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The three operators of the *lac* operon cooperate in repression

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We tested the effect of systematic destruction of all three *lac* operators of the chromosomal *lac* operon of *Escherichia coli* on repression by Lac repressor. Absence of just one 'pseudo-operator' O_2 or O_3 decreases repression by wild-type tetrameric Lac repressor ~2- to 3-fold; absence of both 'pseudo-operators' decreases repression >50-fold. O_1 alone represses under these conditions only ~20-fold. Dimeric active Lac repressor (i^{adi}) represses the wild-type *lac* operon to about the same low extent. This indicates that cooperative interaction between *lac* operators is due to DNA loop formation mediated by tetrameric Lac repressor. Under conditions where loop formation is impossible, occupation of O_3 but not of O_2 may lead to weak repression. This suggests that under these conditions CAP activation may be inhibited and that stopping transcription at O_2 does not significantly contribute to repression.

Key words: Escherichia coli/lac operator/Lac repressor/DNA loops/repression

Introduction

Regulation of the *lac* operon of *Escherichia coli* is a paradigm for negative control of gene expression at the level of transcription (Jacob and Monod, 1961). Control of the *lac* operon is unusually effective in comparison with other negatively regulated bacterial systems. In the presence of inducer, the level of the *lac* enzymes may increase ~1000-fold, whereas the *gal* operon is only induced <20-fold (Buttin, 1963). It has been assumed that repression of the *lac* operon is achieved essentially through direct steric hindrance of binding of RNA polymerase to the *lac* promoter (Gilbert *et al.*, 1975; Galas and Schmitz, 1978) or, as recently suggested, through halting RNA polymerase in a pretranscriptional complex with the promoter by Lac repressor bound to the first *lac* operator O_1 (Straney and Crothers, 1987).

The discovery of additional Lac repressor binding sites in the *lac* operon, the so called 'pseudo-operators' O_2 and O_3 did not essentially change this view. Their affinities for Lac repressor are relatively low (Pfahl *et al.*, 1979; Winter and v. Hippel, 1981; Fried and Crothers, 1981) and their distances from the promoter are such that no significant contribution to repression was expected. O_2 is located 401 bp downstream (Reznikoff *et al.*, 1974) and O_3 92 bp upstream of O_1 (Gilbert *et al.*, 1976). Experimental data suggested that O_1 and O_3, located on the same DNA fragment act independently in repressor binding (Fried and Crothers, 1981) and that the dissociation constant of Lac repressor bound to a DNA fragment carrying all three *lac* operators is essentially unaltered, when this fragment lacks either O_2 or O_3 (Pfahl *et al.*, 1979).

It has been proposed that one Lac repressor tetramer is able to bind simultaneously to two operator like sequences (Kania and Müller-Hill, 1977). *In vitro* experiments indeed showed that Lac repressor molecules simultaneously bind two *lac* operator bearing DNA fragments (O'Gorman *et al.*, 1980; Culard and Maurizot, 1981) or two *lac* operator sequences located on one DNA fragment, thereby forcing the intervening DNA into a loop structure (Krämer *et al.*, 1987, 1988). These *in vitro* studies demonstrated that the extent of DNA loop formation depends on the absolute and relative molarities of repressor and operator and on the superhelical density of the operator bearing DNA. DNA supercoiling can promote cooperative binding between two wild-type *lac* operators (Sasse-Dwight and Gralla, 1988; Whitson *et al.*, 1987a,b). Several *in vivo* studies hinted that loop formation plays a role in the repression of the *lac* operon, but estimates about the degree of contribution in the wild-type situation did not exceed a factor of 3 to 6 (Besse *et al.*, 1986; Mossing and Record, 1986; Eismann *et al.*, 1987; Flashner and Gralla, 1988). However, the hybrid *mac* promoters, containing only part of the *lac* promoter and the first *lac* operator, are just ~5-fold repressed by Lac repressor (Vidal-Ingigliari and Raibaud, 1985). In other bacterial systems additional operators have been shown to contribute ~10- to 20-fold to full repression, such as in the *deo* operon (Dandanell *et al.*, 1987), the *ara BAD* operon (Dunn *et al.*, 1984) and the *gal* operon (Irani *et al.*, 1983; Fritz *et al.*, 1983; Haber and Adhya, 1988). In the *gln ALG* operon additional operators have been shown to increase activation up to 60-fold (Reitzer and Magasanik, 1986).

In order to analyse the contribution of each particular *lac* operator to repression, we measured *in vivo* repression of all possible combinations of active and inactivated operators on the chromosome in an otherwise wild-type *lacZ* construction. A phage lambda expression vector system (Sieg *et al.*, 1989) was used to introduce these constructs in single copy into the bacterial chromosome. To investigate whether the contribution of each of the three operators to repression is due to DNA looping or not, we used the mutant *lacI* gene i^{adi}. This allele codes for a dimeric Lac repressor protein which is fully active in operator binding (Lehming *et al.*, 1987, 1988). We show that cooperativity between the three *lac* operators, mediated through tetrameric Lac repressor, is crucial for repression of the *lac* operon.

Results

We constructed a set of eight plasmids, each encompassing the *lacZ* gene under control of the natural *lac* promoter and the three *lac* operators (O_1, O_2 and O_3) either active or inactivated by site directed mutagenesis (Figure 1) in all possible combinations. These constructs were each cloned in phage λIPI (Sieg *et al.*, 1989) to yield phages λEwt123 to λEwt000 (Figure 2). Strain CSH 9 rec A with the relevant genotype i^+z^- was infected with these phages, lysogenic colonies were isolated and checked for stable integration. β-galactosidase activities were assayed under repressed and under induced conditions (1 mM IPTG) and repression values determined.

The data of Figure 2 show that in the presence of three active operators expression of β-galactosidase is repressed by a factor of 1300 and that destruction of O_1 leads to an almost total loss of repression. Inactivation of either O_2 or of O_3 results in a slight decrease of repression. However, the combined loss of both 'pseudo-operators' O_2 and O_3 leads to a severe (~70-fold) decrease of repression.

