H	3.2 (0.2)
*E.c glnA*p2	11.5 (0.5)
IC-44	3.8 (0.3)
IC-50	2.1 (0.2)
IC-53	2.2 (0.2)
IC-54	3.5 (0.3)
IC-55	2.9 (0.2)
IC-57	1.8 (0.1)
IC-59	1.6 (0.1)
IC-61	4.2 (0.3)
IC-64	3.6 (0.2)
IC-69	2.7 (0.2)
IC-55&87	1.1 (0.1)
nifU Mutant-1	3.0 (0.3)
nifU Mutant-2	2.4 (0.2)

All promoters contain an IHF-binding site except *glnA*p2, *nifLA*p, generic DNA and two *nifU* mutants. The 210-bp generic DNA was chosen from λ DNA from nucleotide 29 853–30 057. The location of the center of the IHF-binding site has been moved from –50 to –69, in the series of IC-*glnA*p2 derivatives. IC-55&87 contains two IHF-binding sites centered respectively at –55 and –87. In *NifU* mutant-1 and 2, the IHF-binding site of *nifU* promoter was replaced by DNA from generic DNA or *glnA*p2. The *j*-factor value represents the average of three independent assays, and the standard deviations are shown in the parenthesis. E.c, *Escherichia coli*; K.p, *Klebsiella pneumoniae*; R.m, *Rhizobium meliloti*.

As shown in Table 2, the *j*-factor of the generic DNA is about 7 nM, in agreement with the previous values (13). The *j*-factor of *nifLA*p is similar to the generic DNA, while that of *glnA*p2 is higher. Significantly, the *j*-factors of all promoters containing IHF-binding site are much lower than the generic DNA (3–9-fold lower). These results suggest that IHF-binding sites might play a role in decreasing the flexibility of the DNA fragments containing them.

To test whether IHF-binding site can affect the cyclizability of a given DNA fragment, we replaced the middle part of the generic DNA by a 22-bp IC site. The construct was named generic-IC (sequence as in Supplementary Table 1). Here, this 22-bp IC sequence was chosen from the H′ site of phage λ (5′-ttgctTATCAAtttgTTGcacc), one of the best-characterized IHF-binding sites (22, 23) which is fully consistent with the IHF consensus binding sequence WATCARXXXXTTR (W is A or T; X is A, T, C or G; R is A or G) (6). As shown in Table 2, the IC insertion decreases the *j*-factor of generic DNA by 3.6-fold, from 6.9 to 1.9 nM. As described previously (13), this generic DNA contains no known curved short DNA elements. Its *j*-factor is similar to the values from other reported 'typical' DNA fragments with similar

length and can be viewed as a standard *j*-factor value for a B-type DNA. Insertion of an IC sequence into this generic DNA significantly decreases its *j*-factor.

To further test whether an IHF binding site could affect loop formation between enhancer and core promoter in a native context, we replaced part of the *glnA*p2 intervening DNA region between enhancer and core promoter by the same 22-bp IC sequence. The IC is centered at –55 and the construct was named IC-55 (sequence as in Table 1). As shown in Table 2, the *j*-factor of *glnA*p2 dropped from 11.5 to 2.9 nM. To further verify the effects of IHF binding site on the DNA looping formation, we inserted a second IC sequence into the constructed IC-55. The second IC sequence is centered at –87 and the new construct was named as IC-55&87 (sequence as in Supplementary Table 1). The *j*-factor then decreased from 2.9 (IC-55) to 1.1 nM (IC-55&87) (Table 2).

