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Recruitment of RNA Polymerase Is a Rate-limiting Step for the Activation of the σ^{54} Promoter *Pu* of *Pseudomonas putida**

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The activity of the σ^{54}-promoter *Pu* of *Pseudomonas putida* was examined *in vitro* with a DNA template lacking upstream activating sequences, such that RNA polymerase can be activated by the enhancer-binding protein XylR only from solution. Although the transcription activation pathway in this system lacked the step of integration host factor (IHF)-mediated looping of the XylR·DNA complex toward the prebound RNA polymerase, IHF still stimulated promoter activity. The positive effect of IHF became evident not only with XylR from solution, but also with other σ^{54}-dependent activators such as NtrC and NifA. Furthermore, an equivalent outcome was shown for the nonspecific DNA-binding protein HU. This stimulation of transcription in the absence of the enhancer was traced to the recruitment of RNA polymerase (*i.e.* increased efficiency of formation of closed complexes) brought about by IHF or HU binding. Thus, under limiting concentrations of the polymerase, the factor-mediated binding of the enzyme to *Pu* seems to enter a kinetic checkpoint in the system that prevents the XylR-mediated formation of an open complex.

Transcription initiation is a sequential multistep process involving promoter DNA recognition by RNA polymerase (RNAP),[1] formation of an initiation-competent RNAP·DNA complex, formation of initial phosphodiester bonds, and escape of RNAP from the initial binding site to elongation (1, 2). From a kinetic point of view, the overall rate of transcription initiation of a given promoter depends on the slowest phase in the process, so that favoring one nonlimiting step does not result in an increase of the total transcription rate (1, 3). Transcriptional activators generally act on these limiting steps to increase promoter output (for review, see Ref. 3). This rule is generally true for the prokaryotic RNAP containing the major sigma factor σ^{70} (σ^{70}-RNAP). Because positively regulated σ^{70} promoters generally fail to form stable closed complexes (4), activator-mediated binding of σ^{70}-RNAP to cognate promoters is often a limiting step, which, similarly to the eukaryotic counterpart (4, 5), is subjected to regulation.

The one exception to this rule is the group of promoters transcribed by the RNA polymerase containing the alternative factor σ^{54} (σ^{54}-RNAP). In this case, the enzyme is believed to form a stable closed complex with the target DNA sequences at −12 and −24 sites (6, 7). On the contrary, isomerization to an open complex is strongly stimulated by the action of cognate regulators, generically known as prokaryotic enhancer-binding proteins (8), that bind to upstream activating sequences (UASs) located at >100 bp from the σ^{54}-RNAP binding site (6). Interactions between σ^{54}-RNAP bound to the −12/−24 region and the regulatory protein associated with the UAS are often facilitated by the bending of the intervening DNA by the integration host factor (IHF). IHF is believed to assist the looping out of the region between the RNAP and the activator, thus increasing the overall rate of transcription initiation (9–13).

Although these notions might be true for most σ^{54}-dependent promoters, we have recently shown that the *Pu* promoter of the TOL plasmid of *Pseudomonas putida* (Fig. 1) can barely form a closed complex with its target DNA sequences (14). In this case, the strict dependence of *Pu* activity on IHF *in vivo* (15) and *in vitro* (16) seems to reflect not only the productive geometry of the region brought about by IHF binding but also a more efficient formation of close complexes of σ^{54}-RNAP with the promoter. Such an IHF-mediated "recruitment" of σ^{54}-RNAP seems to involve the interaction of an otherwise distant *cis*-element with the C-terminal domain of the α subunit of σ^{54}-RNAP (14). This nonanticipated role of IHF was observed in the absence of XylR, the activator of the system, so that the actual effect of IHF-mediated recruitment of σ^{54}-RNAP to *Pu* on transcription was not substantiated. In this work, we have sought to ascertain this issue by using an *in vitro* system in which *Pu* is activated by XylR from solution rather than from the UAS. Our data suggest that σ^{54}-RNAP binding is a rate-limiting step in the process of transcription initiation at the *Pu* promoter.

