DNA-binding determinants of the α subunit of RNA polymerase: novel DNA-binding domain architecture

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The *Escherichia coli* RNA polymerase α -subunit binds through its carboxy-terminal domain (α CTD) to a recognition element, the upstream (UP) element, in certain promoters. We used genetic and biochemical techniques to identify the residues in α CTD important for UP-element-dependent transcription and DNA binding. These residues occur in two regions of α CTD, close to but distinct from, residues important for interactions with certain transcription activators. We used NMR spectroscopy to determine the secondary structure of α CTD. α CTD contains a nonstandard helix followed by four α -helices. The two regions of α CTD important for DNA binding correspond to the first α -helix and the loop between the third and fourth α -helices. The α CTD DNA-binding domain architecture is unlike any DNA-binding architecture identified to date, and we propose that α CTD has a novel mode of interaction with DNA. Our results suggest models for α CTD-DNA and α CTD-DNA-activator interactions during transcription initiation.

[Key Words: Promoter; UP element; RNA polymerase; α -subunit; transcriptional activation]

Received September 26, 1995; revised version accepted November 2, 1995.

Escherichia coli RNA polymerase (RNAP) is a large multisubunit enzyme, consisting of two α (37 kD), one β (151 kD), and one β' (155 kD) subunits, and one of a number of alternative σ -subunits. β and β' carry out the catalytic functions of the enzyme (for review, see Chan and Landick 1994). σ is crucial for promoter recognition, with σ^{70} interacting with promoter elements located at approximately -10 and -35 with respect to the transcription start site (Dombroski et al. 1992). α has three known functions (for review, see Busby and Ebricht 1994): (1) It initiates RNAP assembly, (2) it participates in promoter recognition through direct sequence-specific α -DNA interactions, and (3) it is the target of a large set of transcription activator proteins.

The α -subunit binds to a promoter element, the upstream (UP) element, located upstream of the -35 hexamer in rRNA promoters and certain other promoters (Ross et al. 1993; Fredrick et al. 1995; W. Ross, J. Salomon, and R.L. Gourse, unpubl.). UP elements identified to date increase promoter strength as much as 30-fold in vivo and in vitro primarily by increasing the initial equilibrium constant between RNAP and DNA (Rao et al. 1994).

α consists of two independently folded domains: an amino-terminal domain (α NTD; amino acids 8–241) and

a carboxy-terminal domain (α CTD; amino acids 249–329), connected by a flexible interdomain linker (Blatter et al. 1994). α NTD contains the primary determinants for dimerization and interacts with $\beta\beta'$ during assembly, whereas α CTD contains secondary determinants for dimerization and interacts with UP elements and transcription activators during transcription initiation. RNAP reconstituted in vitro from α -subunits lacking α CTD exhibits normal basal transcription but is defective in transcription activation by class I transcription activators and is defective in UP element-dependent transcription (Igarashi et al. 1991; Ross et al. 1993). Purified α CTD is capable of sequence-specific DNA binding (Blatter et al. 1994), but the specific residues in α CTD responsible for DNA binding have not been defined. In this work we identify the individual residues in α CTD responsible for UP element binding and correlate them with individual secondary structure elements determined by nuclear magnetic resonance (NMR).

Results

Two regions of α CTD are essential for UP element function in vivo

We designed a genetic screen to identify regions of α important specifically for UP element function, that is, for defects in UP element-dependent transcription and

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not in other aspects of transcription. The *rpoA* gene encoding α is essential for viability (Ishihama et al. 1980; Hayward et al. 1991). To facilitate mutagenesis and analysis of *rpoA*, we used a partially diploid system, with a mutagenized *rpoA* gene on a multicopy plasmid, a wild-type *rpoA* gene on the chromosome, and a chromosomal promoter-*lacZ* gene fusion as a reporter of UP element function (Fig. 1A; Zou et al. 1992; Tang et al. 1994). Previously, we showed that expression of plasmid-encoded α subunits with carboxy-terminal truncations re-

duced expression from the UP element-dependent rRNA promoter, *rrnB* P1 (Ross et al. 1993).

We generated substitution mutations throughout the plasmid-encoded *rpoA* gene using PCR-mediated random mutagenesis (Zhou et al. 1991; Tang et al. 1994), transformed cells in which *lacZ* was fused to a promoter containing the *rrnB* P1 UP element, and identified colonies with reduced promoter activity on tetrazolium-lactose indicator plates (Fig. 1B). For each such isolate, plasmid DNA was prepared and introduced into a pair of tester strains with *lacZ* fused to promoters with or without an UP element (see Materials and methods). Twenty-seven independent mutants defective specifically for UP element-dependent transcription were identified in this manner. All mapped to α CTD between residues 257 and 311. Twelve of these mutants resulted in eightfold or greater defects in UP element function (Fig. 1C). These were located in two discrete clusters, residues 265–269 (designated region I) and residues 296–301 (designated region II). We conclude that regions I and II are important for UP element-dependent transcription.

