Lysine 188 of the catabolite gene activator protein (CAP) plays no role in specificity at base pair 7 of the DNA half site

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Received December 11, 1989; Revised and Accepted February 21, 1990

ABSTRACT

Two similar, but not identical, models have been proposed for the amino acid-base pair contacts in the CAP-DNA complex ('model I,' Weber, I. and Steitz, T., *Proc. Natl. Acad. Sci. USA*, 81, 3973–3977, 1984; 'model II,' Ebright, *et al.*, *Proc. Natl. Acad. Sci. USA*, 81, 7274–7278, 1984). The most important difference between the two models involves Lys188 of CAP. Model I predicts that Lys188 of CAP makes a specificity determining contact with base pair 7 of the DNA half site. In contrast, model II predicts that Lys188 makes no contact with base pair 7 of the DNA half site. In the present work, we have used site-directed mutagenesis to replace Lys188 of CAP by Asn, an amino acid unable to make the putative contact. We have assessed the specificities of the following proteins, both *in vitro* and *in vivo*: wild-type CAP, [Asn188]CAP, [Val181]CAP, and [Val181;Asn188]CAP. The results indicate that Lys188 makes no contribution to specificity at base pair 7 of the DNA half site. We propose, contrary to model I, that Lys188 makes no contact with base pair 7 of the DNA half site.

INTRODUCTION

The *Escherichia coli* catabolite gene activator protein (CAP; also referred to as the cAMP receptor protein, CRP) is a sequence-specific DNA binding protein involved in transcription regulation; CAP functions by binding, in the presence of the allosteric effector cAMP, to specific DNA sites located at or near promoters (1–3). The three-dimensional structure of CAP in complex with cAMP has been determined to 2.5 Å resolution by x-ray diffraction analysis (4). The protein is a dimer of two chemically identical subunits, each of which is 209 amino acids in length and contains a helix-turn-helix DNA binding motif (see refs. 5,6). A model has been proposed for the alignment of the structure of CAP to the structure of DNA in the CAP-DNA complex (7,8). In the model, the CAP-DNA complex is 2-fold symmetric: one

subunit of the CAP dimer interacts with one half of the DNA site; the other subunit of the CAP dimer interacts in a 2-fold symmetry related fashion with the other half of the DNA site. One contact between an amino acid of the helix-turn-helix motif of CAP and a base pair of the DNA half site has been identified experimentally (9–11): i.e., Glu181 of CAP has been shown to contact base pair 7 of the DNA half site. Substitution of Glu181 by Val or Leu eliminates specificity between G:C, A:T, C:G, and T:A at base pair 7 of the DNA half site.

Two different detailed models have been proposed, listing contacts between amino acids of the helix-turn-helix motif of CAP and base pairs of the DNA half site (10,13). We will refer to the model of Weber and Steitz (13; see also ref. 14) as 'model I'; and to the model of Ebright *et al.* (10; see also ref. 12) as 'model II.' Figure 1 compares the two models. The most important difference between the two models involves Lys188 of CAP. Model I predicts that Lys188 makes a specificity determining contact with base pair 7 of the DNA half site (13); i.e., model I predicts that Lys188 makes H-bonds with the guanine N7 atom and the guanine O⁶ atom of G:C at base pair 7 of the DNA half site. Note that only G:C (not A:T, C:G, or T:A) can make the two putative H-bonds. In contrast, model II predicts that Lys188 makes no contact with base pair 7 of the DNA half site (10). The different predicted roles for Lys188 in the two models are due to essentially arbitrary differences in the side-chain torsion angles used in the model building. (The Lys side chain is long and flexible.) Both models are stereochemically acceptable; it is not possible to choose between the two models by model building.

