The EMBO Journal Vol.17 No.17 pp.5120–5128, 1998

Active recruitment of σ^{54}-RNA polymerase to the *Pu* promoter of *Pseudomonas putida*: role of IHF and αCTD

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The sequence elements determining the binding of the σ^{54}-containing RNA polymerase (σ^{54}-RNAP) to the *Pu* promoter of *Pseudomonas putida* have been examined. Contrary to previous results in related systems, we show that the integration host factor (IHF) binding stimulates the recruitment of the enzyme to the –12/–24 sequence motifs. Such a recruitment, which is fully independent of the activator of the system, XylR, requires the interaction of the C-terminal domain of the α subunit of RNAP with specific DNA sequences upstream of the IHF site which are reminiscent of the UP elements in σ^{70} promoters. Our data show that this interaction is mainly brought about by the distinct geometry of the promoter region caused by IHF binding and the ensuing DNA bending. These results support the view that binding of σ^{54}-RNAP to a promoter is a step that can be subjected to regulation by factors (e.g. IHF) other than the sole intrinsic affinity of σ^{54}-RNAP for the –12/–24 site.

Keywords: αCTD/IHF/NtrC family/*Pseudomonas*/σ^{54}/TOL/XylR

Introduction

The events that make a DNA segment behave as a promoter include a panoply of specific protein–protein and protein–DNA interactions engaging RNA polymerase (RNAP) and activator proteins (Ishihama, 1993; Busby and Ebright, 1994; Stargell and Struhl, 1996; Ptashne and Gann, 1997). Furthermore, intrinsic or protein-induced distortion of promoter DNA often plays a key role in favouring such interactions (Iyer and Struhl, 1995; Geiselmann, 1997; Pérez-Martín and de Lorenzo, 1997). The first requisite for transcription initiation is promoter recognition by transcriptional machinery. Eubacterial RNAP bearing the major sigma factor σ^{70} (σ^{70}-RNAP) can bind and transcribe DNA sequences containing –35 and –10 hexamers, even in the absence of any other factors (Record *et al.*, 1996). For most σ^{70}-RNAP promoters, the closer the sequence of –35 and –10 sites to the consensus, the higher the affinity of RNAP for the promoter. Apart from the specific core sequence of target promoters, RNAP binding is determined in many cases by *cis*- and *trans*-acting elements which recruit the transcriptional machinery to the DNA (Ptashne and Gann, 1997). One additional element which frequently determines promoter recognition by σ^{70}-RNAP is the interaction between the C-terminal domain (CTD) of its α subunit and AT-rich sequences generically known as UP elements (Ross *et al.*, 1993; Blatter *et al.*, 1994; Rao *et al.*, 1994). These are located upstream of the –35 hexamer and can enhance promoter strength up to 30-fold by increasing the initial equilibrium constant between RNAP and DNA (Rao *et al.*, 1994). In other cases, the αCTD of RNAP makes specific protein–protein contacts with cognate activators bound adjacent to or further upstream of the –35 hexamer. This compensates for the absence of a UP element and recruits the entire σ^{70}-RNAP to the corresponding DNA region (Busby and Ebright, 1994).

Promoters depending on alternative sigma factors follow basically the same general rules for promoter recognition, with the only difference being the target sequences whose specificity is determined by the sigma itself (Lonetto *et al.*, 1992). One extreme case of this kind are the promoters transcribed by the form of RNAP bearing the alternative factor σ^{54}. This sigma is structurally unrelated to σ^{70} and the transcriptional activation of σ^{54}-promoters is directed by a different mechanism (Gralla and Collado-Vides, 1996). σ^{54}-RNAP binds a conserved –12/–24 region (consensus: TGGCAC N5 TTGCa/t) located between positions –11 and –26 relative to the transcription start (Merrick, 1993), which can be considered the functional analogue of the –10/–35 core promoter recognized by σ^{70} (Buck and Cannon, 1992). This sequence is the principal element for promoter recognition by σ^{54}-RNAP in the σ^{54}-dependent promoters studied to date. The affinity of σ^{54}-RNAP for the –12/–24 site does vary, depending on the correct distance between the generally invariant GG and GC motifs and the base composition of the spacer between them. In general, an increasing number of T residues between –14 and –20 enhances the stability of the complex between σ^{54} and the promoter (Hoover *et al.*, 1990; Claverie-Martin and Magasanik, 1991; Buck and Cannon, 1992; Santero *et al.*, 1992). Within the current model of functioning of σ^{54}-promoters (Gralla and Collado-Vides, 1996), σ^{54}-RNAP binds spontaneously the –12/–24 sequence but is unable to initiate transcription by itself in the absence of cognate regulatory proteins. Instead, activation requires necessarily the action of a class of prokaryotic enhancer-binding proteins (known as the NtrC family; North *et al.*, 1993). These activators share structural and functional properties which are reflected in the organization of their homologous domains (Kustu *et al.*, 1991; Morett and Segovia, 1993; Shingler, 1996). Unlike σ^{70}-promoters, the targets for these activators (known as upstream activating sequences or UAS) can be located at >100 bp from the σ^{54}-RNAP binding site (Kustu *et al.*, 1991). Such an arrangement of promoter

