Recruitment of $\sigma^{54}$-RNA Polymerase to the Pu Promoter of Pseudomonas putida through Integration Host Factor-mediated Positioning Switch of $\alpha$ Subunit Carboxyl-terminal Domain on an UP-like Element*

The interactions between the $\sigma^{54}$-containing RNA polymerase ($\sigma^{54}$-RNAP) and the region of the Pseudomonas putida Pu promoter spanning from the enhancer to the binding site for the integration host factor (IHF) were analyzed both by DNase I and hydroxyl radical footprinting. A short Pu region centered at position $-104$ was found to be involved in the interaction with $\sigma^{54}$-RNAP, both in the absence and in the presence of IHF protein. Deletion or scrambling of the $-104$ region strongly reduced promoter affinity in vitro and promoter activity in vivo, respectively. The reduction in promoter affinity coincided with the loss of IHF-mediated recruitment of the $\sigma^{54}$-RNAP in vitro. The experiments with oriented-$\sigma^{54}$-RNAP derivatives containing bound chemical nuclease revealed interchangeable positioning of only one of the two $\alpha$ subunit carboxyl-terminal domains ($\alpha$CTDs) both at the $-104$ region and in the surroundings of position $-78$. The addition of IHF resulted in perfect position symmetry of the two $\alpha$CTDs. These results indicate that, in the absence of IHF, the $\sigma^{54}$-RNAP asymmetrically uses only one $\alpha$CTD subunit to establish productive contacts with upstream sequences of the Pu promoter. In the presence of IHF-induced curvature, the closer proximity of the upstream DNA to the body of the $\sigma^{54}$-RNAP can allow the other $\alpha$CTD to be engaged in and thus favor closed complex formation.

Bacterial promoters are modular DNA regions able to establish productive interactions both with subunits of RNA polymerase holoenzyme (RNAP), subunit composition: $\alpha_2\beta\beta'$ $\sigma$ and regulatory proteins (1–4). The core promoter elements are signature tags for $\sigma$ factor selectivity (4). The major sigma factor $\sigma^{70}$ generally directs RNAP to interact with the core DNA elements $-10$ and $-35$ (hexamers with consensus 5'-TATAAT-3' and 5'-TTGACA-3', respectively) (5). The core promoter elements can be both overlapped and flanked by protein-bound DNA sites involved in the fine modulation of promoter activity (2, 4). In the last decade, a considerable amount of attention has been given to a $\alpha$ + T-rich promoter sequence, the UP element, located upstream of the core promoter region and consisting of two distinct subsites, each of which, by itself, can be bound by the carboxyl-terminal domain of the RNAP $\alpha$ subunit ($\alpha$CTD) (3, 6–10). $\alpha$CTD recognizes and interacts with the backbone structure in the minor groove of the UP element. The $\alpha$ + T-rich sequence of the UP element is needed to provide the optimum width of minor groove for interaction with $\alpha$CTD (7, 11). Several lines of evidence showed that the role of the UP elements is to stimulate transcription in an activator protein-independent manner and to a different extent (from 1.5 to $-90$-fold) depending on the similarity with the consensus UP element sequence (3, 9). It is currently believed that the transcription stimulation by an UP element has to be traced mainly to the cooperation of the sigma factor and the $\alpha$ subunit in RNAP binding to the promoter (1, 12). Thus, the presence of an UP element in a promoter plays the major role of increasing the initial equilibrium constant of closed complex formation between RNAP and promoter DNA (13, 9). However, influences of $\alpha$-UP element interaction on later steps in transcription initiation were also reported (13, 14). The location of the UP element with respect to the transcription start site can influence the degree of transcription stimulation (15). In the Escherichia coli rnrB P1 promoter, the UP element is located in a region spanning form the $-40$ and $-60$ positions and is able to increase transcription from 30- to 70-fold (6, 13). The artificial upstream re-location of the rnrB P1 UP element by a single turn of DNA helix decreases but does not prevent transcription stimulation, while further displacements abolish UP element dependent transcription (15). The ability of $\alpha$CTD to contact DNA and/or activator molecules at different locations upstream of the core promoter (8, 15–21) has been attributed to the flexibility of the linker connecting $\alpha$CTD to the $\alpha$ amino-terminal domain ($\alpha$NTD) (8, 22) assembled in the body of RNAP. This linker flexibility also accounts for the ability of the two copies of $\alpha$CTD to function interchangeably with respect to the subsite recognition within the UP element (10). Sufficient length of the linker between $\alpha$CTD and $\alpha$NTD is also needed for UP element-dependent transcription activation. The linker is flexible but structured to a certain extent to facilitate the
positioning of the oCTD to a proper location for interaction with the UP element (23, 15).

