Two operator sites separated by 599 base pairs are required for *deoR* repression of the *deo* operon of *Escherichia coli*

Gert Dandanell and Karin Hammer*

Institute of Biological Chemistry B, University of Copenhagen, Sølvgade 83, DK-1307 Copenhagen, Denmark

*Formerly Karin Hammer-Jespersen

Communicated by A.Munch-Petersen

DeoP1 and *deoP2* promoter fragments from the *deo* operon of *Escherichia coli* have been transcriptionally fused to the galactokinase gene. From single-copy expression of these fusions it is shown that the *deoR* binding site of both *deoP1* and *deoP2* are necessary to achieve full repression of the *deo* operon by the *deoR* repressor. Repression of the promoters can be achieved either by supplying extra *deoR* repressor *in trans* or by introduction of an extra *deoR* binding site at a position between 224 and 997 bp upstream of the promoter. Furthermore, the *deoP2* promoter is shown to be regulated in a cumulative way by both the *deoR* and the *cytR* repressors, while *deoP1* is only regulated by the *deoR* repressor. *DeoP2* is a strong promoter being 20 times stronger than *araP*$_{BAD}$ and four times stronger than *deoP1*.

Key words: repression/repressor binding sites/*deo* operon/promoter strength/*galK*-fusions

Introduction

The *deo* operon of *Escherichia coli* which encodes four enzymes involved in nucleoside catabolism is regulated at the level of transcription initiation, like many other catabolic operons (i.e., *gal, lac, mal, ara* operons, etc.). Transcription of the *deo* operon originates from a promoter region upstream of the first structural gene *deoC* (Albrechtsen *et al.*, 1976, see Figure 1). The operon is subject to a double negative control exerted by the two repressor proteins *deoR* and *cytR* and to a positive control by the cAMP/CRP complex. Since catabolite repression was observed in *cytR* and *deoR,cytR* mutants but not in *deoR* mutants it was proposed that the *deo* operon is regulated at two promoter/operator sites *deoP1* and *deoP2*, the former being regulated by the *deoR* repressor and the latter by both the *cytR* repressor and

Results from both *in vitro* and *in vivo* experiments indicate that the strongest repression of the *deo* operon originates from the *deoR* repressor. Upon introduction of a mutant *cytR* allele the *deoA* level increases 8-fold while introduction of a mutant *deoR* allele causes the *deoA* level to increase 60-fold (reviewed by Hammer-Jespersen, 1983). The precise position of transcription initiation from *deoP1* and *deoP2* has been determined both *in vivo* the cAMP/CRP complex (Albrechtsen *et al.*, 1976; Jørgensen *et al.*, 1977; Svenningsen, 1977; Valentin-Hansen *et al.*, 1978). and *in vitro* (Valentin-Hansen *et al.*, 1982a). S1-nuclease experiments in regulatory mutants led to the proposal that *deoP2* is regulated by both the *deoR* and *cytR* repressors. Short and Singer (1984) have shown that introduction of a *deoR* overproducing plasmid into a *deoR,cytR* mutant results in a near wild-type level of the *deo* enzymes.

As a tool for studying the regulation of the *deo* operon we have used operon fusions to the *galK* gene of *E. coli* in the system developed by McKenney *et al.* (1981). All gene fusions were transferred to λ*gal8*. By studying the expression of operon fusions in single-lysogens we have obtained accurate *in vivo* measurements which are not influenced by plasmid copy number variations or by titration of repressor molecules, the latter being a prerequisite for the present work.

We present evidence that *deoP2* is regulated in a cumulative way by both the *cytR* and *deoR* repressors and that both promoters are dependent on the presence of the *deoR* binding site of the other promoter in order to achieve full repression. When only one promoter is present this repression can also be obtained by supplying extra *deoR* repressor *in trans*. Furthermore no effect on *deoR* repression was observed when the distance separating the two *deoR* binding sites was varied; we therefore suggest that the presence of two repressor binding sites can increase the local concentration of repressor molecules.

Results

Cloning and expression of the deoP1 + P2 region of the deo operon

An 851-bp *Hae*III fragment covering the region between 101 bp upstream of *deoP1* and 151 bp downstream of *deoP2* (see Figure 1) was expected to contain all the regulatory signals needed for the control of *deoP1 + P2*. Cloning of this fragment into plasmid pKB-1 resulted in a *deoP1 + P2-galK* fusion which subsequently was transferred to λ*gal8* by homologous recombination (see Materials and methods). The resulting phage λKT33 was used to lysogenize *recA,galK* strains which contain both repressors (wild-type), only one of them (*cytR* or *deoR* mutants) or neither of them (*deoR,cytR* mutants). The regulation of *deoP1 + P2* could now be studied by measuring the *galK* expression from these lysogens. The results are listed in Table I. The presence of a *cytR* or *deoR* mutation results in a 4- and 34-fold de-repression of *galK*, respectively, while there is a 84-fold de-repression in a *deoR,cytR* mutant, where both repressors are absent. These numbers closely parallel those found for the chromosomal *deo* operon, where the product of the second gene (*deoA*) was measured (Table I). It therefore seems reasonable to believe that the 851-bp *Hae*III fragment contains all the information that is necessary for the native regulation of *deoP1 + P2*.