EXPERIMENTAL PROCEDURES

Plasmids and General Procedures—All plasmids used in the transcription assays are derived from vector pTE103, which adds a strong T7 terminator downstream of the promoters under study (17). The plasmid called pEZ10 carries the entire region between coordinates −208 and +93 of the *Pu* sequence, inserted as an *Eco*RI-*Bam*HI fragment in pTE103. Plasmid pEZ20 carries the variant named *Pu* ΔUAS inserted in the same vector as a 207-bp *Eco*RI-*Bam*HI fragment excised from plasmid pUC-IHF2 (14), which spans the region −114 to +93 of *Pu*. Similarly, a 122-bp fragment from plasmid pUC-d2 (14), containing the region −53 to +93 of *Pu*, was cloned in pTE103 to yield plasmid pEZ30, which bears the *Pu* ΔUAS ΔIHF promoter variant. All cloned inserts and DNA fragments were verified through automated DNA sequencing in an Applied Biosystems device. All the supercoiled DNA templates used for *in vitro* transcription were purified with the Qiagen

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[1] The abbreviations used are: RNAP, RNA polymerase; UAS, upstream activating sequence; IHF, integration host factor; bp, base pair.

system. Other recombinant DNA manipulations were carried out as described previously (18).

Proteins and Protein Techniques—Purified factor σ^{54}, NtrC, NtrB, and native core RNAP from *Escherichia coli* were the kind gift of B. Magasanik. NifA, IHF, and HU proteins were obtained from M. Buck, H. Nash, and T. Baker, respectively. The XylR variant called XylRΔA is identical to the wild-type protein except for the deletion of its N-terminal module (called the A domain). This variant is fully constitutive and can thus activate transcription from *Pu* in the absence of any aromatic inducer (16, 19). XylRΔA was purified to apparent homogeneity by metalloaffinity of the His-tagged protein (16).

In Vitro Transcription Assays—Single-round transcription assays were performed as described before (20). Supercoiled DNA templates were used at 5 nM concentration. 50-μl reactions were set up at 37 °C in a buffer of 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM bovine serum albumin, 10 mM dithiothreitol, and 1 mM EDTA. Unless indicated otherwise, each DNA template was premixed with 25 nM core RNAP, 100 nM σ^{54}, 25 nM IHF or 75 nM HU, and the concentrations of XylRΔA, NtrB·NtrC, and NifA indicated in each case. Linear DNA templates were generated by digesting the corresponding plasmids (pEZ10, pEZ20, and pEZ30; Fig. 1) with *Eco*RI, and they were used at the same concentration and conditions as the supercoiled counterparts. The DNA templates and the proteins were incubated at 37 °C with 4 mM ATP for 20 min to allow open complex formation. A single cycle of transcription was then initiated by adding a mixture of ATP, CTP, GTP (400 μM each), UTP (50 μM), [α-^{32}P]UTP (5 μCi at 3000/mmol), and heparin (0.1 mg/ml), the latter to prevent reinitiation. After incubating 10 min at 37 °C, the reactions were stopped with an equal volume of a solution containing 50 mM EDTA, 350 mM NaCl, and 0.5 mg/ml carrier tRNA. The mRNA extracted and precipitated with ethanol was electrophoresed on a denaturing 7 M urea, 4% acrylamide gel and visualized by autoradiography.

DNase I Footprinting Techniques—DNA-protein interactions were monitored with DNase I footprinting assays performed in a total volume of 50 μl of a buffer consisting of 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 poly[d(I·C)]. The DNA template used was a 474-bp *Bam*HI-*Pvu*II fragment excised from plasmid pEZ9 (11), which contains the entire *Pu* promoter sequence as an *Eco*RI-*Bam*HI insert in pUC18 spanning positions −208 to + 93 (Fig. 1). The fragment was end-labeled in its *Bam*HI site by filling in the overhanging end with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase. Radioactive nucleotides not incorporated to DNA were removed after a brief spin through small Sephadex G-25 columns. After preincubating the end-labeled fragment (5 nM) for 25 min at 30 °C with the proteins indicated in each case, 3 ng of DNase I were added to each sample and further incubated for 3.5 min. Reactions were halted 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% bromphenol blue, and 0.025% xylene cyanol in 20 mM Tris, pH 8) before loading on a 7% DNA sequencing gel. A+G Maxam and Gilbert reactions (21) were carried out with the same fragments and loaded in the gels along with the footprinting samples.

