Location, structure, and function of the target of a transcriptional activator protein.


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Location, structure, and function of the target of a transcriptional activator protein

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We have isolated and characterized single-amino-acid substitution mutants of RNA polymerase α subunit defective in CAP-dependent transcription at the lac promoter but not defective in CAP-independent transcription. Our results establish that (1) amino acids 258-265 of α constitute an “activation target” essential for CAP-dependent transcription at the lac promoter but not essential for CAP-independent transcription, (2) amino acid 261 is the most critical amino acid of the activation target, (3) amino acid 261 is distinct from the determinants for α-DNA interaction, and (4) the activation target may fold as a surface amphipathic α-helix. We propose a model for transcriptional activation at the lac promoter that integrates these and other recent results regarding transcriptional activation and RNA polymerase structure and function.

[Key Words: Transcriptional activation; RNA polymerase; CAP-dependent transcription; lac promoter]

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Escherichia coli catabolite gene activator protein (CAP) activates transcription by binding to upstream DNA sites and enhances binding and transcription initiation by RNA polymerase (RNAP) (for review, see Reznikoff 1992; Ebright 1993; Kolb et al. 1993a). CAP is a dimer of two identical subunits, each consisting of 209 amino acids. Amino acids 156-164 of the promoter-proximal subunit of the CAP constitute an “activating region” essential for transcriptional activation at the lac promoter but not essential for DNA binding by CAP and DNA bending by CAP (Bell et al. 1990; Eschenlauer and Reznikoff 1991; Zhou et al. 1993a,b; Niu et al. 1994). Alanine scanning of the activating region indicates that the side chain of Thr-158—and, apparently, the side chain of no other amino acid within the activating region—is essential for transcriptional activation at the lac promoter (Niu et al. 1994). In the structure of the CAP–DNA complex (Schultz et al. 1991), the activating region forms a prominently exposed, essentially continuous, surface with dimensions of ~11 × 14 Å, and Thr-158 is especially prominently exposed and located at the center of the surface. It has been proposed that transcriptional activation at the lac promoter involves protein–protein interaction between the activating region of the promoter–proximal subunit of the CAP dimer—particularly the Thr-158 side chain of the promoter–proximal subunit of the CAP dimer—and a molecule of RNAP bound adjacent to CAP on promoter DNA (Zhou et al. 1993a,b, Niu et al. 1994).

E. coli RNAP is a multisubunit enzyme with subunit composition α2ββ′σ (for review, see Burgess 1976; Chamberlin 1976; von Hippel et al. 1992). Three lines of evidence suggest that the α subunit contains the target for the proposed protein–protein interaction between CAP and RNAP at the lac promoter (the “activation target”). First, results of protein–protein photo-cross-linking establish that the carboxy-terminal region of α is in direct physical proximity to the activating region of CAP at the lac promoter (Chen et al. 1994). Second, deletion of the carboxy-terminal region of the α subunit (amino acids 236–329 or 257–329; Igarashi and Ishihama 1991; Kolb et al. 1993b), or substitution of amino acid 265 located within the carboxy-terminal region of α (Zou et al. 1992), results in an RNAP derivative defective in CAP-dependent transcription at the lac promoter but only partially inhibit CAP-independent transcription (Rif tina et al. 1990; Venezia and Krakow 1990).

In this work we have defined the boundaries, critical amino acids, and properties of the activation target within α. Our results suggest a detailed model for the structure and function of the activation target.

*Corresponding author.
Results

**Amino acids 258–265 of α constitute an activation target essential for CAP-dependent transcription but not essential for CAP-independent transcription**

