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Recognition helices of *lac* and λ repressor are oriented in opposite directions and recognize similar DNA sequences

(protein–DNA recognition/*Escherichia coli*)

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ABSTRACT **Exchanges in positions 1 and 2 of the putative recognition helix allow *lac* repressor to bind to ideal *lac* operator variants in which base pair 4 has been replaced. We show here that an Arg-22 → Asn exchange in position 6 of the putative recognition helix of *lac* repressor abolishes *lac* repressor binding to ideal *lac* operator. This *lac* repressor variant, however, binds to a variant of the ideal *lac* operator 5′ TT-TGAGCGCTCAAA 3′ in which the original G·C of position 6 has been replaced by T·A. This result and our previous data confirm our suggestion that the N terminus of the recognition helix of *lac* repressor enters the major groove close to the center of symmetry of *lac* operator and that its C terminus leaves the major groove further away from the center of symmetry. The consequences of this model are discussed in regard to various phage and bacterial repressor operator systems.**

Twenty-seven years ago *lac* and λ repressor became the paradigms of negative control (1). They were isolated at about the same time (2, 3), and soon after, it was shown that they both bound to their operator DNA specifically (4, 5). For a while it seemed that the *lac* system was easier to analyze. Overproducers of *lac* repressor led to the isolation of large amounts of this protein (6). This allowed protein sequence analysis of *lac* repressor (7). Genetic analysis identified the N-terminal domain of *lac* repressor as a small DNA and operator binding protrusion (8). The core of *lac* repressor was shown to be responsible for aggregation and inducer binding (9, 10). Active fusions between *lac* repressor and β-galactosidase (11) led to the conclusion that although *lac* repressor is tetrameric a dimer suffices for *lac* operator recognition (12). *lac* operator (13, 14) and various *lac* operator mutants (15) were sequenced early and protection experiments outlined the active parts of *lac* operator (15).

Yet, *lac* repressor did not yield suitable crystals for x-ray analysis. The x-ray structures of λcro protein (16), of *434* repressor–operator complex (17), of cap protein (18), and *trp* repressor (19) gave these systems a tremendous advantage. In particular, the λ, *P22*, and *434* systems became well understood through the combined use of x-ray data and reverse genetics. An outstanding review of the phage repressor work can be found in the recent book of Ptashne (20).

We have recently reported an analysis (21) that may bring *lac* repressor back into focus. We have set up a system that allows detection and selection of specificity changes in the *lac* repressor–operator complex. The system consists of two plasmids that can coexist in an *Escherichia coli* strain carrying a *lac* deletion. The plasmids have different sizes resistance genes and origins of replication. One carries a semisynthetic *lacI* gene. In its 5′ end, which encodes the operator binding domain, short sequences can be replaced by short synthetic DNA double strands. The other plasmid contains a *lac* operon in which the *lac* operator has been deleted and replaced by a unique restriction site. Into this unique restriction site we cloned all possible symmetric variants of symmetric *lac* operator (22, 23). We could demonstrate that symmetric *lac* operator was indeed the best possible ideal operator for *lac* repressor (21).

We looked for strong specificity changes in the *lac* system (21) in which wild-type *lac* repressor would bind less well to mutant operator and mutant repressor would bind better to mutant than to ideal *lac* operator. We used two approaches to isolate such mutants. In both, we assumed the sequence homology in the DNA binding domain of *lac* repressor and the other repressors (for review, see ref. 24) to extend to a homology in tertiary structure, an assumption supported by NMR data (25). For the first approach, we screened repressor–operator systems that looked rather similar to the *lac* system. Then we introduced the remaining small differences into the putative recognition helix of *lac* repressor and into *lac* operator. In the other approach, we constructed small banks that carried, for example, all possible exchanges in residues 1 and 2 of the recognition helix and introduced these banks into hosts carrying plasmids with *lac* operator variants. Both approaches gave positive results. We found replacements in positions 1 and 2 of the recognition helix of *lac* repressor, which permit binding to two particular variants of base pair 4 of *lac* operator (21).

