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Quality and position of the three *lac* operators of *E.coli* define efficiency of repression

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Repression of the *lac* promoter may be achieved in two different ways: either by interference with the action of RNA polymerase or by interference with CAP activation. We investigated cooperative repression of the *Escherichia coli lac* operon by systematic conversion of its three natural operators (O1, O2 and O3) on the chromosome. We find that cooperative repression by tetrameric Lac repressor increases with both quality and proximity of the interacting operators. A short distance of 92 bp allows effective repression by two very weak operators (O3, O3). The cooperativity of *lac* operators is discussed in terms of a local increase of repressor concentration. This increase in concentration depends on flexible DNA which allows loop formation.

Key words: cooperativity/DNA loops/*lac* operator/Lac repressor/local concentration

Introduction

Cooperation between two distant DNA sites is frequently observed in transcriptional regulation in pro- and eukaryotic organisms. It occurs in both negative and positive regulation. For *Escherichia coli*, it has been shown that at least two operators are necessary for full repression of the *araBAD* operon (Dunn *et al.*, 1984), the *deo* operon (Dandanell *et al.*, 1987), the *gal* operon (Haber and Adhya, 1988) and the *lac* operon (Oehler *et al.*, 1990). In the *glnALG* operon, activation depends on at least two NR binding sites (Reitzer and Magasanik, 1986).

The effect of a repressor on expression from a particular promoter may be increased by cooperative interaction. This implies that deletion or inactivation of a distant operator may have a negative effect on the action of a promoter-proximal operator. A simple quantitative explanation for such an observation is that a promoter-distal operator increases the occupation of the proximal operator by DNA looping (Mossing and Record, 1986; Schleif, 1988, 1992; Adhya, 1989, Law *et al.*, 1993). The regulatory protein, which possesses two independent DNA binding sites, is then trapped in a 'chelate-like' complex (Hammer and Dandanell, 1989) between the two operators while the intervening DNA loops out. DNA loops have been shown to form *in vitro* for all of the above mentioned regulatory systems (Krämer *et al.*, 1987, 1988; Ninfa *et al.*, 1987; Amouyal *et al.*, 1989; Lobell and Schleif, 1990), except for the *gal* operator–Gal repressor interaction (Brenowitz *et al.*, 1990).

We have previously shown that the auxiliary operators O2 (Reznikoff *et al.*, 1974), which lies 401 bp downstream of O1, and O3 (Gilbert *et al.*, 1976), which lies 92 bp upstream of O1, contribute significantly to the repression of the *lac* operon, by cooperation with O1 (Eismann *et al.*, 1987; Oehler *et al.*, 1990; see Figure 1). Simultaneous binding of one Lac repressor tetramer to two *lac* operators (Borowiec *et al.*, 1987) is necessary for full repression of ~1000-fold (Oehler *et al.*, 1990). A DNA loop with either O1 and O2 or O1 and O3 is formed. Interestingly, the cooperative effect of each of the two auxiliary operators is about equally high, despite their different quality: O2 is weaker than O1 (Pfahl *et al.*, 1979; Winter and von Hippel, 1981; this work) and O3 is weaker than O2 (Pfahl *et al.*, 1979; Fried and Crothers, 1981; Winter and von Hippel, 1981; this work). The repression exerted by a particular operator depends on its affinity for Lac repressor and on the concentration of Lac repressor.

It has been shown that Lac repressor binds to the *lac* promoter region simultaneously with RNA polymerase (Schmitz and Galas, 1979; Straney and Crothers, 1987). Thus, the occupancy of an operator by Lac repressor at the position of O1 is directly correlated with its affinity for Lac repressor. For the influence of competition on binding by RNA polymerase see Lanzer and Bujard (1988). Repression of expression is defined as constitutive expression divided by expression under negative control of repressor. In a simple model, assuming that expression is proportional to the fraction of operator molecules free from repressor, the repression value F is related to the occupancy ($[RO]/[O]$) of an operator in the following way: $F = 1 + ([RO]/[O])$ (Sadler and Novick, 1965). Thus, repression values of 10 or greater can in practice be seen as directly correlated with the occupancy of an operator.

Here we present a detailed analysis of the influence of the qualities and distances of the *lac* operators on cooperative repression which, in our interpretation, depends on DNA loop formation.