We have used PCR-mediated random mutagenesis (Zhou et al. 1991, 1993a) followed by application of a screen to isolate single-amino-acid substitution mutants of RNAP α subunit specifically defective in CAP-dependent transcription—that is, defective in CAP-dependent transcription but not defective in CAP-independent transcription. We designate such mutants rpoA_{pct-CAP}, where rpoA denotes the gene encoding α, pct denotes positive control defective, and CAP denotes CAP-dependent transcription. Our screen tested two phenotypes on a single agar plate: (1) defect in CAP-dependent transcription, and (2) absence of defect in CAP-independent transcription. To test phenotype 1, the screen scored expression of the lac and rbs operons. [The lac and rbs operons are required for utilization of lactose and ribose, respectively (Beckwith 1978; Iida et al. 1984; Lopilato et al. 1984). The lac and rbs promoters are CAP-dependent promoters with identical distances between the center of the DNA site for CAP and the transcription start point (61.5 bp) but with different −35-region and −10-region sequences (Dickson et al. 1977; Bell et al. 1986; Zhou et al. 1993a).] To test phenotype 2, the screen scored viability. Mutants lacking CAP are viable on rich media (Sabourin and Beckwith 1974). Therefore, we reasoned that mutants of α specifically defective in CAP-dependent transcription would be viable on rich media. In contrast, we reasoned that mutants of α defective in both CAP-dependent and CAP-independent transcription would be inviable or would exhibit significantly reduced growth rates.

To facilitate mutagenesis and screening, the screen was performed in strains containing mutagenized rpoA gene on a multicopy plasmid and wild-type rpoA gene on the chromosome. Immunoblotting with anti-α antibody indicated that under the conditions of the screen plasmid-encoded α was overproduced ~2.5-fold relative to chromosome-encoded α (see Materials and methods). Based on this level of overproduction, assuming equal efficiencies of assembly into RNAP of plasmid-encoded α and chromosome-encoded α, it was expected that ~90% of RNAP would have at least one plasmid-encoded α subunit, and ~50% of RNAP would have two plasmid-encoded α subunits. For one plasmid-encoded α derivative, [Gly-261]α, we have verified directly that this level of overproduction and assembly into RNAP occurs, by isolation of RNAP followed by two-dimensional electrophoresis/isoelectric-focusing (data not shown). Therefore, it was expected that the phenotype of plasmid-encoded α would be dominant or partly dominant.

Plasmids containing mutagenized rpoA structural gene were introduced by transformation into lac+ rbs+ rpoA+ tester strains, and transformants were plated to double-sugar lactose/ribose/tetrazolium/ampicillin indicator agar. RpoA_{pct-CAP} clones were identified as red colonies [Lac− Rbs−; viable]. Two tester strains were used: XE54 [thi] and XE4 [Δgal165 thi]. XE54 required a strong Lac− phenotype to yield red colonies on lactose/ribose/tetrazolium/ampicillin indicator agar and, therefore, permitted a stringent screen. Because of Δgal165 marker, XE4 required only a moderate Lac− phenotype to yield red colonies on lactose/ribose/tetrazolium/ampicillin indicator agar and, therefore, permitted a less stringent screen (cf. Ebright et al. 1984). Sixty-five independent mutagenesis reactions were performed, and ~30,000 mutagenized transformants were screened in each tester strain (Table 1). From these, 11 independent rpoA_{pct-CAP} candidate clones were identified in high-stringency-screen strain XE54, and 10 independent rpoA_{pct-CAP} candidate clones were identified in low-stringency-screen strain XE4 (Table 1).

For each candidate rpoA_{pct-CAP} clone, plasmid DNA was prepared and introduced by transformation into strains XE54 [thi] and XE56 [lacPL8–UV5 thi], and CAP-dependent transcription at the lac promoter and CAP-independent transcription at the lacPL8–UV5 promoter were assessed in quantitative in vivo assays. [The lacPL8–UV5 promoter is a CAP-independent derivative of the lac promoter (Silverstone et al. 1970; Beckwith et al. 1972; Arditti et al. 1973); the lacPL8–UV5 promoter contains a substitution in the DNA site for CAP and two substitutions in the −10 region (Gilbert 1976; Dickson et al. 1977)]. The results confirmed that all 21 candidate rpoA_{pct-CAP} clones were defective in CAP-dependent transcription at the lac promoter (two- to threefold; Fig. 1A) but not defective in CAP-independent transcription (Fig. 1B).

