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lac repressor mutants with double or triple exchanges in the recognition helix bind specifically to *lac* operator variants with multiple exchanges

Jürgen Sartorius, Norbert Lehming, Brigitte Kisters, Brigitte von Wilcken-Bergmann and Benno Müller-Hill

Institut für Genetik der Universität zu Köln, Weyertal 121, D5000 Köln 41, FRG

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Several *lac* repressor mutants have been isolated which repress β-galactosidase synthesis in *Escherichia coli* up to 200-fold. They do so by binding specifically to particular symmetrical *lac* O^c operator variants. The mutations in the *lac* repressor are localized in two separate parts of the recognition helix comprising (i) residues 1 and 2 which interact with base pairs 4 and 5 of *lac* operator and (ii) residue 6 which recognizes operator base pair 6. Mutations of residues 1 and 2 may be combined with a mutation of residue 6. The resulting mutant protein binds specifically to an operator variant with three symmetric exchanges in base pairs 4, 5 and 6.

Key words: lactose system/*lac* repressor/recognition helix/protein DNA recognition/*E.coli*

Introduction

Proteins which regulate transcription have to recognize specific DNA sequences in order to produce specific effects upon distinct genes. Sequence-specific recognition may be realized by α-helices interacting with specific structures in the major groove of B-DNA. Such α-helices are generally part of a so-called helix-turn-helix motif. The first evidence for this structure came from the X-ray analysis of the dimeric *Lambda cro* protein (Ohlendorf *et al.*, 1982). Here two helix-turn-helix motifs, each provided by one monomer, are spaced such that the antiparallel recognition helices fit into two adjacent major grooves on the same side of B-DNA. Similar structures have been predicted to occur in many other DNA binding proteins (reviewed in Pabo and Sauer, 1984). X-ray analysis of *434 cI* repressor/operator co-crystals proved that its recognition helices indeed recognize details of the major groove of *434* operator (Anderson *et al.*, 1987). On the other hand *trp* repressor/operator co-crystals yielded no evidence for specific contacts between amino acid side chains of the recognition helix and base pairs of the operator (Otwinowsky *et al.*, 1988).

lac repressor also possesses a helix-turn-helix motif (Matthews *et al.*, 1982). So far no crystals suitable for X-ray analysis are available. NMR data obtained from a complex of *lac* repressor headpiece and a fragment of *lac* operator suggested that the orientation of the recognition helix of *lac* repressor in the major groove of its operator is opposite to the direction reported for *Lambda* and other phage repressors (Boelens *et al.*, 1987). We recently presented *in vivo* and *in vitro* evidence supporting this model (Lehming *et al.*, 1988).

During the past five years several changes of the specific recognition between helix-turn-helix motifs and their targets have been reported. In the *cap* protein (Ebright *et al.*, 1984), the *434 cI* repressor (Wharton and Ptashne, 1987) and the *trp* repressor (Bass *et al.*, 1988) mutants have been found which no longer recognize their natural targets but which bind to variant targets not recognized by the wild-type proteins. These mutants carry amino acid exchanges in their recognition helices. In all three systems the specificity has not just been broadened but actually altered by such manipulations.

Similarly, we have mutationally altered the specificity of the *lac* repressor–*lac* operator interaction such that mutant repressors bind better to variant than to ideal *lac* operator (Lehming *et al.*, 1987, 1988). Wild-type *lac* repressor binds less well to all of these variant operators. We have established a system for the detection and selection of such *lac* repressor mutants with altered specificities. It consists of two plasmids with different origins of replication and different antibiotic resistance genes which permit their co-existence in one bacterial cell carrying a deletion of the *lac* system. One of the plasmids carries the *lacZ* gene as an indicator for repression. *lac* operator has been deleted and a unique *Xba*I restriction site has been introduced in its place 3′ of the promoter. Ideal symmetrical *lac* operator (Sadler *et al.*, 1983; Simons *et al.*, 1984) or symmetrical operator variants have been introduced into the *Xba*I restriction site (Lehming *et al.*, 1987, 1988). Within limits repression can be regarded as proportional to the binding constant of the repressor–operator complex. The second plasmid carries a *lacI* gene in which the 5′ end encoding wild-type headpiece has been replaced by synthetic DNA saturated with unique restriction sites (Lehming *et al.*, 1987). Thus small segments of DNA of the *lacI* gene can be exchanged easily in order to test the effect of distinct amino acid exchanges of *lac* repressor on the repression of the *lacZ* gene. Likewise small libraries can be constructed coding for variable amino acids at distinct positions of the recognition helix of *lac* repressor.