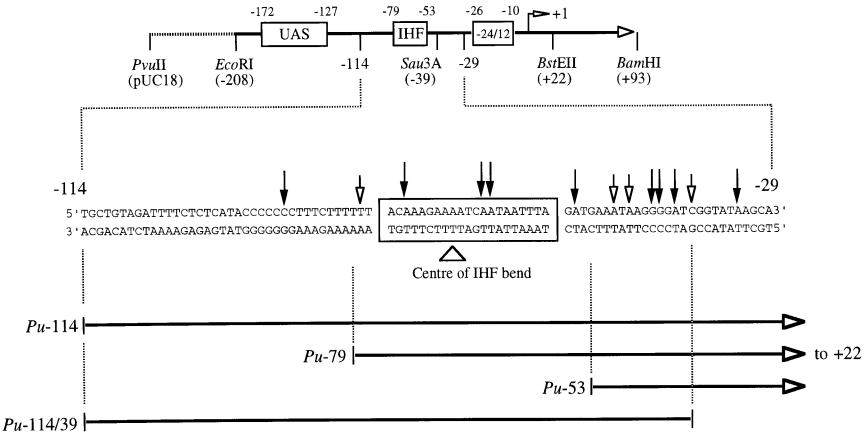

Fig. 1. Organization of the *Pu* promoter of TOL plasmid. The scheme at the top shows the 312 bp *Eco*RI–*Bam*HI insert of plasmid pEZ9 with the distribution of the functional *cis*-elements in respect to the transcription start site (+1). These include the sequence recognized by σ^{54}-RNAP (–12/–24 motif), the binding site for the integration host factor (IHF) and the upstream activating sequences (UAS) which are the target of the activator of the system, XylR. The sequence below is an expansion of the *Pu* region spanning positions –29 to –114, around the IHF binding site. The nucleotides of the upper strand that are either protected or hypersensitive to DNase I in the presence of σ^{54}-RNAP are indicated, respectively, by open or closed arrows (see text for explanation). The predicted centre of the curvature caused by IHF binding is inferred from the known crystal structure of IHF–DNA complexes (Rice *et al.*, 1996). In the lower part of the figure, the extension and names of the promoter-containing DNA fragments used in band-shift assays are indicated.

elements makes transcriptional activation require looping out of the intervening region between the UAS and –12/–24 in order to permit contacts between the activator and σ^{54}-RNAP (Su *et al.*, 1990). This looping is assisted, in most cases, by the integration host factor (IHF) which binds and bends a target sequence located at the intervening region (Pérez-Martín and de Lorenzo, 1997).

In this work we have examined the elements which determine the binding of σ^{54}-RNAP to the σ^{54}-dependent *Pu* promoter of *Pseudomonas putida* (Figure 1). This promoter drives transcription of TOL plasmid upper operon for the degradation of toluene (Ramos *et al.*, 1997). *Pu* includes UAS for the activator protein XylR (Pérez-Martín and de Lorenzo, 1996b), an IHF binding site (de Lorenzo *et al.*, 1991) and a T-poor –12/–24 region (TGGCATG-GCGGTTGCT) for σ^{54}-RNAP binding. Our results show that recognition of *Pu* promoter by σ^{54}-RNAP involves not only the –12/–24 sequence but also a second upstream element that is a functional equivalent of the UP elements of σ^{70} promoters. In addition, we show that IHF participates in the recruitment of σ^{54}-RNAP to *Pu* through a mechanism which includes the correct spatial positioning of the UP element with respect to –12/–24 site caused by the IHF-induced DNA bending. These results reveal a novel role of IHF in σ^{54} promoters which is different from that of simply assisting the contacts between the activator and σ^{54}-RNAP.

Results

The activity of Pu promoter in vivo is affected by the deletion of the αCTD of σ54-RNAP

It is generally believed that recognition of the –12/–24 sequences by σ^{54}-RNAP holoenzyme is determined by the σ factor itself and that the rest of the enzyme (core RNAP) plays little or no role other than assisting such a primary interaction (Cannon *et al.*, 1993). However, since the *Pu* promoter shows a significantly high AT content between positions –45 and –86 upstream of the predicted σ^{54}-RNAP binding site (Figure 1), we considered whether the αCTD of σ^{54}-RNAP could also participate in promoter recognition. To this notion, we monitored *Pu* activity *in vivo* in conditions of overexpression of the α subunit variant α-235, in which the CTD is deleted (Hayward *et al.*, 1991). To this end, *Escherichia coli* JM109(DE3) was transformed with low-copy number plasmid pMCP2, which bears a *Pu–lacZ* fusion along with the gene for a constitutively active form of XylR named XylRΔA (Fernández *et al.*, 1995). The resulting strain was then transformed independently with compatible plasmids directing the IPTG-dependent overproduction of either the wild-type α subunit of RNAP (borne by plasmid pHTTf1–NHα) or α-235 (borne by plasmid pHTTf1–NHα1-235). Addition of IPTG to these strains is anticipated transiently to make the α subunits encoded by the plasmids take over most intracellular RNAP which otherwise would bear the wild-type subunit coded by the chromosome (Hayward *et al.*, 1991). As a control, the same strain was transformed with a *lacZ* fusion to the *PlacUV5* promoter, which is known to be independent of αCTD for activity (Igarashi and Ishihama, 1991). As shown in Figure 2, overexpression of α-235 caused a 5-fold reduction in the accumulation of β-galactosidase in the strain bearing the *Pu–lacZ* fusion as compared with the levels of the enzyme detected in the strain overproducing the wild-type α subunit. This inhibition effect was not due to a general deleterious effect of α-235 overexpression, since the *PlacUV5–lacZ* fusion borne by a similar reporter plasmid showed virtually identical patterns of β-galactosidase accumulation upon induction of both the wild-type α subunit and the α-235 variant. Intracellular levels of IHF, σ^{54} and XylRΔA remained unchanged during overexpression of the α subunits as detected with Western blot (data not shown). Therefore, the decrease in *Pu* activity appeared to be related to the rise of the intracellular pool of σ^{54}-RNAP containing α-235. Although, by themselves, these data were not conclusive, they did hint at the possibility of an involvement of αCTD in *Pu* promoter activity and

