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Transcription Activation at Class II CAP-Dependent Promoters: Two Interactions between CAP and RNA Polymerase

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Summary

At Class II catabolite activator protein (CAP)-dependent promoters, CAP activates transcription from a DNA site overlapping the DNA site for RNA polymerase. We show that transcription activation at Class II CAP-dependent promoters requires not only the previously characterized interaction between an activating region of CAP and the RNA polymerase α subunit C-terminal domain, but also an interaction between a second, promoter-class-specific activating region of CAP and the RNA polymerase α subunit N-terminal domain. We further show that the two interactions affect different steps in transcription initiation. Transcription activation at Class II CAP-dependent promoters provides a paradigm for understanding how an activator can make multiple interactions with the transcription machinery, each interaction being responsible for a specific mechanistic consequence.

Introduction

Escherichia coli catabolite activator protein (CAP; also referred to as the cAMP receptor protein, CRP) is a structurally characterized transcription activator protein (Kolb et al., 1993a). CAP functions by binding, in the presence of the allosteric effector cAMP, to specific DNA sites located near or in CAP-dependent promoters.

Simple CAP-dependent promoters (those that require only CAP for transcription activation) can be grouped into two classes (Ebright, 1993). In Class I CAP-dependent promoters, the DNA site for CAP is located upstream of the DNA site for RNA polymerase (RNAP). The best characterized Class I CAP-dependent promoters are the *lac* promoter and the artificial promoter CC(−61.5), each of which has a DNA site for CAP centered at position −61.5. In contrast, in Class II CAP-dependent promoters, the DNA site for CAP overlaps the DNA site for RNAP. The best characterized Class II CAP-dependent promoters are the *gal*P1 promoter and the artificial promoter CC(−41.5), each of which has a DNA site for CAP centered at position −41.5.

Recent work has established that amino acids 156–164 of CAP constitute an "activating region" essential for transcription activation at both Class I and Class II CAP-dependent promoters, but not essential for DNA binding and DNA bending (Bell et al., 1990; Eschenlauer and Reznikoff, 1991; Zhou et al., 1993a, 1994a; Niu et al., 1994). The activating region functions through protein–protein interaction with RNAP at both Class I and Class II CAP-dependent promoters, interacting with the RNAP α subunit C-terminal domain (αCTD) and facilitating binding of αCTD to DNA adjacent to CAP (Igarashi and Ishihama, 1991; Kolb et al., 1993b; Chen et al., 1994; Zhou et al., 1994b; Attey et al., 1994; Belyaeva et al., 1996; αCTD reviewed by Busby and Ebright, 1994).

At Class I CAP-dependent promoters, interaction between the activating region and αCTD (and concomitant recruitment of RNAP to promoter DNA) appears to be the entire basis of transcription activation. Thus, at Class I CAP-dependent promoters, removal of αCTD eliminates transcription activation (Igarashi and Ishihama, 1991).

At Class II CAP-dependent promoters, the mechanism of transcription activation is more complex. At Class II CAP-dependent promoters, interaction between the activating region and αCTD overcomes an inhibitory effect of αCTD, facilitating the positioning of αCTD at a neutral noninhibitory location on DNA (positions −74 to −58, immediately upstream of the DNA site for CAP) (Zhou et al., 1994b; Attey et al., 1994; Belyaeva et al., 1996). Whereas this "anti-inhibition" effect is essential for transcription activation at Class II CAP-dependent promoters, it is not the entire basis of transcription activation at Class II CAP-dependent promoters. Thus, removal of αCTD eliminates the requirement for anti-inhibition but does not eliminate transcription activation at Class II CAP-dependent promoters (Igarashi et al., 1991; Kolb et al., 1993b; West et al., 1993). In a previous report, we have proposed that transcription activation at Class II CAP-dependent promoters has two components: a first component involving anti-inhibition, mediated by the activating region, and a second component involving direct activation (Zhou et al., 1994b).

In this report, we show that transcription activation at Class II CAP-dependent promoters requires a second, Class II–specific activating region, define the critical residues of the second activating region, define the subunit orientation of the second activating region, present evidence that the second activating region functions through interaction with RNAP, and define the target in RNAP. In addition, we show that the previously characterized and second activating regions affect different steps in transcription.

Results

CAP Has a Class II–Specific Activating Region ("AR2")

The objective of the first set of experiments was to determine whether CAP contains a second activating region specifically required to activate transcription at Class II CAP-dependent promoters, and, if so, to define its location within CAP. Our approach was to perform random mutagenesis of the gene encoding CAP, followed by a screen, to isolate mutants specifically defective in transcription activation at Class II CAP-dependent promoters, i.e., defective in transcription activation at Class II CAP-dependent promoters, but not defective in

Table 1. Mutants Defective in Transcription Activation at Class II and Class I CAP-Dependent Promoters: *crp*^{pc,I}

Amino Acid Substitution	Codon Substitution	Number of Isolates	Activation, II (%)[a]	Activation, I (%)[a]	Repression (%)[a]
158 Thr→Ala	ACT→GCT	2[b]	15	2.0	120
158 Thr→Ile	ACT→ATT	1	13	5.7	120
159 His→Arg	CAC→CGC	1	8.8	4.6	120
160 Pro→Thr	CCG→ACG	1[c]	23	20	140
162 Gly→Asp	GGT→GAT	1	5.3	1.9	110
162 Gly→Val	GGT→GTT	1	5.2	3.4	160
164 Gln→Arg	CAA→CGA	1	6.7	10	110

[a]Activation and repression were measured in vivo and are expressed as percentages of activation and repression by wild-type CAP.
[b]One isolate contained a second substitution: Lys-101→Glu (AAA→GAA).
[c]The isolate contained a second substitution: Gly-132→Asp (GGC→GAC). Data for activation and repression are for a single-substitution mutant constructed by site-directed mutagenesis.

transcription activation at Class I CAP-dependent promoters, and not defective in DNA binding. We designate such mutants "*crp*^{pc,CAP,II}," where "*crp*" denotes the gene encoding CAP, "pc" denotes positive-control-defective, and "CAP, II" denotes Class II CAP-dependent promoters.

For our screen, we used strain XE82/CC(−41.5). This strain contained a deletion of *crp* and contained two reporter constructs. The first reporter construct had *lacZ* fused to the Class II CAP-dependent promoter CC(−41.5) and served as an indicator of Class II CAP-dependent transcription; the second reporter fusion had *lacZ* fused to the artificial CAP-repressed promoter *lacPUV5-O*^{CAP} (Irwin and Ptashne, 1987; Zhou et al., 1993a) and served as an indicator of repression, and thus of DNA binding.

We performed random mutagenesis of the *crp* gene of plasmid pYZCRP using error-prone PCR, introduced the mutagenized plasmid DNA into strain XE82/pRW 2CC(−41.5), and identified transformants defective in Class II CAP-dependent transcription but not defective in DNA binding as red colonies on lactose/tetrazolium agar.

We performed 60 independent mutagenesis reactions, screened 15,000 transformants, and isolated 21 independent mutants defective in Class II CAP-dependent transcription but not defective in DNA binding. For each mutant, we prepared plasmid DNA, introduced the plasmid DNA into strains XE65.2/pRWCC(−41.5), XE65.2/pRWCC(−61.5), and XE82, and performed in vivo assays of Class II CAP-dependent transcription, Class I CAP-dependent transcription, and DNA binding. Based on the results, the mutants could be divided into two groups. The first group, consisting of eight mutants, was defective in both Class II and Class I CAP-dependent transcription. The second group, consisting of 11 mutants, was defective solely in Class II CAP-dependent transcription.

Table 1 summarizes the sequences and phenotypes of the first group of mutants, i.e., those defective in both Class II and Class I CAP-dependent transcription (*crp*^{pc,CAP,I}). These mutants map to the previously defined activating region (Zhou et al., 1993a, 1994a; Niu et al., 1994). Thus, substitutions were obtained at amino acids 158, 159, 160, 162, and 164. Each of these mutants is defective in Class II CAP-dependent transcription, defective in Class I CAP-dependent transcription, but not defective in DNA binding.

