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Participation of IHF and a distant UP element in the stimulation of the phage λ P_L promoter

Hilla Giladi,[1] Simi Koby,[1] Gali Prag,[1] Manuel Engelhorn,[2] Johannes Geiselmann[2] and Amos B. Oppenheim[1]*

[1]*Department of Molecular Genetics, The Hebrew University – Hadassah Medical School, PO Box 12272, Jerusalem, 91120, Israel.*

[2]*Department of Molecular Biology, University of Geneva, 30 Quai Ernest Ansermet, CH-1211 Geneva 4, Switzerland.*

Summary

We have previously identified a UP element in the phage λ P_L promoter, centred at position −90 from the transcription start site. Integration host factor (IHF), a heterodimeric DNA-binding and -bending protein, binds upstream of the λ P_L promoter in a region overlapping the UP element. Stimulation of transcription by IHF requires an intact αCTD and affects the initial binding of RNA polymerase to the promoter. We propose a model for the stimulation of P_L by IHF in which IHF bends the DNA to bring the distal UP sequence in closer proximity to the promoter core sequences to allow the docking of the αCTD of RNA polymerase. Furthermore, IHF may also participate in protein–protein interactions with the αCTD. In support of this model, we found that alanine substitutions in αCTD at positions 265, 268, 270 and 275 reduced P_L promoter activity. Mutations in the IHF DNA binding site, as well as IHF mutant proteins exhibiting a decreased ability to bend the DNA, were both defective in stimulating the P_L promoter. In addition, some of the mutated IHF residues are clustered at a protein surface that interacts with the UP DNA sequence. These residues may also participate in protein–protein interactions with the αCTD.

Introduction

IHF is a heterodimeric DNA binding–bending protein that binds to specific sequences. The crystal structure of IHF bound to DNA shows that the DNA wraps around the protein and bends it by over 160° (Nash, 1996; Rice *et al*., 1996). IHF functions as an accessory factor in a variety of processes including site-specific recombination, replication and transcription initiation (Friedman, 1988). In many of these processes, IHF has been shown to act as an architectural element that facilitates the formation of DNA–protein complexes (Nash, 1996). IHF is involved in the regulation of transcription of a number of σ^{54}-dependent promoters in several bacterial species. Its role is to bend the DNA between the promoter and the activator binding site and to help create a loop that is required to bring the activator in contact with RNA polymerase (Hoover *et al*., 1990; de Lorenzo *et al*., 1991; Claverie-Martin and Magasanik, 1992; Perez-Martin and de Lorenzo, 1997).

Three σ^{70} promoters whose expression is directly stimulated by IHF have been studied extensively: the phage Mu early Pe promoter, the bacterial ilvP_G promoter of the *ilvGMEDA* operon and the phage λ early P_L promoter (Krause and Higgins, 1986; Giladi *et al*., 1990; Giladi *et al*., 1992a; Parekh and Hatfield, 1996). Sequence alignment of these promoter regions revealed that the non-symmetrical IHF recognition sequence in all three has the same orientation relative to the core promoter sequences (Goosen and van de Putte, 1995). Nevertheless, a uniform mechanism for IHF activity in these promoters has not been found. In the Mu Pe promoter, IHF was found to have two roles, one indirect to eliminate the repression exerted by H-NS (van Ulsen *et al*., 1996), and one direct to increase the initial binding of RNA polymerase to the promoter (K_B). This requires an intact αCTD and correct phasing between the IHF site and the promoter (Goosen and van de Putte, 1995; van Ulsen *et al*., 1997). In the ilvP_G promoter, IHF was found to increase open complex formation. The effect of IHF is dependent upon DNA supercoiling and is independent of the face of the helix. It has been proposed that binding of IHF upstream of this promoter causes structural changes in the DNA, which are transmitted downstream to the −10 region in a way that facilitates open complex formation at that promoter (Pagel *et al*., 1992; Parekh and Hatfield, 1996).

IHF has been found to participate in the regulation of a growing number of promoters (Goosen and van de Putte, 1995; Hill *et al*., 1997; Jovanovic and Model, 1997; Porter and Dorman, 1997; Pratt *et al*., 1997; Rondon and Escalante-Semerena, 1997). Recently, it was found that IHF can activate transcription of a modified *malT* promoter in which the Crp binding site was replaced by an IHF binding site (Dethiollaz *et al*., 1996). It appears that IHF stimulates this promoter by affecting its geometry.

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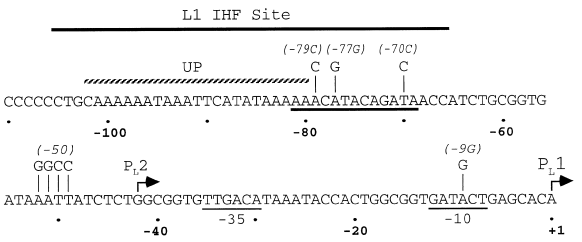

Fig. 1. A map of the P_L promoter region. The nucleotide sequence of the P_L promoter region from −110 to +1. The regions protected from DNase I digestion by IHF (L1) and by the α-subunit (UP), are shown by a solid upper line and a hatched line respectively. The IHF recognition sequence is underlined. The different promoter mutations and mutations in the IHF recognition sequence are shown above the sequence. Transcription start sites of the major P_L and the minor P_L2 promoters are shown by arrows.