Dimeric active Lac repressor

We next addressed the question about the mechanism through which O_2 and O_3 contribute to repression. To discriminate between mere direct action at the respective operator sites and cooperativity between operators one has to create a situation in which loop formation is impossible while binding of operators is unimpaired. For this purpose, we used the mutant *lacI* gene i^{adi} which has lost the first basepair of codon 330 (Lehming *et al.*, 1988). This frameshift leads to a shortened Lac repressor protein of 345 residues with wild-type protein sequence up to residue 329 and an additional 16 residues deviating from wild-type (sequence in Materials and methods). We determined the molecular weight of native *lac* i^{adi} encoded repressor by gel filtration and found it to be 78 kd which corresponds well with the calculated value of 75 kd for dimeric mutant repressor (Figure 3).

In order to prove that this repressor is capable of operator binding but not of DNA loop formation, we performed a gel retardation assay with a 452 bp DNA fragment containing two ideal *lac* operators (Sadler *et al.*, 1983; Simons *et al.*, 1984) at a distance of 158 bp. This fragment has already been shown to be suitable to demonstrate loop formation with Lac repressor *in vitro* by Krämer *et al.* (1987). At high repressor concentrations both tetrameric wild-type and mutant dimeric (i^{adi}) repressor proteins bind the DNA fragment in a 'tandem complex' with both operators occupied by individual repressor molecules (Figure 4). When the concentration of wild-type repressor is lowered, a slower migrating band predominates, which is typical for the very stable loop complex, where one repressor molecule binds simultaneously to both operators (Krämer *et al.*, 1987). In contrast, lowering the concentration of mutant repressor gives rise to a faster migrating double band, indicating that only one of the two operators is occupied by one dimeric Lac repressor molecule. The two possible complexes move with different speed since Lac repressor is bound either in the middle or close to the end of the DNA fragment to the respective operator (Zwieb *et al.*, 1989). We recall here that the half-life of the repressor–operator complex was shown to be the same for dimeric active and tetrameric Lac repressor (Lehming *et al.*, 1987, 1988).

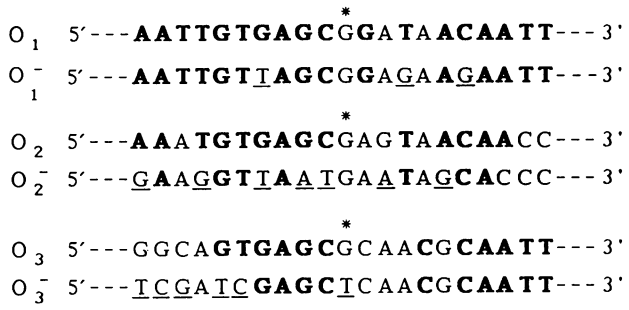

Fig. 1. Sequences of the destroyed *lac* operators (O_1^-, O_2^-, O_3^-) aligned with the respective wild-type sequences (O_1, O_2, O_3). The centres of symmetry are indicated by asterisks (*). The O_2 sequences are given as the lower strand sequences of the *lacZ* gene to make them comparable with the other operators. Bold letters are identical in ideal *lac* operator (Sadler *et al.*, 1983; Simons *et al.*, 1984; Lehming *et al.*, 1988). Altered residues in the O^- sequences are underlined. The exchanges were introduced by site directed mutagenesis.

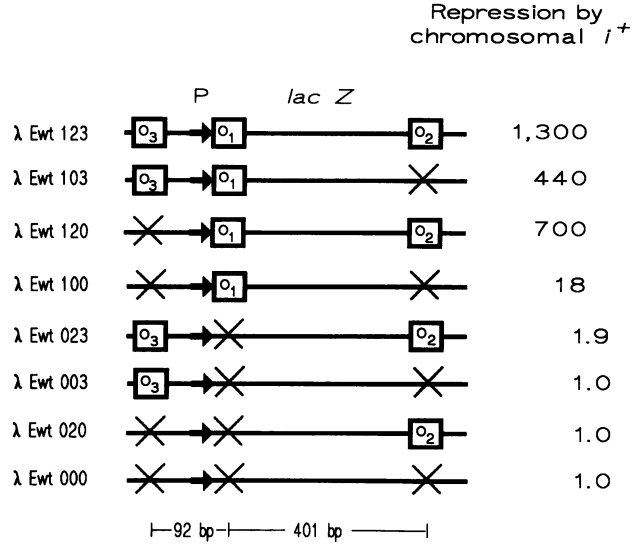

Fig. 2. Repression values for the phage λEwt harboured *lacZ* genes, determined in i^+ background. The phages are integrated as prophages in the chromosome of *E.coli* strain CSH 9 rec A (i^+ z^-). The combination of *lac* operators for each construct is given schematically. The *lac* operators are drawn as open boxes, destroyed operators are indicated by crossed bars. An arrow represents the *lac* promoter (P). Repression is defined as specific activity of β-galactosidase in the presence of inducer (1 mM IPTG) divided by the specific activity of β-galactosidase in the absence of inducer. Repression factors are the mean values of at least three independent determinations.

Probing cooperative repression

The fact that O_2 and O_3 can compensate mutually for the abolition of each other suggests that both of them are able to cooperate with O_1. To examine this hypothesis, we measured repression of the λEwt constructs in the presence of equal amounts of subunits of tetrameric and dimeric Lac repressor. For this purpose we constructed two pACYC derived otherwise identical low copy plasmids carrying the respective *lacI* alleles under control of a very weak synthetic promoter. The amount of Lac repressor in cells harbouring these plasmids is higher than in the presence of a chromosomal i^+ gene, but lower than in the presence of an i^q gene. This can be concluded from the fact that the wild-type *lac* operon is more efficiently repressed by plasmid