Results

We constructed plasmids which put the *lacZ* gene under control of the natural *lac* promoter and the three *lac* operators (O1, O2 and O3). They were either unchanged or altered by site-directed mutagenesis. Each construct was cloned in phage λIP1 (Sieg *et al.*, 1989). Single lysogens of an *E.coli* strain carrying deletions of the *lacZ*

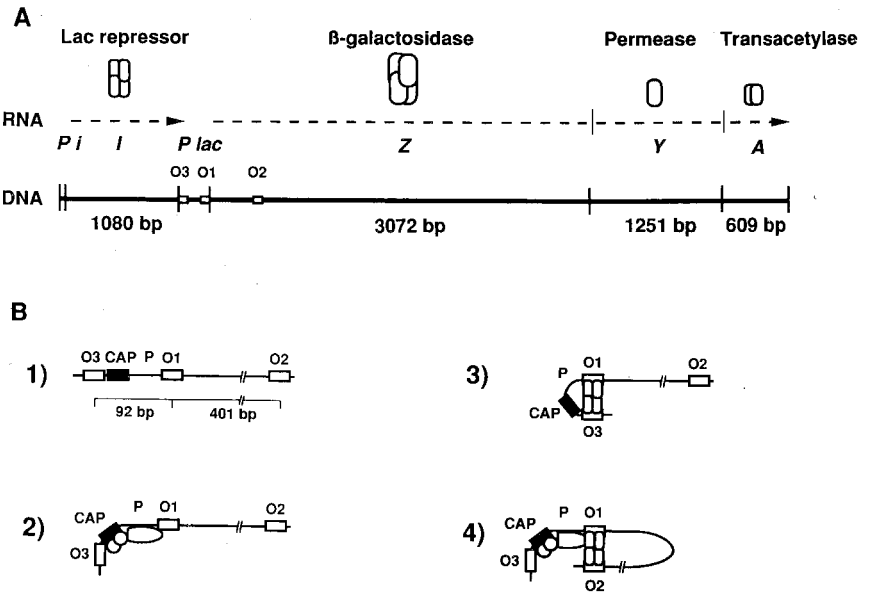


Fig. 1. The organization and control of the *E.coli* lac operon. (**A**) The *lacZ* (β-galactosidase), *lacY* (Lac permease) and *lacA* (Lac transacetylase) genes are transcribed from the *lac* promoter (P *lac*) as a polycistronic mRNA. Expression from the *lac* promoter is negatively controlled by the three *lac* operators O1, O2 and O3. The *lacI* gene, coding for Lac repressor is expressed from its own promoter (*Pi*) and lies upstream of the *lac* promoter. The length of the coding sequence (including the start codon) of each gene is given in bp. (**B**) The control elements of the *lac* promoter (1). The *lac* promoter is flanked upstream by a CAP binding site and downstream by O1. O3 lies immediately upstream of the CAP binding site and O2 is located in the coding region of *lacZ*. The distances from centre to centre of the *lac* operators are given in bp. Transcription, which is activated by the CAP protein, is constitutive in the absence of Lac repressor (2). Tetrameric Lac repressor forms DNA–protein–DNA loops in the absence of inducer. It binds simultaneously to O1 and O3 (3) or to O1 and O2 (4) and thereby blocks transcription efficiently.

and *lacI* genes (BMH 8117 F′) were then generated with these phages. Specific β-galactosidase activities were determined in bacteria grown in the absence and in the presence of different amounts (five times or 90 times i^+) of plasmid-encoded Lac repressor.

Comparison of the relative in vivo affinities of lac operators for Lac repressor

In order to evaluate the relationship between operator quality and its contribution to DNA loop formation *in vivo*, the relative quality of the operators had to be determined *in vivo*. To that purpose, we measured repression of β-galactosidase expression by Lac repressor in the presence of just O3, O2 or O1 at the position of O1 (O2 and O3 have been destroyed at their natural positions; see legend to Figure 2).

The natural auxiliary operators (O2 and O3) were inactivated by site-directed mutagenesis to make any cooperative interaction impossible. The first *lac* operator O1 was replaced by O2 or O3 or the fully symmetric 'ideal' *lac* operator (Sadler *et al.*, 1983; Simons *et al.*, 1984; Lehming *et al.*, 1987). See Figure 2 for the operator sequences. Figure 3 gives the repression values when about five times or ~90 times the i^+ amounts of dimeric or wild-type tetrameric repressor respectively are present. Dimeric Lac repressor is not encoded by the i^{adi} allele here, which we have described previously (Lehming *et al.*, 1988; Oehler *et al.*, 1990). Preliminary experiments indicate that i^{adi}-encoded dimeric Lac repressor dissociates into monomers at low concentrations (data not shown). We therefore constructed another more stable (data not shown) Lac repressor variant (Lac R 331 Stop), by the introduction of a stop codon at position 331 of the coding sequence of the *lacI* gene. This removes the 30 C-terminal

