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Molecular basis of DNA sequence recognition by the catabolite gene activator protein: Detailed inferences from three mutations that alter DNA sequence specificity

(DNA–protein interaction/helix-turn-helix motif/cyclic AMP receptor protein)

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ABSTRACT **Previously, we reported that substitution of Glu-181 of the catabolite gene activator protein (CAP) by lysine, leucine, or valine results in a protein that has specificity for A·T base pairs at positions 7 and 16 of the DNA recognition site, rather than G·C base pairs as is the case with the wild-type CAP. In this paper, we deduce from these genetic data both (*i*) the specific chemical interactions by which amino acid side chains at position 181 interact with base pairs 7 and 16 and (*ii*) the precise alignment between the structures of the CAP and DNA in the intermolecular CAP–DNA complex. Our analysis supports the idea that the two symmetry-related F α-helices of the CAP dimer interact with successive major grooves of right-handed B-type DNA [Pabo, C. & Lewis, M. (1982) *Nature (London)* 298, 443–447; and Steitz, T., Weber, I. & Matthew, J. (1983) *Cold Spring Harbor Symp. Quant. Biol.* 47, 419–426].**

The three-dimensional structures of three proteins that bind to specific DNA sequences have been determined recently—i.e., the catabolite gene activator protein (CAP) complex with cAMP (1), cro (2), and the amino-terminal fragment of the λ repressor (ref. 3; reviewed in ref. 4). Because the structures determined were those of the uncomplexed proteins, efforts to date to elucidate the structures of the protein–DNA complexes have relied on model building (1–8). The models proposed in the cases of cro and the λ repressor and one of four models in the case of CAP exhibit common features. Each model postulates that a pair of 2-fold related α-helices, one from each subunit of the protein, interacts with successive major grooves of right-handed B-type DNA (2, 3, 7). As discussed below, in each instance the α-helix proposed to contact DNA is the second α-helix of a characteristic helix-turn-helix structural motif (9–13). In this paper, we discuss the implications for this analysis of our genetic results (14), which we believe identify a direct contact between an amino acid of a DNA binding protein and the base pair it contacts in the target DNA sequence.

The results discussed in this paper were obtained with CAP (also referred to as the cAMP receptor protein, CRP; reviewed in ref. 15). Eighteen DNA sites to which CAP binds have been identified by nuclease-protection experiments, and a comparison of these sites yields the 14-base-pair consensus sequence A-A-N-T-G-T-G-A-N-N-T-N-N-N-T-C-A-N-A-T-W, where W signifies a position that can be either adenine or thymine (ref. 14; cf. refs. 5 and 15). The recognition sequence has been characterized in detail using chemical-protection and modification experiments (16, 17) and also by sequencing single-nucleotide mutations that decrease the affinity of the site for CAP (18–20). These data are summarized in Fig. 1. It is worth noting that the sequence of the consensus recognition site exhibits an imperfect but substantial 2-fold symmetry, with the axis of symmetry located between base pairs 11 and 12 (14). We also note that without exception the points of putative CAP–DNA contact identified by the chemical experiments reflect this symmetry.

We have described a genetic strategy that enables us to identify individual points of contact between specific amino acids of a protein and specific base pairs in the recognition sequence (14). The basis of this approach is to isolate and sequence mutations that alter the DNA sequence specificity of the protein. In the experiments described in ref. 14, we isolated three mutations that alter the sequence specificity of CAP such that the protein binds tightly to the *lacL8* sequence [A-A-N-T-G-T-A-A-N-N-T-N-N-N-T-C-A-N-A-T-W (14)]. The mutant proteins also interact tightly with the *lacL29* sequence (A-A-N-T-G-T-G-A-N-N-T-N-N-N-T-T-A-N-A-T-W), which is symmetrically related to *lacL8*, but they do not bind with normal affinity to either (*i*) DNA sites altered by mutation at other points or (*ii*) the wild-type recognition site. All three mutations were found to substitute Glu-181 of CAP, converting it to lysine, leucine, or valine. These data indicate that residue 181 of CAP is involved in determining the specificity of the protein for G·C vs. A·T at symmetric positions 7 and 16. In the structure of CAP as determined by Steitz and co-workers (1, 21), Glu-181 is completely exposed to solvent, and it is located in an α-helix that protrudes from the surface of the protein. The absence of interactions between the side chain of amino acid 181 and other residues of CAP suggests that the amino acid substitutions in the three mutant proteins would not perturb CAP conformation (cf. ref. 22). Consequently, we propose that the three mutations define a *direct* contact between amino acid 181 of CAP and base pairs 7 and 16 of the recognition site (14). In this paper, we report that these data allow us to infer both (*i*) the chemical interactions by which base pairs 7 and 16 are recognized by CAP and (*ii*) the alignment between the structure of CAP and the structure of DNA.

