J. Mol. Biol. (1996) **257**, 21–29

JMB

Repression of *lac* Promoter as a Function of Distance, Phase and Quality of an Auxiliary *lac* Operator

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The tetrameric Lac repressor can bind simultaneously to two *lac* operators on the same DNA molecule, thereby including the formation of a DNA loop. We investigated the phasing dependence of DNA loop formation between *lac* operator O_1 and an auxiliary ideal *lac* operator (O_{id}) on the bacterial chromosome, with inter-operator distances varying from 57.5 to 1493.5 bp. Repression of a CAP-independent *lac* UV5 promoter by O_1 at its natural position increased up to 50-fold in the presence of an optimally positioned auxiliary O_{id}. Repression values alternated between local maxima and minima with a periodicity of 11.0 to 11.3 bp, suggesting that the chromosomal helical repeat is in this range *in vivo*. Repression increased significantly with decreasing inter-operator DNA length, indicating that the local Lac repressor concentration at O_1 is crucial for tight repression. Maximal repression, attributed to stable DNA loop formation, was obtained at an operator spacing of 70.5 bp. Other repression maxima occurred at operator distances of 92.5 and 115.5 bp, corresponding to natural operator spacings in the *lac* and in the *gal* operon, respectively. Substitution of the auxiliary O_{id} with the weaker binding *lac* operator O_3 lowered repression efficiency, presumably due to the reduced local concentration of Lac repressor.

Keywords: DNA looping; repression; *lac* operator; Lac repressor; DNA helical repeat

Introduction

The formation of protein-mediated DNA loops plays an important role in transcriptional control in both prokaryotes and eukaryotes. Well documented examples in *Escherichia coli* include the operons of *ara*BAD (Dunn *et al*., 1984; Lee & Schleif, 1989), *deo* (Dandanell *et al*., 1987), and *lac* (Mossing & Record, 1986; Krämer *et al*., 1987; Oehler *et al*., 1990, 1994; Law *et al*., 1993). Induction of the *lac* operon leads to an increase of about 1000-fold in the level of β-galactosidase. Here, effective downregulation of transcription in the absence of inducer is mediated by the promoter–proximal operator O_1 and the two auxiliary operators O_2 and O_3, located 401 base-pairs (bp) downstream of O_1 within the coding region of the *lacZ* gene and 92 bp upstream of O_1, respectively. The operators are not of equal quality: the *in vivo* affinity of the Lac repressor for O_2 is tenfold lower than the affinity for O_1, while the Lac repressor binds O_3 300 times more weakly than O_1 (Oehler *et al*., 1990). Full repression can only be obtained by the simultaneous binding of one tetrameric Lac repressor to the strong operator O_1 and to either O_2 or O_3, creating one of two alternative DNA loops. In the presence of O_1 alone, when no DNA loop can be formed, repression decreases about 50-fold (Oehler *et al*., 1990). Interestingly, when two very weak operators (O_3) are placed 92 bp apart, with one of them replacing O_1, repression is stronger than with one individual operator O_2 in the place of O_1 (Oehler *et al*., 1994). The observation that cooperative interaction between two weak operators, both of which exert almost no repression by themselves, may repress a promoter to the same or an even higher extent than an isolated stronger operator, is best explained by thermodynamic considerations. According to this concept, the occupation of an auxiliary operator with Lac repressor increases the local concentration

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Abbreviation used: IPTG, isopropyl-β-D-thio-galactopyranoside.

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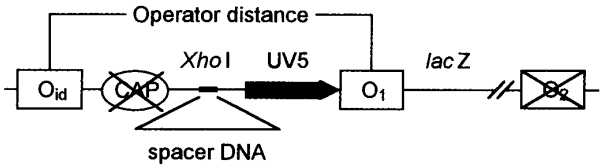


Figure 1. Representation of the *lac* constructs integrated in the bacterial chromosome (not drawn to scale). Active *lac* operators are drawn as boxes and the inactivated O_2 is symbolised by a crossed box. O_{id} is the ideal *lac* operator (Sadler *et al*., 1983; Simons *et al*., 1984). The inactivated CAP binding site is shown as a crossed oval. The CAP-independent UV5 *lac* promoter is represented by an arrow. Operator distance is counted from centre to centre of O_{id} and O_1. The length of spacer DNA between the two active operators was varied by inserting DNA sequences of different length into the unique *Xho*I restriction site.

