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DNA-protein recognition: demonstration of three genetically separated operator elements that are required for repression of the *Escherichia coli deoCABD* promoters by the DeoR repressor

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The sequences required for full repression of the *Escherichia coli deo*P1 and P2 promoters by the *deo*R repressor (DeoR) have been analyzed *in vivo*. Using recombinant techniques, we have constructed a set of *deo-lacZ* fusions which contain different parts of the sequences involved in the regulation of *deo* expression on low copy number fusion vectors. Since these vectors are present in only one copy per chromosome at temperatures below 37°C, this vector system allows very accurate studies of gene control signals. Our results show that three DeoR operator sites exist in the *deo*P1-P2 regulatory region. Two of these loci overlap the initiation sites for *deo*P1 (O_1) and *deo*P2 (O_2) transcription located 599 bp apart, whereas the third site (O_E) is present ~270 bp upstream of P1. DeoR repression of both P1 and P2 transcription is weak on promoter fragments which only contain one operator site (O_1 or O_2). Enhanced repression by *deo*R is observed on promoter fragments containing two operator sites. However, all three sites are needed for full repression. These findings are discussed with respect to upstream and downstream control regions of eukaryotic genes.

Key words: DNA-protein recognition/multiple operators/transcriptional-translational control/deo operon/low copy number fushion vectors

Introduction

In prokaryotes interaction between the gene and sequence-specific DNA binding proteins has been shown to play an essential part in the control of gene expression. Control of transcriptional initiation typically depends on a short sequence upstream of a structural gene. This promoter region contains a binding site for RNA polymerase and may also contain nearby or overlapping targets for regulatory proteins. Repressor proteins most often bind to targets in promoter regions close enough to block the binding of RNA polymerase. The regulatory region of the *lac* operon is the classic example of such a regulatory unit. However, our picture of prokaryotic control regions has changed recently. Thus, two separated operators in *gal, ara* and in *deo* are required for repression (Irani *et al.*, 1983; Fritz *et al.*, 1983; Majumdar and Adhya, 1984; Dunn *et al.*, 1984; Hahn *et al.*, 1984; Dandanell and Hammer, 1985). The finding of an additional regulatory site located at a considerable distance from the promoter target illustrates a new interesting example of how efficient regulation can be achieved.

Structure and regulation of the deo operon

The *deo* operon of *Escherichia coli* consists of four structural genes encoding nucleoside and deoxynucleoside catabolizing enzymes; for review see Hammer-Jespersen (1983). Initiation of transcription occurs from two promoters located 599 bp apart in front of the operon (Valentin-Hansen *et al.*, 1982a), and from an internal promoter (Valentin-Hansen *et al.*, 1984). Thus, two long messages, mRNA1 and mRNA2, are transcribed for all four genes, while the two distal genes are also transcribed into a short mRNA3 (Figure 1).

Initiations from P1 have been shown to be controlled negatively by the DeoR repressor, whereas initiations from P2 are dependent on the cAMP/CRP complex and are negatively controlled by both the CytR and DeoR repressor (Valentin-Hansen, 1985). The structure of the *deo*P1-P2 regulatory region is outlined in Figure 2. Two CRP binding sites at the *deo*P2 promoter are centered ~40 bp (CRP-1) and 90 bp (CRP-2) upstream from the P2 transcriptional initiation site (Valentin-Hansen, 1982). The CRP-2 target is not required for a high expression level; however, if present it increases significantly the rate by which transcription initiation takes place (Valentin-Hansen, 1985). Based on deletion analysis of the P2 regulatory region the binding site for CytR has been mapped to overlap the CRP-2 target (Valentin-Hansen, 1985). One of the most striking features in the nucleotide sequence of the P1-P2 regulatory region is a segment of 16 bp that overlaps the Pribnow boxes and transcriptional initiation sites for P1 and P2 mRNA synthesis (Valentin-Hansen *et al.*, 1982a). Out of 16 bp only one differs between the two repeats and, in addition, the P1 sequence is a perfect palindrome. Due to the sequence homology we have suggested that these repeats are the operator sites for DeoR. Recently, O^c mutants in the P1 target

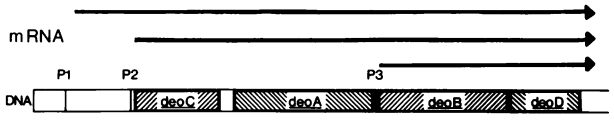

Fig. 1. Schematic map of the *deo* operon. The structural *deo* genes code for the following enzymes: *deoC*, deoxyriboaldolase; *deoA*, thymidine phosphorylase; *deoB*, deoxyribomutase and *deoD*, purine nucleoside phosphorylase. Three promoter regions have been demonstrated: at P_1 and P_2 transcription is repressed by the *deoR* repressor and induced by deoxyribose-5-P; at P_2 transcription is also repressed by the *cytR* repressor, induced by cytidine or adenosine, and depends on the CRP/cAMP complex; at P_3 transcription is controlled by unknown protein factor(s) and induced by inosine or guanosine.

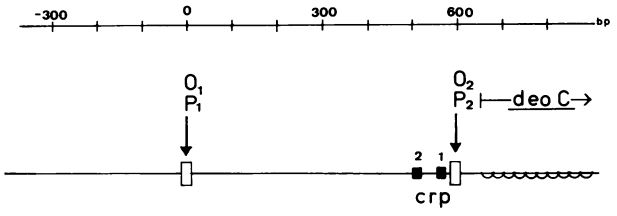

Fig. 2. Map of the *deo*P1-P2 regulatory region. The DNA binding sites for the DeoR repressor (*deo*O_1, *deo*O_2) and the cAMP/CRP complex are indicated by white and black boxes, respectively. The O_1 and O_2 operators overlap the initiation sites for P1 and P2 transcription.

