An intersubunit contact stimulating transcription initiation by *E. coli* RNA polymerase: interaction of the α C-terminal domain and σ region 4

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The C-terminal domain of the *Escherichia coli* RNA polymerase (RNAP) alpha subunit (α CTD) stimulates transcription initiation by interacting with upstream (UP) element DNA and a variety of transcription activators. Here we identify specific substitutions in region 4.2 of sigma 70 (σ^{70}) and in α CTD that decrease transcription initiation from promoters containing some, but not all, UP elements. This decrease in transcription derives from a decrease in the initial equilibrium constant for RNAP binding (K_B). The open complexes formed by the mutant and wild-type RNAPs differ in DNase I sensitivity at the junction of the α CTD and σ DNA binding sites, correlating with the differences in transcription. A model of the DNA- α CTD- σ region 4.2 ternary complex, constructed from the previously determined X-ray structures of the *Thermus aquaticus* σ region 4.2-DNA complex and the *E. coli* α CTD-DNA complex, indicates that the residues identified by mutation in σ region 4.2 and in α CTD are in very close proximity. Our results strongly suggest that α CTD, when bound to an UP element proximal subsite, contacts the RNAP σ^{70} subunit, increasing transcription. Previous data from the literature suggest that this same σ - α CTD interaction also plays a role in transcription factor-mediated activation.

[**Keywords:** RNA polymerase; promoter; UP element; α subunit; σ subunit; transcription activation]

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The *Escherichia coli* RNA polymerase (RNAP) core enzyme ($\alpha_2\beta\beta'\omega$) is capable of transcription elongation, but only the holoenzyme ($\alpha_2\beta\beta'\omega\sigma$) containing one of the seven σ factors can carry out specific transcription initiation. Promoter recognition by the holoenzyme containing the major σ factor ($E\sigma^{70}$) occurs through interactions of σ with up to three promoter modules. The -10 hexamer (consensus sequence 5'-TATAAT-3') is recognized by σ region 2.3-2.4 (Gross et al. 1998); the extended -10 region (consensus 5'-TGTGn-3') is recognized by σ region 3.0 (Burr et al. 2000; Murakami et al. 2002b); and the -35 hexamer (consensus 5'-TTGACA-3') is recognized by σ region 4.2 (Campbell et al. 2002). In addition, the C-terminal domains of the two α subunits (α CTDs) are flexibly tethered to the α N-terminal domains (α NTDs; Blatter et al. 1994) and at some promoters interact with specific sequences referred to as UP elements located upstream of the -35 hexamer (Ross et al. 1993, 1998; Gourse et al. 2000).

The UP element consensus sequence was determined by in vitro selection (full UP element consensus; Estrem

et al. 1998). These results and other data suggested that UP elements can consist of one or two subsites, proximal and distal (Fig. 1). Consensus sequences for the proximal and distal subsites, each of which can interact with one of the two α CTDs, were then identified individually by in vitro selection (Estrem et al. 1999). Extensive genetic analyses by random and alanine scanning mutagenesis identified seven amino acid side chains in α CTD critical for DNA binding (Gaal et al. 1996; Murakami et al. 1996). These residues reside in two helix-hairpin-helix (HhH) motifs (Shao and Grishin 2000) that interact with UP element DNA in and across the minor groove (Ross et al. 2001; Yasuno et al. 2001). A high resolution X-ray structure of α CTD bound to DNA confirmed the roles of the two HhH motifs of α CTD in DNA recognition, and of five of the seven crucial side chains (R265, N268, G296, K298, S299) in direct or water-mediated DNA contacts (Benoff et al. 2002).

The α CTD also plays a critical role in transcription activation by serving as a target of transcription factors [e.g., cyclic AMP receptor protein (CRP), Fis] that bind upstream of the core promoter region (for review, see Hochschild and Dove 1998; Busby and Ebright 1999), either adjacent to the -35 hexamer (Class II promoters; Savery et al. 1998; McLeod et al. 2002), or further up-

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Figure 1. DNA sequences from -27 to -59 (with respect to the transcription start site) for *rrn* P1 promoter constructs. The *top* group of promoters contains the *rrnB* P1 core promoter, and the *bottom* group contains the *rrnD* P1 core promoter. The sequences designated "4547" and "4549" contain the consensus proximal subsites derived by in vitro selection (Estrem et al. 1999). "*rrnB* P1 proximal" contains the *rrnB* P1 natural proximal subsite and an *Eco*RI linker upstream of position -50 (Rao et al. 1994) and was used in a previous study of effects of σ^{70} region 4.2 mutants (Lonetto et al. 1998). "*rrnB* P1 full" contains the natural *rrnB* P1 full UP element. "Consensus full" contains the full UP element derived by in vitro selection (Estrem et al. 1998). "*rrnB* no UP" contains the "SUB" sequence fused to the *rrnB* P1 core promoter (Rao et al. 1994). "*rrnD* P1 full" contains the natural *rrnD* P1 full UP element. "*rrnD* no UP" is the *rrnD* P1 core promoter; sequence upstream of the *Eco*RI linker is derived from pRLG770 (Ross et al. 1998; Table 1). The -35 hexamer is shown in lowercase and boldface. UP element sequences are in uppercase and boldface.

		-50	-40	-30
4547:		gactgcagtggtta AAAAAAGT actt gtcagg cc		
4549:		gactgcagtggtta AAAAAAGA actt gtcagg cc		
<i>rrnB</i> P1 Proximal:		tagaattcc TTTTAAATTTCT ctt gtcagg cc		
<i>rrnB</i> P1 Full:		ag AAAAATTATTTTAAATTTCT ctt gtcagg cc		
Consensus Full:		gg AAAAATTTTTC CAAA AGT actt gtcagg cc		
<i>rrnB</i> No UP:		gactgcagtggttacctaggaatctt gtcagg cc		
<i>rrnD</i> P1 Full:		ag AAAAAAGATCAAAAAATA actt gtgc aaaa		
<i>rrnD</i> No UP:		tcgtcttcaagaattccgaatactt gtgc aaaa		
		Distal Subsite	Proximal Subsite	-35
		UP Element Region		

stream (Class I promoters; Aiyar et al. 2002; Savery et al. 2002). In addition to (or instead of) interacting with α CTD, some activators binding at a Class II position can interact with σ^{70} region 4 (Kuldell and Hochschild 1994; Li et al. 1994; Kim et al. 1995; Lonetto et al. 1998; Landini and Busby 1999; Rhodius and Busby 2000; Nickels et al. 2002; Pande et al. 2002) or with the α N-terminal domain (NTD; Niu et al. 1996). In both Class I and II activation complexes, α CTD can interact in a non-sequence-specific fashion with DNA adjacent to the activator binding site.

In addition to solution and X-ray structures of α CTD and the α CTD-DNA complex (Jeon et al. 1995, 1997; Wada et al. 2000; Benoff et al. 2002), structures are available of the RNAP holoenzymes from *Thermus aquaticus* (Murakami et al. 2002a) and *Thermus thermophilus* (Vassilyev et al. 2002), of the *T. aquaticus* holoenzyme bound to a short promoter fragment (Murakami et al. 2002b), and of *T. aquaticus* region 4 of σ bound to a DNA fragment containing the -35 element (Campbell et al. 2002). These structures provide detailed information about many of the intersubunit interactions and RNAP-promoter interactions in the transcription initiation complex. However, because the flexibly tethered α CTD is not resolved in any of the RNAP core or holoenzyme X-ray structures, no structural information is available concerning potential interactions of α CTD with other RNAP subunits or with DNA in the context of an RNAP-promoter complex.