Chemistry of Recognition at Base Pairs 7 and 16

Our genetic results suggest that glutamate at position 181 hydrogen bonds or otherwise interacts with G·C base pairs whereas lysine, leucine, or valine at this position interact selectively with A·T base pairs (14). Here, we analyze the potential chemical interactions that could explain this pattern of specificity. It is known that CAP protects the guanine N-7 atom at base pairs 7 and 16 from reaction with dimethyl sulfate (ref. 16; Fig. 1*A*). This result indicates that CAP interacts with the DNA *major groove* (in which the N-7 atom is located) at these two base pairs (16). Therefore, we have re-

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Abbreviation: CAP, catabolite gene activator protein.

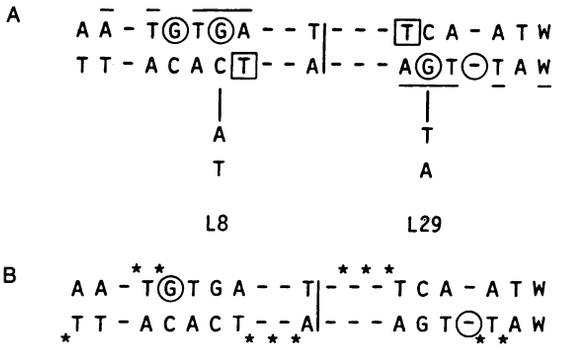

FIG. 1. Consensus recognition site for CAP (14), where W signifies a position that can be either adenine or thymine. Note that the sequence is partly symmetric, with the axis of 2-fold sequence symmetry located between base pairs 11 and 12. (*A*) Genetic and chemical-protection data. The nucleotide substitutions in the *lacL8* and *lacL29* mutations (18, 19) are indicated beneath the sequence. Circles identify guanine residues at which CAP protects the N-7 atom from methylation by dimethyl sulfate (16). Squares mark positions where CAP prevents the strand scission caused by UV irradiation of bromouracil-substituted DNA; this technique probes for close approach of the protein to the 5-position of thymine (17). (*B*) Chemical-modification data (16). Circles indicate guanine residues at which introduction of a methyl substituent at the N-7 position strongly reduces affinity of the site for CAP (effect greater than 1.5-fold). Asterisks identify backbone phosphates at which ethylation reduces affinity for CAP.

stricted the analysis that follows to those base-pair atoms that are exposed to solvent in the major groove of DNA (see ref. 8).

Fig. 2*A* illustrates our conclusion regarding the wild-type interaction: i.e., the interaction between Glu-181 and the G·C base pair. The major-groove edge of the G·C base pair contains three atoms able to make hydrogen bonds: specifically, guanine N-7, guanine O^6, and cytosine N-4. The carboxylate side chain of glutamate is a hydrogen bond *acceptor*. Consequently, the only evident major-groove contact this side chain could make to G·C is a hydrogen bond between one glutamate oxygen atom and the cytosine N-4 atom. In Fig. 2*A* we have illustrated the glutamate side chain in its most likely conformation and at the optimal angle and distance to make this contact.

The contact illustrated in Fig. 2*A* is consistent with the inability of Glu-181 to interact tightly with A·T base pairs (14). In the structure of the A·T base pair, the atom spatially equivalent to cytosine N-4 is a hydrogen bond acceptor (thymine O-4; see Fig. 2*B*). Inspection of the A·T structure indicates that Glu-181 therefore could not hydrogen bond to A·T without movement of the glutamate α- and β-carbon atoms by at least 4 Å from the positions proposed in Fig. 2*A*. We note that movement of this magnitude would require either (*i*) disruption of the α-helical peptide backbone at residue 181 or (*ii*) dislocation of the entire F α-helix with respect to the recognition site, and therefore we consider it unlikely. We consequently propose that the basis of specificity of Glu-181 of CAP to G·C vs. A·T in fact is a single hydrogen bond to the cytosine N-4 position.