For each of the 21 rpoA_{pct-CAP} mutants, the substitution responsible for the phenotype was mapped by marker rescue using XbaI–HindIII and HindIII–BamHI rpoA DNA fragments, which contain codons 1–229 and 230–329, respectively. In each case, the nucleotide substitution responsible for the phenotype mapped to codons 230–329. For each mutant, the nucleotide sequence of codons 230–329 was determined, and the amino acid sequence of the α derivative was inferred.

Remarkably, the amino acid substitutions in the 21 rpoA_{pct-CAP} mutants mapped to a single 8-amino-acid region of α—that is, amino acids 258–265 (Table 2). One substitution was obtained at amino acid 258; two substitutions were obtained at amino acid 261, one substitution was obtained at amino acid 264; and one substitution was obtained at amino acid 265 (Table 2). Substitutions at amino acid 265 [Arg-265 → Cys and Arg-265 → His] have been shown previously to reduce CAP-dependent transcription at the lac promoter (Zou et al. 1992). The fact that we have reisolated a substitution at amino acid 265 confirms the importance of this amino acid sequence. DNA fragments, which contain codons 1–229 and 230–329, respectively. In each case, the nucleotide substitution responsible for the phenotype mapped to codons 230–329. For each mutant, the nucleotide sequence of codons 230–329 was determined, and the amino acid sequence of the α derivative was inferred.

### Table 1. Random mutagenesis and screen

<table>
<thead>
<tr>
<th>Mutagenesis reactions</th>
<th>65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total candidates (XE54)</td>
<td>33,000</td>
</tr>
<tr>
<td>Lac− Rbs− candidates (XE54)</td>
<td>11</td>
</tr>
<tr>
<td>Total candidates (XE4)</td>
<td>27,000</td>
</tr>
<tr>
<td>Lac− Rbs− candidates (XE4)</td>
<td>10</td>
</tr>
</tbody>
</table>
acid for CAP-dependent transcription at the lac promoter. The fact that we also have isolated substitutions at amino acids 258, 261, and 264 indicates for the first time the importance of these amino acids for CAP-dependent transcription at the lac promoter. We conclude that amino acids 258–265 constitute a region of α that is essential for CAP-dependent transcription at the lac and rbs promoters but not essential for CAP-independent transcription. We designate this region the activation target.

The statistics of the analysis are informative. In the high-stringency screen (the screen using strain XE54), we obtained 11 independent mutants substituted at amino acid 261 and no mutants substituted at any other amino acid. These statistics indicate that the high-stringency screen has approached, or reached, saturation of available targets. That is, these statistics indicate that there are few, or no, other amino acids at which a substitution is typically positive and negative substitutions. Second, alanine scanning yields a chemically consistent set of substitutions, including both phenotypically positive and negative substitutions. Third, and most important, alanine scanning yields side-chain truncation substitutions; alanine substitution eliminates all side-chain atoms beyond Cβ, and all interactions made by side-chain atoms beyond Cβ.

We used site-directed mutagenesis to substitute each amino acid from 255 to 270 of α, one-by-one, by alanine. For each resulting α derivative, we then assessed CAP-dependent transcription at the lac and rbs promoters in vivo. The results are presented in Table 3. Alanine substitution of Glu-261 resulted in a large defect in CAP-dependent transcription at the lac and rbs promoters [as large a defect as Gly or Lys substitution; cf. Tables 2 and 3]. Alanine substitution of Asp-258 or Asp-259 resulted in a small, but reproducible, defect. Strikingly, alanine substitution of no other amino acid resulted in a defect.