Next we wanted to compare the regulation of each of the promoters separately with that found for *deoP1 + P2*. Therefore λKT31 (*deoP1*) and λKT32 (*deoP2*) were constructed (see Figure 2). To our surprise we found that the total de-repression of the *deoP1* and *deoP2* fusions was only 3- and 16-fold, respectively. In both cases a 10-fold elevated basal level (wild-type) accounts for the observed decrease in regulation.

Several possibilities could explain this inability to repress the *galK* activities in λKT31 and λKT32. (i) A mutation generating a new promoter or destroying the operator might have been selected during the DNA manipulation. (ii) Artificial promoters could

have been created at the fusion points in λKT31 and λKT32.
(iii) Both promoter fragments are needed to obtain full repression.

The first possibility was ruled out by DNA sequencing which revealed no deviations from the native DNA sequence. The second possibility did not seem very likely either because both λKT31 and λKT32 contain one fusion point which is identical to one of the fusion points of λKT33 (see Figure 2 and Table II). Experiments were performed, however, to test both hypotheses (ii) and (iii).
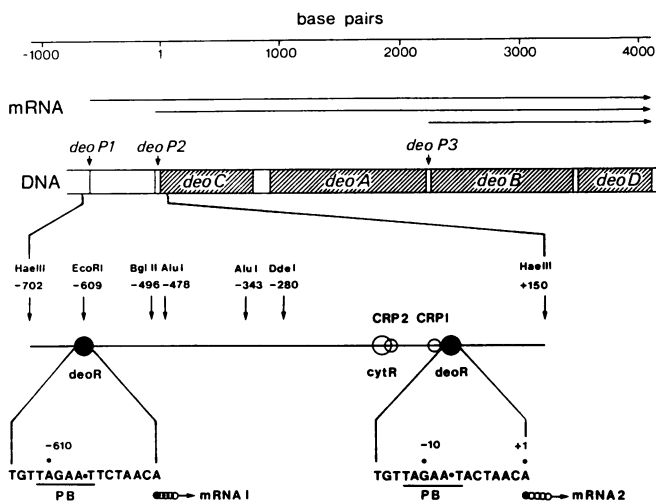
Fig. 1. Structure of the *deo* operon. The *deo* genes code for deoxyribo-aldolase (*deoC*), thymidine phosphorylase (*deoA*), phosphopentomutase (*deoB*) and purine nucleoside phosphorylase (*deoD*). Transcription initiating from three promoters *deoP1*, *deoP2* and *deoP3* has been demonstrated (Valentin-Hansen *et al.*, 1982a, 1984). An 851-bp region containing *deoP1* and *deoP2* is shown enlarged. The position of restriction endonuclease sites used in this site are indicated and numbered relative to the start of mRNA2. The proposed position of the *deoR* binding sites is indicated by black circles and that of the *cytR* binding site by a large open circle while by the two CRP binding sites are indicated by small open circles (Valentin-Hansen, 1982; Valentin-Hansen *et al.*,1982), CRP denotes cyclic AMP receptor protein. The two palindromes (*deoR* binding sites) containing the Pribnow box (PB) are shown enlarged, together with the mapped position of transcription initiations of mRNA1 and mRNA2. The dot in the sequence represents the center of symmetry.

Two *deoR* binding sites are necessary for full repression

To test the possibility of artificial promoters in λKT31 and λKT32 new *deoP-galK* fusions were constructed. λKT36 and λKT37 both contain ends which differ from those in λKT31. As seen in Figure 2 de-repression is still 3-fold due to the elevated basal levels. A new *deoP2* fusion (λKT35) gave the same result with respect to the lack of repression of the basal level. It is therefore concluded that the high basal level in the *deoP-galK* fusions is not caused by the creation of artificial promoters at the fusion points. This leaves us with the third possibility, that both promoter fragments are needed to achieve full repression.

Evidence has been presented indicating that the *deoR* repressor regulates *deoP2* (Valentin-Hansen *et al.*, 1982a; Short and Singer, 1984). *DeoP1* and *deoP2* each contain the same 16-bp palindromic sequence overlapping the Pribnow box (see Figure 1). We have isolated mutants in *deoP1* with reduced affinity for *deoR* and, furthermore, shown that these mutants contain a single or double base-pair change in the palindromic sequence (manuscript in preparation). This indicates that the palindrome constitutes or is part of the *deoR* binding site and, since both promoter fragments are needed to achieve full repression, we assume that in fact two *deoR* binding sites are necessary for full repression of both *deoP1* and *deoP2*.

To test this hypothesis we first introduced a *deoR* binding site upstream of *deoP2* in λKT35. This was done by cloning the 236-bp *deoP1* fragment of λKT31 in opposite orientation into the *Hind*III site located 16 bp upstream of the *deoP2* fragment. The opposite-orientated *deoP1* fragment in the resulting phage λKT38 therefore only provides the palindromic *deoR* binding site but no *deoP1* promoter activity to *deoP2*. As can be seen from Figure 2, these manipulations result in a complete repression of the wild-type levels and a total 310-fold de-repression. The homologous plasmid containing *deoP1* in the same orientation as *deoP2* was easily obtained, but we were not able to transfer this fusion to λ*gal8*.