Table 2 summarizes the sequences and phenotypes of the second, new group of mutants, i.e., those defective solely in Class II CAP-dependent transcription (*crp*^{pc,CAP,II}). These mutants map to the N-terminal cAMP binding domain of CAP, far from the previously defined activating region. Thus, substitutions were obtained at amino acids 19, 21, and 101. Each of these mutants is defective in Class II CAP-dependent transcription, but not defective in Class I CAP-dependent transcription and DNA binding. Each of these mutants is defective in Class II CAP-dependent transcription at all Class II promoters tested (CC[−41.5], *melR*, and *gal*P1 [Table 2 and data not shown; V. Rhodius and S. Busby, personal communication]).

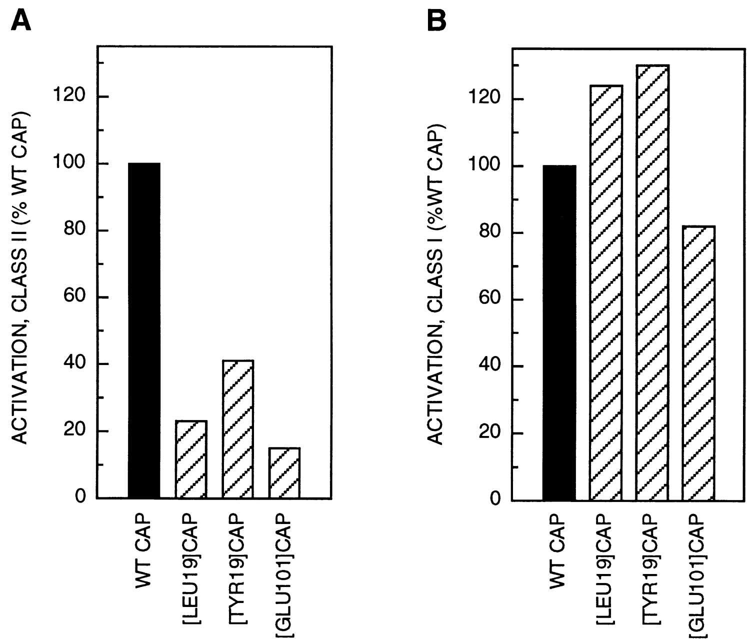
To confirm the results of the in vivo assays, we purified three of the mutant CAP derivatives and assessed Class II CAP-dependent transcription, Class I CAP-dependent transcription, DNA binding, and DNA bending in vitro.

To assess Class II and Class I CAP-dependent transcription, we performed abortive initiation in vitro transcription experiments with the CC(−41.5) promoter and the CC(−61.5) promoter, respectively. The results in Figures 1A and 1B show that the mutant CAP derivatives are defective in Class II CAP-dependent transcription,

Table 2. Mutants Defective in Transcription Activation at Class II CAP-Dependent Promoters but not Defective in Transcription Activation at Class I CAP-Dependent Promoters: *crp*^{pc,II}

Amino Acid Substitution	Codon Substitution	Number of Isolates	Activation, II (%)[a]	Activation, I (%)[a]	Repression (%)[a]
19 His→Leu	CAC→CTC	2	6.6	170	120
19 His→Tyr	CAC→TAC	3	10.3	170	120
21 His→Leu	CAT→CTT	1	7.2	150	180
101 Lys→Glu	AAA→GAA	5[b]	4.9	120	120

[a]Activation and repression were measured in vivo and are expressed as percentages of activation and repression by wild-type CAP.
[b]Two isolates contained second substitutions: Thr140→Ala (ACG→GCG) and Asp111→Val (GAC→GTC).



C

Protein	K_{obs} (M^{-1})
CAP	5 (±2) x 10^{10}
[Leu19]CAP	4 (±2) x 10^{10}
[Tyr19]CAP	4 (±2) x 10^{10}
[Glu101]CAP	7 (±2) x 10^{10}

D

Protein	DNA Bend Angle
CAP	122 (±2)°
[Leu19]CAP	121 (±5)°
[Tyr19]CAP	123 (±2)°
[Glu101]CAP	118 (±2)°

E

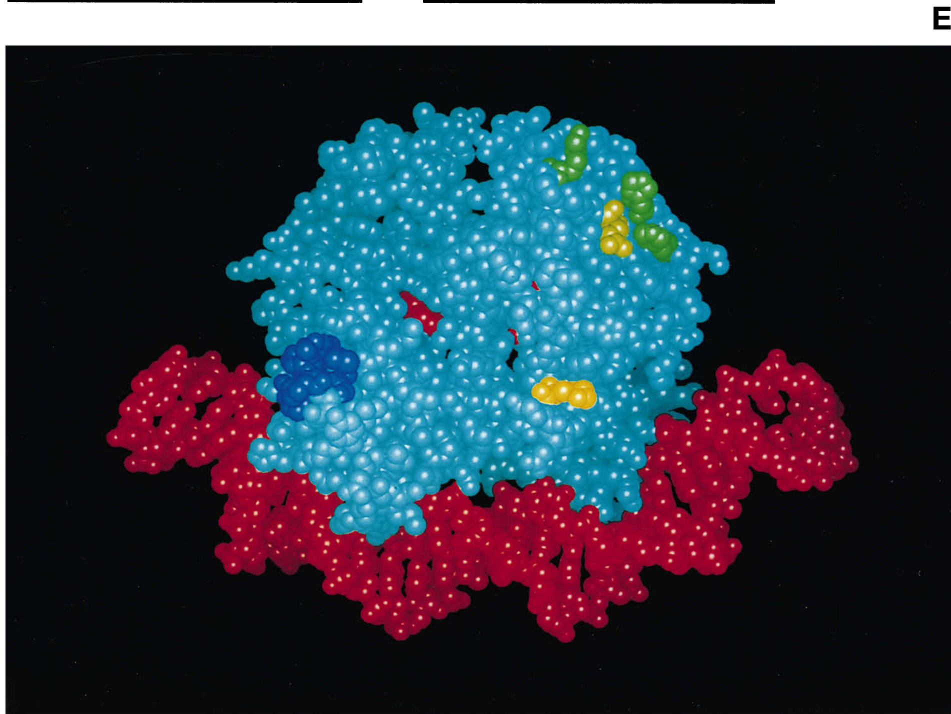


Figure 1. CAP Has a Class-II-Specific Activating Region ("AR2")

(A) Transcription activation at the Class II CAP-dependent promoter CC(−41.5) (in vitro).
(B) Transcription activation at the Class I CAP-dependent promoter CC(−61.5) (in vitro).
(C) DNA binding (means ± 2 SD).
(D) DNA bending (means ± 2 SEM).
(E) Structure of the CAP–DNA complex showing activating regions. The figure illustrates the crystallographic structure of the CAP–DNA complex at 2.5 Å resolution (Schultz et al., 1991; Parkinson et al., 1996). CAP is in light blue. DNA and cAMP are in red. AR1 is in blue (amino acids 156–164). AR2 is in green (amino acids 19, 21, and 101 in dark green; amino acid 96 in light green). Amino acid 52, a site at which substitutions result in improved transcription activation at Class II CAP-dependent promoters (Bell et al., 1990; Williams et al., 1991), is in yellow.

with defects from 2- to 7-fold, but are not defective in Class I CAP-dependent transcription.

To assess DNA binding, we performed equilibrium DNA binding experiments with a DNA fragment containing the consensus DNA site for CAP. The results in Figure 1C show that wild-type CAP and the mutant CAP derivatives exhibit indistinguishable DNA binding affinities.

To assess DNA bending, we performed electrophoretic mobility shift experiments with four circularly permuted DNA fragments containing the consensus DNA site for CAP. The results in Figure 1D show that wild-type CAP and the mutant CAP derivatives result in indistinguishable DNA bend angles.