O_1 5'- **AATTGTGAGC*****G**G**A**T**AACAATT**
O_1^- 5'- g**A**a c t a c**A** t **C** c t c c g c t **A** g g **T**

O_2 5'- **AA**A**TGTGAGC*****G**A**G**T**AACAA**C C
O_2^- 5'- g**A**A g **GT** t **A** a t G A a T A g **CA** c C C

O_3 5'- G G C A**GTGAGC*****G**C A A C**G**C**AATT**

O_{id} 5'- **AATTGTGAGC** * **GCTCACAATT**

Figure 2. The sequences of the *lac* operators. Natural *lac* operators O_1 and O_2 are aligned with the respective inactivated operators and with the ideal *lac* operator. The O_3 sequence was not destroyed but deleted. Centres of symmetry are indicated by asterisks. Positions in which the inactivated operators differ from the respective wild-type sequences are marked by lower case letters. Positions in which the wild-type *lac* operators are identical with the ideal *lac* operator (O_{id}) are typed in bold letters.

of repressor relative to the promoter–proximal operator site, and thus increases the probability of occupation of that operator. The result is a tighter repression than if only one operator was available (Mossing & Record, 1986; Schleif, 1987; Schleif, 1987; Law *et al*., 1993; Oehler *et al*., 1994).

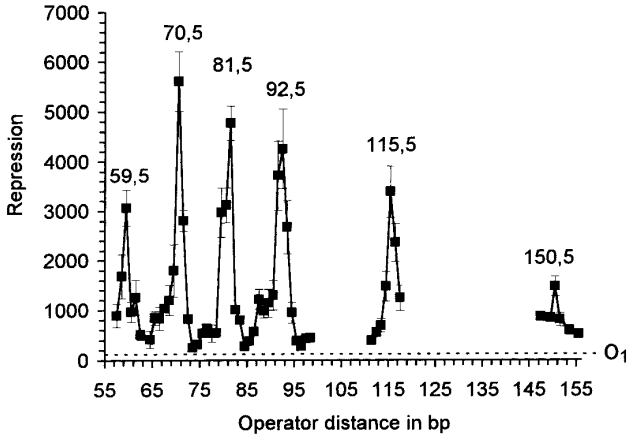
In addition to the energy required to bend the DNA into a loop structure, torsion energy is necessary when two interacting operators are located on opposite sides of the DNA surface, so that loop formation is energetically favoured when the operators are in phase (Wang & Giaever, 1988). Therefore, upon successively changing the distance between two operators, an alternation between repression maxima and minima is expected *in vivo*. The repression maxima are presumably caused by the formation of stable DNA loops, with a periodicity that indicates the alignment of the two operators on the same side of the DNA surface or, in other words, the helical repeat of the DNA stretch examined. A periodic *in vivo* repression with Lac repressor and two *lac* operators, separated by 100 to 200 bp, has been reported (Bellomy *et al*., 1988; Bellomy *et al*., 1988; Law *et al*., 1993; Law *et al*., 1993). Since these studies were performed using plasmids and high amounts of Lac repressor, it was desirable to analyse repression under conditions closer to the natural situation. In a detailed approach, we have systematically varied the distance between two *lac* operators from 57.5 bp to about 1500 bp on the bacterial chromosome. In addition, at a given spacing, the operators were altered in their quality. In both cases *in vivo* repression was examined with tetrameric Lac repressor ranging from two to five times the wild-type amounts.

Results

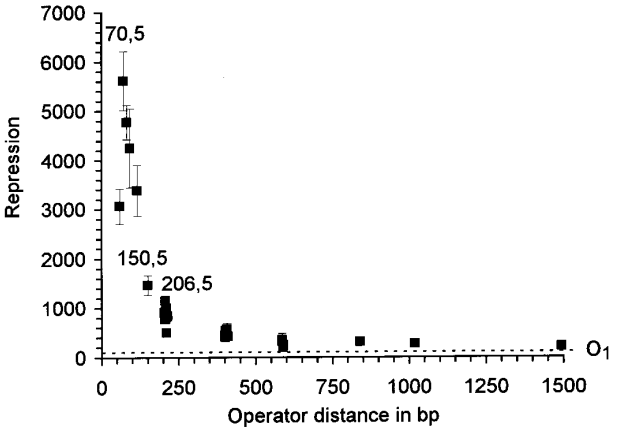
We constructed a set of *E. coli* strains, each bearing a *lac*Z gene under the control of the CAP-independent UV5 promoter (Figure 1). The *lac*Z constructs were integrated into the bacterial chromosome on λ prophages. The CAP binding site of the promoter was deleted and the auxiliary *lac* operator O_3 replaced by an ideal *lac* operator (O_{id}) at various distances from O_1. The *lac* operator O_2 within the coding region of *lac*Z is also absent. The DNA sequences of the *lac* operators are shown in Figure 2. We favoured a CAP-independent promoter for this series of experiments, to avoid any possible interference with DNA loop formation by the Lac repressor caused by DNA binding, bending and transcriptional activation by the CAP protein.

