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DNA Sequence Determinants for Binding of the *Escherichia coli* Catabolite Gene Activator Protein*

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Angelo Gunasekera, Yon W. Ebright, and Richard H. Ebright‡

From the Department of Chemistry and Waksman Institute, Rutgers University, New Brunswick, New Jersey 08855

The consensus DNA site for binding of the *Escherichia coli* catabolite gene activator protein (CAP) is 22 base pairs in length and is 2-fold symmetric: 5′-AAATGTGATCTAGATCACATTT-3′. Positions 4 to 8 of each half of the consensus DNA half-site are the most strongly conserved. In this report, we analyze the effects of substitution of DNA base pairs at positions 4 to 8, the effects of substitution of thymine by uracil and by 5-methylcytosine at positions 4, 6, and 8, and the effect of *dam* methylation of the 5′-GATC-3′ sequence at positions 7 to 10. All DNA sites having substitutions of DNA base pairs at positions 4 to 8 exhibit lower affinities for CAP than does the consensus DNA site, consistent with the proposal that the consensus DNA site is the ideal DNA site for CAP. Specificity for T:A at position 4 appears to be determined solely by the thymine 5-methyl group. Specificity for T:A at position 6 and specificity for A:T at position 8 appear to be determined in part, but not solely, by the thymine 5-methyl group. *dam* methylation has little effect on CAP·DNA complex formation. The thermodynamically defined consensus DNA site spans 28 base pairs. All, or nearly all, DNA determinants required for maximal affinity for CAP and for maximal thermodynamically defined CAP·DNA ion pair formation are contained within a 28-base pair DNA fragment that has the 22-base pair consensus DNA site at its center. The quantitative data in this report provide base-line thermodynamic data required for detailed investigations of amino acid-base pair and amino acid-phosphate contacts in this protein-DNA complex.

The *Escherichia coli* catabolite gene activator protein (CAP)[1] (also referred to as the cAMP receptor protein) is a sequence-specific DNA-binding protein involved in transcription regulation; CAP functions by binding to specific DNA sites located at or near promoters (1, 2). More than 25 examples of specific DNA sites for CAP have been identified in nuclease protection experiments (2–5). Comparison of these DNA sites yields the consensus sequence 5′-AAATGTGATCTAGATCACATTT-3′ (3–5). The consensus DNA site is 22 base pairs in length and exhibits perfect 2-fold sequence symmetry. Positions 4 to 8 of each half of the consensus DNA site (underlined above) have the highest sequence information content; *i.e.* are the most strongly conserved.[2] A synthetic consensus DNA site (6–8) exhibits an extremely high affinity for CAP (450-fold higher affinity than the *E. coli lac* DNA site for CAP; Ref. 6) and functions in the stimulation of transcription initiation by CAP (8).

The three-dimensional structure of CAP has been determined to 2.5 Å resolution by x-ray diffraction analysis (9). CAP is a dimer of two identical subunits, each of which is 209 amino acids in length and contains a helix-turn-helix DNA binding motif (see Refs. 10 and 11). The three-dimensional structure of the CAP·DNA complex has been determined to 3.0 Å resolution by x-ray diffraction analysis (12; Fig. 1). The CAP·DNA complex is 2-fold symmetric: one subunit of CAP interacts with one half of the DNA site; the other subunit of CAP interacts in a 2-fold symmetry related fashion with the other half of the DNA site. Amino acids of the helix-turn-helix motif of CAP contact the DNA base pairs at positions 5, 7, and 8 of the DNA half-site (12–18; Fig. 1). Two contacts between amino acids of the helix-turn-helix motif of CAP and DNA base pairs of the DNA half-site have been demonstrated experimentally (12–16): Arg-180 of CAP has been shown to contact the DNA base pair at position 5 of the DNA half-site, and Glu-181 of CAP has been shown to contact the DNA base pair at position 7 of the DNA half-site.

Important basic information regarding the chemistry and thermodynamics of protein-DNA complex formation can be obtained by measuring the effects of substitution of DNA base pairs, one by one, of the DNA site. Takeda and co-workers (19, 20) have termed this experimental approach "systematic base pair substitution analysis."

Several studies of the DNA sequence determinants for binding of CAP have been reported previously. Ebright and co-workers (15, 17, 18) have examined the effects of substitution of DNA base pairs at position 7 of the *E. coli lac* DNA site for CAP and at positions 6 and 7 of the consensus DNA site for CAP. Jansen *et al.* (21) have examined, semiquantitatively, the effects of substitutions of DNA base pairs at positions 5, 7, and 8 of a DNA site intermediate in sequence between the *E. coli lac* DNA site for CAP and the consensus DNA site for CAP. In this report, we analyze the effects of substitution of DNA base pairs at positions 4 to 8 of the consensus DNA half-site, the effects of substitution of thymine by uracil and by 5-methylcytosine at positions 4, 6, and 8 of the consensus DNA half-site, the effect of *dam* methylation (22) of the 5′-GATC-3′ sequence at positions 7 to 10 of the consensus DNA half-site, and the effects of DNA fragment

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‡ To whom correspondence should be sent: Waksman Institute, Rutgers University, New Brunswick, NJ 08855. Tel.: 908-932-5179; Fax: 908-932-5735.

[1] The abbreviations used are: CAP, catabolite gene activator protein; 5-MeC, 5-methylcytosine; HPLC, high performance liquid chromatography; MOPS, 4-morpholineethanesulfonic acid.

[2] In this report, positions within the DNA site for CAP are numbered as in Refs. 2, 3, 5–8, and 13–18. A different numbering convention is used in Ref. 12.

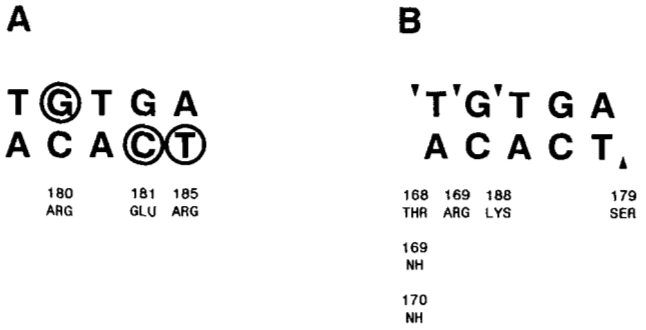

FIG. 1. **Summary of contacts between CAP and positions 4 to 8 of the DNA half-site as observed in the crystallographic structure of the CAP·DNA complex at 3.0 Å resolution (12).** *A*, H-bonds with DNA base pairs. *Circles* indicate the contacted DNA bases. *B*, H-bonds and ion pairs with DNA phosphates. *Arrows* indicate the contacted DNA phosphates.

length. The results provide base-line thermodynamic information required for physical, biochemical, and genetic studies of amino acid-base pair and amino acid-phosphate contacts in this protein-DNA complex.