Although the proposed intrinsic DNA bending sequence of *glnA*p2 (CCCTTTT) is only 7-bp long and centred at –73 (9), it is possible that other intrinsic DNA-bending sequences are located around –55, and the decreased *j*-factors of IC-55 and IC-55&87 may be due to the loss of an intrinsic DNA-bending sequence. In order to eliminate this possibility, we constructed nine *glnA*p2 derived promoters containing the 22-bp IC at different locations between the enhancer and the core promoter, without changing the linear distance between these two elements (for sequences, see Table 1). In total, these 11 IC-constructs have respective mutations between the enhancer and core promoter of *glnA*p2, occurring every precise location. Since wild-type *glnA*p2 does not contain phased A tracts which are intrinsically bent, it is very unlikely that the entire DNA sequence between enhancer and core promoter is required to direct intrinsic DNA bending. Therefore, these IC-constructs can be used to identify whether the inhibition of the DNA loop formation is a direct effect of the IHF-binding site. If IC-insertions decrease the *j*-factor by replacing intrinsic DNA bending element, one would expect the inhibitory effect only happens at specific position(s). On the other hand, if the inhibition is a direct effect due to the physical properties of IHF-binding site itself, it should be position independent.

As shown in Table 2, every IC-construct has a much-reduced *j*-factor compared with *glnA*p2 fragment. For the constructs containing one IC sequence, the measured *j*-factor varies from 1.6 (IC-59) to 3.8 nM (IC-44) while the wild-type *glnA*p2 has a *j*-factor 11.5 nM. Since all the *j*-factor values of IC-constructs are lower than the generic DNA, results are unlikely to be due to a replacement of an intrinsic bending sequence. Rather, results indicate that the inhibitory effect of IC on the DNA loop formation could dominate over the stimulatory effect of intrinsic bend on the DNA looping, likely a direct effect due to its special physical properties.

If the IHF-binding site inhibits the DNA loop formation in native promoters, the substitution of the IHF binding site with other DNA sequences is expected to increase the *j*-factors of the target DNA fragments. In order to test this hypothesis, the IHF-binding sites of *K.pneumoniae nifU* promoter and *E.coli glnH*p2 were mutated (sequences as in Supplementary Table 1). Table 2 shows that these mutations increased the *j*-factor of the DNA containing *nifU* promoter (from 1.6 to 2.4 or 3.0 nM), indicating that the native IHF-binding site sequence reduced the ability of DNA loop formation. However, all of the four kinds of mutations of *glnH*p2 did not increase its *j*-factor as much (from 1.1 to up to 1.8 nM, sequence as in Supplementary Table 1). The restoration of *j*-factor is clearer on *nifU* derivatives compared with *glnH*p2 derivatives. In both cases, even the *j*-factors of the mutated sequences are lower than the generic DNA, indicating that the DNA segments flanking IHF-binding site may also contribute to DNA loop formation.

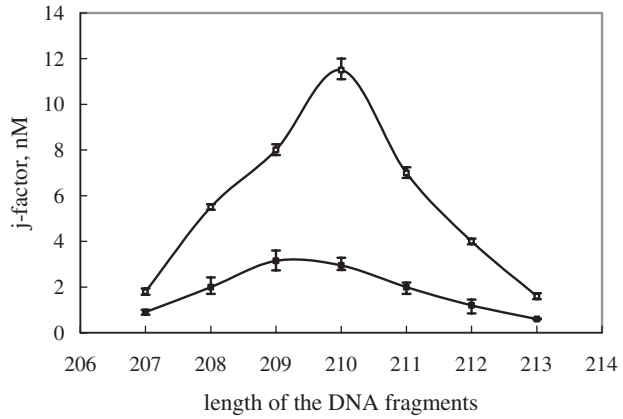

Figure 2. Dependence of *j*-factors on the length of DNA fragments derived from wild-type *glnA*p2 and IC-55. *glnA*p2-derived fragments were marked by open square and IC-55-derived fragments were marked by filled square. Value represents the average of three independent ligation assays, and error bars are shown.

