J. Mol. Biol. (1985) 186, 295–305

Thermodynamic Origins of Specificity in the *lac* Repressor–Operator Interaction

Adaptability in the Recognition of Mutant Operator Sites

Michael C. Mossing and M. Thomas Record, Jr

Departments of Chemistry and Biochemistry
University of Wisconsin-Madison
Madison, WI 53706, U.S.A.

(Received 23 April 1985, and in revised form 13 June 1985)

A system has been developed for facile generation and characterization of mutant *lac* operator sites, free of competing pseudo operator sequences. The interaction of *lac* repressor with these sites has been investigated by the nitrocellulose filter binding assay. The equilibrium binding affinity for each of three single-site changes was reduced by more than three orders of magnitude relative to the wild-type operator under standard assay conditions. The free-energy changes associated with single base-pair substitutions are not additive. We propose that adaptations in the recognition surface of the repressor involving significant trade-offs between electrostatic *versus* non-electrostatic interactions and between enthalpic *versus* entropic contributions to the binding free energy occur, in order to achieve the most stable complex with a given DNA sequence.

1. Introduction

How do proteins recognize DNA sequences? This question has been addressed repeatedly over the years and the degree of precision with which it can be answered has been refined on many levels. The ultimate goal in many studies has been a structure–function relationship. A basic but often overlooked intermediate in correlating structure, as investigated by X-ray crystallography and chemical modification experiments, with function, as studied by genetic and biochemical techniques, is the physical chemistry of the interaction in question. The thermodynamics and kinetics of non-covalent interactions of proteins with nucleic acids provide the fundamental criteria that determine which interactions take place and as such are of critical importance in understanding the control of gene expression.

The *lac* repressor has served as the prototype DNA binding protein in a number of these investigations (Miller & Reznikoff, 1980). Genetically, mutants in both the protein (Miller, 1984) and the DNA (Smith & Sadler, 1971; Gilbert *et al.*, 1976) that affect the formation of a functional complex have been identified. Structurally, *lac* operator DNA is thought to exist as a normal *B* form helix, which undergoes only a slight unwinding (50°) upon complex formation (Wang *et al.*, 1974). Although at present no crystal structure is available, the *lac* repressor has been implicated as a member of a class of structurally similar DNA binding proteins by sequence comparison and genetic evidence (Matthews *et al.*, 1982; Weber *et al.*, 1982), and by nuclear magnetic resonance measurements (Zuiderweig *et al.*, 1983). The location of the protein on the DNA, as well as qualitative information about the recognition surface, has been obtained by chemical protection and DNase footprinting (Ogata & Gilbert, 1979; Galas & Schmitz, 1978). The kinetic aspects of this interaction have been thoroughly studied (Winter *et al.*, 1981; Barkley, 1981) and have been used to illustrate mechanisms of facilitated diffusion in DNA site location by proteins. The thermodynamics of the interaction of repressor with operator and with non-specific DNA have been investigated in detail (see, e.g., Riggs *et al.*, 1970*a*,*b*; Record *et al.*, 1977; Winter & von Hippel, 1981; Barkley *et al.*, 1981). Under standard ionic conditions the equilibrium specificity ratio for the binding of repressor to wild-type operator over non-operator sites is 10^8. The two classes of sites show large and characteristically different salt dependences (Record *et al.*, 1977). Formation of the non-specific complex involves the release of the thermodynamic equivalent of 11 ± 2 monovalent ions (deHaseth *et al.*, 1977; Revzin & von Hippel, 1977) while formation of the specific complex at the wild-type operator releases the thermodynamic equivalent of only $6 \pm 1{\cdot}5$ ions from the vicinity of the DNA (cf. Table 1 of Winter &

0022-2836/85/220295-11 $03.00/0

von Hippel, 1981). The equilibrium binding parameters observed *in vitro* are sufficient to describe repression and induction of the *lac* operon *in vivo* (von Hippel, 1979), providing evidence that control of the repressor–operator interaction is at the thermodynamic level in *Escherichia coli*. The present thorough understanding of this system prompted us to begin a series of experiments aimed at delineating the molecular and thermodynamic bases of selectivity in the binding reaction.

In this paper we examine the thermodynamics of the interaction of wild-type *lac* repressor with various mutant DNA sites. We have measured equilibrium constants for the binding of repressor to operator mutants as a function of solution conditions to elucidate the thermodynamic basis of recognition of a specific site in the midst of a vast excess of non-specific DNA. Advantages of using the DNA sequence as the primary variable rather than the protein sequence include the fact that the secondary structures of the mutant and wild-type operators should be quite similar, and that a single protein sample can be used in experiments comparing several operators, thereby minimizing variable activity of repressor protein in experiments *in vitro* as a source of experimental uncertainty.

Previous work characterizing the interaction of repressor with variant operators has fallen into three classes: (1) genetic studies mapping and quantifying the *in vivo* phenotypes of operator-constitutive (O^c) mutations (Smith & Sadler, 1971); (2) *in vitro* studies of O^c operators on ϕ80plac phage (Jobe *et al.*, 1974); and (3) *in vitro* studies of synthetic operator variants (Caruthers, 1980). Qualitatively different binding behavior has been observed in the interaction of repressor with small synthetic operator fragments and operator-containing DNA macromolecules. Recent studies have emphasized the importance of two secondary binding sites in the *lac* operon originally reported by Reznikoff *et al.* (1974) and Gilbert *et al.* (1976). Estimates of the repressor binding affinities of these sites (Winter & von Hippel, 1981; Fried & Crothers, 1981) are higher than those of many O^c mutations.

Using these studies as a starting point we have set out to characterize more fully the interactions between the *lac* repressor and the individual base-pairs in its DNA site. Here we report a system that facilitates the isolation of operator DNA fragments containing O^c mutations. These fragments are obtained free of competing pseudo operator sites and are of defined size for physical studies. In addition we report a thermodynamic characterization of the interaction of repressor with three of the strongest single-site O^c mutations, as well as with the wild-type operator.

2. Materials and Methods

(a) *Bacterial strains, plasmids and phages*

Media and plates and standard bacterial methods have been described by Miller (1972). *In vitro* manipulations of DNA (restriction enzyme digestions, ligations, labeling, etc.) followed the procedures of Maniatis *et al.* (1982). The strains used are listed in Table 1.