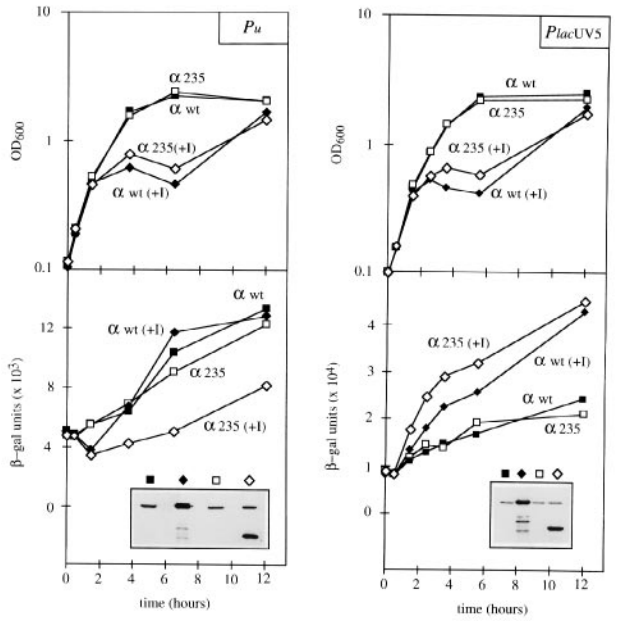

Fig. 2. Involvement of αCTD in *Pu* promoter activity *in vivo*. *E.coli* JM109(DE3) strain bearing the *Pu–lacZ* reporter plasmid pMCP2 was transformed, separately, with plasmids pHTTf1–NHα (encoding wild-type α subunit of RNAP downstream of a T7 promoter) and pHTTf1–NHα(1-235) [encoding the α(1-235) variant containing only the 235 N-terminal amino acids]. As a control, the same strain was transformed with *PlacUV5–lacZ* plasmid pJM–UV5 and the same inducible constructs encoding the wild-type or truncated α subunits. Each of the strains was grown at 30°C in LB medium (Sambrook *et al.*, 1989) with ampicillin (200 μg/ml) and streptomycin (20 μg/ml) until cultures had an OD_{600} of 0.2. IPTG (0.5 mM) was then added to each culture and the incubation continued for 12 h. Accumulation of β-galactosidase in each strain after IPTG addition is shown. Note the loss of activity of the strain bearing the *Pu–lacZ* fusion when the α(1-235) variant is overproduced. A Western blot with anti-α subunit antibodies on culture samples taken at ~4 h from the start of the growth is shown in the insert. ■, α(wt) no IPTG; ◆, α(wt) plus IPTG; □, α(1-235) no IPTG; ◇, α(1-235) plus IPTG. +I indicates samples to which IPTG was added.

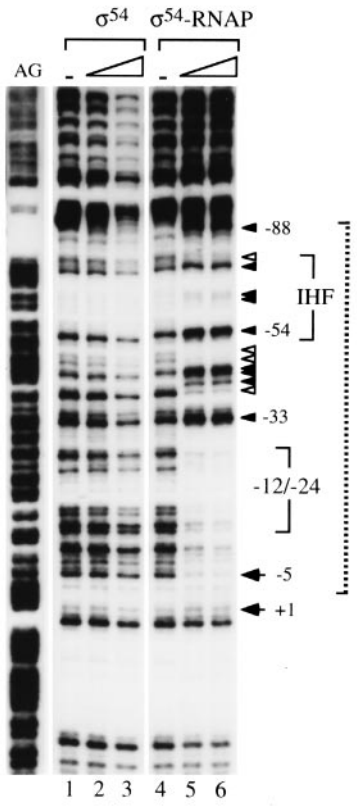

Fig. 3. DNase I footprinting of the *Pu* promoter with purified σ^{54} and σ^{54}-RNAP. The DNA template used was a 474 bp *Bam*HI–*Pvu*II fragment containing the whole *Pu* promoter and end-labelled with ^{32}P at its *Bam*HI site. The protein added to the samples were: lanes 1 and 4, controls, no protein; lane 2, 300 nM σ^{54}; lane 3, 600 nM σ^{54}; lane 5, 300 nM σ^{54} and 100 nM core RNAP; lane 6, 600 nM σ^{54} and 200 nM core RNAP. The A+G Maxam and Gilbert reaction of the same DNA fragment was used as a reference. The location of the IHF site as well as the extended region engaged in interactions with the σ^{54} polymerase (approximately –5 to –90) is indicated at the right. The nucleotides within the region upstream of the –12/–24 sequence which become protected or hypersensitive to DNase I in the presence of σ^{54}-RNAP are marked, respectively, with open or closed arrows (see Figure 1).

prompted us to examine *in vitro* the pattern of interactions of various forms of RNAP with the –12/–24 region.

σ^{54}-RNAP binding to Pu requires contacts with DNA upstream of the –12/–24 region

In order to identify the DNA sequences around the –12/–24 region of the *Pu* promoter which interact with σ^{54}-RNAP, we carried out DNase I footprinting assays on a DNA fragment bearing the entire *Pu* in the presence of either purified σ^{54} factor or the reconstituted σ^{54}-RNAP holoenzyme. As shown in Figure 3, while σ^{54} alone could not bind the *Pu* promoter, the σ^{54}-containing holoenzyme protected a region between –5 and –29 which included the –12/–24 motifs. In this respect, the *Pu* promoter behaves as other σ^{54}-dependent systems (Buck and Cannon, 1992). However, the same footprinting experiment of Figure 3 also showed DNase I-protected and DNase I-hypersensitive bases located upstream of the –12/–24 site, in a region spanning position –33 to –88 (Figures 1 and 3). The footprint of σ^{54}-RNAP in the sequences further upstream of the –12/–24 motif seems to be unique to *Pu*. DNase I protections previously reported for other σ^{54}-promoters did not generally exceed a region approximately between –5 and –33 (Popham *et al.*, 1989; Claverie-Martin and Magasanik, 1992; Cannon *et al.*, 1995; Tintut *et al.*, 1995a,b) and only in one instance (Lee *et al.*, 1993) was an interaction detected up to –46.

