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Effects of Mutations in the Regulatory Region on Transcriptional Regulation of *glpD* Gene

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Expression of the adjacent but divergently transcribed *glpD* and *glpE* gene is positively regulated by cAMP-CRP. In this study, we constructed several mutants in which a CRP-binding site is placed at different distances upstream of the *glpD* promoter. The effect of the spacer length on transcription activation by cAMP-CRP was tested *in vivo* by β-galactosidase. The cAMP-CRP complex activated transcription from *glpD* when bound at a number of positions, all of which lay on the same face of the DNA helix, although the degree of activation varied with the length of the spacer. By contrast, the insertion of spacer length with non-integral turns of the DNA helix extremely inhibited the activation of transcription. The observed transcription activation by cAMP of the *glpD* promoter was influenced by the distance between the CRP binding site and the transcription start point.

The *glpD* gene encoding gly-3-P dehydrogenase is essential for the aerobic growth of *E. coli* on glycerol or gly-3-P (20). The *glpD* and *glpE* genes were cloned and their promoter regions and entire structures were analyzed (2). The *glpE* gene, the function of which is unknown, is transcribed divergently with respect to the *glpD* gene. Expression of the adjacent but divergently transcribed *glpD* and *glpE* genes is positively regulated by the cAMP-CRP complex. Furthermore, our results suggest that the cAMP-CRP complex binds to a single site in the regulatory region of the *glpD* and *glpE* genes and the single CRP-binding site is a *cis*-acting element involved in the positive regulation of the expression of both *glpD* and *glpE* (3).

In *E. coli* and other related bacteria, cAMP receptor protein (CRP) forms a complex with cAMP, and plays an important role in the regulation of gene expression by binding to highly specific sites on DNA (17, 19). When complexed with its allosteric effector cAMP, CRP elicits confomational transition and binds to specific sites within or near promoters to activate or repress transcription (7). The DNA regions of the cAMP-CRP binding sites in various genes are generally about 22-bp in length, in which a conserved motif 5'TGTGA3' and a less conserved inverted repeat, composed of pentamer, are contained. The less conserved sequences are, however, important for CRP-binding even though they are not symmetric to the motif (6).

In a previous study (4), several mutations including substitution, deletion and insertion were introduced into the CRP binding site in the regulatory region of the *glpD* and *glpE* genes. These mutants affect the expression of the two genes at different levels. The CRP binding sites lie at different locations relative to the transcription start site in the different promoters (6). Although direct contact between CRP and RNA polymerase bound to DNA was believed to play an important role in transcriptional activation, the variety of locations of the CRP binding site seemed to be incompatible with a simple protein-protein interaction model (18).

In this study, for precise investigation of this problem, *glpD* mutants in the regulatory region with altered spacing between the CRP binding site and the promoter region have been characterized.

Our data clearly indicates that activation by cAMP-CRP is strictly dependent on the helical phase between the sites in the *glpD* regulatory region.

MATERIALS AND METHODS

Materials

E. coli JM 109 (*recA1, lac, pro, endA1, gyrA96, thi, hsdR17, relA1, F' traD36, proAB*) and TP2010 (*xyl,*

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cya, argH, lacX74, recA, ilv, Srl::Tn10) were used and propagated in LB and M9 media. Restriction endonucleases, T4 DNA ligase and Klenow enzyme were purchased from Bethesda Research Laboratories or Boehringer Mannheim Biochemicals. All sequencing reactions were performed using DNA sequencing kit (Sequenase 2.0 kit). Radioactively labeled materials were from Amersham International. All other chemicals were obtained from Sigma.

Oligonucleotides and Plasmids

The plasmid pHKC9 carries the sequence of *glpD* and *glpE* genes from *E. coli* (2). Plasmids were normally purified according to the procedure described by Sambook *et al.* (13). However, for small-scale preparation of plasmids, the rapid isolation method by Davis *et al.* (5) was used. For preparation of DNA fragments, the plasmids were digested with appropriate restriction endonucleases, and the resulting fragments were purified by electrophoresis on 5% polyacrylamide gel in TBE buffer.