We have been studying the participation of IHF in the expression of the λ P_L promoter. We found that IHF binds at two sites upstream of the promoter and stimulates transcription 2.5- to 3-fold both *in vivo* and *in vitro* by increasing K_B. The activity of IHF is dependent on correct phasing between the IHF site and the promoter and requires an intact αCTD (Giladi *et al*., 1992b; Giladi *et al*., 1996). The P_L region contains a second minor promoter, P_L2, which initiates transcription 42 bp upstream of the major P_L promoter (Fig. 1). P_L2, in contrast to the major P_L, is repressed by IHF (Giladi *et al*., 1992a). The role of this secondary promoter in the phage λ life cycle is not known.

In addition to the −10 and −35 elements, *Escherichia coli* promoters may possess a third critical element, the UP element (Ross *et al*., 1993; Blatter *et al*., 1994). We have identified a UP element located at a distance upstream of P_L nested within the promoter proximal region protected by IHF from DNase I digestion. This UP sequence has been shown to bind the α-subunit of RNA polymerase and to be essential for the basal transcription of P_L2. In addition, the UP sequence could function as an independent element, capable of enhancing promoter activity when placed adjacent to the −35 region of the Plac or the P_L promoters. Mutations in αCTD were found to affect P_L2 activity (Giladi *et al*., 1996).

The αCTD has been shown to be essential for the activity of a number of transcriptional activators (Ishihama, 1992; Russo and Silhavy, 1992; Blatter *et al*., 1994; Tang *et al*., 1994; Tao *et al*., 1995; Artsimovitch *et al*., 1996; Wood *et al*., 1997; Yang *et al*., 1997), and its structure has been recently elucidated by NMR spectroscopy (Jeon *et al*., 1995; Gaal *et al*., 1996). It has been shown that the binding of αCTD to UP improves the binding of RNA polymerase to the promoter (Blatter *et al*., 1994). Extensive mutational analysis of αCTD helped to identify several residues, between amino acids 258 and 275 and between 291 and 299 that are important for activator-dependent and for UP element-dependent transcription (Tao *et al*., 1995; Gaal *et al*., 1996; Murakami *et al*., 1996; Wood *et al*., 1997; Yang *et al*., 1997; Negre *et al*., 1998).

In this work, we show that the geometry of the P_L promoter, as dictated by the binding of IHF, is a key element in the stimulation of the promoter by IHF. IHF mutants altered in their ability to bend DNA were defective in transcription stimulation. We suggest that the DNA architecture induced by IHF is required for the establishment of contacts between αCTD and the UP element, and possibly also with IHF. These contacts increase the affinity of RNA polymerase to the P_L promoter.

Results

The importance of the DNA upstream sequence containing the UP element for P_L activity

In order to assess the importance of upstream sequences for P_L activity, we constructed a number of deletions from the 5′ end of the promoter, fused them to the *lacZ* reporter gene and assayed for constitutive promoter activity in the absence of CI repressor. Two promoter constructs were used, one carrying the wild-type P_L promoter and the other carrying the mutant P_L-9G promoter. The wild-type P_L–*lacZ* fusions cannot be maintained on a plasmid without the presence of the CI repressor and were therefore introduced into the bacterial chromosome as single-copy λ lysogens. Plasmids carrying the weaker −9G promoter can be maintained in the absence of repressor.

As seen in Table 1, deletion of sequences up to −106, which removes the promoter-distal IHF binding site but retains the promoter-proximal L1 site (Fig. 1) (Giladi *et al*., 1990), had only a small effect on promoter activity. A further deletion to −78 or to −40, removing the UP sequence and the IHF binding site, led to a significant reduction in promoter activity. These results demonstrate that the region between −40 and −106, which includes the IHF binding site and the UP element, is required for maximal promoter activity.

IHF binds upstream of the promoter and stimulates P_L transcription (Giladi *et al*., 1990). When expression was measured in the absence of IHF (in *ihf* mutant cells; Table 1), all wild-type P_L promoter constructs showed similar activity. We conclude that the presence of IHF is essential for the stimulation of transcription from the P_L promoter upstream sequences.

We repeated the same experiments described above with plasmids carrying the P_L-9G–*lacZ* fusions. The results show that the P_L-9G promoter is more responsive to the presence of the upstream sequence than the wild-type promoter and that IHF is essential for the stimulation of transcription. Both sets of experiments demonstrate that the region between −40 and −106, which includes the IHF binding site and the UP element, is required for maximal promoter activity.

Table 1. The importance of the Up sequence for P_L activity.