In previous experiments we found several mutants of *lac* repressor with exchanges in positions 1 and 2 of the recognition helix which specifically recognize *lac* operator variants with different base pairs in position 4 (Lehming *et al.*, 1987). We note here that we number the base pairs of all operators in the same systematic manner: the central base pair, if it exists, is base pair 0, the next to the right or left is base pair 1 and so on. One of these mutants, the His1 repressor, is a *lac* repressor which carries a histidine in position 1 of the recognition helix instead of the wild-type tyrosine (Tyr17). It was selected from a *lac* repressor library with a limited number of random exchanges in all codons of the recognition helix. The other mutants were constructed

so as to be analogous in part to other repressor–operator systems. Thus the *lac* repressor mutant Val1-Ala2 is similar to *gal* repressor (von Wilcken-Bergmann and Müller-Hill, 1982) and binds specifically to the *lac* operator variant 341 which like *gal* operator has an A–T base pair in position 4 (Adhya and Miller, 1979; Lehming *et al.*, 1987). Furthermore *lac* repressor mutant Gln1-Met2 resembles *deo* repressor and recognizes specifically *lac* operator variant 344 which resembles the *deo* operator in that it has a T–A base pair in position 4 (Valentin-Hansen *et al.*, 1985; Lehming *et al.*, 1987). Finally *lac* repressor mutant Asn6 was designed to be analogous to phage *16-3* repressor (Dallmann *et al.*, 1987) and shown to recognize the *lac* operator variant 364 which has a T–A pair in position 6 (Lehming *et al.*, 1988).

Here we present evidence that a variety of different amino acids introduced into positions 1 and 2 of the *lac* repressor recognition helix will recognize *lac* operator variants with different base pairs at positions 4 and 5. Furthermore we show that the amino acids in positions 1 and 2 and in position 6 of the *lac* repressor recognition helix act independently in determining the correct recognition of *lac* operator. If we combine mutants at positions 1 and 2 of the recognition helix with a mutant at position 6, the resulting mutants with triple amino acid exchanges specifically recognize operators with predicted double or triple base pair exchanges.

Results

Residues 1 and 2 of the recognition helix of lac repressor interact with base pairs 4 and 5 of lac operator

We constructed a library of ampicillin-resistant plasmids coding for *lac* repressor with random amino acids inserted at positions 1 and 2 of the recognition helix. We used this library to search for further *lac* repressor mutants which could recognize *lac* operator variants with exchanges in position 4. This library was introduced into *Escherichia coli* DH 5α carrying a *lac* deletion and harbouring plasmid pWB 300. pWB 300, a derivative of pACYC 184, confers tetracycline resistance and expresses β-galactosidase under the control of one of the constitutive operator variants 341, 342 or 344 (see Table I for the numbering system).

Cells carrying the two plasmids were screened on minimal agar plates containing ampicillin, tetracycline and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal). *lac* repressor mutants which bind to the respective *lac* operator variants repress the expression of β-galactosidase. Such colonies appear lighter blue on these indicator plates when compared to those in which β-galactosidase is expressed constitutively. The lightest blue colonies were isolated and purified. In each case the capacity of the repressor mutant to down-regulate β-galactosidase expression was measured. In addition, the headpiece encoding regions of the mutant *lacI* genes were sequenced. The results are shown in Table I.