Taken together, our results establish that amino acids 19, 21, and 101 of CAP are critical for Class II CAP-dependent transcription, but are not critical for Class I CAP-dependent transcription, DNA binding, and DNA bending. The simplest interpretation of the results is that Class II CAP-dependent transcription requires a second interaction between CAP and RNAP, in addition to the interaction by the previously defined activating region, and that amino acids 19, 21, and 101 constitute the determinant of CAP for this second interaction.

In the primary structure of CAP, amino acids 19 and 21 are distant from amino acid 101. However, in the three-dimensional structure of CAP, amino acids 19, 21, and 101 are adjacent to each other (Figure 1E). Amino acids 19, 21, and 101 are distant from the amino acids of CAP involved in DNA binding and DNA bending and are located in a prominently accessible and protruding portion of CAP (Figure 1E).

West et al. (1993) have shown that substitution of amino acid 96 of CAP improves transcription activation at Class II CAP-dependent promoters. Amino acids 19, 21, and 101 are adjacent to amino 96, and with this amino acid, form a surface with dimensions of 20 × 8 Å (Figure 1E). We designate these four amino acids "activating region 2" (AR2), and we redesignate the previously defined activating region "activating region 1" (AR1). We propose that both AR1 and AR2 interact with RNAP in transcription activation at Class II CAP-dependent promoters.

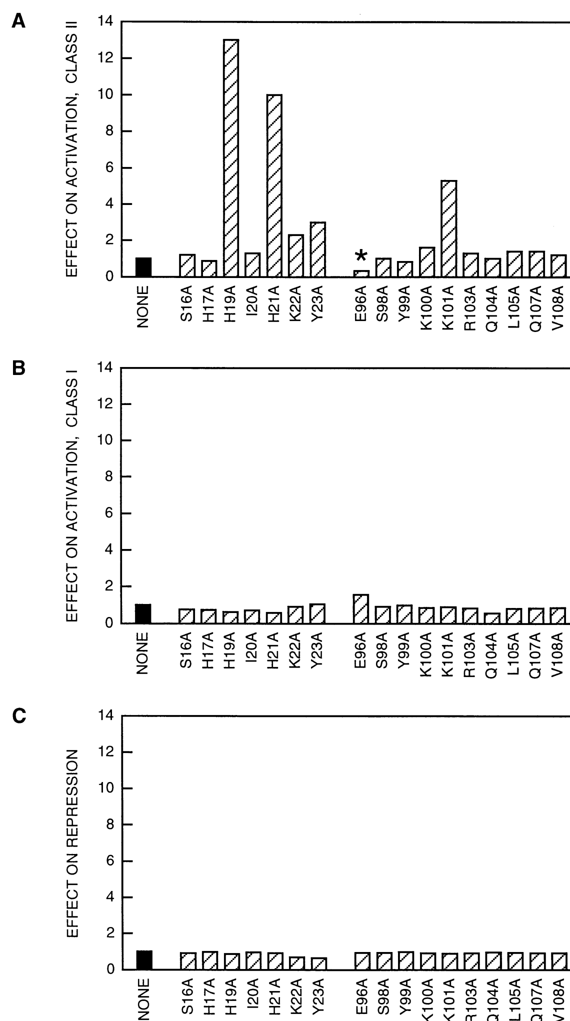


Figure 2. AR2 Requires Positive Charge

(A) Effects of alanine substitution on transcription activation at the Class II CAP-dependent promoter CC(−41.5) ($activation_{CAP}/activation_X$). Substitution of His-19, His-21, or Lys-101 results in a >5-fold defect in transcription activation; substitution of Glu-96 results in a ≥3-fold improvement in transcription activation (asterisk).

(B) Effects of alanine substitution on transcription activation at the Class I CAP-dependent promoter CC(−61.5) ($activation_{CAP}/activation_X$).

(C) Effects of alanine substitution on transcription repression at an artificial CAP-repressed promoter ($repression_{CAP}/repression_X$).

AR2 Requires Positive Charge

To identify critical side-chain determinants within AR2, we performed alanine scanning (Cunningham and Wells, 1989). We substituted each surface amino acid from 16–23 and 96–108 of CAP, one-by-one, by alanine. We then assessed Class II CAP-dependent transcription, Class I CAP-dependent transcription, and DNA binding in vivo.

The results for Class II CAP-dependent transcription are presented in Figure 2A. Alanine substitution of amino acids 19, 21, and 101 resulted in ≥5-fold defects in Class II CAP-dependent transcription. We conclude that, for each of these amino acids, side-chain atoms beyond C^{β} make favorable interactions in Class II CAP-dependent transcription. Alanine substitution of amino acid 96, an amino acid at which non-alanine substitutions improve Class II CAP-dependent transcription (West et al., 1993), resulted in a 2- to 3-fold increase in Class II CAP-dependent transcription. We conclude that, for this amino acid, side-chain atoms beyond C^{β} make unfavorable interactions in Class II CAP-dependent transcription. Alanine substitution of no other amino acid in the region tested had a significant, reproducible effect. We conclude that for no amino acid other than 19, 21, 101, and 96 do side-chain atoms beyond C^{β} make significant interactions in Class II CAP-dependent transcription.

The results for Class I CAP-dependent transcription and DNA binding are presented in Figures 2B and 2C. Alanine substitution of no amino acid in the region tested had a significant, reproducible effect on Class I CAP-dependent transcription or DNA binding, confirming that this region is not involved in these processes.

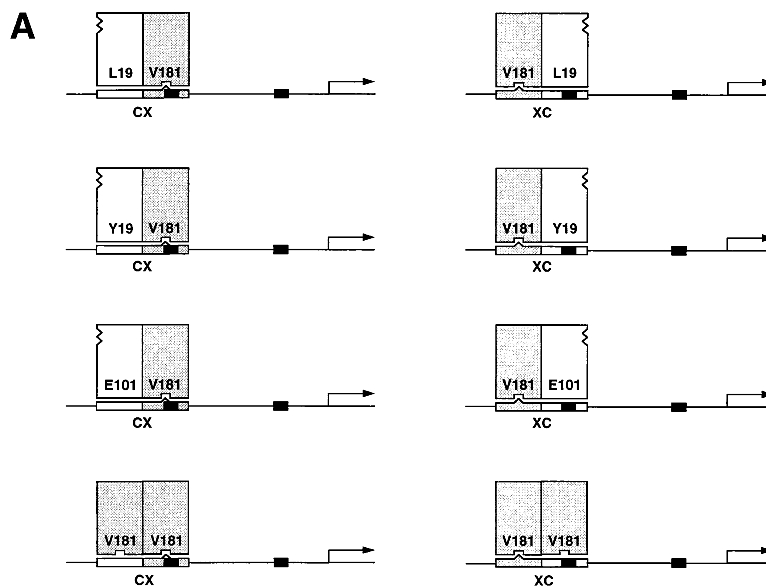


Figure 3. AR2 Functions in the Downstream Subunit of CAP

(A) Expected orientations of CAP heterodimers at the CX(−41.5) and XC(−41.5) promoters (see Zhou et al., 1993b, 1994b). L19, Y19, and E101 denote [Leu-19]CAP, [Tyr-19] CAP, and [Glu-101]CAP subunits (nonfunctional AR2, wild-type DNA-binding specificity). V181 denotes the [Val-181]CAP subunit (functional AR2, non-wild-type DNA-binding specificity). Promoter −35 and −10 elements are shown as bars; transcription start points are shown as arrows.

(B) Transcription activation by oriented heterodimers (determined in vivo as in Zhou et al., 1994b).