Promoter	5′ end of promoter	β-Galactosidase units[a] IHF$^+$	IHF$^-$
(A) P_L wt (single copy)	−228	2039	1158
	−106	1850	1097
	−78	1050	923
	−40	941	1044
(B) P_L-9G (multicopy)	−228	7320	1625
	−106	6115	1711
	−40	1080	875

a. Average of at least two independent experiments, each carried out with two cultures of each strain.
A. The strains are A6826 (IHF$^+$) and its *hip*Δ3::Cat derivative (IHF$^-$) carrying a λ lysogen with the P_L–*lacZ* transcription fusion of the wild-type P_L promoter, extending to +115 at the 3′ end and extending to −228, −106, −78 and −40 at the 5′ end, relative to the P_L transcription start site.
B. Plasmids carrying the P_L-9G–*lacZ* transcription fusion with promoter fragments extending to +115 at the 3′ end and to −228, −106 and −40 at the 5′ end were introduced into strain A6826 (IHF$^+$) and its *hip*Δ3::Cat derivative A6844 (IHF$^-$).
Overnight cultures were diluted 1:300 into LB medium containing 25 μg ml^{-1} (in experiment A) or 50 μg ml^{-1} (in experiment B) ampicillin, grown at 37°C to an OD_{600} of 0.2–0.3 and assayed for β-galactosidase levels.

Mutations in the IHF recognition sequence prevent P_L promoter stimulation

To test whether IHF acts *in vivo* directly by binding to the promoter region, we introduced point mutations in the IHF recognition sequence. The IHF recognition sequence upstream of the P_L promoter, 5′-AAACATacagATA is a strong binding site (Giladi *et al*., 1996). This sequence is 3 bases off the consensus WATCAAnnnnTTR (where W represents A or T, R represents A or G and n represents A, T, G or C) (Goodrich *et al*., 1990). The mutations −70C, −77G and −79C (see Fig. 1) were previously isolated as mutations that reduced P_L2 repression by IHF and were all found to be localized within a region that defined the IHF recognition sequence (Giladi *et al*., 1996). To study the effect of these mutations without P_L2, the mutations were transferred to a P_L-9G-50–*lacZ* promoter fusion. This promoter carries, in addition to the −9G mutation analysed in the previous section, a mutation inactivating P_L2 at its −10 region, mutation −50 (see Fig. 1). Thus, only the major P_L is active. Transformed cells were assayed for β-galactosidase activity in the presence or absence of IHF. The results (Table 2) show that IHF was unable to stimulate P_L-9G-50 carrying the −70C or the −77G mutation. The −70C mutation has been shown previously to reduce greatly the binding of IHF to DNA (Table 2) (Giladi *et al*., 1996). As expected, the weaker mutation −79C had a small effect on P_L activity. These results demonstrate that IHF binding to its site is a prerequisite for P_L stimulation.

Correct phasing of the promoter with the IHF binding site and UP is required for the stimulation of transcription by IHF

We have demonstrated previously that correct phasing of the IHF binding site with the core promoter is important for IHF stimulation of promoter activity. This was done by constructing P_L promoters carrying 5 bp and 11 bp inserts at position −67 and analysing their expression by *in vitro* transcription assays (Giladi *et al*., 1992a). In order to test the effect of these insertions on P_L activity *in vivo*, these modified promoters were fused to *lacZ*, and the fusions were introduced into the bacterial chromosome via a λ lysogen. We found that, in the authentic promoter arrangement and in the promoter with the 11 bp insert, *lacZ* expression was strongly dependent upon the presence of IHF (Table 3), whereas in the fusion carrying the 5 bp insert, IHF stimulation was abolished. Moreover, in this arrangement, IHF reduced promoter activity by about twofold. These results provide further evidence for the importance of promoter architecture for IHF stimulation *in vivo*.

IHF mutants with reduced DNA bending are defective in the stimulation of transcription from P_L

Next, we tested IHF mutants that are impaired in DNA bending for their ability to stimulate transcription from

Table 2. The effect of mutations in the IHF recognition sequence on P_L activity.

Strain	IHF[a]	Plasmid	Mutation[b]	β-Galactosidase units[c]	Relative binding affinity[d]
A8449	+	pHG270	wt	1424 ± 176	1.00
A8453	−			513 ± 13	
A9273		pHG303	−77G	692 ± 13	ND
A9274	−			560 ± 155	
A8450	+	pHG304	−70C	747 ± 80	>0.02
A8454	−			746 ± 127	
A8451		pHG305	−79C	1229 ± 75	0.40
A8455	−			432 ± 25	

a. IHF genotype.
b. Mutation in the IHF recognition sequence upstream of P_L.
c. Average of at least two independent experiments, each carried out with two cultures of each strain.
d. The relative binding affinities of IHF were determined previously (Giladi *et al*., 1996). ND, not done.
Strain A6826 (IHF$^+$) and its *ihfB*Δ3::Cat derivative A6844 (IHF$^-$) were transformed with plasmid pHG270 (wt) carrying the P_L-9G-50 promoter (extending from −130 to +115) fused to the *lacZ* gene or with pHG270 derivatives pHG303, 304 and 305, carrying point mutations in the IHF recognition sequence at positions −77, −70 and −79, respectively, relative to the P_L1 transcription start site (see Fig. 1). Cells were grown at 37°C in LB supplemented with 50 μg ml^{-1} ampicillin to mid-logarithmic phase and assayed for β-galactosidase activity.