Table 1

Strains, plasmids and phages used in this study

Name	Genotype and comments	Origin
Strains		
JM83	Δ(*lac-pro*) *ara strA thi* (ϕ80d*lac* Z M15)	Messing (1979)
JM103	Δ(*lac-pro*)SupE *thi strA endA sbc*B15 *hsd* R4 F_1' *tra*D36 *proA*$^+$ B$^+$ *lac* I^Q Z M15)	Messing *et al.* (1981)
JM103/F'lac$^+$proA$^+$B$^+$	Tester strain for operator titration	This study, transferred by conjugation
JM103 pox38-Gen	*lac* Δhost for purification of phage	W. S. Reznikoff
CSH26Sm^R	Δ(*lac-proAB*) *ara thi* Sm^R	W. S. Reznikoff
Xac	Δ(*lac-pro*) *sup*B Nal^R Rif^R/F'*lac* I^Q ZU118 *pro*$^+$	W. S. Reznikoff
CSH26 recA/pox38-Gen	Gentamycin-resistant derivative of pox38 (Guyer, 1980)	Carol Gross
CAG318	Δ(*lac-pro*)$_{XIII}$ *thi* Sm^R/F'*lac*$^+$ *proA*$^+$ B$^+$	W. S. Reznikoff
K802Δ(OZ)	Δ*lac* (O-Z) *gal*$^-$ *met*$^-$ *sup*E *lac*Y	Lab. collection
Plasmids		
Col3A	A pVH51 derivative containing the *Hin*cII 789 bp *lac*$^+$ fragment	W. S. Reznikoff
pKO4		McKenny *et al.* (1981)
pKO$^+$	*lac* 300 O$^+$Z$^+$ inserted in *Eco*RI site of pKO4	This study
pKO ZU118	*lac* 300 O$^+$Z$^-$ inserted in *Eco*RI site of pKO4	This study
Phages		
ϕ80plac O^{c}D630	Class IIa; G to A at +11	Maquat *et al.* (1980)
ϕ80plac O^{c}D640	Class IIb; G to A at +5	Maquat *et al.* (1980)
ϕ80plac O^{c}D666	Class IIIb; A to G at +8	Maquat *et al.* (1980)
M13ΔE101		Kim *et al.* (1981)
M13ΔE3 O$^+$	M13ΔE101 with *lac* 300 of the designated genotype inserted in the *Eco*RI site	This study
M13ΔE3 Z$^-$		
M13ΔE3 O^c 630		
M13ΔE3 O^c 640		
M13ΔE3 O^c 666		

Cloning of the *Hha*I 282 base-pair *lac* fragment (hereafter referred to as the *lac* 300 fragment: see Fig. 1) into the *Eco*RI site of pKO4 was accomplished as follows. A total *Hha*I digest of Col3A was treated with bacteriophage T4 DNA polymerase and all 4 dNTPs (to render the fragments blunt-ended), ligated to *Eco*RI linkers and subsequently ligated into the *Eco*RI site of pKO4. The resulting plasmids were transformed into JM83, plated on M9 glucose plates containing ampicillin, IPTG, and Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), and blue (α-complementing) colonies were observed. The plasmid designated pKO$^+$ was found to contain a single copy of the *lac* 300 fragment, oriented such that transcription proceeds rightward through the galactokinase gene.

A complementation-negative derivative of this plasmid was obtained by recombination with an F′ *lac* ZU118 episome. Xac (an F′ZU118 strain) was transformed with pKO$^+$. The resulting strain was crossed with CSH 26 Sm^R. Ampicillin and streptomycin-resistant transconjugants were selected with a frequency of about 10^{-6} per recipient cell plated. Plasmid DNA from these pooled colonies was transformed into JM83 and screened for a Lac$^-$ phenotype. Two classes of Amp^R Sm^R *lac* Z$^-$ transconjugants were observed. More than 90% contained a gamma–delta insertion in the *lac* 300 fragment. One Z$^-$ plasmid with no detectable change in restriction pattern was designated pKOZ$^-$.

In order to facilitate generation and characterization of a variety of single-site O^c molecules, the *lac* 300 region was transferred to M13ΔE101. This phage consists of wild-type M13 with a unique *Eco*RI site inserted near the origin of replication. The Lac$^+$ 300 bp† fragment was ligated into this site and the DNA was transformed into JM103. Six α-complementing plaques were picked. All were determined to be in the plus orientation (i.e. the *lac* coding strand is packaged in the virion).

A Z$^-$ M13 phage isolate (M13ΔE3Z$^-$) was obtained by marker rescue, since direct cloning of the Z$^-$ fragment would yield a product molecule phenotypically indistinguishable from M13ΔE101. In order to construct a heteroduplex for transformation, pKOZ$^-$ and M13ΔE101 RF were digested with *Eco*RI and mixed with single-stranded M13ΔE30$^+$ in the molar ratio 10:3:1, sealed in a capillary, immersed in a boiling water bath for 5 min, slowly cooled to 70°C, and reannealed. The resulting mixture was transformed into JM103. Clear plaques were picked and analyzed by restriction mapping. Subsequently, 3 O^c Z$^+$ derivatives were obtained by similarly annealing single-stranded M13ΔE3Z$^-$ DNA, *Eco*RI-digested M13ΔE101 and *Hha*I-cut φ80plac O^c DNA (1:3:4 molar ratio, see Fig. 2).

To verify the presence of the specific O^c mutations, Lac phenotypes were checked in JM103 (for α-complementation) and JM103/F′ *lac*$^+$ *pro*A B (for operator titration) on M9 glucose Xgal plates. Positive clones were subsequently sequenced by the dideoxy method (Sanger *et al.*, 1980).

(b) *DNA preparation and* lac *repressor protein*

Plasmid and M13RF DNAs were prepared by boiling, lysis and CsCl/ethidium bromide gradient centrifugation (Maniatis *et al.*, 1982). φ80plac was grown in K802 and DNA was prepared according to Reznikoff *et al.* (1974). Concentrations were determined by ultraviolet light absorbance ($\varepsilon_{260} = 1{\cdot}3 \times 10^4$ l cm^{-1} mol^{-1} (bp)). *Bam*HI digestion of the M13ΔE3 series yields a single linear 6600 bp fragment; the 300 bp *lac* insert was obtained by *Eco*RI digestion followed by precipitation with 5% polyethylene glycol, 0·5 M-NaCl (Lis, 1980) to remove selectively the 6300 bp vector DNA. The supernatant was subsequently precipitated with ethanol to recover the *lac* 300 fragment. DNA fragments were labeled by the replacement synthesis method using T4 DNA polymerase and [α-^{32}P]dCTP. After labeling, the DNA was extracted with phenol, phenol/chloroform (1:1, v/v) and chloroform, and purified by gel filtration. The 6600 bp DNA was purified on Sephacryl S-300; 300 bp DNA on Sephadex G-100.

lac repressor protein was a gift from Dr Kathleen Matthews, Rice University. Concentrations were determined spectrophotometrically at 280 nM using an extinction coefficient of 9×10^4 l cm^{-1} mol^{-1} (Butler *et al.*, 1977). The protein was approximately 60% active as determined by titration against wild-type operator (on either 300 or 6600 bp fragments) under stoichiometric binding conditions (cf. Riggs *et al.*, 1970*a*). (Filtration of an equimolar mixture of *lac* repressor protein and DNA at concentrations greater than 10-fold above the dissociation constant resulted in retention of only 60% of the plateau level of radioactivity. The plateau is itself approximately 50% of the total radioactivity applied to the filter: this represents the inherent efficiency of retention of the complex on the filter.)