To ascertain the role, if any, of the upstream contacts of σ^{54}-RNAP in promoter recognition, we ran gel retardation assays with DNA fragments bearing either the *Pu* sequence up to –114 (i.e. spanning all the upstream sites revealed by DNase I footprint; Figure 3) or a shorter sequence extending only to –53 (i.e. including the –12/–24 motifs, but lacking most of the upstream contact sites). As shown in Figure 4A (lanes 1 and 2), the mixture of the *Pu*-114 fragment with σ^{54}-RNAP created a distinct slow-migrating complex. To verify that such a complex faithfully reflected the binding σ^{54}-RNAP to promoter sequences, we carried out the control experiment of Figure 4B. In this instance, we ran in parallel *Pu*-114 with the fragment named *Pu*-114/39 (Figure 1) which had been deleted for the –12/–24 sequences. The results of Figure 4B indicated that: (i) only the assembled polymerase originated a retarded band with *Pu*-114; and (ii) formation of the slow-migrating σ^{54}-RNAP–DNA complex required the presence

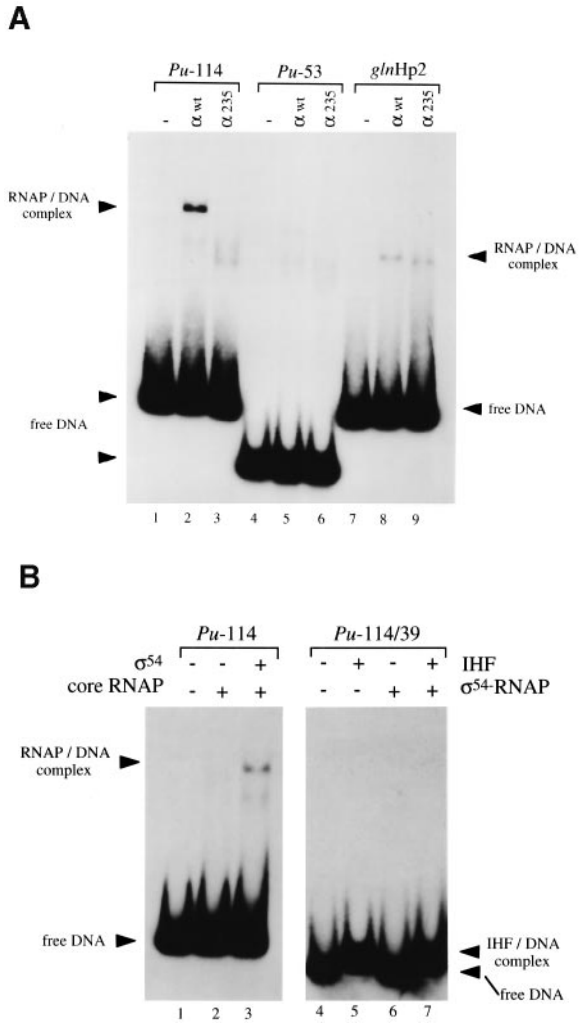

Fig. 4. Band-shift assay of complexes formed between *Pu* fragments and reconstituted σ^{54}-RNAPs. (**A**) Binding of promoter segments to polymerases bearing wild-type and truncated α subunits. Two sequences of the *Pu* promoter spanning positions –114 to +22 (*Pu*-114) or –53 to +22 (*Pu*-53) and a control DNA fragment bearing the *glnHp2* promoter were end-labelled with ^{32}P, mixed with either reconstituted wild-type σ^{54}-RNAP or σ^{54}-RNAP α(1-235) as indicated, and run in a gel retardation assay as explained in Materials and methods. The polymerases were added at a concentration of 100 nM of each of the core enzymes and 300 nM of purified σ^{54}. (**B**) Specificity of *Pu*–σ^{54}-RNAP interactions. The same *Pu* segment *Pu*-114 was run along with the fragment *Pu*-114/39, which spans promoter sequences –114 to –39 (Figure 1) and is therefore deleted of the –12/–24 motif. To this end, both labelled fragments were mixed separately as indicated with core RNAP (70 nM), with or without σ^{54} (200 nM) and IHF (100 nM) and loaded in a gel retardation assay as before. The location of the different complexes is indicated at the sides.

of the –12/–24 sequences. On this basis, we compared the interactions of σ^{54}-RNAP with *Pu*-114 and *Pu*-53. Comparison of lanes 2 and 5 in Figure 4A revealed that the loss of the region between –53 and –114 decreased by 7-fold the number of complexes between σ^{54}-RNAP and the promoter. The same happened when the –53 to –114 region was substituted by a heterologous sequence (not shown), thus suggesting that specific nucleotides upstream of –53 were required to strengthen the interaction between the σ^{54}-RNAP and the target DNA. These results indicated that the contacts of σ^{54}-RNAP with the upstream region were not a residual extension of a predominant interaction with –12/–24, but rather that they did determine binding affinity.

Pu promoter occupation is affected by the loss of the αCTD of σ^{54}-RNAP

In view of the previous results *in vivo* and *in vitro*, it became plausible that the upstream *Pu*–σ^{54}-RNAP contacts could be traced to the α subunit of the enzyme. To examine this possibility, we compared the binding ability of reconstituted σ^{54}-containing polymerases bearing either the wild-type α subunit or the truncated α-235 variant. These σ^{54}-RNAPs were employed in gel retardation assays on DNA fragments containing the entire upstream sequence (up to –114; *Pu*-114) or deleted between –53 and –114 (*Pu*-53) as before. As a control, we also assayed the band shifts caused by the reconstituted polymerases on a different σ^{54}-promoter (*gln*Hp2) known not to interact with RNAP beyond the –12/–24 motifs (Claverie-Martin and Magasanik, 1991). Figure 4 shows (lanes 2 and 3) that the ability of σ^{54}-RNAP to produce stable complexes with *Pu*-114 was far lower with the polymerase bearing the truncated αCTD than with the wild-type enzyme. This was in contrast to the equally retarded product originated by binding of either polymerase to the control promoter *gln*Hp2 (Figure 4, lanes 8 and 9). In no case was a significant interaction of the polymerases detected with promoter variant *Pu*-53 which lacks the upstream sequences (Figure 4, lanes 5 and 6). Taken together, these results suggested that the *Pu* promoter contained an upstream sequence recognized by the αCTD which involves nucleotides between –114 and –53. Since the situation is reminiscent of that of RNAP binding to some σ^{70}-promoters, we hereafter refer to this region as a UP element.