The effect of two *deoR* binding sites on *deoP1* repression was also measured in λKT41 which contains two 224-bp *deoP1* fragments from λKT36 cloned in tandem with both promoters reading into *galK* (Figure 2). Here also we find a complete repression of *galK* expression in the wild-type strain and a total 115-fold de-repression. A construction with the two *deoP1* fragments in different orientations was not obtained; this can be
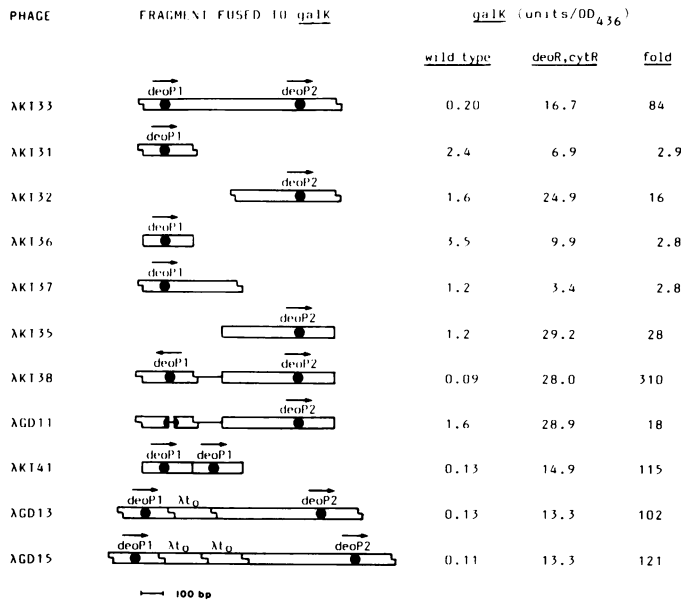
Table I. Single-copy expression of *deoP-galK* fusions

Strain	Phage	*galK* (units/O.D.₄₃₆)						*deoA* units/O.D.₄₃₆
		deoP1+P2 λKT33	*deoP1* λKT31	*deoP2* λKT32	*deoP1,P2* λKT38	*deoP1*,P2* λGD11	*deoP1,P1* λKT41	
SØ3430	Wild-type	0.20 (1.0)	2.4 (1.0)	1.6 (1.0)	0.09 (1.0)	1.6 (1.0)	0.13 (1.0)	3.1 (1.0)
SØ3431	*cytR*	0.69 (3.5)	2.3 (1.0)	15.5 (10)	1.1 (12)	17.6 (11)	0.11 (1.0)	25.5 (8.3)
SØ3418	*deoR*	6.8 (34)	6.6 (2.8)	2.1 (1.3)	2.1 (23)	2.3 (1.4)	15.0 (115)	202 (66)
SØ3432	*deoR,cytR*	16.7 (84)	6.9 (2.9)	24.9 (16)	28.0 (310)	28.9 (18)	14.9 (115)	405 (132)
SØ3430 +pKH31	Wild-type	n.d.	0.09	0.60				
SØ3431 +pKH31	*cytR*	n.d.	0.07	0.90				

Promoter-*galK* fusions were constructed in plasmids pGD4 or pKB-1 and transferred to λ*gal8* by homologous recombination as described in Materials and methods. Single lysogens of the phages in the four regulatory mutants were assayed for galactokinase (*galK*) and thymidine phosphorylase (*deoA*) (triplicate assays with a variation <10%). In each case the *galK* expression from the vector phage λKT34 (0.09 units/O.D.₄₃₆) was substracted from the *galK* expression. The numbers in parentheses indicate the regulation (n-fold) relative to wild-type.
n.d.: not determined.

explained by the creation of a perfect 448-bp palindrome, which has been shown to be unstable in *E. coli* (Collins, 1981; Goodchild *et al.*, 1985).

The final proof that two *deoR* binding sites are a necessity for full repression was provided by site-directed mutagenesis of the *deoP1* fragment in λKT38. This was done in the corresponding plasmid by taking advantage of the *Eco*RI site in the center of the 16-bp palindrome in *deoP1* (Figure 1). After cutting with

*Eco*RI we filled the ends using DNA polymerase (Klenow fragment) and re-ligated, thus disrupting the *deoR* recognition site. λGD11 which contains this construction gave the expected result (Figure 2): the *galK* level in the wild-type strain is elevated thereby causing a decrease in the total de-repression to that found for λKT35. This proves that the presence of the extra *deoR* binding site in λKT38 is responsible for the repression of the wild-type levels.

Regulation of *deoP1* and *deoP2*

More detailed analysis of the regulation of selected *deo-galK* fusions was obtained by measuring *galK* expression in lysogens of otherwise isogenic *recA*-wild-type, *-cytR*, *-deoR* and *-deoR*, *cytR* strains. As seen from Table I, *deoP1* (λKT31) is only regulated by the *deoR* repressor and not by the *cytR* repressor. The evidence for this statement is even better when the tandem *deoP1* (λKT41) is used; the *deoR* de-repression for this strain is 115-fold (*deoR* compared with wild-type and *cytR* compared with *deoR*, *cytR*), while no *cytR* de-repression is seen. The regulation of *deoP1+P2* (λKT33) is very similar to that of the chromosomal *deoA* gene (Table I).