(c) *Filter binding*

All filter binding experiments were performed in 10 mM-Hepes (pH 7·4), 1 mM-EDTA, 5% glycerol, 50 μg bovine serum albumin/ml, 1 mM-dithiothreitol and NaCl to bring the total monovalent cation concentration to the desired level. Wash buffer was identical except for the omission of glycerol, bovine serum albumin and dithiothreitol. Schleicher and Schuell BA-85 nitrocellulose filters were presoaked in 0·4 M-KOH and rinsed extensively in deionized water. Equilibrium experiments were initiated by mixing equal volumes of freshly diluted protein with DNA. Duplicate or triplicate samples were filtered through filters equilibrated with wash buffer. Samples between 50 μl and 1 ml were filtered and washed immediately with 0·5 ml wash buffer. Filters were dried and Čerenkov radiation was counted in a Beckman LS8000 scintillation counter. Data were corrected for background (filter retention in the presence of 1 mM-IPTG) and normalized to the total number of input counts. Equilibrium constants at different temperatures were determined in pairs from a single set of protein-DNA solutions, which was divided and allowed to equilibrate at the desired temperatures. Scatchard analysis of the data obtained from titration of a fixed concentration of DNA with increasing amounts of *lac* repressor protein yielded binding constants as well as efficiencies of filter retention. Equilibrium constants obtained in a single series of experiments (e.g. the temperature experiments listed in Table 2) had an error of approximately 10 to 30%. Agreement between experiments done over longer periods was within ±50%.

3. Results

(a) *Plasmid and phage constructions*

As illustrated in Figure 1, *lac* 300 fragment contains all of the classical *lac* regulatory elements

† Abbreviation used: bp, base-pairs; IPTG, isopropyl-β-D-thiogalactoside.

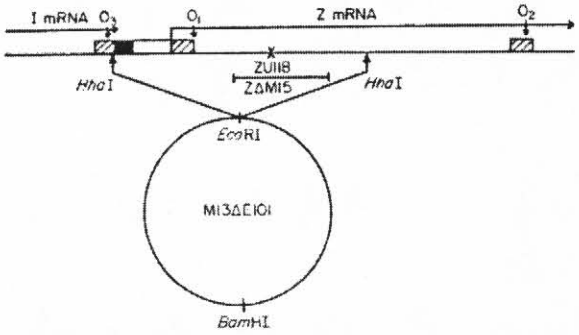

Figure 1. Operator molecules used in this study. The region of the *lac* operon immediately surrounding the regulatory loci. The *Hha*I 282 bp *lac* fragment used in these studies contains all of the classical *lac* regulatory elements (CAP, filled box; promoter, open box; operator, O_1) but excludes the pseudo operator sequences (O_2 and O_3) that have been identified in this region. The amber mutation ZU118 (×) and the extent of the M15 deletion present in the α-accepting host strain JM103 are indicated. The M13 phage vector used in these experiments is also shown. M13ΔE101 has a unique *Eco*RI site into which the *lac* 300 DNA was inserted, as well as a unique *Bam*HI site. Cleavage with *Bam*HI generates the 6600 bp fragment used in some of the studies described in the text.

and enough of the *Z* gene for intragenic complementation of β-galactosidase in a ZΔM15 strain (α-complementation), but does not include either of the pseudo operator sequences previously identified in the region.

A Z^- *lac* 300 bp fragment for marker rescue was obtained by recombination. Because of the small region of homology in which the recombination could occur, a selection was developed involving transfer of a fused recombination intermediate involving the plasmid and a coresident F′ carrying the desired Z^- allele. The selection for recombination by replicon fusion was complicated by two factors: (1) the low frequency of this event (Amp^R, Sm^R transconjugants were obtained at a frequency of 10^{-6}), and (2) the presence of a competing reaction (transposition of gamma–delta from the F′ onto the pKO^+ plasmid; Guyer, 1978). Of the lac^- plasmids obtained by this procedure the majority turned out to have a 2×10^3 base insertion into the *lac* region characteristic of gamma–delta. The few plasmids with no detectible change in restriction maps were assumed to be ZU118 point mutants. One of these (designated $pKOZ^-$) was utilized in subsequent experiments.

A marker rescue technique using M13, modeled after work of Kramer *et al.* (1983), proved to be a very facile way to generate variant operators (Fig. 2). A crude *Hha*I digest of φ80plac O^c DNA and linear M13ΔE101 replicative form DNA were annealed to single-stranded M13ΔEZ^- DNA, resulting in heteroduplex molecules containing mismatches at both the operator and the *Z* gene lesion and two small gaps at the *Eco*RI sites. Appropriate host strains were transformed with the annealed mixture and lac^+ plaques were selected. At the ratios of φ80 to M13 DNA that we used, roughly 30% of the resulting plaques had the Lac^+ phenotype. Subsequent analysis of 16 individual clones showed that each had the expected O^c phenotype in the tester strains, contained both *Eco*RI sites present in the parental M13 phage, and (ultimately) possessed the single base-pair change predicted from the O^c allele. Once the Lac phenotype was verified all subsequent manipulations were carried out in the *lac* deletion strain JM103/pox38-Gen to eliminate the possibility of recombination with a different resident *lac* allele.

Here we report the physical-chemical characterization of the interaction of wild-type repressor with the three O^c mutations: 630 (G to A at +11), 640 (G to A at +5), 666 (A to G at +8). These O^c mutants are three of the strongest mutations originally isolated by Smith & Sadler (1971). (The original mutation numbers are O^c 120, O^c 1 and O^c 17, respectively). They are all located in the left half of the operator (Fig. 3).