The location of the proximal UP element subsite, where α CTD binds centered at approximately -42 (Newlands et al. 1991; Estrem et al. 1999; Ross et al. 2001), suggested that α CTD might, like some activators at Class II promoters, interact with the region of σ^{70} bound to the -35 hexamer (region 4). Although a previous study using a promoter containing an *rrnB* P1 UP element did not support the model that α CTD- σ^{70} region 4 interactions are important for UP element function (Lonetto et al. 1998), we have reexamined this issue in the context of more recently identified UP element sequences consisting of the proximal subsites isolated by in vitro selection (Estrem et al. 1999). Hydroxyl radical protection and missing base interference footprinting studies suggested

that these consensus proximal subsites were protected better by α CTD when in an UP element lacking a distal subsite (Estrem et al. 1999; Ross et al. 2001; data not shown) and have larger stimulatory effects on transcription than the UP element tested previously (~170-fold compared with ~20-fold in vivo; Estrem et al. 1999).

Here, we report the identification of specific substitutions in σ^{70} region 4 and in α CTD that reduce the function of certain UP elements in vivo and in vitro. We identify a position in promoter complexes containing these UP elements where accessibility to DNase I cleavage is altered by the α and σ mutants. This position is at the junction of the α and σ binding sites. We construct a model for the α CTD-DNA- σ^{70} region 4 ternary complex based on our previous identification of the precise position where α CTD interacts with the proximal subsite in the RNAP holoenzyme-promoter complex (Ross et al. 2001), on the X-ray structure of *T. aquaticus* σ region 4 bound to DNA (Campbell et al. 2002), and on the x-ray structure of *E. coli* α CTD bound to DNA (Benoff et al. 2002). Our genetic, biochemical, and modeling studies strongly suggest there is a functionally important interaction between specific surface-exposed residues in α CTD and σ^{70} . Together with data from the literature concerning residues in α CTD required for transcription activation at Class I promoters, our work suggests that this interaction can contribute not only to the mechanism of UP element function, but also to activation by transcription factors.

Results

Substitutions in σ^{70} region 4 reduce UP element function in vivo

We assessed the role of σ^{70} in UP element function by testing a previously described library of single alanine substitutions in region 4.2 (Lonetto et al. 1998). Strains were constructed in which a λ prophage carried *lacZ* fused to either an *rrnB* P1 promoter containing a consensus proximal subsite (UP element 4547; Fig. 1) or an *rrnB* P1 core promoter lacking an UP element. [The UP element examined in a previous study (Lonetto et al. 1998),

the *rrnB* P1 natural proximal subsite, is also shown in Fig. 1.] The host chromosome also contained *rpoD* fused to the *trp* promoter, resulting in control of wild-type σ^{70} expression by the *trp* repressor (Lonetto et al. 1998). These cells were then transformed with plasmids expressing mutant or wild-type σ^{70} (pGEX-2T σ^{70} and derivatives; see Materials and Methods).

β -Galactosidase activities from the promoter-*lacZ* fusions were determined in the *rpoD* plasmid-containing strains when the chromosomally encoded σ^{70} was repressed (see Materials and Methods). Effects of 14 alanine substitutions in σ on UP element function were determined by measuring the ratio of activities of promoters with and without UP elements and then calculating the fraction of the UP element effect in a strain expressing mutant σ versus wild-type σ (Fig. 2; see also Materials and Methods). One σ^{70} substitution, R603A, reduced the effect of the consensus proximal subsite by ~75%. Two substitutions, K593A and K597A, reduced the function of this UP element by ~25%–35%.

Two other σ^{70} mutants, E591A and R596A, had phenotypes not specific to UP element function. E591A reduced transcription from reporter fusions both containing and lacking UP elements by ~25% (data not shown). Although the corresponding residue in *T. aquaticus* σ^A (E416) does not contact DNA directly in the crystal structure (Campbell et al. 2002), it has been proposed that this substitution alters σ interactions with the -35 hexamer indirectly (Nickels et al. 2002). The effect of R596A on UP element function could not be evaluated in vivo, because this substitution led to induction of λ prophages carrying the promoter-*lacZ* fusions and cell lysis. R596 has been implicated previously in activation of the λP_{RM} promoter by λ CI (Li et al. 1994; Nickels et al.

2002). Therefore, the effect of this mutant σ on UP element function was examined only in vitro (see following).

The effects of the σ mutants on the function of a different UP element, the consensus full UP element (Estrem et al. 1998), were also measured. None of the σ mutants reduced the function of this UP element by >20% (data not shown).

Substitutions in σ^{70} region 4 reduce UP element function in vitro

To determine whether the effects of σ R603A, K593A, or K597A on proximal subsite function in vivo were direct, we examined transcription by RNAPs containing these mutant σ subunits in vitro. Figure 3 shows the results from in vitro transcription experiments in which the same preparation of core RNAP was saturated with wild-type or mutant σ subunits (see Materials and Methods for details). Similar results were obtained with RNAPs containing σ subunits with histidine (his) tags or GST tags (data not shown).

Transcription was measured from promoters containing or lacking UP elements (transcripts from representative gels are pictured in Fig. 3A; see Fig. 1 for promoter sequences). The UP element effects with the σ mutant RNAPs were then calculated as ratios of the UP element effects with the wild-type RNAP (Fig. 3B–E). σ R603A reduced the effects of each of two different consensus proximal subsites (UP elements 4549 and 4547) by ~50%–60% and of the natural *rrnD* P1 full UP element by almost 40%. In contrast, R603A did not reduce the effects of the natural *rrnB* P1 or consensus full UP elements (Fig. 3B). Thus, the results obtained in vitro were fully consistent with the results obtained in vivo, indicating that the effect of σ region 4 on UP element function is direct.

The effects of σ substitutions K597A (Fig. 3C) and K593A (Fig. 3D) on UP element function in vitro were smaller than the effects of R603A, reducing 4549 and 4547 UP element function by only ~15%–30%, and the effects of σ K597A and σ K593A on *rrnD* P1 UP element function were not different from the effect of wild-type σ within error. Like σ R603A, σ K597A and σ K593A did not decrease the effect of the consensus full or *rrnB* P1 full UP elements. Because we were not able to test the effect of σ R596A in vivo (see earlier), we measured its effect on transcription in vitro. σ R596A did not decrease UP element function in vitro (Fig. 3E).

In a few cases, effects of the *rrnB* full or consensus full UP elements were reproducibly enhanced by the σ substitutions both in vitro (Fig. 3) and in vivo (data not shown). We will return to this subject in the Discussion.

Identification of α CTD mutants causing defects in UP element proximal subsite function

The identification of σ R603 as a residue important for the function of some (but not all) UP elements suggested

Figure 2. Effects of σ^{70} region 4 alanine substitutions on UP element function in vivo. β -Galactosidase activities were determined from promoter-*lacZ* fusions in strains with *rrnB* P1 constructs containing either proximal subsite 4547 or the "SUB" sequence (no UP element) and with plasmids encoding either wild-type or mutant σ . Expression of the host *rpoD* gene was repressed (see Materials and Methods). The "UP element effect" in the presence of the plasmid-encoded mutant σ was determined as the ratio of activities of promoters with or without the UP element, expressed as a fraction of the UP element effect in the presence of plasmid-encoded wild-type σ (see Materials and Methods).