RESULTS AND DISCUSSION

Rationale for Separating Structural Effects of IHF from Recruitment of the σ^{54}-RNAP in the Pu Promoter—IHF protein has been shown to produce two effects on the *Pu* promoter. On one hand, it provides a structural aid to bring about contacts between the upstream UAS·XylR complex and the σ^{54}-RNAP bound to −12/−24 (11, 13). On the other hand, it augments the affinity of σ^{54}-RNAP for the promoter (14). As a consequence, the observed stimulatory effect of IHF in *Pu* activity (11, 13) should originate from both the optimization of promoter geometry and the increased efficiency of formation of closed complexes. To separate these two effects, we produced a variant of the *Pu* promoter in which UAS DNA was deleted up to the −114 site (*Pu*-114; Fig. 1). Transcription from such a promoter is predicted to miss the step of looping out of the intervening sequence and to rely only on the direct contact between the activator from solution and the σ^{54}-RNAP bound to the −12/−24 site. Thus, we set out to compare *Pu*-114 activation both in the absence and in the presence of IHF in single-round transcription assays with either the intact promoter region (*Pu*) or a *Pu* variant deleted of both the UAS and the IHF site (*Pu*-53). To avoid the addition of an aromatic inducer (*e.g.* toluene) to the *in vitro* assays, these templates were added with XylRΔA, a constitutively active form of XylR that is deleted of its N-terminal module (the so-called A domain; Ref. 16). We also predicted that XylRΔA could activate transcription from the templates deleted of UAS at a higher protein concentration than full-length *Pu*, as has been observed for σ^{54}-RNAP activation from solution in other σ^{54}-dependent regulators (12, 22–25). Under these conditions, any effect of IHF in transcription must reflect exclusively the efficiency of formation of closed complexes, because any geometrical effect to bring about XylR-σ^{54}-RNA contacts is ruled out.

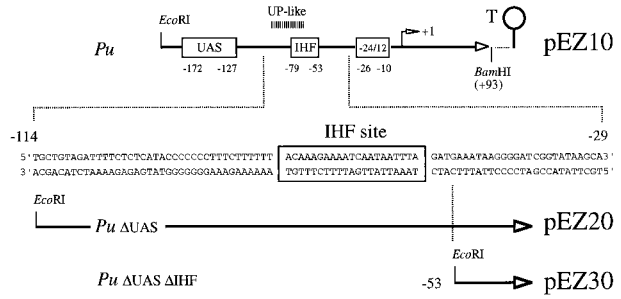

FIG. 1. **Organization of the *Pu* promoter of the TOL plasmid.** The *scheme* at the *top* shows the distribution of the functional cis-elements of the wild-type *Pu* segment (coordinates −208 to +93) included in plasmid pEZ10 with respect to the transcription start site. These include the sequence recognized by σ^{54}-RNAP (−12/−24 motif), the binding site for the IHF, and the UASs, which are the targets of the activator of the system, XylR. The location of an UP-like sequence overlapping part of the IHF site and extending further upstream (14) is also indicated. In addition, the vector pTE103 places a T7 terminator (*T*) downstream of the promoter, so transcripts originated at *Pu* and its derivatives are 394 nucleotides in size. The *bottom schemes* show the *Pu* variants inserted also in pTE103 and used in this study as transcription templates along with the names of the corresponding plasmids. Their inserts span positions −114 to +22 (*Pu* ΔUAS) and −53 to +22 (*Pu* ΔUAS ΔIHF), respectively. The sequence around the IHF site (−114 to −53) is shown for reference.

IHF Stimulates Activation of σ^{54}-RNA by XylRΔA from Solution—To ascertain whether the increased binding of σ^{54} RNA to *Pu* caused by IHF (14) was in fact translated into a higher transcriptional rate, we ran *in vitro* assays with supercoiled plasmids bearing wild type *Pu*, *Pu* ΔUAS (*Pu*-114), or *Pu* ΔUAS ΔIHF (*Pu*-53). These templates were incubated with subsaturating concentrations of σ^{54}-RNAP and IHF, along with XylRΔA, the latter in a 10-fold excess when using templates devoid of the UAS. As expected (16), transcription in any of the conditions tested was absolutely dependent on the presence of the XylRΔA protein (data not shown), a common feature of all σ^{54}-dependent activators known so far (6, 7). Because assays were carried out in the presence of heparin to prevent reinitiation, the transcripts originated from single rounds, and their levels were proportional to the amount of the open complexes formed under different conditions. As shown in Fig. 2*A*, *Pu* ΔUAS could be efficiently transcribed in the presence of XylRΔA (16) by simply increasing approximately 10-fold the amount of the activator added to the assays compared with the wild-type *Pu* template. In addition, it became evident that IHF maintained a strong stimulatory effect on transcription of *Pu* ΔUAS, not unlike that observed with the complete *Pu* promoter. This effect was entirely dependent on IHF bound to its site within the −29/−114 region, as indicated by the control experiment with the *Pu* ΔUAS ΔIHF template, which lacked any stimulation by the factor (Fig. 2*A*). The *Pu* ΔUAS ΔIHF DNA was, in fact, a poor template for transcription, most likely because of the loss of the UP-like element, which overlaps the IHF-binding sequence (Ref. 14 and Fig. 1). That the increased