Several kinds of oligonucleotide chemically synthesized with an automatic DNA synthesizer (Applied Biosystems Inc. Model 380A) contained one-base pair substitution in the regulatory region of the *glpD* and four kinds of linker. The synthetic oligonucleotides used in this study are as follows:

8-mer: 5'-GGT CGA CC-3' (*Sal*I linker)
10-mer: 5'-CGG TCG ACC G-3' (*Sal*I linker)
12-mer: 5'-CCG GTC GAC CGG-3' (*Sal*I linker)
15-mer: 5'-GTT ACC TTA AGC GCG-3' (*Afl*II site)
16-mer: 5'-GGC CGG TCG ACC GGC C-3' (*Sal*I linker)

Construction of Mutants

The synthesized oligonucleotides were phosphorylated with ATP and T4 polynucleotide kinase before use. The phosphorylated oligonucleotides were annealed to the single-stranded recombinant phage DNA, which was composed of *Bam*HI-*Bgl*II 323 bp fragment inserted into the *Bam*HI site in M13mp19. Site directed mutagenesis was then carried out according to the procedure of Amersham's oligonucleotide-directed *in vitro* mutagenesis system. Base substitution of the directed site was confirmed by cleavage pattern of the relevant restriction endonucleases and by sequence analysis.

DNA sequences were determined by a dideoxy chain termination method (10, 14). The reaction mixture was analyzed by 8% denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Enzyme Assay

E. coli strains TP2010 (*Δcya, Δlac*) were transformed with fusion plasmids and used for β-galactosidase assay. M9 medium supplemented with 5% casamino acids and 50 μg/ml ampicillin was also used.

β-galactosidase activity was estimated by the method of Miller (11) and the activity represents the average value of three experiments with standard deviation. Cell growth was measured spectrophotometrically at 610 nm.

RESULTS AND DISCUSSION

Construction of Mutants in the Regulatory Region

The *Bam*HI-*Bgl*II 323 bp fragment of plasmid pHKC9 containing the *glpD* regulatory region was inserted into *Bam*HI site in M13mp19. One-bp substitution was introduced into the regulatory region of the *glpD* gene by oligonucleotide directed mutagenesis as described in Materials and Methods. The T→A substitution at the base position of 89 in the *Bgl*II-*Nru*I fragment created an *Afl*II site (Fig. 1). The spacing between the CRP binding site and the *glpD* promoter sequences was then varied by the introduction of *Sal*I linkers and Klenow treatment into the intervening *Afl*II restriction site. For four-base insertion, the cohesive end generated upon cleavage of the resulting mutant with the *Afl*II site was filled by Klenow. We selected the mutant by cutting with the restriction endonuclease of *Afl*II. The sequence determined by the dideoxy method showed that the *Afl*II site of the A4 mutant had been filled correctly (Fig. 2).

This construction was arranged so that a single *Afl*II site was left between the CRP binding site and the -35 region of the *glpD* promoter. The spacing between the CRP binding site and the *glpD* promoter se-

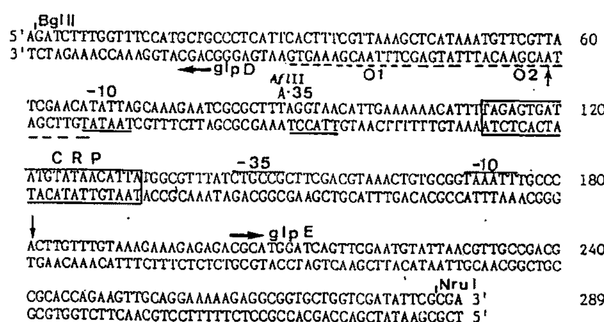
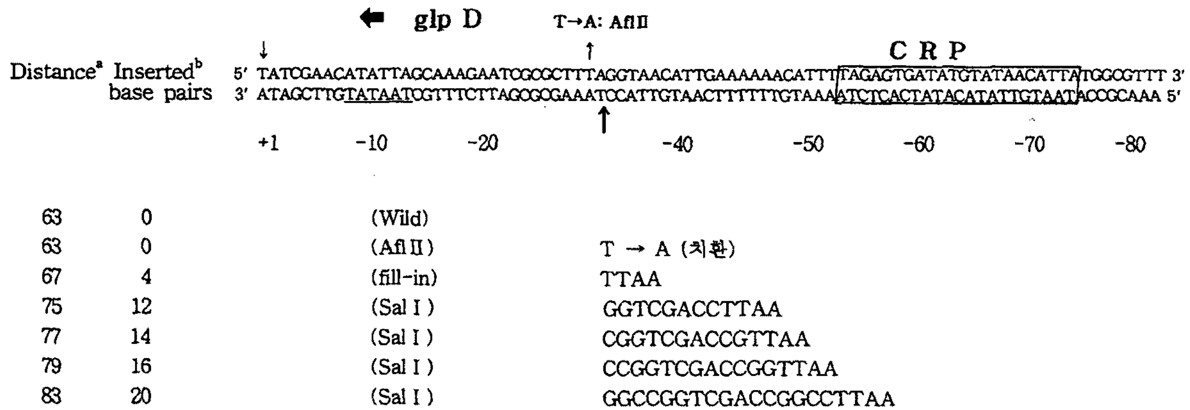