We screened 5000 colonies each carrying one of the operator plasmids 341, 342 or 344. In the case of operator 341 which carries an A–T base pair in position 4 of *lac* operator, ~20% of the colonies were light blue on the indicator plates. Forty of 50 colonies examined exhibited reduced expression of β-galactosidase. Fourteen of these were repressed a 100-fold or more (Table I). Ten candidates carried a serine in position 1 and an alanine in position 2 of the recognition helix specified by different codons. The other four repressor mutations coded for Ser1-Thr2, Pro1-Thr2, Ile1-Thr2 and His1-Val2. The Val1-Ala2 and Ala1-Ala2 mutants also presented in Table I had been synthesized previously (Lehming *et al.*, 1987) and were not found in this search. *lac* repressor mutant Ile1-Ala2 was designed to be analogous to *trp* repressor (Gunsalus and Yanowsky, 1980).

Screening of 5000 colonies harbouring the plasmid with operator 344 which carries a T–A pair in position 4 revealed ~10% light blue colonies. Thirty of 50 of these light blue colonies showed definitely repressed β-galactosidase activities and nine candidates had repression factors of 50 or more (Table I). Seven of these carried a histidine in position 1 and an isoleucine in position 2 of the recognition helix. We found both histidine codons and two of three possible codons for isoleucine. The other two repressor mutations coded for Pro1-Met2 and Gly1-Met2. The Gln1-Met2, Gln1-Ser2 and the His1 mutant included in Table I were not found in this search but had been synthesized previously (Lehming *et al.*, 1987). *lac* repressor mutant Gln1-Ser2 is analogous to *Lambda* repressor and *cro* protein (Ptashne *et al.*, 1976; Hsiang *et al.*, 1977).

In the same library we could not detect a *lac* repressor mutant which would repress β-galactosidase specifically and strongly by binding to operator 342 which carries a C–G pair in position 4. However, two of the mutants selected for binding to operator 341, Ser1-Ala2 and Ser1-Thr2, also interact rather weakly with operator 342 (Table I).

All mutants characterized so far distinctly prefer one operator variant for binding, but their specificities are broadened for position 4 and to a lesser extent also for position 5 of *lac* operator: some of the *lac* repressor mutants with amino acid exchanges in positions 1 or 2 of the recognition helix interact with *lac* operator variants which differ from ideal *lac* operator in position 5 (Table I). This prompted us to construct the operator double variants 341-51, 341-52 and 341-53 which carry an A–T pair in position 4 and an A–T pair (341-51) or a C–G pair (341-52) or a G–C pair (341-53) in position 5 (Table I). All repressor mutants which recognize the operator variant 341 also show significant binding to the operator double variants 341-51 and 341-53. Mutant Gln1-Ser2 binds to the double variant 341-51 even better than to either the single variant 341 or 351 (Table I). On the other hand, the repressor mutants which do not recognize operator variant 341 (His1, Gly1-Met2, Pro1-Met2 and Gln1-Met2) do not bind to the operator double variants 341-51, 341-52 and 341-53, despite their binding to the single variants 351 or 353. All these examples suggest that the specific binding of the *lac* repressor mutants with amino acid exchanges at positions 1 and 2 of the recognition helix is mainly directed towards base pair 4 and to a lesser extent towards base pair 5 of *lac* operator.

Residue 6 of the recognition helix of lac repressor interacts specifically with base pair 6 of lac operator

The second specific contact between the recognition helix and the major groove of the operator DNA involves the amino acid in position 6 of the recognition helix which is an arginine in wild-type *lac* repressor. If arginine 6 is exchanged for asparagine the mutant *lac* repressor exclusively recognizes *lac* operator variant 364 with a T–A base pair in position 6 (Table II and Lehming *et al.*, 1988). We have found previously that the His1 exchange in the