Finally, the results with λKT38 show the regulation of the isolated *deoP2* promoter when provided with an extra *deoR* binding site. As can be seen from Table I, introduction of a *cytR* mutation increases *deoP2* expression 12-fold (wild-type to *cytR* and also *deoR* to *deoR*, *cytR*), while introduction of a *deoR* mutation causes a 23 to 25-fold increase in *deoP2* expression (wild-type to *deoR* and *cytR* to *deoR*, *cytR*).

We may thus conclude that *deoP2* is subject to cumulative repression by the *deoR* and *cytR* repressors. That an extra *deoR* binding site is necessary for full *deoR* repression becomes evident when the results obtained with λKT38 containing *deoP1+P2* are compared with those of λKT32 which contains *deoP2* alone, and those of λGD11 where the *deoR* binding site in *deoP1* is destroyed (Table I). In the absence of *deoR* repressor, the enzyme levels in λKT32, λGD11 and λKT38 are identical, as shown for the *deoR* and *deoR*, *cytR* mutants in Table I. This supports our hypothesis that the *deoP1* fragment donates a *deoR* binding site in λKT38 but does not in any other way interfere with the expression from *deoP2*.

Fig. 2. Single-copy expression of repressed (wild-type) and de-repressed (*deoR*,*cytR*) *deoP-galK* fusions. The fragments fused to *galK* are indicated with either blunt ends (cloned in the *Sma*I site), or with sticky ends (cloned in the *Hind*III site). Construction of the phages is outlined in Table II. The direction of transcription from the promoters is indicated by an arrow (*galK* is positioned to the right of the fragments). Black circles indicate the position of the *deoR* binding sites. The sizes of the fragments are drawn to scale except in λKT38 and λGD11 where the spacing between the *deoP1* and *deoP2* fragment is 16 bp. *GalK* was measured in single lysogens of the phages in SØ3430 (wild-type) and SØ3432 (*deoR*,*cytR* mutant) as described in Materials and methods. The *galK* expression of the vector phage λKT34 is subtracted from each level (0.09 units/O.D.$_{436}$).

Table II. Construction of plasmids and phages

| Phage | Plasmid | DNA fragment inserted | | | *Hind*III linker | Cloning vector/site |
		Function	Size (bp)	Ends		
λKT34	pGD4	–	10	*Bam*HI linker	–	pKB-1/*Sma*I
λKT33	pKH19	*deoP1+P2*	851	*Hae*III/*Hae*III	+	pKB-1/*Hind*III
λKT31	pGD17	*deoP1*	224	*Hae*III/*Alu*I	+	pGD4/*Hind*III
λKT32	pGD19	*deoP2*	430	*Dde*I/*Hae*III	+	pGD4/*Hind*III
λKT36	pGD27	*deoP1*	224	*Hae*III/*Alu*I	–	pGD4/*Sma*I
λKT37	pGD25	*deoP1*	425	*Hae*III/*Dde*I	+	pGD4/*Hind*III
λKT35	pGD21	*deoP2*	492	*Alu*I/*Hae*III	–	pGD4/*Sma*I
λKT38	pGD23	*deoP1*	224	*Hae*III/*Alu*I	+	pGD21/*Hind*III
λKT41	pGD28	2 × *deoP1*	2 × 224	*Hae*III/*Alu*I	–	pGD4/*Sma*I
λGD11	pGD29	–	4	*Eco*RI site in pGD23 filled up by Klenow		
λGD12	pGD30	–	4	*Bgl*II site in pKH19 filled up by Klenow		
λGD13	pGD31	λt$_o$	199	*Bam*HI/*Bam*HI	–	pKH19/*Bgl*II
λGD15	pGD32	2 × λt$_o$	2 × 199	*Bam*HI/*Bam*HI	–	pKH19/*Bgl*II
–	pKH31	*deoR*$^+$	3200	*Bam*HI/*Bam*HI		pBR322/*Bam*HI

All phages are derivatives of λ*gal*8 and were derived from the corresponding plasmids as described by McKenney *et al.* (1981). The promoter fragments were all isolated from pVH17 (see Materials and methods). When a *Hind*III linker was ligated to the fragment the indicated size is increased by 12 bp. The resulting structures are also presented in Figure 2. The position of the relevant restriction sites is shown in Figure 1.

Effects of increasing the intracellular deoR repressor concentration

In wild-type and *cytR* strains we increased the intracellular concentration of *deoR* repressor by introducing the *deoR*[+] gene cloned in pBR322 (pKH31). This resulted in an increase in repression of *deoP1* in λKT31 which equals that observed for λKT41 where an extra *deoR* binding site was introduced (Table I). Increasing the *deoR* concentration also reduces expression from *deoP2* (λKT32) in a wild-type cell ~3-fold, but the repression of *deoP2* is not as efficient as that seen in λKT38 (0.09 compared with 0.60). If the corresponding *cytR* strains are compared, a dramatic effect of increasing the *deoR* repressor concentration is seen on the *galK* expression in λKT32 where a reduction from 15.5 to 0.90 units/O.D.$_{436}$ is observed. This reduced *galK* level is identical to that found in the *cytR* strain lysogenized with λKT38. Again we find that increasing the *deoR* repressor concentration has the same effect on *deoP2* as introduction of an additional *deoR* binding site.