B

Binding-Competent Dimer	β-Galactosidase Synthesis		
	CX	XC	CX/XC
[Leu19]CAP/[Val181]CAP	23	4.4	5.2
[Tyr19]CAP/[Val181]CAP	25	5.2	4.8
[Glu101]CAP/[Val181]CAP	23	3.7	6.2
[Val181]CAP/[Val181]CAP	62	57	1.1

It is striking that all three amino acids that make favorable interactions are positively charged (His-19, His-21, and Lys-101), and that the amino acid that makes an unfavorable interaction is negatively charged (Glu-96). Based on the correlation of positive charge with function, we suggest that the most important structural feature of AR2 is positive charge and that AR2 is likely to interact with a target having a complementary charge, i.e., a negative charge.

AR2 Functions in the Downstream Subunit of CAP

To determine which subunit of the CAP dimer functionally presents AR2, we performed "oriented-heterodimer" analysis (Figure 3A; Zhou et al., 1993b, 1994b). We constructed CAP heterodimers having one subunit with a functional AR2 and one subunit with a nonfunctional AR2, we oriented the heterodimers on Class II promoter DNA using appropriate DNA-binding specificity mutants in protein and DNA, and we assessed the abilities of the oriented heterodimers to activate transcription.

We performed oriented-heterodimer analysis for each of three AR2$^-$ mutants: [Leu-19]CAP, [Tyr-19]CAP, and [Glu-101]CAP (Figure 3). In each case, the result was the same. In the orientation in which the functional AR2 was in the downstream subunit, the heterodimer was functional in transcription activation (CX in Figure 3); in contrast, in the orientation in which the nonfunctional AR2 was in the downstream subunit, the heterodimer was nonfunctional in transcription activation (XC in Figure 3). We conclude that Class II CAP-dependent transcription requires a functional AR2 in only one subunit, i.e., the downstream subunit. Consistent with this conclusion, the super-activating effect of substitution of amino acid 96 occurs when the substitution is present in only one subunit, i.e., the downstream subunit (Williams et al., 1996).

AR2 Functions through Protein–Protein Interaction with RNAP

To test directly the hypothesis that AR2 interacts with RNAP, we measured CAP–RNAP interaction in solution.

In published work, we have shown using fluorescence-anisotropy measurements that a binary complex of CAP and a fluorochrome-labeled short DNA fragment containing the DNA site for CAP and containing no specific determinants for binding of RNAP is able to interact with RNAP to form a ternary complex (Heyduk et al., 1993). The interaction exhibits an equilibrium binding constant of $\approx 5 \times 10^7$ M^{-1} and requires AR1 (Heyduk et al., 1993; Figure 4). In subsequent work, we have shown that the interaction is strengthened 6-fold upon substitution of Lys-52 by asparagine (a substitution that results in improved transcription activation at Class II, but not

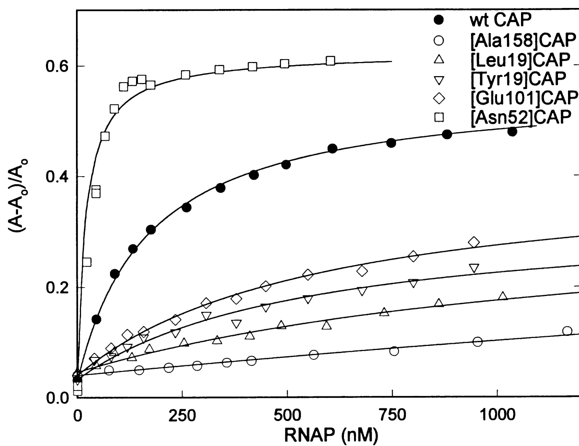


Figure 4. AR2 Functions through Protein–Protein Interaction with RNAP

Fluorescence anisotropy analysis of interaction of RNAP with a preformed binary complex of CAP and a fluorescein-labeled DNA fragment containing the consensus DNA site for CAP (DNA fragment ICAP42FL of Heyduk et al., 1993). Data are shown for wild-type CAP (filled circles), [Ala-158]CAP (AR1$^-$; open circles), [Leu-19]CAP (AR2$^-$; open triangles), [Tyr-19]CAP (AR2$^-$; open inverted triangles), [Glu-101]CAP (AR2$^-$; open diamonds), and [Asn-52]CAP (a mutant superfunctional in transcription activation at Class II CAP-dependent promoters [Bell et al., 1990; Williams et al., 1991]; open squares). Data are expressed as $(A - A_0)/A_0$, where A denotes fluorescence anisotropy in the presence of RNAP and CAP, and A_0 denotes fluorescence anisotropy in the absence of RNAP and CAP.

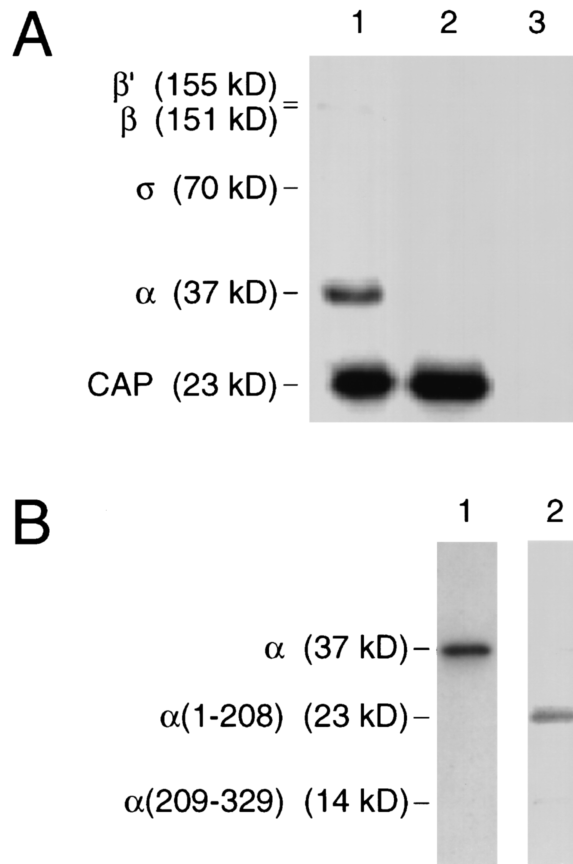


Figure 5. AR2 Interacts with the RNAP α Subunit N-Terminal Domain

(A) Site-specific protein–protein photocross-linking followed by cleavage and radiolabel transfer. Lane 1, photocross-linking reaction; lane 2, control reaction omitting RNAP; lane 3, control reaction omitting UV-irradiation. Radiolabeled CAP is the product of intramolecular cross-linking; radiolabeled α is the result of intermolecular CAP→α photocross-linking.

(B) Proteolytic mapping. Lane *1*, radiolabeled α (see A); lane 2, hydroxylamine digest of radiolabeled α.

Class I, CAP-dependent promoters [Bell et al., 1990; Williams et al., 1991; see Discussion]), indicating that the ternary complex analyzed in fluorescence-anisotropy measurements is a Class II ternary complex (Figure 4).

In the current work, we performed analogous experiments with each of three AR2$^-$ mutants: [Leu-19]CAP, [Tyr-19]CAP, and [Glu-101]CAP (Figure 4). The AR2$^-$ mutants exhibited 8- to 20-fold defects in interaction with RNAP. We conclude that AR2 makes direct, favorable protein–protein interaction with RNAP and that amino acids 19 and 101 contribute ≈1–2 kcal/mol each toward CAP–RNAP interaction.

AR2 Interacts with the RNAP α Subunit N-Terminal Domain

As a first step to identify the target of AR2 within RNAP, we performed site-specific protein–protein photocross-linking (Chen et al., 1994). We constructed a CAP derivative having a photoactivatible cross-linking agent site-specifically incorporated at a single, defined amino acid adjacent to AR2, i.e., amino acid 17. We then formed the ternary complex of CAP derivative, RNAP, and Class II CAP-dependent promoter CC(−41.5), UV-irradiated the ternary complex to initiate cross-linking, and determined the site at which cross-linking occurred. To facilitate identification of the site at which cross-linking occurred, we used a photoactivatible cross-linking agent that contained a radiolabel and that was attached to CAP through a disulfide linkage. This permitted, after UV-irradiation, cleavage of the cross-link and transfer of radiolabel to the site at which cross-linking occurred.