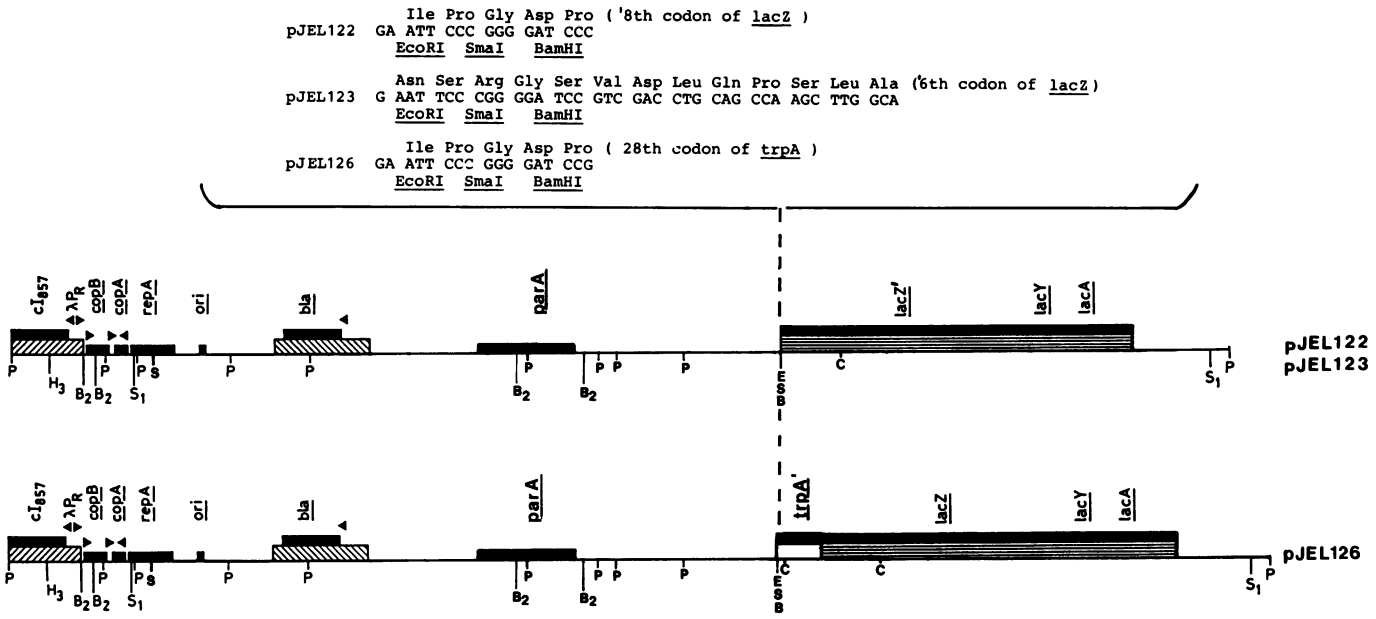

Fig. 3. Structure of the protein fusion vectors pJEL122, pJEL123 and the operon fusion vector pJEL126. The positions of the *cI* gene and P_R promoter from phage λ, the basic replicon from R1 (*copB, copA repA ori*), the ampicillin-resistance gene (*bla*) and the *parA* gene from R1 are shown together with the location of the lactose operon structural genes (*lacZ,Y,A*). Restriction enzyme sites are indicated as follows: *Bam*HI (B), *Bgl*II (B_2), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H_3), *Sal*I (S_1), *Sma*I (S). Shown at the top are the translational phasing of the *Eco*RI, *Sma*I and *Bam*HI restriction sites relative to the 8th codon of *lacZ* (pJEL122) the 6th codon of *lacZ* (pJEL123) and the 28th codon of trpA (pJEL126).

have been isolated, which strongly indicates that the DeoR recognition sites encompass the Pribnow box sequence of each of the two promoters (Dandanell and Hammer, 1985).

Here we present a further analysis of the *deo* system which reveals an interesting example of long range interaction between a repressor−operator complex and targets located 270−870 bp outside the promoter regions. Our analysis of various fusions between the *deo*P1 and *deo*P2 promoters and the *lac* genes shows the existence of three binding sites for the DeoR repressor in *deo* and that all three targets are required for proper regulation of both promoters by this regulatory protein. Moreover, we show that the first structural gene (*deoC*) is translated more efficiently from the longer *deo*P1 messenger than from transcripts from P2.

Results

Low copy vectors for studying gene control signals

Gene fusion has been one of the most important techniques used for studying signals involved in control of gene expression. Several high copy number vector gene fusion systems have been developed recently (McKenney *et al.*, 1981; Casadaban *et al.*, 1980). However, although very useful these systems have certain limitations when used in *in vivo* experiments. Due to the presence of many copies of these vectors per cell various ancillary factors can become limiting; overproduction of the proteins encoded by the recombinant plasmids can be toxic to the cell or make the plasmids extremely unstable. To avoid these and other potential problems (for discussion see Casadaban *et al.*, 1983; Rosenberg *et al.*, 1982; Raibaud *et al.*, 1984) we have created our fusions on low copy number vectors. These vectors (Figure 3) are mini-derivatives of R1 carrying an insertion of the λP_R promoter and the cI857 gene in front of the R1 *cop* genes (Løve Larsen *et al.*, 1984), in which the *Eco*RI, *Sma*I, *Bam*HI−*lacZYA* fragment of pMC1403, pSKS106 or pMC903, respectively, has been inserted (Casadaban *et al.*, 1980, 1983). At low temperatures (30−37°C) the plasmid copy number is one per genome. If the temperature is increased to 42°C the replication of the plasmids is uncontrolled, resulting in accumulation of up to a thousand copies per cell. Furthermore, these plasmids contain the *parA* function from R1 and are, therefore, faithfully segregated into each daughter cell during cell division (Nordström *et al.*, 1981).