Figure 3. Effects of σ^{70} region 4 alanine substitutions on UP element function in vitro. Promoters containing or lacking UP elements were transcribed in vitro with wild-type or σ mutant RNAPs. (A) Transcript bands from representative gels. Templates contained either the *rrnB* P1 or *rrnD* P1 core promoter and the UP element indicated above the images of the transcripts (see Fig. 1 for sequences). RNAP holoenzymes were reconstituted from core RNAP and the purified wild-type or mutant σ indicated at the left of each set of transcripts. Duplicate reactions are shown for most of the promoters. (B–E) Effects of σ mutant RNAPs on UP element function. To calculate the “UP element effect” for each RNAP (mutant and wild type), we compared the amount of transcript from a promoter with an UP element to that from the same promoter lacking an UP element (“no UP”). The bars in the histograms represent the UP element effects obtained with the mutant holoenzyme divided by that with the wild-type holoenzyme. Error bars indicate standard deviations from at least three independent experiments. A ratio of 1.0 (horizontal line) indicates no difference in the UP element effect using the wild-type and mutant holoenzyme. (B) σ RA603A RNAP. (C) σ KA597A RNAP. (D) σ KA593A RNAP. (E) σ RA596A RNAP.

that σ region 4 might interact with α CTD, but that this interaction might occur (or have functional consequences) only in some contexts. To identify residues in α CTD that might interact with σ region 4, we used our library of *rpoA* constructs coding for single alanine substitutions at each position in the α CTD (α residues 255–329; Gaal et al. 1996; Kainz and Gourse 1998). We screened for mutants with a phenotype similar to that observed for the σ mutants described earlier: a defect in consensus proximal subsite function and not in *rrnB* P1 full UP element function. Effects of a subset of the alanine library on *rrnB* P1 full UP element function were reported previously (residues 255–273 and 291–302; Gaal et al. 1996; Murakami et al. 1996). Effects of the remaining substitutions in α CTD on full UP element function, as well as effects of the entire library on consensus proximal subsite function, have not been reported previously.

Plasmids carrying wild-type or mutant *rpoA* genes were transformed into strains containing one of three chromosomal promoter–*lacZ* fusions (*rrnB* P1 with the consensus proximal subsite, with the native *rrnB* P1 full UP element, or without an UP element). Effects of α substitutions on UP element function were determined by measuring the ratio of activities from promoters with and without UP elements and then determining the ratio of these UP element effects in strains expressing mutant versus wild-type α from the *rpoA* plasmids (Fig. 4; see also Materials and Methods).

Twelve substitutions in plasmid-encoded α reduced the effect of consensus proximal subsite 4547 by at least 40% (D259A, E261A, T263A, R265A, N268A, C269A, L290A, L295A, G296A, K298A, S299A, and E302A; Fig. 4A), despite moderation of the effect of the mutant subunit by the presence of the wild-type *rpoA* gene on the

Figure 4. Effects of single alanine substitutions in α CTD on UP element function in vivo. Strains contained plasmids encoding either wild-type or mutant *rpoA* alleles and *rrnB* P1 promoter-*lacZ* fusions containing an UP element [the consensus proximal subsite 4547 (A) or the native *rrnB* P1 full UP element (B)] or lacking an UP element. The “UP element effect” for each α mutant was determined as the ratio of β -galactosidase activities from promoters with and without an UP element. The bars in the figure indicate the UP element effects with each plasmid-encoded α mutant as a fraction of the UP element effect with plasmid-encoded wild-type α (see Materials and Methods). Striped bars indicate a class of α mutants (D261A or E259A) reducing the effect of the consensus proximal subsite approximately twofold, but not reducing the effect of the full UP element. Dark bars indicate alanine substitutions that reduced the effects of both the consensus proximal subsite and the *rrnB* P1 full UP element. The effects of these substitutions on transcription are attributable to defects in UP element DNA binding (see text). Open bars indicate positions that are alanine residues in wild-type α . Asterisks indicate positions where the plasmids contained either additional mutations or (for 312) wild-type *rpoA* (see Materials and Methods).

host chromosome. Two of these substitutions, D259A and E261A, reduced consensus proximal subsite function in vivo by 50%–60% but had little if any effect on *rrnB* P1 full UP element function (i.e., <10% different from wild-type α ; Fig. 4B) or on consensus full UP element function (data not shown). α E261A also reduced *rrnD* P1 and 4549 UP element function by about 40% (data not shown), whereas D259A had smaller effects on the function of these UP elements (15%–25%; data not shown). D259A and E261A therefore constitute a distinct phenotypic class whose effects on UP element function are similar to those of σ R603A. In the α CTD–UP element complex, D259 and E261 are surface exposed and relatively distant from the DNA (Ross et al. 2001; Benoff et al. 2002). Furthermore, it was shown previously that substitutions for E261 do not affect DNA binding (Tang et al. 1994; Savery et al. 1998). Therefore, these residues are candidates for direct interaction with σ^{70} region 4.

The other 10 substitutions reduced the effect not only of the consensus proximal subsite, but also of the *rrnB* full UP element (Fig. 4B). Effects of all 10 substitutions on DNA binding can be rationalized from the positions of these residues in the X-ray structure of the α CTD–DNA complex (Benoff et al. 2002), and most have been reported previously to disrupt binding of α to UP element DNA in vitro (Gaal et al. 1996; Murakami et al. 1996).

Several other alanine substitutions in α CTD (D258A,

L260A, L262A, V264A, S266A, I303A, and V306A) reduced consensus proximal subsite function by ~30%, that is, by less than the substitutions listed earlier. Like α D259A and α E261A, α D258A reduced proximal subsite 4547 (but not full UP element) function in vivo. The other substitutions reduced both 4547 and full UP element function, and generally affected full UP element function more than proximal subsite function (Fig. 4). We suggest that these residues may have indirect effects on DNA binding that are most prominent in the context of full UP elements. L314A, a residue that is surface exposed but located far from either the DNA binding surface of α CTD or from D259 and E261, also reduced full UP element function (by ~50%) and had a slight effect on proximal subsite function. Further characterization revealed that the plasmid encoding L314A contained an additional, previously undetected, mutation coding for an arginine substitution for L281. L281 is not surface exposed. We suspect that introduction of a large basic side chain at this position disrupts the (HhH)₂ fold sufficiently to alter the DNA binding surface indirectly.