O1	5′- AATTGTGAGCGGATAACAATT -3′
O1⁻a	5′- gATTGTtAGCGGAgAAgAATT -3′
O1⁻b	5′- gAactacAtCctccgctAggT -3′
O2	5′- AAaTGTGAGCGagTAACAAcc -3′
O2⁻	5′- gAatGTtAatGaATAgCAccc -3′
O3	5′- ggcaGTGAGCGcAacgCAATT -3′
O3⁻	5′- AAcctcGAGCtcAacgCAATT -3′
Oid	5′- **AATTGTGAGC GCTCACAATT** -3′

Fig. 2. The sequences of the wild-type *lac* operators (O1, O2, O3) and of the 'ideal' *lac* operator Oid are aligned with the sequences of the destroyed operators (O1⁻a, O1⁻b, O2⁻, O3⁻). The destroyed operator O1⁻b has been introduced into construct λEwt 001 (Figure 5), but not λEwt 003 (Figure 5). Asterisks mark the centres of symmetry. The O2 sequences are written as the sequences of the lower strand of *lacZ* to align them with O1 and O3. Exchanges to create O2⁻ were restricted to silent mutations of the *lacZ* coding sequence. Of the possible exchanges, those that are most deleterious to repressor binding (Lehming *et al.*, 1987) were introduced. Lower case letters mark the positions in which the operators deviate from O1, except for Oid, which is shown in bold letters.

residues of Lac repressor which encompass both leucine heptad repeats (Oehler *et al.*, 1990; Alberti *et al.*, 1991). The heptad repeats of wild-type Lac repressor form a four helical bundle and thus enable wild-type Lac repressor to aggregate to tetramers (Alberti *et al.*, 1993).

The repression values of Figure 3 show that the affinity of O2 for Lac repressor is on average ~10-fold lower and

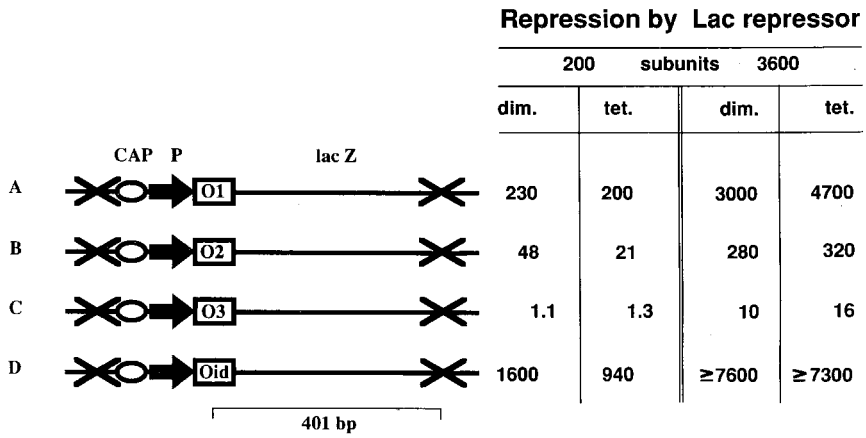


Fig. 3. Repression values for chromosomal (see Materials and methods) *lacZ* genes. The source of low amounts of Lac repressor (~200 monomers/cell) was pSO 331 Stop for dimeric (dim.) and pSO 1010-P1 for wild-type tetrameric (tet.) Lac repressor. The source of high amounts of repressor (~3600 monomers/cell) was pSO 3310 Stop for dimeric (dim.) and pSO 1000 for wild-type tetrameric (tet.) Lac repressor. Plasmid pSO 1000 ΔA carries an i^- allele (see Materials and methods). The combination of operators in each construct is shown schematically. The arrow represents the *lac* promoter. The ovoid symbolizes the CAP binding site. The active *lac* operators are drawn as open boxes, inactivated operators are indicated by crossed bars. Repression is defined as specific activity of β-galactosidase in the absense of active Lac repressor divided by the specific activity in the presence of the indicated Lac repressor (Miller, 1972). A, λEwt 100; B, λEwt 200; C, λEwt 300; D, λEst i00.