The results are presented in Figure 5A. CAP→RNAP cross-linking occurred primarily in the RNAP α subunit (efficiency ≈ 20%). (Smaller amounts of cross-linking occurred in the RNAP β or β' subunit [or in both] [efficiency ≈ 4–5%], and much smaller amounts of cross-linking occurred in the RNAP σ subunit [efficiency ≈ 1%].) Control experiments established that CAP→RNAP cross-linking required UV-irradiation, promoter DNA, RNAP, and cAMP (the allosteric effector required for specific DNA binding by CAP) (Figure 5A and data not shown).

To define the site in α at which cross-linking occurs, we performed proteolytic mapping with hydroxylamine, which cleaves α into fragments consisting of amino acids 1–208 and 209–329 (Figure 5B). The results establish that cross-linking occurs within amino acids 1–208. This region of α corresponds to the α N-terminal domain (αNTD; amino acids 8–235; Blatter et al., 1994; Busby and Ebright, 1994).

We conclude that, in the ternary complex of CAP, RNAP, and Class II CAP-dependent promoter, AR2 of CAP is in direct physical proximity to αNTD, and we propose that AR2 interacts with αNTD.

AR2 Interacts with Amino Acids 162–165 within the RNAP α Subunit N-Terminal Domain

To define further the target of AR2, we performed random mutagenesis of the gene encoding α and screened for mutants specifically defective in Class II CAP-dependent transcription, i.e., defective in Class II CAP-dependent transcription but not defective in Class I CAP-dependent transcription and CAP-independent transcription. We designate such mutants "*rpoA*^{pct,CAP,II}," where "*rpoA*" denotes the gene encoding α, "pct" denotes positive-control-target-defective, and "CAP,II" denotes Class II CAP-dependent transcription.

Our screen tested two phenotypes: first, defect in Class II CAP-dependent transcription, and second, absence of a defect in CAP-independent transcription. To test the first phenotype, the screen scored *lacZ* expression from a $P_{CC(-41.5)}$–*lacZ* fusion. To test the second phenotype, the screen scored viability. We reasoned that mutants of α specifically defective in Class II CAP-dependent transcription, like mutants lacking CAP (Sabourin and Beckwith, 1975), would be viable on rich media, but that mutants of α defective in both Class II CAP-dependent and CAP-independent transcription would be inviable (cf. Tang et al., 1994).

We performed random mutagenesis of the entire *rpoA* gene of plasmid pREIIα using error-prone polymerase chain reaction (PCR), introduced the mutagenized plasmid DNA into strain XE3000, and identified transformants defective in Class II CAP-dependent transcription but not defective in CAP-independent transcription as red colonies on lactose/tetrazolium agar. We performed 60 independent mutagenesis reactions, screened 100,000 transformants, and isolated three independent mutants.

All three mutants mapped to a single amino acid within αNTD and resulted in the same substitution: Glu-165→Lys (Figure 6A). Based on in vivo assays, the mutants were defective in Class II CAP-dependent transcription (assayed at CC[−41.5]), but not defective in Class I CAP-dependent transcription (assayed at CC[−61.5], *lac*, and *rbs*), and not defective in CAP-independent transcription (assayed at *lacPL8-UV5*) (Figure 6A and data not shown).

To confirm the results of the in vivo assays, we reconstituted [Lys-165]α-RNAP from purified [Lys-165]α and wild-type β, β', and σ^{70}, and we analyzed transcription in vitro. The results are presented in Figures 6B–6D. [Lys-165]α-RNAP was 5-fold defective in Class II CAP-dependent transcription, but not defective in Class I CAP-dependent transcription and CAP-independent transcription. We conclude that amino acid 165 within αNTD is essential for Class II CAP-dependent transcription, but not for Class I CAP-dependent transcription and CAP-independent transcription.

Glu-165 is a negatively charged amino acid, and the Glu-165→Lys substitution results in charge reversal, replacing this negatively charged amino acid by a positively charged amino acid. The negative charge of Glu-165 is consistent with the hypothesis that this amino acid interacts with AR2, which carries, and requires, positive charge.

Glu-165 is located in a string of four consecutive negatively charged amino acids, i.e., Glu-162, Glu-163, Asp-164, and Glu-165 (Figure 6A). For each of these amino acids, we constructed a single alanine substitution and analyzed the effect on Class II CAP-dependent transcription at CC(−41.5) (Figure 6). In each case, alanine substitution resulted in a small, but reproducible, defect in Class II CAP-dependent transcription. Simultaneous alanine substitution of all four amino acids resulted in a large defect in Class II CAP-dependent transcription, a defect comparable with that upon charge reversal at amino acid 165. We conclude that, for each of these four consecutive negatively charged amino acids, side-chain atoms beyond C^{β} are important for Class II CAP-dependent transcription, and we propose that it is the negative charge of the side-chain atoms beyond C^{β} that is the critical functional determinant.

Based on the correspondence between the photocross-linking results demonstrating that AR2 of CAP is in direct physical proximity to αNTD in the ternary complex of CAP, RNAP, and Class II CAP-dependent promoter (Figure 5), the genetic results demonstrating that amino acids 162–165 within αNTD are important for Class II CAP-dependent transcription (Figure 6), and the charge complementarity between AR2 and amino acids 162–165 within αNTD, we conclude that transcription activation at Class II CAP-dependent promoters involves interaction between AR2 and amino acids 162–165 within αNTD.

AR1 and AR2 Affect Different Steps in Transcription Initiation

We have analyzed the effects of substitution of AR1 and AR2 on the kinetics of Class II CAP-dependent transcription (methods as in McClure, 1980; Figure 7). Substitution of AR1 resulted in an 8-fold decrease in the binding constant, K_B, for interaction of RNAP with promoter DNA to form the closed complex. Substitution of AR2 resulted in a 10-fold decrease in the rate constant, k_f, for isomerization of the closed complex to the open complex with single-stranded DNA in the RNAP active site. Similar results have been obtained with at least one other Class II CAP-dependent promoter (*melRcon*; V. Rhodius and S. Busby, personal communication). We conclude that AR1 facilitates formation of closed complex and that AR2 facilitates isomerization of closed complex to open complex.

Discussion

CAP–RNAP Interactions at Class II CAP-Dependent Promoters

Previous results establish that transcription activation at Class II CAP-dependent promoters involves interaction between amino acids 156–164 (activating region 1, AR1) of the upstream subunit of CAP and a target within RNAP αCTD (Bell et al., 1990; Williams et al., 1991; Zhou et al., 1994a, 1994b; Attey et al., 1994; Belyaeva et al., 1996). The present results establish that transcription activation at Class II CAP-dependent promoters also involves interaction between amino acids 19, 21, 96, and 101 (activating region 2, AR2) of the downstream subunit of CAP and amino acids 162–165 within RNAP αNTD.