To define the individual amino acid side chain determinants for UP element function, we performed alanine scans (Jin et al. 1992; Tang et al. 1994) in and flanking regions I and II. Alanine scans permit evaluation of every position within a targeted region of a protein and yield a chemically consistent set of substitutions in which all side chain atoms beyond C β (and interactions made by these atoms) are eliminated.

Plasmids encoding single alanine substitutions in residues 255–274 or 291–302 of α were introduced into reporter strains containing promoter-*lacZ* fusions with or without the UP element. The extent of the defect in UP element function caused by each substitution is shown in Figure 2. Alanine substitutions of four amino acids in region I (L262A, R265A, N268A, and C269A) and of three amino acids in region II (G296A, K298A, and S299A) reduced UP element-dependent transcription more than fourfold. We conclude that these amino acids are especially important for UP element-dependent transcription. Several substitutions at nearby positions had smaller effects. R265A had an effect nearly as great as deletion of the entire α CTD (15-fold; data not shown; Ross et al. 1993), suggesting that this substitution results in a complete loss of UP element-dependent transcription.

We tested the complete set of alanine substitution mutants for their ability to complement an *rpoA*^{ts} mutant (*rpoA112*; Ishihama et al. 1980; Hayward et al. 1991). Strikingly, there was an exact correlation between the seven mutants with fourfold or greater effects on UP element-dependent transcription and those unable to restore viability to the *rpoA*^{ts} strain at the nonpermissive temperature (Fig. 2). Six of these seven amino acid residues are invariant in all known bacterial α -subunit sequences (Gebhardt et al. 1993; Gu et al. 1995; GenBank accession nos. U32762 and L42023). The seventh amino acid, C269, is invariant in all but the *Mycoplasma* α sequence (Tan et al. 1995). These data strongly suggest that UP element-dependent transcription is essential for

Figure 1. Random mutagenesis. (A) Screening procedure. The plasmid carrying the mutagenized *rpoA*^{*} (*rpoA*^{*}) gene and the host chromosome containing the wild-type *rpoA* gene and a promoter-*lacZ* reporter fusion are indicated. (B) Two promoter-*lacZ* fusions used for screening effects of plasmid-encoded α mutants. The fusions contained the same core promoter with or without the *rrnB* P1 UP element. (C) The seven substitutions resulting in the strongest effects (eightfold or greater) on UP element function mapped to two regions of α , one including amino acids 265–269, and the other including amino acids 296–301. “Fold defect in UP function” is described in Materials and methods. The following 15 mutants displayed more modest defects (less than fourfold) in UP element-dependent transcription, and the number of independent isolates obtained for each mutant is indicated in parentheses: V257I (1), T263I (1), V264A (5), N268D (1), A272P (1), E273D (1), I278T (2), L295P (1), and G311E (2). Although G311E exhibited a slight colony color phenotype, it did not decrease *rrnB* P1 promoter activity significantly in β -galactosidase assays.

Figure 2. Alanine scan. Alanine substitutions were constructed in regions I and II of the α CTD. The α CTD sequence is displayed on the x-axis, and the y-axis label is described in Materials and methods. Amino acids 267, 272, and 274 are alanines in the wild-type protein and were not changed. An alanine substitution at amino acid 278 was also tested and had little or no effect on UP element-dependent transcription. Asterisks indicate the alanine mutants that failed to complement an *rpoA*^{ts} strain at the nonpermissive temperature (see Materials and methods).

viability. However, we cannot rule out other essential roles for these amino acids (e.g., see Liu and Hanna 1995).

Two regions of α CTD are essential for UP element-dependent transcription in vitro

To confirm and quantify the effects of the mutants leading to the strongest phenotypes in vivo, we examined wild-type α and six of the mutant α proteins in vitro, including three each from region I (R265A, N268A, and C269A) and region II (G296A, K298A, and S299A). The α subunits were overexpressed, purified, and reconstituted into RNAP in vitro with purified wild-type β , β' , and σ (Tang et al. 1995). The reconstituted enzyme preparations had the same subunit composition as RNAP purified by conventional methods and were free of wild-type α - α dimers.

In vitro transcription experiments were performed to evaluate the ability of the reconstituted enzymes to utilize the *rrnB* P1 UP element. Templates carrying *rrnB* P1 promoters with or without the UP element were transcribed by each RNAP, and the products were analyzed by denaturing gel electrophoresis (Fig. 3). Transcription by wild-type reconstituted RNAP was stimulated by the UP element. However, transcription by the holoenzymes containing R265A or G296A was not stimulated, and transcription by the N268A, C269A, K298A, and S299A mutant holoenzymes was stimulated only slightly (Fig. 3). The mutant RNAPs were also defective in UP element-dependent transcription from other promoters (data not shown). We conclude that the strong defects of these mutant RNAPs in transcription in vitro explain the strong phenotypes observed in vivo.

