The three operators of the lac operon cooperate in repression

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Communicated by B. Müller-Hill

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' O2 or O3 decreases repression by wild-type tetrameric Lac repressor ~2-3-fold; absence of both 'pseudo-operators' decreases repression >50-fold. O1 alone represses under these conditions only ~20-fold. Dimeric active Lac repressor (iaa) 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 O1 but not of O2 may lead to weak repression. This suggests that under these conditions CAP activation may be inhibited and that stopping transcription at O2 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 <200-fold (Büttin, 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 O1 (Straney and Crothers, 1987).

The discovery of additional Lac repressor binding sites in the lac operon, the so-called 'pseudo-operators' O2 and O3 did not essentially change this view. Their affinities for Lac repressor are relatively low (Pflahl 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. O2 is located 401 bp downstream (Reznikoff et al., 1974) and O3 92 bp upstream of O1 (Gilbert et al., 1976). Experimental data suggested that O1 and O3, 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 O2 or O3 (Pflahl 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 (Krämmer 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-Ingiigliari 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 pMut. 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 (O₁, O₂, and O₃) either active or inactivated by site directed mutagenesis (Figure 1) in all possible combinations. These constructs were each cloned in phage λPI (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 O₁ leads to an almost total loss of repression. Inactivation of either O₂ or of O₃ results in a slight decrease of repression. However, the combined loss of both ‘pseudo-operators’ O₂ and O₃ leads to a severe (~70-fold) decrease of repression.

Dimeric active Lac repressor

We next addressed the question about the mechanism through which O₂ and O₃ 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⁺d 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⁺d 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⁺d) 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).

Repression by chromosomal i⁺

![Figure 2](https://example.com/figure2.png)

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 O₂ and O₃ can compensate mutually for the abolition of each other suggests that both of them are able to cooperate with O₁. 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⁺ gene. This can be concluded from the fact that the wild-type lac operon is more efficiently repressed by plasmid
encoded tetrameric Lac repressor than by a chromosomal $i^+$. However, this amount of Lac repressor is not detectable by IPTG equilibrium dialysis, whereas $n$ 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 1A 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 unpressed $\beta$-galactosidase activity, since high amounts of Lac repressor do not allow 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

| Table 1. Repression values for the phage $\lambda$Ewt harboured lacZ genes in the presence of low (A) and high (B) amounts of tetrameric or dimeric Lac repressor |
|---------------------------------|------------------|------------------|
| | A ~ 200 subunits of lac repressor per cell | B ~ 3600 subunits of lac repressor per cell |
| | Dimer | Tetramer | Dimer | Tetramer |
| $\lambda$Ewt123 | 110 | 6700 | 9000 | 16 000 |
| $\lambda$Ewt103 | 90 | 3900 | 10 000 | 15 000 |
| $\lambda$Ewt120 | 80 | 1400 | 2600 | 3600 |
| $\lambda$Ewt100 | 60 | 140 | 2500 | 2700 |
| $\lambda$Ewt023 | 1.2 | 4.4 | 0.9 | 2.9 |
| $\lambda$Ewt003 | 1.1 | 1.9 | 5.5 | 21 |
| $\lambda$Ewt020 | 1.1 | 1.1 | 1.2 | 1.2 |
| $\lambda$Ewt000 | 1.0 | 1.1 | 1.2 | 1.3 |

The $\lambda$Ewt phages (see Figure 2) are integrated as prophages in the chromosome of E.coli strain BMH8117 Nal F' (lac pro). (A) Repression values were determined for cells harbouring plasmid pSO1010-P1 which codes for low levels of wild-type Lac repressor or harbouring plasmid pSO310-P1 which codes for low levels of dimeric lac repressor ($\lambda^{MD}$). 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 ($\lambda^{MD}$). Specific $\beta$-galactosidase activity for constitutive expression was measured for cells harbouring plasmid pSO1000A:Apa that carries an $i^-$ gene. Repression is defined as specific activity of $\beta$-galactosidase in the absence of Lac repressor divided by specific activity of $\beta$-galactosidase in the presence of Lac repressor. Repression factors are the mean values of at least three independent determinations.
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 $O_3$ (Borowicz 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' 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 $5 \times 10^{-10}$ to $1 \times 10^{-9}$ M.