Insertions of 57.5 to 117.5 bp between an auxiliary ideal *lac* operator and O_1 display a strong repression periodicity

Since the intervening DNA between two *lac* operators was not shorter than 115 bp in previous *in vivo* studies (Bellomy *et al*., 1988; Law *et al*., 1993), we first concentrated on phasing analysis of smaller inter-operator distances. The intervening DNA length was varied between 57.5 bp and 117.5 bp. A spacing of less than 57.5 bp can not be examined in our test system, as at this distance the auxiliary O_{id} abuts the –35 box of the promoter. Repression values of constructs O_{id} 57.5 O_1 to O_{id} 117.5 O_1 are shown as a function of the operator distance in Figure 3(A). The observed repression displays a strong phasing dependence, consistent with the spacing requirements of the formation of operator–protein–operator complexes (DNA loops). Unfavourable operator spacing causes repression to drop to nearly the same low level that is observed with O_1 in the absence of any auxiliary operator. Maxima of repression are found at inter-operator distances of 59.5, 70.5, 81.5, 92.5 and 115.5 bp. Maximum repression is seen at 70.5 bp, corresponding to a 50-fold increase of repression, while the



(A)



(B)

Figure 3. Repression values for chromosomal *lac*Z genes under control of an O_1 at its natural position and an auxiliary O_{id} at the distances indicated. Five times wild-type amounts of Lac repressor (50 tetramers / cell) were encoded by plasmid pSO1010-P1. Repression is given as specific activity of β-galactosidase in the absence of active Lac repressor (encoded by plasmid pSO1000ΔA) divided by specific activity of β-galactosidase in the presence of active Lac repressor. Error bars represent standard deviation. The broken line indicates the extent of repression with a single O_1 at its natural position. (A) Inter-operator distances are 57.5 to 98.5 bp; 111.5 to 117.5 bp; 147.5, 149.5, 150.5, 151.5, 153.5, and 155.5 bp. (B) Inter-operator distances in bp: 57.5; 70.5; 81.5; 92.5; 115.5; 150.5; 203.5, 205.5, 206.5, 207.5, 208.5, 209. 5, 210.5, 212.5, 214.5; 400.5, 401.5, 405.5, 407.5, 409.5; 585.5, 586.5, 588.5, 589.5; 838.5; 1018.5; 1493.5. Repression values of inter-operator distances from 59.5 to 150.5 bp are the same as in (A); for clarity, only repression maxima are shown.

smallest increase is found at 59.5 bp, corresponding to a 25-fold increase of repression.

Influence of inter-operator distances up to 1500 bp on local Lac repressor concentration

Figure 3(A) shows that, beginning with a spacing of 70.5 bp, the maxima of repression decrease with increasing inter-operator distances. Therefore, an upper limit for operator cooperativity is expected. We examined the decrease of repression at inter-operator distances of up to 1493.5 bp. Repression values are displayed in Figure 3(B). Cooperativity drops sharply with increasing DNA length, decreasing from 50-fold at 70.5 bp to 15-fold at 150.5 bp and to threefold at around 600 bp inter-operator distance. A twofold increase of repression remains for all distances exceeding 600 bp and is still observed at 1493.5 bp, the longest distance examined. Repression at a spacing of around 200 bp is phase dependent, varying by about twofold depending on whether operators are in phase (206.5 bp) or not (Figure 3(B)). For distances exceeding 400 bp, phasing was no longer observed. We therefore restricted the analysis of longer inter-operator distances to single constructs.

Table 1. Repression values and percentage repression upon substitution of an auxillary idea *lac* operator with natural operators

Chromosomal construct			5 Times i^+ LacR (Repression)	(%)	2 Times i^+ LacR (Repression)	(%)
Oid	91,5	O1	3900	100	950	100
O1	92	O1	4200	108	1100	116
O2	92	O1	3300	85	700	74
O3	92	O1	1300	33	140	15

The auxillary O_{id} at the natural position of O_3 (92 bp inter-operator distance) was replaced by the natural *lac* operators O_1, O_2 and O_3 by PCR-directed mutagenesis. Repression values were determined with five or two times the wild-type amounts of wild-type Lac repressor, as indicated.