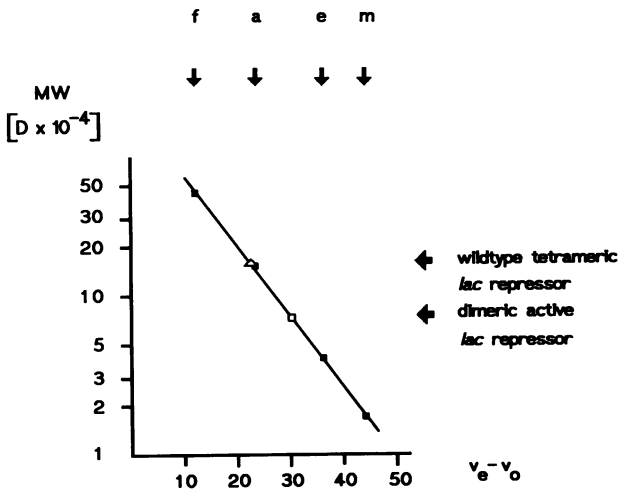

Fig. 3. Determination of molecular weight of dimeric active Lac repressor by gel filtration. A Sephacryl S-300 column (90 × 1.5 cm) was calibrated with a mixture of standard molecular weight proteins, 0.5 mg each. Their molecular weight is plotted against their elution position indicated by vertical arrows. It was measured by absorbance and given as $v_e - v_o$ (ml), where v_e is the elution volume and v_o is the void volume. Samples of 0.5 mg of either dimeric or tetrameric Lac repressor were applied in separate runs. Their molecular weights were deduced from their elution position as determined by absorbance and indicated by horizontal arrows. The molecular weight determined for wild-type tetrameric Lac repessors is 180 kd (calculated value from amino acid composition: 154 kd) and for dimeric active Lac repressor 78 kd (calculated value; 75 kd). The conditions of column runs are described in Materials and methods. ■, molecular weight marker proteins (f = ferritin; a = aldolase; e = albumin, egg; m = myoglobine, equine); □, dimeric active Lac repressor, △, wild-type tetrameric Lac repressor.

encoded tetrameric Lac repressor than by a chromosomal i^+. However, this amount of Lac repressor is not detectable by IPTG equilibrium dialysis, whereas i^q amounts are (data not shown).

If loop formation is necessary for the action of O_2 and O_3, repression by dimeric Lac repressor should be about equally low in comparison to the repression of wild-type *lac* operon by tetrameric Lac repressor for all constructs bearing O_1, irrespective of the presence or absence of the other operators. Table IA shows that this indeed is the case. All four constructs bearing O_1 are poorly repressed by dimeric Lac repressor. Furthermore, the repression rates lie in the same range as repression by tetrameric Lac repressor, mediated only by O_1. The four constructs without O_1 exhibit little or no repression. Thus, cooperative repression is mainly dependent on O_1. A 4-fold repression can be observed with tetrameric repressor in the presence of both O_2 and O_3, indicating an interaction of minor importance between these operators in the absence of O_1.

To further confirm our results, we made use of the fact that high concentrations of repressor abolish loop formation and favour single occupancy of multiple operator sites *in vitro* (Krämer *et al.*, 1987). In an *in vivo* experiment this should lead to a decrease of the difference in repression between dimeric and tetrameric Lac repressor. We constructed a set of three plasmids; two of them express equally high amounts of dimeric or tetrameric Lac repressor, respectively, the third plasmid bears an i^- gene. This plasmid was used to determine unrepressed β-galactosidase activity, since high amounts of Lac repressor do not allow

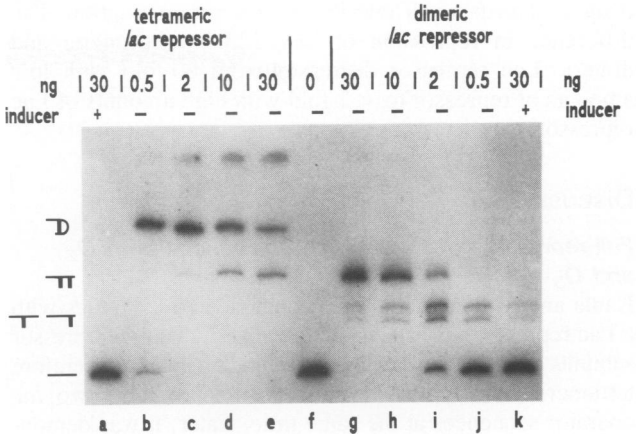

Fig. 4. Titration of a DNA fragment carrying two ideal *lac* operators at a distance of 158 bp with tetrameric and dimeric Lac repressor. The 452 bp DNA fragment was produced by digestion of the plasmid pEE6158 (Krämer *et al.*, 1987) with *Hin*dIII, 3'-end-labelled and gel purified. Approximately 1 fmol of DNA fragment was incubated with the indicated amounts of Lac repressor (above). **(Lanes a–e)** Tetrameric Lac repressor. **(Lanes g–k)** Dimeric Lac repressor. **(Lane f)** No repressor. Incubation was performed in the presence or absence (indicated by + or −) of 5 mM IPTG as inducer. Incubation, electrophoresis and autoradiography were performed as described in Materials and methods. The symbols illustrate the proposed structures (see text).

Table I. Repression values for the phage λEwt harboured *lacZ* genes in the presence of low (A) and high (B) amounts of tetrameric or dimeric Lac repressor

	Repression			
	A ~200 subunits of *lac* repressor per cell		B ~3600 subunits of *lac* repressor per cell	
	Dimer	Tetramer	Dimer	Tetramer
λEwt123	110	6700	9000	16 000
λEwt103	90	3900	10 000	15 000
λEwt120	80	1400	2600	3600
λEwt100	60	140	2500	2700
λEwt023	1.2	4.4	9.8	29
λEwt003	1.1	1.9	5.5	21
λEwt020	1.1	1.1	1.2	1.2
λEwt000	1.0	1.1	1.2	1.3

The λEwt phages (see Figure 2) are integrated as prophages in the chromosome of *E.coli* strain BMH8117 *Nal F'* (*lac pro*$_\Delta$). (A) Repression values were determined for cells harbouring plasmid pSO1010-P1 which codes for low levels of wild-type Lac repressor or harbouring plasmid pSO110-P1 which codes for low levels of dimeric Lac repressor (i^{adi}). Definition of repression as in legend to Figure 2. (B) Repression values were determined for cells harbouring plasmid pSO1000 which codes for high levels of tetrameric Lac repressor or harbouring plasmid pSO100 which codes for high levels of dimeric Lac repressor (i^{adi}). Specific β-galactosidase activity for constitutive expression was measured for cells harbouring plasmid pSO1000ΔApa that carrys an i^- gene. Repression is defined as specific activity of β-galactosidase in the absence of Lac repressor divided by specific activity of β-galactosidase in the presence of Lac repressor. Repression factors are the mean values of at least three independent determinations.