These results did not allow us to orient the putative recognition helix of *lac* repressor. We noticed, however, that the orientation determined for the various phage repressors would impose some strain on the interpretation of the data of the *lac* system. The methylation protection experiments (26), for example, could not be easily explained. We thus proposed that the recognition helix of *lac* repressor may be oriented in the opposite direction to that of λ and other phage repressors (21). This had already been suggested in an NMR study for the complex between *lac* repressor head piece and a *lac* operator fragment (25). Here we present *in vivo* and *in vitro* evidence for this proposition.

MATERIALS AND METHODS

Media, Plasmids, Strains, and General Methods. Media, plasmids, strains, and general methods were as described (21). *E. coli* strain DC41-2 was used for the repression tests. It has the genotype *(lac pro)ΔgalE smR recA*. β-Galactosidase was determined as described (27).

***In Vivo* Footprinting.** Methylation protection was performed essentially as described (28). Cells were grown exponentially at 37°C in 50 ml of minimal medium to a cell density of 2×10^8 cells per ml. Dimethyl sulfate (100 μl) was added, and the cells were aerated for 30 sec at 37°C and then poured on ice. Then, 5 ml of 0.5 M EDTA (pH 8.0) was added. Plasmids were prepared, *Hin*dIII/*Cla* I-digested, and end-

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labeled with [α-^{32}P]dATP, and the corresponding band was eluted from a 6% preparative polyacrylamide gel. After reaction with piperidine, the DNA was analyzed on a 10% polyacrylamide sequencing gel (29).

Retardation Gels. DNAs of the various pWB300 derivatives (21) were digested with *Hin*dIII, end-labeled, and purified from a polyacrylamide gel. The *lac* repressor variant His-1–Asn-6 was purified according to ref. 30. A 520-base-pair fragment of each pWB300 derivative carrying its respective *lac* operator was used for retardation. The *Hin*dIII fragment (0.2 ng) was incubated with 30 ng of His-1–Asn-6 *lac* repressor variant (or 30 ng of wild-type *lac* repressor) in 15-μl samples of binding buffer (10 mM $MgAc_2$/10 mM KCl/0.1 mM EDTA/10 mM Tris·HCl, pH 8/0.1 mM dithiothreitol/50 μg of bovine serum albumin per ml) as described (31). Each sample of repressor was diluted 1:100 in binding buffer before incubation. After 20 min at room temperature, 5 μl of 15% Ficoll in binding buffer with 0.06% bromphenol blue and 0.06% xylene cyanol were added. A 4% polyacrylamide gel was prerun at 12 V/cm for 1 hr. Electrophoresis of the samples was performed for 1 hr. After that, the gel was dried and autoradiographed at −70°C on Kodak X-Omat AR film.

RESULTS

Our previous success in changing the specificity of *lac* repressor depended on the fact that we had found two repressor and operator systems that differed slightly from the *lac* repressor operator system (21). The essential differences between the operators of the *gal*, *deo*, and *lac* systems seemed to be limited to 1 base pair in each operator half site. The essential differences between their putative recognition helices seemed to occur in residues 1 and 2. When we analyzed the corresponding synthetic variants of *lac* repressor (Val-1–Ala-2 and Gln-1–Met-2 in the recognition helix) and the variants of *lac* operator (A·T and T·A replacements in base pair 4) we found that they indeed bound and recognized each other specifically (Fig. 1). Since our success was limited to 1 base pair (no. 4) of *lac* operator and two residues (nos. 1 and 2) of the recognition helix of *lac* repressor, we were unable to use these results to orient the recognition helix. To do so, we needed an exchange further down in the recognition helix that would recognize a *lac* operator variant with an exchange either closer to or further away from the center of symmetry.