Table I. Repression of *lac* operator variants by *lac* repressor mutants carrying exchanges in positions 1 and 2 of the recognition helix

pWB	Operator sequence	Repression by *lac* repressor mutants														
		wt	S1A2	V1A2*	A1A2	I1A2*	S1T2	P1T2	I1T2	H1V2	H1I2	H1	G1M2	P1M2	Q1M2	Q1S2
	9 8 7 6 5 4 3 2 1															
310	A A T T G T G A G C G C T C A C A A T T	≥200	50	20	16	48	75	40	70	20	20	≥200	70	80	27	24
332	A A T T G T G C G C G C G C A C A A T T	2	2	1	1	4	2	1	2	6	6	2	2	2	1	1
333	G C	5	1	1	1	4	1	1	1	1	1	3	1	1	1	2
334	T A	2	3	1	1	4	2	2	2	3	3	1	2	2	1	1
341	A A T T G T A A G C G C T T A C A A T T	5	160	≥200	120	≥200	100	150	120	100	24	2	2	2	1	10
342	C G	6	19	2	1	4	36	8	4	1	1	1	1	1	1	1
344	T A	4	17	11	10	33	25	24	14	36	70	≥200	60	100	100	6
351	A A T T G A G A G C G C T C T C A A T T	16	20	25	60	50	13	3	17	7	3	11	10	28	7	30
352	C G	11	4	2	3	6	5	6	15	1	1	1	3	6	3	4
353	G C	9	30	14	28	28	18	20	14	2	1	3	6	14	12	50
361	A A T T A T G A G C G C T C A T A A T T	3	1	1	1	2	1	1	2	1	1	2	1	1	1	1
362	C G	2	1	1	1	3	1	1	2	1	1	1	1	1	1	1
364	T A	3	1	1	1	2	1	1	3	1	1	3	1	1	1	1
341-51	A A T T G A A A G C G C T T T C A A T T	2	≥200	70	130	≥200	≥200	100	≥200	32	15	1	1	1	1	60
341-52	A A T T G C A A G C G C T T G C A A T T	2	70	15	40	20	90	45	60	7	2	1	1	1	1	20
341-53	A A T T G G A A G C G C T T C C A A T T	2	110	50	80	160	140	90	60	30	7	2	1	1	1	40

Repression is defined as specific activity of β-galactosidase in the presence of the *lac* repressor deletion mutant Δ1 (codons 14–60 of the *lacI* gene are deleted) divided by the specific activity of β-galactosidase in the presence of the respective *lac* repressor mutant. Unavoidable rare loss (0.5%) of *lacI* plasmids carrying an ampicillin resistance gene limits repression to a factor of 200. The *lacZ* gene is under the control of the operator variant indicated (for details see Lehming *et al.*, 1987). The *lac* repressor mutants are named according to the exchanges they carry in their recognition helices. Positions 1 and 2 of the wild-type recognition helix of *lac* repressor correspond to the residues Tyr-17 and Gln-18 of *lac* repressor. Most *lac* repressor mutants presented in this table are active dimers encoded by *lacI* genes with a frameshift mutation in codon 330 (Lehming *et al.*, 1988). *lac* repressor mutants marked with an asterisk are tetrameric *lac* repressors. The defective *lacI* gene encoding dimeric *lac* repressor has been repaired here to yield a *lacI* gene which encodes a tetrameric *lac* repressor with wild-type core. Comparison of repression values obtained with dimeric and tetrameric *lac* repressor and several operator variants indicates that they never differ more than by a factor of two.

Table II. Repression of *lac* operator variants by *lac* repressor mutants carrying exchanges in positions 1, 2 and 6 of the recognition helix