Two regions of α CTD are essential for UP element DNA binding in vitro

The α -subunit is a sequence-specific DNA-binding protein that interacts directly with the UP element [Ross et

al. 1993; Blatter et al. 1994]. Therefore, one explanation for the effects of the mutations on UP element-dependent transcription would be that they are defective in DNA binding. An alternative explanation would be that they reduce UP element-dependent transcription by failing to transmit a conformational change in RNAP induced by DNA binding. To test the DNA-binding characteristics of the six purified mutant α proteins (R265A, N268A, C269A, G296A, K298A, and S299A), we performed electrophoretic mobility-shift experiments using a 35-bp fragment containing the *rrnB* P1 UP element. Wild-type α binds specifically to this DNA fragment with an apparent equilibrium constant of $\sim 1 \times 10^{-7}$ M

Figure 3. In vitro transcription. Arrows indicate the positions of transcripts originating from the *rrnB* P1, RNA I, and RNA II promoters. The RNAP used in each pair of reactions (i.e., on templates containing or lacking the UP element) are identified by the α substitution present in the holoenzyme. Data are presented for the wild type and R265A, N268A, G296A, and K298A. Equivalent results were obtained with the C269A and S299A substitutions.

under our experimental conditions. The purified mutant proteins did not bind detectably to the UP element DNA fragment at concentrations up to 1.5×10^{-6} M (Fig. 4A; data not shown). We conclude that the substitutions with the strongest phenotypes in both regions I and II define positions that are essential for DNA binding.

The strong effects of the mutants on UP element-dependent transcription and the absence of effects on UP element-independent transcription suggested that the mutant holoenzymes would not interact with the UP element but would display relatively normal interactions with the core region of the promoter. To test this prediction, we performed DNase I footprints using wild-type RNAP and the six reconstituted mutant holoenzymes. Representative results are shown in Figure 4B. Wild-type RNAP protected both the core promoter region (from position +23 to approximately -40) and the UP element region (approximately -41 to about -59), and footprints displayed DNase I hypersensitive cleavage at a site within the -35 hexamer. Mutant RNAP protected the core promoter region to the same extent as did the wild-type RNAP, but it failed to protect the UP element and showed less hypersensitivity at the DNase I site in the -35 hexamer. The footprints with mutant RNAP were identical to those obtained previously with holoenzymes lacking α CTD ($\Delta 235$ and $\Delta 256$) (Ross et al. 1993). These results confirm that the defects of the mutants in UP element-dependent transcription are attributable to direct effects on UP element DNA binding.

α CTD contains a nonstandard helix followed by four α -helices

We produced milligram quantities of α CTD (amino acids 245–329) in native, ^{15}N -labeled, $^{13}\text{C}/^{15}\text{N}$ -labeled, and five selectively ^2H -labeled forms to perform multidimensional NMR spectroscopy. We obtained complete residue-specific resonance assignments and determined the secondary structure (Fig. 5).

α CTD contains a nonstandard helix (NSH) (amino acids 252–257) followed by four α -helices: α_1 (amino acids 264–272), α_2 (amino acids 278–283), α_3 (amino acids 286–292), and α_4 (amino acids 300–312). NSH and α_1 are separated by a 6 amino acid turn containing a 3 amino acid extended segment (amino acids 261–263); α_1 and α_2 are separated by a 5 amino acid turn; α_2 and α_3 are separated by a 2 amino acid turn, and α_3 and α_4 are separated by a 7 amino acid unstructured loop.

NSH has a proline at its fifth position (Fig. 5A, lines 1,12). Nonstandard geometry is indicated by the presence of the proline, which interrupts α -helical backbone H-bonding and presumably results in a kink in the helix (kink angle of 20–30° (Barlow and Thornton 1988), and by the unusual chemical shift changes (C_α , H_α) around the proline residue (Fig. 5A, lines 7–9). Numerous examples of proline-kinked α -helices have been identified previously (Barlow and Thornton 1988).

Residues in α_1 exhibit higher amide–proton exchange than residues in the other helices of α CTD (Fig. 5A, line

11). We infer that α_1 exhibits higher dynamic mobility than the other α -helices. Flexibility of α_1 may be important for function.

α_1 , α_2 , possibly α_3 , and α_4 are amphipathic, each with one face consisting exclusively of hydrophobic amino acids and one face consisting primarily of hydrophilic amino acids (Fig. 5B). It is likely that the hydrophobic faces of the α -helices are buried within the core of α CTD, whereas the hydrophilic faces are accessible on the surface. Consistent with this hypothesis, 2 amino acids of the hydrophilic face of α_1 , R265 and C269, have been shown to be solvent accessible (Goff 1984; Selutchenko et al. 1985). The structure indicates that α CTD contains 41% α -helix and 0% β -sheet, which is in excellent agreement with the prediction from CD spectroscopy (Blatter et al. 1994). However, the structure is only in partial agreement with the prediction from helix-wheel analysis that amino acids 255–270 (in the observed structure, amino acids 264–272) constitute an α -helix (Tang et al. 1994).

α CTD interacts with DNA through an α -helix and a loop

The genetic, transcription, and footprinting results described above define two regions within α CTD essential for interaction with UP element DNA. The correspondence of region I to helix α_1 and region II to the α_3 – α_4 loop is striking. Three of the four amino acids in region I at which alanine substitutions result in large defects in DNA binding are on a single facet of α_1 and comprise 100% of the nonalanine residues on this facet. All three of the amino acids in region II at which alanine substitution prevents α CTD–UP element interaction are located in the α_3 – α_4 loop.