The quality of an auxiliary *lac* operator influences local Lac repressor concentration

We analysed the effect of operator quality at a distance of 91.5 bp upstream of O_1, which corresponds to the spacing of O_3 in the wild-type *lac* operon (92 bp). In place of the auxiliary operator O_{id}, the sequences of either O_1, O_2 or O_3 were inserted. Repression of the UV5 promoter for all constructs was determined in the presence of five times and two times the wild-type amounts of Lac repressor. Table 1 gives the repression values as percentage of repression obtained with O_{id} as the auxiliary operator. O_{id} and O_1 show essentially the same repression with either high or low repressor concentration. With high amounts of Lac repressor, repression by an auxiliary O_2 is similar to that obtained with O_{id}, but is reduced with two times the wild-type amounts of repressor. With the weak auxiliary O_3, repression with five times the wild-type amounts of Lac repressor is still about one third as efficient as with O_{id}, whereas with the lower Lac repressor concentration of two times the wild-type amounts, we observe only 15% of the repression obtained with an auxiliary O_{id}.

Discussion

Cooperative repression depends on operator phase

When a tetrameric Lac repressor binds to two *lac* operators on the same DNA molecule, a DNA loop is formed. The efficiency of loop formation has been shown to depend on operator phase both *in vitro* (Krämer *et al*., 1987) and *in vivo* (Bellomy *et al*., 1988; Law *et al*., 1993). *In vivo*, loop formation is demonstrated indirectly by an alternating pattern of repression maxima and minima, where repression maxima are assumed to reflect alignment of the operators on the same side of the DNA (Figure 3(A) and (B)). Since the physical resistance of DNA to torsion decreases with increasing length (Shore *et al*., 1981; Shore & Baldwin, 1983a), the requirement for operator phasing becomes less stringent upon lengthening the intervening DNA. At an operator spacing of about 200 bp, a weak phasing effect is still observed. Phasing disappears for inter-operator distances of 400 bp and longer (Figure 3(B)).

The helical repeat *in vivo* differs from the value *in vitro*

Because repression maxima indicate operator alignment on the same side of the DNA, the repression values presented in Figure 3 allow an estimation of the helical repeat *h* of the chromosomal DNA examined. However, depending on the number of repression peaks considered, different values may be obtained. The repression peaks from 59.5 to 92.5 bp fit a periodicity of 11.0 bp per full turn of the DNA helix. When the additional repression peak at a distance of 115.5 bp is taken into account, the calculated value is 11.1 bp (Figure 3(A)). Further consideration of the repression peaks at 150.5 and 206.5 bp leads to an average value of 11.3 bp (Figure 3(B)), if an error of 0.5 bp for every particular repression peak is taken into account because only integral bp steps are possible.

A value for *h* of 11.0 to 11.3 bp differs considerably from the value of 10.5 bp that was determined for relaxed linear *B*-DNA *in vitro* (Shore & Baldwin, 1983b; Horowitz & Wang, 1984; Goulet *et al*., 1987). However, it resembles other *in vivo* experiments, where a helical repeat of 11.1 bp (Lee & Schleif), 11.2 bp (Haykinson & Johnson, 1993), or 11.3 bp (Law *et al*., 1993) was observed. A deviation of the helical repeat *in vivo* from the *in vitro* value is to be expected as a result of a different DNA topology (Rhodes & Klug, 1981; Bliska & Cozzarelli, 1987; Liu & Wang, 1987; Drlica & Rouviere-Yaniv, 1987; White *et al*., 1988; Wu & Liu, 1991). Furthermore, the helical repeat of a given DNA may not be uniform. Although the value of 11.3 bp for *h* matches all prominent repression maxima between 59.5 and 206.5 bp, we note that this is not necessarily the accurate value for the whole DNA stretch examined. It is an average value over about 150 bp, and there may well be local deviations.

The repression peak at an operator spacing of 59.5 bp (Figure 3(A)) indicates that here the operators are in phase. However, division of 59.5 bp by $h = 11.0$ to 11.3 bp yields 5.2 to 5.4 and not an integral number, which would correspond to full helical turns. The *in vivo* results reported by Record and co-workers (Bellomy *et al*., 1988; Law *et al*., 1993) displayed a similar deviation from integral helix turns, which was not observed *in vitro* (Krämer *et al*., 1987, 1988) and suggest an *in vivo* situation different from that *in vitro*.