(b) *Binding characteristics of operator fragments*

We were initially surprised by the extent of the reduction in binding affinity due to each of these single-site mutations. In each case, the equilibrium binding affinity of repressor was reduced by more than 1000-fold relative to identically prepared O^+ DNA. The affinities were decreased so much that, in general, the equilibrium specificity ratios of the O^c mutants were too low to permit detection of specific binding in the presence of the 6600 non-specific sites of the M13 vector DNA. Furthermore, in attempts to investigate the kinetics of these interactions, we found that both the association and dissociation reactions were too fast to measure ($t_{\frac{1}{2}}$ less than 10 s) at protein concentrations that were sufficient to observe specific binding. Subsequent experiments with these O^c operators on 300 bp fragments showed 666 and 640 to have retention levels significantly above background while 630 was still not specifically retained under a variety of solution conditions tested. The fact that no specific retention was observed for O^c 630 served as a useful control. It demonstrated that there were no other sites on these fragments with sufficient repressor binding affinity to be retained on the filter and that under the conditions investigated the observed effects could be attributed solely to binding at the primary operator.

It was necessary to demonstrate that the presence of the inducer IPTG would reduce specific binding to the O^c mutant operators to a negligible level and therefore yield a valid measure of non-specific retention. In all cases we find that IPTG at 1 mM reduces binding to the O^c operators to the level of background binding of 300 non-specific sites, such that for each O^c the IPTG-insensitive retention shows the same repressor concentration dependence.

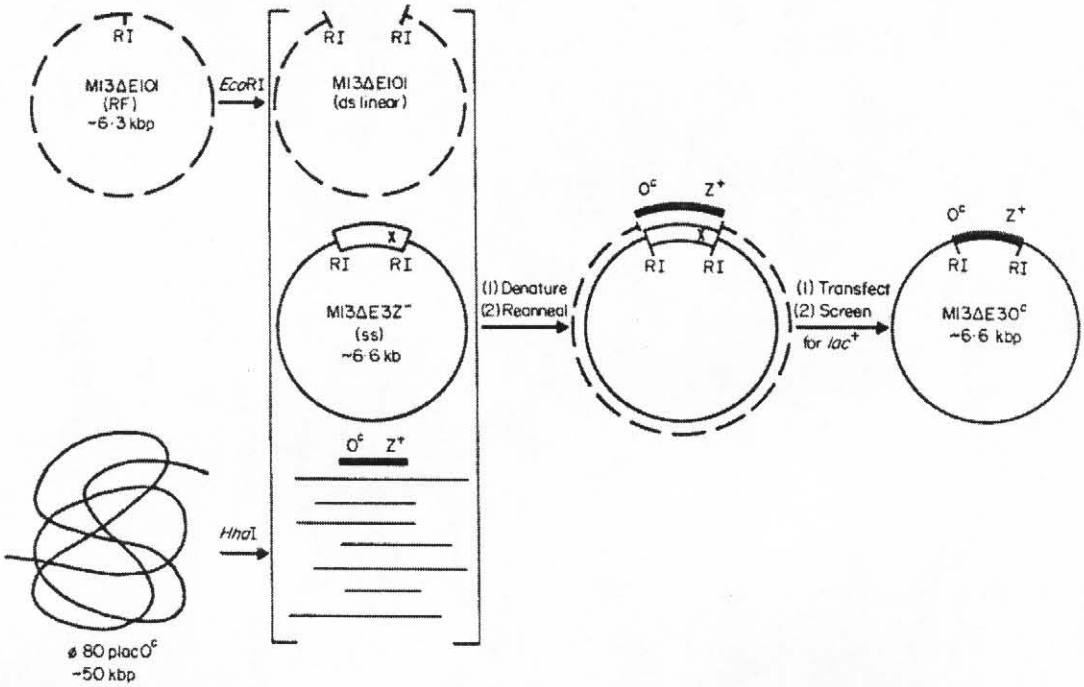

Figure 2. Transfer of *lac* alleles to M13 phage by marker rescue. The transfer of O^c alleles to M13 through rescue of α-complementation activity is illustrated. (An analogous technique was used to transfer the Z$^-$ allele to M13 from pKOZ$^-$). M13ΔE101 was digested with *Eco*RI (RI) yielding a linear molecule with ends corresponding to the points of insertion of the *lac* 300 fragment in the single-stranded M13ΔE3Z$^-$ DNA. ϕ80plac O^c DNA was digested with *Hha*I to give a mixture of fragments including the 282 bp *lac* O^c Z$^+$ fragment. After heating the digested DNA to 70°C for 10 min to inactivate the accompanying restriction enzymes, the M13 replicative form (RF) and single-stranded (ss) DNA and the ϕ80 DNA were mixed, sealed in a capillary, denatured and reannealed. This annealed mixture containing heteroduplex molecules of the type indicated was transformed in JM103 and *lac*$^+$ plaques were selected. ds, double-stranded; kbp, 10^3 base-pairs.

For the wild-type operator and for O^c mutants in cases where the background was acceptable (e.g. for O^c 666 at high salt concentration where the ratio of specific to non-specific binding is high) measurements were made on 6600 bp as well as 300 bp fragments. Contrary to previous results we detected no length dependence of the interaction at the equilibrium level (see below). Scatchard plots such as the one shown in Figure 4 were used to extract equilibrium constants from the binding data. The intercept on the abscissa in Figure 4(b) represents the amount of radioactivity retained at infinite repressor concentration (the inherent filter efficiency of the complex). Filter efficiencies of O^c complexes (~20%) were reproducibly lower than those observed for O$^+$ (~50%). This may be attributable to their faster dissociation rates (see above).

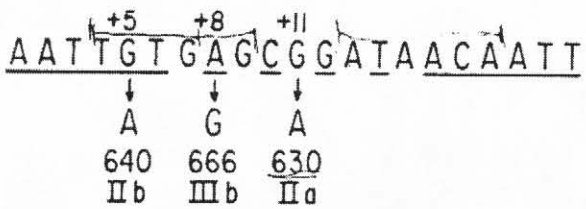

Figure 3. Operator mutants used in this study. Sequence of *lac* operator extending from +1, the start point of transcription, to +21. Regions of dyad symmetry centered around base-pair 11 are indicated by underlines. The 3 O^c mutations investigated here are illustrated at their respective positions beneath the wild-type sequence. The numerical designations are those of Maquat *et al.* (1980). The Roman numeral designations are those of Smith & Sadler (1971). Hyphens have been omitted from the sequence for clarity.