We conclude that for Glu-261, side-chain atoms beyond Cβ are critical for CAP-dependent transcription at the lac and rbs promoters. We conclude that for Asp-258 and Asp-259, side-chain atoms beyond Cβ are important for CAP-dependent transcription at the lac and rbs promoters but, quantitatively, at a lower level of importance. Finally, we conclude that Glu-261, Asp-258, and Asp-259 are the only amino acids of the activation target for which side-chain atoms beyond Cβ are important for CAP-dependent transcription at the lac and rbs promoters. In the random-mutagenesis analysis of the preceding section, substitutions at two other amino acids were shown to result in defects in transcriptional activation at the lac and rbs promoters—that is, Val-264 → Asp and Arg-265 → Cys (Table 2; see also Zou et al. 1992). Based on the alanine-scanning analysis of this section, we sug-

### Table 2. Sequences and phenotypes of rpoA<sup>△<sub>lac</sub></sup>.<sub>CAP</sub> mutants

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>Codon substitution</th>
<th>Number of isolates, (XE54)</th>
<th>Number of isolates (XE4)</th>
<th>CAP-dependent transcription (lac)*</th>
<th>CAP-dependent transcription (rbs)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>--</td>
<td>--</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>258 Asp → Val</td>
<td>GAC → GTC</td>
<td>--</td>
<td>1</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>261 Glu → Gly</td>
<td>GAA → GGA</td>
<td>4</td>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>261 Glu → Lys</td>
<td>GAA → AAA</td>
<td>7</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>264 Val → Asp</td>
<td>GTC → GAC</td>
<td>--</td>
<td>1</td>
<td>+ + +</td>
<td>+ +</td>
</tr>
<tr>
<td>265 Arg → Cys</td>
<td>CGC → TGC</td>
<td>--</td>
<td>1</td>
<td>+ + +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

*Colony colors of XE54 pREII<sub>α</sub> and derivatives on tetrazolium indicator agar. (+ + + +) White; (--) dark red.
Amino acid scanning

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>CAP-dependent transcription (lac)*</th>
<th>CAP-dependent transcription (rbs)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>255 Arg → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>256 Pro → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>257 Val → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>258 Asp → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>260 Leu → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>261 Glu → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>262 Leu → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>263 Thr → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>264 Val → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>265 Arg → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>267 Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>266 Ser → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>268 Asn → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>269 Cys → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>270 Leu → Ala</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
</tbody>
</table>

*Colony colors of XE54 pHTfla and derivatives on tetrazolium indicator agar.

suggest that the Val-264 → Asp and Arg-265 → Cys substitutions affect CAP-dependent transcription indirectly, by introducing non-native side-chains atoms that disrupt the activation target sterically, electrostatically, or conformationally.

The fact that the activation target has only a small number of critical amino acids is reminiscent of the patterns observed with functional epitopes for protein–antibody and protein–receptor interaction (Cunningham and Wells 1989, 1993; Jin et al. 1992; Kelley and O’Connell 1993) and, most important, reminiscent of the pattern observed with the activating region of CAP (Niu et al. 1994).

Amino acid 261 of α is essential for CAP-dependent transcription in vitro

To confirm and quantify the in vivo results, we performed in vitro transcription experiments with wild-type RNAP and with RNAP derivatives containing [Gly-261]α and [Lys-261]α. To prepare mutant RNAP substantially free of contaminating wild-type α, we subcloned the genes encoding [Gly-261]α and [Lys-261]α into T7 RNA polymerase expression vector pET3a (Studier et al. 1990), overproduced [Gly-261]α and [Lys-261]α to the extent of ~30% of total cell protein, purified [Gly-261]α and [Lys-261]α, and reconstituted mutant RNAP from purified mutant α and purified wild-type β, β′, and σ (see Zalenskaya et al. 1990; Borukhov and Goldfarb 1993). Results of MALDI mass spectrometry (Fig. 2) and two-dimensional electrophoresis/isoelectric-focusing [data not shown] established that each purified mutant α and each reconstituted mutant RNAP contained ≤10% contaminating wild-type α.

To analyze CAP-dependent transcription, we performed abortive initiation in vitro transcription experiments with the lac promoter. To analyze CAP-independent transcription, we performed abortive initiation in vitro transcription experiments with the lacPUV5 promoter [Silverstone et al. 1970; Beckwith et al. 1972; Arditti et al. 1973; Malan et al. 1984]. The results are presented in Figure 3. Each mutant RNAP was defective in CAP-dependent transcription at the lac promoter. The mutant RNAP containing [Gly-261]α was ~5-fold defective, the mutant RNAP containing [Lys-261]α was ~10-fold defective. In contrast, each mutant RNAP was fully functional in CAP-independent transcription at the lacPUV5 promoter. We conclude that amino acid 261 of α is essential for CAP-dependent transcription at the lac promoter but not essential for CAP-independent transcription, both in vivo and in vitro.