It is appropriate to recall that the lambdoid $P_R$ promoter is negatively controlled by $C_1$ 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 $C_1$ 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 $i^{\text{del}}$

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 $i^{\text{del}}$ for active dimer. 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-sense 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 interdimer 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 step 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 $P_I$ (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
Cooperation in the lac operon

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 start, i.e. full repression of $\beta$-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 $\sim 3\%$ 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-$\beta$-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside (X-Gal) from Bachem Fine Chemicals (Torrance, USA); ATP, deoxyribonucleoside triphosphates, dithiothreitol, ethidium bromide, and 'Trizmabase' from Sigma Chemie (München, FRG); $\beta$-mercaptoethanol from Fluka AG (Buchs, CH); o-nitro-phenyl-$\beta$-D-galactoside (ONPG) and protein molecular weight standards from Serva Feinbiochimica (Heidelberg, FRG); urea from Bethesda Research Laboratories (Neu-Isenburg, FRG); the chemicals used for automatic DNA synthesis from Applied Biosystems (Pfundstadt, 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 and 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 lac z-dr thri rec A and is a derivative of strain CSH 9 (Miller et al., 1972).

Strain BMH 8117 F' has the genotype: (lac pro)$_3$ malA thi sup E' lac pro F' $\Delta$ Z- $\Delta$ Y'. The episome has been described by Gho and Miller (1974). It carries an l-Z deletion, leaving the 5' end of the $P$ gene intact. Plasmid pE6158 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 EcoRI--HindIII fragment from pWB300 (Lehming et al., 1987), encompassing the tetracycline resistance gene and the p15A derived origin of replication, and (ii) the HindII--Apal fragment from pMC7 containing the N-terminal part of the lacI gene, including the $P$ promoter (Calos, 1978). The HindII site has been transformed to an EcoRI site by adding a linker. The Apal--BglII fragment from pWB100 which carries the C-terminal part of the lacI gene and a transcription termination signal (Lehming et al., 1987). The HindIII generated protruding single strand has been treated with SI nuclease and the BglII generated protruding 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-Tyr-Trp(330)-Arg-Pro-Ile-Arg-Lys-Pro-Pro-Leu-Pro-Ala-Arg-Trp-Pro-Ile-His-C'. pSO100 is identical to pSO100 except that it contains the 3' coding region from pWB100 (Lehming et al., 1988) instead of pWB100 and therefore codes for tetrameric wild-type Lac repressor. A lac I' derivative of pSO100 (pSO100DA) was obtained by introducing a small deletion at the Apal site in the lacI gene. pSO1010-P1 and pSO1010-P1 are derivatives of pSO100 and pSO100, respectively. In both cases the EcoRI--Apal fragments, carrying the lacI promoter are replaced by a HindII--Apal 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, pEwT03, pEwT120 and pEwT123 are derivatives of pEwTO22, pEwTO03, pEwTO20 and pEwTO00 were obtained by destruction of O$_1$ by site-directed mutagenesis, as described by Eismann et al. (1987). The respective Xbal fragments that contain the lacZ gene and the ampicillin resistance gene but not the tetracycline resistance gene nor the origin of replication of all pEw constructs were ligated into the unique Xbal restriction site of XP1 (Sieg et al., 1989) generating phages $\lambda$EwTO00, $\lambda$EwTO03, $\lambda$EwTO20, $\lambda$EwTO23, $\lambda$EwTO100, $\lambda$EwTO120 and $\lambda$EwTO123. 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 Y-T-X-gal plates for stable expression of $\beta$-galactosidase. For each different construct, several independent lysogenic isolates were tested for induced $\beta$-galactosidase levels. Those sporadic lysogens with $\geq$ 2-fold expression than the others were considered to bear more than one prophage and were excluded from further determinations. $\lambda$ 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 Sieberg, 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$_{450}$ was determined. KPG buffer is 0.075 M potassium phosphate, pH 7.2 (KH$_2$PO$_4$/KH$_2$PO$_4$: 5:1), 0.3 mM DTT, 0.1 mM EDTA, 5% (w/v) lactose and 1 mM NaN$_3$. Molecular weight markers used were: ferritin, bovine (4.5 × 10$^6$ daltons); aldolase, rabbit (1.6 × 10$^6$ daltons); albumin, egg (4.5 × 10$^5$ daltons); myoglobin, equine (1.8 × 10$^6$ daltons).

Gel electrophoresis

Gel electrophoresis was performed as described by Krämer et al. (1988), with the following modifications. The 3'-end-labeled HindIII DNA fragment from pE6158 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, 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

$\beta$-Galactosidase assays and IPTG equilibrium dialysis were performed according to Müller 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 EwTO prophages were grown at 32°C in the presence of 400 µg/ml ampicillin and strains harbouring plasmids pSO100, pSO1000, pSO1010-P1 or pSO1010-P1 were grown in the presence of 10 µg/ml tetracycline.

Acknowledgements

We thank B.von Wilcken-Bergmann and B.Jack for critically reading this manuscript, R.Ehring and B.Walter for discussions, the Fritz Thyssen Stiftung for a stipend to H.K. and the Graduiertenförderung Nordrhein-Westfalen for a stipend to S.O. This work was supported by a grant from Deutsche Forschungsgemeinschaft through SFB 245.

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Received on October 24, 1989; revised on January 10, 1990