UP-like elements were also found in promoters recognized by alternative sigma factors, such as the δ54-dependent flagellar promoter of Bacillus subtilis (24) and the δ54-dependent Pu promoter of Pseudomonas putida (Fig. 1) (25, 26). The latter drives transcription of TOL plasmid upper operon for the degradation of toluene (27) and shows the typical modular structure of the δ54-dependent promoters (for review, see Refs. 28 and 29) that consists of: (i) the −12/−24 region (consensus: TGGCAC N5 TTGCa/t located between positions −11 and −26) (30) recognized by δ54 and considered the functional analogue of −10/−35 core promoter bound by σ70 (31), (ii) DNA enhancer sequences (known as upstream activating sequences or UAS) targets for the activators of the δ54-RNAP, usually located at >100 bp from the transcription start site, and (iii) an intervening sequence between UAS and −12/−24 motif that may contain a target site of the IHF (32), which, by its ability to bind and bend DNA sequences, assists the looping out required to bring closer together the δ54-activator prebound at UAS and δ54-RNAP assembled in a closed complex with −12/−24 DNA region. The productive contact between δ54-activator and δ54-RNAP closed complex triggers promoter opening (open complex) and eventually transcription initiation (33, 34).

Within this typical modular scheme for δ54-dependent promoters, the P. putida Pu promoter presents unique features. In fact, our previous results showed the additional IHF role of stimulating the otherwise limiting step of closed complex formation between δ54-RNAP and Pu DNA (26, 35). We also showed that the recognition of Pu promoter by δ54-RNAP involves not only the −12/−24 region but also a functional equivalent of an UP element located in the intervening region, upstream to the IHF binding site. Furthermore, our data strongly suggested that the Pu UP element could play a key role in the IHF-mediated stimulation of closed complex formation by δ54-RNAP. In this work, we closely inspected the interactions, both in the presence and absence of IHF, between δ54-RNAP and the Pu intervening region located upstream the IHF binding site. The data strongly support the notion of a non-canonical arrangement of the stimulating DNA sequences functioning as UP element. δ54-RNAP upstream interactions concentrate on two sites located in the surroundings of positions −104 and −78, respectively, thus being distant about 25 bp. In the absence of IHF and probably due to asymmetrical positioning of the upstream DNA, the two sites can be contacted interchangeably only by one oCTD of the αββ′ δ54 complex constituting the δ54-RNAP. On the contrary, the binding by IHF apparently introduces symmetry to the nucleoprotein complex allowing the other oCTD to interact with the two sites. Thus, the IHF-mediated stimulation of closed complex would result from curvature-dependent increased probability of wide range upstream interactions by δ54-RNAP through the oCTDs.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and General Procedures—Plasmid pE29 (25) contains the entire Pu promoter sequence as a 312-bp EcoRI-BamHI insert in pUC18 spanning positions −208 to +93. P. putida strains KT2442 hom. gyjXyRS and its derivative HPu (Pu: lacZ, xylR) have been described previously (35, 36). KT2424/2PuClau (Pu: XhoClau-lacZ, xylR), KT2424/2PuScr1 (Pu: Scr1-lacZ, xylR), and KT2442/2PuScr2 (Pu: Scr2-lacZ, xylR) carrying mutant Pu: lacZ fusions in the same location of the chromosome as the IF Pu were obtained as follows. The Pu version, cloned in pUC-PuClau-79, derived from pE29 and bearing a Clau site engineered within positions −79 and −94 (26), was subjected to site-directed mutagenesis by the QuikChange™ site-directed mutagenesis kit (Stratagene) to engineer a XhoI site within nucleotides −121 to −126. This procedure generated the plasmid pUC-PuClauXho. The replacement of the 47-bp XhoI-Clau fragment of pUC-PuClauXho for synthetic XhoI-Clau fragments harboring scrambled sequences from nucleotides −105 to −120 and from −95 to −120 gave rise to plasmids pUC-PuScr1 and pUC-PuScr2, respectively. The Pu versions presented in pUC-PuClauXho, pUC-PuScr1, and pUC-PuScr2, respectively, were rescued as 312-bp EcoRI-BamHI fragments, fused to lacZ by cloning in the corresponding sites of pBK16 vector (36) and recombined with the homology fragment inserted in the chromosome of KT2442 hom. gyjXyRS as described previously (36). All cloned DNA fragments and DNA fragments were purified before use by automated DNA sequencing. Recombinant DNA manipulations were carried out according to published protocols (38).

