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

Hong Tang,¹ Konstantine Severinov,² Alex Goldfarb,² David Fenyo,³ Brian Chait,³ and Richard H. Ebright^{1,4}

¹Department of Chemistry and Waksman Institute, Rutgers University, New Brunswick, New Jersey 08855 USA; ²Public Health Research Institute, New York, New York 10016 USA; ³Laboratory for Mass Spectrometry, Rockefeller University, New York, New York 10021 USA

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 $\sim 11 \times 14$ 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 $(\alpha)_2\beta\beta\sigma$ (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 not defective in CAP-independent transcription. Third, certain anti- α monoclonal antibodies strongly inhibit CAP-dependent transcription at the *lac* promoter but only partly inhibit CAP-independent transcription (Rifina 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.

⁴Corresponding 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 $\sim 90\%$ of RNAP would have at least one plasmid-encoded α subunit, and $\sim 50\%$ 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 ($\Delta gal165$ *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 $\Delta gal165$ 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 $\sim 30,000$ 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 \rightarrow Cys and Arg-265 \rightarrow 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

Table 1. Random mutagenesis and screen

Mutagenesis reactions	65
Total candidates (XE54)	33,000
$Lac^- Rbs^-$ candidates (XE54)	11
Total candidates (XE4)	27,000
$Lac^- Rbs^-$ candidates (XE4)	10

Tang et al.

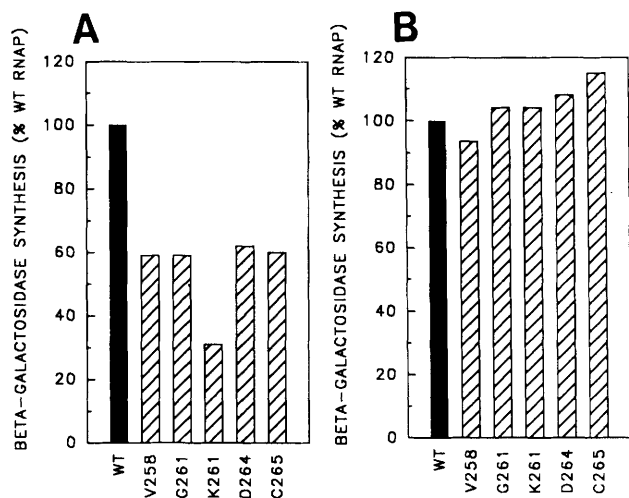


Figure 1. Transcription in vivo. (A) CAP-dependent transcription at the *lac* promoter; (B) CAP-independent transcription at the *lacPL8-UV5* promoter.

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 obtainable by a single-base-pair change can confer a $RpoA^{pct,CAP}$ phenotype able to pass the high-stringency screen. We propose that amino acids 258–265 constitute the only region of α that is essential for CAP-dependent transcription at the *lac* and *rbs* promoters but not essential for CAP-independent transcription.

The side chain of amino acid 261 of α is essential for CAP-dependent transcription

To define individual side-chain determinants for CAP-dependent transcription at the *lac* and *rbs* promoters, we performed an alanine scan (Cunningham and Wells 1989) of the activation target and 8 flanking amino acids. Alanine scanning has three distinct advantages for identification of individual side-chain determinants for protein–protein interaction (see Cunningham and Wells 1989, 1993; Wells 1991; Jin et al. 1992; Kelley and O’Connell 1993; Niu et al. 1994): First, alanine scanning yields a comprehensive set of substitutions, including both phenotypically positive and negative substitutions. Second, alanine scanning yields a chemically consistent set of substitutions. Third, and most important, alanine scanning yields side-chain truncation substitutions; alanine substitution eliminates all side-chain atoms beyond $C\beta$, and all interactions made by side-chain atoms beyond $C\beta$.