full induction by IPTG (Gilbert and Müller-Hill, 1970). Cells harbouring either of the plasmids coding for active Lac repressor exhibit a specific Lac repressor activity of 9 (90-fold more than i^+) according to IPTG equilibrium

dialysis. The data in Table IB confirm our assumption. The difference in repression of λEwt123 by tetrameric and dimeric Lac repressor decreases from 60-fold with low amounts of repressor to <2-fold with high amounts of Lac repressor.

Discussion

Full repression of the lac operon requires both O_2 and O_3

Kania and Müller-Hill (1977) concluded from studies with a Lac repressor−β-galactosidase chimera that two repressor subunits are sufficient for operator binding and that therefore tetrameric Lac repressor must be able to bind two *lac* operator sequences at the same time. Later, it was demonstrated that Lac repressor is indeed able to bind simultaneously to two operator sequences (Krämer *et al.*, 1987) and that Lac repressor may, under certain conditions, form loops by binding simultaneously to O_1 and O_2 or to O_1 and O_3 (Borowiec *et al.*, 1987; Flashner and Gralla, 1988; Sasse-Dwight and Gralla, 1988).

A 3- to 6-fold decrease of repression has been observed when O_2 is inactivated (Eismann *et al.*, 1987; Flashner and Gralla, 1988). Speculations about the significance of O_3 for repression assume minimal if any participation since no *in vivo* binding of repressor to O_3 could be detected in the presence of O_1 and O_2 (Sasse-Dwight and Gralla, 1988). We found the presence of only either O_2 or O_3 to be necessary for almost full repression of the *lac* operon. Abolishing either one of them results in only 2- to 3-fold derepression of the system whereas the elimination of both 'pseudo-operators' reduces repression >50-fold (Figure 2, Table IA). In a certain sense, repression of the *lac* operon is redundant, since O_1 and either one of the 'pseudo-operators' are sufficient to guarantee efficient repression. Addition of a further operator exerts only a minor enhancing effect on repression. This redundancy is also the reason why no O^c mutation has been found which mapped in one of the two 'pseudo-operators'. Finally, the *in vivo* dissociation constant of Lac repressor and O_1 has been estimated to be $1-2 \times 10^{-11}$M, based on the 1000-fold repression of the *lac* operon which has been attributed to the action of O_1 alone (Gilbert and Müller-Hill, 1967). The observed repression value of only ~20 in the absence of the 'pseudo-operators' correspondingly leads to the revised estimate of 5×10^{-10} to 1×10^{-9} M.

It is appropriate to recall that the lambdoid P_R promoter is negatively controlled by C_I repressor to a similar extent as the *lac* promoter is by Lac repressor (Johnson *et al.*, 1981) and that repression at P_R involves cooperative binding of C_I to two operator sites (Johnson *et al.*, 1979). One is led to conclude that interaction between two or more operator sites may be a general means to achieve effective but at the same time rapidly reversible regulation.

Features of i^{adi}

The existence of dimeric Lac repressor mutants that are still able to bind to DNA had been postulated previously (Kania and Brown, 1976). Miller *et al.* (1970) described a dimeric mutant which exhibited binding to operator, but was severely degraded. We characterize here a member of this class of mutations which produces stable active Lac repressor dimers. We call it i^{adi} for *a*ctive *di*mer. It is caused by a frameshift mutation in codon 330 of the *lacI* gene, the first codon of a so called 'silent region', for which no non-sense or mis-sence mutation, causing i^- phenotype is known. It reaches from codon 330 to 360 (Gordon *et al.*, 1988). A unique i^- mis-sense mutation in codon 345 has been reported very recently (Hsia *et al.*, 1989). We propose that this region is responsible for aggregation of active dimers to tetramers. We note that residues 342−356 might form an amphipathic α-helix which could be involved in the inter-dimer interaction. Experiments supporting this hypothesis will be reported elsewhere.

Repression of the lac operon involves loop formation

Dimeric active Lac repressor enabled us to probe the *lac* operon for cooperative repression (Table IA). In the presence of small amounts of dimeric Lac repressor, prevention of loop formation results in as large a de-repression as does destruction of both O_2 and O_3 in the presence of tetrameric Lac repressor. O_2 or O_3 alone exert neither detectable repression with dimeric nor with tetrameric Lac repressor. Only the combination of O_2 and O_3 exhibits a minor effect if tetrameric Lac repressor is present (Figure 2, Table IA). This leads us to the conclusion that the action of the 'pseudo-operators' is *in vivo* in *E.coli* totally dependent on DNA loop formation and almost completely dependent on the presence of O_1.

Recently, dimeric active Lac repressor has been shown to be unable to efficiently repress a modified *gal* operon, in which both *gal* operators are converted into *lac* operators (N.Mandal *et al.*, 1990).

Repression values determined in the presence of high amounts of Lac repressor provide information about effective sites of repression. DNA loop formation leads to an increase of operator occupancy (Mossing and Record, 1986; Schleif, 1987). It might be asked at which operator sites bound repressor directly exerts its effect. O_1 is certainly the main site of repression. λEwt100 in i^+ background is considerably repressed while under the same conditions repression of λEwt020 and λEwt003 cannot be measured (Figure 2). Increasing repressor concentrations should increase operator occupancy as does loop formation (Table IB). Correspondingly, high amounts of repressor increase repression of λEwt100 considerably. The same conditions lead to an ~20-fold repression by O_3 alone (λEwt003). However, unexpectedly, λEwt020 is not detectably repressed. This indicates that stop of transcription at O_2 (Flashner and Gralla, 1988) does not essentially contribute to repression of the *lac* operon. Addition of O_3 also enhances repression of the λEwt constructs bearing O_1 in the presence of high amounts of tetrameric or dimeric Lac repressor when DNA loop formation should be minimal (Table IB). We suggest that occupancy of O_3 interferes with the binding of CAP to its binding site. The CAP site partly overlaps with O_3. Thus the CAP mediated activation of the *lac* promoter may be reduced. This resembles the effect suggested for the upstream *gal* operator O_1 controlling *gal* promoter P_1 (Kuhnke *et al.*, 1986; G.Kuhnke, personal communication).