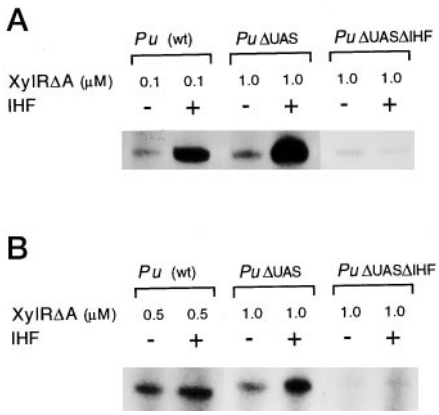

FIG. 2. **Effect of IHF addition in transcription of *Pu* promoter variants lacking upstream sequences.** *A*, Supercoiled DNA templates. Single-round transcription reactions containing 5 nM supercoiled plasmids pEZ10, pEZ20, and pEZ30 (bearing the promoter variants indicated) were assembled with 25 nM core RNAP, 100 nM σ^{54}, and, where indicated (+), 25 nM IHF as well. Purified XylRΔA was entered in the reactions at a concentration of 100 nM for the wild-type *Pu* template (*Pu (wt)*) containing the UAS and in a 10-fold excess (1.0 μM) for those lacking the upstream region (*Pu ΔUAS* and *Pu ΔUAS ΔIHF*). Samples were processed as explained in under "Experimental Procedures." Note the effect of IHF addition in *Pu* and *Pu* ΔUAS, and the lack of any significant activity of *Pu* ΔUAS ΔIHF. *B*, Linear DNA templates. Transcription reactions were set up and run identically as before but using as templates pEZ10, pEZ20, and pEZ30 linearized upon digestion with *Eco*RI. This cleaved the plasmids at sites −208, −114, and −53, respectively, and thus entirely deleted the upstream DNA sequences. The concentration of XylRΔA was increased to 0.5 μM in the control assay with wild type *Pu* to compensate for the loss of affinity of the regulator for relaxed UAS DNA (16). Under these conditions, the effect of IHF on wild-type *Pu* was less pronounced than with the supercoiled counterpart. No transcripts were detected in the absence of XylRΔA in any of the conditions tested (data not shown).

activation of *Pu*ΔUAS with IHF was not caused by nonspecific binding of XylRΔA to DNA upstream of the −114 site in the supercoiled template (Fig. 1) was verified by the experiment shown in Fig. 2*B*. In this case, linear templates entirely deleted of any sequence upstream of −208 (wild-type *Pu*), −114 (*Pu* ΔUAS), or −53 (*Pu* ΔUAS ΔIHF) were passed through the same transcription assays than the supercoiled counterparts. The data of Fig. 2*B* show that although *Pu* ΔUAS could be stimulated by IHF, the *Pu* ΔUAS ΔIHF template could not. Although the ability of XylRΔA to activate *Pu* from solution is reminiscent of that observed in NtrC (12) and NifA (22); such an activation was prevented by the lack of IHF or deletion of the binding site for the factor. The data of Fig. 2 thus strongly suggested that the interaction of σ^{54}-RNAP with *Pu* limited transcription initiation and that the previously described IHF-mediated recruitment of σ^{54}-RNAP (14) could relieve this limitation.