MATERIALS AND METHODS

CAP—CAP was the kind gift of Dr. J. Krakow (Hunter College of CUNY),and had been purified as described in Ref. 23. The fraction of CAP molecules active in sequence-specific DNA binding (0.64) was determined by titration of DNA fragment ICAP (6) under stoichiometric binding conditions; all data are reported in terms of molar concentrations of active CAP dimers.

Synthetic DNA Sites—40-base pair incompletely symmetric double-stranded DNA fragments containing the consensus DNA site for CAP, or substituted derivatives of the consensus DNA site for CAP (sequences in Figs. 2*A*, 3*A*, and 4), were prepared as described in Ref. 6. Uracil, 5-methylcytosine, and N^6-methyladenine were introduced using, respectively, deoxyuridine-β-cyanoethylphosphoramidite (ABN), 5-methyldeoxycytidine-β-cyanoethylphosphoramidite (ABN), and N^6-methyldeoxyadenosine-β-cyanoethylphosphoramidite (Pharmacia LKB Biotechnology Inc.).

The 28-base pair incompletely symmetric double-stranded DNA fragment containing the consensus DNA site for CAP (DNA fragment ICAP28; sequence in Fig. 5) was prepared as follows. 28-residue oligodeoxyribonucleotides corresponding to the top and bottom strands were synthesized using solid-phase β-cyanoethylphosphoramidite chemistry on an Applied Biosystems 380A automated synthesizer. Products were deprotected and were purified using a two-step protocol, consisting of DEAE-5PW (TSK) anion-exchange HPLC followed by PRP-100 (Hamilton) reversed-phase HPLC. The top strand oligodeoxyribonucleotide (15 pmol) was 5′-end labeled using [γ-^{32}P]ATP (200 Bq/fmol) and T4 polynucleotide kinase (24). The labeled top strand oligodeoxyribonucleotide was purified from unincorporated [γ-^{32}P]ATP and T4 polynucleotide kinase by chromatography on a Nensorb-20 mini-Kel-F column (Du Pont). The top-strand and bottom-strand oligodeoxyribonucleotides were annealed to yield double-stranded DNA as follows. 7.5 pmol of labeled top-strand oligodeoxyribonucleotide, 64.5 pmol of unlabeled top-strand oligodeoxyribonucleotide, and 75 pmol of unlabeled bottom-strand oligodeoxyribonucleotide were combined in 50 μl of 25 mM Tris-HCl (pH 8.0) and 500 mM NaCl, heated 10 min at 90 °C, and allowed to cool gradually (30–36 h) to 22 °C. The specific activity obtained was 20 Bq/fmol.

30-base pair or shorter completely symmetric double-stranded DNA fragments containing the consensus DNA site for CAP (DNA fragments SICAP30, SICAP28, SICAP26, SICAP24, and SICAP22; sequences in Fig. 5) were prepared as follows. A self-complementary oligodeoxyribonucleotide having the desired sequence was synthesized using solid-phase β-cyanoethylphosphoramidite chemistry on an Applied Biosystems 380A automated synthesizer. The product was deprotected and was purified using a two-step protocol consisting of DEAE-5PW (TSK) anion-exchange HPLC followed by PRP-100 (Hamilton) reversed-phase HPLC. The self-complementary oligodeoxyribonucleotide (5 nmol) was 5′-end labeled using [γ-^{32}P]ATP (0.8 Bq/fmol) and T4 polynucleotide kinase (24). The labeled self-complementary oligodeoxyribonucleotide was purified from unincorporated [γ-^{32}P]ATP and T4 polynucleotide kinase by chromatography on a Nensorb-20 mini-Kel-F column (Du Pont) The labeled self-complementary oligodeoxyribonucleotide (2.5 nmol) was annealed to yield double-stranded DNA in 10 μl of 25 mM Tris-HCl (pH 8.0) and 500 mM NaCl, by heating 10 min at 90 °C followed by gradual cooling (30–36 h) to 22 °C. Specific activities obtained ranged from 0.4 to 0.8 Bq/fmol.

Nondenaturing polyacrylamide gel electrophoresis followed by autoradiography indicated ≥ 95% duplex formation with the incompletely symmetric DNA fragments and 60–90% duplex formation with the completely symmetric DNA fragments. Under the reaction conditions utilized for the nitrocellulose filter binding experiments (see next section), the presence of 5–50% non-duplex DNA did not affect the measured equilibrium binding constants; therefore, annealed DNA fragments were used without further purification.

DNA concentrations were determined from absorbance at 260 nm; nucleotide extinction coefficients utilized were 9,200 M^{-1} cm^{-1} for single-stranded DNA and 6,500 M^{-1} cm^{-1} for double-stranded DNA (25).

Nitrocellulose Filter Binding Experiments—Filter binding experiments were performed as described in Refs. 6, 17, and 18. Experiments were performed using Schleicher & Schuell BA83 filter membranes (0.22 μm × 25 mm) and a Hoefer filter manifold. Standard experiments were performed in 500 μl of buffer containing 10 mM MOPS/NaOH (pH 7.3), 200 mM NaCl, 0.1 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 0.2 mM cAMP. For studies of salt dependence, the NaCl concentration of the assay buffer was varied to yield the desired monovalent cation concentration. ^{32}P-Labeled DNA (1–30 pM) and CAP (5–50,000 pM; in >5-fold excess over the DNA concentration) were equilibrated at room temperature for 60 min and were then filtered in ≈10–15 s under suction through filters presoaked in assay buffer minus bovine serum albumin. Filters were dried, and Cerenkov radiation was determined in a Beckman LS5000TD scintillation counter. All data were corrected for background (*i.e.* radioactivity retained on the filter in the absence of CAP).

Representative data are presented in Fig. 6. Values of K_{obs} were extracted by nonlinear regression (Marquardt-Levenberg algorithm as implemented in SigmaPlot 4.0 (Jandel Scientific) using the equation

$$B = \frac{B_M[CAP]_F}{(1/K_{obs}) + [CAP]_F} \quad (1)$$

where $[CAP]_F$ denotes the free CAP concentration, B denotes radioactivity retained on the filter, and B_M denotes radioactivity retained on the filter at saturation. $[CAP]_F$ and B were inputs to the nonlinear regression; K_{obs} and B_M were unconstrained outputs. ΔG_{obs}, the binding free energy for CAP·DNA complex formation, was calculated using the equation $\Delta G_{obs} = -RT\ln(K_{obs})$. $\Delta\Delta G_{obs}$, the difference between the binding free energy for CAP·DNA complex formation in the case of DNA site *i versus* the binding free energy for CAP·DNA complex formation in the case of the consensus DNA site, was calculated using the equation $\Delta\Delta G_{obs} = -RT\ln(K_{obs,i}) + RT\ln(K_{obs,consensus})$. High precision data were obtained where $K_{obs} = 1 \times 10^7$ M^{-1} to $K_{obs} = 2 \times 10^{11}$ M^{-1}. Where $K_{obs} < 1 \times 10^7$ M^{-1}, the value $K_{obs} = 1 \times 10^7$ M^{-1} was used to calculate a minimum estimate for $\Delta\Delta G_{obs}$.