Cooperative repression in vivo and speculations about its evolution

The traditional concept of repression of the *lac* operon turns out to be a simplification. Figure 5 shows our present model for repression of the *lac* operon in *E.coli*. The presence of just O_1 yields moderate repression, roughly in the same

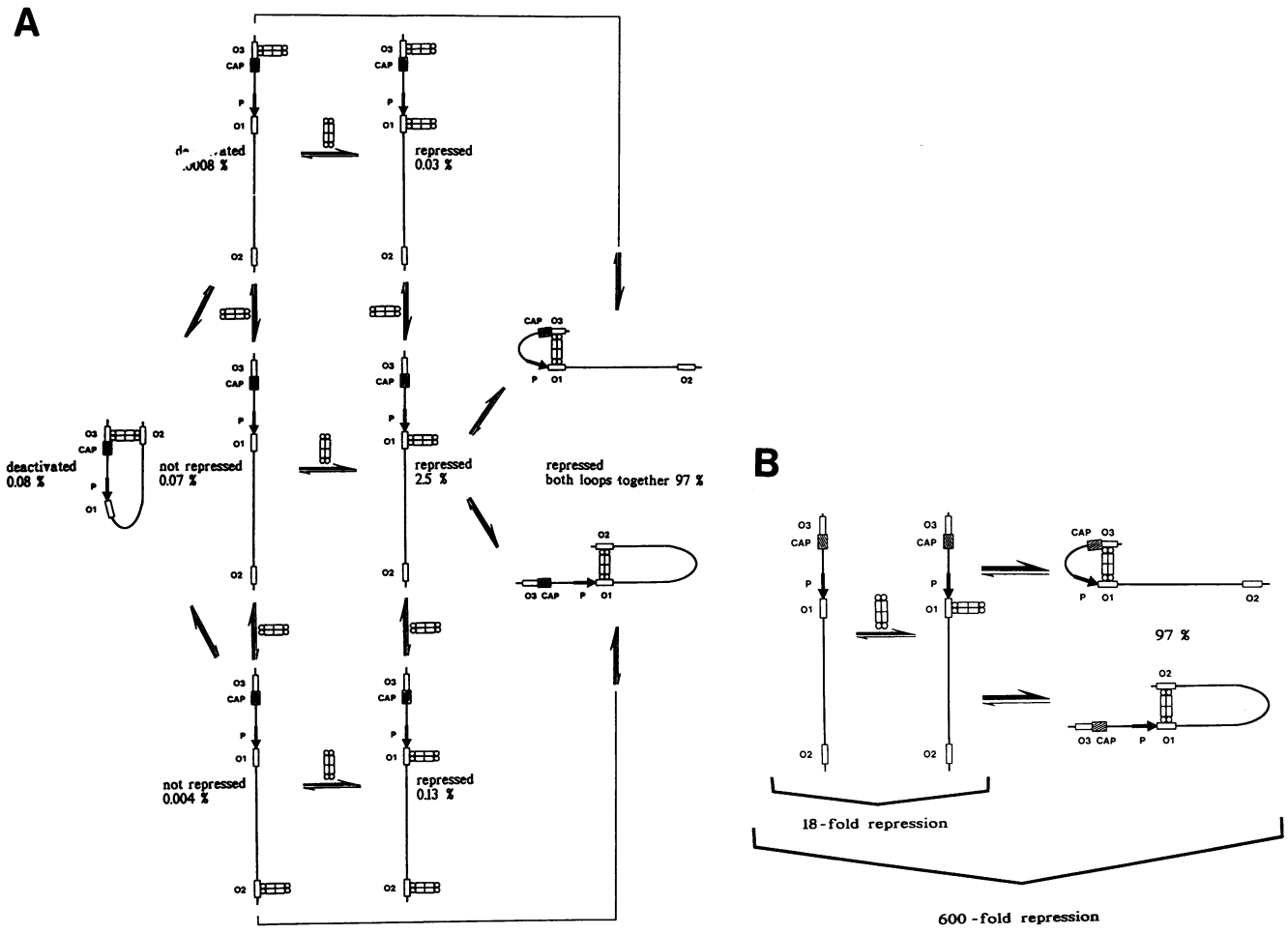

Fig. 5 (**A**) Various states of the repressed wild-type *lac* operon, not drawn to scale. The system is regarded in thermodynamic equilibrium. We assume that occupation of O_1 by Lac repressor leads to a total stop of new transcription starts, i.e. full repression of β-galactosidase synthesis. The contribution of each structure to repression is noted as follows. Repressed = no expression; deactivated = maximal 50-fold repression through interference with CAP activation; not repressed = full expression. The likelihood of presence of each structure is given in percent. To calculate the percentages of the various structures, we used the following information: (i) the wild-type *lac* operon is 1300-fold repressed; (ii) a *lac* operon in which O_2 and O_3 are destroyed is 18-fold repressed, (iii) a *lac* operon in which O_1 is destroyed is 2-fold repressed, (iv) O_2 and O_3 have a 20- and 100-fold lower affinity for Lac repressor than O_1 *in vitro* and (v) *lac* transcription is 50-fold activated by CAP. Note that we disregard various aspects of the system: we do not take into account the presence of CAP protein and of RNA polymerase. We disregard too the repressed form of the *lac* operon in which a Lac repressor molecule is cooperatively bound by O_2 and O_3 while O_1 is occupied by another molecule of Lac repressor. We also neglect the possible tandem occupation of both O_2 and O_3 with one Lac repressor molecule each. It seems to us that such structures will have little functional impact. Open boxes – *lac* operators (O_1, O_2, O_3); hatched box = catabolite activator protein binding site (CAP); arrow = *lac* promoter (P). (**B**) The main states of the repressed wild-type *lac* operon. Single occupation of O_1 by Lac repressor that does not involve DNA loop formation is infrequent. When the system is forced into this structure by mutating the two 'pseudo-operators', repression is only 18-fold. When loop formation between O_1 and either O_2 or alternatively O_3 is allowed, repression is 700- or 440-fold, respectively (approximate mean value of repression: 600). Note that the loops are stable for hours, i.e. for the lifetime of an *E.coli* cell.