Table 3. Influence of promoter geometry on P_L activity.

Strain	IHF	Promoter	β-Galactosidase units[a]
A7880	+	P_L	1238 ± 85
A8283	−	P_L	398 ± 19
A7694	+	P_L+5	325 ± 20
A8284	−	P_L+5	597 ± 31
A7695	+	P_L+11	1548 ± 58
A8285	−	P_L+11	614 ± 32

a. Average of two independent experiments, each performed with two cultures of each strain.
All strains are A6826 derivatives bearing a λ lysogen carrying a *lacZ* transcription fusion of wild-type P_L (extending from −130 to +115) or modified P_L with 5 (P_L+5) or 11 (P_L+11) bp inserted at position −67 relative to the P_L transcription start site (Giladi *et al.*, 1992a). The strains were rendered IHF$^-$ by disruption of the *ihfB* gene (*hip*) by P1 transduction of *ihfB*Δ3::Cat.
Overnight cultures were diluted into LB supplemented with 25 μg ml^{-1} ampicillin, grown at 37°C to an OD_{600} of 0.2–0.3 and then assayed for β-galactosidase levels.

the P_L promoter. These mutants are described in detail in the accompanying paper by Engelhorn and Geiselmann.

IHF-deficient cells carrying the P_L-9G-50–*lacZ* fusion plasmid were transformed with a second compatible plasmid expressing wild-type IHF or the various IHF mutants and assayed for β-galactosidase activity. The assays were performed at 20°C because, at this temperature, the dependence of P_L-9G-50 on IHF is more pronounced (Giladi *et al.*, 1995). The results presented in Table 4 show that IHF mutants 1, 2, 3, 11 and 12 were defective in the stimulation of P_L, while mutant 6 moderately reduced β-galactosidase activity, and mutant 4 yielded wild-type levels. The finding that mutants that were shown to affect DNA bending reduced the level of P_L expression suggests that IHF is required to induce a specific DNA bend for P_L stimulation.

αCTD and IHF act in concert to stimulate P_L activity

We have found previously that an intact αCTD of RNA polymerase is required for the stimulation of P_L transcription (Giladi *et al.*, 1992b). Those *in vitro* transcription experiments were performed using reconstituted RNA polymerase carrying truncated α-subunits. In the following experiments, we tested the importance of specific residues in the αCTD for P_L transcription *in vivo*.

Cells wild type for the *rpoA* gene, which is an essential function, were transformed by plasmids expressing the wild-type *rpoA* or mutants thereof carrying alanine substitutions in αCTD (Murakami *et al.*, 1996). Induction of the cloned *rpoA* by IPTG in these merodiploid cells leads to the substitution of the wild-type α-subunit by mutant α-subunits in a large fraction of RNA polymerase molecules in the cell.

The results summarized in Table 5A show that mutations R265A, N268A, L270A and I275A reduced P_L activity, demonstrating that αCTD is required for the stimulation of the major P_L promoter *in vivo*.

If αCTD function requires IHF, then it would be expected that, in the absence of IHF, mutations in *rpoA* would not affect P_L promoter activity. As seen in Table 5B, in cells lacking IHF, the level of β-galactosidase was not reduced upon induction of the *rpoA* mutants. Similar results were obtained with cells expressing IHF but harbouring a P_L construct carrying the −70C mutation in the IHF binding site, which prevents the binding of IHF (Table 5C). These results show that, in the absence of IHF, αCTD does not participate in the stimulation of P_L and that both αCTD and IHF act in concert to stimulate P_L activity.

The importance of a flexible DNA joint between the sites bound by IHF and by RNA polymerase

The results presented above can be explained by the model in which IHF bends the DNA to bring the distal UP sequence in closer proximity to the promoter core sequences to allow docking of αCTD (Fig. 3). However, as shown in the schematic model, the bending by IHF is not sufficient; a second bend is implied at around −50. We have found previously in DNase I footprinting experiments that, in the simultaneous binding of IHF and RNA

Table 4. IHF mutants altered in their ability to bend DNA are impaired in stimulation of P_L transcription.