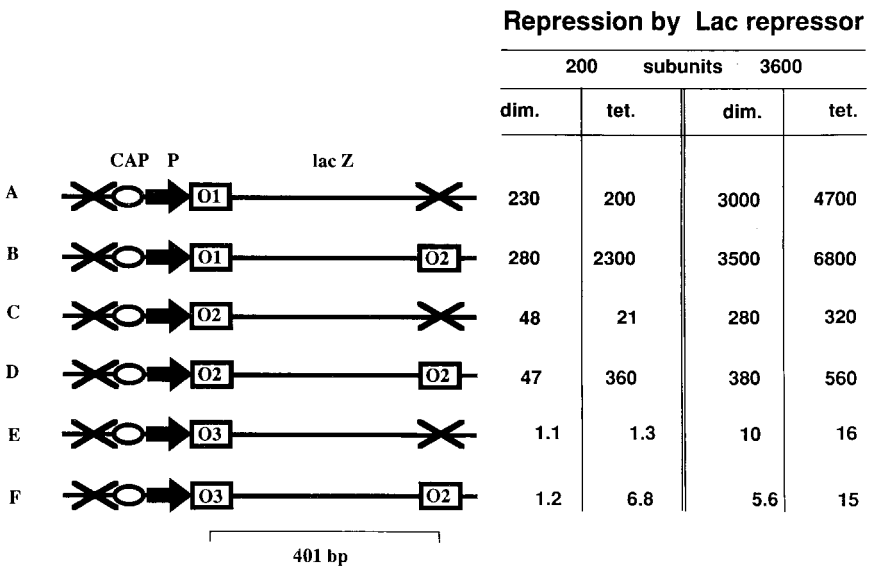


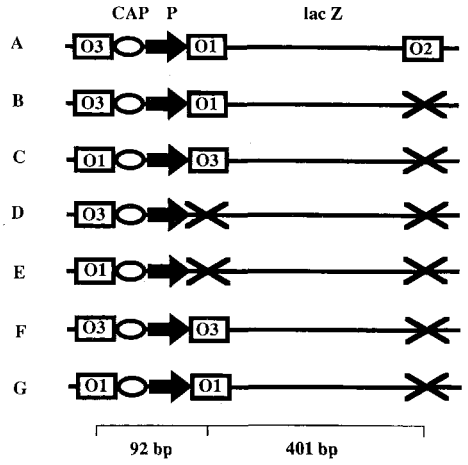
Fig. 4. Repression values for chromosomal (see Materials and methods) *lacZ* genes. Repression values are given as the specific β-galactosidase activity in the absence of active Lac repressor divided by the specific activity in the presence of the indicated Lac repressors. For details see legend to Figure 3. A, λEwt 100 (from Figure 3); B, λEwt 120; C, λEwt 200 (from Figure 3); D, λEwt 220; E, λEwt 300 (from Figure 3); F, λEwt 320.

that of O3 is ~300-fold lower than that of O1. In contrast, Oid binds repressor ~6-fold better than wild-type O1. These results are in general agreement with *in vitro* measurements. The *in vitro* affinity of O2 has been determined to be 1/10 of that of O1 (Pfahl *et al.*, 1979; Winter and von Hippel, 1981). The *in vitro* affinity of O3 has been determined to be between 16- and 1000-fold lower than that of O1 (Pfahl *et al.*, 1979; Fried and Crothers, 1981; Winter and von Hippel, 1981). The *in vitro* affinity of the ideal operator was found to be ~8-fold greater than that of O1 (Sadler *et al.*, 1983; Simons *et al.*, 1984).

The role of O2 in repression

We have shown that the natural O2 alone, in the absence of O1 and O3, works very inefficiently, if at all, as a roadblock for RNA polymerase (Oehler *et al.*, 1990). Therefore, the effect which O2 exerts in the presence of a second operator at the position of O1 is predominantly due to cooperative interaction, i.e. DNA loop formation. An observed increase in repression by the addition of O2 should result from cooperative increase of occupation of O1 or any other operator at the position of O1. Figure 4 gives the repression values of O1, O2 and O3 at the natural location of O1 in the presence of O2. For comparison, the values for the constructs without the natural O2 from Figure 3 are also listed.