(c) *Salt dependence of repressor–operator interactions*

To determine the extent to which O^c mutations affected the electrostatic component of the interaction, the dependence of the equilibrium extent of binding on the monovalent salt concentration was investigated. A plot of log K_{eq} *versus* log NaCl yields the stoichiometry of release of small ions upon formation of the repressor–operator complex. As illustrated in Figure 5, linear plots of this type were obtained for both O$^+$ and O^c operators. The slopes of these log–log plots are 5 ± 1 for O^c 666 and O$^+$ (within the range of published results for O$^+$) while that obtained for O^c 640 is 10 ± 1. The salt dependence of the O^c 640–repressor interaction is in the range observed for non-specific binding (Revzin & von Hippel, 1977; deHaseth *et*

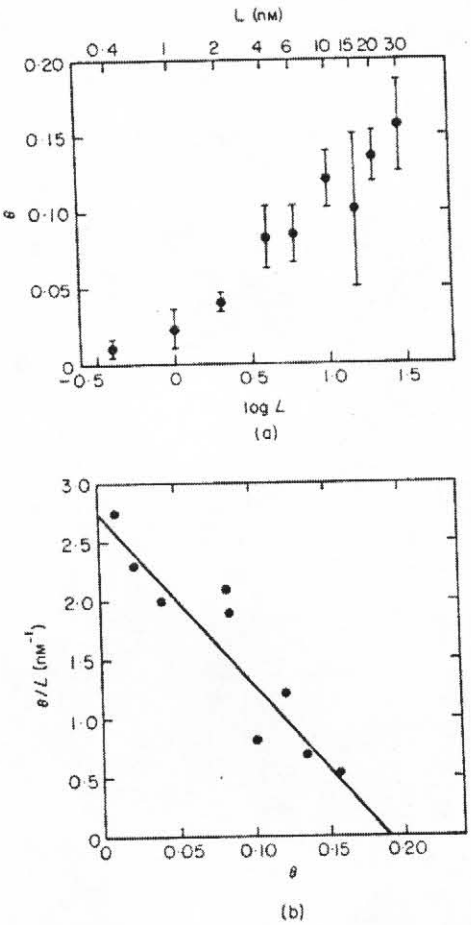

Figure 4. Equilibrium titration of O^c 666 300 bp DNA with repressor at 0·2 M-KCl and 15°C. (a) Repressor titration of O^c 666 DNA. Repressor protein was in excess over DNA (20 pM) so that in all cases the total ligand concentration (L) was equal to the free ligand concentration. On the ordinate are the counts per minute retained on the filter, corrected for background (as measured by filter retention in the presence of 1 mM-IPTG) and normalized by the total input radioactivity minus the background (θ). The points plotted are the averages of 2 independent experiments utilizing duplicate samples for each. Standard deviations are indicated by error bars. (b) Modified Scatchard plot of the same data. The slope of the line is the negative of the equilibrium constant (when corrected for 60% activity of the protein, $K_{obs} = 2{\cdot}0 \times 10^8$ M^{-1}) and the intercept on the abscissa is the efficiency of retention of the complex on the filter (0·19).

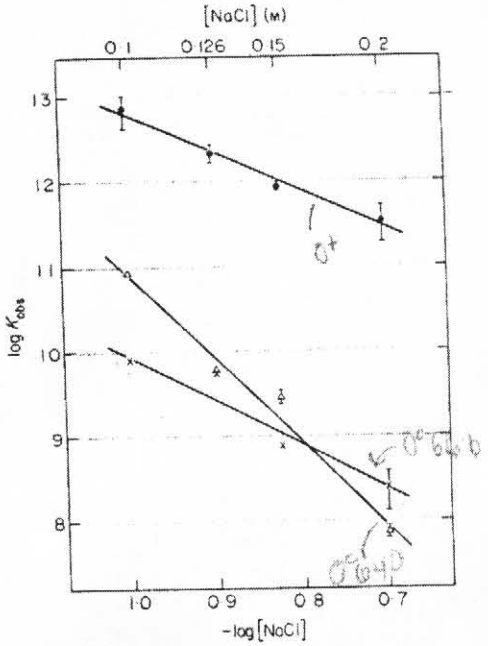

Figure 5. Salt dependences of repressor–operator interactions. Plot of the log of the equilibrium constant at 23°C (obtained as in Fig. 4(a) and (b)) *versus* the log of the NaCl concentration for 300 bp fragments of O^+, O^c 640 and O^c 666 between 0·1 M-NaCl and 0·2 M-NaCl. Error bars, where present, indicate the standard deviation of multiple independent determinations. The slopes of the linear least-squares fits to the data are equal to the numbers of ions released upon complex formation (5 ± 1 for O^+ (●) and O^c 666 (×), 10 ± 1 for O^c 640 (△)).

al., 1977). As a result the relative affinities of repressor for O^c 666 and O^c 640 change dramatically over the range of salt concentrations investigated.

(d) *Temperature dependence of repressor–operator interactions*

The apparent van't Hoff enthalpy (ΔH°_{obs}) of complex formation is obtained from the temperature dependence of the equilibrium constant. We have determined equilibrium constants for O^+, O^c 666 and O^c 640 at temperatures between 5°C and 23°C (see Table 2). Equilibrium constants for O^+ and O^c 640 increase significantly with increasing temperature in this range, yielding van't Hoff enthalpies of 14 ± 5 kcal/mol (1 cal = 4·184 J) for O^+ and 16 ± 5 kcal/mol for O^c 640. (The result that we observe for O^+ is somewhat larger than the value of 8·5 kcal/mol determined by Riggs *et al.* (1970*b*).) By contrast, the equilibrium constant for the interaction of repressor with O^c 666 increases only slightly with increasing temperature, yielding a van't Hoff enthalpy of $4{\cdot}5 \pm 2$ kcal/mol.

4. Discussion

(a) *Cloning strategy*

Previous studies of repressor–operator interactions indicated the importance of using operator-containing DNA molecules that were free of competing pseudo-operator sites. Utilizing M13 as a source of single-stranded DNA for hybridization and also for characterization by DNA sequencing, we have generated a family of molecules containing the O^c and O^+ sites of

Table 2

Thermodynamic parameters of repressor–operator interactions for 300 bp fragments

	K_{obs} (M^{-1})			0·2 M-NaCl		
	5°C	15°C	23°C	ΔH°_{obs} (kcal/mol)	ΔS°_{obs} (cal/mol deg.)	$-\Delta G^\circ_{obs}$(23°C) kcal/mol
O$^+$(0·2 M-NaCl)	$3{\cdot}2\pm0{\cdot}5\times10^{10}$	$7{\cdot}3\pm1{\cdot}5\times10^{10}$	$(1{\cdot}5\pm0{\cdot}5\times10^{11})$†	14 ± 5	100 ± 20	15 ± 1
O^c 630 (0·2 M-NaCl)	—	—	$<2\times10^7$	—	—	<10
O^c 640 (0·15 M-NaCl)	$4{\cdot}6\pm0{\cdot}2\times10^8$	$1{\cdot}3\pm0{\cdot}2\times10^9$	$2{\cdot}7\pm0{\cdot}3\times10^9$	(16 ± 5)‡	(90 ± 20)‡	(11 ± 1)‡
O^c 666 (0·2 M-NaCl)	$1{\cdot}4\pm0{\cdot}3\times10^8$	$2{\cdot}0\pm0{\cdot}3\times10^8$	$2{\cdot}5\pm0{\cdot}7\times10^8$	$4{\cdot}5\pm2$	50 ± 10	11 ± 1

† The equilibrium constant listed here was determined in a self-consistent set of experiments as outlined in Materials and Methods. It falls in the lower range of values obtained under these conditions in general.