In Fig. 2*B* we present our proposals regarding the interaction between lysine, leucine, and valine at position 181 and the A·T base pair (14). To construct Fig. 2*B*, we simply superimposed the α- and β-carbon atoms of the three mutant side chains onto the α and β positions proposed in Fig. 2*A* for Glu-181. Our assumption in this step is that the peptide backbone conformation at residue 181 will be essentially constant, irrespective of whether the side chain at this position is glutamate, lysine, leucine, or valine. The striking result is that lysine, leucine, and valine are all capable from this orientation of making stereochemically feasible specific interactions with atoms of the A·T base pair (Fig. 2*B*). Specifically, the lysine ε-nitrogen atom, which is a hydrogen bond donor, is placed in a position appropriate to make a hydrogen bond to the thymine O-4 atom, which is a hydrogen bond acceptor. In addition, the hydrophobic portion of the lysine side chain (in particular the γ and ε methylene groups) is in a position to make a potentially significant van der Waals interaction with the thymine methyl substituent. Leucine and valine are hydrophobic residues without the capacity to make hydrogen bonds; however, in the arrangement shown, each is located so as to make a van der Waals interaction with the methyl substituent at the thymine position-5 atom (cf. refs. 23 and 24). We point out that the proposed contacts are consistent with the inability (14) of lysine, leucine, and valine at position 181 to interact as tightly with G·C base pairs. In particular, lysine cannot hydrogen bond to the cytosine N-4 atom of G·C, which is a hydrogen bond donor (see Fig. 2*A*; although plausibly the lysine side chain could reach to adjacent base pairs to make alternative contacts). Likewise, leucine and valine, which we propose inter-

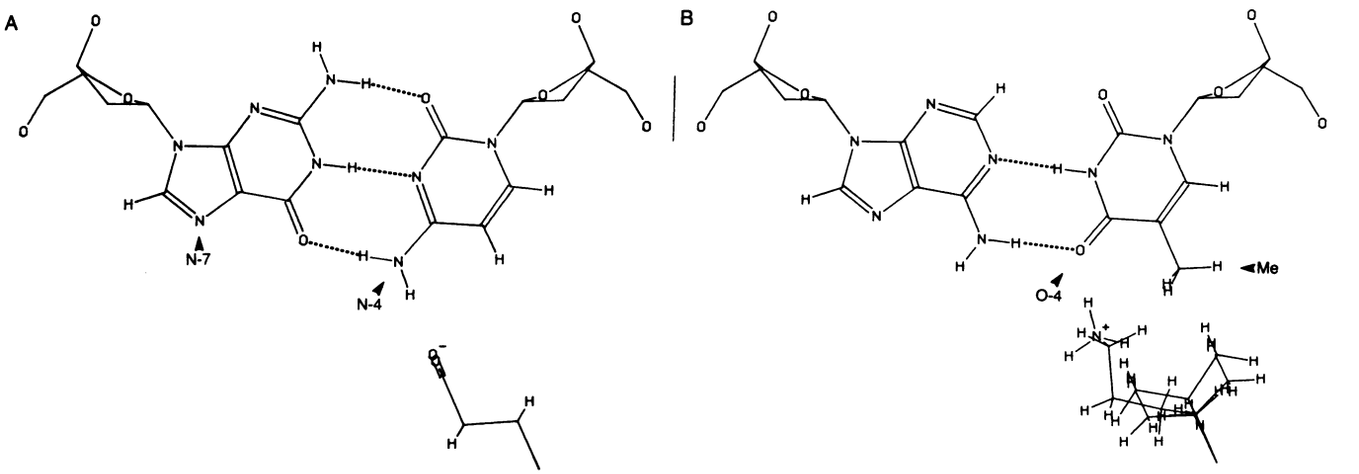

FIG. 2. Proposed contacts between residue 181 of CAP and base pairs 7 and 16 of the recognition site. (*A*) Interaction between Glu-181, present in wild-type CAP, and the G·C nucleotide pair. The minor groove of the nucleotide pair is at the top, the major groove is at the bottom. (*B*) Interaction between lysine, leucine, and valine at position 181 [the side chains present in the three altered-specificity mutants (14)] and the A·T nucleotide. The α- and β-carbon atoms of the three side chains are positioned in exactly the same orientation with respect to the nucleotide pair as are the α and β carbons in *A*.

act with the methyl moiety in A·T, cannot interact comparably with G·C, which contains no hydrophobic substituent.