Law *et al*. (1993) quantitatively analysed the cooperative repression of plasmid borne *lac*Z genes under the control of two *lac* operators, with inter-operator distances varying between 127 and 197 bp. This analysis led to a description of physical parameters of the inter-operator DNA. Applying their method to our data for operator spacing varying between 57.5 and 117.5 bp, we obtain strikingly similar values (the values of Law *et al*. (1993) are given in parentheses) for the helical repeat, $h = 11.2$ bp (11.28 ± 0.04), the torsional rigidity, $C = 1 \times 10^{-19}$ erg cm ($(1.1 \pm 0.1) \times 10^{-19}$ erg cm), and the apparent persistence length, $a_{app} = 20$ bp (19 ± 8 bp). Only the fractional twist, $\phi = 0.29$ (0.074 ± 0.044), differs significantly from that obtained by Law *et al*. (1993). This difference is possibly due to their constructs being based on a CAP-dependent, wild-type promoter on plasmids, while our constructs contain a CAP-independent *lac* UV5 promoter located on the bacterial chromosome. It is highly unlikely that these promoters will share the same geometry *in vivo*.

The most stable DNA loop is formed at an inter-operator distance of 70.5 bp

A tetrameric Lac repressor bound to an auxiliary operator is located within a distance relative to the primary operator that is determined by the length of the intervening DNA. To estimate the probability of binding of the second Lac repressor DNA binding domain to the primary operator, one may simply assume that the repressor bound to the auxiliary operator is trapped in a sphere with a radius corresponding to the inter-operator distance. The resulting local repressor concentration relative to O_1, as a function of the distance of an auxiliary operator, is shown in Figure 4(A). The predicted increase of local repressor concentration is consistent with the increasing repression values shown in Figure 3(B). Upon addition of the auxiliary O_{id}, repression is enhanced from twofold at around 600 bp, over 15-fold at 150.5 bp, to 50-fold at 70.5 bp operator distance. However, further shortening of the intervening DNA to 59.5 bp yields a twofold decrease of repression. We assume that this is due to the geometry of the tetrameric Lac repressor. If a length of 130 Å for the Lac repressor (Weber *et al*.,

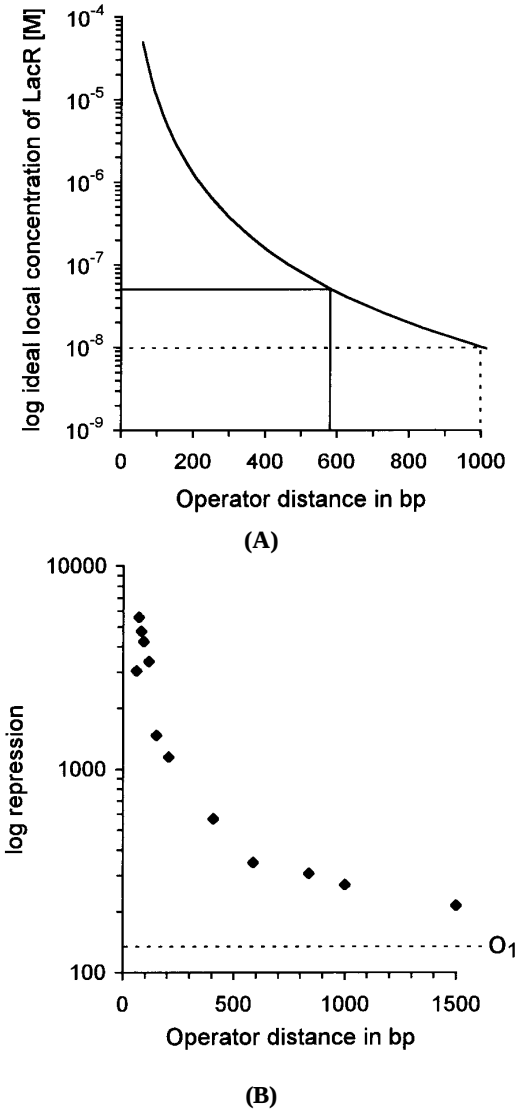


Figure 4. (A) The calculated local Lac repressor concentration as a function of the inter-operator distance under ideal conditions. The operator distance where local Lac repressor concentration equals the overall cellular Lac repressor concentration is indicated by a broken line for wild-type amounts (1×10^{-8} M) or a continuous line for five times the wild-type amounts (5×10^{-8} M) of Lac repressor tetramers, respectively. (B) Repression values for chromosomal *lac*Z genes under control of an O_1 at its natural position and an auxiliary O_{id}. Operator distances and repression values are the same as in Figure 3, but only local repression maxima are shown. The broken line indicates the extent of repression with a single O_1 at its natural position.