Effect of the distance between the two deoR binding sites

From Figure 2 it can be seen that full repression can be obtained in the presence of two *deoR* binding sites when these are separated by 224 bp (λKT41), 449 bp (λKT38) or 599 bp (λKT33). To test whether or not *deoP2* could still be repressed with increasing distances between the binding sites we constructed λGD13 and λGD15. In λGD13 we inserted a 199-bp *Bam*HI fragment containing the λ t$_0$ terminator into the *Bgl*II site of pKH19. In λGD15 two inserts are placed in tandem, thus increasing the distance by 398 bp. The results in Figure 2 clearly show that full repression in wild-type strains is also obtained when the distance between the two *deoR* binding sites is 798 bp and 997 bp.

Discussion

The *galK* vector system allows a comparison of promoter strength since all fusions contain the same untranslated leader sequence of at least 73 bp before the initiation codon for *galK* translation. The de-repressed *galK* level in the *deoR,cytR* strains is therefore a good estimate of the relative promoter strength of the *deo* promoters. From the results in Figure 2 *deoP2* is calculated to be four times stronger than *deoP1*. Furthermore, expression from *deoP1*+*P2* is lower than that from *deoP2* alone and only half of the sum of *deoP1* and *deoP2* expression (16.7 units/O.D.$_{436}$ as compared with 31.8 units/O.D.$_{436}$). These results suggest that *deoP2* expression is inhibited in λKT33 and therefore also in the native *deo* operon. The results with λGD13 and λGD15 show that it is not transcription from *deoP1* which inhibits *deoP2*, since insertion of a transcriptional terminator between the two promoters did not result in elevated de-repressed levels, i.e., the expression from λGD13 and λGD15 in a *deoR,cytR* mutant is expected to be like that of λKT32 where transcription only originates from *deoP2* if transcription from *deoP1* inhibits *deoP2* expression.

A comparison of the *galK* expression in the *deoR* strains suggests that at least 6.8 − 2.1 = 4.7 units/O.D.$_{436}$, and probably more, originates from *deoP1* when both promoters are present as in the native *deo* operon. In Figure 2 a rather large variation in promoter strength of *deoP1* was noticed: 3.5 − 10 units/O.D.$_{436}$. The calculation performed above suggests that λKT31 reflects the native *deoP1* promoter strength quite well (6.9 units/O.D.$_{436}$).

DeoP2 is a strong promoter with a measured activity of 25 units/O.D.$_{436}$, which corresponds to 56 units/O.D.$_{650}$. In the same *galK* system the activity from *araP*$_{BAD}$ was found to be

2.7 units/O.D.$_{650}$ when induced with arabinose (Dunn *et al.*, 1984; Hahn *et al.*, 1984). *DeoP2* is thus 20 times stronger than *araP*$_{BAD}$. The λ P$_L$ promoter which is expected to be one of the strongest procaryotic promoters was found to be only 2 − 3 times stronger than *deoP2* (manuscript in preparation). The strength of the *deo* promoters is also demonstrated by the finding that the growth of N100 strains containing plasmid *deoP-galK* fusions is inhibited in the presence of galactose (see Materials and methods).

With respect to the regulation of the *deo* operon, the *deoP1*- and *deoP2-galK* fusions provide clear evidence for the following two conclusions. (i) *DeoP1* is regulated by the *deoR* repressor but not by the *cytR* repressor. (ii) *DeoP2* is regulated both by the *deoR* and the *cytR* repressors in a cumulative way.

Finally, the results show that the presence of two *deoR* binding sites is necessary for full *deoR* repression of both *deoP1* and *deoP2*. The two *deoR* binding sites were shown to be functional even at completely different distances from each other: 599 bp in λKT33 and the native *deo* operon, 224 bp in λKT41, 449 bp in λKT38, 798 bp in λGD13 and 997 bp in λGD15. However, altering the distance from 599 bp to 603 bp in *deoP1*+*P2* (λKT33) by filling out the *Bgl*II site did not alter the repressed level (data not shown), while changing the 16-bp palindromic sequence by filling out the *Eco*RI site in *deoP1* (λKT38) destroyed the operator activity. This confirms both the location of the *deoR* binding site and the hypothesis of two *deoR* binding sites being necessary for full repression of *deoP2*.

The occurrence of two or several repressor binding sites in operons in *E. coli* is not a new observation. In the *gal* operon an operator site necessary for repression was found within the first structural gene *galE*, at a position 110 bp downstream of the *gal* promoter/operator region (Irani *et al.*, 1983; Majumdar and Adhya, 1984). Also in the *ara*$_{BAD}$ operon a second operator site was found 200 bp upstream of the *araI* site, and here also it was demonstrated that both sites are required in order to obtain full repression of the operon (Dunn *et al.*, 1984; Hahn *et al.*, 1984).