IHF binding enhances recruitment of σ^{54}-RNAP to the Pu promoter

The region of *Pu* involved in the interaction with αCTD overlaps the IHF site present in the promoter (Figure 1). This fact suggested to us that IHF binding and σ^{54}-RNAP recruitment could influence each other either positively or negatively. To address this issue, we incubated labelled *Pu*-114 DNA with increasing amounts of σ^{54}-RNAP in the presence or absence of subsaturating concentrations of purified IHF protein, and analysed the resulting complexes in band shift assays. As shown in Figure 5A, IHF stimulated the formation of low-mobility DNA–protein complexes containing σ^{54}-RNAP at least 7-fold. The specific effect of IHF on σ^{54}-RNAP binding to *Pu* could be also monitored by DNase I footprinting. The gel of Figure 5B shows that at a σ^{54}-RNAP concentration that barely protected the –12/–24 region, IHF addition strongly enhanced the distinct protection of σ^{54}-RNAP throughout the downstream –5/–29 region. Some of the DNase I-protected or hyper-reactive bands caused by higher concentrations of σ^{54}-RNAP on the upstream region which were detected in the absence of IHF (Figure 3) could also be observed, although many were hindered by the strong protection of IHF on its binding site (Figure 5B).

IHF binding enhances the interactions between αCTD and the UP element

In order to distinguish whether IHF-mediated recruitment of σ^{54}-RNAP to *Pu* was due to new interactions of IHF with the polymerase, or resulted from the improvement of already existing contacts with upstream DNA, we generated a new DNA fragment which kept the IHF binding site intact (i.e. extended up to –79; *Pu*-79) but lacked any further upstream sequence. This fragment was incubated with increasing concentrations of σ^{54}-RNAP in the presence or absence of IHF and was submitted to a gel retardation assay as before. As controls, we employed *Pu*-114 and *Pu*-53 DNA. The gels (Figure 6) clearly indicated that promoter sequences perfectly able to bind IHF fail to stimulate recruitment of σ^{54}-RNAP to –12/–24 if they do not also bear the 5′ region adjacent to the IHF site. The consequence of this experiment is that IHF binding to *Pu* does not enhance *per se* the recruitment of σ^{54}-RNAP, but rather facilitates contacts of the polymerase with upstream sequences.

In view of the preceding results, we set out to ascertain whether the IHF-mediated enhancement of recruitment of σ^{54}-RNAP could be traced to improved interactions of the αCTD with the UP-like element described above, or to different interactions of other domains of the polymerase with DNA further upstream. The latter situation could originate from productive contacts between the DNA and the back side of RNAP, as has been described in other systems (Dethiollaz *et al.*, 1996). To clarify this issue, we carried out the experiment shown in Figure 7. In this case, gel retardation assays were run with labelled *Pu*-114 DNA and σ^{54}-RNAPs reconstituted with either the wild-type α subunit or the α-235 variant. The results of this assay were that IHF fails to stimulate binding of σ^{54}-RNAP (α-235) to the promoter. This suggested that IHF strengthens the interaction of the αCTD with the UP element, and that the effect is mediated by changes in the DNA of the region brought about by IHF binding and bending of its target site in *Pu.*

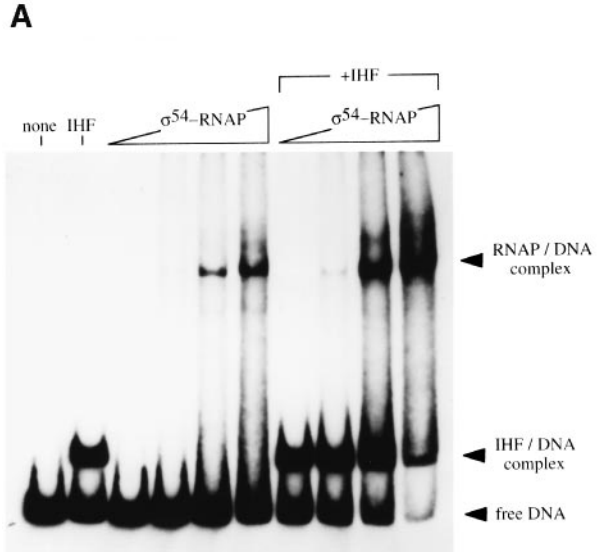

Fig. 5. Effect of IHF on recruitment of σ^{54}-RNAP to *Pu.* (**A**) Band-shift assays. An end-labelled DNA fragment of *Pu* promoter spanning positions –114 to +22 (as in Figure 4) was mixed with the proteins indicated in each case and run in a non-denaturing gel under the same conditions as described in Figure 4. The concentration of IHF was 100 nM. Increasing concentrations of RNAP contained 20, 60, 120 and 200 nM of the core enzyme mixed, in all cases, with a 3-fold molar excess of purified σ^{54}. The location and nature of the migrating complexes is indicated at the right. (**B**) DNase I footprinting. The same end-labelled fragment as Figure 3 was subjected to nicking with the nuclease in the presence of the proteins indicated at the top, using conditions as in Figure 3. IHF was added at 100 nM. Purified core RNAP and σ^{54} concentrations in the samples indicated were, respectively, 15 nM and 50 nM. The location of relevant sequences is indicated at the left, using as a reference the Maxam and Gilbert A+G reaction.

Discussion

Unlike σ^{70}-RNAP, the polymerase bearing σ^{54} is unable to isomerize to an open complex because of the inhibition caused by σ^{54} itself (Wang *et al.*, 1995). This inhibition is only overcome by the enhancer-binding proteins which act in concert with σ^{54}-RNAP (Wang *et al.*, 1995, 1997; Wang and Gralla, 1996). The molecular target of enhancer-binding proteins appears to be σ^{54} itself and the rest of the polymerase may not be limiting for open complex formation (Lee *et al.*, 1993). This is in contrast with the σ^{70}-RNAP, in which not only the σ subunit interacts with activators (Makino *et al.*, 1993; Kuldell and Hochschild, 1994; Li *et al.*, 1994) but also α and β′ subunits are targets for transcriptional activation (Ishihama, 1993; Ebright and Busby, 1995; Busby and Ebright, 1997; Miller *et al.*, 1997). In this respect, although the αCTD of σ^{54}-RNAP is dispensable for open complex formation (Lee *et al.*, 1993), this domain is still present in the holoenzyme and