IHF Facilitates Activation of Pu by Other Enhancer-binding Proteins—To ensure that the stimulatory effect of IHF on *Pu* activation from solution was not restricted only to XylRΔA, we also assayed two proteins of the family of enhancer binding factors, NtrC and NifA (26, 8), known to activate, respectively, the *glnHp2* and *PnifH* promoters from solution (12, 22). Because the wild-type *Pu* does not have binding sites for NtrC or NifA, the assays were made using the complete promoter rather than the version lacking the UAS (27). To this end, purified NtrC and NifA were mixed separately with the *Pu* template and added or not with IHF before running single-round transcription assays. The reaction with NtrC was amended with purified NtrB protein, which is needed for the activation of NtrC by phosphorylation (28). It was also required to add twice as much of NtrC and NifA to the assays than it was of XylRΔA, perhaps reflecting some difference in the intrinsic

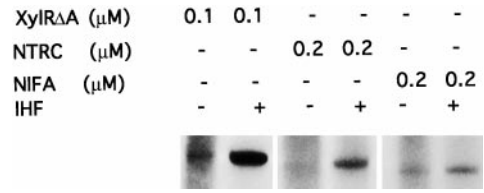

FIG. 3. **Activation of the *Pu* promoter by NtrC or NifA proteins in the presence of IHF.** Single-round transcription reactions contained 5 nM supercoiled plasmid pEZ10, which bears the wild-type *Pu* promoter. This was mixed with 25 nM core RNAP, 100 nM σ^{54}, and, where indicated (+), 25 nM IHF. Purified XylRΔA was entered in the control sample at a concentration of 100 nM, whereas NifA was added at 200 nM. In the case of NtrC, the protein at 200 nM was combined with a 15 nM concentration of its partner kinase NtrB to phosphorylate the regulator in the presence of ATP (28). Note in all cases the positive effect of IHF addition.

activities of the regulators. In any case, as shown in Fig. 3, the presence of IHF was necessary to produce significant amounts of open complexes with any of the proteins tested. These results provided further evidence that IHF stimulation of open complex formation was independent of the UAS and could be traced to an increased occupation of the promoter by σ^{54}-RNAP.

Promoter Occupation by σ^{54}-RNAP Limits Pu Activation from Solution—The data above indicated that IHF stimulates transcription initiation from *Pu* even in conditions in which looping effects between σ^{54}-RNAP and XylRΔA bound to distant sites are ruled out. Because IHF allows the *Pu* promoter to be occupied at lower concentrations of the polymerase (14), the mechanism for such an activation could imply an increased binding of the enzyme and a subsequent increase in the stability of the closed complexes. The prediction is then that an excess of σ^{54}-RNAP concentration should bypass the need of IHF for full transcriptional activity. To test this issue, we carried out *in vitro* transcription assays in which the *Pu* ΔUAS promoter was mixed with growing concentrations of σ^{54}-RNAP and activated from solution by XylRΔA in the absence or in the presence of IHF. As shown in Fig. 4, the amount of open complexes in the absence of IHF increased with the concentration of σ^{54}-RNAP added, such that they appeared to be limited only by the occupation of the promoter by the enzyme. As shown in Fig. 4 also, IHF addition did overcome such a limitation, because the system became saturated at lower σ^{54}-RNAP concentrations than without the factor.

HU Enhances Activation of the Pu Promoter in trans by XylRΔA—Although the data presented above seems to substantiate that IHF increases the binding σ^{54}-RNAP to the *Pu* promoter, the mechanism might not be trivial. Increasing formation of a closed complex may be the result of protein-protein interactions between IHF and σ^{54}-RNAP. Alternatively, recruitment may result from the change of DNA geometry caused by IHF binding, so that an otherwise distant UP-like sequence is brought into the proximity of the −12/−24 motif (14). To discriminate between these two possibilities, we used the activation-from-solution assay described above using HU rather than IHF to examine any potential stimulatory effect. HU has been shown to replace IHF in a variety of assays involving DNA bending (29, 30, 31). Therefore, if IHF-mediated recruitment of σ^{54}-RNAP were caused by specific protein-protein interactions between the factor and the C-terminal domain of the α subunit of σ^{54}-RNAP, then HU could not replace IHF for the stimulatory effect. On the contrary, if the main effect of IHF were caused exclusively by the indirect structural outcome of binding to the promoter region, then HU could substitute functionally its positive influence. To bring these possibilities into a test, the activities of wild-type *Pu* and *Pu* ΔUAS were compared under various combinations of IHF and HU with an excess of

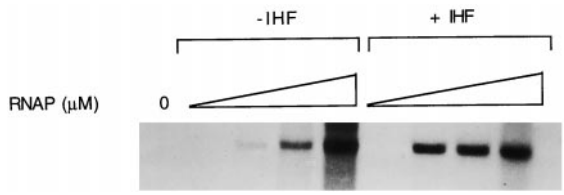

FIG. 4. **Effect of IHF on activation of *Pu* ΔUAS with growing concentrations of σ^{54}-RNAP.** Shown is the result of single-round transcription reactions containing 5 nM supercoiled plasmid pEZ20, which bears the *Pu* ΔUAS promoter. Besides including in all cases 1 μM XylRΔA, the reactions included 25 nM IHF where indicated (+ *IHF*) and growing concentrations of σ^{54}-RNAP (0.05, 0.2, 0.4, and 0.8 μM) of the core enzyme mixed with a 3-fold molar excess of purified σ^{54}.