As an independent test of the proposal that α_1 and the α_3 – α_4 loop are involved in UP element binding, we have used ^{15}N -labeled heteronuclear single quantum correlation (HSQC) NMR spectroscopy to identify amino acids of α CTD whose backbone–amide–proton resonances exhibit perturbations upon interaction with DNA. We find that the resonances of amino acids 263, 267, 268, 271, 272, and 295 are selectively broadened and exhibit large changes in chemical shift (≥ 0.1 ppm) upon interaction with DNA, and that the resonances of the remaining amino acids in the segments 260–276 and 290–300 are selectively broadened to the extent that they become undetectable in the ^{15}N -HSQC spectrum upon interaction with DNA (data not shown). We attribute selective broadening to fast exchange between free α CTD and the α CTD–DNA complex on the NMR time scale, with concomitant “chemical exchange broadening” of the backbone–amide–protein resonances of amino acids that occupy different environments in the α CTD–DNA complex (Baleja et al. 1994; Baumann et al. 1995). The NMR spectroscopic results define two regions of α CTD as potentially involved in interaction with DNA, one centered on α_1 and the other on the α_3 – α_4 loop.

The results obtained from the genetic and NMR studies are in complete agreement in defining the two deter-

Figure 4. UP element binding in vitro. (A) DNA binding by isolated α in bandshift assays. Arrows indicate the positions of free DNA and the α -DNA complex. (B) DNA binding by RNAP in DNase I footprint assays. Holoenzymes are identified by their α allele. The extent of the core promoter and UP element sequences are indicated with brackets. The solid bar illustrates the extent of protection by the wild-type RNAP; the stippled bar illustrates the extent of protection by the R265A and G296A holoenzymes. Equivalent results were obtained with N268A, C269A, K298A, and S299A.

pretation that the two determinants affect DNA binding directly. Therefore, we propose that α_1 of the α CTD constitutes a "recognition α -helix," that the α_3 - α_4 loop constitutes a "recognition loop," and that these two secondary structure elements in α make direct sequence-specific contacts with DNA in the RNAP-promoter complex.

Discussion

α CTD has a novel DNA-binding domain architecture

Our results indicate that α CTD has a DNA-binding domain architecture consisting of a NSH followed by four α -helices, with the first α -helix and the loop between the third and fourth α -helices most critical for UP element interaction.

There are >50 structurally characterized, sequence-specific DNA-binding proteins. These proteins can be grouped into classes based on DNA-binding architecture. Only two previously defined DNA-binding architectures consist of four or five α -helices. One is the so-called phage repressor fold, present in λ cI (Pabo and Lewis 1982), 434 repressor (Anderson et al. 1987), 434 Cro (Wolberger et al. 1988), P22 repressor (Sevilla-Sierra et al. 1994), Tet repressor (Hinrichs et al. 1994), and Oct-1 POU-specific domain (Assa-Munt et al. 1993; Dekker et al. 1993). In this architecture, the third α -helix serves as the recognition helix involved in direct sequence-specific protein-DNA interaction, clearly different from the situation in α CTD. The other is the architecture present in FIS (factor for inversion stimulation) (Kostrewa et al. 1991; Yuan et al. 1991), where the fourth α -helix is the recognition helix involved in direct sequence-specific protein-DNA interaction. Once again, this pattern is clearly different from the one that occurs in α CTD. Therefore, we conclude that α CTD defines a previously unknown DNA-binding architecture and a novel mode of interaction with DNA.

Implications for α CTD-DNA interactions

In most structurally characterized protein-DNA complexes, α -helices making sequence-specific contacts with DNA interact with the DNA major groove, and

minants of DNA binding. We cannot exclude the possibility that one or both of these determinants affect DNA binding indirectly (e.g., they could be required for a conformation or oligomerization change prerequisite for DNA binding). Nevertheless, we favor the simple inter-

A

B

Figure 5. Secondary structure of α CTD. (A) Secondary structure determination. Line 1 presents the sequence of α CTD, lines 2–10 present NMR spectroscopic data for α CTD (sequential NOEs, $C\alpha$ and $H\alpha$ chemical shift dispersions from random-coil values, $^3J_{HN-H\alpha}$ coupling constants, and amide-proton exchange with solvent), and line 11 presents the inferred secondary structure. For sequential NOEs, decreasing bar heights indicate strong, moderate, weak, and very weak intensities as observed in high-resolution two-dimensional NOESY and three-dimensional ^{15}N -labeled NOESY–HSQC spectra. For $^3J_{HN-H\alpha}$ coupling constants, solid squares, half-filled squares, and open squares indicate $J < 6.0$, $6.0 < J < 7.5$, and $J > 7.5$, respectively. For amide-proton exchange, solid circles indicate positions with amide-proton resonances observable after 20 min in 2H_2O . (B) Helix wheel analysis. The shaded regions represent the hydrophobic faces of the α -helices (T263, A267, L270 in α_1 ; I278, L281, and V282 in α_2 ; V287 and L290 in α_3 ; and L300, I303, L307, and G311 in α_4).