Table I. DeoP1 operon fusion[a]

Strain	Genotype	β-Galactosidase activity[b]			
		pVH143	pVH144	pVH145	pVH146
S0928	wt	0.9 (1)	0.8 (1)	0.07 (1)	0.08 (1)
S0929	cytR⁻	0.9 (1)	0.8 (1)	0.07 (1)	0.08 (1)
S0930	deoR⁻	2.4 (2.6)	2.3 (2.9)	2.3 (33)	2.4 (30)
S0931	deoR⁻,cytR⁻	2.4 (2.6)	2.3 (2.9)	2.3 (33)	2.4 (30)

[a]For the structure of the plasmids, see Figure 4.
[b]The enzyme levels are measured after exponential growth at 36°C with glycerol as carbon source. Specific activities of β-galactosidase are expressed as OD_{420}/OD_{450}/min/ml culture.
The numbers in brackets indicate the regulation (n-fold) relative to wild-type.

The protein fusion vectors pJEL122 and pJEL123 (Figure 3) contain the entire *lac* operon but are missing the promoter/operator and translational initiation sites as well as the first six or eight non-essential codons of the *lacZ* gene. They contain three restriction endonuclease cleavage sites (*Eco*RI, *Sma*I and *Bam*HI), in various translation phases with the *lacZ* codons, into which DNA fragments containing promoter and translational initiation sites can be inserted to form gene fusions (see Figure 3). The operon fusion vector pJEL126 has the entire *lac* operon intact, except for the promoter/operator region, and contains part of the *trpA* gene between the *Eco*RI, *Sma*I and *Bam*HI restriction sites and *lacZ*. Expression of β-galactosidase from this vector is only dependent on insertion of DNA fragments carrying a promoter. All the fusions constructed for this study have *deo*

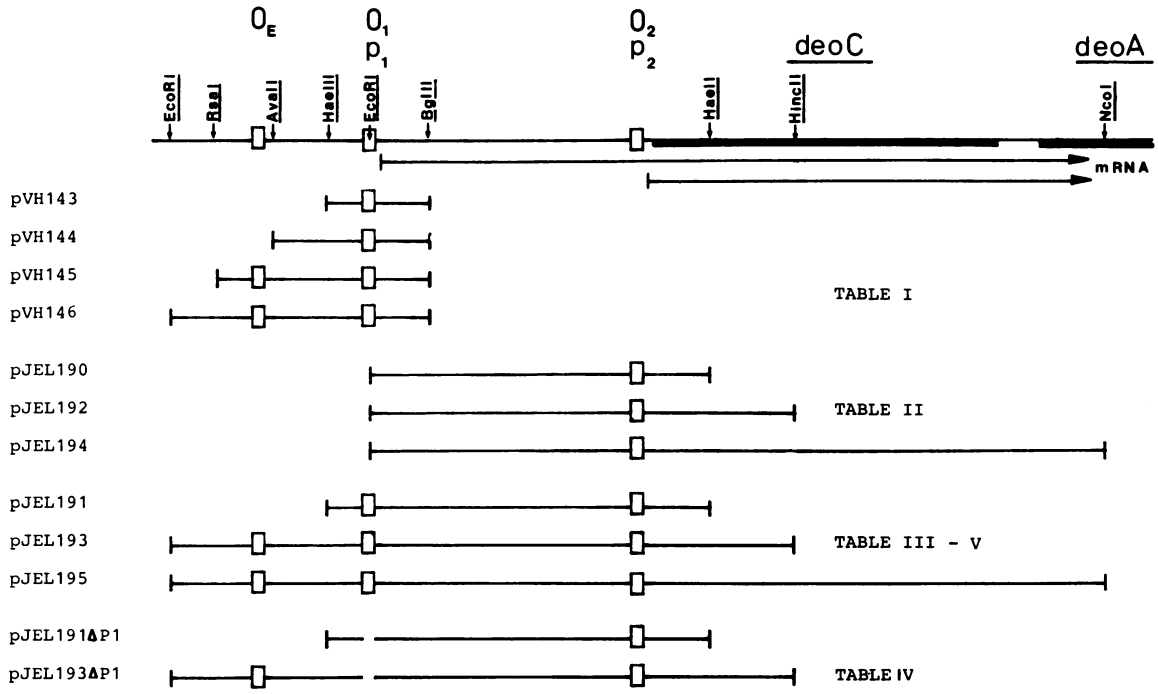

Fig. 4. *deo* fragments fused on the *lacZ* gene of pJEL126 and/or pJEL122/pJEL123. A schematic map of the *deo* P1-P2 regulatory region, the first structural gene *deoC* and the first part of *deoA* is shown at the top along with the restriction sites used in construction of *lacZ* fusions. The DeoR binding sites O_1 and O_2 and the *deo*O_E sequences are indicated by open boxes. The O_1 and O_2 targets overlap the P1 and P2 promoter regions.