Amino acid 261 of α is not essential for α–DNA interaction in vitro

Recently, it has been shown that RNAP α is a sequence-specific DNA-binding protein involved directly in promoter recognition (Ross et al. 1993; Blatter et al. 1994). RNAP α recognizes a 15-bp adenine/thymine-rich DNA sequence present immediately upstream of the −35 region in certain strong promoters, notably the rrrB P1 promoter, but absent in the lac promoter (the upstream element or UP element; Ross et al. 1993; Rao et al. 1994).

Figure 2. Purities of α derivatives and RNAP derivatives. Data are from endoproteinase Glu-C digestions followed by MALDI mass spectrometry. Peptide α(230–261) [molecular mass, 3805 daltons] is produced upon endoproteinase Glu-C digestion of wild-type α, but not upon endoproteinase Glu-C digestion of [Gly-261]α and [Lys-261]α and, therefore, is diagnostic of the presence of wild-type α. Control peptide α(289–319) [molecular mass, 3379 daltons] is produced upon endoproteinase Glu-C digestion of wild-type α, [Gly-261]α, and [Lys-261]α. Data are ratios of peptide α(230–261) to control peptide α(289–319), normalized to values with homogeneous wild-type α or homogeneous wild-type RNAP. (A) Wild-type α, [Gly-261]α, and [Lys-261]α; (B) wild-type RNAP, [Gly-261]α-RNAP, and [Lys-261]α-RNAP.
We conclude that amino acid 261 is not essential for α-DNA interaction. We conclude further that amino acid 261 of α is distinct from the determinants for α–DNA interaction.

**Discussion**

**Structure of the activation target**

Previous work has established that RNAP α consists of (1) an independently folded, independently dimerized amino-terminal domain containing determinants for interaction with the remainder of RNAP, (2) an independently folded, independently dimerized, carboxy-terminal domain containing determinants for interaction with DNA, and (3) an unstructured and/or flexible interdomain linker [Hayward et al. 1991; Igarashi et al. 1991; Kolb et al. 1993b; Ross et al. 1993; Blatter et al. 1994]. The activation target defined in this work (amino acids 258–265) is located entirely within the α carboxy-terminal domain (amino acids 249–329).

No high-resolution information is available regarding the structure of the α carboxy-terminal domain. Nevertheless, because 4 of the 5 amino acids at which substitutions conferring an RpoA △ CAP phenotype were obtained are charged amino acids—that is, Asp-258, Asp-259, Glu-261, and Arg-265—we predict that the side chains of these amino acids are exposed to solvent on the surface of RNAP and are available to interact with other molecules. [The side chains of 80% of Asp residues, 80% of Glu residues, and >90% of Arg residues are solvent exposed in globular proteins [Chothia 1976; Janin 1979].] We note further that Arg-265 is the target for bacteriophage T4 ADP ribosylation of RNAP [for review, see Goff 1984]. We infer that the side chain of this amino acid is sufficiently exposed on the surface of RNAP to interact with 70,000- and 26,000-dalton enzymes [the products of the bacteriophage T4 alt and mod genes [Goff 1984]].

Circular dichroism spectroscopy indicates that the α carboxy-terminal domain contains ~40% α-helix and ~0% β-sheet [Blatter et al. 1994]. On the basis of secondary structure prediction [Rost and Sander 1993] and helix wheel analysis [Schiffer and Edmundson 1967], we suggest that amino acids 256–270 fold as an amphipathic α-helix, with one face consisting exclusively of hydrophobic amino acids and one face consisting primarily of hydrophilic amino acids [Fig. 5]. We suggest further that the amphipathic α-helix lies on the surface of the α carboxy-terminal domain, with the side chains of the hydrophobic amino acids interacting with the core of the domain and with the side chains of the hydrophilic amino acids solvent exposed and available to interact with other molecules [Fig. 5]. According to these sugges-
Target of a transcriptional activator protein

A

B

Figure 5. Proposed structure of the activation target. The hydrophobic and hydrophilic faces of the proposed surface amphipathic α-helix are shaded and unshaded, respectively. The 5 amino acids at which substitutions conferring an RpoA<sup>per</sup> phenotype were obtained are underlined. [A] Helix-wheel representation (Schiffer and Edmundson 1967); [B] helix-net representation.