Since we could not find a suitable candidate among bacterial repressor–operator systems, we looked at phage repressor–operator systems. If our prediction about the orientation were correct, the *434*, *P22*, and *16-3* repressor systems (32–36) looked interesting. In these repressors, a Gln or Asn residue is found in position 6 of the recognition helix. The phage operator regions recognized by their recognition helices differ from the corresponding *lac* operator region by a G·C to T·A exchange in the position of base pair 6 (see Fig. 4). Thus, we synthesized the *lacI* gene variants encoding Asn-6 or Gln-6 in the recognition helix and tested their binding to *lac* operator variant 64 (Fig. 1). We found that the Asn-6 *lac* repressor variant indeed repressed exclusively the operator variant of plasmid 364 but not the ideal *lac* operator or any other *lac* operator variant (Fig. 1). The Gln-6 variant on the contrary did not repress any of our *lac* operator variants (data not shown).

Next we tried to see whether the Asn-6 *lac* repressor variant could bind *in vitro* to *lac* operator variant 64. We first did an *in vivo* footprint experiment (Fig. 2). Wild-type *lac* repressor completely protects G-4 and G-6 of ideal *lac* operator. The Asn-6 variant did not protect ideal *lac* operator at all. On the other hand, wild-type *lac* repressor did not protect G-4 of operator variant 64 (in which G-6 is replaced by T), while the Asn-6 variant did (Fig. 2).

Then we asked whether we could combine the previously obtained Val-1–Ala-2 or Gln-1–Met-2 or His-1 variants with the new variant Asn-6 and whether these repressor mutants would repress the corresponding double operator variants. Fig. 1 shows the *in vivo* results. It indicates that indeed these two specificity changes can be combined. To determine whether these effects can also be seen *in vitro*, we did gel retardation experiments. As expected, the double *lac* repressor variant His-1–Asn-6 binds to *lac* operator variant 64 and

		Repression by variant of lac Repressor-tetramer							
pWB	Operator-sequence	wt	V_1A_2	Q_1M_2	H_1	N_6	H_1N_6	$Q_1M_2N_6$	$V_1A_2N_6$
	987654321								
310	AATTGTGAGC GCTCACAATT	≥200	20	60	≥200	1	6	1	1
332	AATTGTGCGC GCGCACAATT	5	1	1	3	1	1	1	1
333	G C	6	1	1	1	1	1	1	1
334	T A	4	1	1	2	1	1	1	1
341	AATTGTAAGC GCTTACAATT	14	≥200	2	2	1	1	1	1
342	C G	12	2	1	1	1	1	1	1
344	T A	7	11	100	≥200	1	1	1	1
351	AATTGAGAGC GCTCTCAATT	28	25	25	23	1	1	1	1
352	C G	15	2	10	3	1	1	1	2
353	G C	10	14	28	7	1	1	1	1
361	AATTATGAGC GCTCATAATT	6	1	1	2	1	10	1	1
362	C G	4	1	1	1	1	5	1	1
364	T A	8	1	1	4	100	≥200	2	3
371	AATAGTGAGC GCTCACTATT	11	2	2	10	2	2	2	2
372	C G	20	1	1	11	1	1	1	1
373	G C	40	1	2	28	1	2	1	1
34164	AATTTTAAGC GCTTAAAATT	4	1	1	1	1	1	1	100
34464	AATTTTTAGC GCTAAAAATT	4	1	1	4	1	≥200	25	2

FIG. 1. Repression of *lac* operator variants by *lac* repressor variants. *lac* repressor and its variants are encoded on the plasmid pWB1000, which carries the gene for ampicillin resistance and the origin of replication from pBR322 (details in ref. 21). The plasmid pWB300 with the origin of replication from pACYC184 and the gene for tetracycline resistance carries the *lacZ* gene under the control of the different operator variants (21). The *lac* repressor variants are named after the amino acid exchanges they carry in the recognition helix. The standard one-letter code is used. The numbers indicate the positions in the recognition helix. Tyr-17, Gln-18, Arg-22 of wild-type (wt) *lac* repressor are residues 1, 2, or 6 of the recognition helix. Repression is defined as specific activity of β-galactosidase in the presence of the mutant *lac* repressor Δ1 (codons 14–60 of *lacI* are deleted) divided by the specific activity of β-galactosidase in the presence of the respective *lac* repressor variants.