Dimeric Lac repressor is not able to form DNA loops (Oehler *et al.*, 1990). Consequently, it shows virtually the same repression with and without the second operator, at both low and high Lac repressor concentrations. In contrast, repression by wild-type tetrameric Lac repressor



Repression by Lac repressor

	200 subunits		3600 subunits	
	dim.	tet.	dim.	tet.
A	460	8100	8500	≥ 19000
B	730	6100	≥ 11000	≥ 21000
C	18	890	144	3900
D	1.3	1.7	4.0	25
E	6.3	18	11	28
F	2.9	38	96	960
G	3900	≥ 12000	≥ 24000	≥ 46000

Fig. 5. Repression factors for chromosomal (see Materials and methods) *lacZ* genes. Repression values are given as the specific β-galactosidase activity in the absence of active Lac repressor divided by the specific activity in the presence of the indicated Lac repressors. For details see legend to Figure 3. A, λEwt 123; B, λEwt 103; C, λEwt 301; D, λEwt 003, this construct carries the operator mutant O1⁻a; E, λEwt 001, this construct carries the operator mutant O1⁻b which has been inserted to exclude any residual operator activity; F, λEwt 303; G, λEwt 101.

increases at low concentration with the addition of the second operator. With high concentrations of repressor, little effect of O2 can be seen. This is in agreement with the fact that at high repressor concentrations, DNA loops break up and cooperativity is lost (Krämer *et al.*, 1987).

The cooperative effect of the second operator on O1 or O2 at the position of O1 is quite similar, namely 12- and 18-fold. The increase of repression by O3 at the position of O1 through cooperation with O2 cannot be directly determined, since repression of construct λEwt 300 (Figure 4, line E) by five times i^+ amounts of repressor is too low to be accurately measured. Ninety times i^+ amounts give a 16-fold repression with O3 at the position of O1 (λEwt 300, Figure 4, line E). Since the occupancy $[RO]/[O]$ of an operator will increase linearly with an increase of Lac repressor, we can estimate that repression with five-fold i^+ amounts should be 1.8. Then, the cooperativity of the second operator with O3 is ~7-fold, which is not far from the effect with O1 or O2.

The role of O3 in repression

When O2 at its natural position is inactivated, repression by low amounts of tetrameric repressor does not drop, because of the interaction between O1 and O3 (λEwt 123, Figure 5, line A and λEwt 103, Figure 5, line B). Naively, one might expect that, since cooperation is mutual, O1 and O3 could be exchanged without any effect on repression. But Figure 5, line C (λEwt 301) shows that repression drops ~6-fold, although it still remains relatively high compared with repression with dimeric Lac repressor. Repression of λEwt 001 (Figure 5, line E) reveals that part of the repression of λEwt 301 (Figure 5, line C) is obviously due to interference with CAP activation by Lac repressor bound at the position of O3.

This construct (λEwt 001, Figure 5, line E) indeed illuminates the difference between true repression and deactivation. While repression increases linearly with increasing amounts of Lac repressor, deactivation can maximally abolish the effect of an activator. In this case, deactivation by high amounts of Lac repressor is ~30-fold, which is close to the 50-fold reported value of CAP activation of *lac* promoter (Hopkins, 1974). Interestingly, dimeric repressor deactivates less well than wild-type tetrameric Lac repressor at both low and high concentration. This could be explained by reduced steric hindrance of CAP binding by the smaller repressor protein. We have previously shown that high amounts of Lac repressor exert repression even with the natural O3 in the absence of both O1 and O2 (Oehler *et al.*, 1990). This apparent repression is in fact deactivation by competition with binding of CAP to its target site immediately downstream of O3. In the absence of the CAP protein there is no repression of construct Ewt 001 by Lac repressor (Sundarp, 1993). If we take an ~20-fold deactivation into account, an ~40-fold true repression of construct λEwt 301 (Figure 5, line C) remains. Obviously, in a DNA loop the operator which blocks polymerase, rather than the overall strength of the cooperative interaction, determines repression. This reflects the fact that the loop is asymmetrically breathing. When O1 is now restored to its natural position (λEwt 101, Figure 5, line G), repression is increased with respect to the natural situation (λEwt 103, Figure 5, line B). Repression by dimeric repressor, where there should be no cooperativity, is also considerable here. Thus, in construct λEwt 101 also (Figure 5, line G), both real repression and deactivation contribute to apparent repression. What if O3 is now restored to its natural position and combined with another O3 at the position of O1 (λEwt 303, Figure 5, line F)? Cooperativity can still be observed.

It is interesting to note that while each O3 alone exerts no measurable effect (λEwt 300, Figure 4, line E and λEwt 003, Figure 5, line D), repression by the combination of both (38-fold) is even better than the effect of one O2