range that has been determined for other bacterial regulatory systems. Only when cooperative binding to a further, remote operator sequence is involved, is full repression observed. Loop formation predominates by far. Single occupation of O_1 results in an 18-fold repression. Since the apparent repression of the *lac* operon is 1300-fold, single occupation can maximally account for ~3% of the repression. Note that half of those forms unoccupied at O_1 show cooperative binding of Lac repressor to O_2 and O_3. *In vitro* measurements show that the dissociation rate of a complex between wild-type Lac repressor and plasmid DNA which contains all three *lac* operators is extremely low (Whitson *et al.*, 1987; Eismann and Müller-Hill, 1990). We suppose that a DNA loop between two *lac* operators remains stable for a whole cell cycle. Thus, two alternative loops, both involving O_1 and each of which is stable throughout a cell cycle result in full repression.

The *lac* system seems to us remarkable with regard to its evolution. The primordial system of negative control may well have comprised only Lac repressor and the first operator. The presence of a rather simple device at the C-terminus of Lac repressor allows the formation of tetramers from dimers. This may have permitted the selection of mutants containing the 'pseudo-operators'. Thus the first operator did not reach its limit of possible excellence since the two 'pseudo-operators' came to its help. Neither the first operator nor the 'pseudo-operators' needed to have a perfect sequence. Repressor binding to them could be rather weak though the effect of such binding would still be very strong. Here, as elsewhere, evolution rather than favouring the

perfection of a simple system (here the dimeric Lac repressor and the dyadic symmetric operator) has instead favoured a cooperative system (here tetrameric Lac repressor and three *lac* operators). The 'pseudo-operators' betray their name and should be called auxiliary operators.

Materials and methods

Chemicals, media and enzymes

[^{32}P]Deoxyribonucleoside triphosphates were obtained from Amersham Buchler (Braunschweig, FRG); isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) from Bachem Fine Chemicals (Torrance, USA); ATP, deoxyribonucleoside triphosphates, dideoxyribonucleoside triphosphates, dithiothreitol, ethidium bromide and 'Trizmabase' from Sigma Chemie (München, FRG); β-mercaptoethanol from Fluka AG (Buchs, CH); *o*-nitro-phenyl-β-D-thiogalactoside (ONPG) and protein molecular weight standards from Serva Feinbiochemica (Heidelberg, FRG); urea from Bethesda Research Laboratories (Neu-Isenburg, FRG); the chemicals used for automatic DNA synthesis from Applied Biosystems (Pfungstadt, FRG); all other chemicals were obtained from Merck (Darmstadt, FRG) or Sigma Chemie (München, FRG). Agarose, acrylamide and *N,N'*-methylenebisacrylamide were obtained from Bethesda Research Laboratories (Neu-Isenburg, FRG); Sephacryl S-300 from Pharmacia Fine Chemicals (Uppsala, Sweden). Restriction endonucleases and other enzymes were obtained from Boehringer (Mannheim, FRG), New England Biolabs (Bad Schwalbach, FRG) and Bethesda Research Laboratories (Neu-Isenburg, FRG).

Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer and purified on denaturing polyacrylamide gels (Maniatis *et al.*, 1982). Lac repressor was purified according to Müller-Hill *et al.* (1971).

Bacterial strains, plasmids and phages

Strain CSH 9 rec A has the genotype: *trp lacZ strA thi rec A* and is a derivative of strain CSH 9 (Miller *et al.*, 1972).

Strain BMH 8117 F′ has the genotype: $(lac\ pro)_{\Delta}$ *nalA thi sup E* F′ lac pro I^{q-} Z^{-} Y^{+}. The episome has been described by Gho and Miller (1974). It carries an *I−Z* deletion, leaving the 5′ end of the I^{q} gene intact.

Plasmid pEE6158 has been described (Krämer *et al.*, 1988). All plasmids were constructed according to standard procedures (Maniatis *et al.*, 1982). Plasmid pSO100 codes for dimeric active Lac repressor. It is composed of three restriction fragments: (i) the *Eco*RI−*Hin*dIII fragment from pWB300 (Lehming *et al.*, 1987), encompassing the tetracycline resistance gene and the p15A derived origin of replication, and (ii) the *Hin*cII−*Apa*I fragment from pMC7 containing the N-terminal part of the *lacI* gene, including the I^{q} promoter (Calos, 1978). The *Hin*cII site has been transformed to an *Eco*RI site by adding a linker. The *Apa*I−*Bgl*II fragment from pWB100 which carries the C-terminal part of the *lacI* gene and a transcription termination signal (Lehming *et al.*, 1987). The *Hin*dIII generated protruding single strand has been treated with S1 nuclease and the *Bgl*II generated protruding single strand has been filled in with Klenow's large fragment of DNA polymerase (Maniatis *et al.*, 1982) in order to ligate the resulting blunt ends. pWB100 and pSO100 carry a deletion of the first base of codon 330 of the *lacI* gene, and thus code for a Lac repressor with an altered and shortened C-terminus (Lehming *et al.*, 1988). The deduced C-terminal amino acid sequence deviates from wild-type residue 330. The mutant protein ends with residue 345. The altered amino acid sequence is: N′-Trp(330)-Arg-Pro-Ile-Arg-Lys-Pro-Pro-Leu-Pro-Ala-Arg-Trp-Pro-Ile-His-C′. pSO1000 is identical to pSO100 except that it contains the 3′ coding region from pWB1000 (Lehming *et al.*, 1988) instead of pWB100 and therefore codes for tetrameric wild-type Lac repressor. A *lac* i^{-} derivative of pSO1000 (pSO1000ΔA) was obtained by introducing a small deletion at the *Apa*I site in the *lacI* gene. pSO110-P1 and pSO1010-P1 are derivatives of pSO100 and pSO1000, respectively. In both cases the *Eco*RI−*Apa*I fragments, carrying the *lacI* promoter are replaced by a *Hin*fI−*Apa*I fragment from pWB100-P1. This plasmid is a derivative of pWB100 whose *lacI* gene is under the control of a weak synthetic promoter (J.Sartorius, personal communication).