Figure 6. Proposed function of the activation target. [A] CAP-dependent transcription at the lac promoter; [B] CAP-independent transcription at the lac<sup>PUV5</sup> promoter.

Function of the activation target

Our results establish that (1) amino acids 258–265 of α constitute an activation target essential for CAP-dependent transcription at the lac promoter but not essential for CAP-independent transcription, (2) amino acid 261 is the most critical amino acid of the activation target, (3) amino acid 261 is distinct from the determinants for α-DNA interaction, and (4) the activation target may fold as a surface amphipathic α-helix. Previous results establish that amino acids 209–329 of α are in direct physical proximity to the activating region of CAP in the ternary complex of RNAP, CAP, and lac promoter (Chen et al. 1994). We propose that transcriptional activation at the lac promoter requires protein–protein interaction between the activation target of α and the activating region of CAP. We suggest that at the lac promoter the entire 8-amino-acid activation target of α—folded as a surface amphipathic α-helix—is in direct physical proximity to the entire 9-amino-acid activating region of CAP. We suggest further that at the lac promoter the most critical amino acid of the activation target of α, Glu-261, makes direct side-chain contact with the most critical amino acid of the activating region of CAP, Thr-158 (see Niu et al. 1994).

Figure 6 presents a model that integrates our results and other recent results regarding transcriptional activation and RNAP structure and function (Kolb et al. 1993b; Ross et al. 1993; Zhou et al. 1993a,b; Blatter et al. 1994; Busby and Ebright 1994; Chen et al. 1994; Rao et al. 1994). The model proposes that at the lac promoter the α carboxy-terminal domain makes two interactions: (1) a protein–protein interaction involving the activation target of α and the activating region of CAP; and (2) a non-specific protein–DNA interaction involving determinants of α distinct from the activation target—or at least
**Table 5. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>XE4</td>
<td>lac⁻ Δgal165 thi</td>
<td>Ebright et al. (1984)</td>
</tr>
<tr>
<td>XE54</td>
<td>lac⁻ thi</td>
<td>this work</td>
</tr>
<tr>
<td>XE56</td>
<td>lacPL8-UV5 thi</td>
<td>this work</td>
</tr>
</tbody>
</table>

All strains were constructed from strain X7029 (ΔlacX74 thi; Ebright 1985). The Δgal165 marker was from bacteriophage κθ857–gal313Δ165 (S. Adhya, unpublished), the lac⁻ and lacPL8–UV5 markers were from strains CA8439 (Sabourin and Beckwith 1974) and E8027 (Arditti et al. 1973), respectively.

distinct from Glu-261—and promoter DNA between the DNA site for CAP and the −35 region. The protein–protein interaction compensates for the absence in the lac promoter of a specific, high-affinity DNA site for α. The protein–protein interaction increases the binding constant for RNAP–promoter interaction in addition, the protein–protein interaction recruits the α carboxy-terminal domain to DNA resulting in possibly activatory changes in RNAP conformation and RNAP–promoter organization.

This model accounts for the observation that in the presence of CAP, intact RNAP, but not an RNAP derivative lacking the α carboxy-terminal domain, protects the DNA segment between the DNA site for CAP and the −35 region from DNase I digestion (Kolb et al. 1993b). This model also accounts for the observation that CAP-dependent transcription requires structural integrity of the DNA segment between the DNA site for CAP and the −35 region (Ryu et al. 1994).

This model, in which α carboxy-terminal domain serves as an activator-binding and DNA-binding module flexibly tethered to the remainder of RNA polymerase, has similarities to developing models for function of co-activators and TBP-associated factors (TAFs) in eukaryotic transcription activation (Purnell et al. 1994; Tjian and Maniatis 1994; Verrijzer et al. 1994).