α E261A and D259A reduce UP element proximal subsite function in vitro

To determine whether E261A and D259A affect UP element function directly, we reconstituted RNAPs using purified wild-type α , α E261A, or α D259A, and transcription was measured in vitro from promoters contain-

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Figure 5. Effects of α E261A and α D259A on UP element function in vitro. Promoters containing or lacking UP elements were transcribed in vitro with wild-type or α mutant RNAPs. (A) Transcript bands from representative gels. Templates contained either the *rrnB* P1 or *rrnD* P1 core promoter and the UP element indicated above the images of the transcripts (see Fig. 1 for sequences). RNAP holoenzymes were reconstituted from purified subunits using the wild-type or mutant α indicated at the left of each set of transcripts (Materials and Methods). Duplicate reactions are shown. (B–C) Effects of α mutant RNAPs on UP element function. To calculate the “UP element effect” for each RNAP (mutant and wild type), we compared the amount of transcript from a promoter with an UP element to that from the same promoter lacking an UP element (“no UP”). The bars in the histograms represent the UP element effects obtained with the mutant holoenzyme divided by the UP element effects obtained using the wild-type holoenzyme. Error bars indicate standard deviations from at least three independent experiments. A ratio of 1.0 (horizontal line) indicates no difference in UP element effect using the wild-type and mutant holoenzyme. (B) α E261A RNAP. (C) α D259A RNAP.

ing or lacking UP elements (Fig. 5A). Consistent with the results obtained in vivo, E261A reduced the function of the consensus proximal subsites (4547 and 4549) by ~40% and of the *rrnD* P1 full UP element by ~30%, and had little or no effect on the function of the *rrnB* P1 full and consensus full UP elements (Fig. 5B). Although the effects of E261A were relatively small, they were very reproducible in multiple experiments and with different RNAP preparations. Although only a subset of these templates was tested with D259A RNAP, it had similar, albeit not identical, effects on UP element function as E261A RNAP (Fig. 5C). As in the in vivo experiments, D258A RNAP had smaller effects on proximal subsite function than either E261A or D259A RNAP (data not shown).

σ R603A decreases initial binding of RNAP (K_B)

Previous kinetic analyses indicated that the native *rrnB* P1 UP element increased the overall association rate (k_a) of RNAP with the *rrnB* P1 promoter ~20–30 $\times$ in vitro (Rao et al. 1994). Most of this stimulation resulted from increasing the initial binding constant, K_B , but there also appeared to be an increase in the rate of isomerization to the open complex, k_f (Rao et al. 1994). Later studies showed that an UP element sequence fused to a synthetic promoter could facilitate isomerization (Strainic et al. 1998). We carried out kinetic studies to determine the step(s) responsible for the effect of σ R603A on UP element function.

Concentrations of active wild-type and σ R603A reconstituted RNAPs were determined, and the rates of formation of heparin-stable complexes on a promoter containing a consensus proximal subsite (UP element 4549) were measured as a function of active enzyme concentration using a filter binding assay (Roe et al. 1984; Rao et al. 1994; Barker et al. 2001). The composite association rate constant, k_a , has contributions from both initial binding (K_B) and subsequent isomerization (k_f) steps ($k_a = K_B k_f$). k_a and k_f were determined from both linear and nonlinear analysis of k_{obs} as a function of RNAP concentration (see Materials and Methods). A linear representation of the data [Tau plot; $1/k_{obs}$ vs. $1/[RNAP]$; McClure 1980] is shown in Figure 6.

The value of k_a for σ R603A RNAP was eightfold smaller than for wild-type RNAP under these solution conditions, even though isomerization was slightly faster (Fig. 6 legend). As a consequence, the equilibrium binding constant K_B ($K_B = k_a/k_f$) for σ R603 RNAP was 14-fold smaller than for wild-type RNAP. Kinetic parameters determined for RNAPs reconstituted with α E261A versus wild-type α indicated that α E261A reduced the

Figure 6. Effects of σ R603A on association of RNAP with the *rrnB* P1 promoter containing the consensus proximal subsite 4549. Association rate constants, k_{obs} , were determined at different RNAP concentrations, and the results are shown as a Tau plot (McClure 1980). The kinetic parameters, $k_a = K_B k_f$ (determined from the nonlinear fit described in Materials and Methods), indicated that σ R603A reduced k_a , the observed second order association rate constant, by eightfold, all of which was caused by a decrease in the initial binding constant, K_B . Kinetic parameters: k_a (wild-type RNAP) = $9.7 \pm 0.8 \times 10^6$ ($M^{-1}sec^{-1}$); k_a (R603A RNAP) = $1.2 \pm 0.1 \times 10^6$ ($M^{-1}sec^{-1}$); k_f (wild-type RNAP) = $1.3 \pm 0.1 \times 10^{-2}$ (sec^{-1}); k_f (R603A RNAP) = $2.3 \pm 0.6 \times 10^{-2}$ (sec^{-1}).

association rate 2.7-fold, and, as with σ R603A RNAP, the reduction resulted largely from a change in K_B (data not shown). The complexes formed with either wild-type or σ R603A polymerase showed no dissociation over the time course of the experiments (data not shown). The combination of these association and dissociation kinetic data demonstrate that the σ region 4- α CTD interaction primarily affects recruitment of RNAP to the promoter, rather than subsequent steps in the transcription mechanism. The larger defect caused by σ R603A in Figure 6 versus Figure 3 could derive either from the greater sensitivity of the kinetic assay or from an UP element-independent component contributing to the effect of the mutant. Studies addressing this issue are in progress.

σ R603A and α E261A alter the structure of the transcription initiation complex

We asked whether α E261A or σ R603A affected the structure of the RNAP-promoter complex using DNase I footprinting. A selection of these footprints is shown in

Figure 7A–D. On the 4547 promoter (a template where these mutants reduced UP element function both in vitro and in vivo), the footprints with α E261A and wild-type RNAP were virtually identical, except at position –38 on the template strand (numbering refers to the phosphodiester bond connecting –38 and –39). This position, which is located at the junction of the proximal subsite and the –35 element, was more accessible to DNase I in the α E261A RNAP complex (Fig. 7A) than in the wild-type RNAP complex (Fig. 7A, inset). Likewise, on the 4549 promoter (another template where these mutants reduced UP element function both in vitro and in vivo), template strand position –38 was more accessible to DNase I in the σ R603A (Fig. 7B, see inset) or α E261A (data not shown) RNAP complex than in the wild-type RNAP complex. In contrast, cleavage at –38 was not altered in promoter complexes containing the mutant RNAPs and the *rrnB* P1 full UP element (σ R603A RNAP, Fig. 7C; α E261A RNAP, data not shown), where these mutants did not affect UP element-dependent transcription. Similarly, altered cleavage by DNase I was not

Figure 7. DNase I footprints of promoters in the presence of wild-type and mutant RNAPs. Footprints were performed on *rrnB* P1 containing UP element 4547 (A), UP element 4549 (B), the *rrnB* P1 full UP element (C), or the *lacUV5* promoter (D; see Materials and Methods). The regions in DNA protected from DNase I cleavage and the regions corresponding to the UP elements are indicated under the gel images. The RNAP used in each lane is indicated at the left of the gel images: wild-type RNAP (WT), α E261A RNAP (α E261A), σ R603A RNAP (σ R603A), or no RNAP (-). Superimposed scans of gel lanes are shown above the images. Scans corresponding to footprints with mutant RNAPs are red, footprints with wild-type RNAP are blue, and footprints without RNAP are gray. Red arrows indicate position –38, and this region is magnified in the inset in each panel. Footprints of an E261A RNAP–4549 promoter complex were very similar to those shown in A, and footprints on an E261A RNAP–*rrnB* P1 full UP element promoter complex were very similar to those shown in C (data not shown). On the 4547 (A) and 4549 (B) promoters, position –38 was more sensitive to DNase I cleavage in the complexes formed with the mutant RNAPs than with the wild-type RNAP.