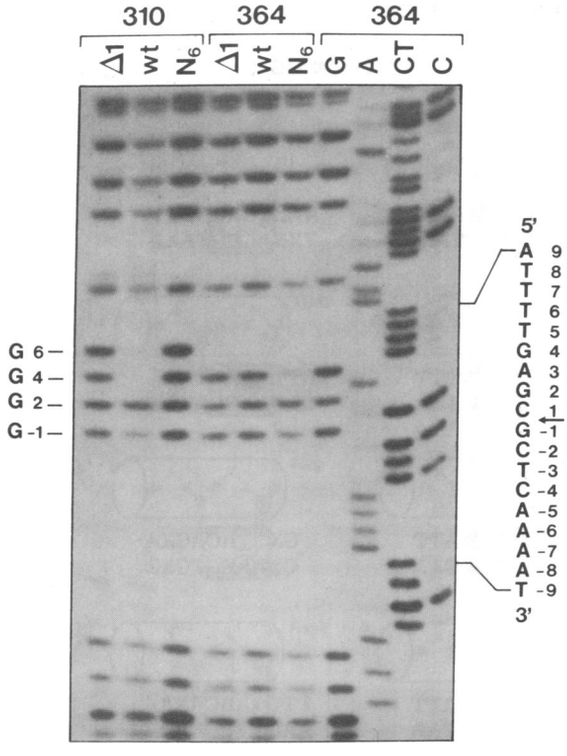

FIG. 2. *In vivo* methylation protection of ideal *lac* operator and *lac* operator variant 64 by wild-type (wt) *lac* repressor and *lac* repressor variant N-6. The wt *lac* repressor protects G-4 and G-6 of ideal *lac* operator (from plasmid 310) but not G-4 of *lac* operator variant 64 (see Fig. 1 for explanation). Substitution of arginine in position 6 of the recognition helix by asparagine changes the specificity of binding and protection. *lac* repressor variant N-6 binds and protects G-4 of *lac* operator variant 64 but shows no affinity to ideal *lac* operator. As a control serves the *lacI* gene deletion mutant Δ1 (codons 14–60 of *lacI* are deleted). The DNA sequence analysis of *lac* operator variant 64 was performed according to ref. 29, and the *in vivo* methylation protection was performed according to ref. 28.

to the *lac* operator variant with the double exchange 44-64 (Fig. 3).

We emphasize the fact that all our *lac* repressor variants were tetramers (Fig. 1). Wild-type *lac* repressor is indeed known to be a tetramer (2). Recently, however, we found that the *lacI* (repressor) gene cloned in plasmids pWB100 produces a functioning *lac* repressor dimer. Its *I* gene has accidentally acquired a frameshift mutation in codon 330. Thus, all *lac* repressor variants and the wild type analyzed in

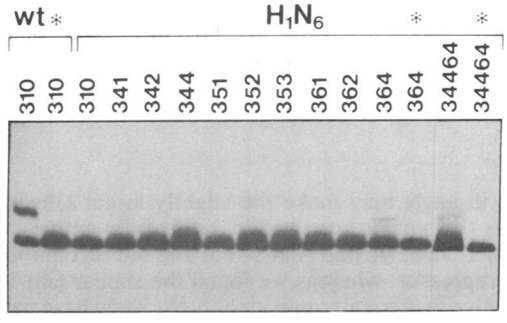

FIG. 3. Specific gel retardation of DNA fragments carrying the *lac* operator variants 64 or 44-64. The *lacI* gene variant encoding the *lac* repressor variant His-1–Asn-6 was recloned in plasmid pWBP4 carrying a strong synthetic promoter. The *lac* repressor variant was purified according to ref. 30. Nomenclature of *lac* operator variants is as in Fig. 1. Asterisks indicate the presence of 10^{-3} M isopropyl β-D-thiogalactoside as inducer.

ref. 21 were actually active dimers. Here we have repaired this defect. Comparison between the repression values obtained with dimeric and tetrameric *lac* repressor and all variants so far tested indicates that they never differ by more than a factor of 2, with the tetrameric repressor being the more effective. The properties of the active dimer of *lac* repressor will be reported elsewhere.