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 $C\beta$ 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 $C\beta$ 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 $C\beta$ 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 \rightarrow Asp and Arg-265 \rightarrow 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 $rpoa^{pct,CAP}$ mutants

Amino acid substitution	Codon substitution	Number of isolates, (XE54)	Number of isolates (XE4)	CAP-dependent transcription (<i>lac</i>) ^a	CAP-dependent transcription (<i>rbs</i>) ^a
None	None	—	—	++++	++++
258 Asp \rightarrow Val	GAC \rightarrow GTC	—	1	++	+
261 Glu \rightarrow Gly	GAA \rightarrow GGA	4	6	+	+
261 Glu \rightarrow Lys	GAA \rightarrow AAA	7	1	—	—
264 Val \rightarrow Asp	GTC \rightarrow GAC	—	1	+++	++
265 Arg \rightarrow Cys	CGC \rightarrow TGC	—	1	+++	+++

^aColony colors of XE54 pREII α and derivatives on tetrazolium indicator agar. (++++) White; (–) dark red.

Table 3. Alanine scanning

Amino acid substitution	CAP-dependent transcription (<i>lac</i>) ^a	CAP-dependent transcription (<i>rbs</i>) ^a
None	++++	++++
255 Arg → Ala	++++	++++
256 Pro → Ala	++++	++++
257 Val → Ala	++++	++++
258 Asp → Ala	++	+
259 Asp → Ala	++	++
260 Leu → Ala	++++	++++
261 Glu → Ala	-	-
262 Leu → Ala	++++	++++
263 Thr → Ala	++++	++++
264 Val → Ala	++++	+++
265 Arg → Ala	++++	+++
267 Ala	++++	++++
266 Ser → Ala	++++	++++
268 Asn → Ala	++++	++++
269 Cys → Ala	++++	++++
270 Leu → Ala	++++	++++

^aColony colors of XE54 pHTf1 α and derivatives on tetrazolium indicator agar.

gest that the Val-264 → Asp and Arg-265 → Cys substitutions affect CAP-dependent transcription indirectly, by introducing non-native side-chain 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 \leq 10% contaminating wild-type α .

To analyze CAP-dependent transcription, we performed abortive initiation in vitro transcription experi-

ments 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 *rnnB* P1 promoter, but absent in the *lac* promoter (the upstream element or UP element; Ross et al. 1993; Rao et al. 1994).

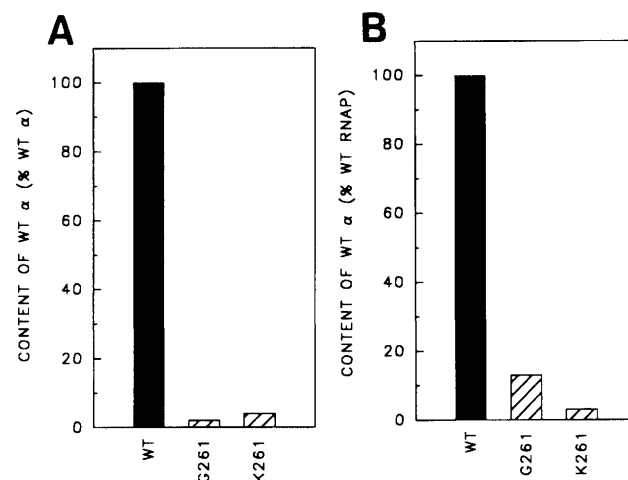


Figure 2. Purities of α derivatives and RNAP derivatives. Data are from endoproteinase Glu-C digestions followed by MALDI mass spectrometry. Peptide $\alpha(230-261)$ (molecular mass, 3805 daltons) is produced upon endoproteinase Glu-C digestion of wild-type α , but not upon endoproteinase Glu-C digestion of [Gly-261] α and [Lys-261] α and, therefore, is diagnostic of the presence of wild-type α . Control peptide $\alpha(289-319)$ (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 $\alpha(230-261)$ to control peptide $\alpha(289-319)$, 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.