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observed with σ R603A versus wild-type RNAP on the *lacUV5* promoter (which does not contain an UP element; Ross et al. 1998; Fig. 7D). These results suggest that the RNAP substitutions alter the structure at the junction of the binding sites for α CTD and σ region 4, but only at promoters where the mutant RNAPs affect UP element-dependent transcription (see also Discussion).

Structure-based identification of an interaction between α CTD and σ region 4

Our studies suggest that an interaction between σ R603 and α D259 and/or E261 plays an important role in UP element-dependent transcription at certain promoters. High resolution X-ray structures of *E. coli* α CTD bound to a DNA fragment containing an A-tract (Benoff et al. 2002) and of *T. aquaticus* σ^A domain 4.2 bound to a DNA fragment containing a -35 hexamer (Campbell et al. 2002) indicate that these residues are surface exposed and relatively distant from the DNA. (*T. aquaticus* σ^A region 4.2 is 79% similar or identical to *E. coli* σ^{70} region 4.2.) In order to determine whether σ R603 and α D259 and/or E261 are likely to be in close proximity, we juxtaposed the structures of the α CTD–DNA complex and the σ region 4–DNA complex to model a ternary complex, positioning the center of the α CTD binding site precisely 6 bp from the upstream end of the -35 hexamer (promoter position -42 ; sequence numbering from *rrnB* P1). This placement of α CTD relative to the -35 element was based on detailed information provided by protection and interference footprinting studies of RNAP on *rrnB* P1 promoters containing consensus proximal sub-sites (UP elements 4547 and 4549; Ross et al. 2001).

Figure 8 indicates that when the α CTD and σ region 4 binding sites are thus aligned, residues D259 and E261 of α CTD are in very close proximity to R429 of *T. aquaticus* σ^A (which corresponds to R603 of σ^{70} ; σ^{70} residue numbers are used on the figure), in complete agreement with their proposed interaction. σ^{70} Residues K593 and K597 (corresponding to σ^A K418 and K422, respectively), which had small effects on UP element-dependent transcription (Figs. 2, 3), are not in the proposed σ – α interface and are discussed following. Position -38 , whose accessibility to DNase I was increased when the proposed functional interaction between α and σ was altered by mutation, is on the DNA surface opposite to the binding sites of α and σ . This suggests that the functional interaction between α and σ distorts the DNA, altering the width of the minor groove, and thereby reducing access to cleavage by DNase I (see Discussion).

Discussion

Interaction of α CTD and σ^{70} region 4.2

The genetic and biochemical results presented here strongly suggest there is a functional interaction between acidic side chains D259 and E261 on α CTD and basic side chain R603 on σ^{70} . The proposed interaction is

based on several criteria: (1) the identification of alanine substitutions at these positions, leading to defects in UP element function in vivo and in vitro at the same subset of promoters; (2) the identification of a structural alteration (detected by altered DNase I sensitivity of promoter position -38) in complexes containing either α E261A RNAP or σ R603A RNAP; (3) the proximity of α residues D259 and E261 to σ residue R603 in a structure-based model of the ternary complex formed by α CTD, σ region 4, and DNA.

The structure-based model (Fig. 8) provides a clear explanation for our genetic and biochemical observations. However, we emphasize that the model is based on a complex containing region 4.2 from *T. aquaticus* σ^A , not from *E. coli* σ^{70} . Therefore, although the σ^{70} and σ^A sequences are very similar, confirmation of the model awaits solution of a structure of a ternary complex with subunits derived from the same bacterium.

Although α D259, α E261, and σ R603 are positioned appropriately to interact, our model of the ternary complex suggests that two other nearby residues in σ that slightly affected UP element-dependent transcription (K593 and K597) are unlikely to interact directly with α CTD. In the -35 hexamer–*T. aquaticus* σ^A region 4 complex (Campbell et al. 2002), K418 (σ^{70} K593) contacts the DNA backbone at the promoter position corresponding to -39 (see also Fig. 8 legend). K593 mutants also reduce activator-dependent transcription (e.g., Lonetto et al. 1998; Landini and Busby 1999; Nickels et al. 2002), suggesting that the same K593–DNA backbone interaction plays a role in both UP element function and in activation by transcription factors. *T. aquaticus* σ^A residue K422 (σ^{70} K597) does not contact DNA in the crystal structure (Campbell et al. 2002). Although the mechanism responsible for its small effect on UP element function therefore remains to be determined, one possibility is that it alters DNA binding indirectly by affecting K593. Our results do not exclude potential roles for other nearby residues in σ , although the in vivo data suggest that these effects are small, if they occur.

Our data suggest that α CTD residues E261 and D259 contact σ region 4. α D258 could also be part of the patch contacting σ because the alanine substitution at this position slightly reduced proximal subsite 4547 (but not full UP element) function. However, in our model of the complex (Fig. 8), α D258 is not as close to the σ interface as E261 and D259, suggesting that either the DNA is distorted in the actual complex, slightly changing the orientation of D258 with respect to σ , or that D258A affects UP element function indirectly by altering the E261 and/or D259 interaction with σ R603. It is possible that α subunits containing E261A, D259A, and D258A would cause greater defects in UP element-dependent transcription than the single-substitution mutants.

Context dependence of the α CTD– σ interaction

The proposed α CTD– σ interaction affects use of three different UP elements tested here: two consensus proxi-

Figure 8. Structure-based model for the interaction between α CTD and σ region 4.2. The structure of the *E. coli* α CTD–DNA complex is from Benoff et al. (2002), and the structure of the *T. aquaticus* σ^A region 4.2 to σ^A region DNA complex is from Campbell et al. (2002). α CTD is shown centered at -42 , 6 bp upstream of the upstream boundary of the -35 hexamer (position -36 ; numbering from *rrnB* P1), based on results from extensive footprinting studies of a complex containing RNAP and *rrnB* P1 promoters with UP elements 4547 and 4549 (Ross et al. 2001). DNA from -49 through -38 is from the α CTD–DNA structure, and DNA from position -37 through -28 is from the σ^A region 4–DNA complex. α CTD is shown in ribbon form in white, with α D259 and α E261 in spacefill in blue. *T. aquaticus* σ^A region 4.2 is shown in ribbon form in green (labeled with corresponding *E. coli* amino acid numbers). σ R603 (spacefill in red) corresponds to σ^A R429, and σ K593 and σ K597 (spacefill in light pink) correspond to σ^A K418 and K422, respectively. DNA is shown in stick form. The views in A and B differ by a 90° rotation. The models were constructed using Insight II. The phosphodiester linkages on both strands between positions -38 and -37 , the junction of the α CTD–DNA and σ^A region 4.2–DNA structures, are not modeled. The two complexes are positioned to produce a continuous B-form DNA helix because the degree of DNA bending, if any, at their junction is not known. The DNase I cleavage site on the template strand between -38 and -39 (referred to here as -38), which is affected by the proposed α - σ interaction (Fig. 7 and text), is indicated by a magenta sphere. The upstream DNA in the structure of σ bound to the -35 region (Campbell et al. 2002) is bent toward σ , and K593 interacts with nontemplate strand position -39 [-38 in the promoter used by Campbell et al. (2002) corresponds to -39 in *rrnB* P1]. This DNA distortion is not modeled in our complex (because the upstream DNA comes from the α CTD–DNA structure), and σ K593 is closer to -38 than to -39 .