Plasmids pEwt100, pEwt103, pEwt120 and pEwt123 are derivatives of pEWO100, pEWO103, pEWO120 and pEWO123 (Eismann and Müller-Hill, 1990), respectively. The numbers 1, 2 or 3 stand for the presence of the first, second or third *lac* operator, respectively, whereas a 0 indicates the absence of a particular operator. The *Nhe*I−*Nde*I restriction fragments that carry the complete *lac* promoter and the major part of the *lacZ* gene up to codon 990 were isolated from the pEWO plasmids and inserted between the *Spe*I and *Nhe*I restriction site of a pJK 2 (Sieg *et al.*, 1989) derived plasmid, with an additional *Spe*I site 5′ of the *lac* promoter. Plasmids pEwt023, pEwt003, pEwt020 and pEwt000 were obtained by destruction of O_1 by site-directed mutagenesis, as described by Eismann *et al.* (1987). The respective *Xba*I fragments that contain the *lacZ* and the ampicillin resistance gene but not the tetracycline resistance gene nor the origin of replication of all pEwt constructs were ligated into the unique *Xba*I restriction site of λIP1 (Sieg *et al.*, 1989) generating phages λEwt000, λEwt003, λEwt020, λEwt023, λEwt100, λEwt103, λEwt120 and λEwt123. Isolation of lambda phages and of lambda DNA and *in vitro* packaging and infection of bacteria has been performed according to standard procedures (Maniatis *et al.*, 1982). Lysogenic colonies were isolated and checked on YT-X-gal plates for stable expression of β-galactosidase. For each different construct, several independent lysogenic isolates were tested for induced β-galactosidase levels. Those sporadic lysogens with ≥2-fold expression than the others were considered to bear more than one prophage and were excluded from further determinations. λ DNA was isolated from prophage bearing bacteria and the *lacZ* constructs have been recloned, using the poT plasmid (Sieg *et al.*, 1989) in order to subsequently verify the operator sequences by sequence analysis (Sanger *et al.*, 1977; Chen and Seeburg, 1985).

Gel filtration

Sephacryl S-300 was equilibrated with 0.075 M KPG and a 90 × 1.5 cm column prepared according to the manufacturers recommendations. Proteins (~500 μg each) were applied in a total volume of 300 μl of 0.075 M KPG containing 6 mg/ml dextran blue and 5% glycerol. The column was eluted with a constant flow rate of 20 ml/h. Fractions of 1 ml were collected and the OD_{220} was determined. KPG buffer is 0.075 M potassium phosphate, pH 7.2 (K_2HPO_4:KH_2PO_4, 5:1), 0.3 mM DTT, 0.1 mM EDTA, 5% (w/v) glucose and 1 mM NaN_3. Molecular weight markers applied were: ferritin, bovine (4.5×10^5 daltons); aldolase, rabbit (1.6×10^5 daltons); albumin, egg (4.5×10^4 daltons); myoglobine, equine (1.8×10^4 daltons).

Gel electrophoresis

Gel electrophoresis was performed as described by Krämer *et al.* (1988), with the following modifications. The 3′-end-labeled *Hin*dIII DNA fragment from pEE6158 was purified on a polyacrylamide gel (Maniatis *et al.*, 1982). Approximately 1 fmol was incubated in 20 μl binding buffer (BB) with the indicated amounts of dimeric active or tetrameric Lac repressor in the presence or absence of 5 mM IPTG. Binding buffer was 10 mM Tris−HCl (pH 8.0), 10 mM KCl, 10 mM $MgAc_2$, 0.1 mM EDTA, 0.1 mM DTT, 50 μg/ml bovine serum albumin and 25 μg/ml pBR322 DNA. After 20 min at room temperature, 5 μl BB with 15% Ficoll, 0.06% bromphenol blue and 0.06% xylene cyanol were added. The samples were loaded on a 4% polyacrylamide gel (acrylamide:bisacrylamide, 29:1) in 45 mM Tris−borate, 1.5 mM EDTA (pH 8.3). The gel was pre-run for 4 h with 12 V/cm at room temperature. Electrophoresis was performed for 1 h under the same conditions. The dried gel was autoradiographed with a Fuji RX 100 film at −70°C.

Other methods

β-Galactosidase assays and IPTG equilibrium dialysis were performed according to Miller *et al.* (1972). Minimal medium contained 0.4% glycerol and 20 μg/ml thiamine and for strain CSH 9 rec A additionally 50 μg/ml tryptophane. Strains with λEwt prophages were grown at 32°C in the presence of 400 μg/ml ampicillin and strains harbouring plasmids pSO100, pSO1000, pSO110-P1 or pSO1010-P1 were grown in the presence of 10 μg/ml tetracycline.

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References

Besse,M., Wilcken-Bergmann,B.v. and Müller-Hill,B. (1986) *EMBO J.*, **5**, 1377−1381.

Borowiec,J., Zhang,L., Sasse-Dwight,S. and Gralla,J.D. (1987) *J. Mol. Biol.*, **196**, 101−111.

Buttin,G. (1963) *J. Mol. Biol.*, **7**, 164−182.

Calos,M. (1978) *Nature*, **274**, 762−765.