In this paper, we have tested experimentally the role of Lys188 in specificity at base pair 7 of the DNA half site. Our approach was to replace Lys188 by an amino acid unable to contact base pair 7, and, then, to ask whether this replacement affects specificity at base pair 7. This general approach has been designated the 'loss-of-contact approach,' and has been used successfully in investigation of amino acid-base pair contacts by CAP, Lac repressor, λ repressor, λ cro, AraC protein, and the

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Figure 1. The two models for contacts between amino acids of the helix-turn-helix motif of CAP and base pairs of the DNA half site. Base pairs 4 through 8 of the DNA half site are illustrated. *A*, Model I (13; see also ref. 14). Model I predicts that Lys188 ('K188') contacts base pair 7 of the DNA half site. *B*, Model II (10,12).

σ subunit of *E. coli* RNA polymerase (11,12,15–19).

To eliminate the ability of amino acid 188 to contact a DNA base pair, we have replaced Lys188 by Asn. We chose Asn for two reasons: First, at position 188 of CAP, the Asn side chain is too short to contact a DNA base pair (in the context of either model I or model II). Second, in four of the eight proteins known from structural studies to contain the helix-turn-helix motif, Asn is the amino acid at the position corresponding to position 188 of CAP (5,6); this suggests that replacement of Lys188 of CAP by Asn is not likely to disrupt the helix-turn-helix motif. Site-directed mutagenesis was used to construct two substituted CAP variants: [Asn188]CAP and [Val181;Asn188]CAP. [Asn188]CAP lacks the putative contact by Lys188. [Val181;Asn188]CAP lacks both the known contact by Glu181—the amino acid previously demonstrated to contact base pair 7 (9–11; see above)—and the putative contact by Lys188; we reason that the absence of the contact by Glu181 would permit detection of even a slight contribution by amino acid 188 to specificity at base pair 7.

We have measured the specificities of wild-type CAP and of the substituted CAP variants with respect to G:C, A:T, C:G, and T:A at base pair 7 of the DNA half site. DNA binding experiments were performed both *in vitro* and *in vivo*. [Asn188]CAP exhibited the same specificity at base pair 7 as did wild-type CAP. [Val181;Asn188]CAP exhibited the same specificity at base pair 7 as did [Val181]CAP. These results indicate that Lys188 makes no contribution to specificity at base pair 7. We propose, contrary to model I (13), that Lys188 makes no contact with base pair 7.

MATERIALS AND METHODS

Bacterial Strains

A list of *E. coli* K-12 strains constructed in this work is presented in Table 1. The *E. coli* K-12 strains are derivatives of strain XA102 (Δ lacproABX111 *argEam* *metB* *ara* *rpoB* *nal* Su2; ref. 20). The *crp* and *strA* markers are from strain CA8445 (HfrH

Δ crp45 Δ cya854 *strA* *thi*; ref. 21). The *pcnB80* and *zad::Tn10* markers are from strain MRi93 (*pcnB80* *zad::Tn10* Δ rrbs7 Δ lacU169 *araD139* *motA* *strA* *thi*; ref. 22).

Strains XAE300, XAE371, XAE372, XAE373, XAE374, XAE375, and XAE376 were constructed as follows: Strain XAE13 (Δ crp45 *strA* *pcnB80* *zad::Tn10* Δ lacproABX111 *argEam* *metB* *ara* *rpoB* *nal* Su2 pHA5) was lysogenized with λ i434*plac5* and derivatives of λ i434*plac5* having substitutions at base pairs –66 and/or –57 of *lacP1* (Table 2); lysogens were identified by blue colony color on 5-bromo-4-chloro-3-indolyl- β -D-galactoside indicator plates. The resulting lysogens were grown in liquid culture in the absence of ampicillin selection; isolates that had lost plasmid pHA5 were identified by red colony color on maltose-tetrazolium indicator plates. The isolates utilized were established to be single-copy lysogens, based on segregation pattern, and based on the level of CAP-independent β -galactosidase expression.

Bacteriophage

A list of bacteriophage used or constructed in this work is presented in Table 2.