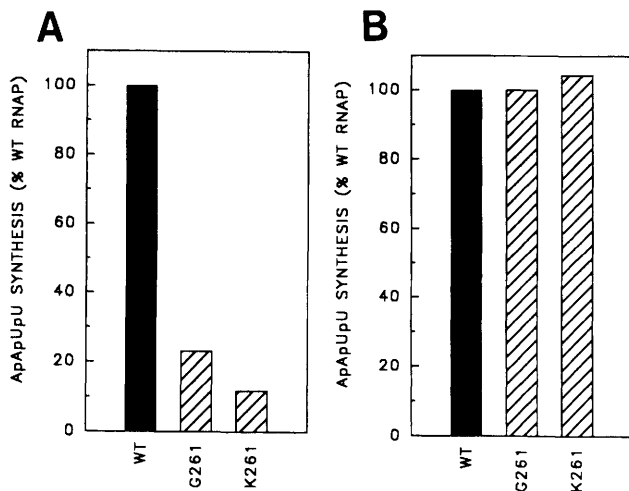


Figure 3. Transcription in vitro. (A) CAP-dependent transcription at the *lac* promoter (150 nM RNAP or derivative, 40 nM CAP); (B) CAP-independent transcription at the *lacPUV5* promoter (150 nM RNAP or derivative, 0 nM CAP).

The determinants for α -DNA interaction are contained within amino acids 245–329 of α .

As a first step in defining the relationship between activation target function and α -DNA interaction, we assessed the effect of substitution of the most critical amino acid of the activation target—amino acid 261—on α -DNA interaction. We performed electrophoretic mobility shift DNA-binding experiments using a 19-bp DNA fragment containing a specific DNA site for α (positions –57 to –47 of the *rnnB* P1 promoter) and using purified wild-type α , [Gly-261] α , and [Lys-261] α . The results are presented in Figure 4 and Table 4. Wild-type α binds to the DNA fragment with an apparent equilibrium binding constant of $2.1 \times 10^5 / M^{-1}$. [Gly-261] α and [Lys-261] α exhibit apparent equilibrium binding constants equal to, or slightly higher than, that of wild-type α . Consistent with the absence of a negative effect of substitution of amino acid 261 on α -DNA interaction in vitro, we find that substitution of amino acid 261 has little or no negative effect on upstream-element-dependent transcription at the *rnnB* P1 promoter in vivo (T. Gaal, W. Ross, R. Gourse, H. Tang, and R. Ebright, unpubl.). We conclude that amino acid 261 is not essential for α -DNA interaction. We conclude further that amino

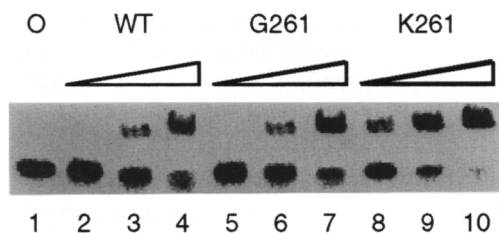


Figure 4. α -DNA interaction in vitro. (Lane 1) No α ; (lanes 2–4) 1.25, 2.5, and 5 μ M wild-type α ; (lanes 5–7) 1.25, 2.5, and 5 μ M [Gly-261] α ; (lanes 8–10) 1.25, 2.5, and 5 μ M [Lys-261] α .

Table 4. α -DNA interaction in vitro

Protein	K_{app} (M^{-1}) ^a
α	$2.1 (\pm 0.7) \times 10^5$
[Gly-261] α	$2.2 (\pm 0.7) \times 10^5$
[Lys-261] α	$6.3 (\pm 0.4) \times 10^5$

^aMean \pm 1 S.D.

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^{Pct,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-

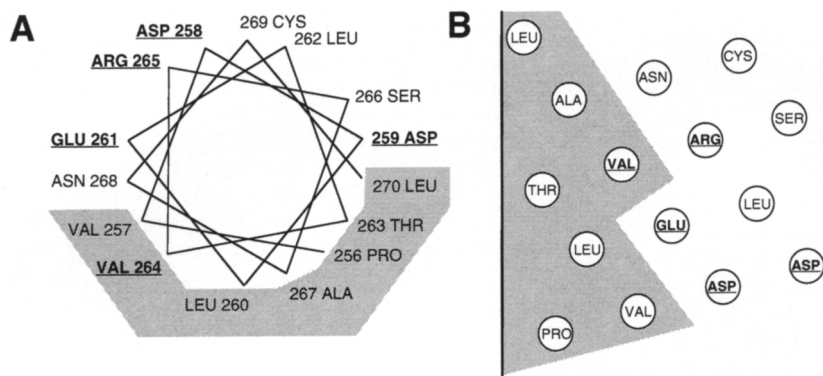


Figure 5. Proposed structure of the activation target. The hydrophobic and hydrophilic faces of the proposed surface amphipathic α -helix are shaded and unshaded, respectively. The 5 amino acids at which substitutions conferring an RpoA^{pct,CAP} phenotype were obtained are underlined. (A) Helix-wheel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

tions, the 5 amino acids at which substitutions conferring an RpoA^{pct,CAP} phenotype were obtained—that is, Asp-258, Asp-259, Glu-261, Val-264, and Arg-265—form a cluster, with 4 of the 5 amino acids solvent exposed and with the most critical amino acid—that is, Glu-261—centrally located and prominently solvent exposed (Fig. 5B).