Chen,E.J. and Seeburg,P.H. (1985) *DNA*, **4**, 165–170.
Culard,F. and Maurizot,J.C. (1981) *Nucleic Acids Res.*, **9**, 5175–5184.
Dandanell,G., Valentin-Hansen,P., Løve Larsen,J.E. and Hammer,K. (1987) *Nature*, **325**, 823–826.
Dunn,T.M., Hahn,S., Ogden,S. and Schleif,R.F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5017–5020.
Eismann,E., Wilcken-Bergmann,B.v. and Müller-Hill,B. (1987) *J. Mol. Biol.*, **195**, 949–952.
Eismann,E.R. and Müller-Hill,B. (1990) *J. Mol. Biol.*, in press.
Flashner,Y. and Gralla,J.D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8968–8972.
Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
Fritz,H.-J., Bicknäse,H., Gleumes,B., Heibach,C., Rosahl,S. and Ehring,R. (1983) *EMBO J.*, **2**, 2129–2135.
Galas,D.J. and Schmitz,A. (1978) *Nucleic Acids Res.*, **5**, 3157–3170.
Gho,D. and Miller,J.H. (1974) *Mol. Gen. Genet.*, **131**, 137–146.
Gilbert,W. and Müller-Hill,B. (1967) *Proc. Natl. Acad. Sci. USA*, **57**, 2415–2421.
Gilbert,W. and Müller-Hill,B. (1970) In Beckwith,J.R. and Zipser,D. (eds), *The Lactose Operon*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 93–110.
Gilbert,W., Gralla,J., Majors,J. and Maxam,A. (1975) In Sund,H. and Blauer,G. (eds), *Protein–Ligand Interactions*. de Gruyter, Berlin, pp. 193–210.
Gilbert,W., Majors,J. and Maxam,A. (1976) In *Dahlem Workshop on Chromosomes*. Abakon Verlagsgesellschaft, Berlin, pp. 167–178.
Gordon,A.J., Burns,P.A., Fix,D.F., Yatagai,F., Allen,F.L., Horsfall,M.J., Halliday,J.G., Bernelot-Moens,C. and Glickman,B.W. (1988) *J. Mol. Biol.*, **200**, 239–251.
Haber,R. and Adhya,S. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9683–9687.
Hsia,H.C., Lebkowski,J.S., Leong,P.-M., Calos,M. and Miller,J.H. (1989) *J. Mol. Biol.*, **205**, 103–113.
Irani,M.H., Orosz,L. and Adhya,S. (1983) *Cell*, **32**, 783–788.
Jacob,F. and Monod,J. (1961) *J. Mol. Biol.*, **3**, 318–356.
Johnson,A.D., Meyer,B.J. and Ptashne,M. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5061–5065.
Johnson,A.D., Poteete,A.R., Lauer,G., Sauer,R.T., Ackers,G.K. and Ptashne,M. (1981) *Nature*, **294**, 217–223.
Kania,J. and Brown,D.T. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 3529–3533.
Kania,J. and Müller-Hill,B. (1977) *Eur. J. Biochem.*, **79**, 381–386.
Krämer,H., Niemöller,M., Amouyal,M., Revet,B., Wilcken-Bergmann,B.v. and Müller-Hill,B. (1987) *EMBO J.*, **6**, 799–803.
Krämer,H., Amouyal,M., Nordheim,A. and Müller-Hill,B. (1988) *EMBO J.*, **7**, 1481–1489.
Kuhnke,G., Krause,A., Heibach,C., Gieske,U., Fritz,H.-J. and Ehring,R. (1986) *EMBO J.*, **5**, 167–173.
Lehming,N., Sartorius,J., Genenger,G., Wilcken-Bergmann,B.v. and Müller-Hill,B. (1987) *EMBO J.*, **6**, 3145–3153.
Lehming,N., Sartorius,J., Oehler,S., Wilcken-Bergmann,B.v. and Müller-Hill,B. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7947–7951.
Mandal,N., Su,W., Haber,R., Adhya,S. and Echols,H. (1990) *Genes Dev.*, **4**, 410–418.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miller,J.H., Platt,T. and Weber,K. (1970) In Beckwith,J.R. and Zipser,D. (eds), *The Lactose Operon*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 343–352.
Mossing,M.C. and Record,T.M.,Jr (1986) *Science*, **233**, 889–892.
Müller-Hill,B., Beyreuther,K. and Gilbert,W. (1971) *Methods Enzymol.*, **21**, 483–487.
O'Gorman,R.B., Dunaway,M. and Matthews,K.S. (1980) *J. Biol. Chem.*,**255**, 10100–10106.
Pfahl,M. (1981) *J. Mol. Biol.*, **147**, 1–10.
Pfahl,M., Gulde,V. and Bourgeois,S. (1979) *J. Mol. Biol.*, **127**, 339–344.
Reitzer,L.J. and Magasanik,B. (1986) *Cell*, **45**, 785–792.
Reznikoff,W.S., Winter,R.B. and Hurley,C.K. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 2314–2318.
Sadler,J.R., Sasmor,H. and Betz,J.L. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6785–6789.
Sanger,F., Nicklen,S. and Coulsen,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5468.
Sasse-Dwight,S. and Gralla,G.D. (1988) *J. Mol. Biol.*, **202**, 107–119.
Schleif,R. (1987) *Nature*, **327**, 369–370.
Sieg,K., Kun,J., Pohl,I., Scherf,A. and Müller-Hill,B. (1989) *Gene*, **75**, 261–270.
Simons,A., Tils,D., Wilcken-Bergmann,B.v. and Müller-Hill,B. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1624–1628.
Straney,S.B. and Crothers,D.M. (1987) *Cell*, **51**, 699–707.
Vidal-Ingigliardi,D. and Raibaud,O. (1985) *Nucleic Acids Res.*, **4**, 1163–1172.
Whitson,P.A., Hsieh,W.T., Wells,R.D. and Matthews,K.S. (1987a) *J. Biol. Chem.*, **262**, 4943–4946.
Whitson,P.A., Hsieh,W.T., Wells,R.D. and Matthews,K.S. (1987b) *J. Biol. Chem.*, **262**, 14592–14599.
Winter,R.B. and Hippel,P.H.v. (1981) *Biochemistry*, **20**, 6948–6960.
Zwieb,C., Kim,J., and Adhya,S. (1989) *Genes Dev.*, **3**, 606–611.

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