unstructured loops making sequence-specific contacts with DNA interact with the DNA minor groove (Pabo and Sauer 1992). [There are, however, exceptions: α -He-

lices interact with the minor groove in the PurR–DNA and high mobility group (HMG)–DNA complexes (Schumacher et al. 1994; Werner et al. 1995); an unstructured

loop interacts with the major groove in the p53–DNA complex (Cho et al. 1994).] We tentatively propose that α CTD binds to UP element DNA with α_1 interacting with a major groove and with the α_3 – α_4 loop interacting with an adjacent minor groove.

The α -binding site in the *rrnB* P1 UP element is located between approximately –40 and –60. Hydroxyl radical footprinting analysis (Newlands et al. 1991, 1992), deletion analysis (Rao et al. 1994), and base substitution analysis (S. Estrem, T. Gaal, W. Ross, and R.L. Gourse, unpubl.) indicate that the UP element consists of two critical DNA determinants (subsites) centered at about –42 and –52.

Two models for the α CTD–UP element interaction appear most consistent with the available information and are useful for guiding future studies. For simplicity, the features of each model include (1) interaction of α CTD with DNA as a twofold symmetric dimer, as α CTD dimerizes in solution (Blatter et al. 1994); (2) interaction with two regions of DNA, as RNAP protects two distinct regions in the UP element against hydroxyl radical attack (Newlands et al. 1991, 1992); (3) α CTD monomer-binding sites consisting of a major groove (interacting with the α_1 helix of α CTD) and a flanking minor groove (interacting with the α_3 – α_4 loop of α CTD). (However, we emphasize that it has not been demonstrated that the carboxy-terminal domains of both α monomers are essential for UP element-dependent transcriptional activation, that both α monomers interact with DNA, that α CTD dimerizes when bound to DNA, or that the interactions are twofold symmetric.)

In model I (Fig. 6A), both α subunits recognize a central major groove and one of the flanking minor grooves. In this model, the –42 and –52 regions of the UP element are protected simultaneously by one bound α CTD homodimer. In model II (Fig. 6B), both α CTD monomers interact with a central minor groove, and each monomer recognizes a separate flanking major groove. In this model each of the two protected regions in the footprint reflects an alternative functional dimer binding site.

Figure 6. Models for α CTD–UP element interaction. A and B are alternative models for the α CTD–UP element interaction. Regions of the DNA minor grooves centered at about –42 and –52 that are protected in hydroxyl radical footprints (Newlands et al. 1991) are shaded. The α_1 recognition helices in the α CTD monomers are represented by rectangles, and the α_3 – α_4 recognition loops are indicated by short, bold, curved lines. For discussion of the models, see the text.

Implications for α CTD–DNA–activator interactions

Catabolite activator protein (CAP) activates transcription at the *lac* promoter by directly contacting α CTD and thereby recruiting α CTD to promoter DNA, despite the absence of an UP element (Ebright 1993; Busby and Ebright 1994). Alanine scanning indicates that amino acids D258, D259, and E261 of α CTD (and not the residues essential for UP element binding) are those most critical for CAP-dependent transcription at the *lac* promoter (Tang et al. 1994). Alanine substitutions at positions 258–261 do not impair UP element-dependent transcription (Fig. 2) or DNA binding (Tang et al. 1994). Therefore, it has been proposed that CAP contacts amino acids 258–261 (Tang et al. 1994). These amino acids are located in the turn immediately adjacent to the proposed DNA-recognition α -helix, α_1 , of α CTD (Fig. 7). The contacting region in CAP is also immediately adjacent to its DNA-binding motif (Ebright 1993). We envision that CAP and α CTD bind to adjacent sites at the *lac* promoter, with CAP making sequence-specific protein–DNA interactions, α CTD making solely nonspecific protein–DNA interactions (Kolb et al. 1993), and CAP and α CTD making protein–protein interactions through determinants immediately adjacent to their respective DNA-binding motifs.

Determinants in α CTD located adjacent to, but not overlapping with, the residues necessary for DNA binding are critical for interaction with several other activator proteins (e.g., AraC, MelR, CysB, Ogr, Russo and Silhavy 1992; Ishihama 1993; Ebright 1993). We envision that these activators and α CTD, like CAP and α CTD, contact each other through determinants adjacent to their respective DNA-binding motifs. This arrangement is also reminiscent of that at the λP_{RM} promoter, where the λ CI protein and σ^{70} bind to adjacent DNA sites and make protein–protein interactions through determinants immediately adjacent to their respective DNA-binding motifs (Kuldell and Hochschild 1994; Li et al. 1994). Thus, the use of adjacent determinants for protein–DNA and protein–protein interactions may be a common theme for transcription activators and their targets.