**Materials and methods**

**Strains, media, and microbiological techniques**

A list of *E. coli* K-12 strains used in this work is presented in Table 5. Standard media were prepared and standard genetic manipulations were performed as described in Miller (1972). Lactose/ribose/tetrazolium/ampicillin indicator agar plates contained 1% ribose, 1% lactose, and 200 μg/ml of ampicillin.

**Plasmids**

A list of plasmids used in this work is presented in Table 6. Plasmids pREIlα, pHTH1α, and derivatives encode α under control of the lpp–lacUV5 tandem promoter. Plasmid pHTT7α and derivatives encode α under control of the bacteriophage T7 gene 10 promoter. Each plasmid has a unique XhoI site preceding the rpoA Shine–Delgarno sequence, a unique EcoRI site at codon 168, a unique HindIII site at codon 229, a unique SacI site at codon 288, and, in plasmids pREIlα, pHTT7α, and derivatives, a unique BamHI site immediately following the rpoA stop codon. For each plasmid, the DNA–nucleotide sequence of the entire rpoA gene was verified.

**Mutagenesis**


**Determination of levels of α in vivo**

To determine levels of α in cells grown on agar, 20–30 colonies were suspended in 1 ml of M63 medium (Miller 1972), suspensions were adjusted to an OD₆₀₀ of 0.6, and aliquots of serial dilutions were analyzed by SDS-PAGE followed by immunoblotting. Immunoblotting was performed according to the procedure of Harlow and Lane (1988) using rabbit polyclonal anti-α antibody (prepared by Lampire Laboratories, Inc., Pipersville, PA), alkaline-phosphatase-conjugated goat anti-rabbit-immunoglobulin antibody (Bio-Rad, Inc.), and nitrocellulose membranes (Bio-Rad, Inc.).

**Measurement of transcription in vivo**

To measure transcription in vivo in the presence of α derivative X, the plasmid encoding α derivative X was introduced into tester strains XE54 (lac⁺, CAP-dependent transcription) and XE56 (lacPL8–UV5, CAP-independent transcription), and the differential rates of β-galactosidase synthesis in the resulting plasmid-bearing strains were determined (method in Miller 1972), except that cultures were grown in Luria–Bertani (LB) medium containing 150 μg/ml of ampicillin and 5 mm isopropyl-thio-B-D-galactoside; three independent determinations).

**Preparation of α**

RNAP α and derivatives were produced in strain BL21(DE3) (Studier et al. 1990, Novagen, Inc.) transformed with plasmid pHITT7α and derivatives (production to the level of ~30% of total cell protein). Following cell lysis by sonication, RNAP α and derivatives were localized ~50% in the soluble cell fraction, and ~50% in the insoluble cell fraction. RNAP α and derivatives were purified from the soluble cell fraction by polyethyleneimine precipitation, ammonium sulfate precipitation, and hydrophobic interaction chromatography on phenyl-Toyopearl (Toyoba, Inc.) (modification of the procedure of Borukhov and Goldfarb 1993). Yields of RNAP α and derivatives were 3–4 mg/liter of culture, and purities were >90%. RNAP α and derivatives were stored in aliquots at −20°C in 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.5 mM EDTA, and 50% glycerol.

**Preparation of β, β’, and α**

RNAP β, β’ and α were prepared as described (Borukhov and Goldfarb 1993; Severinov et al. 1993).