mal subsites, originally identified by in vitro selection (Estrem et al. 1999), and the native UP element from the *rrnD* P1 promoter. The proximal subsite in the *rrnD* P1 UP element more closely resembles the consensus proximal subsites than the proximal subsites in the other three promoter constructs tested (which were unaffected by the proposed α CTD- σ interaction; *rrnB* P1 full, consensus full, or *rrnB* P1 proximal; Fig. 1). Although further studies will be required to understand the context dependence of the α CTD- σ interaction, we propose two potential (nonexclusive) explanations:

(1) The context dependence of the α CTD- σ interaction reflects differing contributions of the proximal subsite to overall UP element function. This model is sug-

gested by results from interference footprinting experiments, in which missing bases in the proximal subsite greatly reduced RNAP binding to a promoter containing only the consensus proximal subsite, but did not affect binding to promoters containing the native *rrnB* P1 full UP element or the consensus full UP element (Ross et al. 2001; data not shown). Furthermore, the proximal subsite was less strongly protected from hydroxyl radical attack by α CTD in promoter complexes containing both subsites than in complexes containing only the proximal subsite (Newlands et al. 1991; Estrem et al. 1999).

It is possible that, in some full UP elements, a reduced contribution of the proximal subsite to transcription re-

sults from a decrease in α CTD occupancy of this subsite, thereby reducing effects of the proposed α - σ interaction. The mechanism by which the distal subsite might reduce occupancy of the proximal subsite remains unclear, however. Perhaps the distal subsite in some UP elements binds both α CTDs at closely adjacent positions along the minor groove, as observed in the crystal of the α CTD-DNA complex (Benoff et al. 2002). This configuration would explain why the length of the protected region is greater for the distal subsite than for the proximal subsite in hydroxyl radical footprints and missing base interference footprints of full UP elements (Newlands et al. 1991; Estrem et al. 1998, 1999; Ross et al. 2001). We speculate that, at some promoters containing two UP element subsites, transcription might be favored by having both α CTDs positioned at the distal subsite. The slight transcription enhancement observed at these promoters by mutations that inhibit α - σ interactions might be explained by reduced competition for α CTD at the distal subsite by α CTD bound at the proximal subsite (see Results).

(2) The context dependence of the effect of the α CTD- σ interaction could also reflect effects of local DNA sequence near the junction of the α and σ binding sites. Slight differences in the mode of DNA binding might affect the spatial separation of the relevant side chains in α and σ . For example, differences in the proximal subsite DNA sequence could affect the trajectory of the DNA and thereby the orientation of the bound α CTD in the promoter complex. Binding of σ region 4.2 to the -35 hexamer induces a bend in the DNA of about 36° (Campbell et al. 2002), facilitating interactions between σ and the DNA backbone on the nontemplate strand immediately upstream of the -35 hexamer. It is conceivable that differences in local DNA sequence could affect these σ -DNA backbone interactions, thereby affecting the orientation of σ R603 at the α - σ interface.

We note that the extent of DNase I cleavage of template strand position -38 in wild-type RNAP-consensus proximal subsite promoter complexes differs from that in some other promoter complexes, where cleavage at this position is enhanced by binding of RNAP (Fig. 7C,D; Ross et al. 1993; Craig et al. 1995). We suggest that the major DNA bend proposed to occur at this position in many promoter complexes (Craig et al. 1995) is less pronounced when there is an interaction between α CTD and σ^{70} region 4.

α CTD- σ interactions at other promoters

It is likely that the α CTD- σ interactions described here are important for many bacterial promoters. UP elements are present in promoters transcribing rRNAs, tRNAs, and mRNAs, and good proximal subsites are more frequent than good distal subsites or good full UP elements in *E. coli* promoters (Estrem et al. 1999). In this regard, it has been reported that substitutions for E261 reduce factor-independent transcription from the

metE promoter in vitro and in vivo (Jafri et al. 1995, 1996).

The same α CTD- σ interaction that facilitates UP element proximal subsite function apparently also plays a role in activation by transcription factors at Class I promoters, where α CTD is at the same position relative to σ as at promoters with proximal subsites (Busby and Ebright 1999). α E261A reduces Class I CRP-dependent activation of the *lac* promoter (Tang et al. 1994; Savery et al. 2002) and Class I TyrR-dependent activation of the *mtr* promoter (Yang et al. 1997). *E. coli* strains haploid for E261K also display a variety of other phenotypes (Jafri et al. 1995, 1996), suggesting that this surface on α CTD plays a role in multiple cell functions.

Residues in σ^{70} region 4.2 have also been identified as activation determinants at several Class II promoters, for example, *alkA* (Landini and Busby 1999), *melRcon* (Lonetto et al. 1998), *narG* and *dmsA* (Lonetto et al. 1998), and λP_{RM} (Nickels et al. 2002), where transcription factors bind just upstream of the -35 hexamer. Not surprisingly, different amino acid side chains in σ may interact with different activator proteins at these promoters: R603 for CRP, FNR, and Ada (Lonetto et al. 1998; Landini and Busby 1999) and R588 for λ cI (Nickels et al. 2002). Therefore, at Class II promoters, σ region 4 interactions with activator proteins might function like σ - α CTD interactions at the UP element-dependent promoters described here.

It was suggested previously that σ R603A might cause a general transcription defect (Lonetto et al. 1998) because its effects on Class II CRP-dependent transcription did not depend on the putative σ interaction surface on CRP, it affected transcription from all promoters tested, and strains expressing R603A displayed reduced growth rates in the defined media used. As indicated earlier, it is possible that R603A has general effects in addition to its specific effects on UP element-dependent transcription (Fig. 3), although it did not reduce the growth rate of our strain in the rich medium used (data not shown).

Amino acid sequences C-terminal to σ^{70} R599 diverge in the alternative σ factors (Gruber and Bryant 1997). Future studies will be required to determine whether alternative σ - α interactions can contribute to transcription by other holoenzymes.

In conclusion, our results indicate that a σ region 4- α CTD interaction plays a role in transcription from a subset of UP element-dependent promoters, where an α CTD protomer binds to the DNA minor groove adjacent to the -35 hexamer, adjacent to σ region 4.2 bound in the DNA major groove. We suggest that this same interaction participates in activation by transcription factors at Class I promoters, where an α CTD protomer binds at a comparable position in the activation complex.

Ebright and colleagues (H. Chen and R. Ebright, unpubl.) independently have proposed that an interaction between the 261 determinant of α CTD and region 4 of σ^{70} plays roles in activator-dependent and UP element-dependent transcription.

Materials and methods

Bacterial strains and plasmids

Strains and plasmids are listed in Table 1. Effects of *rpoA* and *rpoD* mutations on promoter activities were measured in strains carrying promoter-*lacZ* fusions on a single copy λ prophage. Effects of *rpoA* mutations were measured in derivatives of NK5031 (Gaal et al. 1989), and effects of *rpoD* mutations were measured in derivatives of VH1000 (Gaal et al. 1997). The *trp* promoter-*rpoD* chromosomal fusion coding for wild-type σ^{70} was introduced into λ lysogens by P1 transduction from CAG20153 (Lonetto et al. 1998), with selection on LB agar containing chloramphenicol (25 μ g/mL), indole-3-acrylic acid (IAA; 0.2 mM; Sigma), and sodium citrate (50 mM). Fresh transductants were used for each set of experiments.