1982) is regarded as the diameter of the semicircle formed by a DNA loop, the circumference of that semicircle is then about 200 Å or 60 bp. Loop formation with a DNA of this length would be energetically unfavourable and therefore less probable than with a longer intervening DNA, which is in agreement with a model deduced from X-ray analysis of an isopropyl-β-D-thiogalactopyranoside (IPTG)-complexed Lac repressor core (Friedman *et al*., 1995). Krämer *et al*. (1987) obtained a DNase sensitivity pattern characteristic of loop formation with two operators separated by six helical turns with linear DNA *in vitro*, while only a weak pattern was observed for 52 bp, corresponding to five helical turns. In the same study, looped DNA–Lac repressor complexes were detected in gel retardation experiments with an inter-operator distance of 63 bp, but not 52 bp.

In contrast to previous *in vitro* studies examining self-ligation efficiency as a function of DNA length (Shore *et al*., 1981; Shore & Baldwin, 1983a), our *in vivo* results suggest, through the existence of stable DNA loops at an operator distance of 70.5 bp, a high degree of flexibility of the intervening DNA. Small DNA loops were also observed in other studies (Bellomy *et al*., 1988; Law *et al*., 1993). Several factors may influence DNA flexibility *in vivo* that are not involved in the *in vitro* ligation-rate experiments.

In a Lac repressor-mediated loop, the DNA is connected by a protein bridge and therefore would have to be bent to a reduced extent. In addition, DNA is highly complexed with polyamines and histone-like proteins *in vivo* (Gosule & Schellmann, 1978; Bloomfield, 1991; Pettijohn, 1982; Drlica & Rouviere-Yaniv, 1987). Binding of these factors increases DNA flexibility and promotes the cyclisation of DNA fragments with a length of about 100 bp (Hodges-Garcia *et al*., 1989), as well as facilitating recombination processes (Johnson *et al*., 1986; Haykinson & Johnson, 1993). Moreover, negative supercoiling leads to an increase in DNA flexibility, and an increased superhelical density supports the formation of DNA loops (Borowiec *et al*., 1987; Whitson *et al*., 1987a,b; Sasse-Dwight & Gralla, 1988; Krämer *et al*., 1988; Eismann & Müller-Hill, 1990).

Local Lac repressor concentration determines DNA loop formation

The primary effect of an auxiliary operator is to enhance the occupation of the primary operator O_1 by increasing the local Lac repressor concentration (Bellomy & Record, 1990; Oehler *et al*., 1994). At wild-type Lac repressor concentration (10^{-8} M; Gilbert & Müller-Hill, 1966) and an inter-operator distance of 92 bp, the increase in local Lac repressor concentration due to full occupation of the auxiliary operator is 1300-fold above the wild-type repressor level if bending resistance of the DNA is neglected (Figure 4(A)). At the distance of O_2, 401 bp from O_1, the maximal possible local increase of repressor is less than 20-fold. At a distance of 1000 bp, cooperative operator interaction can still lead to an increase in the local repressor concentration of twofold in the wild-type situation. With the five times higher Lac repressor amounts we used in this study (5×10^{-8} M), a similar effect is expected, although the cooperativity between the operators is reduced due to the overall increase of repressor

concentration. Under ideal conditions, with five times wild-type amounts of Lac repressor, a twofold concentration increase is calculated for an inter-operator distance of 586 bp. Experimentally, we observed an approximately threefold increase of repression at this distance and a twofold increase for all longer inter-operator distances (Figure 4(B)). Similarly, with two times wild-type amounts of Lac repressor, cooperativity over long distances is enhanced compared with five times Lac repressor amounts (data not shown). The small deviation from the predicted ideal value may be attributed to the nucleoid organisation of the bacterial chromosome, which consists of about 50 large DNA folds (Pettijohn, 1982). This folding physically divides the DNA into discrete domains that may hinder complete dissociation of Lac repressor once bound to an operator.

Interestingly, repression maxima are observed at operator distances that resemble the natural situation in the *lac* and *gal* operons. In the *lac* operon, O_1 and O_3 are separated by 92 bp (Gilbert *et al*., 1976), while the *gal* operators O_E and O_I are separated by a distance of 114 bp (Irani *et al*., 1983; Fritz *et al*., 1983). We report repression maxima at inter-operator distances of 92.5 and 115.5 bp, respectively (Figure 3(A)). One would expect that in the *lac* operon, a weak operator like O_3 should be located in an optimal position to interact with O_1. Considering that the presence of the CAP binding site in the natural situation (– 61.5 bp upstream from the start of transcription) precludes a smaller inter-operator distance, nature has indeed established the best possible position for O_3 within the spatial limits. The location of O_2 within the coding region of *lac*Z is not subjected to phasing constraints, as indicated in Figure 3(B).