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      -471        -461        -451        -441        -431        -421        -411        -401        -391
EcoRI
GAATTCACAC CGCGCATCGA TGATGGCGAT CTGCTGCTGG CAGTAGTGCG GTAGAGTGCG CGTAAAGAAG TCATCCAGCG ATTCGCTGCG

      -381        -371        -361        -351        -341        -331        -321        -311        -301
                              RsaI
CTCGGCAATG GCTTTCAGGT TAAGGCGTAC CAGCGTGCTG CCACCACCCG CCAGCGGCAG TGAGTTGTAA CAGCTCACAA TCCCGTAGCC

      -291        -281        -271        -261        -251        -241        -231        -221        -211
                              AvaII
CCCTTTTGTG AAAATTTTAT CATGCACCGG ACCGTTGGCG ATGTGCGGTT TGCTACATTC ACAGATGTTC TTCGCCACTT CCAGCAGCAG

      -201        -191        -181        -171        -161        -151        -141        -131        -121
GTCATCAGGG GTGATTTCAG GATCGTAGAT AAAGGTCAGG TTCGGTGAAA CCTGCTTCAA CTCTGCATCT GCACGTAAGA TCGCGCGGGT

      -111        -101          91          -81          -71          -61          -51          -41          -31
                  HaeIII
AATGGGCGAA TCAGACGGGC CGATATTGGC GTGCATAAAG GCGTCTGGCA GGGTTCTGTC GAGGTAACGC CAGAAACGTT TTATTCGAAC

       -21          11          -1
                              EcoRI
ATCGATCTCG TCTTGTGTTA GAATTCTAAC ⟶
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Fig. 5. The nucleotide sequence of the 460 bp *Eco*RI fragment located upstream of the P1 promoter (see Figure 4). The P1 DeoR binding site and the *deo*O_E sequences are underlined. The start site for P1 transcription is indicated by an arrow, and the restriction endonuclease sites used for construction of *lacZ* fusions are overscored. The DNA sequence strategy is discussed in Materials and methods.

fragments inserted into the *Sma*I or *Eco*RI–*Sma*I sites of these three vectors, so the inserts are flanked by unique *Eco*RI and *Bam*HI sites for further manipulations (see Materials and methods).

Regulation of the deoP1 promoter

To elucidate the role of DeoR on *deo*P1 expression we inserted fragments containing different lengths of *deo* DNA upstream from the *deo*P1 promoter into pJEL126, Figure 4. Table I summarizes expression data for these operon fusions in four Δ*lac*, *deo*R, *cyt*R regulatory strains. In accordance with previous results these data show that initiation from P1 is negatively controlled by the DeoR repressor. However, the basal levels of β-galactosidase were ~12 times higher from the two fusions (pVH143, pVH144) which contain only 100 bp and 270 bp of *deo* DNA upstream of the P1 start site compared to those containing 360 and 480 bp (pVH145, pVH146). Since all four fusions produce the same messenger, and the derepressed levels are equal, these results suggest that sequences between −270 and −360 are involved in DeoR repression.

DNA sequence analysis of deo DNA upstream of P1

The 470 bp *Eco*RI–*Eco*RI fragment of pVH146 (Figure 4) was subjected to DNA sequence analysis, Figure 5. The sequence

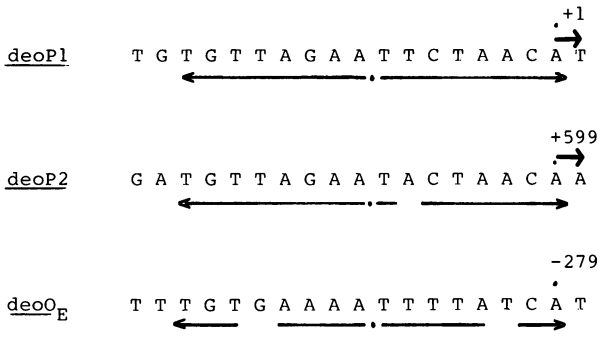

Fig. 6. Comparison of the DeoR operators (O_1, O_2) with that of *deo*O_E. The symmetrical sequences are underlined and the initiation sites for P1 and P2 transcription are indicated by arrows.

between −5 and −113 matches the previously determined P1 promoter sequence from pVH17 (Valentin-Hansen *et al.*, 1982a). Between the two sites used for construction of pVH144 and pVH145 (*Ava*II at −272 and *Rsa*I at −364) there exists a segment, *deo*O_E, which shares extensive homology with the known *deo*P1 and *deo*P2 targets for DeoR (Figure 6). This sequence homology together with the regulatory features of the P1 operon fusions discussed above strongly suggests that this palindrome also functions as an operator for DeoR and that this target in analogy with what has been found in *ara* and *gal* is required for repression of the *deo*P1 promoter by DeoR.

Regulation of deoP2 lacZ protein fusions

To examine whether *deo* DNA upstream or downstream of *deo*P2 affects the regulatory pattern of this promoter, we constructed fusions that join the transcriptional and translational regulatory signals of *deo*P2 to the *lacZ* gene. Based on the nucleotide sequence and the known start sites for *deo* transcription and *deo*C or *deo*A translation (Valentin-Hansen *et al.*, 1982a, b; 1984) three *deo*P2 fragments were inserted into pJEL122 or pJEL123 in such a way that a fused polypeptide is expressed (Figure 4, pJEL190, 192, 194).