Since IHF-binding sites might have special secondary structural architecture used to be readout indirectly by IHF protein, it is possible that IHF-binding sites have specific helical pitch other than typical B-type DNA. If the helical pitch of the 22-bp IHF-binding site could be as high as 12.0, the DNA length needed to form 20 helical repeats will increase to 213 bp; if the helical pitch of the 22-bp IHF-binding site could be as low as 9.0, the DNA length needed to form 20 helical repeats will decrease to 207 bp. Since DNAs longer or shorter than an optimal length need to under- or overtwist to allow end-base pairing, they will cyclize with increased energetic cost and decreased equilibrium probability (17,23,25), leading to lower *j*-factor compared to the DNA with optimal length. To exclude the possibility that the IHF-binding site changed the optimal length for monomer circles formation, we measured *j*-factor for two sets of five fragments from *glnA*p2 and IC-55. The longest fragments consisted of 213 bp and the shortest fragments consisted of 207 bp. They were constructed by adding or removing one, two or three base-pairs from the end of the *glnA*p2 or IC-55 (sequences as in Supplementary Table 1). As shown in Figure 2, the peak of *j*-factor from the *glnA*p2-derived set of DNA locates at 210 bp, in agreement with the proposal that the helical pitch is 10.5. The peak of *j*-factor from the IC-55-derived set of DNA

Table 3. Quantification of enhancer-dependent, and enhancer-independent, single round transcription on supercoiled or relaxed DNA templates

	Supercoiled template				Relaxed template	
	NtrC-P		PspFΔHTH		NtrC-P	
	−IHF	+IHF	−IHF	+IHF	−IHF	+IHF
*glnA*p2	100	112	100	93	10	27
IC-44	15	36	83	35	2	130
IC-50	20	36	75	73	2	40
IC-53	15	34	60	65	1	24
IC-54	15	60	95	75	0	127
IC-55	21	84	130	73	0	140
IC-57	19	66	80	78	2	25
IC-59	22	33	75	75	2	33
IC-61	20	55	76	75	4	120
IC-64	17	82	105	45	3	140
IC-69	20	94	90	92	2	115

Transcription initiation was activated by 50-nM enhancer-dependent activator NtrC-P or 160-nM enhancer-independent activator PspFΔHTH. When present, the concentration of IHF was 150 nM. The transcription activities of supercoiled *glnA*p2 template are the same using 50 nM NtrC-P or 160 nM PspFΔHTH and the absolute value under these two conditions was taken as standard (100%). The relative percentages of transcription activities under other conditions were shown. All experiments were at least triplicated and the standard deviation of each set of experiments is <15% of the mean. The sample transcription gels were shown in Supplementary Figure 2.

is slightly different from the *glnA*p2 set and locates around 209 bp. However, all *j*-factors from the IC-55-derived set of DNA are still much lower than the one of original *glnA*p2, indicating that the decreased cyclization of IC-55 compared to native *glnA*p2 is not due to the change of the DNA helical pitch. It is interesting to notice that the *j*-factor of any IC-55 derivative is always 2–4-fold lower than the one from a same size *glnA*p2 derivative, the reason is not clear but is very likely due to the specific physical properties of the IHF-binding site itself.

In vitro transcription assays

Ligation-mediated cyclization assays showed that IHF-binding site could inhibit the DNA loop formation when located between enhancer and core promoter of the *glnA*p2. We then checked whether the reduction in DNA looping formation could decrease transcription initiation at the *glnA*p2 promoter. We first examined whether the binding of RNAP to core promoter and the binding of EBP to enhancer are affected by the introduction of IHF-binding sites on *glnA*p2 derivatives. Results obtained from DNase I footprints and gel shift assays showed that IHF protein binds to its binding site and the closed complex formation as well as activator binding were unaffected on IC-constructs (see Supplementary Figure 1 for sample gels, and data not shown), providing us the opportunity to investigate the effect of an IHF-binding site on open complex formation at *glnA*p2 IC constructs.