λ i434*plac5*-P1L162 is a derivative of λ i434*plac5* (15) having the L162 promoter mutation (J. Beckwith, personal communication); λ i434*plac5*-P1L162 yields colorless plaques on 5-bromo-4-chloro-3-indolyl- β -D-galactoside indicator plates. Derivatives of λ i434*plac5* having substitutions at base pairs –66 and/or –57 of *lacP1* were constructed by homologous recombination between λ i434*plac5*-P1L162 and appropriate M13mp2 derivatives (11,23,24; method in ref. 26); recombinants were identified by light blue plaque color on 5-bromo-4-chloro-3-indolyl- β -D-galactoside indicator plates.

Plasmids Encoding CAP and CAP Derivatives

Plasmids pHA5, which encodes wild-type CAP, and pPC181V, which encodes [Val181]CAP, have been described previously (11,27).

Plasmids encoding [Asn188]CAP (plasmid pTK188N) and

TABLE 1. *E. COLI* K12 STRAINS CONSTRUCTED IN THIS STUDY

STRAIN GENOTYPE	
XAE300	XA102 Δ crp45 strA pcnB80 zad::Tn10 λ i434plac5
XAE371	XA102 Δ crp45 strA pcnB80 zad::Tn10 λ i434plac5-P1(-66A;-57T)
XAE372	XA102 Δ crp45 strA pcnB80 zad::Tn10 λ i434plac5-P1(-66C;-57G)
XAE373	XA102 Δ crp45 strA pcnB80 zad::Tn10 λ i434plac5-P1(-66T;-57A)
XAE374	XA102 Δ crp45 strA pcnB80 zad::Tn10 λ i434plac5-P1(-66A)
XAE375	XA102 Δ crp45 strA pcnB80 zad::Tn10 λ i434plac5-P1(-66C)
XAE376	XA102 Δ crp45 strA pcnB80 zad::Tn10 λ i434plac5-P1(-66T)
XAE300/CRP	XAE300 pHA5
XAE371/CRP	XAE371 pHA5
XAE372/CRP	XAE372 pHA5
XAE373/CRP	XAE373 pHA5
XAE374/CRP	XAE374 pHA5
XAE375/CRP	XAE375 pHA5
XAE376/CRP	XAE376 pHA5

To analyze the profile of specificity of CAP variant *X*, the plasmid encoding *X* was introduced into each of the following: XAE300, XAE371, XAE372, XAE373, XAE374, XAE375, and XAE376 (see Materials and Methods). As an example, the set of strains constructed in order to analyze the specificity of wild-type CAP is listed.

[Val181;Asn188]CAP (plasmid pTK181V188N) were constructed by use of site-directed mutagenesis to introduce substitutions into M13mp18-CRP (method in ref. 28), followed by subcloning of the 3.5 kb *Bam*HI-*Bam*HI *crp* fragment into the *Bam*HI site of plasmid pBR322 (29). For each plasmid, the DNA-nucleotide sequence of the complete *crp* structural gene and promoter was verified. Plasmids were constructed and maintained in strain CA8445 (HfrH Δ crp45 Δ cya854 strA thi; ref. 21).

CAP and CAP Derivatives

CAP and CAP derivatives were purified as described in ref. 30. For each preparation, the fraction of molecules active in sequence-specific DNA binding (0.14 to 0.64) was determined by titration with 5.0 nM [³²P]-ICAP; all data are reported in terms of molar concentrations of active dimers.

Synthetic DNA Fragments

40 base pair double-stranded DNA fragments containing the consensus DNA site for CAP, or substituted derivatives of the consensus DNA site for CAP (sequences in Figure 2), were synthesized, purified, and radiolabelled as described in ref. 8.

In Vitro DNA Binding Experiments

In vitro DNA binding experiments were performed using the nitrocellulose filter binding technique as described in ref. 8. The technique yields high-precision data for $K_D = 1 \times 10^{-12}$ M to $K_D = 1 \times 10^{-7}$ M (precision of estimate of K_D typically within a factor of 1.5 to 2). Where $K_D > 1 \times 10^{-7}$ M, the value $K_D = 1 \times 10^{-7}$ M was used to calculate a minimum estimate for the ratio $K_D/K_{D,CONS}$.