In the structure of the CAP–DNA complex, AR1 of one subunit and AR2 of the other subunit are located on a

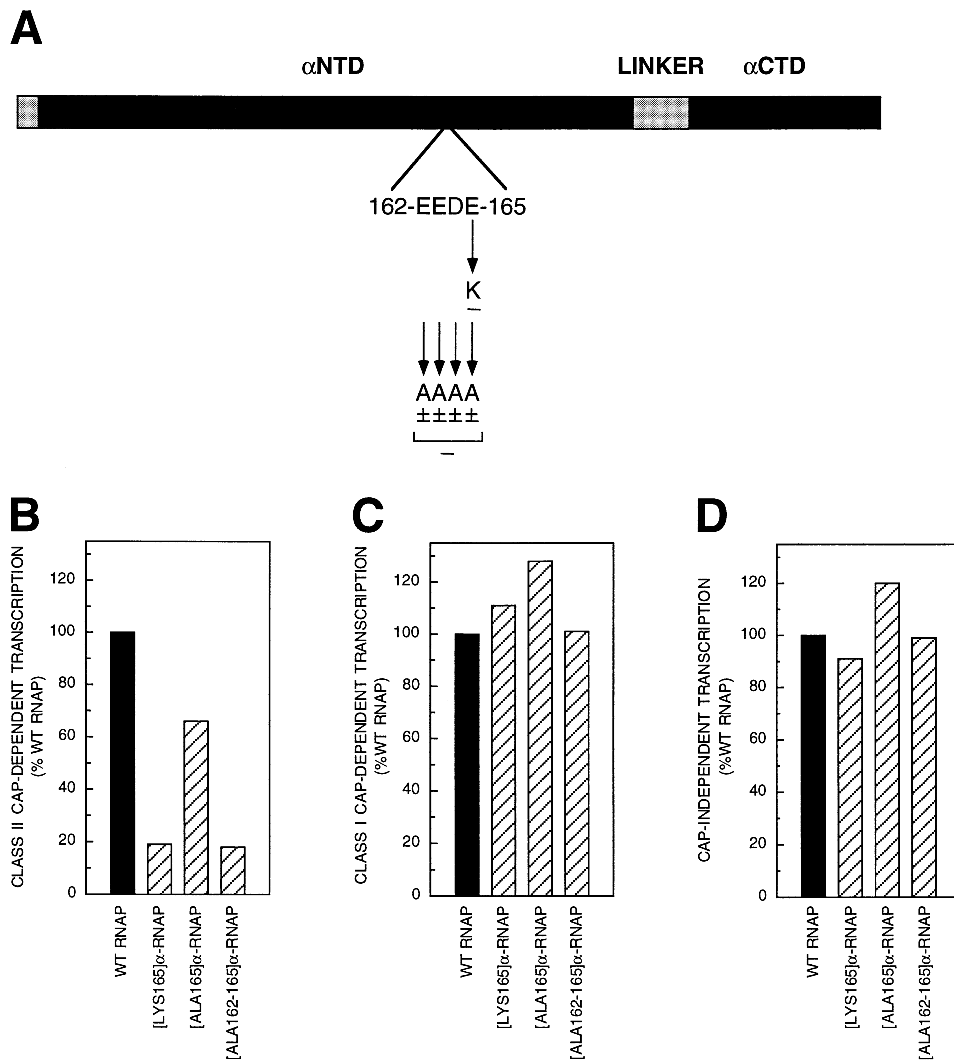


Figure 6. AR2 Interacts with Amino Acids 162–165 of RNAP α Subunit N-Terminal Domain

(A) Substitutions within RNAP α subunit that result in specific defects in Class II CAP-dependent transcription (Glu-165→Lys, Glu-162→Ala, Glu-163→Ala, Asp-164→Ala, Glu-165→Ala; −, large defect in vivo; ±, small, but reproducible, defect in vivo). αNTD, αCTD, and linker denote the α N-terminal domain, the α C-terminal domain, and the interdomain linker, respectively (Blatter et al., 1994; Busby and Ebright, 1994).

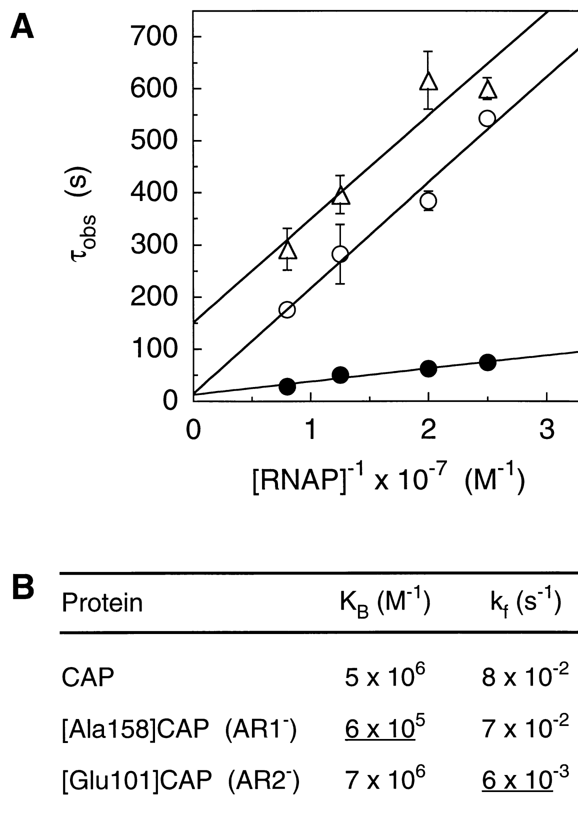
(B) Class II CAP-dependent transcription at CC(−41.5) (in vitro).

(C) Class I CAP-dependent transcription at CC(−61.5) (in vitro).

(D) CAP-independent transcription at *lacUV5* (in vitro).

single face of the CAP dimer, but are separated by nearly the full length of this face (50 Å; Figure 1E). We infer that, in the ternary complex of CAP, RNAP, and a Class II CAP-dependent promoter, RNAP interacts with a single face of the CAP dimer and extends across the full length of this face, with αCTD contacting AR1 of the upstream subunit and αNTD contacting AR2 of the downstream subunit (Figure 8).

Substitution of Lys-52 of the downstream subunit of CAP results in improved transcription activation at Class II CAP-dependent promoters (Bell et al., 1990; Williams et al., 1991, 1996). Lys-52 of the downstream subunit of CAP is located on the same face of the CAP–DNA complex as AR1 of the upstream subunit and AR2 of the downstream subunit (Figure 1E), but is distant from AR1 and AR2 (30 Å from each; Figure 1E). In the context of wild-type CAP, the region containing Lys-52 plays little or no role in transcription activation. Thus, random mutagenesis and screening for mutants defective in transcription activation yields no mutants affecting this region (Tables 1 and 2), and targeted mutagenesis yields no strong mutants (Williams et al., 1991; X. Zhang and R. H. E., unpublished data). It appears likely that substitution of Lys-52 improves transcription activation promoters by creating a new, non-native favorable interaction with residues of RNAP close to Lys-52 in the ternary complex of CAP, RNAP, and Class II CAP-dependent promoter. Several lines of evidence suggest that the residues of RNAP close to Lys-52 in the ternary complex are residues of σ^{70} (Jin et al., 1995; M. Lonetto, V. Rhodius, S. Busby, and C. Gross, personal communication; Figures 1E and 8).



Protein	K_B (M^{-1})	k_f (s^{-1})
CAP	5×10^6	8×10^{-2}
[Ala158]CAP (AR1$^-$)	$\underline{6 \times 10^5}$	7×10^{-2}
[Glu101]CAP (AR2$^-$)	7×10^6	$\underline{6 \times 10^{-3}}$

Figure 7. AR1 and AR2 Affect Different Steps in Transcription Initiation

(A) τ-plot comparing Class II CAP-dependent transcription with wild-type CAP (filled circles), [Ala-158]CAP (AR1$^-$; open circles), and [Glu-101]CAP (AR2$^-$; open triangles). Data plotted are means of three independent determinations (± 1 SD). Slopes = $(K_B k_f)^{-1}$; y-intercepts = $(k_f)^{-1}$ (McClure, 1980).

(B) Values of K_B and k_f.

Mechanism of Transcription Activation at Class II CAP-Dependent Promoters

The AR1–αCTD interaction and the AR2–αNTD interaction have different mechanistic consequences. The AR1–αCTD interaction overcomes an inhibitory effect of αCTD on Class II CAP-dependent transcription (West et al., 1993; Zhou et al., 1994b) and manifests itself as an increase in the binding constant, K_B, for interaction of RNAP with promoter DNA to form closed complex (Figure 7). The AR2–αNTD interaction increases the rate constant, k_f, for isomerization of closed complex to open complex (Figure 7). Both interactions—and both mechanistic components—are essential for Class II CAP-dependent transcription.