In vitro single round transcriptional assays with *glnA*p2 derivatives were carried out on supercoiled and relaxed templates (26). When Nitrogen Regulatory protein C-phosphate (NtrC-P) was used as the activator at low concentrations, it needs to bind enhancer DNA and contact the promoter bound RNAP-σ^{54} via DNA looping. The transcription results showed that NtrC-P dependent activities of all supercoiled IC-constructs in the absence of IHF protein were reduced to 15% to 21% compared to the wild-type *glnA*p2, regardless the location of the IHF-binding site (Table 3, and Supplementary Figure 2A). On relaxed templates, under the same conditions, transcripts could hardly be detected from the IC-constructs (0–4% compared to the expression from supercoiled wild-type *glnA*p2 template, see Table 3, and Supplementary Figure 2B). As a control, transcription from relaxed wild-type *glnA*p2 template is about 10% compared to its supercoiled counterpart (Table 3, and Supplementary Figure 2B). Taken together, the presence of IHF-binding site reduces NtrC-P mediated transcriptional activation of *glnA*p2 derivatives on both supercoiled and relaxed templates. The effect observed is independent of the location of IHF-binding site.

To further verify that IHF-binding site inhibits the transcription initiation via inhibiting DNA loop formation, the DNA-binding defective form of PspF (PspFΔHTH), a homologue of NtrC (18,27), was used in the transcription assays. Importantly, PspFΔHTH cannot bind to the enhancer and it can activate transcription initiation via direct contact with the closed complex from solution in a DNA-looping-independent manner. Results showed that, in the absence of IHF, PspFΔHTH-mediated transcriptional activities of IC-constructs were at the same level as that from the wild-type *glnA*p2 on supercoiled and relaxed templates respectively (Table 3). The NtrC-P and PspFΔHTH results indicate that the presence of consensus IHF binding site, regardless of its location, inhibits DNA loop formation between enhancer and promoter and prevents enhancer–promoter communication at *glnA*p2 derivatives. As the inhibitory effect is independent of the IHF-binding site's location, the flanking DNA sequence does not play the major role in preventing DNA loop formation, strongly indicating that the IHF-binding sequence itself is the negative regulatory element in this case. Taken together, our data using purified components demonstrate that the IHF-binding site can itself inhibit enhancer-dependent transcription initiation.

The role of IHF-mediated DNA bending

The *j*-factors of the 210-bp DNA fragments containing the enhancer and promoter of IC-55 and native *glnA*p2 were measured in the presence of different concentrations of IHF. Results showed that the cyclization of IC-55 is increased up to 3-fold by IHF while the wild-type *glnA*p2 is not affected by IHF (data not shown). This indicates that IHF restores the cyclization of DNA fragment containing its target site and helps the DNA loop formation, in agreement with the previous observation that short fragments bent by IHF readily form a DNA loop or circular monomer (28,29). One simple prediction is that IHF should stimulate transcription activation of the IC-constructs.

To test the prediction, single-round transcription assays of IC-constructs together with their native *glnA*p2 counterpart were carried out on both supercoiled and relaxed templates respectively. The results showed that, regardless the location of the IHF-binding site, IHF provided a general restoration of enhancer-binding (NtrC-P)-dependent transcriptional activation of all the IC-constructs, when compared with their native *glnA*p2 counterpart (up to 5-fold activation for supercoiled templates and up to 100-fold for relaxed templates, see Table 3 and Supplementary Figure 2).

DISCUSSION

Transcriptional activation at σ^{54}-dependent promoters requires looping of the upstream DNA between enhancer and core promoter to allow productive contacts between enhancer bound activator and promoter bound RNAP-σ^{54}. Therefore, the loop formation ability of the DNA upstream of the RNAP binding site at the promoter determines the efficiency of DNA loop formation in the absence of a DNA bender such as IHF.

It has been proposed that IHF-independent promoter *glnA*p2 contains an intrinsic DNA-bending sequence CCC TTTT centered at –73 (9). Here, the *j*-factor of *glnA*p2 is higher than the generic DNA, supporting this proposal. This DNA fragment is essential for *in vitro* transcription with relaxed *glnA*p2 template, but plays no role when transcription is initiated from supercoiled *glnA*p2 template (9). In agreement, when Huo *et al*. (18) replaced the native *glnA*p2 fragment at a series of precise positions between enhancer and promoter by a fixed size (22 bp) CRP consensus binding site, the transcriptional activity was not affected by the DNA replacement in the absence of CRP with all supercoiled *glnA*p2 derivatives both *in vitro* and *in vivo*. Since the distance between enhancer and promoter is similar for IHF-dependent and IHF-independent promoters, some unknown elements among those IHF-dependent promoters must inhibit the DNA loop formation in the absence of IHF.