DISCUSSION

The assumption that there may be a code that governs the specific interactions between the amino acid side chains of an α-helix with the bases in the deep groove of B-DNA (8) has been almost abandoned in the last few years. First, the structure of B-DNA was shown to be sequence dependent (for example, see refs. 37 and 38); second, the recognition α-helices were shown to enter the deep grooves at widely different angles (see discussion in ref. 24); third, only a few specificity changes have so far been reported (20, 39, 40), some of which are completely uninterpretable (40); moreover, attempts to change the specificity have often failed. The *lac* system now provides a means to study such specificity changes.

The absence of any x-ray data in the *lac* system may at first seem to be a tremendous disadvantage. We think, however, that successful specificity changes of the type we report not only make up for the lack of x-ray data but even give insights into the interaction that a low-resolution x-ray analysis would not yield. We have found the *in vivo* repression values to be reliable measures for the binding constants of the repressor–operator complexes. Wherever we have controlled the *in vivo* data by *in vitro* measurements, such as filter binding assays, methylation protection tests, and gel retardation assays (ref. 21 and this study), we have found them to be qualitatively or quantitatively accurate.

Our conclusions are based on the proposition that a base pair and an amino acid interact directly, when the exchange of a single residue of the recognition helix leads to the better recognition of an operator variant differing from ideal *lac* operator in a single base pair at each half side. We concluded previously (21) that residues 1 and 2 of the recognition helix of *lac* repressor interact with base pair 4 of ideal *lac* operator. We report here that an exchange of residue 6 of the recognition helix of *lac* repressor leads to better recognition of a variant of ideal *lac* operator with an exchange in base pair 6. Thus, we conclude that residue 6 of the recognition helix of *lac* repressor interacts with base pair 6 of ideal *lac* operator. This conclusion is reinforced by model building, which excludes more complicated interactions. Alanine in position 2 (Fig. 1) and asparagine in position 6, for example, are too small to possibly reach across 2 base pairs.

We conclude from these experiments that the recognition helix of *lac* repressor is oriented in opposite direction compared to the recognition helix of the various phage repressor systems for which x-ray data exist (Fig. 4). Our *in vivo* data are supported by Kaptein's NMR data (25), which indicate that the head piece of *lac* repressor binds in a similar manner to a half *lac* operator fragment *in vitro*.

Furthermore, our experiments suggest not only the orientation but also a particular alignment of the recognition helix with the basis of the major groove. If we compare the alignment reported for the complex between λ consensus operator and λ repressor or λcro protein, we observe the already reported (21) fact that *lac* and λ repressor are apparently able to recognize the same bases in the major groove of B-DNA (Fig. 4). Whereas the recognition helix of *lac* repressor supports Gln-1–Ser-2 without too much loss of binding capacity to ideal *lac* operator (21) Lys or Ala, the residues found in λ repressor or cro protein in position 6 give rise to *lac* repressor variants that are inactive with any *lac*