	pWB	Operator sequence	Repression by *lac* repressor mutants							
			wt	H1	N6	H1N6	L6	H1L6	V1A2	V1A2N6
		9 8 7 6 5 4 3 2 1								
(A)	310	A A T T G T G A G C G C T C A C A A T T	≥200	≥200	1	6	1	4	20	1
	341	A A T T G T A A G C G C T T A C A A T T	14	2	1	1	1	1	≥200	1
	342	C G	12	1	1	1	1	1	2	1
	344	T A	7	≥200	1	1	1	1	11	1
	351	A A T T G A G A G C G C T C T C A A T T	28	23	1	1	1	1	25	1
	352	C G	15	3	1	1	1	2	2	1
	353	G C	10	7	1	1	1	1	14	1
	361	A A T T A T G A G C G C T C A T A A T T	6	2	1	10	1	2	1	1
	362	C G	4	1	1	5	1	7	1	1
	364	T A	8	4	100	≥200	2	21	1	3
(B)	341-51	A A T T G A A A G C G C T T T C A A T T	3	1	1	1	1	1	70	1
	341-52	A A T T G C A A G C G C T T G C A A T T	4	1	1	1	1	1	15	1
	341-53	A A T T G G A A G C G C T T C C A A T T	4	1	1	1	1	1	50	1
	341-64	A A T T T T A A G C G C T T A A A A T T	4	1	1	1	1	1	1	100
	344-64	A A T T T T T A G C G C T A A A A A T T	4	4	1	≥200	1	60	1	2
	341-53-64	A A T T T G A A G C G C T T C A A A T T	6	1	1	1	1	1	1	70

Repression is defined as in Table I. All *lac* repressor mutants in this table are tetrameric (see Table I). In Part A repressions obtained with related single, double and triple *lac* repressor mutants and single operator variants are shown. Part B shows the repression of these mutants observed with double and triple operator variants.

recognition helix of *lac* repressor increased its binding to ideal *lac* operator at least 100-fold (Lehming *et al.*, 1987). We reasoned that combining His1 with exchanges in residue 6 of the recognition helix such as Asn6 might increase the binding. Thus we joined together the two independent amino acid exchanges from tyrosine to histidine in position 1 and from arginine to asparagine in position 6 of the recognition helix to form the double mutant His1-Asn6. This double mutant indeed binds better to operator variant 364 than the single mutant Asn6, but it also weakly recognizes the variants 361, 362 and ideal operator, 310 (Table II).

We observed a similar effect when a leucine was introduced in position 6 of the recognition helix instead of the wild-type arginine. The Leu6 mutant was predicted to bind to operator variant 364 from model building. Furthermore, a leucine occurs in position 6 of the recognition helix of the *cro* protein of phage *434* (Grosschedl and Schwartz, 1979). The single *lac* repressor mutant Leu6 does not recognize any operator variant but the double *lac* repressor mutant His1-Leu6 binds to operator variant 364 and to a lesser extent to variant 362 (Table II). The N-terminal histidine indeed tightens the binding of this altered recognition helix thereby shifting the border of resolution attainable in this *in vivo* system by more than one order of magnitude.

New sequence specific recognition helices can be created by combining mutations in positions 1 and 2 with mutations in position 6 of the recognition helix of lac repressor

The double *lac* operator variant 344-64 where the G−C pairs in positions 4 and 6 have been replaced by T−A pairs is well recognized by the double *lac* repressor mutants His1-Asn6 and His1-Leu6 (Table II). The single mutant His1 recognizes this double operator variant very weakly and the single mutants Asn6 or Leu6 do not recognize it at all. Similarly, the double *lac* operator variant 341-64 is only recognized by the combined repressor mutant Val1-Ala2-Asn6 but not by the mutants Val1-Ala2 or Asn6 (Lehming *et al.*, 1988 and Table II).

Furthermore, the triple *lac* repressor mutant Val1-Ala2-Asn6 binds to an operator variant with three adjacent base pair exchanges: 341-53-64 (A−T in position 4, G−C in position 5 and T−A in position 6, see Figure 1). This variant is a combination of *lac* operator double variant 341-53 which is recognized by the repressor mutant Val1-Ala2 and operator variant 364 which is bound by the repressor mutant Asn6 (Table II). These results suggest that the interactions of *lac* operator base pairs 4 and 5 with amino acids 1 and 2 are independent of the interactions of base pair 6 with residue 6 of the recognition helix of *lac* repressor.

Discussion

Our *in vivo* system is well suited to detect *lac* repressor mutants with moderate affinities to *lac* operator. It is, however, not suited to characterize extremely high-affinity repressor−operator complexes because on the average 0.5% of the cells lose the repressor encoding plasmid pWB 100 during growth (Lehming *et al.*, 1987). Likewise very low affinities cannot be detected because such weak repressor−operator complexes do not inhibit RNA polymerase.