In this study, the cyclizability of the 210-bp DNA fragments from eight native promoters, 10 *glnA*p2-derived promoters containing an IHF-binding site, one generic DNA and several IHF binding site variants were measured by the ligase-mediated cyclization method. Results showed that the upstream DNA from IHF-dependent promoters has much less cyclizability than those from IHF-independent promoters, suggesting that the overall cyclizability of the intervening region between enhancer and promoter is a key factor in determining whether the promoter is IHF dependent or not. Furthermore, for the first time our results showed that the IHF-binding site itself plays a negative regulatory role in DNA loop formation. Therefore, an IHF-binding site could block the enhancer-promoter communication in the absence of IHF whereas the presence of IHF provided a general restoration of the DNA loop formation and so increased enhancer–promoter communication. The reason why IHF-binding site itself inhibits the DNA loop formation is not clear. One possibility is that the IHF-binding site has a strong distortion or writhe [based on the reports that DNA deformation energy is low for IHF-binding sites (30,31)], which guides its flanking DNA fragments into different planes, potentially reducing enhancer–promoter communication.

To establish the relevance of our *in vitro* results to *in vivo* promoter activities we also investigated the effect of IHF-binding site on the activities of *glnA*p2 IC derivatives *in vivo*. In both *himA* (IHF subunit alpha) and *himD* (IHF subunit beta) minus strains, the activities of the *glnA*p2 derivatives are about 60–80% of the native *glnA*p2 (see Supplementary Figure 3), not as greatly reduced as *in vitro*. On the other hand, the *in vivo* binding of IHF protein to the IHF-binding sites generally increases the promoter activities of all IC-constructs as observed *in vitro*, although an up- and down regulatory pattern was observed (see Supplementary Figure 3). The precise reasons for the difference between *in vivo* and *in vitro* results are not clear. One possibility could be that the DNA segments flanking consensus IHF-binding site play an important, but undefined role *in vivo*. Another possibility could be that other histone-like proteins, such as HU (a protein in the same protein family of IHF) may display a partial complementation of the DNA-bending functionality of IHF. The partial overlapping functionality of chromatin-related proteins has been observed previously (9), making their functional roles difficult to demonstrate genetically. Although HU is not reported to be able to replace IHF for activating the expressions of σ^{54}-dependent promoters *in vivo*, HU protein might partially replace IHF for the general restoration effect of the *glnA*p2 derivatives under *in vivo* conditions (see Supplementary Figure 3). Biochemistry and *in vitro* assays are likely more powerful tools to gain insight into the precise mechanisms associated with these chromosome organising proteins.

At some σ^{54}-dependent promoters, IHF can also work as an active suppressor (restrictor) of the promiscuous activation (cross-activation) by heterologous EBPs, and thus increase the specificity and fidelity of these promoters (32,33). In this study, we noticed that when IHF binding was centered at –44 and –64, it repressed the PspFΔHTH-dependent expression of *glnA*p2 IC-constructs by a factor of 2 (Table 3). Since the insertion of IHF-binding site at these two positions does not affect the closed complex formation (data not shown), our results might be explained by the proposal that the sharp IHF-induced

DNA bending could direct a segment of upstream DNA to occlude the access of promiscuous EBP to the promoter bound RNA polymerase (32,34).

Taken together, we propose that one role of IHF at σ^{54}-dependent promoters is to antagonize the negative regulatory effects of IHF target sites and their flanking DNA sequence in DNA loop formation, thus providing a general restoration of the productive enhancer–promoter communication. One extension of our results is that the antagonism between IHF and the DNA segments it binds puts the expression of most σ^{54}-dependent promoters under the control of the intracellular concentration of IHF protein, which is only accumulated maximally in stationary phase.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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