Materials and methods

Random mutagenesis

Random mutagenesis was performed by PCR of a 1-kb *Xba*I–*Bam*HI *rpoA* fragment and insertion into pREII α or pHTf1 α as described (Tang et al. 1994). Plasmids were transformed into RLG3101, a derivative of NK5031 (Gaal et al. 1989) carrying a λ prophage (from RLG1817; Rao et al. 1994) containing a promoter–*lacZ* fusion. This is a hybrid promoter in which an *rrnB* P1 UP element fused to the *lac* core promoter increases transcription 30-fold, that is, to the same extent as it increases transcription from the *rrnB* P1 core promoter (Rao et al. 1994) and was used as a reporter of UP element-dependent transcription to avoid potential complications arising from regulation of the *rrnB* P1 core promoter (Ross et al. 1993).

Transformants of RLG3101 with decreased β -galactosidase activity were identified as red or pink colonies on nutrient agar

Figure 7. Locations of amino acids important for interaction with UP element and with CAP. Numbers refer to amino acid residues in α CTD. The dots denote the positions of single alanine substitutions that most strongly reduce UP element-dependent transcription (this work) or CAP-dependent transcription (Tang et al. 1994).

plates containing 1.5% lactose, 100 μ g/ml of ampicillin, 40 μ g/ml of tetrazolium chloride, 2.0 grams/liter of NaCl (pH 6.0) (adjusted with HCl). Plasmid DNA was purified from the transformants and was used to retransform RLG3101 to confirm that the phenotype derived from the plasmid. The plasmid DNA was also transformed into RLG3100, an NK5031 derivative with the same promoter-*lacZ* fusion as RLG3101, but lacking an UP element, to confirm that the effects of the mutations were specific to UP element function and did not reduce basal transcription. Candidate mutations were mapped by restriction fragment replacement with a *Xba*I-*Bam*HI fragment (containing the entire *rpoA* gene) and with a *Hind*III-*Bam*HI fragment (containing codons 230–329). All mapped to the *Hind*III-*Bam*HI fragment. The entire DNA sequence corresponding to residues 230–329 was determined from double-stranded plasmid DNA.

Site-directed mutagenesis

Site-directed mutagenesis for the alanine scans was performed using synthetic oligonucleotide primers and primer extension according to Kunkel et al. (1991). pHTf1 α derivatives encoding single alanine substitutions at amino acids 255–271, 273, and 302 were constructed as described (Tang et al. 1994). Alanine substitutions from 291A–301A were constructed analogously in M13mp19 α , an M13mp19 derivative containing the *Hind*III-*Bam*HI fragment from pREII α (codons 230–329). The mutated *Hind*III-*Bam*HI fragments were then used to replace the analogous segment in pREII α . All constructs were confirmed by DNA sequencing.

Assay of UP element utilization in vivo

The effect of expression of the α substitutions from pREII α or pHTf1 α on UP element function was quantified in vivo using the promoter-*lacZ* fusions described above and also using reporters constructed from wild-type *rnnB* P1 core promoters with an UP element (RLG957 with promoter sequences –61 to +50 from *rnnB* P1), or without an UP element (RLG2263 containing –41 to +50 from *rnnB* P1) (Rao et al. 1994). The data presented in Figures 1 and 2 were from the set of reporters derived from the *rnnB* P1 core promoter. β -Galactosidase assays were performed according to Miller (1972), except that the cultures were grown in LB medium supplemented with 100 μ g/ml of ampicillin. At least three independent cultures were assayed for each α mutant, and the standard errors were within 10%. Fold defect is a measure of the reduction in UP element function of an *rnnB* P1 promoter in the presence of plasmid-encoded mutant α . For example, in the presence of only wild-type α (chromosomally encoded with or without plasmid-encoded wild-type α) the UP element increases transcription 30-fold (from 31 Miller units for the UP element-lacking core promoter to 945 Miller units for the UP element-containing promoter). However, in the presence of mutant plasmid-encoded α and chromosomally encoded wild-type α , the effect of the UP element is reduced. For example, in the presence of R265C the UP element increases tran-

scription only threefold (from 105 to 310 Miller units). Thus, R265C reduces UP element function 10-fold. The increase in core promoter activity in cells expressing R265C (from 31 to 105 Miller units) most likely results from feedback derepression of rRNA transcription, as described by Ross et al. (1993).

Previously existing chromosomal *rpoA* alleles (obtained from G. Christie, Medical College of Virginia, Richmond) were introduced into strains containing the promoter-*lacZ* fusions by transduction with P1 *vir*. None of the substitutions tested (G3S, L28F, P240S, K271E, L290H, P322S, P323L, and P323S) (Russo and Silhavy 1992) had a significant effect on UP element-dependent transcription (data not shown).

Haploviability assay

The viability of strains encoding the complete set of alanine substitutions in the absence of wild-type *rpoA* was tested by introducing pREII α or pHTf1 α plasmid derivatives into the temperature-sensitive strain HN317 (= HN198, *rpoA*112^{ts}, *Str*^r, Ishihama et al. 1980; obtained from G. Christie). Transformants were plated on LB containing ampicillin (100 μ g/ml) at the permissive temperature (30°C) and then screened on the same media but at the restrictive temperature (42°C). Equivalent results were obtained when the efficiency of plating was determined by directly plating aliquots of transformants at 30°C and 42°C. Plating efficiency <0.02 compared to cells with the wild-type plasmid *rpoA* allele was scored as inability to complement the *rpoA*^{ts} allele.