RESULTS

Effects of Substitution of DNA Base Pairs

We have performed equilibrium DNA binding experiments to investigate the effects of substitution of DNA base pairs at positions 4 to 8 of the consensus DNA half-site. We have used as ligands a 40-base pair DNA fragment having the consensus DNA site (DNA fragment ICAP; Ref. 6) and 15 40-base pair DNA fragments having derivatives of the consensus DNA site with substitutions of DNA base pairs at positions 4 to 8 of each DNA half-site (sequences in Fig. 2*A*). The results obtained are presented in Table I and Fig. 2*B*.

Position 4—The consensus base pair at position 4 of the DNA half-site is T:A (3–5). CAP exhibits moderate specific-

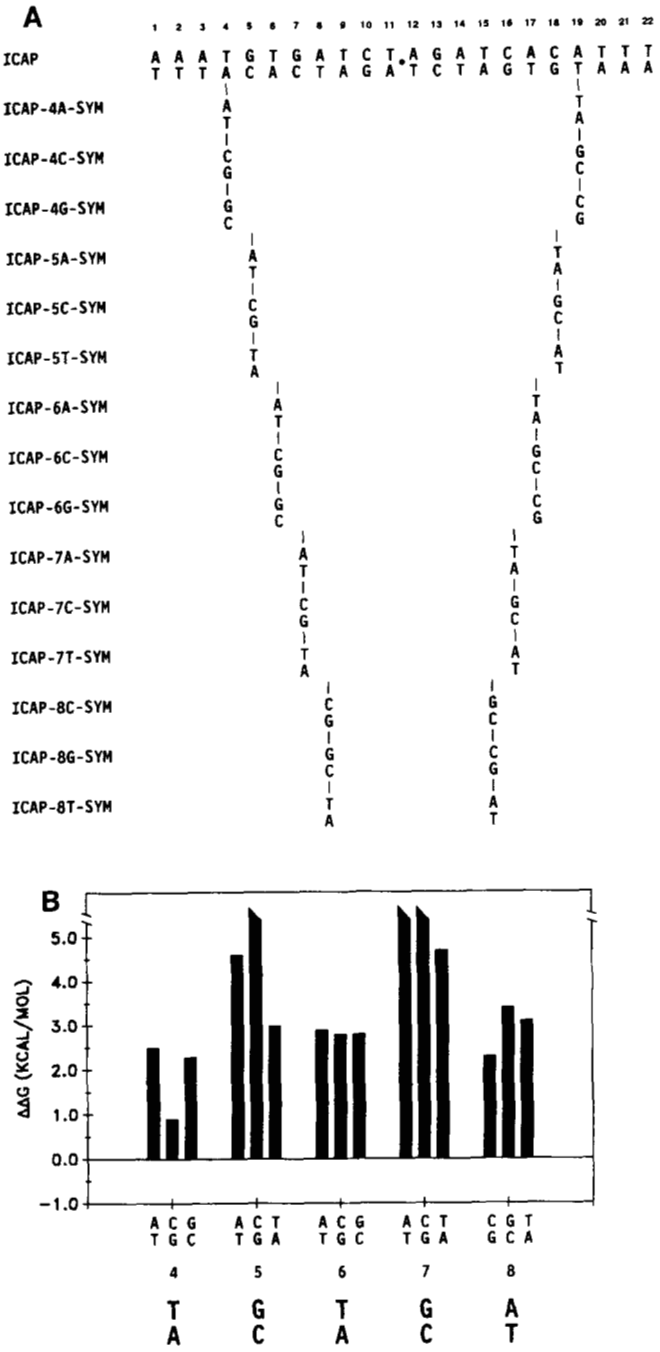

FIG. 2. *A*, DNA sites used to assess the effects of substitution of DNA base pairs. DNA sites are present on 40-base pair DNA fragments; sequences flanking the DNA sites are identical to the sequences flanking the *E. coli lac* DNA site for CAP (6). DNA fragment ICAP has the consensus DNA site for CAP (3–6). *B*, binding free energy changes ($\Delta\Delta G_{obs}$) upon substitution of DNA base pairs. The sequence in *large characters* corresponds to positions 4 to 8 of the consensus DNA half-site. *Solid bars* show the binding free energy changes upon the indicated substitutions. Binding free energy changes are for DNA sites substituted in *both* DNA half-sites.

ity for T:A at position 4. CAP prefers T:A to nonconsensus base pairs by 0.5–1.3 kcal/mol/DNA half-site. The rank order of preference is T:A > C:G > G:C ≈ A:T.

Position 5—The consensus base pair at position 5 of the DNA half-site is G:C (3–5). CAP exhibits strong specificity for G:C at position 5. CAP prefers G:C to nonconsensus base pairs by 1.5 to > 2.6 kcal/mol/DNA half-site. The rank order of preference is G:C >> T:A > A:T > C:G.

Position 6—The consensus base pair at position 6 of the DNA half-site is T:A (3–5). CAP exhibits moderate specificity for T:A at position 6. CAP prefers T:A to nonconsensus base pairs by 1.4–1.5 kcal/mol/DNA half-site. The rank order of preference is T:A > A:T ≈ C:G ≈ G:C.

Position 7—The consensus base pair at position 7 of the DNA half-site is G:C (3–5). CAP exhibits strong specificity for G:C at position 7. CAP prefers G:C to nonconsensus base pairs by 2.4 to > 2.6 kcal/mol/DNA half-site. The rank order of preference is G:C >> T:A > A:T ≈ C:G.

Position 8—The consensus base pair at position 8 of the DNA half-site is A:T (3–5). CAP exhibits moderate to strong specificity for A:T at position 8. CAP prefers A:T to nonconsensus base pairs by 1.2–1.7 kcal/mol/DNA half-site. The rank order of preference is A:T > C:G > G:C ≈ T:A.

TABLE I

Effects of substitution of DNA base pairs

Values of K_{obs} and ΔG_{obs} were calculated as described under "Materials and Methods" and are reported ±1 S.D.