‡ The repressor binding affinity of O^c 640 at 0·2 M-NaCl was insufficient to obtain reliable equilibrium constants at 5°C and 15°C. The enthalpy obtained at 0·15 M-NaCl was used to estimate the ΔS°_{obs} and ΔG°_{obs} at 0·2 M-NaCl.

interest. These molecules, identical except for single base-pair changes, are obtainable in different lengths. The fact that repair of the lesion in the *Z* gene is the primary screen for recombination makes it possible to transfer operator alleles of any phenotype into the M13ΔE3 series for study. Furthermore, the *in vitro* hybridization technology lends itself readily to directed changes, once a picture of the interactions responsible for specific recognition starts to emerge.

(b) *Comparison with previous work*

Previous studies *in vitro* and *in vivo* predict substantially smaller effects of single-site O^c mutations than those that we observe. Jobe *et al.* (1974) measured the affinity of repressor for O^c operators on intact ϕ80dlac transducing phages *in vitro*. They found that the lower limit of repressor affinity for O^c mutations was approximately 5% of O$^+$; O^c 666, 640 and 630 were all at this level and could not be distinguished *in vitro* by wild-type repressor. With the benefit of hindsight, we believe that Jobe *et al.* were measuring binding to the secondary operator site located in the *Z* gene. On the other hand the *in vivo* studies of Jobe *et al.* did distinguish the constitutive phenotypes of the three alleles. Here operator affinities of 1·8% 1·1% and 1% of wild type, as measured by ratios of constitutive to induced levels of β-galactosidase, were reported for 666, 640 and 630, respectively. We observe the same order of affinities *in vitro* (under solution conditions that may approximate the complex ionic environment *in vivo*) but the reductions in repressor binding affinity at 0·2 M-NaCl (0·1%, 0·05% and <0·02% of wild type for 666, 640 and 630, respectively) are substantially more pronounced than those observed *in vivo* (see below).

We see no dependence of the binding constant on length for 300 bp and 6600 bp DNA molecules. Winter & von Hippel (1981) found a five- to tenfold lower binding affinity for the interaction of repressor with a 203 bp fragment (containing O_1 and O_3) than for a 6000 bp fragment (containing O_1, O_2 and O_3). Winter & von Hippel interpreted this difference as a length dependence. Their result and ours can be reconciled if the apparent binding constant for the primary operator site is increased by the presence of the secondary operator site. The possibility that *lac* repressor interacts with (for further discussion of this point, see Pfahl *et al.*, 1979) more than one DNA site at a time has been proposed on the basis of experiments with repressor–β-galactosidase fusion proteins (Kania & Muller-Hill, 1977). Recent work on the *gal* repressor (Majumdar & Adhya, 1984) and the *ara*C protein (Hahn *et al.*, 1984) indicate that these proteins interact with pairs of sites separated by distances of 90 and 200 base-pairs, respectively. Such a co-operative model may also account for the apparent discrepancy between our *in vitro* (single operator) binding constants and the *in vivo* (multiple operator) phenotypes (Jobe *et al.*, 1974). This type of effect should be readily detected in the case where association at a weak binding site can be monitored independently of association at a neighboring stronger binding site. Since the secondary operator site in the *Z* gene has been shown to be ineffective in repressing transcription *in vivo* (Reznikoff & Beckwith, 1969), this situation pertains in the case of an O^c operator at the locus of effective repression and the stronger *Z* gene pseudo-operator located downstream. Experiments are in progress to investigate the nature of this proposed co-operativity.

Physical characteristics of the interaction of repressor with operator sites on short synthetic DNA molecules (21 to 29 bp) differ significantly from the observations reported here and from all of the published work on operator-containing molecules larger than 100 base-pairs. The binding constants obtained with synthetic fragments are lower by 20 to 1000-fold (depending on solution conditions) than those obtained on larger molecules. In addition, the salt dependence of the interaction of repressor with O$^+$ on a synthetic fragment ($-\partial \log K_{obs}/\partial \log NaCl = 2$), reported by Goeddel *et al.* (1977) and O'Gorman *et al.* (1980), is significantly smaller in magnitude than that observed for macromolecular DNA ($-\partial \log K_{obs}/\delta \log NaCl = 6$ for molecules >100 bp). One of the

mutants studied here, O^c 666, has also been investigated as a 21 bp synthetic fragment (Fisher & Caruthers, 1979). The *in vitro* analysis of the interaction of repressor with this fragment was limited to measurement of the dissociation rate constant at a single (low) salt concentration. By assuming the association rates were independent of the base sequence of the operator, Fisher & Caruthers estimated that this mutation reduced the equilibrium constant for repressor binding by a factor of only five to eightfold, as compared to a similarly sized O^+ fragment. By contrast, we observe a greater than 1000-fold reduction in the binding constant due to this mutation (at 23°C independent of salt concentration).

In spite of their distinct differences from larger operator fragments, these synthetic operators are recognized by repressor with high specificity. Although the energetics of the interaction are apparently perturbed by the absence of flanking sequences, it is reasonable to assume that key recognition features responsible for specificity of the interaction are similar on both the synthetic fragments and the larger molecules. The studies of Caruthers on synthetic operator variants provide "yes-or-no" answers as to the importance of individual functional groups for recognition. However, inferring the forces responsible for the interaction (function) at a given base-pair from the individual functional groups (structure) critical for recognition is far from straightforward, as our results demonstrate.