The specific activity of β-galactosidase expressed from the fusions was measured in the various regulatory strains with glycerol as carbon source. As shown in Table II, initiation of transcription from P2 is regulated strongly by CytR (10-fold) but only weakly by DeoR (2-fold) in all three fusions. These results are similar to those obtained with a *deo*P2 fusion, which only contains 110 bp upstream of P2 and a small part of the first structural gene (*deo*C) (Valentin-Hansen, 1985) indicating that neither sequences between the P1 promoter and the second CRP binding site of P2 nor sequences in the first structural gene have any effect on *deo*P2 regulation.

Fusion of the deoP1-P2 region to lacZ

To study the interaction of DeoR with the 'intact' *deo*P1-P2 promoter region we constructed three types of protein fusions. First, we inserted an 851 bp *Hae*III fragment covering the region between 101 bp upstream of *deo*P1 and 151 bp downstream of P2 into pJEL123. This fusion (pJEL191) contains the same sequences upstream of P1 as the operon fusion pVH143 (Figure 4). Next, we reconstructed the *deo*P1 promoter region on the P2 fusions pVH192 and pVH194, by cloning the 470 bp *deo* *Eco*RI−*Eco*RI fragment located upstream from the P1 initiation site for transcription (Figure 4). The resulting plasmids pVH193 and pVH195 contain the O_E sequences in addition to the DeoR O_1 and O_2 targets and differ in sequence only in the promoter downstream region, as shown in Figure 4.

Table II. DeoP2 protein fusion[a]

Strain	Genotype	β-Galactosidase activity[b]		
		pJEL190	pJEL192	pJEL194
S0928	wt	0.5 (1)	0.8 (1)	0.6 (1)
S0929	cytR$^-$	5.6 (11)	7.6 (9)	6.5 (11)
S0930	deoR$^-$	0.9 (2)	1.5 (2)	1.0 (2)
S0931	deoR$^-$,cytR$^-$	6.7 (13)	10.0 (12)	8.5 (14)

[a]For the structure of the plasmids, see Figure 4.
[b]See Table I.
The numbers in brackets indicate the regulation (n-fold) relative to wild-type.

Table III shows the specific activity of β-galactosidase expressed from these fusions in the various regulatory strains with glycerol as carbon source. It can be seen that all fusions display a marked reduction in β-galactosidase expression in *deoR*$^+$ strains relatively to the *deo*P2 fusions described above (Table II). Moreover, the two fusions (pJEL193 and pJEL195) which contain the upstream element (O_E) show a 3- to 4-fold higher derepression ratio than the fusion pVH191 without this element. This indicates that (i) two DeoR binding sites are necessary for high repression of both *deo*P1 and *deo*P2 by DeoR, (ii) sequences upstream of the *deo*P1 promoter are required for full repression of the *deo* regulatory region. The data in Table III also show that the regulation of fusions pVH193 and pVH195 by *cytR* and *deoR* parallel very closely the regulatory patterns found for the chromosomal *deoC* and *deoA* genes, respectively (Table III).

Regulation of the P2 promoter in presence of the O_E target

To investigate the specific role of the O_E sequences in DeoR repressor binding to the P2 promoter we constructed P1 deletion derivatives of pVH191 and pVH193 (Figure 4). By means of S1 nuclease a 4-bp deletion was introduced in the *Eco*RI site present in the O_1 region of P1, see Materials and methods. This restriction site also overlaps the Pribnow box sequences of P1, Figure 5 (Jørgensen *et al.*, 1977; Valentin-Hansen *et al.*, 1982a). The specific activities of β-galactosidase expressed from the resulting plasmids (pJEL191ΔP1, pJEL193ΔP1) in the various regulatory strains are shown in Table IV together with the P-2 fusions pJEL190 and pJEL192. It can be seen that only the activity of the P2 promoter is altered specifically in *deo*R$^+$ strains harbouring pVH193ΔP1 (5- to 6-fold reduction). Since the regulatory response and the enzyme levels expressed from pVH191ΔP1 and the *deo*P2 fusion pVH190 are very similar we can conclude that the small deletion introduced in the *Eco*RI site of pJEL191 and pJEl193 inactivates both the P1 promoter as well as the O_1 target. These results clearly show that DeoR repression of P2 can be enhanced by the O_E target located more than 860 bp upstream of this promoter.

Discoordinate expression of the deoC and deoA genes

In the experiments described above we have not differentiated between transcriptional and translational control of gene expression. To discriminate between these two levels of control we constructed a set of transcriptional gene fusions. The *Eco*RI−*Eco*RI−*Bam*HI *deo* fragment from the protein fusion vectors pJEL191, pJEL193 and pJEL195 (Figure 4) was inserted into the unique *Eco*RI−*Bam*HI sites of the operon fusion vector pJEL126. The data in Table V show that the expression of β-galactosidase from pVH191-t is several fold lower than from the two larger transcriptional fusions (pVH193t and pVH195-t). Presumably this difference is due to transcriptional polarity, since

Table III. Deo P1-P2 protein fusion[a]

Strain	Genotype	β-Galactosidase activity[b]			Deoxyriboaldolase[c] (*deoC*)	Thymidine phosphorylase[c] (*deoA*)
		pJEL191	pJEL193	pJEL195		
S0928	wt	0.25 (1)	0.14 (1)	0.08 (1)	0.02 (1)	0.05 (1)
S0929	cytR$^-$	0.75 (3)	1.3 (9)	0.9 (11)	0.15 (7.5)	0.64 (12)
S0930	deoR$^-$	6.5 (25)	11 (78)	5.5 (69)	1.4 (70)	3.5 (70)
S0931	cytR$^-$,deoR$^-$	9.0 (36)	15 (107)	13 (163)	2.3 (115)	9.4 (188)

[a]For the structure of the plasmids, see Figure 4.
[b]See Table I.
[c]Enzyme levels expressed from the chromosomal *deo* genes. Data from Hammer-Jespersen (1982). Enzyme activities are expressed as units/mg protein. The numbers in brackets indicate the regulation (n-fold) relative to wild-type.