It remains to be determined how the AR2–αNTD interaction increases k_f. Based on the structure of the CAP–DNA complex and the position of the DNA site for CAP at a Class II CAP-dependent promoter, the AR2–αNTD interaction occurs 30 Å from promoter DNA and >70 Å from the RNAP active site (Figures 1C and 8). Therefore, the effect of the AR2–αNTD interaction on k_f must be a long-range effect. In principle, two mechanisms are possible: first, the AR2–αNTD interaction may trigger an allosteric change between inactive and active RNAP conformations; or second, the AR2–αNTD interaction may stabilize the transition state between closed and open complex. We favor the second mechanism, since it requires only that the AR2–αNTD interaction become stronger in the transition state between closed and open complex (something easily envisioned given the large changes in RNAP and promoter conformation in the transition state [see Schickor et al., 1990]). We note that the AR2–αNTD interaction appears to be an electrostatic interaction, i.e., an interaction that increases linearly in strength with decreasing distance and, thus, an interaction that could become stronger with decreased AR2–αNTD distance in the transition state.

General Implications

Our results have three general implications.

First, our identification of a promoter-class-specific activating region and activation target establishes that mechanisms of transcription activation depend on promoter architecture.

Second, our results establish that αNTD can serve as an activation target. This brings to three the number of identified activation targets within the prokaryotic transcription machinery: σ^{70} region 4 (Busby and Ebright, 1994), αCTD (Busby and Ebright, 1994), and αNTD. We speculate that the requirements for function as an activation target are not stringent. We speculate that nearly any exposed surface of the transcription machinery can serve as the target for an activator that increases K_B (see also Busby and Ebright, 1994; Barberis et al., 1995), and that nearly any exposed surface of the transcription machinery that undergoes changes in position or conformation in the transition from closed complex to open complex can serve as the target for an activator that increases k_f.

Third, our results establish that an activator can interact with multiple targets within the transcription machinery and thereby affect multiple steps in transcription initiation. The eukaryotic activator VP16 has been reported to interact with at least five components of the eukaryotic transcription machinery, but the functional significance of the interactions has not been established—and is controversial (Tjian and Maniatis, 1994). Transcription activation at Class II CAP-dependent promoters provides a paradigm for understanding how an activator can make multiple interactions with the transcription machinery, with each interaction being responsible for a specific mechanistic consequence.

Experimental Procedures

Strains

Strains are listed in Table 3. Strains XE3000 and XE3020 were constructed in two steps: first, ligation of the BglII–BstEII $P_{CC(-41.5)}$ *lacZYA* and $P_{CC(-61.5)}$–*lacZYA* segments of pRW2CC(−41.5) and pRW 2CC(−61.5) with the BamHI–BstEII *att*λ Km^r segment of pTAC3466 to prepare reporter-fusion-bearing *ori*$^-$ *att*λ Km^r minicircles, and second, introduction of the minicircles into strain X7029/pINT-ts, selecting Km^r at 37°C, and screening Ap^s at 37°C (see Atlung et al., 1991; Hasan et al., 1994).

Plasmids

Plasmids are listed in Table 4. pREII-NHα, which encodes N-terminally hexahistidine-tagged α under control of tandem *lpp* and

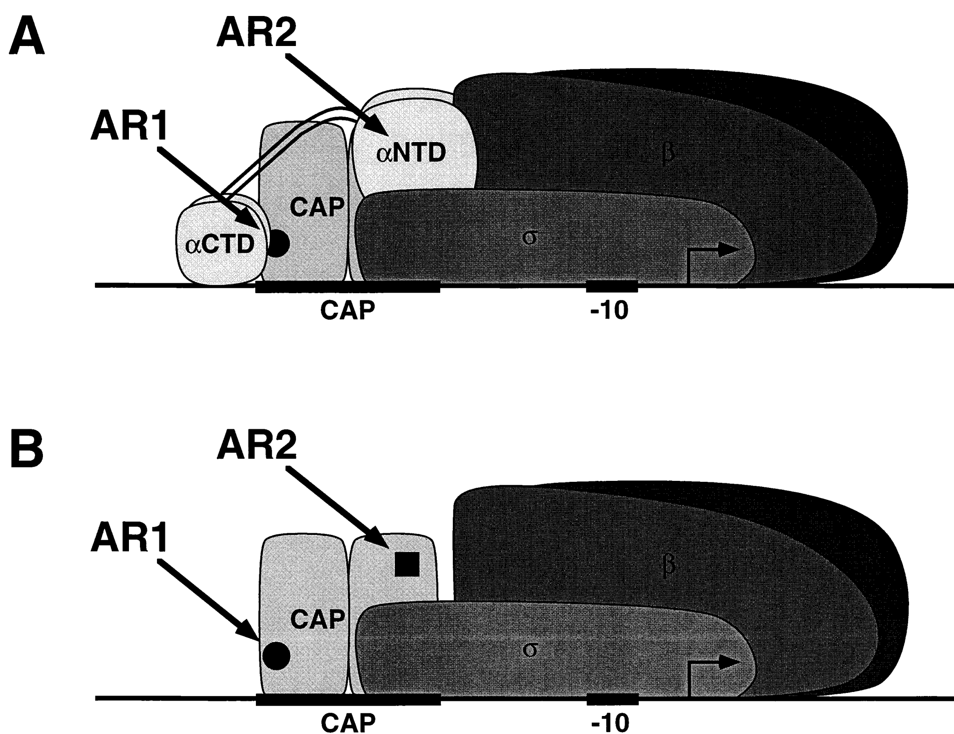


Figure 8. Mechanism of Transcription Activation at Class II CAP-Dependent Promoters

There are two mechanistic components to transcription activation at Class II CAP-dependent promoters: anti-inhibition (mediated by interaction of AR1 with αCTD) and direct activation (mediated by interaction of AR2 with αNTD).

(A) Model of the ternary complex of CAP, RNAP, and a Class II CAP-dependent promoter. AR1 (functional in upstream subunit of CAP) is indicated by a filled circle. AR2 (functional in downstream subunit of CAP) is not visible in this orientation but would be located directly beneath the "N" of "αNTD."

(B) As (A), but omitting RNAP α subunit.

'lacUV5 promoters, was constructed by replacing the XbaI–BamHI *rpoA* segment of pREIIα by the equivalent segment of pHTT7f1-NHα.

CAP
CAP was purified as in Zhou et al. (1993a).

RNAP
For experiments with mutants of CAP, RNAP was purified as in Heyduk et al. (1993). For experiments with mutants of α, RNAP was reconstituted from C-terminally hexahistidine-tagged α, β, β', and σ^{70} as in Tang et al. (1995, 1996). For photocross-linking experiments, RNAP was purified as follows: strain XE54/pREII-NHα in 6 L 4×LB containing 170 mM NaCl, 5 mM IPTG, and 200 μg/ml ampicillin was shaken at 37°C until OD_{600} = 1.5, and harvested by centrifugation (5,000 × g; 15 min at 4°C). Cell lysis, polymin P precipitation, and ammonium sulfate precipitation were performed as in Burgess and Jendrisak (1975). The sample was suspended in 30 ml buffer A (20 mM Tris–HCl [pH 7.9], 500 mM NaCl, and 5% glycerol), adsorbed onto 4 ml Ni^{++}–NTA agarose (Qiagen) in buffer A, washed with 40 ml buffer A, and eluted with 3 × 4 ml each of buffer A containing 2.5 mM, 5 mM, 10 mM, 20 mM, and 40 mM imidazole. Fractions containing RNAP were pooled, dialyzed against 10 mM Tris–HCl (pH 7.9), 250 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol, further purified by Mono-Q chromatography (Borukhov and Goldfarb, 1993), dialyzed against 25 mM Tris–HCl (pH 7.9), 100 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 50% glycerol, and stored at −20°C.