(c) *Thermodynamic analysis*

(i) O^+

The wild-type allele has been studied extensively over the years, primarily in the presence of the secondary operator sites. Our results mostly bear out the previous work, although some differences of potential significance exist. Under the ionic conditions investigated, the reaction is entropically driven and accompanied by an unfavorable van't Hoff enthalpy; the ΔH°_{obs} is positive. Analysis of the salt dependence of the reaction indicates that approximately five small ions are released upon formation of the complex. Extrapolation of the linear log–log plot to the 1 M standard state reveals that at 0·2 M-NaCl approximately half of the net interaction free energy is electrostatic in origin. The remainder of the entropic contribution may arise from the release of water from the DNA and protein recognition surfaces that accompany the formation of the complex. Differences do exist at the qualitative level between our results and those obtained with DNA molecules containing pseudo-operator sites. Compared at the same ionic conditions and temperature, the repressor binding affinities of multi-operator DNA molecules are larger than those that we observe by a factor of approximately 10. In addition we find an unfavorable enthalpy change that is somewhat larger than that observed by Riggs *et al.* (1970*b*). These differences are being investigated in the light of the co-operative model discussed above.

(ii) O^c 666

This mutation (an A·T to G·C at +8) is at the position in which the functional group involved in recognition of O^+ is best defined. Extensive substitutions of modified bases at this siste indicated that high binding affinity correlated only with the presence of the thymine-methyl group (or a methyl group on a base analog in the equivalent position) in the major groove (Caruthers, 1980). Caruthers postulated that a hydrophobic contact between the protein and this particular methyl group was responsible for recognition at this site. Consequently, we have asked whether the thermodynamics of recognition of this O^c differ from the thermodynamics observed for O^+ in the manner expected for the loss of a hydrophobic interaction.

Studies on model compounds (cf. Tanford, 1980) suggest that the contribution of a single methyl group to the ΔG of transfer from a hydrocarbon to an aqueous environment is approximately 1 kcal/mol and that this is primarily an entropic effect in the temperature range investigated here. Studies on the thermodynamics of binding of amino acids by tRNA synthetases (Fersht *et al.*, 1980) indicate that the absence of a methyl group on the ligand results in a decrease of $\sim$3 kcal/mol in the binding free energy. This effect can be explained in terms of the release of structured water, not only from the vicinity of the methyl group on the ligand but from the protein binding site as well, and is very similar to the effect we observe for O^c 666. Studies on a mutant *mnt* repressor of phage P22 (Youderian *et al.*, 1983) have shown a similar decrease in affinity due to the loss of methyl groups from its binding site in the major groove. The operator recognized by this protein contains symmetrically disposed recognition sites for the *dam* methylase of *E. coli*. When operator DNA is prepared from *dam*$^-$ cells the binding affinity of the protein is reduced by approximately 500-fold from that observed when the sites are methylated. Although this effect is comparable to the effect that we observe, it results from a difference of four methyl groups between the *dam*$^+$ *versus dam*$^-$ *mnt* operator as opposed to a single methyl group in the case of O^+ *versus* O^c 666.

While the effect of O^c 666 on the standard free energy of complex formation is of the magnitude expected for the loss of a hydrophobic contact, closer examination of the enthalpic and entropic contributions to the binding free energies of O^c 666 and O^+ suggest that much more than the loss of a hydrophobic interaction is involved. The electrostatic term in the free energy is the same for the interaction of repressor with wild-type operator and O^c 666. However, the van't Hoff enthalpy of the interaction is more favorable than for O^+ (although still positive) by roughly 10 kcal/mol. Since enthalpies of transfer of model non-polar

solutes to water are invariably small in this temperature range, this large decrease in the ΔH_{obs} is incompatible with a simple hydrophobic explanation for the effect of this single base-pair change on the standard binding free energy.

(iii) *O^c 640*

The thermodynamics of complex formation at O^c 640 make it quite distinct from the behavior of O$^+$. The steep salt dependence of this reaction (the thermodynamic equivalent of 10 ± 1 ions are released upon binding) is reminiscent of the non-specific interaction. Analysis of the temperature dependence of the interaction shows a large positive enthalpy of approximately 16 kcal/mol between 5°C and 23°C. This is similar to that observed for the wild-type operator.

Caruthers' (1980) work on variant synthetic operator sequences suggested that the specific interaction of the protein with this base-pair could be attributed to hydrogen bonds involving either the four amino groups of cytosine or the six carbonyl groups of guanine. Perturbation of hydrogen bonding interactions might have a range of effects depending on the details of the exchange reactions between the free solvated protein and DNA molecules and the complex. In the extreme case of a net loss of one hydrogen bond (e.g., a group on the protein hydrogen bonded to water in the free protein and to a DNA base in the wild-type complex, which is buried without a partner in the mutant complex) the cost in free energy could be as large as 4 kcal to 5 kcal (Fersht *et al.*, 1985). The large change in the electrostatic component of the interaction upon mutation of this site, however, is not explainable by any scenario involving changes only in hydrogen bonding interactions.

At the simplest level of interpretation, the twofold increase in the stoichiometry of ion release (from 5 ions for O$^+$ to 10 ions for O^c 666) implies a corresponding twofold increase in the number of phosphate charges on the DNA neutralized by binding of the protein (from approximately 6 phosphates for O$^+$ to 12 phosphates for O^c 666). The loss of a single protein–DNA contact at the site of the O^c mutation could not in and of itself result in this more global change in the electrostatic component of the interaction. Steric distortion of the structure of the complex due to misaligned functional groups of mispositioned hydrogen-bonded water (which would be replaced by protein–DNA contacts in the complex with O$^+$) should, if anything, reduce the neutralization of the DNA phosphates and result in a reduced dependence on salt concentration (a smaller stoichiometry of ion release). We propose that *lac* repressor compensates for the loss of a key non-electrostatic interaction site on the DNA by adapting its binding surface to introduce new electrostatic contacts with the DNA phosphates. The fact that the observed salt dependence is that characteristic of the non-specific complex suggests that perhaps the protein is adopting a compromise conformation between the well-characterized extreme of specific and non-specific binding.

(d) *Different operators show distinct modes of recognition*

The interactions of *lac* repressor with O$^+$ and non-specific DNA show characteristically different dependences of salt, temperature and inducer concentrations (von Hippel, 1979, and references therein). The *lac* repressor protein has evolved to be adaptable, at least in these extremes. In order to obtain the needed degree of specificity (against an *in vivo* background of 10^7 non-specific sites), the protein binds to the operator site in a conformation that utilizes a substantial number of non-electrostatic contacts (comprising approximately 50% of the binding free energy at 0·2 M-NaCl). In interacting with the relatively featureless phosphate lattice of non-specific DNA, the protein appears to utilize a predominantly electrostatic binding mode in which the stoichiometry of ion release is roughly twice as large as that observed for O$^+$ (Record *et al.*, 1977). Our present results on the mutant operators, taken together, suggest a similar adaptability of recognition on a finer scale.