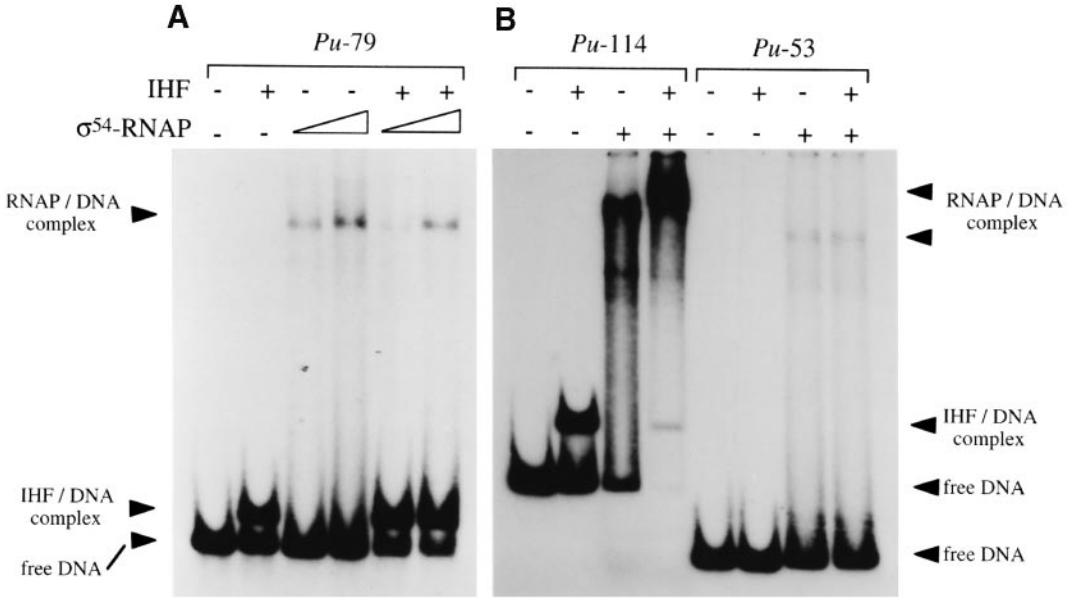

Fig. 6. Mapping upstream contacts of σ^{54}-RNAP within *Pu* with band-shift assays. The binding mixtures included the end-labelled DNA segments and the proteins indicated in each case. (**A**) DNA fragment spanning positions –79 to +22 (*Pu*-79; intact IHF site and –12/–24 region, but lacking the sequence upstream of IHF). The IHF concentration was 100 nM. RNAP was added to 120 or 200 nM core polymerase with a 3-fold molar excess of purified σ^{54}. (**B**) DNA fragments spanning positions –114 to +22 (*Pu*-114; control, IHF site with adjacent 5′ sequence and –12/–24 region) and –53 to +22 (*Pu*-53; IHF site deleted, only downstream –12/–24 region). The IHF concentration was 100 nM. RNAP was used at 200 nM core enzyme with a 3-fold molar excess of purified σ^{54}. The location of the migrating complexes is indicated at the sides of the gels.

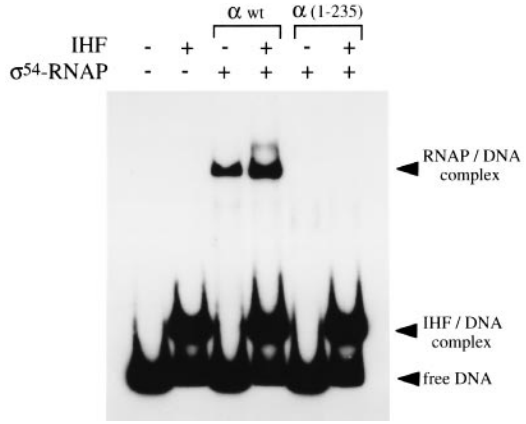

Fig. 7. IHF does not stimulate recruitment of σ^{54}-RNAP lacking the αCTD. *Pu*-114 DNA, bearing positions –114 to +22 of *Pu*, was mixed with either reconstituted wild-type σ^{54}-RNAP or σ^{54}-RNAP α(1-235) in the presence or absence of IHF as indicated, and run in a gel retardation assay. The polymerases were added at a concentration of 100 nM of each of the core enzymes and 300 nM of purified σ^{54}. The IHF concentration was 100 nM The location of the different complexes is indicated at the right of the gel.

does not necessarily lose its ability to bind upstream DNA, or to interact with other proteins for increasing the initial equilibrium constant between RNAP and promoter DNA. In this study, we show that this is precisely the case in the *Pu* promoter of *P.putida*. In systems containing a suboptimal –12/–24 region, the interactions between αCTD and upstream DNA may constitute a second recognition element for σ^{54}-RNAP, reminiscent of the UP elements of σ^{70} promoters, which strengthens the binding of the polymerase to the primary site. In fact, such a binding may become limiting in some promoters, since recruitment of the σ^{54}-RNAP is a prerequisite for further transcriptional activity.

The distance between functional sequences in σ^{54} promoters makes them particularly dependent on the overall geometry of the DNA segment encompassing the enhancer and the RNAP-binding site (Gralla and Collado-Vides, 1996; Geiselmann, 1997; Pérez-Martín and de Lorenzo, 1997). The looping out of the intervening region is thus a key event in the mechanism of activation of this class of promoters. Since IHF can assist such a bending, but does not influence directly open complex formation, its role in σ^{54}-dependent systems has been considered that of a co-regulator (Pérez-Martín and de Lorenzo, 1997). In this context, we have shown previously that DNA bending caused by IHF binding to *Pu* is required for full promoter activity *in vivo* (Pérez-Martín *et al.*, 1994) and *in vitro* (Pérez-Martín and de Lorenzo, 1996a). In addition to these structural effects, the results presented in this paper show that IHF binding to *Pu* strongly favours the recruitment of σ^{54}-RNAP. All our data indicate consistently that IHF binding strengthens the interaction of αCTD with a DNA region which extends upstream of the IHF site. This conclusion is justified as follows: (i) IHF does not enhance recruitment of σ^{54}-RNAP to *Pu* in the absence of the region –79 to –114; (ii) the lack of αCTD abolishes all effects of IHF in binding of the polymerase to DNA; (iii) the footprint of σ^{54}-RNAP on the sequence upstream of –12/–24, as revealed with DNase I, overlaps the IHF site but includes also the adjacent 5′ DNA; and (iv) no evidence was found of protein–protein interactions between IHF and σ^{54}-RNAP.