Proteins and Protein Techniques—Accumulation of β-galactosidase raised by lacZ fusions was measured in P. putida KT2442 cells permeabilized with chloroform and sodium dodecyl sulfate (SDS) as described by Miller (39) under the conditions specified in each case. Purified δ54-RNAP was kindly provided by B. Magasinsingh, respectively; native core RNAP was purchased from Epitheric Technol. Reconstitution of RNAP carrying (p-bromocetamidobenzoyl)-EDTA(Fe-BABE) on β-associated α(Fe)ββ′, β′-associated α, αββ′(Fe), and simultaneously on both a subunits, α(Fe)ββ′(Fe), was carried out as described by Murakami et al. (17, 40).

DNA Binding Assays—The DNA fragments used in DNA footprinting experiments of Fig. 2 (A and B) and Fig. 5 were excised from pE29 as 390-bp EcoRI-PouI and end-labeled at their EcoRI sites by in-filling the overhanging ends as described below. The collection of Pu promoter sequences (Pu-126, Pu-114, Pu-105, Pu-85) having the same downstream end at +45 and different upstream ends at −126, −114, −105, and −85 were generated by separate PCR amplification from pE29 with the common reverse primer 5′-GTCTTGATGCATCCTCCCTC-3′ and forward primers 5′-GCTCTTATGagaXbaI/TACACGCGCCG- GTTGCTTGAAGAGGTTCATCTTTCTTCAT-AC3′, 5′-GCTCTTATGagaXbaI/TCTCTTCTTCATACCCCT-3′, 5′-GCTCTTATGagaXbaI/TCTCTTCTTCATACCCCT-3′, and 5′-GCTCTTATGagaXbaI/TCTCTTCTTCATACCCCT-3′, respectively.

For gel retardation assays, the PCR-amplified fragments described above were end-labeled with [32P]ATP and T4 polynucleotide kinase. Radioactive nucleotides not incorporated in DNA were removed by centrifuging briefly in small Sephadex G-25 columns. Binding reactions were performed in a total volume of 25 μl of transcription buffer containing 35 mm Tris acetate, 70 mm KAc, 5 mm MgAc2, 20 mm NaAc, 2 mm CaCl2, 1 mm dithiothreitol, 3% glycerol, and 40 μg/ml of poly[dI-C]/. Labeled fragments, added to the buffer at a final concentration of 5 μM, were incubated with 100 μM HFe60, 60 μM core RNAP, and a 3-fold molar excess of δ54 factor for 25 min at 30 °C. The entire reaction volume was loaded on 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 MgCl2), electrophoresed at 12 m M at 4 °C for 6 h, and dried. Banding was visualized and quantified by Typhoon 8600 variable exposure autoradiography. DNA footprinting assays were performed in a total volume of 50 μl with similar concentrations of end-labeled fragments and proteins used in the gel mobility-shift assays. For DNase I footprinting, after precipitation of end-labeled Pu DNA and proteins in transcription buffer for 25 min at 30 °C, 3 μg 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 μM EDTA, pH 8, 0.8% SDS, 1.6 μM NH4Ac, and 300 μg/ml sonicated salmon sperm DNA. Nuclease acids were precipitated with 175 μl of ethanol, lyophilized and directly resuspended in denaturing loading buffer (7 μl urea, 0.025% bromophenol blue, and 0.025% xylene cyanol in 20 μl Tris, pH 8) prior to loading on a 7% DNA sequencing gel. A + G Maxam and Gilbert reactions (41) were carried out with the same fragments and loaded onto the gels along with the footprinting samples. For hydroxyl radical footprinting, after precipitation of end-labeled Pu DNA and proteins for 25 min at 30 °C in hydroxyl radical buffer containing 25 mM HEPES, 70 mm KAc, 5 mm MgAc2, 19 mm NaAc, 0.7 mM DTT, 1% glycerol, and 40 μg/ml of poly[dI-C], 5 μl each of [Fe(EDTA)]125 (125 mm [Fe(EDTA)], 20 μl, 250 mM peroxide), and 0.84% hydrogen peroxide were added to the samples and then incubated for a further 5 min. Reactions were stopped by addition of 15 μl of 0.1 M thiourea and 25 μl of STOP buffer (as above), respectively. Nuclease acids were precipitated with 200 μl of ethanol and treated for separation and visualization as described above for DNase I footprinting. For DNA footprinting in the presence of (Fe-BABE)-RNAPs, after precipitation of end-labeled Pu DNA and proteins for 25 min at 30 °C in hydroxyl radical buffer, 1 μl each of 100 mM ascorbate and 0.6% hydrogen peroxide were added to the samples and then incubated for a further 15 min. Reactions were stopped by addition of 10 μl of 0.1 M thiourea and 25 μl of STOP buffer (as above), respectively. Nuclease acids were pre-