**Reconstitution of RNAP holoenzyme**

RNAP holoenzyme and derivatives were reconstituted from purified subunits and were purified by size-exclusion chromatog-
Mass spectrometry

RNAP α and derivatives, prepared as described above, or prepared from RNAP and derivatives by denaturing centrifugal ultrafiltration [Centrificon-100 filter units (Amicon, Inc.) 1000 g, for 40 min at 4°C in 8 μM guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 150 mM KCl, and 1 mM EDTA], were digested with endonuclease Glu-C, and products were analyzed by MALDI mass spectrometry. Reaction mixtures (30 μl) contained 10 μM RNAP α or derivative, 0.2 μg of endonuclease Glu-C (Boehringer Mannheim GmbH), 50 mM ammonium bicarbonate (pH 8.0), and 15 mM NaCl. After 1 hr at 22°C, 1-μl aliquots were withdrawn, mixed with 9 μl of 50 μM α-cyano-4-hydroxycinnamic acid in formic acid/water/isopropanol (1:3:2, vol/vol/vol), and analyzed by MALDI mass spectrometry (procedure of Beavis and Chair 1990).

Transcription experiments

Abortive initiation in vitro transcription experiments were performed as described by Zhang et al. [1992]. Experiments were performed using as templates 203-bp EcoRI–EcoRI DNA fragments of plasmids pBR-203-lac and pBR-203-lacPUV5. Reaction mixtures contained (25 μl): 150 mM NaCl or NaCl derivative, 0 or 40 mM CAP (purified as described by Zhang et al., 0.5 mM DNA fragment, 0.5 mM ApA (ICN Biomedicals, Inc.), 50 nM [α-32P]UTP (30 Bq/mole), 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 5% glycerol, and 0.2 mM cAMP. Reaction components except ApA and [α-32P]UTP were pre-equilibrated for 10 min at 37°C. Reactions were initiated by addition of ApA and [α-32P]UTP and were allowed to proceed for 15 min at 37°C. Reactions were terminated by addition of 5 μl 0.5 M EDTA. The reaction product [α32P]ApApUpU was resolved by paper chromatography in water/saturated ammonium sulfate/isopropanol (18:80:2, vol/vol) and quantified by Cerenkov counting.

DNA-binding experiments

Electrophoretic mobility shift DNA-binding experiments were performed using a 19-bp DNA fragment containing a specific DNA site for α (positions 57 to 47 of the rpoB PI promoter upstream element, 5’-TCACAAAATTATTTCTCGG-3’/5’-CCGAAAATAAATTCTCTGA-3’). Reaction mixtures contained (20 μl): 0 to 75 μM α or derivative, 125 nm [32P]-labeled DNA fragment (10 Bq/μmole), prepared as described by Ebert et al. [1989], 10 mM MOPS–NaOH (pH 7.0), 50 mM NaCl, 10 mM MgCl2, and 5% glycerol. Reaction mixtures were incubated for 1 hr at 30°C. Reaction mixtures then were applied to 5% polyacrylamide, 2.7% glycerol slab gels (9 × 7 × 0.15 cm), and electro-

Table 6. Plasmids

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<thead>
<tr>
<th>Plasmid</th>
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<td>Zhou et al. [1993a]</td>
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*Plasmid pHTF1a was constructed by insertion of the 0.5-kb BglII–BglII ori-fl segment of plasmid pND118B [Heitman et al. 1989] at the BamHI site of plasmid pREIlA. Replication initiating at ori-fl yields single-stranded DNA containing the antisense strand of rpoA.
Electrophoretic mobility shift DNA-binding experiments yield results for α-DNA interaction that are quantitatively equivalent to those of DNase I footprinting (cf. Ross et al. 1993; Blatter et al. 1994). Control experiments establish that the observed interactions are specific. Thus, under identical conditions, wild-type α, [Gly-261]α, and [Lys-261]α exhibit ~30-fold lower affinities for DNA fragments not containing a specific DNA site for α (5'-TCAGTTTTAT-GCAGC-3'/5'-GCTGGATGACGTCATAGGCG-3') and ~10-fold lower affinities for a DNA fragment containing a scrambled version of the specific DNA site for α (5'-TCAGTTTTATTAAACGGG-3'/5'-CCCGTTTTAATAAAACTGA-3').

For each preparation of α or derivative, the fraction of molecules active in sequence-specific DNA binding was determined by titration of the DNA fragment under stoichiometric binding conditions (100 μM DNA fragment; 50–400 μM α or derivative), all data are reported in terms of molar concentrations of active dimers.

Acknowledgments

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References