No other major-groove contact evidently could account for the A·T specificity of *either* lysine, leucine, or valine. In principle, a part of the ability of the substitutions to restore interaction with the A·T-substituted site might be explained simply by the elimination of the bad Glu–A·T interaction. However, for both energetic and structural reasons, it seems unlikely to us that the relief of a bad contact is the most important factor in the observed binding specificity. We therefore propose the following additional rules of specificity: (*i*) lysine at position 181 of CAP hydrogen bonds to the O-4 atom of the base thymine (and may make a van der Waals interaction with the thymine methyl) and (*ii*) leucine and valine at position 181 interact hydrophobically with the 5-methyl moiety of the base thymine.

The analysis in Fig. 2 is supported by the data of the chemical-modification experiments of Majors (16). In particular, he found that at G·C base pair 7 neither (*i*) methylation of the guanine N-7 atom nor (*ii*) *complete removal* of the guanine base affected the affinity for CAP of the recognition site significantly. These results are in sharp contrast to the effects of methylation or depurination at base pairs 5 and 18 and also to the effect of the *lacL8* mutation, which is the G→A substitution at base pair 7. [Under identical experimental conditions these latter treatments abolish detectable specific binding (16).] The data therefore argue that the contact CAP makes to base pair 7 does not involve an essential interaction with the guanine base (16). We note that this result is consistent with our own analysis, which implies that the CAP hydrogen bonds to the cytosine N-4 atom but makes no contact to the guanine (Fig. 2*A*).

Several additional implications of our analysis are worth noting. For example, it is possible to identify other amino acids that in principle could interact from position 181 in a way analogous to those in Fig. 2. Glutamine (using the amide oxygen atom), aspartate, and possibly asparagine could make a contact to the G·C base pair that is similar to that proposed for Glu-181. Amino acids that might be expected from Fig. 2*B* to interact with A·T base pairs include isoleucine, and possibly alanine and threonine. We also suggest from Fig. 2*B* that the lysine-substituted protein should interact more tightly with a synthetic site containing an A·U base pair at position 7 or 16 than with a site containing a G·methyl-C base pair (cf. ref. 23). This prediction follows from our conclusion that Lys-181 interacts with thymine principally through a hydrogen bond to the O-4 atom (which is present also in uracil, but not in methylcytosine) and interacts only in ways of lesser importance with the thymine methyl substituent. In contrast, we expect that the leucine- and valine-substituted proteins should exhibit the opposite pattern of specificity.

Structure of the CAP–DNA Complex

The data in ref. 14 indicate that Glu-181 of CAP makes an *identical* contact to base pair 7 of the recognition site and to the symmetric position, base pair 16 (14). We conclude therefore that CAP exploits structurally the 2-fold symmetry in its recognition sequence. The symmetry of the "contacts" identified in the chemical-protection and -modification experiments (refs. 16 and 17; Fig. 1) provides additional strong support for this conclusion. In principle CAP could exploit the symmetry in its target sequence in one of only two ways.

(*i*) CAP might bind to its target sequence in two alternative symmetry-related orientations, each functional in activating transcription. By this hypothesis, Glu-181 would contact base pair 7 in one orientation and base pair 16 in the other.

(*ii*) The two identical subunits of the CAP dimer might each contact one half of the symmetric recognition site. This hypothesis has been a critical assumption in most attempts to date to model the DNA sequence recognition properties of CAP (refs. 1, 3, 7, 16, and 19; however, see refs. 5 and 6). Our results indicate that, by this hypothesis, Glu-181 of one CAP subunit would contact base pair 7 and Glu-181 of the other subunit would contact base pair 16.