Mutant[a]	IHF on plasmid	Activity[b] (%)	Dissociation[c] constant
−	Wt IHF	100	4
1	IhfA: K45N; IhfB: P64L, A90V	25	8
2	IhfA: K45E, F13I; IhfB: E5D, H16R	42	5
3	IhfB: K27E	32	ND
4	IhfA: K24N, K45I	101	ND
6	IhfA: K45N	85	5
11	IhfB: K3A, S4E	26	20
12	IhfB: K3E, S4M	31	ND

a. Mutant number as appears in the accompanying paper by Engelhorn and Geiselmann.
b. The percentage activity was calculated by first subtracting from all the results the 712 β-galactosidase units expressed in the presence of the vector only with no IHF and then calculating the percentage activity relative to wt IHF (2962 units). The results are an average of three independent experiments each performed with two cultures of each transformation.
c. Determined in the accompanying paper by Engelhorn and Geiselmann. Strain JG385 mutated in both IHF genes was transformed with plasmid pHG270 carrying the P_L-9G-50–*lacZ* transcription fusion and with a second compatible plasmid p99kanIHF expressing both IHF subunits (IhfA and IhfB) or their mutant derivatives as indicated (see accompanying paper by Engelhorn and Geiselmann).
Cultures were grown in LB supplemented with 40 μg ml^{-1} kanamycin and 50 μg ml^{-1} ampicillin, at 37°C to an OD_{600} of 0.1–0.2. The cultures were transferred to 20°C, and β-galactosidase assays were performed after 3 h (one generation time) at 20°C.

Table 5. The effect of mutations in the αCTD on P_L activity.

Strain	αCTD mutation on *rpoA* plasmid	Activity (%)
(A) A7506 (IHF^+)		
	Wt	100
	D258A	104
	D259A	103
	L260A	89
	E261A	114
	L262A	83
	T263A	99
	V264A	86
	R265A	51
	S266A	108
	N268A	67
	C269A	80
	L270A	72
	K271A	98
	E273A	100
	I275A	63
(B) A7507 (IHF^-)	Wt	100
	R265A	102
	N268A	95
(C) A9438 (IHF^+)		
	Wt	100
	R265A	105

A. Strain A7506 is an A6826 derivative harbouring an integrated λ prophage carrying the P_L-9G-50–*lacZ* fusion. The cells were transformed with plasmids expressing the wild type or mutant *rpoA* genes, carrying alanine substitutions at the positions indicated, under an IPTG-inducible promoter (Murakami *et al.*, 1996).
B. Strain A7507 (IHF^-), a *hip*Δ3::Cat derivative of strain A7507, was transformed with the *rpoA* plasmids.
C. Strain A9438 is a derivative of A7506 in which the P_L-9G-50 promoter fused to *lacZ* carries the −70C point mutation at the IHF site upstream of the promoter.
The cells were grown at 37°C in LB supplemented with 50 μg ml^{-1} ampicillin to an OD_{600} of 0.2. IPTG (0.5 mM final concentration) was added, and β-galactosidase was measured after 90 min of IPTG induction.

polymerase to the promoter region, an interval of 27 bp (from −37 to −64) remains unprotected (Giladi *et al.*, 1992a). Furthermore, DNase I hypersensitive sites, spaced approximately 10 bp apart, were created in this interval. The induced DNase I hypersensitive sites are indicative of looping and bending, suggesting that this region acts as a flexible DNA joint.

We performed computational prediction of the P_L promoter DNA region, according to the matrix of Calladine *et al.* (1988). The analysis suggests that the IHF binding sequence and the sequence around −50, which we propose to serve as a DNA joint, are highly bendable (Fig. 2). As predicted, mutating the AATT sequence to GGCC at the −50 region reduced DNA bendability (Fig. 2). We have assayed the GGCC mutation for its effect on transcription *in vivo* using a P_L-50–*lacZ* fusion (Table 6). The −50 mutation was found to reduce promoter activity greatly, much more than the −9G mutation, which is expected to

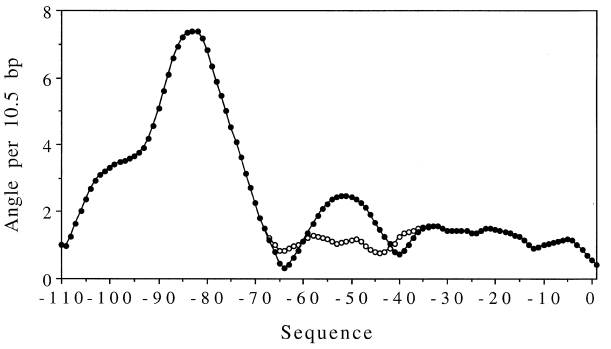

Fig. 2. DNA bending model of the P_L promoter region. The prediction of DNA bending was done on P_L DNA spanning the region from +1 to −110, according to the matrix of Calladine *et al.* (1988) (http://www.icgeb.trieste.it/dna/curve_it.html) using a window size of 20 bp. The angle (in degrees) is given per DNA turn (10.5 bp). Filled circles represent wild-type P_L sequence; open circles represent the same DNA region carrying the GGCC mutation at −50.

reduce the binding of σ^{70} to the promoter. The combination of both the −50 and the −9G mutations further decreased promoter activity. These results support our model for the presence of a flexible DNA joint. As the GGCC region overlaps the −10 region of P_L2, our experiments cannot exclude additional indirect effects of this mutation on P_L activity.