In Vivo DNA Binding Experiments: Experimentation

To analyze the profile of specificity of CAP variant *X*, the plasmid encoding *X* was introduced into each of the following: XAE300, XAE371, XAE372, XAE373, XAE374, XAE375, and XAE376. Note that each of the resulting strains was identical except for one base pair, or two base pairs, in *lacP1*. Therefore, in each of the resulting strains the intracellular concentration of CAP was equal.

For each strain, the differential rate of β -galactosidase synthesis was determined by the method of Miller (33). Cultures were

TABLE 2. BACTERIOPHAGE USED IN THIS STUDY

BACTERIOPHAGE	SOURCE
λ i434plac5	14
λ i434plac5-P1L162	This work
λ i434plac5-P1(-66A;-57T)	This work
λ i434plac5-P1(-66C;-57G)	This work
λ i434plac5-P1(-66T;-57A)	This work
λ i434plac5-P1(-66A)	This work
λ i434plac5-P1(-66C)	This work
λ i434plac5-P1(-66T)	This work
M13mp2-lacP1(-66A;-57T)	11
M13mp2-lacP1(-66C;-57G)	11
M13mp2-lacP1(-66T;-57A)	11
M13mp2-lacP1(-66A)	23
M13mp2-lacP1(-66C)	24
M13mp2-lacP1(-66T)	24
M13mp18	25
M13mp18-CRP	This work
M13mp18-188N	This work
M13mp18-181V;188N	This work

grown in LB medium (33) containing 100 μ g/ml ampicillin. Data were corrected for background: i.e., for the differential rate of β -galactosidase synthesis in the absence of CAP or CAP variant.

In Vivo DNA Binding Experiments: Data Reduction

The fractional occupancy of *lacP1* by CAP is calculated as follows (see refs. 12,15,34,35):

$$\theta = Z/Z_M \quad (1)$$

where *Z* denotes the measured differential rate of β -galactosidase synthesis, and *Z_M* denotes the maximum differential rate of β -galactosidase synthesis. By rearrangement of the equilibrium relationship, it can be shown that (see refs. 12,15,34,35):

$$\theta = \frac{[C]}{K_D + [C]} \quad (2)$$

where [*C*] denotes the intracellular concentration of unbound CAP dimers, and *K_D* denotes the equilibrium dissociation constant. For two strains such that [*C*]₁ = [*C*]₂, the ratio of the

Figure 2. DNA sites utilized in this study. *A*, The consensus DNA site for CAP (8,11,31). The symmetric A:T, C:G, and T:A substitutions at base pairs 7 of the DNA half site are indicated beneath the sequence. *B*, The wild-type *lacP1* DNA site for CAP (base pairs -72 to -50 with respect to the start point of the *lacP1* promoter [32]). The symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site, and the single A:T, C:G, and T:A substitutions at base pair 7 of the left DNA half site, are indicated beneath the sequence.

substitutions at base pair 7 of the DNA half site (fragments ICAP7AS, ICAP7CS, and ICAP7TS; sequences in Figure 2A). The data are presented in Table 3. The data in Table 3 are expressed as the ratio $K_D/K_{D,CONS}$: i.e., as the equilibrium dissociation constant for the interaction of CAP variant X with a substituted DNA site, divided by the equilibrium dissociation constant for the interaction of the identical CAP variant X with the consensus DNA site. The role of Lys188 is inferred from comparison of the profile of specificity exhibited by wild-type CAP to the profile of specificity exhibited by [Asn188]CAP, and from comparison of the profile of specificity exhibited by [Val181]CAP to the profile of specificity exhibited by [Val181;Asn188]CAP.

Wild-Type CAP and [Asn188]CAP

Wild-type CAP exhibited $K_{D,CONS}$ equal to $2.6 (\pm 0.3) \times 10^{-11}$ M for interaction with the consensus DNA site. Wild-type CAP interacted at least 3000 times more tightly with the consensus DNA site than with the derivatives of the consensus DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site. This corresponds to a specificity free energy of at least 4.7 kcal/mol. These results demonstrate that wild-type CAP has robust specificity for G:C at base pair 7 of the DNA half site.