To select between these two hypotheses, we have undertaken to correlate our genetic data to the available structural information. We consider that it is acceptable for this purpose to use the known x-ray diffraction structures of uncomplexed CAP (1, 21) and of right-handed B-type DNA, noting that circular dichroism and unwinding experiments indicate that neither molecule is altered substantially on formation of the CAP–DNA complex (25–27). Using coordinates for the structure of CAP obtained from the data of McKay and Steitz (refs. 1 and 21; see legend to Fig. 3), we conclude that it is not possible to reconcile hypothesis *i* to the structural information. However, we find that we are able to construct a model for the structure of the CAP–DNA complex that does satisfy the requirements of hypothesis *ii*. This model is illustrated in Fig. 3.

To construct the model illustrated, we translated and rotated the structure of CAP with respect to the structure of DNA, so as to place Glu-181 of one CAP subunit in contact with base pair 7 and Glu-181 of the other subunit in contact with base pair 16; we specified in addition that the Glu-181 side chain should be oriented so as to contact the cytosine N-4 atom located at base pairs 7 and 16 (see above). The figure shows that it clearly is possible to do this.

Three key features of the alignment in Fig. 3 are worth noting. First, in the proposed complex, the 2-fold axis of the CAP dimer coincides with the axis of 2-fold symmetry of the recognition sequence. Consequently, every amino acid–DNA contact will be similar or identical on either side of the common 2-fold axis. Second, in the alignment illustrated, the F α-helix of each CAP subunit penetrates the major groove of DNA and it lies tangent to the groove for $\approx$12 Å. Third, the model satisfactorily accounts for the pattern of major-groove and DNA-backbone "contacts" identified in the chemical experiments (refs. 16 and 17; Figs. 1 and 3). In particular, there is an exact correlation between the 10 innermost phosphate positions at which ethylation interferes with CAP binding (16) and the proposed points of CAP-phosphate contact (Fig. 1*B*).

The alignment between the structure of CAP and the structure of DNA illustrated in Fig. 3 was deduced based on the genetic data. It is striking therefore that this alignment is identical in its geometry to a model proposed based on different criteria by Steitz and co-workers (7, 29). The analysis of Steitz *et al.* had two steps. In step 1, the 2-fold axis of the CAP dimer was superimposed on the 2-fold axis of the recognition site (7). In step 2, CAP was rotated about the common 2-fold axis, the final angle of rotation being that judged to give optimal overlap between CAP and DNA electrostatic fields. The resulting model, illustrated in figure 10 of ref. 7, can be superimposed on our Fig. 3*B*. The similarity of the present model of Steitz *et al.* to our own proposal extends even to the contact between Glu-181 of the CAP and the cytosine N-4 atom at base pairs 7 and 16 (ref. 30; however, a different contact is indicated in ref. 29). The excellent correspondence between the genetic approach and that used by Steitz and co-workers, we believe, provides strong support for the assumptions inherent in each. It should be noted that Pabo and Lewis (3) were the first to propose the possibility that the NH_2-terminal ends of the F α-helices in CAP might interact with right-handed B-type DNA. This idea originated by analogy to their model for the structure of the λ repressor–DNA complex (ref. 3; see detailed discussion below). Although these workers did not present a figure of the predicted CAP–DNA interaction, it is apparent from their de-