Fig. 1. Oligonucleotide-directed mutagenesis in the CRP-binding site of *glpD* gene.
Mutagenesis was attempted to substitute one base at the target site. The alteration resulted in a newly created cleavage site of the endonuclease, *Afl*II. The CRP-binding site is boxed. Sequences -10 and -35 of *glpD* and *glpE* genes are underlined. The initiation sites of transcription are indicated by the vertical arrows. The transcription start point and nucleotide sequence were determined by Ye and Larson and Choi *et al.* (2, 20) Primer used in the site directed mutation; 5'-GTTACCTTAAGCGCG-3'.



Distance[a]	Inserted[b] base pairs		
63	0	(Wild)	
63	0	(AflII)	T → A (치환)
67	4	(fill-in)	TTAA
75	12	(Sal I)	GGTCGACCTTAA
77	14	(Sal I)	CGGTCGACCGTTAA
79	16	(Sal I)	CCGGTCGACCGGTTAA
83	20	(Sal I)	GGCCGGTCGACCGGCCTTAA

Fig. 2. Nucleotide sequence of the *glpD* regulatory region and their insertional mutants.
a; The distance between the center of symmetry of the CRP binding site and the transcription start point is given in the first column, b; Various linkers were cloned into the *AflI* site of a mutated *glpD* promoter (indicated by the vertical arrow). The mutant of fill-in was treated by Klenow fragment. The *glpD* promoter sequence is numbered with respect to the transcription start point taken as nt +1. The probable -10 sequence is underlined. The CRP binding site is boxed. Each promoter region was transferred to the *lacZ* fused vector pMS437C.

quences was then altered by the introduction of a series of *SalI* linkers, 8-mer, 10-mer, 12-mer and 16-mer, into the intervening *AflII* restriction site which was treated by Klenow.

The family of insertions produced in this way are shown in Fig. 2. Each mutant was named according to the size of the insertion between the CRP binding site and *glpD* promoter sequences.

Construction of *glpD-lacZ* Operon Fusion Plasmids

The 0.3 kb *HincII-SmaI* fragments containing all mutations, which had been created by site-directed mutagenesis and insertional mutations, were isolated from the replicative form of the mutant phages and ligated into the unique *SmaI* site in plasmid pMS437C (8). The *E. coli* strain MC4100 (Δlac) containing the recombinant plasmids was pheotypically Lac^+ on MacConkey agar plates. Constructed *glpD-lacZ* operon fusion plasmids were carrying the inserts each in the correct orientation, by cutting with restriction endonucleases (Fig. 3).

Transcrption Activation of Spacing Mutants by cAMP-CRP

We constructed *glpD-lacZ* operon fusion plasmids, carrying the different size insertions, 4, 12, 14, 16, and 20-bp in distance. β-Galactosidase levels were measured in *E. coli* TP2010 (Δlac, Δcya) growing in minimal media in the presence of 1 mM cAMP or absence of cAMP. The levels of β-galactosidase activity specified by each of the mutants in Fig. 2 is shown diagrammatically in Fig. 4.

In each case the β-galactosidase activity measured in the Δcya strain is similar to that directed by the original regulatory region (wild type), implying that the sequence inserted has little or no effect on promoter

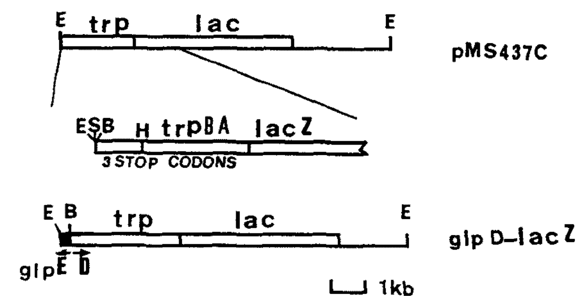


Fig. 3. Maps of the plasmids carrying *glpD-lacZ* operon fusion genes.
The 0.3 kb fragments of *HincII-SmaI* restriction enzyme containing all mutations were ligated into the *SmaI* site in plasmid pMS437C. Plasmid pMS437C, which is derived from both pMC1403 and pMC 81, possesses three stop codons in different reading frames in the *trpB* region to eliminate all possible translational read through into the *trpA-lac* hybrid gene. Abbreviations; E: *EcoRI*, B: *BamHI*.