[Asn188]CAP exhibited $K_{D,CONS}$ equal to $3.9 (\pm 0.5) \times 10^{-10}$ M for interaction with the consensus DNA site. [Asn188]CAP interacted at least 300 times more tightly with the consensus DNA site than with the derivatives of the consensus DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site. This corresponds to a specificity free energy of at least 3.3 kcal/mol. These results demonstrate that [Asn188]CAP has the same qualitative profile of specificity at base pair 7 of the DNA half site as does wild-type CAP: i.e., robust specificity for G:C at base pair 7 of the DNA half site.

We have also analyzed the profiles of specificity of wild-type CAP and [Asn188]CAP with respect to base pairs 5 and 6 of the DNA half site. The experiments were performed using as ligands derivatives of the consensus DNA site with the symmetric A:T, C:G, and T:A substitutions at base pair 5 of the DNA half site, and derivatives of the consensus DNA site with the symmetric A:T, C:G, and G:C substitutions at base pair 6 of the DNA half site. The results demonstrate that [Asn188]CAP has the same profile of specificity at base pairs 5 and 6 as does wild-type CAP (data not shown).

In addition to the above experiments, we have performed analogous *in vitro* DNA binding experiments, using the gel-retardation assay technique (11,36,37), and using as ligands a 42 base pair DNA fragment having the wild-type *lacP1* DNA

site for CAP and three 42 base pair DNA fragments having derivatives of the *lacP1* DNA site for CAP with the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site (sequences in Figure 1 of ref. 11; R.H.E., A. Kolb, and H. Buc, unpublished data). The results were in agreement with the above results. However, due to apparent instability of the [Asn188]CAP-DNA complexes under the conditions of the gel-retardation assay, it was not possible to obtain high-precision data for [Asn188]CAP. The cause of the apparent instability of the [Asn188]CAP-DNA complexes is not known.

[Val181]CAP and [Val181;Asn188]CAP

[Val181]CAP exhibited $K_{D,CONS}$ equal to $6.5 (\pm 1) \times 10^{-11}$ M for interaction with the consensus DNA site. [Val181]CAP interacted essentially equally with the consensus DNA site and with the derivatives of the consensus DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site; the mean value of $K_D/K_{D,CONS}$ was 1. These results, in agreement with previously reported results (11), demonstrate that [Val181]CAP has no specificity at base pair 7 of the DNA half site (i.e., no ability to discriminate between G:C, A:T, C:G, and T:A at base pair 7 of the DNA site).

[Val181;Asn188]CAP exhibited $K_{D,CONS}$ equal to $1.8 (\pm 0.2) \times 10^{-9}$ M for interaction with the consensus DNA site. [Val181;Asn188]CAP interacted essentially equally with the consensus DNA site and with the derivatives of the consensus DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site; the mean value of $K_D/K_{D,CONS}$ was 0.5. These results demonstrate that [Val181;Asn188]CAP has the same qualitative profile of specificity at base pair 7 of the DNA half site as does [Val181]CAP: i.e., no specificity at base pair 7 of the DNA half site.

We have also analyzed the profiles of specificity of [Val181]CAP and [Val181;Asn188]CAP with respect to base pairs 5 and 6 of the DNA half site. The experiments were performed using as ligands derivatives of the consensus DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 5 of the DNA half site, and derivatives of the consensus DNA site containing the symmetric A:T, C:G, and G:C substitutions at base pair 6 of the DNA half site. The results demonstrate that [Val181;Asn188]CAP has the same profile of specificity at base pairs 5 and 6 as does [Val181]CAP (data not shown).