Our results reveal a phenomenon by which the DNA geometry induced by IHF binding enhances the recruitment of σ^{54}-RNAP to the *Pu* promoter (Figure 8). Within this scheme, the distance between the –12/–24 site and the UP element might disfavour simultaneous binding to σ^{54}-RNAP. An IHF-induced bending could, however, bring the two elements into a proximity. The resulting promoter

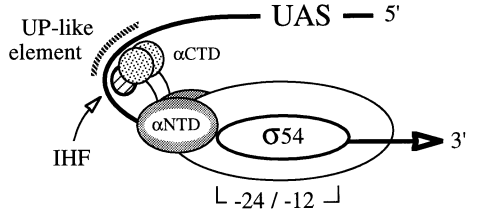

Fig. 8. Model of IHF-assisted docking of the σ^{54}-RNAP in the *Pu* promoter. The scheme summarizes a plausible picture of how IHF can assist the interaction of σ^{54}-RNAP holoenzyme with its target sequences at –12/–24. The main feature is that the αCTD of the polymerase interacts with an upstream sequence (a UP-like element) which partially overlaps the IHF site and extends further upstream. The promoter geometry caused by IHF binding to DNA and the ensuing bending may favour the proximity of the UP-like element to αCTD and perhaps also the strength of the contacts. This is independent of the architectural effect of IHF in bringing the upstream activating sequences (UAS) bound to XylR into contact with the polymerase. The shape and volume of the different proteins are symbolic.

conformation could stabilize αCTD and σ^{54} interactions with the UP element and –12/–24 site, respectively. In addition, local distortions in DNA structure caused by IHF could in itself improve αCTD binding to the UP-like element. Interestingly, IHF produces a sharp bend of the helix axis resulting in the widening of the major DNA groove, which remains accessible for interaction with other proteins (Rice *et al.*, 1996).

IHF-mediated stimulation of transcription has been substantiated in a number of σ^{70}-promoters, including *pL* of bacteriophage λ (Giladi *et al.*, 1992, 1996), the *Pe* promoter of Mu (van Ulsen *et al.*, 1996, 1997) and the P_G2 promoter of the *ilv* operon of *E.coli* (Pagel *et al.*, 1992; Parekh and Hatfield, 1996). In contrast, the role of IHF in stimulating the binding of RNAP to a σ^{54}-promoter is a case which, to our knowledge, has not been reported before. Instead, IHF was previously shown only to assist the firing contact between the activator and σ^{54}-RNAP (Hoover *et al.*, 1990; Claverie-Martin and Magasanik, 1991; Santero *et al.*, 1992). In view of the new data reported here, it seems that IHF not only facilitates the contacts of XylR with σ^{54}-RNAP in the *Pu* promoter, but it is also able to stimulate RNAP binding. We speculate that such dependency on IHF for RNAP binding may not be peculiar only to the *Pu* promoter and might represent a transcriptional bottleneck in other σ^{54}- and IHF-dependent promoters having suboptimal –12/–24 sites.

Materials and methods

Bacterial strains, plasmids and general procedures

Escherichia coli JM109(DE3) is a *lacZ*-minus strain which expresses bacteriophage T7 RNA polymerase upon induction with IPTG as described by Studier *et al.* (1990). Plasmids pHTTf1–NHα and pHTTf1–NHα(1-235) encode, respectively, the wild-type α subunit of RNAP and the α(1-235) variant (Tang *et al.*, 1995). Both α and α(1-235) are expressed in these plasmids through a T7 gene 10 promoter as fusions to an N-terminal hexahistidine motif. pMCP2 is a low-copy number plasmid selectable with streptomycin which bears a reporter cassette (named MAD2) containing a transcriptional *Pu–lacZ* and the gene encoding a constitutive variant of the XylR protein (named XylRΔA) under the control of its native promoter (Fernández *et al.*, 1995). pJM–UV5 is an equivalent construct carrying a *PlacUV5–lacZ* transcriptional fusion. The *PlacUV5* promoter present in this plasmid was obtained from strain *E.coli* JM109(DE3) by amplifying the DNA segment between positions +12 and –117 of this promoter with the polymerase chain reaction (PCR) using synthetic primers 5′-CGGAATTC(*Eco*RI)GACA-GGTTTCCCGACTGGAAAG-3′ and 5′-GCGGATCC(*Bam*HI)GCT-CACAATTCCACACATTATA-3′. The resulting DNA product was cloned as an *Eco*RI–*Bam*HI fragment in the corresponding sites of the *lacZ* vector pUJ8 (de Lorenzo *et al.*, 1990) and the whole fusion transferred to the low-copy number vector as before. All cloned inserts and DNA fragments were verified before use by automated DNA sequencing in an Applied Biosystems device. Recombinant DNA manipulations were carried out according to published protocols (Sambrook *et al.*, 1989).

Proteins and protein techniques

Accumulation of β-galactosidase raised by *lacZ* fusions was measured in *E.coli* cells permeabilized with chloroform and sodium dodecyl sulphate (SDS) as described by Miller (1972) under the conditions specified in each case. Purified σ^{54} factor and native core RNAP were the kind gift of B.Magasanik; IHF protein was a gift from A.Oppenheim. Reconstitution of wild-type RNAP and its variant bearing the deletion α(1-235) in its α subunit core RNAP from separate subunits was carried out as described previously (Igarashi and Ishihama, 1991). The presence of the wild-type α and α(1-235) proteins, σ^{54}, IHF and XylRΔA was detected in crude extracts by Western blot with the corresponding pre-adsorbed polyclonal rabbit antibodies as described by Fernández *et al.* (1995).