DNA site	K_{obs}	$-\Delta G_{obs}$	$\Delta\Delta G_{obs}$
	M^{-1}	*kcal/mol*	*kcal/mol*
ICAP	$7.0 \pm 0.3 \times 10^{10}$	14.64 ± 0.02	[0]
ICAP-4A-SYM	$9.5 \pm 0.7 \times 10^{8}$	12.12 ± 0.04	2.5
ICAP-4C-SYM	$1.5 \pm 0.1 \times 10^{10}$	13.73 ± 0.04	0.9
ICAP-4G-SYM	$1.5 \pm 0.2 \times 10^{9}$	12.38 ± 0.07	2.3
ICAP-5A-SYM	$3.0 \pm 0.4 \times 10^{7}$	10.09 ± 0.07	4.6
ICAP-5C-SYM	$<1.0 \times 10^{7}$	<9.45	>5.2
ICAP-5T-SYM	$4.0 \pm 1.0 \times 10^{8}$	11.61 ± 0.13	3.0
ICAP-6A-SYM	$5.3 \pm 1.0 \times 10^{8}$	11.78 ± 0.10	2.9
ICAP-6C-SYM	$6.0 \pm 1.1 \times 10^{8}$	11.85 ± 0.10	2.8
ICAP-6G-SYM	$6.3 \pm 0.7 \times 10^{8}$	11.88 ± 0.06	2.8
ICAP-7A-SYM	$<1.0 \times 10^{7}$	<9.45	>5.2
ICAP-7C-SYM	$<1.0 \times 10^{7}$	<9.45	>5.2
ICAP-7T-SYM	$2.3 \pm 0.5 \times 10^{7}$	9.94 ± 0.12	4.7
ICAP-8C-SYM	$1.3 \pm 0.3 \times 10^{9}$	12.30 ± 0.12	2.3
ICAP-8G-SYM	$2.0 \pm 0.4 \times 10^{8}$	11.20 ± 0.11	3.4
ICAP-8T-SYM	$3.3 \pm 0.7 \times 10^{8}$	11.50 ± 0.11	3.1

Effects of Substitution of Thymine Residues by Uracil and by 5-Methylcytosine

The thymine 5-methyl group projects into the DNA major groove (27, 28). The thymine 5-methyl group can be a critical determinant for specificity for A:T or T:A in protein-DNA interaction. It has been shown experimentally that the thymine 5-methyl group is the sole determinant of specificity for A:T at one position in the DNA half-site for Lac repressor (29) and that the thymine 5-methyl group is the sole determinant of specificity for T:A at one position in the DNA half-site for [Ala-28]434 repressor (30).

We have performed equilibrium DNA binding experiments to investigate the effects of substitution of thymine by uracil and by 5-methylcytosine at positions 4, 6, and 8 of the consensus DNA half-site. Uracil does not have a 5-methyl group but is otherwise identical to thymine; 5-methylcytosine has a 5-methyl group but is otherwise identical to cytosine. We have used as ligands a 40-base pair DNA fragment having the consensus DNA site (DNA fragment ICAP; Ref. 6), three 40-base pair DNA fragments having derivatives of the consensus DNA site with substitutions of thymine by uracil at positions 4, 6, or 8 of each DNA half-site (sequences in Fig. 3*A*), and three 40-base pair DNA fragments having derivatives of the consensus DNA site

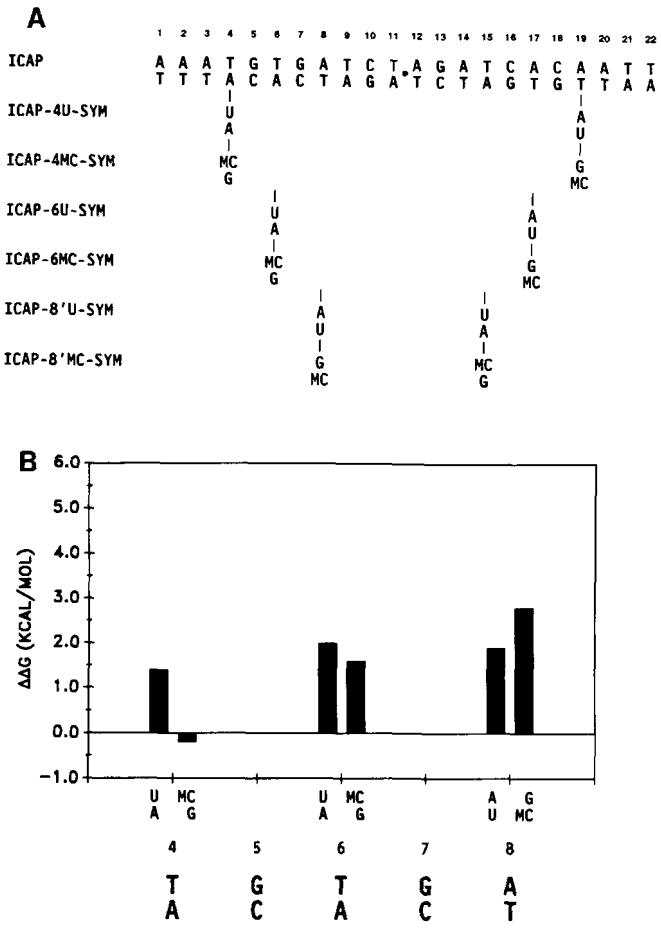

FIG. 3. *A*, DNA sites used to assess the effects of substitution of thymine residues by uracil and by 5-methylcytosine. DNA sites are present on 40-base pair DNA fragments; sequences flanking the DNA sites are identical to the sequences flanking the *E. coli lac* DNA site for CAP (6). DNA fragment ICAP has the consensus DNA site for CAP (3–6). *U* denotes uracil. *MC* denotes 5-methylcytosine. *B*, binding free energy changes ($\Delta\Delta G_{obs}$) upon substitution of thymine residues by uracil and by 5-methylcytosine. The sequence in *large characters* corresponds to positions 4 to 8 of the consensus DNA half-site. *Solid bars* show the binding free energy changes upon the indicated substitutions. Binding free energy changes are for DNA sites substituted in *both* DNA half-sites.

TABLE II

Effects of substitution of thymine residues by uracil or 5-methylcytosine

Values of K_{obs} and ΔG_{obs} were calculated as described under "Materials and Methods" and are reported ±1 S.D.