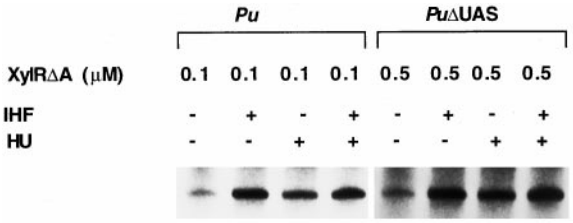

FIG. 5. **Transcriptional co-activation of *Pu* and *Pu* ΔUAS by IHF, HU, or both.** Single-round reactions containing 5 nM supercoiled plasmids pEZ10 (*Pu*) or pEZ20 (*PuΔUAS*) were mixed with 25 nM core RNAP, 100 nM σ^{54}, and, as indicated (+), 25 nM IHF, 75 nM HU, or both. Purified XylRΔA was added to the reactions at a concentration of 0.1 μM for the wild-type *Pu* template and 1.0 μM for *Pu* ΔUAS. Note the similar effects of IHF and HU addition.

XylRΔA. As shown in Fig. 5, HU indeed had a positive effect on the activation of *Pu* by XylRΔA in trans, albeit less pronounced than IHF. Similar also to the results of Fig. 2, HU had no effect on the transcription of a DNA template deleted of the region upstream of −53 (data not shown), suggesting that, like IHF, its stimulatory effect required the presence of the UP-like element (Fig. 1). Simultaneous addition of the two factors did not appear to further increase the degree of stimulation achieved with IHF alone. These data support the notion that the recruitment of the polymerase brought about by IHF is caused by indirect structural effects (*i.e.* approaching an otherwise distant UP-like element), and that protein-protein interactions may not play a significant role.

HU Promotes Occupation of Pu by σ^{54}-RNAP—The notion that HU produces the same effect as IHF on *Pu* regarding the recruitment of the polymerase was tested directly with a DNase I footprinting assay. To this end, a DNA fragment bearing the entire *Pu* was mixed with subsaturating concentrations of σ^{54}-RNAP holoenzyme and either purified IHF or HU proteins. The results in Fig. 6 show that the same effect of IHF in promoting σ^{54}-RNAP binding to −12/−24 (as revealed by the protection of the sequence from DNase I digestion) could also be achieved by HU. Interestingly, because HU does not interact with an specific DNA sequence but rather promotes the flexibilization of the sequence through transient contacts with the minor groove (32), the recruitment of the enzyme becomes evident without an occupation of the upstream IHF site. Interestingly, the distinct pattern of protected and overdigested bands observed in the region upstream and adjacent to the −24/−12 sequence remains the same. This suggests that the same interactions of the σ^{54}-RNAP with the upstream region operatively designated a UP-like element (Ref. 14 and Fig. 1) are facilitated equally well by either of the two proteins. These results favor the notion that it is the structural effect of IHF binding to *Pu* and not the contacts between the proteins that causes the observed increase in σ^{54}-RNAP affinity and the resulting stabilization of the closed complexes.

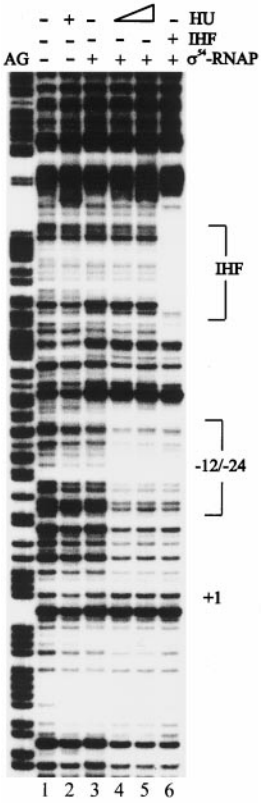

FIG. 6. **DNase I footprinting of the *Pu* promoter with purified σ^{54}-RNAP, HU, and IHF proteins.** The DNA template used was a 474-bp *Bam*HI-*Pvu*II fragment from plasmid pEZ10 containing the entire *Pu* promoter and labeled with ^{32}P at its *Bam*HI end. The proteins were added to the samples as indicated at the above the gels at the following concentrations: HU, 50 and 100 nM; IHF, 100 nM; and polymerase, 15 nM core enzyme/50 nM σ^{54}. The A+G Maxam and Gilbert reaction of the same fragment was used as a reference. The locations of the IHF binding site, the −12/−24 motif, and the transcription start site (*+1*) are indicated to the *right*.