**RESULTS**

$\sigma^{54}$-RNAP Per Se Can Contact Sequence Elements Located Upstream the IHF Binding Site of Pu Promoter—The affinity of $\sigma^{54}$-RNAP for Pu promoter DNA lacking the sequence region located upstream the position −79 is strongly reduced (Fig. 1, A–D). These data suggested that $\sigma^{54}$-RNAP could utilize per se additional DNA affinity elements located far upstream of the −12/−24 region involved in the interaction with $\sigma^{54}$. Since αCTD-deleted derivatives of $\sigma^{54}$-RNAP showed reduced promoter affinity (26), we also suggested that αCTD could be directly involved in the recognition of such upstream DNA, reminiscent, in this case, of the UP elements of α70 promoters. Furthermore, an αCTD/UP-like interaction also seemed to be involved in the mechanism behind the stimulation of closed complex formation between Pu DNA and $\sigma^{54}$-RNAP on IHF binding and bending, which we referred to as IHF-mediated recruitment of $\sigma^{54}$-RNAP to the Pu promoter (26). To map the contact sites of $\sigma^{54}$-RNAP upstream −79 position and to reveal any possible influence of IHF-induced bending on these upstream contacts, we carried out either Dnase I and hydroxyl radical footprinting assays on end-labeled DNA fragments bearing the entire Pu mixed with increasing amounts of $\sigma^{54}$-RNAP, both in the presence and in the absence of sub-saturating concentrations of purified IHF. As shown in Fig. 2A (lanes 1–3), the footprint of the $\sigma^{54}$-RNAP in the region spanning approximately from −83 to −108 consisted in a generalized hypersensitivity to Dnase I. The addition of IHF (Fig. 2A, lanes 4 and 5) caused a further slight increase of upstream DNA reactivity to Dnase I, with the exception of positions −90 and −91, appearing to be more protected from Dnase I cleavage than when in the presence of $\sigma^{54}$-RNAP alone. Similar conditions of labeled Pu DNA/protein ratio were employed to perform hydroxyl radical footprinting assays (Fig. 2B). The inspection of the Pu DNA sequence upstream of the IHF binding site revealed both protected and hypersensitive sites in a region spanning from −95 to −109. In particular, as shown in Fig. 2B (lanes 1–4), the hydroxyl radical footprint of $\sigma^{54}$-RNAP consisted of three hypersensitive positions, −103 to −105, which seemed to be flanked by short protected regions. Apparently, the addition of IHF protein (Fig. 2B, lanes 5–7) caused minimal changes to the pattern of hydroxyl radical cutting. Examined together, these results strongly indicated that, even when unassisted, $\sigma^{54}$-RNAP can establish contacts to DNA sites located far upstream of the −12/−24 region. In the case of the hydroxyl radical footprint, the DNA region involved in interactions with $\sigma^{54}$-RNAP appeared to be limited to a short sequence in the surroundings of position −104, which we named UP-like $p_{I}^{\alpha}$. In our previous work (26), to explain the mechanism behind the IHF-mediated recruitment of $\sigma^{54}$-RNAP, we suggested that the distance between the −12/−24 site and the UP element(s) might disfavor either the formation or the maintenance of simultaneous binding by $\sigma^{54}$-RNAP through $\sigma^{54}$ and αCTD, respectively. Thus, the key recruiting action of IHF-induced bending would have consisted of increasing $\sigma^{54}$-RNAP affinity for Pu DNA by bringing the −12/−24 site and the UP element(s) into a closer proximity. Apparently, from the footprinting analysis presented in Fig. 2, the ability of $\sigma^{54}$-RNAP to establish contacts with the Pu region located upstream of the IHF binding site seemed to be enhanced to a limited extent only by the addition of IHF. However, further analysis with free radical-delivering $\sigma^{54}$-RNAPs (see below) showed more clearly that IHF-induced bending can cause increased occupancy by αCTD of the region upstream to the IHF binding site.

The Integrity of the UP-like $p_{I}^{\alpha}$ Site Is Required Both for Full Promoter Affinity and IHF-mediated Recruitment of $\sigma^{54}$-RNAP—The footprinting experiments presented above allowed
FIG. 2. Footprinting of the Pu promoter with \( \sigma^{54}\)-RNAP and IHF. The 390-bp EcoRI-PvuII fragment containing the whole Pu promoter and end-labeled with \( ^{32}P \) at its EcoRI site was mixed with the proteins indicated at the top, treated with DNase I (A) or hydroxyl radicals (B), and run in sequencing gels. The A + G Maxam and Gilbert reaction of the same DNA fragment was used as a reference. The location of the IHF binding site, some coordinates along the promoter sequence, and the region corresponding to the UP-like \( \sigma_{\text{Pu}} \) site are indicated with empty arrows. The IHF concentration was 100 nM in each case. \( \sigma^{54}\)-RNAP was added as core polymerase with 3-fold molar excess of purified \( \sigma^{54}\). Core polymerase concentrations were 30 or 60 nM in A and 15, 30, or 60 nM in B.