The specific β-galactosidase activities obtained with different operator variants depend on promoter strength and mRNA stability, which in turn may both be influenced by the particular operator sequence (see also Lehming *et al.*, 1987). The use of repression rates (defined as ratios of β-galactosidase activities in the absence and in the presence of *lac* repressor mutants, see also legend to Table I) largely eliminates these differences. The intracellular levels of the various *lac* repressor mutants may also vary due to possibly different protein stabilities. Thus the measurable repression rates may not be strictly proportional to the affinities but instead represent minimal values. However, the relative repression factors determined for one repressor mutant and several operator variants can be considered to reflect qualitatively the affinities of this mutant for the respective operator sequences.

We present here a variety of mutant *lac* repressors with changed but not simply broadened specificities. Most of these *lac* repressor mutants bind substantially tighter to *lac* operator variants with different base pairs in positions 4 and 5 than to ideal *lac* operator (310). Concomitantly, wild-type *lac* repressor binds substantially weaker to these *lac* operator variants than to ideal *lac* operator (see also Lehming *et al.*, 1987). All these mutant *lac* repressors carry exchanges in positions 1 and 2 of the recognition helix which correspond to Tyr-17 and Gln-18 of *lac* repressor. The mutants may be classified into three groups.

The first group comprises the mutants that specifically recognize the A−T pair in position 4 of *lac* operator variant 341. Four of them have an alanine in position 2 of the recognition helix (Ser1-Ala2, Val1-Ala2, Ala1-Ala2 and Ile1-Ala2, Table I), another three carry a threonine in position 2 of the recognition helix (Ser1-Thr2, Pro1-Thr2 and Ile1-Thr2, see Table I) and one a valine (His1-Val2, see Table I). This indicates that the ability to recognize an A−T pair in position 4 of the operator is similar for alanine, threonine and valine in position 2 of the recognition helix (compare in particular Ser1-Ala2 and Ser1-Thr2 or Ile1-Ala2 and Ile1-Thr2 in Table I). We recall that the G−C base pair 4 in ideal *lac* operator is protected by wild-type *lac* repressor but not by Ala1-Ala2 repressor mutant in a methylation protection experiment (Lehming *et al.*, 1987).

The second group consists of three *lac* repressor mutants which carry a methionine in position 2 of the recognition helix instead of a glutamine. They recognize specifically the T−A pair in position 4 of the operator variant 344 (Gly1-Met2, Pro1-Met2 and Gln1-Met2, Table I). These observations suggest that the residue in position 2 of the *lac* recognition helix contributes more to the specificity of the recognition of base pair 4 of *lac* operator than residue 1 (compare Pro1-Thr2 and Pro1-Met2 in Table I).

This view is further supported by the three repressor mutants of the third group in which the tyrosine in position 1 of the recognition helix is replaced by a histidine. The single mutant His1 (which retains the wild-type glutamine in position 2) binds strongly to operator variant 344 and to ideal *lac* operator (310) both *in vivo* and *in vitro* (Lehming *et al.*, 1987). It does not bind at all to operator variant 341 (Table I). But a valine in position 2 together with a histidine in position 1 of the recognition helix (mutant His1-Val2, Table I) switches the specificity of recognition to operator variant 341. In these cases the second residue of the recognition helix seems to determine the specificity.

In contrast we present here too the Gln1-Ser2 *lac* repressor mutant which is characterized by a broadened specificity with

respect to the recognition of base pairs 4 and 5 of *lac* operator. All *lac* operator variants with base pair exchanges in positions 4 and 5 are bound almost equally well. We recall that *Lambda* repressor and *Lambda cro* protein both have Gln1-Ser2 as first residues of their recognition helices (Ptashne *et al.*, 1976; Hsiang *et al.*, 1977).