A strong operator should exert a higher cooperativity than a weaker sequence at the same position. As presented in Table 1, the substitution of the auxiliary ideal operator at the natural position of O_3 with *lac* operators O_1 or O_2, which are occupied 95 to 100% at five times wild-type amounts of Lac repressor (Oehler *et al*., 1994), results in virtually unchanged repression values compared to O_{id} (the slightly increased repression with the comparable strong O_1 instead of O_{id} may be due to a more favourable operator spacing). However, the presence of the weak O_3, with an occupancy of about one third under these conditions, leads to a correspondingly lower repression. This effect is increased when reduced repressor amounts are used. With two times wild-type amounts of Lac repressor, the relatively strong operator O_2 is still occupied most of the time, while occupancy of O_3 is only around 15%. This is reflected in the repression efficiency that remains approximately 80% for O_2 but drops sharply for O_3 (Table 1).

Comparison of the predicted local increase of Lac repressor (Figure 4(A)) with the observed repression values (Figure 4(B)) shows that these values lie within a factor of 2 for inter-operator spacings larger than 200 bp (Figure 5), reflecting a large lateral flexibility of the intervening DNA. Conversely, for shorter distances the disparity rises reciprocally with the operator distance from a factor of 5 at 115.5 bp up to a factor of 13 at 70.5 bp. This divergence indicates the increasing resistance of shorter DNA to bending, suggesting severe physical constraints for DNA of that length. An even higher disparity factor of 37 is obtained for the distance of 59.5 bp. However, it is not clear whether the reduced loop formation efficiency is caused by constraints originating from the geometry of the Lac repressor, from an insufficient DNA flexibility, or from both. It remains to be shown to what extent loop formation at this distance contributes to repression at all.

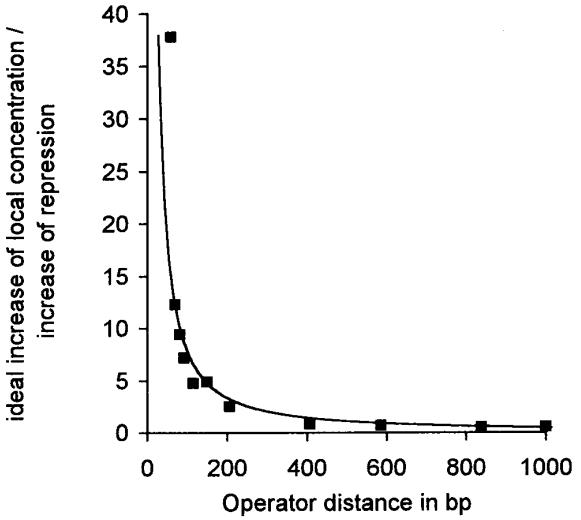


Figure 5. Deviation of the calculated increase of local Lac repressor concentration from the observed repression values as a function of operator distance. The deviation is the ideal increase of local repressor concentration divided by the measured increase of repression upon addition of an auxiliary O_{id}.

Materials and Methods

Chemicals, media and general methods

Chemicals, media and general methods have been described by Oehler *et al*. (1990). β-Galactosidase assays were performed according to Miller (1972). Repression is given as the specific activity of β-galactosidase in the absence of active Lac repressor divided by the specific activity of β-galactosidase in the presence of active Lac repressor. At least three colonies from at least two independent transformations were assayed for specific activity of β-galactosidase. All repression values are mean values with a general standard deviation of 20% or less.

Plasmids were constructed according to standard procedures (Sambrook *et al*., 1989). The sequences of all constructs were confirmed by DNA sequence analysis according to the method of Sanger *et al*. (1977) or using an Applied Biosystems 373A sequencer. Oligonucleotide synthesis was performed with an Applied Biosystems 380 synthesiser.