us to identify an upstream DNA element, UP-like \( \sigma_{\text{Pu}} \), which is contacted by \( \sigma^{54}\)-RNAP in the closed complex with the Pu promoter. To evaluate the role of the contacts with UP-like \( \sigma_{\text{Pu}} \) in determining the affinity of \( \sigma^{54}\)-RNAP for the Pu promoter, we ran gel retardation assays on the nucleoprotein closed complexes formed by \( \sigma^{54}\)-RNAP with DNA fragments bearing either the Pu sequence up to position −126 (Pu−126; Fig. 1) or progressively shorter Pu sequences extending up to −114, −105, and −85, respectively (Pu−114, Pu−105, Pu−85; Fig. 1). Side-by-side comparison of the amounts of complex assembled by \( \sigma^{54}\)-RNAP with Pu−105 were strongly reduced with respect to Pu−114 (Fig. 3, lanes 5 and 6). A more extended deletion up to position −85 (Pu−85) (Fig. 3, lane 4) did not decrease further the amounts of nucleoprotein complex with \( \sigma^{54}\)-RNAP.

To test the requirement of UP-like \( \sigma_{\text{Pu}} \) integrity for the IHF-mediated recruitment of \( \sigma^{54}\)-RNAP, we added a sub-saturating concentration of purified IHF protein to the mixtures of \( \sigma^{54}\)-RNAP with Pu−85, Pu−105, and Pu−114, respectively. As shown in Fig. 3 (lanes 4–9), while the binding of \( \sigma^{54}\)-RNAP to Pu−114 could be stimulated by IHF as shown previously (26), IHF failed to enhance closed complex formation with Pu−105 and Pu−85, respectively. Thus, these results strongly indicated that the interactions established by \( \sigma^{54}\)-RNAP with UP-like \( \sigma_{\text{Pu}} \) site in the closed complex are instrumental in determining promoter affinity. In addition, as the integrity of UP-like \( \sigma_{\text{Pu}} \) is also required to observe IHF-mediated enhancement of \( \sigma^{54}\)-RNAP recruitment, we speculated that the promoter architecture imposed by IHF binding contributes to the interactions between \( \sigma^{54}\)-RNAP and the UP-like \( \sigma_{\text{Pu}} \) site.

The Scrambling of UP-like \( \sigma_{\text{Pu}} \) DNA Region Affects Pu Performance in Vivo—In view of the previous results in vitro, the disruption of the integrity of UP-like \( \sigma_{\text{Pu}} \) site was expected to affect Pu activity. To address this issue, we aimed to monitor in vivo the consequences of altering the sequence spanning the UP-like \( \sigma_{\text{Pu}} \) on Pu expression pattern. To this end, we introduced progressive sequence scrambling into the DNA region from −120 to −93 sites (Fig. 1) by replacement with synthetic double-stranded oligomers for the wt DNA sequence located between the XhoI and ClaI sites that were opportunely engineered within positions −126 and −121, and −84 and −79, respectively, in the Pu variant, PuXhoCla (Fig. 1). From this procedure, we obtained two Pu derivatives, PuScraI and PU-Scre2 (Fig. 1), that, along with their parental PuXhoCla, were fused to lacZ and recombined into the chromosome of P. putida KT2442 hom.fg/xyRS as described previously (36). As shown in Fig. 4A, the comparison of accumulation of \( \beta\)-galactosidase...
respectively, are shown. The performances of absence of induction with toluene, respectively. 

\[ \text{PuScra1-lacZ} \]

\[ \text{Cla-lacZ} \]

in vivo.

\[ \text{Pu} \]

\[ \text{13.2\%} \]

respectively. The vertical arrow indicates the position of the \( \sigma^{54} \)-RNA polymer-DNA complexes. The percentage of volume under each peak with respect to the total volume of the lane profile was calculated. The value close to the profiles of each promoter indicates the ratio between the percentage volume of the peaks corresponding to the \( \sigma^{54} \)-RNA polymer-DNA complexes in the presence and in the absence of IHF, respectively. For Pu-85, Pu-105, and Pu-114, the absolute percentage values of peak volumes for \( \sigma^{54} \)-RNA polymer-DNA complexes in the absence of IHF resulted 6.5, 6.4, and 13.2\%, respectively.