The collection of *lac* repressor mutants presented here is most likely incomplete. We assume that further screening of the library coding for *lac* repressor with random amino acids in positions 1 and 2 might yield other repressor mutants that recognize the operator variants 341 and 344. None of the *lac* repressor mutants so far identified by this approach carries the amino acids Val1-Ala2, Ala1-Ala2, Ile1-Ala2 or Gln1-Met2 in positions 1 and 2 of the recognition helix which had been constructed before (see Table I and Lehming *et al.*, 1987). Since all these mutants repress β-galactosidase with either operator 341 or 344 >50-fold, their absence indicates that the library used was unbalanced and incomplete, despite its size of 3×10^4 independent clones. This observation strongly suggests that random DNA synthesis does not yield fairly equal distributions of all possible oligonucleotide sequences. It is highly unlikely that we could have missed, for example, the Val1-Ala2 repressor mutant in the screening procedure if an adequate proportion of the 16 possible hexamers had been present in the library. The same holds true for repressor mutant His1.

An interesting property of the histidine in position 1 of the recognition helix of *lac* repressor is the general enhancement of specific interactions between residue 6 of the recognition helix and base pair 6 of *lac* operator which can be observed in the case of Asn6 or Leu6 combined with His1 (Table II). One possible explanation, which we have tested by model building, is that in the case of the His1 mutant the angle between helix 1 and helix 2 (the recognition helix) can be diminished without distortion of the overall complex structure. This results in the ability of the recognition helix to enter the major groove at slightly different angles. It seems conceivable that an adjustment of the angle between protein helix and major groove might be advantageous for some of the amino acid−base pair interactions. That this enhancement needs the presence of specific, although weak, interactions is indicated by the finding that some amino acids in position 6 (i.e. histidine or isoleucine, data not shown) when combined with His1 do not bind to any operator variant at base pair 6. That this is not simply due to proteolytic degradation of the particular *lac* repressor mutants was excluded by Western blot experiments (data not shown).

All *lac* repressors mutated in positions 1 and 2 of the recognition helix except Gln1-Ser2 strongly prefer one particular base pair at position 4 (341 or 344) for binding. The binding to the variants at base pair 5 is weaker and less specific. In contrast, the repressor mutants Leu6 and Asn6 (which recognizes operator variant 364) do not bind at all to operator variants at base pair 5 (Table II). Thus base pair 5 plays no role in the binding of residue 6 of the recognition helix to base pair 6 of the operator.

Since *lac* repressor mutants Asn6 and Leu6, even in combination with His1, do not recognize operator variants with altered base pairs at positions 4 and 5 (Table II) and since all repressor mutants at positions 1 and 2 of the recognition helix fail to bind to operator variants at base pair 6 (Table I) we conclude that the recognition of base pairs 4 and 5 is totally independent of the recognition of base