DNA site	K_{obs}	$-\Delta G_{obs}$	$\Delta\Delta G_{obs}$
	M^{-1}	*kcal/mol*	*kcal/mol*
ICAP	$7.0 \pm 0.3 \times 10^{10}$	14.64 ± 0.02	[0]
ICAP-4U-SYM	$6.4 \pm 0.3 \times 10^{9}$	13.24 ± 0.03	1.4
ICAP-4MC-SYM	$1.0 \pm 0.1 \times 10^{11}$	14.85 ± 0.06	−0.2
ICAP-6U-SYM	$2.2 \pm 0.1 \times 10^{9}$	12.61 ± 0.03	2.0
ICAP-6MC-SYM	$4.3 \pm 1.0 \times 10^{9}$	13.00 ± 0.12	1.6
ICAP-8'U-SYM	$2.9 \pm 0.4 \times 10^{9}$	12.77 ± 0.08	1.9
ICAP-8'MC-SYM	$5.5 \pm 0.5 \times 10^{8}$	11.80 ± 0.05	2.8

with substitutions of thymine by 5-methylcytosine and adenine by guanine at positions 4, 6, or 8 of each DNA half-site (sequences in Fig. 3*A*). The results obtained are presented in Table II and Fig. 3*B*.

Position 4—The consensus base pair at position 4 of the DNA half-site is T:A (3–5). CAP prefers T:A to U:A at position 4 of the DNA half-site by 0.7 kcal/mol/DNA half-site. The magnitude of the preference for T:A *versus* U:A at position 4 of the DNA half-site is comparable to the magnitude of the preference for T:A *versus* A:T, C:G, or G:C (0.5–1.3 kcal/mol/DNA half-site; Table I and Fig. 2*B*). These results indicate that the 5-methyl group is a determinant of specificity for T:A at position 4 of the DNA half-site.

CAP does not prefer T:A to 5-MeC:G at position 4 of the DNA half-site. The absence of a preference for T:A *versus* 5-MeC:G at position 4 of the DNA half-site is in marked contrast to the preference for T:A *versus* C:G (0.5 kcal/mol/DNA half-site; Table I and Fig. 2*B*). These results further indicate that the 5-methyl group is a determinant of specificity for T:A at position 4 of the DNA half-site.

It is striking that, within experimental error, the apparent incremental binding free energy contribution of the 5-methyl group is equal in the comparison of T:A *versus* U:T (0.7 kcal/mol/DNA half-site) and in the comparison of 5-MeC:G *versus* C:G (0.6 kcal/mol/DNA half-site). Taken together, the results indicate that the 5-methyl group is the *sole* determinant of specificity for T:A at position 4 of the DNA half-site.

Position 6—The consensus base pair at position 6 of the DNA half-site is T:A (3–5). CAP prefers T:A to U:A at position 6 of the DNA half-site by 1.0 kcal/mol/DNA half-site. The magnitude of the preference for T:A *versus* U:A at position 6 of the DNA half-site is comparable to the magnitude of the preference for T:A *versus* A:T, C:G, or G:C (1.4 to 1.5 kcal/mol/DNA half-site; Table I and Fig. 2*B*). These results indicate that the 5-methyl group is a determinant of specificity for T:A at position 6 of the DNA half-site.

However, CAP also prefers T:A to 5-MeC:G at position 6 of the DNA half-site, by 0.8 kcal/mol/DNA half-site. This result indicates that the 5-methyl group is not the sole determinant of specificity for T:A at position 6 of the DNA half-site. The apparent incremental binding free energy contribution of the 5-methyl group is significantly different in the comparison of T:A *versus* U:T (1.0 kcal/mol/DNA half-site) and in the comparison of 5-MeC:G *versus* C:G (0.6 kcal/mol/DNA half-site). Taken together, the results indicate that the role of the thymine 5-methyl group in specificity at position 6 of the DNA half-site is complex.

Position 8—The consensus base pair at position 8 of the DNA half-site is A:T (3–5). CAP prefers A:T to A:U at position 8 of the DNA half-site by 1.0 kcal/mol/DNA half-site. The magnitude of the preference for A:T *versus* A:U at position 8 of the DNA half-site is comparable to the magnitude of the preference for A:T *versus* C:G, G:C, or T:A (1.2 to 1.7 kcal/mol/DNA half-site; Table I and Fig. 2*B*). These results indicate that the 5-methyl group is a determinant of specificity for A:T at position 8 of the DNA half-site.

However, CAP also prefers A:T to G:5-MeC at position 8 of the DNA half-site, by 1.4 kcal/mol/DNA half-site. This result indicates that the 5-methyl group is not the sole determinant of specificity for A:T at position 8 of the DNA half-site. The apparent incremental binding free energy contribution of the 5-methyl group is significantly different in the comparison of A:T *versus* A:U (1.0 kcal/mol/DNA half-site) and in the comparison of G:5-MeC *versus* G:C (0.3 kcal/mol/DNA half-site). Taken together, the results indicate that the role of the 5-methyl group in specificity at position 8 of the DNA half-site is complex.

Effect of dam Methylation

In *E. coli*, 5′-GATC-3′ sequences are methylated on both DNA strands at the N^6 atom of adenine, by action of the *E. coli dam* methylase (22). *dam* methylation can have large effects on protein-DNA complex formation (31, 32). The consensus DNA site for CAP contains one 5′-GATC-3′ sequence in each DNA half-site (positions 7 to 10 of the DNA half-site). We have performed equilibrium DNA binding experiments to investigate the effect of *dam* methylation of the consensus DNA site. We have used as ligand a 40-base pair DNA fragment having a derivative of the consensus DNA site with N^6-methyladenine at positions 8 and 9 of each DNA half-site (Fig. 4). The results obtained are presented in Table III. The results indicate that CAP exhibits nearly equal affinities for the consensus DNA site and for the derivative of the consensus DNA site with N^6-methyladenine at positions 8 and 9 of each DNA half-site.

Effects of DNA Fragment Length

Crothers and co-workers (33), using primer extension mapping and gel mobility shift experiments, have established that the *E. coli lac* DNA site for CAP spans 28–34 base pairs. CAP-induced DNA bending brings the outer segments of the DNA site into contact with the flanks of the CAP dimer, enabling the CAP dimer (≈55 Å in diameter) to contact an extended DNA site (≥95 Å in length if not bent; 12, 33, 34).