activity. This data also shows that the amount of CRP-dependent promoter activity is strongly influenced by the distance between the CRP binding site and the transcription start point. The insertion of 0, 12 and 20-bp resulted in at least 70% recovery of cAMP-CRP mediated induction of *glpD-lacZ* fusion genes. These altered spacing lengths correspond to the integral or near-integral turns of the DNA double helix (12, 16).

However, the insertion of roughly half-integral turns of the helix structure corresponding to the insertion of 4, 14 or 16 bp extremely inhibites the cAMP-CRP mediated induction.

The observed transcription activation by cAMP of the *glpD* regulatory region is strongly influenced by the distance between the CRP binding site and the transcriptional start point.

This result is in agreement with findings obtained

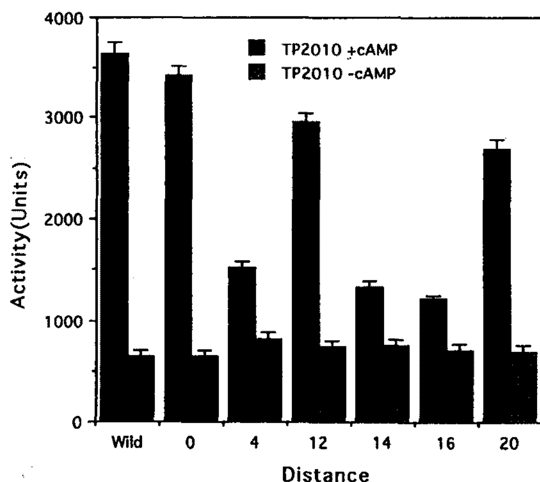


Fig. 4. Expression of β-galactosidase under control of insertional mutants in the distance between the center of CRP binding site and the transcription start point of *glpD*.
The distance is the number of bases inserted in the newly created *Afl*II site. Each of the strains containing *glpD-lacZ* operon fusion genes was incubated at 37°C until the A_{610} reached 0.1~0.2, and then 1 mM cAMP was added to each sample. Each sample was further incubated until the A_{610} was 0.6, and β-galactosidase activity was measured and the data represent the average value of three experiments with standard deviation.

by Mandecki and Caruthers (9) and Straney *et al.* (15). Mandecki and Caruthers (9) showed that a 5 bp insertion between the *lac* CRP binding site and the promoter region abolished the activation by cAMP-CRP, but that an 11 bp insertion resulted in a limited restoration of activation. Straney *et al.* (15) have shown, by using similar spacing mutants, that stabilization of CRP binding to the *lac* DNA by RNA polymerase occured in parallel with CRP activation. Aiba *et al.* (1) showed that the *lac* CRP binding site positioned 61 bp upstream of the *galP1* promoter, or 60 bp upstream of *galP2,* could bring about some activation of transcription. Our results also indicate that the distance between the center of the CRP site and the transcription start point has a strong effect corresponding to those seen in the *glpD* and *lac* promoters, 62 and 63 bp, respectively.

Ushida and Aiba (18) also showed that transcription activation by CRP was dependent on the helical phase between the CRP binding site and the promoter region. In addition, it has been shown that activation by CRP decreases rapidly with deviation from the optimum spacer length.

All of our in-phase mutants, which preserve the integral helical spacing between the CRP binding site and the promoter region, have been shown to retain, more or less, the ability to be activated by cAMP-CRP.

These data support the view that the interaction between CRP and RNA polymerase bound on the same side of the DNA helix is important for cAMP-CRP action.

A CRP binding site on the *glpD* and *glpE* regulatory region is a *cis*-acting element involved in the positive regulation of the expression of both *glpD* and *glpE* genes. Therefore, we also constructed *glpD-lacZ* fusion plasmids with all the mutated DNA fragments. The levels of β-galactosidase activity specified by each mutants showed no effect on *glpE* promoter acivity, respectively (data not shown). This result indicates that spacing mutation in the *glpD* region has no effect on *glpE* promoter activity by cAMP-CRP.

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