Discussion

DNA bending facilitates the interaction of proteins or protein domains with DNA sites that are widely separated. For example, in supporting site-specific recombination of phage λ, IHF allows two protein domains of integrase to interact concurrently with two distantly located DNA recognition sequences (Kim *et al.*, 1990). Likewise, in the activation of the σ^{54} promoters, IHF binds and bends the DNA to bring together RNA polymerase bound to the core promoter and activator protein bound to a distal enhancer sequence. We propose a similar function for IHF in the

Table 6. The activity of mutant P_L promoters.

Strain	Promoter	β-Galactosidase units[a]
A7655	$P_L$wt	2306 ± 75
A8046	P_L-9G	1405 ± 60
A7879	P_L-50	385 ± 14
A7521	P_L-9G-50	137 ± 9

a. Average of at least three experiments, each performed with two cultures of each strain.
All strains are A6826 derivatives carrying a λ lysogen with the P_L–*lacZ* transcription fusion of the wild-type P_L extending from −228 to +115 and mutants thereof (see Fig. 1). The cells were grown in LB supplemented with 25 μg ml^{-1} ampicillin at 37°C to mid-logarithmic phase and assayed for β-galactosidase activity.

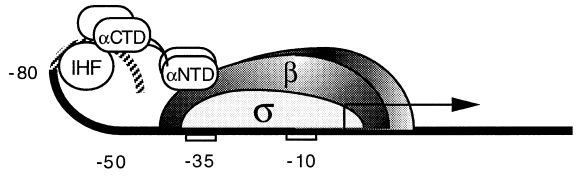

Fig. 3. A model for the interaction of IHF and αCTD with the P_L upstream region. IHF bends the DNA to allow αCTD of RNA polymerase bound at P_L to make contact with UP (hatched line) and thereby increase the affinity between the promoter and RNA polymerase. αCTD also possibly interacts with IHF.

stimulation of P_L (Fig. 3). In this model, IHF bends the DNA to bring the distal UP element, centred at −90 from the transcription start site, in closer proximity to RNA polymerase. We postulate that this geometry allows the docking of αCTD onto the UP element. In addition, IHF may establish protein–protein contacts with αCTD.

The UP sequence is nested within the region protected by IHF from DNase I digestion. It is therefore difficult to determine whether both IHF and αCTD proteins occupy the DNA region simultaneously. We attempted to resolve this question by DNA gel retardation experiments. However, we were unable to obtain evidence for the binding of both proteins to P_L simultaneously. We note, however, that the affinity of the α-subunit to the AT-rich UP sequence present in the P_L promoter is rather low (Giladi *et al.*, 1996; unpublished results).

A computer analysis of 27 IHF binding sites (Goodrich *et al.*, 1990) revealed an extended AT-rich sequence that was consistently found 5′ to the WATCAA part of the IHF consensus sequence (Goodrich *et al.*, 1990). Some of these IHF sites are located near promoters and, thus, it is possible that their AT-rich sequences constitute potential binding sites for αCTD and that IHF works in concert with αCTD to modulate the activity of these promoters.

It has been shown previously that IHF could be replaced by LEF-1 in the process of λ integration and in the expression of the $ilvP_G$ promoter (Giese *et al.*, 1992; Parekh and Hatfield, 1996). LEF-1 is a DNA-bending mammalian transcription factor that shares no amino acid sequence similarity with IHF. To learn whether IHF can be replaced functionally by LEF-1 in P_L activation, we substituted the IHF recognition sequence with that of LEF-1 (CCTTTGAA). We found that, in both orientations, LEF-1 was incapable of replacing IHF in stimulating P_L (data not shown). It is possible that the LEF-1 site was not positioned optimally in our constructs or that the degree of DNA bending exerted by LEF-1 is not sufficient for P_L stimulation. An alternative possibility is that LEF-1 was unable to establish protein–protein contacts with αCTD required for P_L activation.

Analysis of the IHF mutants used in this study indicated that they reduce the ability of IHF to wrap and thereby bend DNA (Engelhorn and Geiselmann, 1997). Unfortunately, these mutant proteins carry more than one amino acid change, making it difficult to attribute specific contributions of individual residues. Mutations changing residues βK3, βS4 and βK27 in IhfB cluster in regions in which IHF interacts non-specifically with DNA (see Fig. 4). In the IhfA subunit, the corresponding amino acid cluster participates in specific binding to DNA. This fact may explain why no mutants were picked by Engelhorn and Geiselmann in this region. Residue βE5 is also in this cluster but does not contact the DNA. Residue βP64 in IhfB probably introduces a kink in the DNA, facilitating the wrapping of the DNA (Rice *et al.*, 1996). Mutations affecting residues αK24 and αK45 of IhfA and residue βK27 of IhfB may affect binding to DNA or, more probably, change the interactions between the two subunits and indirectly reduce the bending of DNA. We have found previously that the mutation βA90D of IhfB reduced the binding affinity of IHF to DNA, possibly by affecting IHF folding and/or monomer–monomer interaction (Mengeritsky *et al.*, 1993). However, we suspect that mutation βA90V, present in mutant no. 1, probably has a small effect as Ala or Val are represented almost equally at this position in IHF from various Gram-negative bacteria (Highlander *et al.*, 1997).