*deo*C′ and *trp*A′ are joined out of frame in pVH191-t (see Figure 3, pJEL191 is a derivative of pJEL123). Therefore, the main part of the 5′ end of the messenger from this fusion is untranslated. However, taking into consideration only the regulatory response and not the actual enzyme levels the results in Table III (protein fusions) and Table V (transcriptional fusions) show that (i) the regulatory patterns of the translational *deo*A fusion (pJEL195) and the transcriptional *deo*C and *deo*A fusions (pVH193-t and pVH195-t) are similar, (ii) expression of the *lacZ* gene from the three transcriptional fusions (Table V) and from the *deo*A protein fusion pJEL195 (Table III) is 2- to 3-fold higher in the *deo*R *cyt*R mutant than in the *deo*R mutant, whereas the corresponding increase in expression for the *deo*C translational fusions pJEL191 and pJEL193 is much lower (1.3×). A similar discoordinated expression of the two first genes has been observed for the chromosomal *deo* operon (Table III, *deo*C, *deo*A). Since the P2 promoter is stronger than P1 and the majority of initiations in *deo*R$^-$ mutants and in *cyt*R$^-$ *deo*R$^-$ mutants (with glycerol as carbon source) start at P1 and P2, respectively (Valentin-Hansen *et al.*, 1982a; Dandanell and Hammer, 1985), the lack of full response of translational *deo*C fusions to derepression of the P2 promoter indicates that *deo*C is translated better from the longer P1 message than from the P2 message.

A similar observation has been described for the *gal* operon where the first structural gene (*gal*E) is translated better from the five nucleotide longer S-2 message than from the shorter S-1 transcript (Queen and Rosenberg, 1981; Dreyfus *et al.*, 1985).

Table IV. Effects of the O_E target on expression from *deoP-2*[a]

Strain	Genotype	β-Galactosidase activity[b]			
		pJEL191ΔP1	pJEL190	pJEL193ΔP1-	pJEL192
S0928	wt	0.7 (1)	0.6 (1)	0.2 (1)	1
S0929	cytR$^-$	6.5 (9)	6.0 (10)	2.2 (11)	10
S0930	deoR$^-$	1.2 (1.7)	1.2 (2)	1.6 (8)	1.6
S0931	cytR$^-$,deoR$^-$	8.2 (12)	8.0 (13)	12.5 (63)	12

[a]For the structure of the plasmids, see Figure 4.
[b]See Table I.
The numbers in brackets indicate regulation (n-fold) relative to wild-type.

Table V. deoP1-P2 operon fusion

Strain	Genotype	β-Galactosidase activity[a]		
		pVH191-t	pVH193-t	pVH195-t
S0928	wt	0.1	0.25	0.25
S0929	cytR$^-$	0.3	3.2	2.8
S0930	deoR$^-$	3	15	15
S0931	cytR$^-$,deoR$^-$	7	34	34

[a]See Table I.

Discussion

Here low copy number fusion vectors carrying various fusions of *deo* DNA to the *lacZ* gene were used to study the *cis*-acting transcriptional regulatory elements involved in DeoR regulation of the *deo* P1-P2 promoters. We have recently shown that both P1 and P2 are regulated by the DeoR repressor and O^c mutations as well as sequence homology strongly indicate that the DeoR recognition sites overlap the Pribnow box sequences of each of the two promoters (Valentin-Hansen *et al.*, 1982a; Valentin-Hansen, 1985; Dandanell and Hammer, 1985).

The experiments described here have revealed interesting new features of *deo* regulation. The properties of P1-*lacZ* operon fusions which contain different parts of *deo* DNA upstream of the promoter region have identified an additional element required for proper DeoR repression. This upstream element is located between 270 and 360 bp from the start point of P1 transcription. Since this segment of DNA contains a palindrome which shares extensive homology with the known DeoR targets in *deo*P1 and *deo*P2 (Figure 6), the simplest interpretation is that this palindrome is a target for DeoR. This new element is termed *deo*O_E, because it most likely functions as an operator which enhances repressor binding. All fusions between *deo*P1 or *deo*P2 and *lacZ* which only contain one operator (O_1 or O_2 which overlaps the P1 and P2 Pribnow boxes, respectively) are regulated weakly by DeoR. Increased repression by DeoR is observed in fusions harbouring two operators. Our results clearly show, however, that the presence of all three targets is necessary for full repression of the P1 and P2 promoters by DeoR. Thus, the upstream element (O_E) is able to enhance DeoR repression of *deo*P1 *lacZ* fusions 12-fold; *deo*P1-P2 fusions ~3- to 4-fold and *deo*P2 *lacZ* fusions with a defective O1 region ~5-fold.

Our results with the operon fusion vector pVH191-t (Table V, Figure 4) are in agreement with recent results of Dandanell and Hammer (1985). These authors have fused different parts of the 851-bp *Hae*III fragment covering the P1-P2 region but not the upstream element (O_E) to the *galK* gene of *E. coli* and have studied the expression of *deo-galK* transcriptional fusions in single lysogens. Moreover, the regulatory features of the *deoC*- and *deoA-lacZ* protein fusions pJEL193 and pJEL195 (Figure 4) parallel very closely those found for the chromosomal genes

(Table III). Therefore, we believe that the low copy number fusions used in this study and in Valentin-Hansen (1985) reflect the normal *in vivo* functions of the cloned regulatory signals. Furthermore, these fusions have defined the DNA sequence within the *deo* operon that are necessary for *in vivo* regulation by DeoR, CytR and the cAMP/CRP complex.

