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

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Manuel Carmona‡, Víctor de Lorenzo‡‡, and Giovanni Bertoni¶

From the ‡Department of Microbial Biotechnology, Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas, Campus de Cantoblanco, 28049 Madrid, Spain and ¶Dipartimento di Genetica e Biologia dei Microrganismi, Università degli Studi di Milano, via Celoria 26, 20133 Milan, Italy

The activity of the $\sigma^{54}$-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 $\sigma^{54}$-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), 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 $\sigma^{70}$ ($\sigma^{70}$-RNAP). Because positively regulated $\sigma^{70}$ promoters generally fail to form stable closed complexes (4), activator-mediated binding of $\sigma^{70}$-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 $\sigma^{54}$ ($\sigma^{54}$-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 $\sigma^{54}$-RNAP binding site (6). Interactions between $\sigma^{54}$-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 $\sigma^{54}$-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 vitro (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 $\sigma^{54}$-RNAP with the promoter. Such an IHF-mediated “recruitment” of $\sigma^{54}$-RNAP seems to involve the interaction of an otherwise distant cis-element with the C-terminal domain of the $\sigma$ subunit of $\sigma^{54}$-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 $\sigma^{54}$-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 $\sigma^{54}$-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 EcoRI-BamHI fragment in pTE103. Plasmid pEZ40 carries the variant named Pu ΔUAS inserted in the same vector as a 207-bp EcoRI-BamHI fragment excised from plasmid pUC-IHF2 (14), which spans the region −114 to +93 of Pu. Similarly, a 122-bp fragment from plasmid pUC-IHF2 (14), containing the region −93 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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were performed as described before (20). Single-round transcription assays were the kind gift of B. Magasanik. NifA, IHF, and HU proteins were obtained from M. Buck, aromatic inducer (16, 19). XylR and native core RNAP from Escherichia coli indicated otherwise, each DNA template was premixed with 25 nM core (5 nM) for 25 min at 30 °C with the proteins indicated in each case, 3 ng Sephadex G-25 columns. After preincubating the end-labeled fragment Klenow fragment of DNA polymerase. Radioactive nucleotides not in-

buffer (7m urea, 0.025% bromphenol blue, and 0.025% xylene cyanol in 10 mM MgAc2, 2m M NH4Ac, and 300 mM MgCl2, 10 mM MgCl2, 0.1 mM EGTA, pH 8, 0.8% SDS, 1.6M NH4Ac, and 300 μg/ml carrier tRNA. The mRNA extracted and precipitated with ethanol was electro-

horesed on a denaturing 7 m urea, 4% acrylamide gel and visualized by autoradiography.

DNAase I Footprinting Techniques—DNA-protein interactions were monitored with DNAase 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 MgAc2, 20 mM NH4Ac, 2 mM CaCl2, 1 mM DTT, 3% glycerol, and 40 μg/ml poly(dIC)]. The DNA template used was a 474-bp EcoRI-fragment excised from plasmid pEZ9 (11), which contains the entire Pu site by filling in the overhanging end with [32P]dATP and the

7m terminal module (the so-called A domain; Ref. 16). We also included in plasmid pEZ10 with respect to the transcription start site.

Pu derivatives are 394 nucleotides in size. The DNA was, in fact, a poor template for transcription, most likely because of the loss of the UP-like element, which overlaps the

Pu promoter sequence as an EcoRI-BamHI insert in pUC18 spanning positions −208 to +93 (Fig. 1). The fragment was end-labeled in its BamHI site by filling in the overhanging end with [32P]dATP and the Klenow fragment of DNA polymerase. Radioactive nucleotides not incorpo-

ted to DNA were removed after a brief spin through small Sephadex G-25 columns. After preincubating the end-labeled fragment (5 ns) for 25 min at 30 °C with the proteins indicated in each case, 3 ng of DNAase I were added to each sample and further incubated for 3.5 min. Reactions were halted by adding 25 μl of STOP buffer containing 0.1 mM EDTA, pH 8, 0.8% SDS, 1.6 mM NH4Ac, 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 α54-RNAP to 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 α54-RNAP bound to −12/−24 (11, 13). On the other hand, it augments the affinity of α54-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, 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 α54-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 α54-RNAP activation from solution in other σ54-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-α54-RNA contacts is ruled out.

IHF Stimulates Activation of α54-RNA by XylRΔA from Solution—To ascertain whether the increased binding of α54-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 IHF (Pu-53). These templates were incubated with subsaturating concentrations of α54-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 σ54-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. 2A, 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. 2A). 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
activation of PuΔUAS 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. 2B. 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. 2B 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 σ54-RNAP with Pu limited transcription initiation and that the previously described IHF-mediated recruitment of σ54-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 PnuH2 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 for XylRΔA, perhaps reflecting some difference in the intrinsic 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 σ54-RNAP.

Promoter Occupation by σ54-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 σ54-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 σ54-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 σ54-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 σ54-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 σ54-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 σ54-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 σ54-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 σ54-RNAP was caused by specific protein-protein interactions between the factor and the C-terminal domain of the a subunit of σ54-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
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 α54-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 promoter and labeled with 32P at its BamHI 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 α54. 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.

Recruitment of α54-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 α54 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 $\sigma^{54}$-RNAP-DNA complex to an open complex has been generally considered 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 $\sigma^{54}$-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 $\sigma^{54}$ 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 promotor also favors the binding of $\sigma^{54}$-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 $\sigma^{54}$-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 XylR-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 sigma 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 $\sigma^{54}$-RNAP and stimulate $Pu$ transcription in a $\Delta$UAS 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 $\alpha$ subunit of $\sigma^{54}$-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 $\sigma^{54}$-RNAP recruitment.

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