Purification of α and reconstitution of RNAP in vitro

Wild-type and mutant α -subunits were prepared using pHTT7f1-NH α (Tang et al. 1995) or derivatives after replacement of the *Hind*III-*Bam*HI fragment with fragments encoding alanine substitutions. Overproduction of amino-terminal hexahistidine-tagged (his-tagged) proteins and purification by metal ion affinity chromatography were performed as described (Tang et al. 1994). Purification of the other RNAP subunits and reconstitution into holoenzyme was performed after overexpression from plasmid pLHN126 for σ (L. Nguyen and R. Burgess, unpubl.), pMKSe2 for β , and pT7 β' for β' (Tang et al. 1995).

In vitro transcription assays

Multiple-round in vitro transcription assays were performed using 0.6 nM RNAP, supercoiled plasmid templates (0.2 nM) containing *rnnB* P1 promoters with and without the UP element (pRLG862 and pLR14, respectively; Rao et al. 1994), and [α ³²P] UTP at 40 Ci/mMole as described (Ross et al. 1993). Typically, autoradiographs were exposed for ~14 hr using intensifying screens.

DNA-binding assays

Electrophoretic mobility shift DNA-binding assays were performed using a DNA fragment containing the UP element from

pLR1 (−61 to −28 of *rrnB* P1) (Gaal et al. 1994). Reaction mixtures (20 μ l) contained the 32 P-3'-end-labeled DNA fragment (~0.01–0.1 nm and 10^7 cpm/pmol), 20 mM Tris-HCl (pH 7.9), 20 mM NaCl, 1 mM EDTA, 10% glycerol, 5 μ g/ml of sonicated salmon sperm DNA, and 0.15 nM–1.5 μ M α . In the experiment shown in Figure 4A, the α concentration was 1.5 μ M. Reactions were incubated 15 min at 22°C, applied to 6% polyacrylamide, 10% glycerol slab gels in 0.5 $\times$ TBE buffer (Peacock and Dingman 1968), electrophoresed at 20 V/cm at 6°C for 90 min, and the gels were dried and autoradiographed. Equilibrium constants were estimated from the concentration of α required to shift 50% of the labeled fragment. At least 15-fold higher concentrations of wild-type α failed to shift DNA fragments lacking UP element sequences.

DNase I footprinting of RNAP on a promoter fragment containing *rrnB* P1 sequences from −88 to +50 with a 1-bp deletion at −72 (Bokal et al. 1995), 32 P-3'-end-labeled at a *Hind*III site at +50, was performed as follows; the binding reaction (25 μ l) contained 60 nM RNAP, ~10 nM DNA fragment (~ 10^7 cpm/pmol), 30 mM potassium glutamate, 10 mM Tris-acetate (pH 7.9), 10 mM MgCl₂, 1 mM DTT, 100 μ g/ml of BSA, 500 μ M ATP, and 50 μ M CTP (Ross et al. 1993). DNase I (0.4 μ g/ml) digestion for 30 sec was terminated by the addition of 20 mM EDTA (pH 8.0) on ice; the RNAP–DNA complex was separated from free DNA by electrophoresis on a 6% acrylamide gel in 1/2 $\times$ TBE, and the complex was located by autoradiography. The DNA was eluted from the gel by diffusion, extracted with phenol, and analyzed by denaturing gel electrophoresis (10% polyacrylamide, 7 M urea) followed by autoradiography for ~14 hr with an intensifying screen.

Preparation of α CTD for NMR spectroscopy

Plasmid pEBT7- α CTD encodes one non-native amino acid (M1), followed by amino acids 245–329 (α CTD) under the control of the bacteriophage T7 gene 10 promoter. pEBT7- α CTD was constructed by replacement of the *Nde*I–*Bam*HI segment of plasmid pET21a (Novagen) with an *Nde*I–*Bam*HI *rpoA* DNA fragment by “add-on PCR.” BL21(DE3)pLysS (Novagen) containing pEBT7- α CTD was shaken at 37°C in LB medium (Miller 1972) containing 100 μ g/ml of ampicillin and 35 μ g/ml of chloramphenicol to an OD₆₀₀ of 0.7. Production of α CTD was then induced by addition of isopropyl-thio- β -D-galactoside to 1 mM, and shaking was continued for an additional 3 hr at 37°C. Cultures were harvested and cells were lysed as described by Burgess and Jendrisak (1975). α CTD was purified by polyethyleneimine precipitation (0.2% supernatant), ammonium sulfate precipitation (80% supernatant; 100% pellet), and size-exclusion chromatography on Superdex-75 (Pharmacia) in buffer A (50 mM sodium phosphate (pH 6.0), 0.05 mM EDTA, 0.05 mM DDT, and 0.05% sodium azide). The yield was 10–20 mg/liter of culture, and the purity was >99%. When necessary, α CTD was concentrated to 1–4 mM by centrifugal ultrafiltration at 5000g for 3–6 hr at 4°C [Microsep 10K filter units prewashed with 3 ml of 0.1 N NaOH followed by 25 ml of buffer A (Filtron, Inc.)] and stored in aliquots at 4°C in buffer A.