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

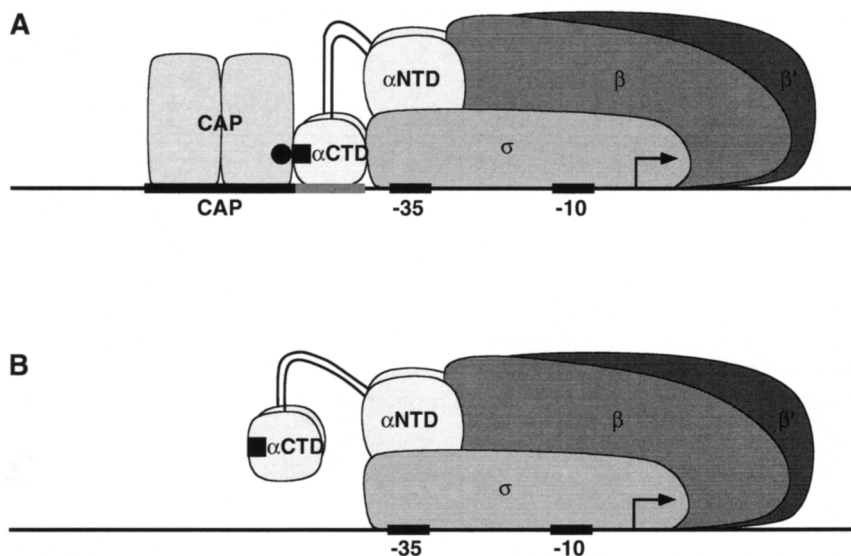


Figure 6. Proposed function of the activation target. α CTD and α NTD denote the α carboxy-terminal domain (two per RNAP molecule) and the α amino-terminal domain (two per RNAP molecule), respectively. (■) The activation target of α ; (●) the activating region of CAP. Black and shaded bars denote specific protein–DNA interactions and nonspecific protein–DNA interactions, respectively. Recent work regarding the domain organization and structure of α indicates that (1) the α carboxy-terminal domain is an independently folded, independently dimerized DNA-binding domain, and (2) the α carboxy-terminal domain is connected to the remainder of α and, thus, to the remainder of RNAP, through a ≥ 13 -amino-acid unstructured and/or flexible linker (≥ 44 Å if fully extended; Blatter et al. 1994). Alternative positioning of the α carboxy-terminal domain is proposed to be facilitated by the linker and, possibly, by bending of the intervening DNA. (A) CAP-dependent transcription at the *lac* promoter; (B) CAP-independent transcription at the *lacPUV5* promoter.

main is proposed to be facilitated by the linker and, possibly, by bending of the intervening DNA. (A) CAP-dependent transcription at the *lac* promoter; (B) CAP-independent transcription at the *lacPUV5* promoter.

Tang et al.

Table 5. Bacterial strains

Strain	Genotype	Source
XE4	<i>lac</i> ⁺ Δ <i>gal165 thi</i>	Ebright et al. (1984)
XE54	<i>lac</i> ⁺ <i>thi</i>	this work
XE56	<i>lacPL8-UV5 thi</i>	this work

All strains were constructed from strain X7029 (Δ *lacX74 thi*; Ebright 1985). The Δ *gal165* marker was from bacteriophage λ *CI857-gal313 Δ 165* (S. Adhya, unpubl.); 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 μ g/ml of ampicillin.