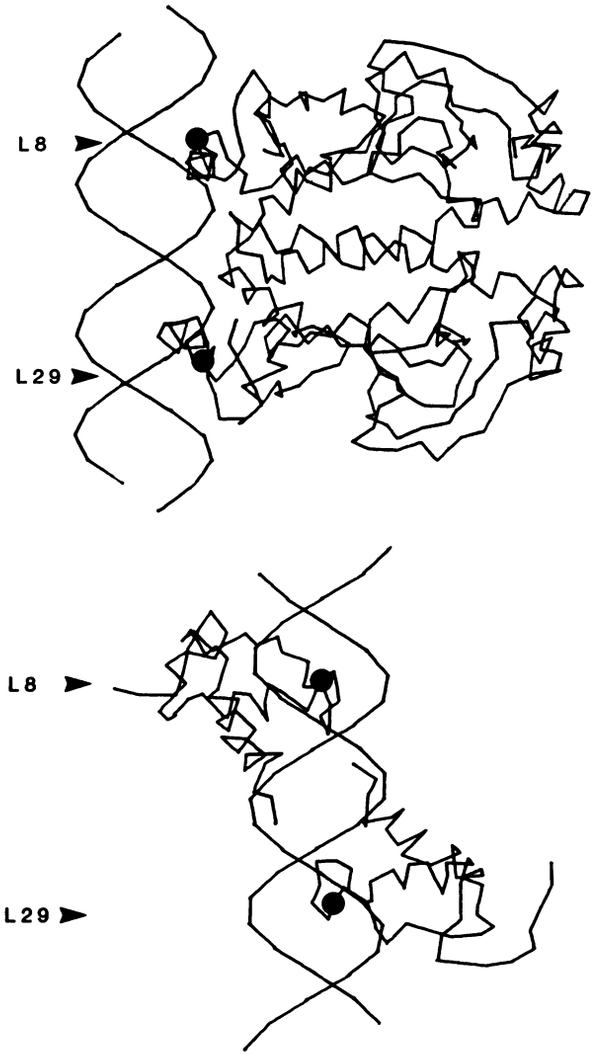

FIG. 3. Proposed structure of the CAP–DNA complex. (*A*) View perpendicular to the 2-fold axis of the CAP dimer. Solid circles identify the α-carbon atom of Glu-181 in each subunit of the CAP dimer. Arrows indicate the position in the DNA site of the *lacL8* and *lacL29* mutations (base pairs 7 and 16). Coordinates for the phosphates of right-handed B-type DNA were obtained from S. Arnott and R. Chandrasekaran (personal communication). Coordinates for the α-carbon atoms of the CAP dimer were extracted from the data of McKay and Steitz (1), using the algorithm of Rossmann and Argos (28); the α-carbon atom of Glu-181 was identified by relating these coordinates to the refined structure as illustrated in ref. 21. (*B*) Same as *A*, but the view is parallel to the 2-fold axis. Amino acids 1–135 of CAP have been omitted for clarity.

scription that their model would be similar or identical to that proposed subsequently in Fig. 3 and by Steitz *et al.* (ref. 7).

We believe that no model without the features shown in Fig. 3 can satisfactorily account for our genetic results. In particular, three published models are *not* compatible with the data: binding to left-handed B-type DNA (ref. 1; I. Weber and T. Steitz, personal communication), single-subunit binding using helix D (5), and single-subunit binding using helix F (6). The model that invokes single-subunit contacts by helix F merits additional comment. This model, unlike the other two, is consistent in principle with an interaction between Glu-181 and either base pair 7 or base pair 16. However, our analysis indicates that, in the resulting CAP–DNA complex, CAP would interact at most with one-half of the recognition site, and therefore this model is not consistent with the factor of 70 reduction in affinity produced by the *lacL8* and *lacL29* mutations (refs. 16 and 31; each of which alters only one-half of the site). We consequently consider this model to be unlikely. Variations in the model in Fig. 3 that would remain consistent with the genetic information apparently include only the following: (*i*) rotation of CAP by $\leq 5°$ about the common 2-fold axis, (*ii*) small alterations in CAP conformation, and (*iii*) certain alterations in DNA structure—for example, bending, kinking, or local heterogeneities in twist.

The model shown in Fig. 3 also appears to rule out the hypothesis (32) that CAP-bound cAMP interacts directly with a DNA base pair in the recognition site. Although the results of crystallographic (21) and solution (33, 34) experiments conflict regarding the glycosidic conformation of CAP-bound cAMP, both types of experiment agree that the cAMP binding site is located in a β-roll structure comprised of amino acids 18–97 of CAP (21, 34, 35). In the alignment illustrated, the cAMP binding site is seen to be a minimum of 25 Å from the DNA, thereby precluding a cAMP–DNA contact. We have made attempts to identify alternative alignments of CAP to DNA that could satisfy the genetic data while placing cAMP in contact with DNA, but we find none; we therefore conclude that the conjectured cAMP–DNA interaction (32) does not take place.