In Vivo DNA Binding Experiments

We have performed quantitative *in vivo* DNA binding assays in order to confirm the profiles of specificity of wild-type CAP,

TABLE 3. *IN VITRO* DATA: RECOGNITION OF DNA SITES SYMMETRICALLY ALTERED AT POSITIONS 7 AND 16

CAP	$K_D/K_{D,CONS}$			
	CONS (7G;16C)	7A;16T	7C;16G	7T;16A
WILD-TYPE CAP	[1]	>3000	>3000	3000
[ASN188]CAP	[1]	>300	>300	>300
[VAL181]CAP	[1]	0.8	2	1
[VAL181;ASN188]CAP	[1]	0.2	0.8	0.4

'CONS' denotes the consensus DNA site; substituted derivatives of the consensus DNA site are identified by the position and sequence of the substitutions (sequences in Figure 2A). Data are from nitrocellulose filter binding assays. Values of $K_{D,CONS}$ were as follows: wild-type CAP, $2.6 (\pm 0.3) \times 10^{-11}$ M; [Asn188]CAP, $3.9 (\pm 0.5) \times 10^{-10}$ M; [Val181]CAP, $6.5 (\pm 1) \times 10^{-11}$ M; [Val181;Asn188]CAP, $1.8 (\pm 0.2) \times 10^{-9}$ M.

TABLE 4. *IN VIVO* DATA: RECOGNITION OF DNA SITES SYMMETRICALLY ALTERED AT POSITIONS 7 AND 16

CAP	$K_D/K_{D,P+}$			
	P ⁺ (7G;16C)	7A;16T	7C;16G	7T;16A
WILD-TYPE CAP	[1]	> 1000	> 1000	800
[ASN188]CAP	[1]	> 900	> 900	500
[VAL181]CAP	[1]	0.2	8	2
[VAL181;ASN188]CAP	[1]	0.1	4	0.8

'+' denotes the wild-type *lacPI* DNA site; substituted derivatives of the *lacPI* DNA site are identified by the position and sequence of the substitutions (sequences in Figure 2).

TABLE 5. *IN VIVO* DATA: RECOGNITION OF DNA SITES ALTERED AT POSITION 7

CAP	P ⁺	$K_D/K_{D,P+}$		
		(7G) 7T	7A	7C
WILD-TYPE CAP	[1]	100	100	20
[ASN188]CAP	[1]	100	100	20
[VAL181]CAP	[1]	0.4	1	0.4
[VAL181;ASN188]CAP	[1]	0.6	1	0.5

See legend to Table 4.

[Asn188]CAP, [Val181]CAP, and [Val181;Asn188]CAP with respect to base pair 7 of the DNA half site. The experiments were performed using as ligands the wild-type *lacPI* DNA site for CAP, three derivatives of the *lacPI* DNA site for CAP with the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site, and three derivatives of the *lacPI* DNA site for CAP with the single A:T, C:G, and T:A substitutions at base pair 7 of the left DNA half site (sequences in Figure 2B). The data are presented in Tables 4 and 5. The data in Tables 4 and 5 are expressed as the ratio $K_D/K_{D,P+}$: i.e., as the equilibrium dissociation constant for the interaction of CAP variant X with a substituted *lacPI* DNA site, divided by the equilibrium dissociation constant for the interaction of the identical CAP variant X with the wild-type *lacPI* DNA site. The role of Lys188 is inferred from comparison of the profile of specificity exhibited by wild-type CAP to the profile of specificity exhibited by [Asn188]CAP, and from comparison of the profile of specificity exhibited by [Val181]CAP to the profile of specificity exhibited by [Val181;Asn188]CAP.

Method

We have developed a quantitative *in vivo* assay technique that enables one to determine the profile of specificity of CAP variant X with respect to the wild-type *lacPI* DNA site for CAP, and with respect to each of the six substituted derivatives of the *lacPI* DNA site for CAP illustrated in Figure 2B. The technique uses seven *E. coli* tester strains: XAE300, XAE371, XAE372, XAE373, XAE374, XAE375, and XAE376 (Table 1). Each tester strain has three important components:

- $\Delta crp45$, a deletion of the gene that encodes wild-type CAP. $\Delta crp45$ is > 5 kb in length; it deletes all homology to the 3.5 kb *crp* insert of the plasmids used in this study (21,38).
- lacZ*, the gene that encodes the assayable product β -galactosidase. In tester strain XAE300, *lacZ* is placed under the control of the wild-type *lacPI* DNA site for CAP; in the remaining six tester strains, *lacZ* is placed under the control of

derivatives of the *lacPI* DNA site for CAP with the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site, or with the single A:T, C:G, and T:A substitutions at base pair 7 of the left DNA half site (sequences in Figure 2B). The *lacZ* gene is present on a λ i434 $plac5$ prophage stably integrated into the bacterial chromosome at *att λ* .