Isotopically labeled α CTD

Uniformly 15 N-labeled and uniformly 13 C/ 15 N-labeled α CTD were prepared (Muchmore et al. 1989) as described for unlabeled α CTD using cultures grown in M9 minimal medium (Miller 1972) containing 1 mg/ml of 15 NH₄Cl [99% atom (Isotec, Inc.)], 0 or 2 mg/ml of 13 C-labeled glucose [99% atom (Isotec, Inc.)], 100 μ g/ml of ampicillin and 35 μ g/ml of chloramphenicol. Selectively 2 H-labeled forms of α CTD were prepared containing

the following sets of 2 H-labeled amino acids (one-letter code): (1) A, D, E, F, G, H, I, K, M, P, R, S, T, V, and Y; (2) A, D, E, F, G, H, I, L, M, P, R, S, V, and Y; (3) A, D, E, F, G, H, K, L, M, R, S, T, V, and Y; (4) A, D, F, G, H, I, K, L, M, P, T, V, and Y; and (5) E, F, H, I, K, L, P, R, S, T, and Y. Samples were grown as above, but when the OD₆₀₀=0.6, cultures were supplemented with 2 H-labeled algal hydrolysate (Isotec, Inc.) to 750 μ g/ml; C, N, Q, and W to 45 μ g/ml each; and L (sample 1), R and T (sample 2), I and P (sample 3), E, R, and S (sample 4), or A, D, G, M, and V (sample 5) in 10-fold excess over the corresponding 2 H-labeled amino acids in the algal hydrolysate. Cultures were shaken an additional 10 min at 37°C, supplemented with adenine, cytosine, and uracil to 50 μ g/ml each, guanosine to 60 μ g/ml, thymine to 20 μ g/ml, and IPTG to 1 mM with continued shaking for an additional 3 hr at 37°C.

NMR spectroscopy

NMR experiments were carried out at 30°C (except where indicated otherwise) on a Varian UNITY-plus 500 spectrometer equipped with a pulse-field-gradient triple-resonance probe. Samples contained 0.1–2.5 mM α CTD in buffer A. Samples for measurement of DNA-induced perturbations of backbone–amide–proton resonances contained in addition 0.5 M equivalents of a 19-bp DNA fragment containing positions −57 to −47 of the *rrnB* P1 upstream element (5'-TCAGAAAT-TATTTTCGGG-3'/5'-CCCCAAAATAATTTTCTGA-3') (see Tang et al. 1994).

Homonuclear and heteronuclear NMR experiments were carried out, including (1) two-dimensional NOESY, three-dimensional 15 N-labeled NOESY–HSQC (Fesik and Zuiderweg 1988), three-dimensional 15 N-labeled TOCSY–HSQC (Bax et al. 1990), three-dimensional 13 C-labeled HSQC–NOESY (Fesik and Zuiderweg 1988; Majumdar and Zuiderweg 1993), and three-dimensional HNCA, HN(CO)CA, and HNCO (Kay et al. 1990; Yamazaki et al. 1994) for assignment of 1 H, 15 N, and 13 C resonances; (2) three-dimensional HNHA and constant-time HMQC-J (Kuboniwa et al. 1994) for measurement of $J_{\text{HN-H}\alpha}$ coupling constants; (3) two-dimensional 15 N-labeled HSQC for measurement of rates of backbone–amide–proton chemical exchange; and (4) two-dimensional 15 N-labeled HSQC at 40°C for measurement of DNA-induced perturbations of backbone–amide–proton resonances. 1 H, 15 N, and 13 C chemical shifts were referenced to external 3-trimethylsilyl-propionate, external 15 NH₄Cl, and external 2,2-dimethyl-2-silapentane-5-sulfonate, respectively. NMR data were analyzed using Felix 2.30 (Biosym Technologies, Inc.) using an SGI Indigo workstation.

Acknowledgments

We thank K. Severinov and R. Burgess for discussions and materials and G. Christie for strains. We also thank A. Ishihama and Y. Kyogoku for sharing NMR information prior to publication. This work was supported by National Institutes of Health grants GM37048 to R.L.G. and GM51527 to R.H.E., by National Science Foundation grant MCB-9506933 to N.A.-M., and by funding from the Lucille P. Markey Charitable Trust and the Mathers Foundation to the La Jolla Cancer Research Foundation.

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Note

“Solution structure of the activator contact domain of the RNA polymerase α subunit” (Y.-H. Jeon, T. Negishi, M. Shirakawa, T.

Yamazaki, N. Fujita, A. Ishihama, and Y. Kyogoku) is in press (*Science*). The secondary structure in that report is consistent with that reported here.

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Genes Dev. 1996, **10**:

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