**Fig. 3.** Band-shift assay of the complexes formed between Pu segments and \( \sigma^{54} \)-RNA polymer. Left, three sequences of the Pu promoter spanning positions -114 to +45 (Pu-114), -105 to +45 (Pu-105), and -85 and +45 were end-labeled with \( ^{32}\text{P} \), mixed with 60 nM \( \sigma^{54} \)-RNA polymer in the absence and in the presence of 100 nM IHF, and run in a gel retardation assay as explained under “Materials and Methods.” The location of the different complexes is indicated to the sides. Right, superimposed profiles from scans of lanes 4 and 7 (Pu-85), 5 and 8 (Pu-105), 6 and 9 (Pu-114), respectively, are shown. The vertical arrow indicates the position of the \( \sigma^{54} \)-RNA polymer-DNA complexes. The percentage of volume under each peak with respect to the total volume of the lane profile was calculated. The value close to the profiles of each promoter indicates the ratio between the percentage volume of the peaks corresponding to the \( \sigma^{54} \)-RNA polymer-DNA complexes in the presence and in the absence of IHF, respectively. For Pu-85, Pu-105, and Pu-114, the absolute percentage values of peak volumes for \( \sigma^{54} \)-RNA polymer-DNA complexes in the absence of IHF resulted 6.5, 6.4, and 13.2\%, respectively.

**Fig. 4.** Involvement of UP-like segment in Pu promoter activity in vitro. *P. putida* KT2442 derivatives bearing the Pu-lacZ, PuXhoCla-lacZ, PuScra1-lacZ, and PuScra2-lacZ transcriptional fusions recombined into the same site of the chromosome, respectively, were tested for the performance of expression of the lacZ reporter gene during the growth at 30 °C in LB medium (53). Each strain was grown until cultures had an absorbance of 0.5 at 600 nm. Toluene was then administered and the incubation continued for the subsequent 3.5 h. Accumulation of \( \beta \)-galactosidase along the time and growth curves of each strain after toluene addition are shown. A, comparison of the performances of Pu-lacZ and PuXhoCla-lacZ in the absence and in the presence of IHF, respectively. B, comparison of the performances of PuXhoCla-lacZ, PuScra1-lacZ, and PuScra2-lacZ, respectively, upon toluene induction.

upon toluene induction of KT2442PuXhoCla, KT2442PuScra1 (PuScra1::lacZ, xylR) and KT2442PuScra2 (PuScra2::lacZ, xylR), respectively, revealed that the promoter activity of PuScra2 was severely impaired. Since the sequence scrambling did not introduce either phase alteration of key regulatory sites (UAS, IHF box and -12/-24 sites) or a predictable drastic variation of promoter DNA curvature (42), we inferred that the reduction of promoter activity of PuScra2 was caused by the destruction of upstream contacts between \( \sigma^{54} \)-RNA polymer and the UP-like Pu region.