(iii) *pcnB80* (22). The presence of the *pcnB80* marker results in a decrease in the copy number of plasmid pBR322 derivatives from approximately 40 copies per cell to approximately 4 copies per cell; this results in an up to 10-fold reduction in the intracellular concentration of proteins encoded by plasmid pBR322 derivatives (22). The presence of *pcnB80* reduces the possibility that a plasmid-encoded CAP variant will be toxic to the cell.

In order to analyze the profile of specificity of CAP variant X, the plasmid encoding X is introduced into each of the seven tester strains. This results in the construction of a set of seven strains each having the identical plasmid encoding X (example in Table 1). The differential rate of β -galactosidase synthesis is determined for each strain of the set, and ratios of equilibrium dissociation constants for the interactions of CAP variant X with different DNA sites are calculated as described in Materials and Methods. Note that each strain of one set is identical except for *lacPI*; one important implication is that in each strain of one set the intracellular concentration of CAP is equal. All calculations are performed using only data from strains within the same set. Therefore, this analysis does not incur complications due to either (i) differing stabilities of substituted vs. wild-type proteins, or (ii) differing rates of synthesis of substituted vs. wild-type proteins.

The equilibrium under analysis is $C + P \rightleftharpoons CP$, where C denotes CAP, P denotes *lacPI*, and CP denotes the CAP-*lacPI* complex. The measured differential rate of β -galactosidase synthesis, Z, is proportional to CP. The maximum differential rate of β -galactosidase synthesis, Z_M (determined independently), is proportional to P_{tot} ($P_{tot} \equiv P + CP$). Therefore, the

occupancy of *lacP1*, θ , is equal to Z/Z_M (equation 1). For two strains in which the intracellular concentration of CAP is equal (e.g., any two strains from within the same set), one can calculate the ratio $K_{D,1}/K_{D,2}$ from the measured values Z_1 and Z_2 (equation 3).

This technique is similar to that used previously to analyze the profile of specificity of Lac repressor and Lac repressor variants (12,15). The technique is rapid and simple; most important, the technique enables one to determine the profile of specificity of CAP variant *X* without purification of the protein. The good agreement between the results of the *in vitro* DNA binding experiments and the results of this *in vivo* technique (see below; cf. Tables 3 and 4) supports the usefulness of this technique.

Wild-Type CAP and [Asn188]CAP

Wild-type CAP interacted 800 to at least 1000 times more tightly with the wild-type *lacP1* DNA site than with the derivatives of the *lacP1* DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site. Wild-type CAP interacted 20 to 100 times more tightly with the wild-type *lacP1* DNA site than with the derivatives of the *lacP1* DNA site containing the single A:T, C:G, and T:A substitutions at base pair 7 of the left DNA half site. These results confirm that wild-type CAP has robust specificity for G:C at base pair 7 of the DNA half site.

[Asn188]CAP interacted 500 to at least 900 times more tightly with the wild-type *lacP1* DNA site than with the derivatives of the *lacP1* DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site. [Asn188]CAP interacted 20 to 100 times more tightly with the wild-type *lacP1* DNA site than with the derivatives of the *lacP1* DNA site containing the single A:T, C:G, and T:A substitutions at base pair 7 of the left DNA half site. These results demonstrate that [Asn188]CAP has *exactly* the same profile of specificity at base pair 7 of the DNA half site as does wild-type CAP: i.e., robust specificity for G:C at base pair 7 of the DNA half site.