Plasmids

A list of plasmids used in this work is presented in Table 6. Plasmids pREII α , pHTf1 α , and derivatives encode α under control of the *lppP*–*lacPUV5* tandem promoter. Plasmid pHTT7 α and derivatives encode α under control of the bacteriophage T7 gene 10 promoter. Each plasmid has a unique *Xba*I site preceding the *rpoA* Shine–Delgarno sequence, a unique *Eco*RI site at codon 168, a unique *Hind*III site at codon 229, a unique *Sac*I site at codon 288, and, in plasmids pREII α , pHTT7 α , and derivatives, a unique *Bam*HI site immediately following the *rpoA* stop codon. For each plasmid, the DNA–nucleotide sequence of the entire *rpoA* gene was verified.

Mutagenesis

Random mutagenesis was performed by error-prone PCR amplification of the 1-kb *Xba*I–*Bam*HI *rpoA* segment of plasmid pREII α (procedure of Zhou et al. 1991, 1993a). The primers used were PIN-XBA-PCR (5'-GGATAACAATTTTCACACAGGAAA-CAG-3') and PIN-BAM-PCR (5'-CATTGCGTTCACGTCGT-TGCTCAG-3'). The procedure yields all four transition substitutions (A:T \rightarrow G:C, T:A \rightarrow C:G, C:G \rightarrow T:A, and G:C \rightarrow A:T), and at least four of eight transversion substitutions (A:T \rightarrow T:A, T:A \rightarrow A:T, G:C \rightarrow T:A, C:G \rightarrow A:T) (Table 2; Zhou et al. 1991, 1993a). Site-directed mutagenesis was performed by primer extension (procedure of Kunkel et al. 1991).

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 μ g/ml of ampicillin and 5 mM isopropyl-thio- β -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 pHTT7 α and derivatives (production to the level of \sim 30% of total cell protein). Following cell lysis by sonication, RNAP α and derivatives were localized \sim 50% in the soluble cell fraction, and \sim 50% in the insoluble cell fraction. RNAP α and derivatives were purified from the soluble cell fraction by polyethylenimine precipitation, ammonium sulfate precipitation, and hydrophobic interaction chromatography on phenyl-Toyoppearl (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 σ

RNAP β , β' and σ 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-

Table 6. Plasmids

Plasmid	Relevant characteristics	Source
pREII α	Ap ^R ; ori-pBR322; <i>IppP</i> '- <i>lacPUV5-rpoA</i>	Blatter et al. (1994)
pREII α 258V	Ap ^R ; ori-pBR322; <i>IppP</i> '- <i>lacPUV5-rpoA258V</i>	this work
pREII α 261G	Ap ^R ; ori-pBR322; <i>IppP</i> '- <i>lacPUV5-rpoA261G</i>	this work
pREII α 261K	Ap ^R ; ori-pBR322; <i>IppP</i> '- <i>lacPUV5-rpoA261K</i>	this work
pREII α 264D	Ap ^R ; ori-pBR322; <i>IppP</i> '- <i>lacPUV5-rpoA264D</i>	this work
pREII α 265C	Ap ^R ; ori-pBR322; <i>IppP</i> '- <i>lacPUV5-rpoA265C</i>	this work
pHTf1 α ^a	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA</i>	this work
pHTf1 α 255A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA255A</i>	this work
pHTf1 α 256A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA256A</i>	this work
pHTf1 α 257A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA257A</i>	this work
pHTf1 α 258A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA258A</i>	this work
pHTf1 α 259A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA259A</i>	this work
pHTf1 α 260A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA260A</i>	this work
pHTf1 α 261A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA261A</i>	this work
pHTf1 α 262A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA262A</i>	this work
pHTf1 α 263A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA263A</i>	this work
pHTf1 α 264A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA264A</i>	this work
pHTf1 α 265A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA265A</i>	this work
pHTf1 α 266A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA266A</i>	this work
pHTf1 α 268A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA268A</i>	this work
pHTf1 α 269A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA269A</i>	this work
pHTf1 α 270A	Ap ^R ; ori-pBR322; ori- <i>f1</i> ; <i>IppP</i> '- <i>lacPUV5-rpoA270A</i>	this work
pHTT7 α	Ap ^R ; ori-pBR322; ϕ 10P- <i>rpoA</i>	Blatter et al. (1994)
pHTT7 α 261G	Ap ^R ; ori-pBR322; ϕ 10P- <i>rpoA261G</i>	this work
pHTT7 α 261K	Ap ^R ; ori-pBR322; ϕ 10P- <i>rpoA261K</i>	this work
pMKSe2	Ap ^R ; ori-pBR322; <i>lacP-rpoB</i>	Severinov et al. (1993)
pT7 β '	Ap ^R ; ori-pBR322; ϕ 10P- <i>rpoC</i>	Zalenskaya et al. (1990)
pMRG8	Ap ^R ; ori-pBR322; λ P _L O _L - <i>rpoD</i>	Gribskov and Burgess (1983)
pBR-203-lac	Ap ^R ; ori-pBR322; <i>lacP</i> ⁺	Malan et al. (1984)
pBR-203-lacPUV5	Ap ^R ; ori-pBR322; <i>lacPUV5</i>	Zhou et al. (1993a)