One other important implication of having defined the detailed structure of the CAP–DNA complex is that we are able to infer from it additional likely points of amino acid–DNA contact. This was done by projecting a standard α-helix, with side chains, onto the α-carbon positions of helix F as aligned to DNA in Fig. 3. The side chain of Glu-181 was positioned to make a hydrogen bond to the cytosine N-4 atom of base pairs 7 and 16, and the potential interactions by adjacent side chains were investigated. Our two strongest predictions follow:

(*i*) Arg-180 (residue 1 of helix F) contacts the G·C base pair at positions 5 and 18 of the recognition site. We predict that the chemistry involved in this contact is a bidentate hydrogen-bonded interaction between the guanidinium side chain of arginine and the N-7 and O^6 atoms of the base guanine (cf. refs. 8 and 36).

(*ii*) Arg-185 (residue 6 of helix F) contacts the A·T base pair at positions 8 and 15 in the site. In principle, the interaction could involve a hydrogen bond by the arginine side chain to either the thymine O-4 or the adenine N-7 atom. However, based on the data of (*a*) the bromouracil/UV-irradiation experiment (ref. 17; Fig. 1*A*) and of (*b*) depurination at positions 8 and 15 (ref. 16; no effect on affinity), we currently favor the idea that the contact made by Arg-185 is to the thymine O-4 atom.

Several other parts of CAP are likely to contact DNA (in these cases, principally the DNA-phosphate backbone): These include (*a*) the turn between helices E and F, (*b*) the NH_2-terminus of helix E, (*c*) the turn between helices C and D, and (*d*) a short sequence near the COOH-terminus of the CAP. These latter contacts are readily apparent in Fig. 3. We note that Weber and Steitz (29, 30) have independently reached similar (but not identical) conclusions from their analysis.

Other Proteins

It has been pointed out that α-helices E and F of CAP comprise a helix-turn-helix motif that is *identical* in its peptide-backbone conformation to a structural feature contained in the λ repressor and cro (9, 10). In addition, CAP helices E and F exhibit amino acid sequence homology to the implicated regions of the λ repressor and cro and also to regions of a variety of DNA-binding proteins whose three-dimensional structures are not yet known, including the Lac repressor

(11–13). Based on these considerations, it has been speculated that the helix-turn-helix motif is a highly conserved structural feature that mediates the sequence-specific interactions to DNA of CAP and other proteins that possess it (9, 11–13). Our results regarding the role of Glu-181, the second residue of α-helix F of CAP, provide strong experimental support for this hypothesis (14).

Models in which the helix-turn-helix motif interacts with DNA have been proposed by Matthews and co-workers for cro (2, 8) and by Pabo, Lewis, and co-workers for the λ repressor, and these models are in agreement with the existing genetic and chemical data for the two proteins (refs. 21, 37, and 38; A. Pakula and R. Sauer, personal communication). We note here that the proposed model for the λ repressor–DNA interaction (3, 24) appears to be extremely similar to that illustrated in Fig. 3 for CAP, both in its geometry and also in its details. The model for cro also exhibits similarities, but in this case the proposed tilt of the helix F homolog is greater, thereby resulting in a nearly parallel orientation of this helix with respect to the major groove (2, 8, 10). It should be mentioned, however, that such differences in detail involve untested assumptions regarding the precise conformation of both the protein and the target DNA site in the complex.

In the absence of additional experimental information, it is interesting to speculate that the λ repressor, cro, and other proteins that contain the helix-turn-helix motif might use it in a way that is identical to the way CAP uses helices E and F. In particular, the λ repressor might make contacts to DNA such that the α- and β-carbon atoms of amino acid 2 of the helix F homolog have the same spatial relationship to a DNA base pair as do the α- and β-carbon atoms of amino acid 181 of CAP. It therefore follows that the chemical rules deduced in this report for amino acid 181 of CAP in principle may also apply to the chemical interactions at residue 2 of the other proteins. An analogous "code" might also exist at other points of helix F–DNA contact: for example, residue 1 of helix F (amino acid 180). The sequences of 11 proteins that contain the helix-turn-helix motif, and of the DNA sequences of the respective recognition sites (to be presented elsewhere), are consistent with this hypothesis.

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