Molecular modelling was used to visualize the spatial position of mutations in IHF, αCTD and in the IHF binding site (Fig. 4). Owing to the asymmetrical nature of the IHF consensus sequence, it is possible to position the mutated residues in IHF with respect to the bent DNA and to the UP sequence. IHF mutations in residues βK3, βS4, βS5, βK27 and αK45 affecting promoter activity are clustered at a protein surface that interacts with the UP DNA sequence. The model further suggests that IHF and αCTD can occupy the P_L promoter region simultaneously and establish protein–protein contacts.

In the accompanying paper, it is suggested that mutations in IHF that reduce its ability to bend DNA lead to an increased *malT* promoter activity. The observation that the same bending mutants of IHF have opposite effects on P_L argues in favour of a model in which a stronger DNA bend is required for maximal P_L activity. It appears that the optimal bending angle at the modified *malT* promoter is less than the 160°–180° induced by IHF, whereas at P_L, the combined bending contributed by IHF and by the curved DNA at −50 exceeds 180°. It is therefore likely that a different contact between RNA polymerase and the upstream DNA region occurs at the two promoters.

The mode of interaction of αCTD with the UP sequence is more difficult to analyse as no co-crystal with DNA is available. In our study (Table 5), we used mutants that had been analysed previously with respect to their effect on factor-dependent and UP-dependent stimulation of transcription. Our experiments provide evidence for the importance of residues R265, N268, L270 and I275 of αCTD for P_L activity. Mutations affecting residues R265