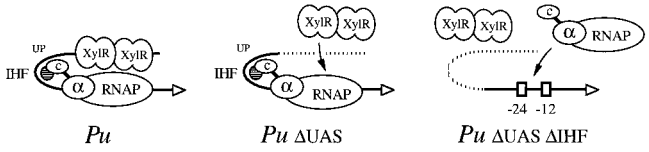

FIG. 7. **Steps controlling transcription rate of *Pu*.** The scheme pictures how IHF and recruitment of σ^{54}-RNAP may become the rate-limiting step for the activation of the σ^{54}-*Pu* promoter. The shape and volume of the different proteins is symbolic. From our data it appears that the promoter geometry caused by IHF binding to DNA and the ensuing bending may favor the proximity of the UP-like element to C-terminal domain of the α subunit of σ^{54}-RNAP and perhaps also increase the strength of the contacts (14). In the absence of such a UP-like element (as is the case with *Pu ΔUAS ΔIHF*), the polymerase does not form a closed complex spontaneously; hence the promoter remains inactive. The sole presence of the IHF site and the resulting DNA bending stimulate the recruitment of the enzyme to −12/−24, allowing the polymerase to be activated by XylR from solution (*Pu ΔUAS*). Such an activation is further increased in the wild-type *Pu* promoter by virtue of the structural effect, which brings the upstream XylR·UAS complex into close proximity to the already bound enzyme.

Recruitment of σ^{54}-RNAP Is a Rate-limiting Step for Pu Activation—The changes in DNA conformation required for assembling an orderly promoter geometry represent a kinetic barrier for transcription initiation and may constitute a rate-limiting step of the whole process (3). This notion is exacerbated in σ^{54} promoters, because their activity is dependent on the shape of the DNA segment encompassing the enhancer and the RNAP binding site (6, 33). Despite this, isomerization of the

closed σ^{54}-RNAP·DNA complex to an open complex has been generally considerered the key bottleneck to be overcome by the cognate activators (6). Once such a barrier is defeated, the transcriptional output depends on the probability of contacts between the activator and the σ^{54}-RNAP bound at distant sites, which, in turn, depends on the intrinsic or protein-induced bending or flexibility of the DNA region involved. The stimulatory effect of IHF in σ^{54} promoters has been interpreted in this context to overcome the hurdle corresponding to this phase. But apart from these geometrical effects, we have observed that the binding of IHF to the *Pu* promoter also favors the binding of σ^{54}-RNAP to its target sequences at $-12/-24$ (Ref. 14 and Fig. 7). On top of this, we have shown now that polymerase binding becomes a rate-limiting checkpoint in the process of *Pu* activation. All our data indicate consistently that IHF-mediated recruitment of σ^{54}-RNAP controls *Pu* output. On this basis, we conclude that formation of a stable closed complex in *Pu* represents a kinetic barrier that, in cases of limiting concentrations of enzyme, becomes more important than the XylRΔA-mediated formation of an open complex. This could be effective under physiological conditions (*e.g.* during the onset of stationary phase) in which the various sigmas compete for a scarce intracellular concentration of core RNAP (34). In this respect, the data of Fig. 4 show that IHF addition and the ensuing recruitment of the enzyme to *Pu* lowers the concentration of the polymerase required for activation. HU protein appeared to both enhance the recruitment of σ^{54}-RNAP and stimulate *Pu* transcription in a ΔUAS promoter, hence reproducing the same stimulatory effect than IHF. This suggests that formation of closed complexes is stimulated by factor-induced changes on the conformation of the DNA, perhaps with little need of protein-protein contacts. It thus appears that although IHF and the C-terminal domain of the α subunit of σ^{54}-RNAP may bind very close or even have overlapping sites in *Pu* (14), the two proteins may not physically contact, or, even if they do, such contacts appear to be irrelevant for σ^{54}-RNAP recruitment.

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