[Val181]CAP and [Val181;Asn188]CAP

[Val181]CAP interacted essentially equally tightly with the wild-type *lacP1* DNA site and with the derivatives of the *lacP1* DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site; the mean value of $K_D/K_{D,P+}$ was 3. [Val181]CAP also interacted essentially equally tightly with the wild-type *lacP1* DNA site and with the derivatives of the *lacP1* DNA site containing the single A:T, C:G, and T:A substitutions at base pair 7 of the left DNA half site; the mean value of $K_D/K_{D,P+}$ was 0.6. These results confirm that [Val181]CAP has essentially no specificity at base pair 7 of the DNA half site.

[Val181;Asn188]CAP interacted essentially equally tightly with the *lacP1* DNA site and with the derivatives of the *lacP1* DNA site containing the symmetric A:T, C:G, and T:A substitutions at base pair 7 of the DNA half site; the mean value of $K_D/K_{D,P+}$ was 2. [Val181;Asn188]CAP also interacted essentially equally tightly with the wild-type *lacP1* DNA site and with the derivatives of the *lacP1* DNA site containing the single A:T, C:G, and T:A substitutions at base pair 7 of the left DNA half site; the mean value of $K_D/K_{D,P+}$ was 0.6. These results confirm that [Val181;Asn188]CAP has the same profile of specificity at base pair 7 of the DNA half site as does [Val181]CAP: i.e., no specificity at base pair 7 of the DNA half site.

DISCUSSION

The results indicate that [Asn188]CAP has the same specificity at base pairs 5, 6, and 7 of the DNA half site as does wild-type CAP. The results indicate that [Val181;Asn188]CAP has the same specificity at base pairs 5, 6, and 7 of the DNA half site as does [Val181]CAP. Based on these results, we conclude that Lys188 of CAP makes no contribution to specificity at base pairs 5, 6, and 7 of the DNA half site.

We propose, contrary to model I (Figure 1A; ref. 13), that Lys188 of CAP does not contact base pair 7 of the DNA half site. This proposal is consistent with the methylation-interference and the depurination-interference data regarding base pair 7 of the DNA half site (39,40). Methylation of the guanine N7 atom of base pair 7 of the left half site of the *lacP1* DNA site for CAP does not detectably affect affinity (39,40). Furthermore, complete removal of the guanine base of base pair 7 of the left half site of the *lacP1* DNA site for CAP does not detectably affect affinity (39). These methylation-interference and depurination-interference data suggest that the guanine base of base pair 7 of the DNA half site—the base that Lys188 is proposed to contact in model I (Figure 1A; ref. 13)—is not involved in an amino acid-base contact.

We propose that Lys188 of CAP either: (i) makes no contact with DNA, or (ii) makes a sequence-independent contact with a DNA phosphate. Model building indicates that formation of a contact between Lys188 of CAP and the top-strand DNA phosphate 5' to base pair 6 of the DNA half site is stereochemically feasible (8). In this report, we have observed that substitution of Lys188 of CAP results in a 20-fold to 30-fold increase in the equilibrium dissociation constant for interaction with the consensus DNA site, $K_{D,CONS}$; this result is consistent with the existence of a contact between Lys188 of CAP and DNA in the CAP-DNA complex*. It should be possible, through comparison of the salt-dependences of [Asn188]CAP-DNA complex formation and CAP-DNA complex formation, to establish definitively whether Lys188 of CAP makes a contact with a DNA phosphate (see refs. 8,42).

ACKNOWLEDGEMENTS

We thank Dr. J. Beckwith for important discussions, and for laboratory space and research support used in construction of the λ i434*plac5* derivatives. We thank Dr. S. Garges for the DNA-nucleotide sequence analysis of pTK188N and pTK181V;188N. We thank Drs. H. Buc and A. Kolb for important discussions. This work was supported by National Institutes of Health grants GM41376 to R.H.E., GM22619 to J.S.K., and GM13017 to Dr. J. Beckwith.

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* Gent *et al.* (41) have reported that substitution of Lys188 of CAP does not reduce affinity for the specific DNA site; however, their experiments were performed with crude extracts not controlled for CAP concentration, and were performed using a non-equilibrium, immunoprecipitation assay.

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