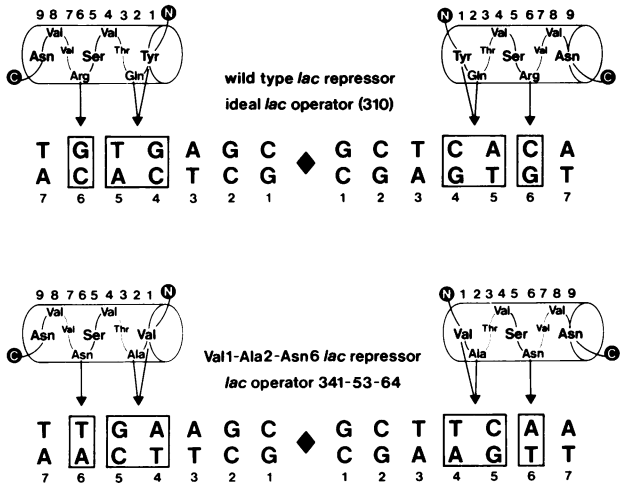

Fig. 1. Model of recognition of *lac* operator by the recognition helices of *lac* repressor. The upper part shows the wild-type recognition helices of *lac* repressor dimer and *lac* operator 310 (ideal operator), the lower part shows the recognition helices of the *lac* repressor mutant Val1-Ala2-Asn6 and *lac* operator triple variant 341-53-64 (see also Table II) in which base pairs 4, 5 and 6 differ from the ideal *lac* operator. The amino acids in positions 1 and 2 of the recognition helix interact with base pairs 4 and 5 of the operator and the amino acid in position 6 of the recognition helix interacts with base pair 6 of the operator. Tyrosine in position 1 of the recognition helix corresponds to Tyr-17 of *lac* repressor, asparagine 9 corresponds to Asn-25 of *lac* repressor. N and C mark the N and C termini respectively. From the symmetric operators the inner 14 base pairs are shown. The black rhombus marks the axes of symmetry.

pair 6. On this basis we predicted that *lac* repressor mutants with particular exchanges of residue 1 and 2 of the recognition helix combined with particular exchanges of residue 6 should recognize *lac* operator variants with the corresponding exchanges of base pairs 4, 5 and 6. The specific binding of the double repressor mutants His1-Asn6 and His1-Leu6 and of the triple mutant Val1-Ala2-Asn6 to their respective predicted operator double or triple variants confirms the model (Table IIB and Figure 1). Thus the independent interactions of residues 1 and 2 of the recognition helix with base pairs 4 and 5 of *lac* operator and of residue 6 with base pair 6 constitute further evidence for the predicted orientation of the recognition helices of *lac* repressor (Lehming *et al.*, 1987, 1988). The results presented here imply that the N termini of the helices are closer to the centre of symmetry of *lac* operator than the C termini (Figure 1).

Materials and methods

Chemicals and enzymes

All chemicals and enzymes used have been described in Lehming *et al.* (1987). The oligonucleotides were synthesized on an Applied Biosystems 380 A DNA synthesizer. After purification on denaturing polyacrylamide gels they were cloned and sequenced according to Maxam and Gilbert (1977).

General methods

For details of the cloning methods see Lehming *et al.* (1987). For the selection experiments the *E.coli* strain DH 5α (*F⁻ endA1 hsdR17(rk⁻, mk⁺) supE44 thi1* λ⁻ *recA gyrA96 relA φ80 dlacZ ΔM15*) was used. For the repression tests the *E.coli* strain DC 41-2 was used $(lac\ pro)_{\Delta}$ *galE smR recA*. The specific activity of β-galactosidase was determined as in Miller (1972).

Construction of the library of lac repressor mutants

Plasmid pWB 100 DNA (Lehming *et al.*, 1987) was digested with *Aat*II and *Xma*I in order to construct a library of *lac* repressor mutants with amino

acid exchanges in positions 1 and 2 of the recognition helix. The 75-bp fragment between the two sites was replaced by synthetic DNA fragments with protruding ends, complementary to the *Aat*II and *Xma*I sites. At the six positions corresponding to codons 1 and 2 of the recognition helix a mixture containing 25% of each nucleotide was offered for the coupling reaction. After purification of the oligonucleotides from a 10% polyacrylamide gel in 7 M urea, a 1:1 mixture was heated to 90°C and cooled down slowly to room temperature to allow hybridization of the correct complementary strands. The hybridized oligonucleotides were ligated to 1 μg pWB 100 DNA to yield 3×10^4 independent transformants.

Detection of lac repressor mutants

Samples of the amplified pWB 100 library were transformed into *E.coli* DH 5α cells carrying plasmid pWB 300 with the operator variants 341, 342 or 344. The transformed cells were plated onto minimal agar containing 2 g/l glucose, 10 mg/l tetracycline, 0.4 g/l ampicillin and 200 mg/l 5-bromo-4-chloro-3-indolyl-β-D-galactoside After 2 days incubation at 37°C, light blue colonies were picked and purified for β-galactosidase assays according to Miller (1972).

Model building and calculations

Model building was done on an Evans and Sutherland PS 330 graphic system hosted by a MicroVAX II with INSIGHT. Calculations were done on a Cray X/MP-48 with DISCOVER. (INSIGHT and DISCOVER from Biosym Inc., San Diego, CA.)

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