^aPlasmid pHTf1 α was constructed by insertion of the 0.5-kb *Bgl*II-*Bgl*II *ori-f1* segment of plasmid pND118B (Heitman et al. 1989) at the *Bam*HI site of plasmid pREII α . Replication initiating at *ori-f1* yields single-stranded DNA containing the antisense strand of *rpoA*.

raphy on Superose 6 (Pharmacia, Inc.) followed by anion-exchange chromatography on Mono Q (Pharmacia, Inc.) (procedure of Borukhov and Goldfarb 1993). RNAP and derivatives were stored in aliquots at -20°C in 10 mM Tris-HCl (pH 7.9), 200 mM NaCl, 0.5 mM EDTA, and 50% glycerol.

Mass spectrometry

RNAP α and derivatives, prepared as described above, or prepared from RNAP and derivatives by denaturing centrifugal ultrafiltration [Centricon-100 filter units (Amicon, Inc.); 1000g; for 40 min at 4°C in 8 M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 150 mM KCl, and 1 mM EDTA], were digested with endoproteinase Glu-C, and products were analyzed by MALDI mass spectrometry. Reaction mixtures (30 μ l) contained 10 μ M RNAP α or derivative, 0.2 μ g of endoproteinase Glu-C (Boehringer Mannheim GmbH), 50 mM ammonium bicarbonate (pH 8.0), and 15 mM NaCl. After 1 hr at 22°C, 1- μ l aliquots were withdrawn, mixed with 9 μ l of 50 mM 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 Chait 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 *Eco*RI-*Eco*RI DNA frag-

ments of plasmids pBR-203-lac and pBR-203-lacPUV5. Reaction mixtures contained (25 μ l): 150 nM RNAP or RNAP derivative, 0 or 40 nM CAP (purified as described by Zhang et al. 1991), 0.5 nM DNA fragment, 0.5 mM ApA (ICN Biomedicals, Inc.), 50 nM [α -³²P]UTP (30 Bq/fmole), 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 μ l 0.5 M EDTA. The reaction product [³²P]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 *rnnB* P1 promoter upstream element; 5'-TCAGAAAATTATTTTCGGG-3'/5'-CCGAAAATAATTTTCTGA-3'). Reaction mixtures contained (20 μ l): 0 to 75 μ M α or derivative, 125 nM [³²P]-labeled DNA fragment (10 Bq/pmol; prepared as described by Ebright 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 \times 7 \times 0.15 cm), and electro-

phoresed in 45 mM Tris-borate (pH 8.0), 5 mM MgCl₂, and 0.1 mM EDTA (20 V/cm; 20 min at 4°C). Following electrophoresis, gels were dried and autoradiographed. Radioactivity was quantified by PhosphorImager analysis (Molecular Dynamics, model 425E). Equilibrium binding constants were calculated as described by Gunasekera et al. (1992).

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'-CGCCGATTGCGCAATCCAGC-3'/5'-GCTGGATTGCGCAATCGGCG-3'; 5'-CGCCTATGACGTCATCCAGC-3'/5'-GCTGGATTGCGTCATAGGCG-3') and ~10-fold lower affinities for a DNA fragment containing a scrambled version of the specific DNA site for α (5'-TCAGTTTAT-TAAAACGGG-3'/5'-CCCCTTTTAATAAAAAGTGA-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 μ M DNA fragment; 50–400 μ M α or derivative); all data are reported in terms of molar concentrations of active dimers.

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