Dunn *et al.* (1984) demonstrated that repression of the *ara*$_{BAD}$ operon is unaffected by insertion of small DNA fragments with an integral turn of the DNA helix (11 and 31 bp) between the two operators, while repression is impaired when half-integral turns of the helix were inserted (5, 15 bp, etc.). Based on these findings they proposed a model where a loop in the DNA is formed by mutual attraction between *araC* molecules bound to the two *araC* binding sites. They furthermore suggest that this could leave the *araC* protein in a state incapable of performing its transcriptional stimulation of the *araP*$_{BAD}$ promoter. Majumdar and Adhya (1984) proposed three mechanisms for the repression of the *gal* operon: (i) steric hindrance of open complex formation; (ii) a conformational change in the DNA and (iii) blocking of RNA polymerase entry sites.

The finding that the *deoR* operator site and the Pribnow box have overlapping locations in both *deoP1* and *deoP2* (Valentin-Hansen *et al.*, 1982a) strongly suggests that *deoR* repression of the *deo* operon is exerted by steric hindrance of open complex formation between the *deo* promoters and RNA polymerase [mechanism (i)]. Since the two *deoR* operator sites function at several different distances from each other, i.e., 224 bp to 997 bp, a mechanism involving a DNA conformational change or blocking of RNA polymerase entry sites caused by the presence of these two operator sites seems unlikely. Two *deoR* binding sites are, however, needed for full repression of the *deo* operon. The results in Table I show that this requirement may be circum-

Table III. Construction of bacterial strains

Strain	Genotype	Construction/source/selection	
N99	F⁻,*pro,galK2,rpsL,*sup⁰,*deoR50,cytR50*	Max Gottesman	
SØ928	Δ*deo,lac,thi,upp,udp,ton,*Φ80ᴿ	This laboratory	
SØ1937	F⁻,*thi,thr,leu,metB,rpsL,argE::tn10*	This laboratory	
YYC194	F⁻,*proA2,trp30,his51,lac28,rpsL101,cm1A1,zbi::tn10*	J.E.Cronan, Jr.	
JC10240	Hfr(PO45),*thr300,ilv318,rspE300,recA56,srlC300::tn10*	S.Short	
SØ3314	X,*clmA1,zbi::tn10,cytR50*	N99 + Plv(YYC194)	tetᴿ
SØ3309	X,*metB,argE::tn10,deoR50*	N99 + Plv(SØ1937)	tetᴿ
SØ3320	X,*metB,deoR50*	SØ3309 + Plv(SØ928)	Arg⁺
SØ3330	X,*clmA1,zbi::tn10,metB*	SØ3320 + Plv(YYC194)	tetᴿ
SØ3297	X,*clmA1,metB*	From SØ3330	tetˢ
SØ3298	X,*clmA1,cytR50*	From SØ3314	tetˢ
SØ3341	X,*metB,deoR51*	From SØ3330, fast growth on IR	
SØ3342	X,*deoR51,cytR50*	SØ3341 + Plv(SØ3298)	Met⁺
SØ3430	X,*clmA1,metB,recA56,srlC300::tn10*	SØ3297 + Plv(JC10240)	tetᴿ
SØ3431	X,*clmA1,cytR50,recA56,srlC300::tn10*	SØ3298 + Plv(JC10240)	tetᴿ
SØ3418	X,*metB,deoR51,recA56,srlC300::tn10*	SØ3341 + Plv(JC10240)	tetᴿ
SØ3432	X,*deoR51,cytR50,recA56,srlC300::tn10*	SØ3342 + Plv(JC10240)	tetᴿ

X indicates the common genotype F⁻,*pro,galK2,rpsL,*sup⁰. SØ3341 was selected as fast growing colonies of SØ3330 on inosine (IR) minimal plates (see Hammer-Jesperson, 1983). Apparently *clmA1,zbi::tn10,deoR*⁺ is deleted from the chromosome since SØ3341 also became clmˢ and tetˢ during selection for *deoR*.

vented if the intracellular concentration of *deoR* repressor is increased by introduction of the *deoR*⁺ gene cloned in pBR322 (pKH31). We therefore suggest that the presence of two *deoR* operators increases the local concentration of *deoR* repressor. It is possible that transfer of *deoR* repressor from *deoP1* to *deoP2* is accomplished by sliding along the DNA helix or by intersegment transfer from one operator site to the other (von Hippel *et al.*, 1984). The latter mechanism would require a DNA loop formation as a transient intermediate. This DNA loop structure, held by one repressor molecule bound to both operator sites could, however, also be a permanent DNA structure present when the *deo* operon is repressed. Such a repressor-DNA complex can be expected to possess an enhanced stability because of the low probability of dissociation from both binding sites at the same time. Dissociation from only one operator site could happen occasionally, but the operator site would soon be caught by the repressor still being bound at the other operator site. This is analogous to the principle used in metal chelates.

If intersegment transfer from one operator site to the other or permanent loop formation is the main mechanism involved in *deoR* repression, we expect the repression to be sensitive to small changes in the distance between the binding sites, i.e., half-integral turns of the DNA helix, provided that the distance between the binding sites is ~ 100 bp or less.

Materials and methods

Materials

Restriction endonucleases, T4 DNA ligase, DNA polymerase (Klenow fragment) and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim or New England Biolabs and were used as recommended by the manufacturers. Synthetic *Hind*III and *Bam*HI linkers were obtained from PL Biochemicals and BRL, respectively. The DNA sequencing kit was from New England Biolabs and was used following the manufacturer's instructions. DE81 filters were purchased from Whatman.