In this report, we have analyzed the effects of DNA fragment length on CAP·DNA complex formation with the consensus DNA site and have determined the minimum effective DNA fragment length for CAP·DNA complex formation with the consensus DNA site. We have used as ligands a 40-base pair incompletely symmetric DNA fragment having the consensus DNA site (DNA fragment ICAP; Ref. 6) and a 28-base pair incompletely symmetric DNA fragment having the consensus DNA site (DNA fragment ICAP28; sequence in Fig. 5). In the incompletely symmetric DNA fragments, the sequences flanking the consensus DNA site were identical to the sequences flanking the *E. coli lac* DNA site for CAP. In addition, we have used as ligands 30-, 28-, 26-, 24-, and 22-base pair completely symmetric DNA fragments having the consensus DNA site (sequences in Fig. 5). In the completely symmetric DNA fragments, the sequences flanking the consensus DNA site were identical to the upstream sequence flanking the *E. coli lac* DNA site for

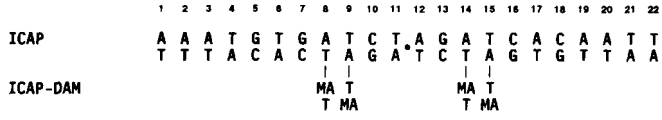

FIG. 4. **DNA sites used to assess the effects of *dam* methylation.** DNA sites are present on 40-base pair DNA fragments; sequences flanking the DNA sites are identical to the sequences flanking the *E. coli lac* DNA site for CAP (6). DNA fragment ICAP has the consensus DNA site for CAP (3–6). *MA* denotes N^6-methyladenine.

TABLE III

Effect of dam methylation

Values of K_{obs} and ΔG_{obs} were calculated as described under "Materials and Methods" and are reported ±1 S.D.

DNA fragment	K_{obs}	$-\Delta G_{obs}$	$\Delta\Delta G_{obs}$
	M^{-1}	*kcal/mol*	*kcal/mol*
ICAP	$7.0 \pm 0.3 \times 10^{10}$	14.64 ± 0.02	[0]
ICAP-DAM	$2.0 \pm 0.2 \times 10^{11}$	15.25 ± 0.06	−0.6

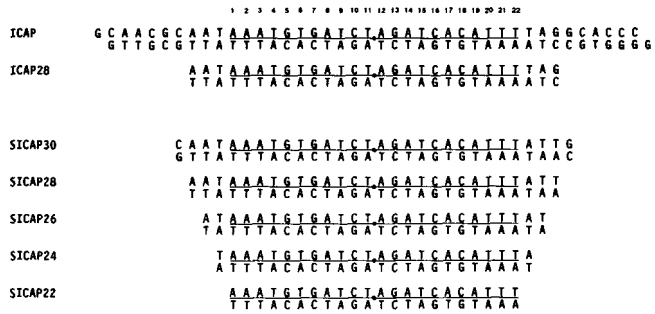

FIG. 5. **DNA fragments used to assess the effects of DNA fragment length.** DNA fragment ICAP and ICAP28 are 40- and 28-base pair incompletely symmetric DNA fragments having the consensus DNA site for CAP. DNA fragments SICAP30, SICAP28, SICAP26, SICAP24, and SICAP22 are 30-, 28-, 26-, 24-, and 22-base pair completely symmetric DNA fragments having the consensus DNA site for CAP.

CAP. The results obtained are presented in Table IV.

CAP·DNA Complex Formation—CAP exhibits nearly equal affinities for the 40-base pair incompletely symmetric DNA fragment and for the 28-base pair incompletely symmetric DNA fragment. In addition, CAP exhibits nearly equal affinities for the 30-base pair completely symmetric DNA fragment and for the 28-base pair completely symmetric DNA fragment. In contrast, CAP exhibits a lower affinity for the 26-base pair completely symmetric DNA fragment ($\Delta\Delta G$ = 1.0 kcal/mol) and much lower affinities for the 24- and 22-base pair completely symmetric DNA fragments ($\Delta\Delta G$ = 2.7 and 3.9 kcal/mol, respectively).

Salt Dependence of CAP·DNA Complex Formation—Record and co-workers (35, 36), based on the polyelectrolyte theory of Manning (37), have shown that for B-DNA, in the absence of anion-release effects

$$\log K_{obs} = \log K_{obs(1.0\,M)} - 0.88\ m' \log[M^+] \qquad (2)$$

where $[M^+]$ denotes the monovalent cation concentration (equal to $[Na^+]$ in this paper); $K_{obs(1.0\,M)}$ denotes K_{obs} at $[M^+]$ = 1.0 M ; and m' denotes the number of protein-DNA ion pairs formed upon protein-DNA complex formation (ion pairs between cationic residues of the protein [Lys, Arg, His, or N terminus] and DNA phosphates of the DNA site).

In previous work, we have shown that CAP makes eight thermodynamically defined ion pairs with a 40-base pair DNA fragment having the consensus DNA site (6).[3] The results in Table IV indicate that CAP likewise makes eight thermodynamically defined ion pairs with a 30- and a 28-base pair DNA fragment. In contrast, CAP makes only seven thermodynamically defined ion pairs with a 26-base pair DNA fragment. It was not possible to quantify the number of thermodynamically defined ion pairs in the cases of the 24- and 22-base pair DNA fragments because of their low affinities for CAP.

DISCUSSION

Specificity at Positions 4 to 8 of the DNA Half-site

Position 4—Our results show that CAP exhibits moderate specificity for the consensus base pair T:A at position 4, and that this specificity is determined *solely* by the thymine 5-

[3] CAP makes only six thermodynamically defined ion pairs with a 40-base pair DNA fragment having the *E. coli lac* DNA site for CAP (6; cf. Ref. 38). The difference in ion pair formation is believed to be due to greater CAP-induced DNA bending in the case of the consensus DNA site than in the case of the *E. coli lac* DNA site (6, 34).

TABLE IV
Effects of DNA fragment length

DNA fragment	K_{obs}[a]	$-\Delta G_{obs}$[a]	$\Delta\Delta G_{obs}$	Number of ion pairs[b]	
				m'	m', integer
	M^{-1}	*kcal/mol*	*kcal/mol*		
ICAP	$7.0 \pm 0.3 \times 10^{10}$	14.64 ± 0.02	[0]	8.4 ± 0.2	8
ICAP28	$4.3 \pm 0.3 \times 10^{10}$	14.35 ± 0.04	0.3	7.5 ± 0.4	8
SICAP30	$2.2 \pm 0.2 \times 10^{10}$	13.96 ± 0.05	[0]	7.8 ± 0.4	8
SICAP28	$1.0 \pm 0.1 \times 10^{10}$	13.50 ± 0.06	0.5	7.5 ± 0.6	8
SICAP26	$3.8 \pm 0.3 \times 10^{9}$	12.93 ± 0.04	1.0	6.8 ± 0.4	7
SICAP24	$2.1 \pm 0.4 \times 10^{8}$	11.23 ± 0.10	2.7	ND	ND
SICAP22	$3.0 \pm 0.4 \times 10^{7}$	10.09 ± 0.07	3.9	ND	ND

[a] Values of K_{obs} and ΔG_{obs} were calculated as described under "Materials and Methods" and are reported ±1 S.D.
[b] m' denotes the number of protein-DNA ion pairs formed upon protein-DNA complex formation. Values of m' were determined from the dependence of $\log K_{obs}$ on $-\log[Na^+]$ at constant pH and temperature (pH = 7.3; temperature = 23 °C) as described in Refs. 6 and 44–46 and are reported ±1 S.E.