Plasmid pGEX-2T σ^{70} (residues 8–613) and derivatives containing individual alanine substitutions in σ region 4.2 (Lonetto et al. 1998) were kindly provided by K. Lamberg and P. Kiley (University of Wisconsin). A new version of the *rpoD* plasmid coding for the L598A substitution was constructed by J. Toplin and W. Ross using a two-step PCR procedure and Pfu DNA polymerase (Invitrogen), in which a fragment bounded by an

XhoI site (*rpoD* codon 528) and an *AatII* site (downstream of the *rpoD* gene in plasmid vector sequence) was replaced with a mutant fragment coding for the L598A substitution to form plasmid RLG4734. Plasmids for purification of N-terminally hexahistidine tagged mutant derivatives of σ^{70} were constructed using the wild-type σ expressing plasmid pHis6 σ^{70} , a derivative of pET28 (Novagen), kindly provided by C. Roberts (Cornell University). σ^{70} Mutant derivatives K593A, K597A, and R603A were constructed by replacing the *BamHI*-*HindIII* fragment from the wild-type plasmid (from σ^{70} codon 434 to vector sequence downstream of *rpoD*) with similarly digested mutant fragments generated by a two-step PCR procedure using Turbo Pfu Polymerase (Invitrogen). Additional details of constructions of plasmids coding for wild-type and alanine-substituted hexahistidine-tagged σ^{70} factors and for GST-tagged σ L598A are available on request. Sequences of all fragments inserted into *rpoD* plasmids were verified by Big Dye sequencing (UW Biotechnology Center).

Plasmids containing the *rpoA* gene coding for single alanine substitutions in the α CTD were derivatives of pHTf1 α or pREII α , as described previously (Gaal et al. 1996; Kainz and Gourse 1998). After the scans of the 74-amino acid α CTD were completed, three of the plasmids used for this purpose were

Table 1. *Strains and plasmids*

Strain	Genotype ^a	Source
VH1000	MG1655 <i>lacZ lacI pyrE+</i>	Gaal et al. 1997
NK5031	<i>lacM5265</i> Nal ^R SupF	Gaal et al. 1989
RLG957	NK5031/ λ <i>rrnB</i> Pl (-61 to +50)- <i>lacZ</i>	Ross et al. 1990
RLG4547	NK5031/ λ <i>rrnB</i> Pl (-66 to +50; proximal UP 4547)- <i>lacZ</i>	Estrem et al. 1999
RLG4549	NK5031/ λ <i>rrnB</i> Pl (-66 to +50; proximal UP 4549)- <i>lacZ</i>	Estrem et al. 1999
RLG2258	NK5031/ λ system II <i>rrnB</i> Pl (-41 to +50)- <i>lacZ</i>	Rao et al. 1994
RLG4755	VH1000/ λ system II <i>rrnB</i> Pl (-41 to +50)- <i>lacZ</i>	This work
RLG5643	VH1000/ λ system II <i>rrnB</i> Pl (-41 to +50)- <i>lacZ ptrp-rpoD</i>	This work
RLG5417	VH1000/ λ <i>rrnB</i> Pl (-66 to +50; proximal UP 4547)- <i>lacZ</i>	This work
RLG5601	VH1000/ λ <i>rrnB</i> Pl (-66 to +50; proximal UP 4547)- <i>lacZ ptrp-rpoD</i>	This work
RLG5600	VH1000/ λ <i>rrnB</i> Pl (-66 to +50; full consensus UP 4192)- <i>lacZ</i>	This work
RLG5602	VH1000/ λ <i>rrnB</i> Pl (-66 to +50; full consensus UP 4192)- <i>lacZ ptrp-rpoD</i>	This work
Plasmid	Description	Source
pRLG770	Transcription Vector	Ross et al. 1990
pRLG4210	<i>rrnB</i> Pl (-66 to +50, with SUB, an upstream sequence lacking UP element function) in pRLG770	Estrem et al. 1999
pRLG4213	<i>rrnB</i> Pl (-66 to +50, with selected proximal subsite UP element 4547) in pRLG770	Estrem et al. 1999
pRLG3289	<i>rrnB</i> Pl (-66 to +50, with selected proximal subsite UP element 4549) in pRLG770	Ross et al. 2001
pRLG3278	<i>rrnB</i> Pl (-66 to +50, with consensus full UP element 4192) in pRLG770	Estrem et al. 1998
pRLG4238	<i>rrnB</i> Pl (-66 to +50, with native UP element) in pRLG770	Estrem et al. 1998
pRLG3266	<i>rrnD</i> Pl (-60 to +1, with native UP element) in pRLG770	Ross et al. 1998
pRLG3267	<i>rrnD</i> Pl (-41 to +1); lacking an UP element) in pRLG770	Ross et al. 1998
pRLG593	<i>placUV5</i> (-60 to +39) in pRLG770	Ross et al. 1990
pGEX-2T σ^{70}	GST- σ^{70} (8-613) fusion pasmid, with wild-type or region 4.2 alanine substitutions	Lonetto et al. 1998
pRLG4734	pGEX-2T σ^{70} (8-613, L598A)	This work
pETHis6- σ^{70}	pET28 with wild-type σ^{70}	Marr and Roberts 1997
pRLG3293	pETHis6- σ^{70} K593A	This work
pRLG3295	pETHis6- σ^{70} K597A	This work
pRLG3296	pETHis6- σ^{70} R603A	This work
pHTf1 α	pHTf1 with wild-type and α 273-329 alanine substitutions	Gaal et al. 1996; Kainz and Gourse 1998; Tang et al. 1994
pREII α	pREII with wild-type or α 255-271 alanine substitutions	Tang et al. 1994; Gaal et al. 1996

^a λ Prophage carrying promoter-*lacZ* fusions are system I (Rao et al. 1994) unless otherwise indicated.

discovered to contain additional substitutions, and one plasmid (thought to code for L312A) was found to be wild type. The plasmid coding for K304A also contains a second mutation, coding for E241V; the plasmid coding for L314A also contains a mutation coding for L281R; and the plasmid coding for M316A also codes for P323S and D328N. None of these substitutions are in regions of α CTD with the potential to interact with σ according to the structure-based model, and none except L281R (see Results) affected transcription in vivo. Furthermore, because the multiple mutations in the plasmids coding for K304A and M316A had no phenotype (Fig. 4), it is unlikely that K304A or M316A when alone would exert any effect on UP element function.

Plasmids used for in vitro transcription were derivatives of pRLG770 (Ross et al. 1990) and are listed in Table 1.