Our finding of an upstream regulatory element in the *deo* operon that is required for repression is the third example of how regulatory sites located at a considerable distance from promoter regions can enhance repression in bacteria. In the case of *gal* and *ara* an additional operator needed for repression has been found within a strucural gene at a position 110 bp downstream and 200 bp upstream of the promoter-operator region (Irani *et al.*, 1983; Majumdar and Adhya, 1984; Dunn *et al.*, 1984; Hahn *et al.*, 1984). The *deo* system, however, reveals an even more complex example of long-range interaction between regulatory signals, since there exists an interaction between operators in two promoters separated by 599 bp with an enhancer target located 270 and 870 bp outside the promoter regions. We strongly suggest that additional targets exist for many other regulatory proteins and that these sites are required to facilitate the transfer of these proteins from non-specific binding sites on the chromosome to the active targets. For example, in the case of *lac* at least two lines of evidence suggest the involvement of pseudo operators (Gilbert *et al.*, 1976) in cooperative repressor binding. First, the effects of single base pair changes in isolated operators on repressor binding are much greater *in vitro* than they are *in vivo* in the context of the *lac* operon (Bourgeois *et al.*, 1975). Second, the activity of a small *lac* promoter/operator fragment fused to the chromosome *malPQ* operon is 30 times less represssed by the *lac* repressor than its natural position (Viral-Ingigliardi and Raibaud, 1985).

How the upstream and downstream operators function remains to be determined. The results of Dandanell and Hammer (1985) show that enhanced repression can be achieved either by increasing the intracellular concentration of repressor or by introduction of an extra operator site upstream of the promoter target. This suggests that there might exist an interaction between repressors bound at different targets or that one repressor is able to bind two operators at the same time. This could enhance repression by keeping the repressor, after its dissociation from the 'active' operator, in the vicinity of the promoter/operator region. Such a model has been proposed for the *lac* repressor to explain the DNA binding properties of hybrids between subunits of chimaeric Lac repressor β-galactosidase protein and the enzyme β-galactosidase (Kania and Müller-Hill, 1977).

During the last few years it has become clear that transcriptional control regions in eukaryotes are more complex and larger than found in bacteria. Sequences important for regulation can lie hundreds of nucleotides downstream or upstream of start points for transcription. At first sight the structure of these new control elements appear quite distinct from what we have seen in prokaryotes and have raised the possibility that prokaryotes and eukaryotes use different mechanisms to control transcriptional initiation. However, the finding of upstream and downstream control regions in bacteria invites to a comparison with regulatory elements in eukaryotic genes. Enhancers are eukaryotic examples of regulatory sites that are distant from gene promoters and yet can affect their activity. Enhancers function in relative independece of orientation and can work at distances of up to several kilobases upstream or downstream from a promoter region (Khoury and Gruss, 1983). The results obtained with the *deo* promoter region have several aspects in common with these *cis*-acting transcriptional regulatory elements. Thus, the *deo*O_1 operator will increase repression of the P2 promoter by DeoR when introduced at a position between 200 and 1000 bp upstream of this promoter (Dandanell and Hammer, 1985) and it is also able to enhance repression although inserted 4000 bp downstream of P2 (our unpublished results). Recently, it has been shown that mouse and human light-chain and heavy-chain immunoglobulin genes contain a regulatory element 70 bp upstream from the site of initiation and an enhancer element located in an intron. Interestingly, a nuclear factor, IgNF-A, which is proposed to be a positive *trans*-acting factor, binds specifically to a conserved segment in both the upstream promoter element and in the downtream enhancer element (Singh *et al.*, 1986). Also RNA polymerase I enhancers from *Xenopus laevis* show homology to sequences in the promoter, and it is suggested that the enhancers act by attracting a transcription factor whose function is to bind to the promoter region and activate transcription (Reeder, 1984). Therefore it is tempting to believe that at least one class of enhancers act in a way similar to that of operator sites located outside promoter regions in bacteria, and a determination of the molecular details of the interaction between regulatory proteins and multiple targets in *ara, gal* and *deo* may well turn out to be the clue to an understanding of upstream and downstream control elements of eukaryotic genes.

Materials and methods

Restriction endonucleases, T4 DNA ligase, S1 nuclease, T4 polynucleotide kinase, bacterial alkaline phosphatase, Klenow fragment of *E. coli* DNA polymerase I and synthetic *Eco*RI linkers were obtained from Boehringer Mannheim or New England Biolabs. α-^{32}P- and γ-^{32}P nucleotides were purchased from NEN.

Isolation of plasmid DNA, cloning, transformation of *E. coli* with plasmid DNA, and gel analysis of recombinant plasmids were carried out as described by Maniatis *et al.* (1982). DNA fragments were isolated as described in Valentin-Hansen *et al.* (1982a).

Bacterial strains and plasmids

E. coli strain K-12 S0928 (Δ*deo*,Δ*lac*), S0929 (Δ*deo*,Δ*lac*,*cytR*$^-$), S0930 (Δ*deo*,Δ*lac*,*deoR*$^-$) and S0931 (Δ*deo*,Δ*lac*,*cytR*$^-$,*deoR*$^-$) (Valentin-Hansen *et al.*, 1978) were used as the host for all experiments described below. Plasmids pVH11 (*deoCA*$^+$) and pVH17 (*deoC*$^+$) are pBR322 derivatives containing *deo* DNA from 600 bp upstream to 2350 bp downstream of *deo*P2 and 490 bp upstream of *deo*P1 to 1040 bp downstream of *deo*P2, respectively (Valentin-Hansen *et al.*, 1982a, 1984).