Plasmids and strains

The promoter cloning vectors pKB-1 and pKO1-t were generous gifts from Dr K.McKenney. pKB-1 contains a region of bacterial DNA ('B region') followed by the tₗ terminator, the cloning sites *Hind*III and *Sma*I, the untranslated leader and the *galK* gene (McKenney, 1982). The presence of the 'B region' which is identical to the DNA found upstream of the *gal* promoter in λ*gal*8 makes it

possible to transfer promoter-*galK* fusions from the plasmid to λ*gal*8 by homologous recombination *in vivo*. The tₗ terminator was inserted in order to terminate a weak transcription initiating in the 'B region' (McKenney, 1982). pVH17, which was kindly given to us by Dr P.Valentin-Hansen, is a pBR322 derivative containing 2130 bp *deo* DNA covering from 490 bp upstream of *deoP1* to 1040 bp downstream of *deoP2* (Valentin-Hansen *et al.*, 1982a). pSS1819, which was kindly given to us by Dr S.Short, contains the *deoR*⁺ gene cloned into the *Bam*HI site of pBR322 (Short and Singer, 1984). All strains used are derivatives of N99 (*E. coli* K12), see Table III. When we tested N99 we found that it had become *deoR,cytR* spontaneously, the *deoR* mutation being leaky (*deoR50*) (N100 which should be the isogenic *recA* derivative is *deoR*⁺,*cytR*⁺). SA494 (*his, ΔgalETK*), which was used to test λ*gal*8 derivatives, was a gift from Dr S.Adhya. λ*gal*8 (λ*galETK*⁺,*c*I857) (Feiss *et al.*, 1972), λW30 (λ*b2,c*I) and λW60 (λ*c*I90,*c*17) were obtained from the NIH collection.

Standard techniques

E. coli transformation, plasmid DNA isolation, digestion of DNA with restriction enzymes, etc., were carried out as described by Maniatis *et al.* (1982) and Silhavy *et al.* (1984). DNA fragments were isolated and purified from agarose or polyacrylamide gels as described by Maniatis *et al.* (1982). DNA sequencing was performed using the dideoxy method of Sanger *et al.* (1977).

Construction of plasmids

Construction of all plasmids used in this work is outlined in Table II. Since the 'B region' in pKB-1 contains a *Sma*I site, pGD4 was constructed by inserting a 10-bp *Bam*HI linker into this *Sma*I site. Therefore two unique sites, *Sma*I and *Hind*III, can now be used for cloning. pGD4 was used as a cloning vector for all constructions except pKH19 where pKB-1 was used.

An 851-bp *Hae*III fragment containing all information necessary for the native regulation of the *deo* operon (see Results) was isolated from pVH17 and from this preparation smaller fragments were isolated (see Figures 1 and 2). Fragments with blunt ends (*Hae*III, *Alu*I) were either inserted directly into the *Sma*I site of pGD4 or could, after addition of a 12-bp *Hind*III linker, be inserted into the *Hind*III site. The *Dde*I 5' overhang was made blunt by filling in with DNA polymerase (Klenow fragment). Plasmid *deoP-galK* fusions were selected in N100 as transformants which grew well on LB plates while being restrained on MacConkey galactose plates (small white colonies). Alternatively, the cloning sites were treated with alkaline phosphatase prior to ligation in order to prevent self ligation of the plasmid (Maniatis *et al.*, 1982). The *Eco*RI site in pGD23 and the *Bgl*II site in pKH19 were destroyed by filling up the protruding ends by DNA polymerase (Klenow fragment) followed by religation (Maniatis *et al.*, 1982). The λ tₒ terminator was isolated from pKO1-t on a 199-bp *Bam*HI fragment (189 bp *Dpn*I fragment + 10 bp *Bam*HI linker; McKenney, 1982) and inserted into the *Bgl*II site between *deoP1* and *deoP2* in pKH19. pKH31 was constructed by cloning the 3.2-kbp *Bam*HI fragment of plasmid pSS1819 containing the *deoR*⁺ gene into the *Bam*HI site in pBR322.

Transfer of gene fusions to λgal8

Transfer of promoter-*galK* fusions from plasmid to λ*gal*8 by *in vivo* recombination

was performed as described by McKenney *et al.* (1981). The phages and corresponding plasmids of all the λ*gal*8 derivatives are outlined in Table II.

Construction of bacterial strains

Four new isogenic strains SØ3430, SØ3431, SØ3418 and SØ3432 were constructed from N99 by P1v transduction (Miller, 1972). The strains were made recA⁻ to ensure that the λ phages would only integrate into the λ attachment site and not by homology. The construction of the strains is outlined in Table III. These four strains termed wild-type, *cytR*, *deoR* and *deoR*,*cytR* were lysogenized with the phages listed in Table II as described by Silhavy *et al.* (1984) using λW30 (λ*b*2,cI) as selector phage. Lysogens were scored as single lysogens by their sensitivity to λW60 (λcI90,c17) in cross-streaking tests, using known single and multiple lysogens as controls (Shimada *et al.*, 1972).