Preparation of DNA fragments containing Pu promoter variants

DNA fragments with different portions of the *Pu* promoter were prepared as inserts in vector pUC18. Plasmid pEZ9 (de Lorenzo *et al.*, 1991) contains the entire *Pu* promoter sequence as an *Eco*RI–*Bam*HI insert in pUC18 spanning positions –208 to + 93 (Figure 1). The DNA fragment used in the experiments of Figures 3 and 5B was excised from pEZ9 as a *Bam*HI–*Pvu*II segment and end-labelled at its *Bam*HI site by in-filling the overhanging ends as described below. *Pu* promoter sequences –114 to +93 and –53 to +93 were separately amplified from pEZ9 with direct primers 5′-GAAGGCCT(*Stu*I)GCTGTAGATTTTCTCTCATAC-3′ and 5′-GAAGGCCT(*Stu*I)GAAATAAGGGGATCGGTATAA3′ and the reverse universal primer 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ for both. The amplified products were digested with *Stu*I and *Bam*HI and cloned in pUC18 digested with *Sma*I and *Bam*HI, thereby originating plasmids pUC–IHF2 and pUC–d2, respectively. DNA fragments used in the experiments of Figures 4, 5A, 6 and 7 were excised from pUC–IHF2 and pUC–d2 as *Eco*RI–*Bst*EII fragments (the *Bst*EII site maps in nucleotide +22 of the *Pu* promoter sequence; Figure 1) and end-labelled at both ends as described below. The *Pu* promoter fragment *Pu*-114/39 (spanning positions –114 to –39, i.e. lacking –12/–24 sequences; Figure 1) was excised from pUC–IHF2 as an *Eco*RI–*Sau*3A segment, taking advantage of the site present in position –39 of the *Pu* promoter sequence. For preparation of additional DNA fragments, a *Cla*I site was engineered within nucleotides –79 to –84 of the *Pu* sequences of pEZ9. To this end, two DNA segments of *Pu* were amplified separately from this plasmid using, in one case, the direct universal primer 5′-AGCGGATAACAATT-TCACACAGGA-3′ and 5′-CCATCGAT (*Cla*I)CCGGGGGGGTATGA-GAGAAAA-3′ and, in the other case, the reverse universal primer 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ and 5′-CCATCGAT(*Cla*I)T-TACAAAG AAAATCAATAATT-3′. The amplified fragments were digested, respectively with *Cla*I–*Eco*RI and *Cla*I–*Bam*HI, mixed with pUC18 cleaved with *Eco*RI–*Bam*HI and allowed to react in a tripartite ligation. The resulting plasmid (named pUC–PuCLA-79) was transformed in the *dam*$^-$ *dcm*$^-$. *Escherichia coli* strain GM48 (Yanisch-Perron *et al.*, 1985), purified and digested with *Cla*I and *Bst*EII to prepare the DNA segment used in Figure 6. A DNA fragment spanning the *glnHp2* promoter of *E.coli* was obtained by PCR of chromosomal DNA with primers 5′-GGAATTC(*Eco*RI)CTGTGTTGAGTGCACAATTTTA-GCGC-3′ and 5′-CGGATCC(*Bam*HI) ATTCACATATATGAAAAAAT-CGTGCC-3′. This amplified segment was cloned as an *Eco*RI–*Bam*HI fragment in pUC18 giving rise to plasmid pUC18–glnHp2. This plasmid was digested with *Eco*RI and *Bam*HI to produce the fragment which, once labelled, was used in one of the samples of the experiment shown in Figure 4.

DNA binding assays

For gel retardation assays, restriction fragments spanning portions of the *Pu* promoter described above were end-labelled by in-filling the overhanging ends left by restriction enzymes with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase. Radioactive nucleotides not

incorporated to DNA were removed by centrifuging briefly through small Sephadex G-25 columns. Binding reactions were performed in a total volume of 25 µl transcription buffer containing 35 mM Tris–acetate, 70 mM KAc, 5 mM $MgAc_2$, 20 mM NH_4Ac, 2 mM $CaCl_2$, 1 mM DTT, 3% glycerol and 40 µg/ml of poly[d(I–C)]. Labelled fragments, added to the buffer at a final concentration of 5 nM, were incubated with 100 nM IHF, wild-type core RNAP or α(1-235) core RNAP (at concentrations indicated in each case) and a 3-fold molar excess of σ^{54} factor for 25 min at 30°C. The entire reaction volume was loaded into non-denaturing 4% polyacrylamide gels (acrylamide:bis ratio, 80:1) in 0.5× TBE buffer (45 mM Tris–borate pH 8.3, 0.1 mM EDTA, 5 mM $MgCl_2$), electrophoresed at 12 mA at 4°C for 6 h, dried and autoradiographed. Relative band intensities were quantitated from exposed X-ray films with Molecular Analyst software (Bio-Rad). DNase I footprinting assays were performed in a total volume of 50 µl of transcription buffer (as above) and with the same concentrations of end-labelled fragments and proteins used in the gel mobility-shift assays. After preincubation of the binding reactions for 25 min at 30°C, 3 ng of DNase I were added to each sample and further incubated for 3.5 min. Reactions were stopped by addition of 25 µl of STOP buffer containing 0.1 M EDTA pH 8, 0.8% SDS, 1.6 M NH_4Ac and 300 µg/ml sonicated salmon sperm DNA. Nucleic acids were precipitated with 175 µl of ethanol, lyophilized and directly resuspended in denaturing loading buffer (7 M urea, 0.025% bromophenol blue, 0.025% xylene cyanol in 20 mM Tris–HCl pH 8) prior to loading on a 7% DNA sequencing gel. A+G Maxam and Gilbert reactions (Maxam and Gilbert, 1980) were carried out with the same fragments and loaded in the gels along with the footprinting samples.

Acknowledgements

The authors are indebted to R.Ebright, W.Niu, M.Carmona, F.Claverie-Martín, B.Magasanik, A.Oppenheim and H.Nash for the kind gifts of valuable materials used in this work. F.Rojo, I.Cases and J.Pérez-Martín are gratefully acknowledged for inspiring discussions. This work was funded by the ENV4-CT95-0141 and BIO4-CT97-2040. Contracts of the EU and by Grant BIO95-0788 of the Comisión Interministerial de Ciencia y Tecnología. G.B. was the recipient of a Fellowship of the Spanish Ministry of Education and Science for foreign PhD visitors.

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Received May 20, 1998; revised and accepted July 9, 1998