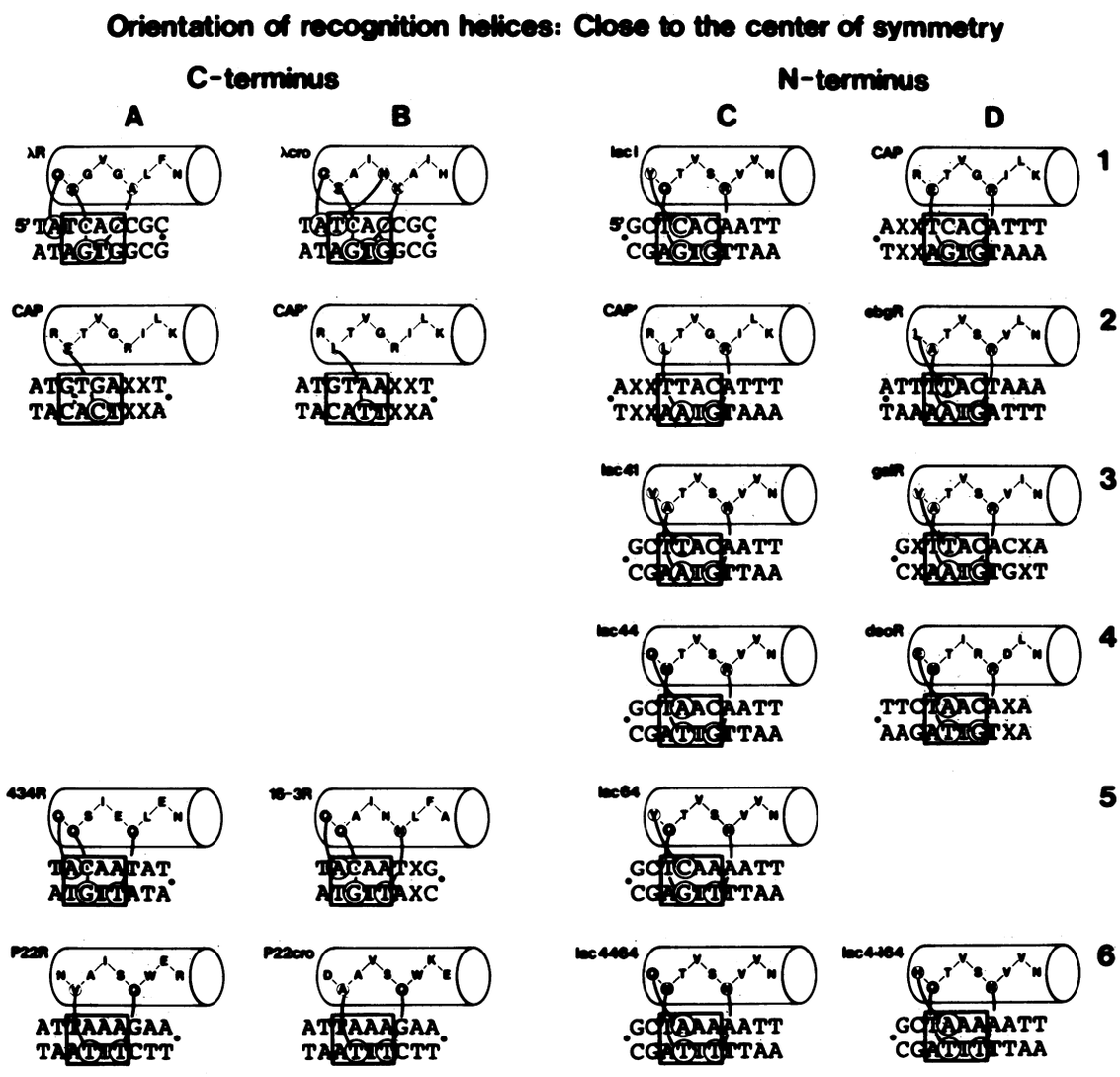

FIG. 4. Models of possible interactions between the recognition helices of various repressor proteins and their operators (targets). (*A1*) λ repressor, Ptashne model (41). (*B1*) λcro protein, Ptashne model (42, 43). (*C1*) *lac* repressor, our model (7, 44). (*D1*) cap protein, our model (45, 46). (*A2* and *B2*) cap protein, Ebright–Steitz model (18, 47, 48, 49). (*C2*) *cap* variant, our model (47, 48). (*D2*) *ebg* repressor, our model (50). (*C3*) *lac* repressor variant V1A2, our model. (*D3*) *gal* repressor, our model (51, 52). (*C4*) *lac* repressor variant 44, our model. (*D4*) *deo* repressor, our model (53). (*A5*) *434* repressor, Anderson–Harrison–Ptashne model (17). (*B5*) *16-3* repressor, our model (36). (*C5*) *lac* repressor variant N-6, our model. (*A6*) *P22* repressor, Ptashne model (20, 35). (*B6*) P22cro protein, Ptashne model (20, 33). (*C6* and *D6*) *lac* repressor variants Q1M2N6 and H1N6, our model. In the case of the phage repressors and of the cap protein, according to the Ebright–Steitz model, the left half of the corresponding operators is shown. For the bacterial repressors, their variants, and the cap protein (our model), the right half of the operators is shown. A dot indicates the center of symmetry of the operator (target). Protein and DNA sequences are written in the conventional way from N to C terminus and from 5′ to 3′, respectively. The standard single-letter amino acid code is used. We neglected that the angles between the DNA helices and the protein α-helices differ vastly in the particular systems. Circled bases indicate direct contact with the residue of the recognition helix established by x-ray analysis (*434R*), by mutants (λ repressor, λcro protein, CAP protein, *lac* repressor) or by homology. The boxed sequences indicate the sequence homologies between the various operators. Note that residue 2 of the recognition helix always points to the same position in the box (base pair 4 of *lac* operator), whereas residue 6 may recognize 2 base pairs (base pairs 5 and 6 of *lac* operator).

operator variant including ideal *lac* operator (data not shown). We assume that the angle of the recognition helices of λ and *lac* repressor may be sufficiently different to explain this result.

That Asn in position 6 of the recognition helix of *lac* repressor interacts with a T·A in position 6 of *lac* operator was predicted from the presumed structure (Fig. 4*A6*) of the complex between the recognition helix and operator of the phage 16-3 repressor system (36). That the Gln found in the same position of *434* repressor, *P22* repressor, and *P22 cro* (Fig. 4 *A5*, *A6*, and *B6*) is inactive in the context of the recognition helix of *lac* repressor supports the idea that a change in angle may make the slightly larger Gln inactive, whereas the similar but smaller Asn is fully active.