Growth of bacteria for enzyme assays

Cells were grown exponentially at 33°C for at least six generations in AB medium (Clark and Maaløe, 1967) with glycerol as carbon source (0.2%) supplemented with the appropriate requirements. Plasmid-containing cells were grown with 150 μg/ml ampicillin. Cells were harvested by centrifugation at a density of 1.5×10^{10} cells/ml and washed in 1/2 volume AB. Finally the cells were resuspended in lysis buffer (100 mM Tris-HCl pH 7.9, 3 mM EDTA, 3 mM DTT). Several dilutions were made for accurate O.D.$_{436}$ measurements and the cells were disrupted by sonication. Cell debris was removed by centrifugation.

Enzyme assays

The assay for thymidine phosphorylase (*deoA*) has been described elsewhere (Munch-Petersen, 1968). Units are expressed as nmol of thymidine converted to thymine per min at 37°C.

Galactokinase (*galK*) was assayed as described by McKenney *et al.* (1981) with the following modifications: 10 μl sonic extract (or proper dilution in lysis buffer) was mixed with 40 μl of reaction mix (10 μl mix1 + 25 μl mix2 + 5 μl mix4, see McKenney *et al.*, 1981) and incubated at 32°C. After 2 and 32 min, 20 μl was transferred to a DE81 filter, washed with distilled water, dried and counted. It ws necessary to use a two-point assay since the background value (blank) varied with the concentration of the extract, and a high concentration was required ($5 \times 10^9 - 10^{10}$ cells/ml) when repressed levels were measured. Galactokinase units are expressed as nmol of galactose phosphorylated per min at 32°C.

Specific activities are expressed as units of enzyme activity per ml of cell at O.D.$_{436}$=1.0. 1 ml cells at O.D.$_{436}$=1 corresponds to 1 ml cells at O.D.$_{650}$=0.44 (measured on a Zeiss M4 QIII spectrophotometer) and to ~0.08 mg/ml of protein when a supernatant of a sonicated extract is subjected to a protein determination according to Lowry *et al.* (1951) (using bovine serum albumin as a standard).

Acknowledgements

References

Albrechtsen,H., Hammer-Jespersen,K., Munch-Petersen,A. and Fiil,N. (1976) *Mol. Gen. Genet.*, **146**, 139-145.

Clark,D.J. and Maaløe,O. (1967) *J. Mol. Biol.*, **23**, 99-112.

Collins,J. (1981) *Cold Spring Harbor Symp. Quant. Biol.*, **45**, 409-416.

Dunn,T.M., Hahn,S., Ogden,S. and Schleif,R. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5017-5020.

Feiss,M., Adhya,S. and Court,D.L. (1972) *Genetics*, **71**, 189-206.

Goodchild,J., Michniewicz,J., Seto-Young,D. and Narang,S. (1985) *Gene*, **33**, 367-371.

Hahn,S., Dunn,T. and Schleif,R. (1984) *J. Mol. Biol.*, **180**, 61-72.

Hammer-Jespersen,K. (1983) in Munch-Petersen,A. (ed.), *Metabolism of Nucleotides, Nucleosides and Nucleobases in Microorganisms*, Academic Press, London, pp. 203-257.

Irani,M.H., Orosz,L. and Adhya,S. (1983) *Gene*, **32**, 783-788.

Jørgensen,P., Collins,J. and Valentin-Hansen,P. (1977) *Mol. Gen. Genet.*, **155**, 93-102.

Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265-275.

Majumdar,A. and Adhya,S. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6100-6104.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.

McKenney,K. (1982) Ph.D. Thesis, The Johns Hopkins University, Baltimore, MD.

McKenney,K., Shimatake,H., Court,D., Schmeissner,U., Brady,D. and Rosenberg,M. (1981) in Chirikjian,J.C. and Papas,T.S. (eds.), *Gene Amplification and Analysis, Vol. II: Analysis of Nucleic Acids*, Elsevier-North Holland/Amsterdam, pp. 383-415.

Miller,H.J. (1972) *Experiments in Molecular Genetics*, published by Cold Spring Harbor Laboratory Press, NY.

Munch-Petersen,A. (1968) *Eur. J. Biochem.*, **6**, 432-442.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.

Shimada,K., Weisberg,R.A. and Gottesman,M.E. (1972) *J. Mol. Biol.*, **63**, 483-503.

Short,S.A. and Singer,J.T. (1984) *Gene*, **31**, 204-211.

Silhavy,T.J., Berman,M.L. and Enquist,L.W. (1984) *Experiments with Gene Fusions*, published by Cold Spring Harbor Laboratory Press, NY.

Svenningsen,B.A. (1977) *Carlsberg Res. Commun.*, **42**, 517-524.

Valentin-Hansen,P. (1982) *EMBO J.*, **1**, 1049-1054.

Valentin-Hansen,P., Svenningsen,B.A., Munch-Petersen,A. and Hammer-Jespersen,K. (1978) *Mol. Gen. Genet.*, **159**, 191-202.

Valentin-Hansen,P., Aiba,H. and Schumperli,D. (1982) *EMBO J.*, **1**, 317-322.

Valentin-Hansen,P., Hammer,K., Larsen,J.E.L. and Svendsen,I. (1984) *Nucleic Acids Res.*, **12**, 5211-5224.

von Hippel,P.H., Bear,D.G., Morgan,W.D. and McSwiggen,J.A. (1984) *Annu. Rev. Biochem.*, **53**, 389-446.