In vivo promoter activity determinations

To determine effects of substitutions in α CTD or σ^{70} on promoter activity, we transformed strains containing promoter-*lacZ* fusions (see earlier) with plasmids encoding α , σ^{70} , or mutant derivatives (see earlier). Competent cells for transformation were prepared as described (Chung et al. 1989), and transformants were selected on LB agar containing ampicillin (100 μ g/mL). For strains encoding the *trp* promoter-*rpoD* fusion, selective plates also contained IAA (0.2 mM). For assay of α or σ function, fresh colonies scraped from the selective agar plates were used to inoculate LB liquid cultures with ampicillin and without IAA. Cultures were inoculated to an OD₆₀₀ ~0.02 and grown for three to four generations at 30°C; β -galactosidase activity was assayed as described (Gaal et al. 1989). Effects of alanine substitutions in α or σ on UP element function were determined by measuring the ratio of activities of promoters with and without UP elements and then calculating the fraction of the UP element effect in a strain expressing mutant versus wild-type α or σ [(activity of UP element-containing promoter in the presence of mutant subunit/activity of SUB sequence-containing promoter in the presence of mutant subunit) $\div$ (activity of UP element-containing promoter in the presence of wild-type subunit/activity of SUB sequence-containing promoter in the presence of wild-type subunit)].

RNA polymerases

RNAP holoenzymes containing wild-type or mutant α subunits were reconstituted as described previously (Gaal et al. 1996; Tang et al. 1996). RNAP holoenzymes containing wild-type or mutant σ subunits were assembled at 30°C by incubating core RNAP (a gift from R. Landick, University of Wisconsin) for 30 min with sufficient σ to saturate the core RNAP (determined by titration; ~10:1 molar ratio of purified σ to core RNAP). Purified GST-tagged σ subunits were gifts from the laboratories of C. Gross and P. Kiley. Hexahistidine tagged σ subunits were purified from the soluble fraction using nickel-agarose (Qiagen) after brief overexpression in BL21 (DE3; Novagen). His tags on σ R603A, σ K597A, σ K593A, and wild-type σ were removed by cleavage with biotinylated thrombin, followed by removal of thrombin using streptavidin agarose (Thrombin Cleavage Capture Kit; Novagen). The RNAP containing σ R596A contained a GST tag and was compared with an RNAP containing a GST-tagged wild-type σ .

In vitro transcription

Supercoiled plasmid DNAs containing promoters inserted upstream of an *rrnB* T1 terminator in vector pRLG770 (Ross et al.

1990; see Table 1) were used as templates. Transcription reactions (25 μ L) contained 0.5 nM plasmid DNA in 10 mM Tris-Cl (pH 7.9); 150 mM NaCl; 10 mM MgCl₂; 1 mM dithiothreitol (DTT); 100 μ g/mL bovine serum albumin (BSA); 200 μ M ATP, GTP, and CTP; 10 μ M UTP; and 2 μ Ci α -³²P-UTP (Perkin Elmer Life Sciences). Reactions were initiated by addition of RNAP (~2 nM) and were terminated after 15 min at 25°C by addition of gel loading solution. Equivalent active concentrations of mutant and wild-type RNAPs were used, as determined from the activities of the enzymes on a promoter lacking an UP element (*lacUV5*). Samples were analyzed by electrophoresis in 5% acrylamide-7M urea gels, and promoter-specific transcripts were quantified by phosphorimaging (Molecular Dynamics). Duplicate reactions were performed in each experiment, and most experiments were carried out at least three times.

DNAse I footprinting

DNA fragments containing the *lacUV5* promoter, the *rrnB* P1 promoters with UP elements 4547 or 4549 (consensus proximal subsites), or the *rrnB* P1 full UP element were prepared by PCR from appropriate plasmid templates (see Table 1 for sequence endpoints). Fragments were ³²P 3' end labeled and gel purified, as described (Ross et al. 2001), and labeled fragments (~0.5 nM) were incubated with 20 nM RNAP for 15 min at 37°C. Footprints comparing the effects of wild-type and σ R603A RNAP used the same core RNAP preparation and σ^{70} lacking hexahistidine or GST tags (see Results). Footprints comparing the effects of wild-type and α E261A RNAP used holoenzymes reconstituted from purified subunits. The 25- μ L reaction contained 10 mM Tris-Cl (pH 7.9), 30 mM KCl, 10 mM MgCl₂, 1 mM DTT, 100 μ g/mL BSA, 500 μ M ATP, and 50 μ M CTP. Heparin (final concentration 10 μ g/mL) was added, and after 30 sec, DNase I (0.4 μ g/mL; Worthington) was added for 30 sec, as described (Ross et al. 1990). Samples were processed and analyzed by electrophoresis on 9% acrylamide-7M urea gels, followed by phosphorimaging, as described (Ross et al. 2001).

Association kinetics

³²P end-labeled DNA fragments [~190 bp containing centrally located promoters, either *rrnB* P1 with UP element 4549 (−66 to +50) or *lacUV5* (−60 to +39)] were prepared by PCR from appropriate plasmid templates, as described earlier for footprinting (Ross et al. 2001). Concentrations of active wild-type or mutant RNAPs (both containing hexahistidine-tagged σ subunits) were determined by titration with a known concentration of *lacUV5* promoter fragment using a filter-binding assay (Roe et al. 1984). The rates of association of wild-type and σ R603A RNAP with *rrnB* P1 containing the consensus proximal subsite were measured in 10 mM Tris-Cl (pH 7.9), 30 mM KCl, 3 mM NaCl (from RNAP storage buffer), 10 mM MgCl₂, 1 mM DTT, and 100 mg/mL BSA at 25°C in the presence of the initiating NTPs (500 μ M ATP, 50 μ M CTP). The complexes formed with the wild-type and mutant RNAPs were stable for longer than 30 min following heparin addition (data not shown), consistent with previous results with the native *rrnB* P1 promoter (Gourse 1988; Borukhov et al. 1993). All experiments were done under pseudo first-order conditions, where RNAP was in large excess over promoter DNA. At time zero, RNAP was added to promoter DNA and NTPs, aliquots were removed at time intervals to tubes containing heparin (final 10 μ g/mL), and samples were filtered after 30 sec through Schleicher and Schuell NC45 nitrocellulose filters. After correcting for background retention in the absence of RNAP, observed counts per minute (cpm_{obs}) were fit to a single exponential to obtain the observed rate constant

k_{obs} : $\text{cpm}_{\text{obs}} = \text{cpm}_{\text{plateau}} - (1 - e^{-k_{\text{obs}}t})$, where $\text{cpm}_{\text{plateau}}$ is the value of counts per minute retained at equilibrium.

Data were linearly fit to $1/k_{\text{obs}}$ versus $1/(\text{RNAP})$ using the equation $1/k_{\text{obs}} = 1/k_a(\text{RNAP}) + 1/k_f$ (McClure 1980) and also fit to the nonlinear form of this equation: $[k_{\text{obs}} = k_a k_f (\text{RNAP})] / [k_a (\text{RNAP}) + k_f]$ for error propagation. In these equations, k_a is a composite second-order association constant, and k_f is the composite first-order isomerization rate constant. In general, fitting of data to the nonlinear expression for k_{obs} is necessary for proper weighting (Saecker et al. 2002). Data points in the nonlinear fit were weighted by $1/(\text{the uncertainty in } k_{\text{obs}})^2$. For this set of data, either method of fitting yielded the same values of k_a and k_f within uncertainty. All data were fit using SigmaPlot 5.0 (SSPS).

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An intersubunit contact stimulating transcription initiation by *E. coli* RNA polymerase: interaction of the α C-terminal domain and ζ region 4

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