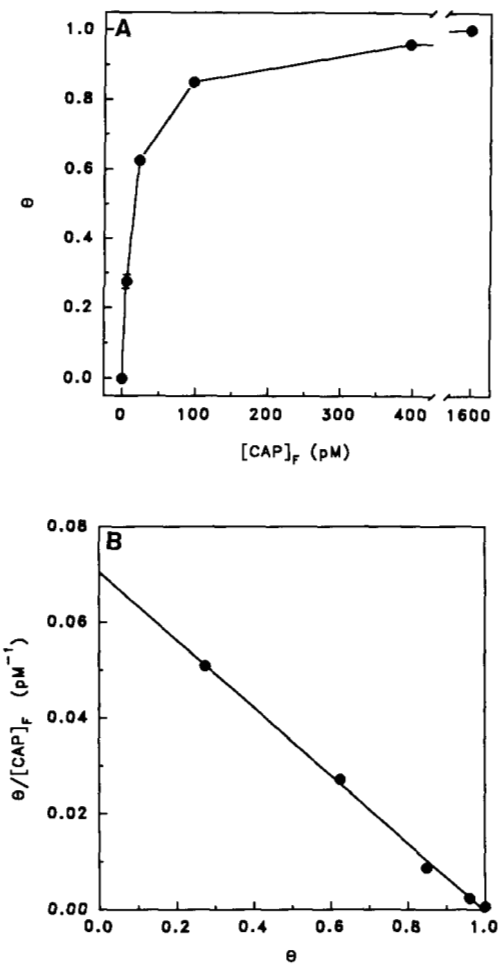

FIG. 6. **Equilibrium titration of DNA fragment ICAP with CAP.** Data are from nitrocellulose filter binding assays performed at [NaCl] = 200 mM; pH 7.3; temperature = 23 °C. *A*, titration data. $[CAP]_F$ denotes the free CAP concentration; θ denotes the fractional saturation of DNA fragment ICAP. Data are means ±1 S.D. of two independent determinations. *B*, modified Scatchard plot of the same data (*cf.* Ref. 26). The line is a linear least-squares fit of the data. The slope of the line is equal to the negative of K_{obs} (7.0×10^{10} M^{-1}).

methyl group. In the structure of the CAP·DNA complex, no amino acid of CAP forms H-bonds with the base pair at position 4 of the DNA half-site (12). We propose that specificity for T:A at position 4 results from either: (i) energetically favorable van der Waals interactions between CAP and the thymine 5-methyl group of T:A at position 4; (ii) effects of the thymine 5-methyl group of T:A at position 4 on the geometry of the DNA phosphates at position 4; (iii) effects of the thymine 5-methyl group of T:A at position 4 on the solvation of the DNA phosphates at position 4; or (iv) effects of the thymine 5-methyl group of T:A at position 4 on DNA twist or DNA bending. These mechanisms are not mutually exclusive. With respect to mechanism i, it is noteworthy that in the structure of the CAP·DNA complex the Cζ, Nη1, and Nη2 atoms of the side chain of Arg-180 of CAP appear to be in van der Waals contact with the thymine 5-methyl group of T:A at position 4 (12). With respect to mechanisms ii and iii, it is noteworthy that in the structure of the CAP·DNA complex the side chain of Thr-168 of CAP and the peptide backbone NH groups of Arg-169 and Gln-170 of CAP form H-bonds with the top strand DNA phosphate 5′ to position 4 and that the side chain of Arg-169 of CAP forms an ion pair with the top strand DNA phosphate 3′ to position 4 (12; Fig. 1*B*). High resolution x-ray crystallographic investigations of B-DNA duplexes indicate that at T:A nucleotide pairs there is a site-bound water molecule immobilized between the thymine 5-methyl group and the top strand DNA phosphate 5′ to the nucleotide pair; in contrast, at U:T, A:T, C:G, or G:C nucleotide pairs there is no equivalent site-bound water molecule (39, 40; see also Ref. 41). This suggests one detailed, although hypothetical, version of mechanism iii, as follows. Upon binding of CAP to a DNA site having T:A at position 4, formation of H-bonds between CAP and the top strand DNA phosphate 5′ to position 4 displaces a site-bound water molecule from position 4; displacement of the site-bound water molecule results in an increase in entropy and a corresponding increment of binding free energy. Upon binding of CAP to DNA sites having U:T, A:T, C:G, or G:C at position 4 this increment of binding free energy is absent.

Position 4 is the first nucleotide pair within the six-nucleotide pair DNA segment in contact with the helix-turn-helix motif of CAP (positions 4 to 9 of the DNA half-site; Ref. 12). We point out that, like CAP, λ repressor and 434 repressor exhibit moderate specificity for T:A at the first nucleotide pair within the six-nucleotide pair DNA segment in contact with the helix-turn-helix motif (20, 42, 43) and that, like in the structure of the CAP·DNA complex, in the structure of the λ repressor-DNA complex and in the structure of the 434 repressor-DNA complex, no amino acid forms H-bonds with the base pair at the first nucleotide pair within the six-nucleotide pair DNA segment in contact with the helix-turn-helix motif (42, 44). It appears likely that CAP, λ repressor, and 434 repressor use similar or identical mech-

anisms to determine specificity at the first nucleotide pair within the six-nucleotide pair DNA segment in contact with the helix-turn-helix motif (43). This may be a general phenomenon among helix-turn-helix motif sequence-specific DNA-binding proteins (43).

Position 5—Our results show that CAP exhibits strong specificity for the consensus base pair G:C at position 5. In the structure of the CAP·DNA complex, the side chain of Arg-180 of CAP forms two H-bonds with G:C at position 5 of the DNA half-site: *i.e.* one H-bond with the guanine N7 atom and one H-bond with the guanine O^6 atom (12, 16). The strong specificity for G:C at position 5 is consistent with the formation of these two H-bonds. The rank order of preference among DNA base pairs at position 5 also is consistent with the formation of these two H-bonds (cf. Refs. 27 and 28). Model building suggests that the side chain of Arg-180 could form one H-bond with T:A at position 5 (with the thymine O^4 atom), one H-bond with A:T at position 5 (with the adenine N7 atom, with unfavorable effects on solvation of the adenine N^6 atom), but zero H-bonds with C:G at position 5.