A primary conclusion of the present work is that the free energy of interaction of *lac* repressor with mutant operators cannot be explained on the basis of additive contributions from independent interactions with individual base-pairs. Adding up the free energy effects of three single base-pair variants investigated to date (neglecting 8 or so other recognition sites shown to be important by mutational analysis), one comes up with a larger effect than that distinguishing the wild-type operator from non-specific DNA. At 0·2 M salt the specific and non-specific binding affinities differ by approximately eight orders of magnitude (11 kcal/mol) (Lin & Riggs, 1975) while O^c 666, 640 and 630 account for 4, 5 and >5 kcal, respectively. Thus, the O^c mutants investigated cannot be considered independent in the sense that they represent simply the loss of a favorable interaction confined to the site of the mutant base-pair. The magnitudes of the effects observed suggest that the O^c mutations may introduce unfavorable interactions that distort the recognition surface of the complex beyond the point of contact with the mutant base-pair and therefore perturb the interaction of the protein with other bases. A corollary to this hypothesis is that the protein (and perhaps the DNA), when confronted with a major loss of interaction free energy due to long-range disruption of contacts, can actually adapt to reduce that loss (i.e. increase the binding affinity for a mutant site through a conformational adjustment; see discussion of O^c 640 and O^c 666 above). Although at this point we can only speculate concerning the nature and the magnitude of the structural differences between repressor–O^c complexes and those at O$^+$, the salient fact is that the differences at the thermodynamic level are very

large. The free energy of the interaction is the important determinant of activity, independent of structural considerations.

The discrepancies between the results obtained with short synthetic operator DNAs and larger operator molecules, noted above, might also be explained in terms of protein adaptability. If indeed the structure of the protein can conform to recognize different sequences, then the presence of flanking sequences, in addition to providing "non-specific" electrostatic contacts, may provide steric constraints that position the central part of the protein binding site optimally for recognition of the operator sequence. Thus there could be substantial shifts in the overall balance of forces due to the "indirect" effect of flanking non-specific DNA. Somewhat analogous behavior is seen in the end-binding modes of RNA polymerase (Melancon *et al.*, 1983) and T4 gene *32* protein (Kowalczykowski *et al.*, 1981) where the absence of flanking sequences leads to a higher affinity of the protein for an end than for an interior non-specific site.

(e) *Conformational adaptability: an integral part of specific recognition*

Nick *et al.* (1982*a*,*b*) have measured the effect of operator binding on ^{19}F tyrosine resonances in both isolated headpiece and intact repressor. The spectra obtained for tyrosines of free headpiece and in the amino terminus of intact repressor are very similar, indicating that the headpieces form essentially separate and highly mobile domains in the intact tetrameric structure. Upon binding a 36 bp operator fragment, the resonances of the intact repressor but not those of the free headpiece are broadened substantially (evidence that these regions of the protein are substantially immobilized in the complex). This broadening is not observed in interactions with similarly sized non-specific DNA fragments. The extent of broadening is too large to be caused merely by the increase in molecular weight due to operator binding. Since the binding of one 36 bp operator molecule per intact repressor is enough to broaden all four headpiece resonances simultaneously, the immobilization of the headpieces must be a consequence of structural changes in the protein rather than direct protein–DNA interactions.

Adaptability of the protein to the DNA site (or *vice versa*) also seems reasonable on purely energetic grounds. Privalov (1979), in a review of calorimetric studies on a number of small globular proteins, found them to be thermodynamically stable at 37°C by only 12 ± 5 kcal/mol. Furthermore, the stability reflects a delicate balance between large opposing enthalpic and entropic effects. Although the stability of *lac* repressor has not yet been determined calorimetrically, if one assumes that the magnitude of the free-energy change noted above is characteristic of small globular protein domains, one sees that it is comparable to the observed free energy of interaction with the wild-type operator. Under physiological conditions one can infer from the ratio of induced to constitutive expression of the wild-type *lac* operon, that the ratio of repressor–operator complex to free operator is approximately 10^3. This results in a free energy of binding (ΔG_b: Creighton, 1983) of -4 kcal/mol at this repressor concentration. Thus it seems quite likely that a compromise between the thermodynamically stable structure of the free protein and the structure optimal for recognition of a particular operator will occur and that the resulting changes in the opposing enthalpic and entropic contributions to stability could explain the complicated thermodynamic effects that we observe. In addition, the DNA site may adapt to the recognition surface of the protein. The stability of one turn of the double helix is also in the range of 10 kcal/mol at 37°C, and it has been demonstrated that repressor binding to O^+ results in a net unwinding of approximately 50° (Wang *et al.*, 1974). However, the complex thermodynamics that we observe appear difficult to explain in terms of the thermodynamics of distortions of DNA helices (cf. Record *et al.*, 1981). Thus we expect that adaptation of the DNA (if it occurs) is secondary to that of the protein.

The concept of adaptability in the recognition of mutant sequences may aid in the resolution of the long-standing controversy over the role of symmetry in the repressor–operator interactions. Chemical protection studies (Gilbert *et al.*, 1976) and binding studies with synthetic operator variants (Caruthers, 1980) have led to the conclusion that the interaction of repressor with operator is decidedly non-symmetric. On the other hand, the observations that two of the four identical subunits of the protein are involved in recognition of the operator (Kania & Muller-Hill, 1977), and recent work showing that a perfectly symmetric *lac* operator binds repressor more strongly than O^+ (Sadler *et al.*, 1983; Simons *et al.*, 1984), argue for the importance of symmetry (of the proper sequences). If adaptation of the protein to the particular sequence of its DNA site is considered to be an integral part of recognition, then it is not surprising that recognition of symmetrical and non-symmetrical operators is qualitatively different. We propose that the symmetrical nature of the protein is only manifest in its interactions with DNA sequences that are similarly symmetrical. The adaptations of the individual subunits to the non-symmetrical sequences of the wild-type or O^c operators are enough to disrupt the inherent symmetry of the protein.

We thank Bill Reznikoff, Lianna Munson, Jerry Yin, Paul Lambert and Debby Siegele for strains and advice. We acknowledge the generous gift of repressor protein from Kathy Matthews of Rice University. We appreciate comments on the manuscript from Bill Reznikoff, Pete von Hippel, Joan Betz and Bob Sauer. We thank Sue Mossing for careful typing of this manuscript. M.C.M. was

an NIH trainee in Cellular and Molecular Biology. This work was supported by National Institutes of Health grant GM23467.

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Edited by C. R. Cantor