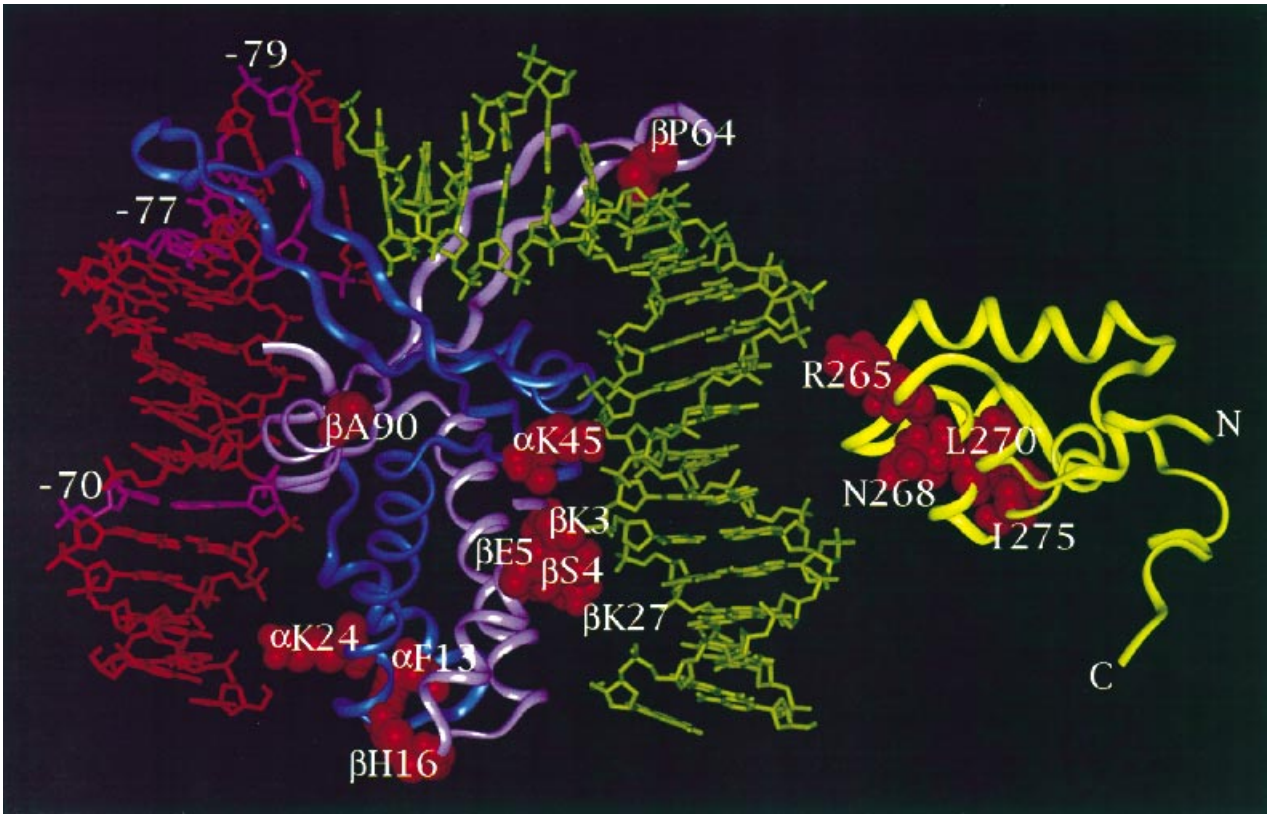

Fig. 4. Structures of the IHF–DNA complex and of αCTD. A ribbon display of the IHF–DNA co-crystal complex (left) and the NMR structure of αCTD (right) (Jeon *et al.*, 1995; Rice *et al.*, 1996), generated using the BIOSYM *Insight* II package programs. The IhfA (α) and IhfB (β) subunits of IHF are coloured in blue and light purple respectively; the αCTD is coloured yellow. Mutated positions in IHF and in αCTD are rendered in space-filling and are coloured red. The DNA is coloured red; mutations at −70C, −77G and −79C in the IHF recognition sequence are coloured pink; the UP sequence is shown in light green.

and N268 were shown to be important for both UP-dependent transcription and Crp-dependent *lac*P1 transcription (Gaal *et al.*, 1996; Murakami *et al.*, 1996). Additional residues that were found to be important for UP-dependent transcription (L260, L262, C269) were found to have a smaller effect on P_L activity. Residues L270 and I275 were also implicated in Crp-dependent *lac*P1 transcription (Murakami *et al.*, 1996; Chugani *et al.*, 1997). Mutations affecting residues R265, N268 and L270 and, to a lesser extent, I275 were found to reduce TyrR activation of the *mtr* promoter (Yang *et al.*, 1997). However, additional residues important for Crp- and TyrR-dependent transcription (258, 259, 261) did not affect P_L activity. The comparison among promoters of the effect of the various αCTD mutations suggests that, in the binding of RNA polymerase to the P_L promoter, the αCTD interacts with both UP and IHF.

Experimental procedures

Strains and plasmids

Strain A6826 is CSH50 F^- *ara* D (*lac-pro*) *rpsL thi*. A6844 is its *ihfBΔ3::Cat* derivative. Strain JG385 (pop2492 *ihfA82*::Tn10 *ihfBΔ3*::Cat; Engelhorn and Geiselmann, 1997) is mutated in both IHF genes. P_L promoter fragments were generated by polymerase chain reaction (PCR) using the following primers: for the 3′ end of the promoter 2011 5′-AAGGATCCAATGCTTCGTTT (position +115) and for the different 5′ ends primers 1921: 5′-AAGAATTCGGGTTTCTTT (position −228); 1815: 5′-TCAGAATTCTCACCTACC (position −130); 2323: 5′-AGAATTCCTGCAAAAAATAAATT (position +106); 1844: 5′-GGAATTCATACAGATAAC (position −78); and 2115 5′-GAATTCGGTGTTGACATAAA (position −40). Mutants −9G and −50 of the P_L promoter have been described previously (Giladi *et al.*, 1992a) and were amplified by PCR and cloned upstream of *lacZ* in pHG86 (Giladi *et al.*, 1992a). To transfer the mutations in the IHF recognition sequence from the P_L2 promoter, where they were isolated originally (Giladi *et al.*, 1996), to the P_L-9G-50 promoter, we used two successive PCR reactions. In the first, we generated a P_L fragment extending from −130 to −50 using primers 1815 and 1842 (5′-GGCCTATCACCGCAGATGG) respectively (primer 1842 carries the −50 mutation at its 3′ end) on templates carrying the different IHF site mutations. In the second reaction, we used as primers, oligonucleotide 2011 and the product of the first PCR reaction, using as a template the P_L-9G-50 promoter. P_L promoter fragments with 5 bp (5′-CATCT) and 11 bp

(5′-CCATCTGATCA) insertions were constructed previously (Giladi *et al*., 1992a), amplified by PCR using primers 1815 and 2011 and fused to *lacZ* in the pHG86 vector. Lysogens carrying the various promoter–*lacZ* fusions were constructed by first recombining the plasmid with phage λB299 (described in Giladi *et al*., 1995) and then lysogenizing strain A6826.

The wild-type and mutant IHF plasmids have been described elsewhere (Engelhorn and Geiselmann, 1997). In these plasmids, both genes coding for the IHF subunits (*ihfA* and *ihfB*) were cloned under the Trc-inducible promoter in the vector p99kan, which was derived from pTrc99A (Pharmacia) by exchanging the origin of replication and the β-lactamase gene for the pACYC origin and the kanamycin-resistant gene. The plasmids carrying the *rpoA* mutants under the Trc-inducible promoter were kindly donated by Akira Ishihama and have been described previously (Murakami *et al*., 1996). Plasmid pGST-LEF-HMG expressing the LEF-1 HMG domain fused to GST was kindly donated by Wesley Hatfield (Parekh and Hatfield, 1996).

Enzymatic assays

β-Galactosidase assays were carried out as described by Miller (1972). PCR was performed with the PWO DNA polymerase (Boehringer Mannheim).

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