Monitoring the Positioning of the Two \( \alpha \) CTDs of \( \sigma^{54} \)-RNA polymer along Upstream Sequences of the Pu Promoter in the Absence and in the Presence of IHF—In the \( \alpha_2\beta_2\sigma^5 \) core RNAP complex, the two identical \( \alpha \) subunits can be distinguished by their arrangement with respect to \( \beta \) and \( \beta' \) subunits. In fact, one \( \alpha \), \( \alpha' \), interacts with the \( \beta \), whereas the other, \( \alpha'' \), interacts with \( \beta' \) (43, 40, 17). To provide a more precise definition of the positioning of the \( \alpha \) CTD of \( \alpha_2^1 \) (\( \alpha \) CTD\( ^{1} \) and \( \alpha \) CTD\( ^{2} \), respectively) along the Pu DNA region upstream the IHF site, we set out to exploit the UP DNA cleavage capability caused by free radicals originated from (\( \beta \)-bromoacetamidobenzyl)-EDTA-Fe (Fe-BABE) attached to the UP contact surface of one or both \( \alpha \) subunits assembled in the RNAP holoenzyme complex (40, 17). We prepared free radical-releasing \( \sigma^{54} \)-RNA polymer by adding a saturating amount of \( \sigma^{54} \) both to oriented-\( \alpha \) and non-oriented (Fe-BABE)-labeled RNA polymer core complexes: \( \alpha_1^1(\text{Fe})/\alpha_2^2 \) and \( \alpha_1^1(\text{Fe})/\alpha_2^2(\text{Fe}) \), in which the (Fe-BABE) moiety is bound to \( \alpha \) CTD\( ^{1} \) and \( \alpha CTD^{2} \), respectively, and \( \alpha_1^1(\text{Fe})/\alpha_2^2(\text{Fe}) \), in which the (Fe-BABE) moiety is bound to both \( \alpha \) CTDs. Such (Fe-BABE)-labeled \( \sigma^{54} \)-RNPs were incubated with end-labeled Pu promoter DNA, and the pattern of Pu DNA fragmentation was analyzed in sequencing gels as in the footprinting experiments presented in Fig. 2 (A and B). To test any differential influence of IHF on upstream DNA occupancy by \( \alpha \) CTD\( ^{1} \) or \( \alpha \) CTD\( ^{2} \), a set of reactions was also incubated in the presence of IHF. As shown in Fig. 5, in the absence of IHF, both \( \alpha_1^1(\text{Fe})/\alpha_2^2(\text{Fe}) \) and \( \alpha_1^1(\text{Fe})/\alpha_2^2(\text{Fe}) \) (lanes 2 and 3) could produce in the UP-like Pu region.
a fragmentation pattern that was very similar to the hydroxyl radical hypersensitivity profile at positions −74 to −79. We named this second αCTD-interacting region, which did not result so evident in the previous footprinting experiments (Fig. 2, A and B), UP-likeⅡ. Unlike α(Fe)/αⅠ(Fe) and α(Fe)/αⅠ(Fe), in the absence of IHF the other oriented-α54-RNAP, α(Fe)/αⅠ(Fe), could not produce any significant fragmentation of the end-labeled Pu DNA (Fig. 5, lane 4). The addition of IHF did not substantially modify the fragmentation pattern by α(Fe)/αⅠ(Fe) and α(Fe)/αⅠ(Fe). On the contrary, the presence of IHF increased the fragmentation by α(Fe)/αⅠ(Fe). In fact, under these conditions, α(Fe)/αⅠ(Fe) was also able to generate a fragmentation pattern (Fig. 5, lane 8) identical to that of α(Fe)/αⅠ(Fe) and α(Fe)/αⅠ(Fe). Taken together, these results clearly revealed that αCTD interaction with the Pu upstream region can occur at two unusually distant sites, UP-likeⅠ and UP-likeⅡ, respectively. Both sites can be bound interchangeably by αCTDⅠ, both in the absence and in the presence of IHF. Unlike αCTDⅠ, and probably due to an asymmetrical arrangement of the DNA upstream the IHF site, αCTDⅠ can efficiently contact both UP-likeⅠ and UP-likeⅡ only in the presence of IHF protein. These results strongly suggest that when the Pu promoter DNA conformation is structured by the binding and bending of IHF, the two αCTD can distribute simultaneously and interchangeably among UP-likeⅠ and UP-likeⅡ.

**DISCUSSION**

The activity of the Pu promoter of *P. putida* is strongly influenced by DNA architecture. Remarkably, the bending activity of IHF strongly augments the probability of interaction between the activator XylR and α54-RNAP (44) and also positively influences the docking of α54-RNAP on the promoter (26). Our previous studies showed the involvement of αCTD in the interaction with a Pu region reminiscent of an UP element located upstream to the IHF binding site (26). Furthermore, our results suggested that the upstream interactions of α54-RNAP by αCTD could play an active role in its IHF-mediated recruitment. However, the relationship between topology of the promoter DNA and topography of such upstream interactions required further clarification. In fact, this might be at the basis of the IHF-mediated enhancement mechanism of closed complex formation. In this study, we investigated the nature and the role of the upstream contacts that α54-RNAP is able to establish with the Pu promoter both in the presence and in the absence of IHF protein. The footprinting experiments presented in Fig. 2 sustained more clearly than our previous works (26) the notion that α54-RNAP per se is able to establish contacts with DNA sequences located upstream to the IHF binding site. In addition, these experiments allowed us to identify a discrete upstream DNA site surrounding position −104, UP-likeⅠ (Fig. 2B), engaged in the interactions with α54-RNAP. Then, we addressed the issue of the functional significance of the α54-RNAP/UP-likeⅠ contacts. At least two lines of evidence indicated that the α54-RNAP contacts with UP-likeⅠ are functional interactions participating in Pu promoter activity. First, alterations of the Pu sequence spanning from −114 to −85 positions (Pu-105 and Pu-85, respectively) affect promoter affinity for α54-RNAP per se (Fig. 3, lanes 4–6). Second, the rearrangement of the sequence surrounding UP-likeⅠ in Pu-Scra2 derivative severely affects Pu activity in vivo. The experiment presented in Fig. 3 also indicated that UP-likeⅠ participates in the IHF-mediated stimulation of closed complex formation by α54-RNAP even though its occupancy by α54-RNAP did not seem to change significantly upon the addition of IHF (Fig. 2B). In our previous work (26), to explain the IHF-mediated recruitment of α54-RNAP, we considered the possibility that IHF binding and bending could strengthen the upstream contacts established by αCTD with Pu DNA. We figured
that this could be accomplished by one or the combination of the following two mechanisms: (i) IHF-induced curvature centered at −68 would bring into closer proximity the −12/−24 region and an UP element located upstream of the IHF binding site, thus favoring the simultaneous interaction of σ^{54}-RNAP with both sites, and (ii) the IHF binding would locally distort the double helix of DNA strengthening the aCTD/UP interaction. However, the idea that IHF could reinforce upstream contacts by aCTD contrasts with the evidence presented in Fig. 2 that IHF addition did not substantially alter the pattern of the upstream footprint of the σ^{54}-RNAP. Furthermore, this had to be reconciled with the fact that the integrity of UP-like{pu} \(^1\) was required both to determine promoter affinity and stimulate IHF-mediated closed complex formation. The experiments with (Fe-BABE)-labeled σ^{54}-RNAPs showed that the hypothesis of the IHF-mediated strengthening of the aCTD upstream contacts was correct. However, the possible scenario (Fig. 6) can be more complex than previously thought (Ref. 26 and see above). First, aCTD can bind to UP-like{pu} \(^1\) and also to another site, UP-like{pu} \(^\text{II}\), located downstream. In the absence of IHF (Fig. 6A), only one aCTD, aCTD\(^\text{II}\) (i.e. aCTD of α that associates with β′), can bind interchangeably to both UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\). These asymmetrical upstream aCTD interactions determine a higher second level of promoter affinity which would underlie the IHF-mediated recruitment of σ^{54}-RNAP to the Pu promoter. The topological shift from asymmetrical to symmetrical aCTD interactions can be attributed to the IHF-induced bending that brings the −12/−24 site and the upstream DNA into closer proximity. In fact, this would favor the σ^{54}-RNAP contacts with the upstream DNA also through the aCTD\(^\text{II}\) domain. Despite this IHF-mediated topological switch, the flexibility of the long unstructured interdomain linker connecting aCTD to the rest of a (8, 22) might also account for the ability of σ^{54}-RNAP to contact interchangeably the UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\) sites located at a distance from the −12/−24 core promoter region.