Bacterial strains, plasmids and λ phages

E. coli strain BMH8117F' has the genotype: (*lac pro*)Δ *nal thi supE* F' *lac* I^{q-}, Z^-, Y^+ *pro* AB^+ (Oehler *et al*., 1990). The episome carries an I-Z deletion, leaving the 5' end of the I gene and the 3' end of the Z gene intact (Gho & Miller, 1974). Lac repressor encoding plasmids pSO 1010-P1 (five times i^+) and pSO 1000ΔA (i^-) are as described by Oehler *et al*. (1990). Plasmid pSO 1000-X11 expresses about two times the wild-type amounts of Lac repressor and is a promoter-defective derivative of pSO1000 (Fickert, 1992). The *lac*Z gene was cloned in a set of plasmids termed pOd O_x N O_x, derived from pEwt103 (Oehler *et al*., 1990). The name of a particular operator is indicated by the letter x, while the letter N stands for the respective operator distance in bp. For example, pOd O_{id} 70.5 O_1 carries an auxiliary O_{id} 70.5 bp upstream of O_1 at its natural position. The pOd plasmids carry a *lac*Z gene under control of the CAP-independent *lac* UV5 promoter and the wild-type O_1 operator at its natural position. The operator O_2 within the coding region of *lac*Z was destroyed by site-directed mutagenesis (Eismann *et al*., 1987). A unique *Xho*I restriction site was introduced by PCR-mediated mutagenesis immediately upstream of the −35 box to obtain pEwt103Xho, with the following substitutions: 5'-CCCCAG to 5'-C**T**C**G**AG.

The sequence between the *Hin*dIII and the *Xho*I restriction sites of pEwt103Xho, including the CAP binding site and the operator O_3, was replaced by *Hin*dIII-*Xho*I restriction fragments from pHK76O^c Tet to pHK98O^c Tet (Krämer, 1989), resulting in a set of plasmids that carry an auxiliary ideal *lac* operator upstream of the *Xho*I restriction site. A *Hin*dIII-*Xho*I restriction fragment from pHK0O^c93Tet was inserted into pEwt103Xho, which bears no auxiliary operator upstream of the *Xho*I site (pOd O_1). The operator O_1 of plasmid pOd O_{id} 70.5 O_1 was destroyed by PCR mutagenesis to obtain pOd O_{id} 70.5 O_1^-. Additional constructs were obtained by inserting a synthetic polylinker into the *Xho*I restriction site and subsequent insertion and deletion of single bp or small oligonucleotides within this region. For large inter-operator distances, the *Hin*dIII-*Nru*I fragment of the tetracycline resistance gene was first removed to allow the use of additional restriction sites in the polylinker. Suitable fragments of the tetracycline resistance gene or DNA derived from intron 2 of human trypsinogen 4 (Wiegand, 1993) were cloned into the corresponding restriction sites within the polylinker regions of tetracycline-sensitive pOd plasmids.

To convert the auxiliary ideal operator of pOd O_{id} 91.5 O_1 to the natural *lac* operators O_1, O_2 or O_3, suitable primers for PCR-mediated mutagenesis were designed. Operator distances are counted from centre of symmetry to centre of symmetry, counting the central bp of the natural *lac* operators as 0.5 bp. Thus, the operator distance in pOd O_{id} 91.5 O_1 increases half a bp upon converting O_{id} without a central bp to the natural *lac* operators. The *Xba*I fragments of all pOd plasmids that contain the *lac*Z and the ampicillin resistance gene, but neither the tetracycline resistance gene nor the origin of replication, were ligated into the unique *Xba*I restriction site of phage λ IP1 (Sieg *et al*.,1989) to generate the respective λ phages.

Isolation of λ phages, λ DNA, *in vitro* packaging of recombinant λ DNA and infection of bacteria was performed as described by Oehler *et al*. (1990). Isolation and confirmation of single lysogens was performed as described previously by Oehler *et al*. (1990). The specific activity of β-galactosidase of individual lysogens in the absence of any repressor plasmid was found to vary between 5000 and 15,000 for constructs with manipulations close to the −35 box and affected all individual isolates of a respective construct. Therefore, these constructs were not considered as bearing more than one prophage. Rather, this effect may be attributed to differences in the UP element upstream of the −35 box that have been shown to affect promoter activity (Ross *et al*., 1993). This variation did not correlate with the efficiency of repression (data not shown). Prophage DNA was amplified from single colonies by PCR and construct sequences were verified as above.

Acknowledgements

We thank Petra Hammes for excellent technical assistance and Brigitte von Wilcken-Bergmann and Andrew Barker for critically reading the manuscript. Intron 2 DNA of human trypsinogen 4 was kindly provided by Ulrich Wiegand. This work was supported by Deutsche Forschungsgemeinschaft through SFB 274.

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Edited by M. Yaniv

(Received 1 September 1995; accepted 18 December 1995)