Position 6—Our results show that CAP exhibits moderate specificity for the consensus base pair T:A at position 6 and that this specificity is determined in part, but not solely, by the thymine 5-methyl group. In the structure of the CAP·DNA complex, no amino acid of CAP forms H-bonds with the base pair at position 6 of the DNA half-site (12, 18). We propose that specificity for T:A at position 6 results from either: (i) energetically favorable van der Waals interactions between CAP and the thymine 5-methyl group of T:A at position 6; (ii) sequence-dependent effects on the geometry of the DNA phosphates at position 6; (iii) sequence-dependent effects on the solvation of the DNA phosphates at position 6; or (iv) sequence-dependent effects on DNA twist or DNA bending. These mechanisms are not mutually exclusive. With respect to mechanisms ii and iii, it is noteworthy that in the structure of the CAP·DNA complex the side chain of Lys-188 of CAP forms an ion pair with the top strand DNA phosphate 5′ to position 6 (12; Fig. 1*B*). With respect to mechanism iv, it is noteworthy that in the structure of the CAP·DNA complex there is a large (≈40°) DNA kink between positions 6 and 7 (12) and that the sequence 5′-TG-3′ has been shown to favor DNA flexibility and DNA bending (45–47).

Position 7—Our results show that CAP exhibits strong specificity for the consensus base pair G:C at position 7. In the structure of the CAP·DNA complex, the side chain of Glu-181 of CAP forms one H-bond with G:C at position 7 of the DNA half-site: *i.e.* one H-bond with the cytosine N^4 atom (12–15). The strong specificity for G:C at position 7 is consistent with the formation of this H-bond. The rank order of preference among DNA base pairs at position 7 also is consistent with the formation of this H-bond (cf. Refs. 27 and 28). Model building suggests that the side chain of Glu-181 could form one H-bond with T:A at position 7 (with the adenine N^6 atom, with unfavorable effects on solvation of the adenine N7 atom), but zero H-bonds with A:T or C:G at position 7.

Position 8—Our results show that CAP exhibits moderate to strong specificity for the consensus base pair A:T at position 8 and that this specificity is determined in part, but not solely, by the thymine 5-methyl group. In the structure of the CAP·DNA complex, the side chain of Arg-185 of CAP forms one H-bond with A:T at position 8 of the DNA half-site: *i.e.* one H-bond with the thymine O^4 atom (12). The moderate to strong specificity for A:T at position 8 is consistent with the formation of this H-bond. The rank order of preference among DNA base pairs at position 8 also is consistent with the formation of this H-bond (cf. Refs. 27 and 28). Model building suggests that the side chain of Arg-185 could form one H-bond with C:G at position 8 (with the guanine O^6 atom, with unfavorable effects on solvation of the guanine N7 atom) but zero H-bonds with G:C or T:A at position 8.

In the structure of the CAP·DNA complex, the $O\epsilon 1$ atom of the side chain of Glu-181 of CAP appears to be in van der Waals contact with the thymine 5-methyl group of A:T at position 8 (12). The effect of the thymine 5-methyl group in specificity at position 8 may be related to this contact.

Effect of dam Methylation

Although the consensus DNA site for CAP contains one *dam* methylation site in each DNA half-site (positions 7 to 10 of the DNA half-site), our results show that *dam* methylation of the consensus DNA site has little effect on CAP·DNA complex formation. In the structure of the CAP·DNA complex (12), no residues of CAP are close to the adenine N^6 atom (the target of *dam* methylation) at positions 8 and 9 of the DNA half-site. The absence of a large effect on binding of CAP of *dam* methylation of the consensus DNA site is consistent with the structure.

Effects of DNA Fragment Length

CAP·DNA Complex Formation—Our results indicate that the minimum effective DNA fragment length for binding of CAP to the consensus DNA site is 28 base pairs. All, or nearly all, DNA determinants required for maximal affinity for CAP are present in a 28-base pair DNA fragment having the 22-base pair consensus DNA site at its center. In contrast, not all DNA determinants required for maximal affinity for CAP are present in a 26-base pair DNA fragment having the 22-base pair consensus DNA site at its center. These results are in agreement with the results obtained by Crothers and co-workers (33) using the *E. coli lac* DNA site for CAP.

In the structure of the CAP·DNA complex, all CAP·DNA contacts are to DNA determinants present in a 28-base pair DNA segment (12). Lys-26 of CAP contacts the bottom strand phosphate 5′ to position −3 of the DNA half-site (present in a 28-base pair DNA fragment but not present in shorter DNA fragments); Lys-166 of CAP contacts the bottom strand phosphate 5′ to position −2 of the DNA half-site (present in a 26-base pair DNA fragment but not present in shorter DNA fragments); and His-199 of CAP contacts the bottom strand phosphate 5′ to position −1 of the DNA half-site (present in a 24-base pair DNA fragment but not present in shorter DNA fragments) (12). It appears likely that the difference in affinity between the 28- and 26-base pair DNA fragments having the consensus DNA site (0.5 kcal/mol) reflects the absence of the contact by Lys-26 of CAP in the case of the 26-base pair DNA fragment, that the difference in affinity between the 26- and 24-base pair DNA fragments having the consensus DNA site (1.7 kcal/mol) reflects the absence of the contact by Lys-166 of CAP in the case of the 24-base pair DNA fragment, and that the difference in affinity between the 24- and 22-base pair DNA fragments having the consensus DNA site (1.2 kcal/mol) reflects the absence of the contact by His-199 of CAP in the case of the 22-base pair DNA fragment.

CAP·DNA Ion Pair Formation—In previous work, we have shown that CAP makes eight thermodynamically defined ion pairs with a 40-base pair DNA fragment having

the consensus DNA site (6).[3] Our present results indicate that CAP likewise makes eight thermodynamically defined ion pairs with 30- and 28-base pair DNA fragments having the consensus DNA site but makes only seven thermodynamically defined ion pairs with a 26-base pair DNA fragment having the consensus DNA site. We conclude that the DNA phosphates that participate in the eight thermodynamically defined ion pairs in the case of the 40-base pair DNA fragment also are present in the 30- and 28-base pair DNA fragments but that at least one of these DNA phosphates is not present in the 26-base pair DNA fragment. However, we caution that end effects may complicate interpretation of salt dependence experiments with short DNA fragments (48, 49)

In the structure of the CAP·DNA complex as reported in Ref. 12, there are nine CAP·DNA ion pairs. The CAP·DNA ion pairs involve Lys-26 of one CAP subunit and Lys-166, Lys-188, Arg-169, and His-199 of both CAP subunits. Lys-26 of one CAP subunit forms an ion pair with the bottom strand phosphate 5′ to position −3 of one DNA half-site (present in a 28-base pair DNA fragment, but not present in shorter DNA fragments). It appears likely that the difference in the number of thermodynamically defined ion pairs between the 28- and 26-base pair DNA fragments having the consensus DNA site (eight *versus* seven) reflects the absence of the ion pair by Lys-26 of CAP in the case of the 26-base pair DNA fragment.

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