Both UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\) sites may resemble the UP element subsites, each of which constitutes a binding site for aCTD (10). The certain assignment of UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\) to one of the two classes, distal- or proximal-type, of UP subsite (10) cannot be deduced from this study. Moreover, it remains to be clarified whether UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\) are arranged adjacently as in the UP element consensus sequence (10) or are separated by turns of DNA helix. Hypothetically, the A-tracts at positions −102 to −105 and −77 to −82 within UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\) (Fig. 1), respectively, could constitute the core subsites (7, 10, 45). In view of this, we suggested that the distance between the core A-tracks is not consistent with a side-by-side arrangement as for the UP subsites in the canonical UP element (10), and there would be turns of helix between UP-like{pu} \(^1\) subsites. Despite the distance between UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\), it would still be possible that the binding of one aCTD to one UP-like{pu} subsite cooperatively assists the second copy of aCTD to bind to the other UP-like{pu} subsite. This could be accomplished by a combination of protein-protein interaction between the two aCTDs and local DNA flexibilization. The hypersensitivity to DNase I of the region upstream to the IHF binding site (Fig. 2A), both in the absence and in the presence of IHF, could trace the DNA bending of the UP-like{pu} arising from the cooperative binding of the two aCTDs. We suggest that even in the absence of IHF, the binding of aCTD\(^1\) to one UP-like{pu} subsite may recruit an otherwise distant aCTD\(^\text{II}\) to the other UP-like{pu} subsite, and this would originate bending of the UP-like{pu}. The promoter bending introduced by IHF\(^0\) that renders the UP-like{pu} region more accessible to aCTD\(^\text{II}\) (see above) may result in mutual and stronger cooperative binding of two the aCTDs. The slight increase in the general DNase hypersensitivity and DNase I protection of positions −90 and −91 in the presence of IHF (Fig. 2A, lanes 3 and 4) might account for the strengthening of the cooperative binding of aCTD to the UP-like{pu} region. Since no evidence for the occupation of other sites different to UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\) resulted from the assay with {α(Fe)}-{α(Fe)} \(\sigma^{54}\) RNAP, we also suggest that the cooperative occupancy of the upstream DNA region involves only UP-like{pu} \(^1\) and UP-like{pu} \(^\text{II}\) subsites.

In summary, the evidence presented in this work strongly supports the notion of IHF-mediated topological switch that governs the occupancy of a promoter by RNAP through the curvature-mediated modulation of aCTD interactions with the upstream promoter region. It was shown previously that, by protein-protein interaction, transcription factors can direct the aCTD positioning on the upstream promoter region (17, 19). In
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a novel way, the positioning of αCTD on the Pu promoter would be directed predominantly by the DNA architecture.

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Recruitment of σ^{54}-RNA Polymerase to the Pu Promoter of Pseudomonas putida through Integration Host Factor-mediated Positioning Switch of α Subunit Carboxyl-terminal Domain on an UP-like Element

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