Growth of bacteria and β-galactosidase activity measurements

Cells were grown exponentially at 36°C in AB medium (Clark and Maaløe, 1967) with glycerol as carbon source (0.2%).

Measurements of β-galactosidase expressed from fusion plasmids were done as described by Miller (1972). Samples for enzyme assays were taken between OD_{450} 0.1 and 0.5. Specific activities of β-galactosidase are expressed as OD_{420}/OD_{450}/ml/min.

DNA sequencing

DNA fragments for sequencing were either labeled at their 5′ ends of strands by polynucleotide kinase or at the 3′ ends of strands by DNA polymerase (*E. coli* large fragment). Plasmid pVH17 was digested with *Eco*RI, labeled at its 5′ or 3′ ends and digested with *Cla*I or *Asu*II [location of restricton sites, see Figure 5: *Eco*RI (−10, −480); *Cla*I (−465); *Asu*I (−37)].

Purification of labelled fragments was performed as described in Valentin-Hansen *et al.* (1984). DNA sequence analysis was carried out by the method of Maxam and Gilbert (1980).

Construction of deo-lacZ fusions (see Figure 4)

Cells harbouring *deo-lacZ* fusions were selected/screened on lactose−McConkey agar containing 30 μg/ml ampicillin (in the following denoted indicator plates).

pJEL190 and pJEL191. An 850-bp *Hae*III fragment containing the *deo*P1-P2 promoters was isolated from pVH17 (Valentin-Hansen *et al.*, 1982a). Intact or *Eco*RI-restricted fragments were inserted into *Sma*I or *Eco*RI/*Sma*I digested pJEL123 and transformed into S0930. Cells harbouring pJEL190 were selected as weak, red colonies on indicator plates after incubation for 12 h at 30°C, whereas cells containing pJEL191 appeared bright red on those plates.

pJEL192, pJEL194, pJEL93 and pJEL195. Plasmid pVH17 was digested with *Eco*RI and *Hin*cII, ligated with *Eco*RI/*Sma*I restricted pJEL122 and transformed into S0929. Plasmid pVH192 was isolated from Ap^r Lac^+ colonies. Plasdmid pVH11 was first restricted with *Nco*I and after filling in the protruding ends with DNA polymerase I (*E. coli* large fragment) digested with *Eco*RI. A small sample was ligated with *Eco*RI/*Sma*I digested pJEL122 and transformed into S0929. pJEL194 was isolated from ampicillin-resistant Lac^+ transformants. pJEL193 and pJEL195 were constructed by insertion of the 470-bp *Eco*RI fragment from pVH17 into *Eco*RI restricted pJEL192 and pJEL194, respectively. Selection and screening after transformation into S0930 was for Ap^r and Lac^+ (bright red) phenotype on indicator plates.

pJEL191ΔP1 and pJEL193ΔP1. The *Eco*RI site located in the P1 promoter of pJEL191 and pJEL193 was destroyed as follows. Partially digested pJEL191 or pJEL193 was subjected to S1 nuclease treatment to remove the protruding ends and after inactivation of the nuclease the DNA was ligated and transformed into S0930. Cells harbouring pJEL191ΔP1 and pJEL193ΔP1 were selected as weak, red colonies after 12 h incubation at 30°C on indicator plates.

pJEL191-t, pJEL193-t and pJEL195-t. Samples of partially *Eco*RI-digested DNA of pJEL191, pJEL193 and pJEL195 were restricted with *Bam*HI and *Pst*I and ligated with *Eco*RI-*Bam*HI digested pJEL126. DNA was isolated from Ap^r Lac^+ transformants of S0930 (bright red colonies after 12 h of incubation at 30°C).

pVH143, pVH144, pVH145 and pVH146. pVH146 was constructed as follows. Partially *Eco*RI digested pVH17 was restricted with *Bgl*II, ligated with *Eco*RI/*Bam*HI digested pJEL126 and transformed into S0930. DNA of pVH146 was isolated from Ap^r Lac^+ transformants.

To construct pVH143 and pVH145 the 470-bp *Eco*RI-*Eco*RI fragment of pVH146 was deleted. The resulting plasmid (pVH146Δ*Eco*RI) was digested with *Eco*RI and ligated with *Eco*RI/*Pst*I-restricted pJEL191. DNA of pVH143 was isolated from Ap^r Lac^+ transformants of S0930.

A 423-bp *Rsa*I fragment (Valentin-Hansen *et al.*, 1982a) covering the region between 362 bp upstream and 61 bp downstream of P1 was ligated with T4 DNA ligase in presence of *Eco*RI linker and restricted with *Eco*RI. A small sample of this DNA was ligated with *Eco*RI digested pVH146Δ*Eco*RI and transformed into S0930. Plasmid pVH145 was isolated from Ap^r Lac^+ colonies.

A purified *Ava*II fragment (Valentin-Hansen *et al.*, 1982a) which contains the *deo*P1-P2 region was used in construction of pVH144. First, the *Ava*II 5′ overhang was made blunt by filling in with DNA polymerase (*E. coli* large fragment) and after a *Bgl*II digest the DNA was ligated with *Sma*I/*Bam*HI restricted pJEL126. Plasmid pVH144 was isolated from Ap^r Lac^+ transformants.

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