Furthermore, we find Asn-1–Val-2 in the recognition helix of *P22* repressor, whereas we found the similar Gln-1–Met-2 to be active to recognize presumably the same base pair in the *deo* system and our corresponding *lac* repressor variant. We tested whether the Asn-1–Val-2 is active too in the *lac* repressor context. We found no binding of Asn-1–Val-2 *lac* repressor to any *lac* operator variant (data not shown). Accurate spacing of the parts of the complex seems again to be absolutely important.

Finally, an inspection of the proposed complex between

cap protein and the cap site (18, 47) suggests the possibility of an alternative alignment (Fig. 4 *A2* and *D1*). The mutant analysis of Ebright *et al.* (39) does not give a clue to the orientation of the recognition helix in the *cap* system. We are unable to say whether the model is conclusive in this respect (18). If, however, we orient the *cap* recognition helix the other way around (Fig. 4*D1*), we see that the structure of this complex is similar to the *lac* complex. Whereas the recognition helix of *lac* repressor begins with Tyr-1–Gln-2, the recognition helix of the cap protein begins with Arg-1–Glu-2. We propose that the positive and negative charges of Arg-1–Glu-2 neutralize each other so that the Glu-2 in this context resembles a Gln-2. Arg-1 *lac* repressor represses ideal *lac* operator quite well (data not shown). Moreover, residues 5 and 6 of the recognition helices of *cap* and *lac* repressor are the same. The DNA sequence recognized by the so oriented *cap* recognition helix is the same as the DNA sequence recognized by the *lac* recognition helix. Preliminary model building suggests that such a complex is possible without much distortion of the DNA (Irene Weber, personal communication).

Assuming that our model is correct, the question that arises is whether it is just a coincidence that the best analyzed bacterial operators (λ, *434*, *P22*, *lac*, *gal*, *deo*, and *cap*) are all variants of a very few DNA sequences as has been suggested (54). Is an α-helix actually that limited in the proper recognition of the deep grooves of B-DNA? Is the difference in specificity of protein–DNA recognition obtained just by different orientations, different spacing from the center of symmetry, and combinations of such very few fundamental sequences? We think, as we proposed before (8), that our results point toward rules (a code) governing protein DNA recognition of protein α-helices embedded in the deep groove of B-DNA.

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