Mutagenesis
Random mutagenesis was performed by error-prone PCR amplification of the 2 kb HindIII–BamHI *crp* segment of pYZCRP or the 1 kb XbaI–BamHI *rpoA* segment of pREIIα (procedures of Zhou et al.,

Table 3. Bacterial Strains

Strain	Genotype	Source
XE82/CC(−41.5)	Δ*crp39 strA thi lacPUV5-O*^{CAP} pRW2CC(−41.5)	This work
XE65.2/CC(−41.5)[a]	Δ*crp39 strA thi* Δ*lacX74* pRW2CC(−41.5)	Zhou et al., 1994a
XE65.2/CC(−61.5)[a]	Δ*crp39 strA thi* Δ*lacX74* pRW2CC(−61.5)	Zhou et al., 1994a
XE032.1/CX(−41.5)	*crp181V thi* Δ*gal65* Δ*lacX74* pRW2CC(−41.5)	Zhou et al., 1994b
XE032.1/XC(−41.5)	*crp181V thi* Δ*gal65* Δ*lacX74* pRW2CC(−61.5)	Zhou et al., 1994b
X7029/pINT-ts	*thi* Δ*lacX74* pINT-ts	This work
XE3000	*thi* Δ*lacX74 att*λ::($P_{CC(-41.5)}$–*lacZYA* Km^R)	This work
XE3020	*thi* Δ*lacX74 att*λ::($P_{CC(-61.5)}$–*lacZYA* Km^R)	This work
XE56	*thi* Δ*lacPL8-UV5*	Tang et. al., 1994
XE54/pREII-NHα	*thi* pREII-NHα	This work

[a]XE65.2/CC(−41.5) and XE65.2/CC(−61.5) originally were designated as XE65.2/CC and XE65.2/CC+20 (Zhou et al., 1994a).

Table 4. Plasmids

Plasmid	Characteristics	Source
pYZCRP	Ap^R; ori-pBR322; ori-f1; *crp*	Zhou et al., 1991
pRW2CC(−41.5)[a]	Tc^R; ori-pRK2; $P_{CC(-41.5)}$−*lacZYA*	Bell et all., 1990
pRW2CC(−61.5)[a]	Tc^R; ori-pRK2; $P_{CC(-61.5)}$−*lacZYA*	Williams et al., 1991
pAA121CC(−41.5)[a]	Ap^R; ori-pBR322; $P_{CC(-41.5)}$	Gaston et al., 1990
pAA121CC(−61.5)[a]	Ap^R; ori-pBR322; $P_{CC(-61.5)}$	Gaston et al., 1990
pBR-203–lacPUV5	Ap^R; ori-pBR322; P_{lacUV5}	Zhou et al., 1993a
pREII-α	Ap^R; ori-pBR322; P_{lpp}−P'_{lacUV5}−*rpoA*	Blatter et al., 1994
pREII-NHα	Ap^R; ori-pBR322; P_{lpp}−P'_{lacUV5}−*rpoA(NH6)*	This work
pHTT7f1-NHα	Ap^R; ori-pBR322; ori-f1; $P_{\phi10}$−*rpoA(NH6)*	Tang et al., 1995
pHTT7f1-CHα	Ap^R; ori-pBR322; ori-f1; $P_{\phi10}$−*rpoA(CH6)*	Tang et al., 1995
pHTT7f1-σ	Ap^R; ori-pBR322; ori-f1; $P_{\phi10}$−*rpoD*	Tang et al., 1995
pTAC3466	Ap^R; Km^R; ori-pBR322; *att*λ	Atlung et al., 1991
pINT-ts	Ap^R; ori-pSC101^{ts}; *int*λ	Hasan et al., 1994

[a]pRW2CC(−41.5), pRW2CC(−61.5), pAA121CC(−41.5), and pAA121CC(−61.5) originally were designated as pRW2CC, pRW2CC+20, pAA121CC, and pAA121CC+20 (Gaston et al., 1990; Bell et al., 1990; Williams et al., 1991).

1991, 1993a; Tang et al., 1994). Site-directed mutagenesis was performed as in Kunkel et al. (1991).

Transcription: In Vivo

Effects of mutants of CAP were measured using derivatives of pYZCRP and strains XE65.2/CC(−41.5), XE65.2/CC(−61.5), and XE82 (procedures of Zhou et al., 1994a). Effects of mutants of α were measured using derivatives of pREIIα and strains XE3000, XE3020, and XE56 (procedures of Tang et al., 1994, except that cultures were grown in LB containing 0 or 5 μg/ml kanamycin, 200 μg/ml ampicillin, and 1 mM IPTG).

Transcription: In Vitro

Reaction mixtures contained the following: 0.5 nM DNA fragment corresponding to positions −82 to +118 of CC(−41.5), −102 to +118 of CC(−61.5), or −140 to +63 of *lacUV5* (prepared by PCR of pAA121CC[−41.5], pAA121CC[−61.5], or pBR-203–lacPUV5); 40 nM RNAP; 40 nM CAP; 0.2 mM cAMP; 0.5 mM ApU (ApA in experiments with *lacUV5*); 50 nM [α^{32}P]-UTP (30 Bq/fmol); 40 mM Tris–HCl (pH 8.0); 100 mM KCl; 10 mM $MgCl_2$; 1 mM dithiothreitol; and 5% glycerol. For experiments in Figures 1 and 6, reaction components except ApU (or ApA) and [α^{32}P]-UTP were incubated 10 min at 37°C, reactions were initiated by addition of ApU (or ApA) and [α^{32}P]-UTP, and reactions were terminated after 15 min at 37°C by addition of 10 μl 0.5 M EDTA. For experiments in Figure 7, reaction components, with the exception of RNAP, were incubated 20 min at 37°C, reactions were initiated by addition of RNAP (40–125 nM), and reactions were sampled and analyzed at 0.5- to 10-min intervals. Reaction products were resolved by paper chromatography in water/saturated ammonium sulfate/isopropanol (18:80:2, v/v) and were quantified by phosphorimaging.

DNA Binding

DNA binding experiments were performed as in Zhou et al. (1993a).

DNA Bending

DNA bending experiments were performed as in Zhou et al. (1993a).

Fluorescence Anisotropy

Fluorescence anisotropy experiments were performed as in Heyduk et al. (1993).

Site-Specific Protein–Protein Photocross-linking

Reaction mixtures contained (250 μl) the following: 4 nM biotinylated DNA fragment corresponding to positions −82 to +118 of CC(−41.5) (prepared by PCR of pAA121CC[−41.5] with one biotinylated primer [synthesized using Biotin Amidite; Applied Biosystems] and one nonbiotinylated primer), 40 nM RNAP, 20 nM [*S*-[2-([^{125}I]iodo-4-azidosalicylamido)ethanethio]-Cys- 17;Ser-178] CAP [25 Bq/fmol; prepared as in Chen et al. (1994); Ebright et al., (1996); verified to retain nearly full ability to activate transcription], 0.2 mM cAMP, 40 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 50 μg/ml bovine serum albumin, and 5% glycerol. After 15 min at 37°C, 8.5 μl assay buffer containing 85 μg streptavidin-coated magnetic beads (Dynal) was added, and, after a further 10 min at 37°C, beads were magnetically pelleted (≈30 s at 37°C). Beads were resuspended in 10 μl assay buffer and UV-irradiated 20 s at 37°C (350 nm; 900 ergs mm^{-2} s^{-1}) in a Rayonet RPR100 photochemical reactor (Southern New England Ultraviolet). Following UV-irradiation, 0.5 μl 800 mM iodoacetamide was added, samples were incubated 15 min at 22°C, 10 μl 80 mM iodoacetamide, 15 M urea was added, and samples were further incubated for 15 min at 22°C. Samples were mixed with 5 μl 0.5 M Tris–HCl (pH 6.8), 50% glycerol, 10% SDS, 10% β-mercaptoethanol, and 0.1 mg/ml bromophenol blue, and were analyzed by SDS–PAGE followed by phosphorimaging. Proteolytic mapping was performed as in Chen et al. (1994).

Acknowledgments

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