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Regulation of Expression of the *crp* Gene of *Escherichia coli* K-12: In Vivo Study

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Expression of the *crp* gene was studied in vivo by use of a *crp-lacZ* gene fusion first constructed on a plasmid and then transferred onto the chromosome. Our in vivo data confirm the in vitro findings that *crp* is negatively autoregulated via the cyclic AMP-catabolite gene activator protein complex. We present evidence that gene *crp* is repressed by glucose.

The catabolite gene activator protein (CAP) complexed with its allosteric effector, cyclic AMP (cAMP), plays a key role in gene expression in *Escherichia coli*. This complex is involved in the regulation of catabolic operons and of genes responsible for the resistance to some antibiotics, in the synthesis of different proteins of the outer or inner membranes, and in the control of bacteriophage lysogeny (reviewed in references 1, 7, and 19). The cAMP-CAP complex binds at specific sites in the promoter of several operons and thereby stimulates or inhibits transcription initiation. The concentration of the cAMP-CAP complex in the cell is an important factor in this regulation. Variations in the concentration of this complex are usually assumed to follow variations in the intracellular cAMP concentrations. We wondered whether CAP concentration could also vary and modulate the level of cAMP-CAP complex.

CAP is encoded by the *crp* gene located at 73 min on the chromosome. This region has been cloned, and the nucleotide sequence of *crp* was determined (3, 6). The transcription initiation site precedes the translation initiation codon of *crp* gene by 167 base pairs (2). Gene *crp* is followed by a sequence homologous to a rho-independent type terminator (3, 6), which suggests that *crp* is the only gene of the transcription unit. Using an in vitro assay, Aiba has shown that the cAMP-CAP complex inhibits transcription of the *crp* gene. By footprinting experiments, he demonstrated the existence of one specific, high-affinity CAP-binding site located between the promoter and the coding region. This was unusual since CAP-binding sites are usually located upstream from the transcription start. Aiba also detected another CAP-binding site located upstream from the transcription start. However, this site was much weaker and had no inhibitory effect on the in vitro transcription (2).

To test whether *crp* is autoregulated in vivo and is subject to other types of regulation, we constructed in vitro a *crp-lacZ* hybrid gene, transferred it onto the chromosome by in vivo recombination, and studied its expression under various conditions. The regulation of *crp* expression could then be studied by measuring β-galactosidase activity.

For unclear reasons, we were unable to obtain any *crp-lacZ* operon fusions by using the Mu d(Ap lac) phage (5), as selection for carbohydrate phenotypes yielded only *cya-lacZ* fusions (carbohydrate-positive phenotype in the presence of cAMP) or fusions with genes of the phosphoenolpyruvate-phosphotransferase system (PTS) (glucose-negative phenotype). Therefore, we decided to construct a *crp-lacZ* fusion in vitro. To avoid the presence of intergenic material with unknown function between *crp* and *lacZ*, we constructed a gene fusion rather than an operon fusion. We used the recombinant plasmid pMC1403 (4). This plasmid contains a truncated *lac* operon, i.e., the controlling region, the promoter, and the first seven codons of the amino-terminal end of *lacZ* are missing. A unique *Bam*HI site is present before the codon 8 of *lacZ*. This plasmid (Fig. 1) allows a very precise construction with well-defined junctions between *lacZ* and the gene of interest. We constructed such a recombinant plasmid with a *crp-lacZ* fusion containing regions homologous to the chromosomal *crp* region on both sides of the gene fusion. This construction allowed a transfer of the gene fusion from the plasmid to the chromosome by in vivo recombination. Hence, we eliminated the possibility of errors of interpretation in the study of *crp* regulation due to some readthrough from a plasmid promoter or to a titration by sites present on the plasmid, such as, in our case, pBRP4 (17), a CAP-binding site present in pBR322.

The *crp* gene had previously been cloned in pBR322, on a 4-kilobase-pairs *Bam*HI fragment (plasmid pBScrp2) (6). At codon 193, we noticed a *Sau*3A site which could be ligated to the *Bam*HI site of the *lac Bam*HI-*Sal*I fragment isolated from pMC1403, and form a gene fusion, *crp-lacZ*, under the control of the *crp* regulatory region. The *Sal*I site located upstream from *crp* is absent in pBScrp3, a derivative of pBScrp2 lacking the 1-kilobase-pair *Hpa*I fragment. We digested this plasmid with *Sau*3A in the presence of ethidium bromide to get a population of plasmids linearized by a single cleavage, with some of them cleaved at the desired site (16). Conditions for this limited digestion (pBScrp3 has more than 50 sites) were carefully tested to obtain a maximum amount of plasmid DNA linearized by a single cut in the digestion mixture, as tested by agarose gel electrophoresis. A digestion by *Sal*I was then performed. This digestion mixture, ligated with the *lac* fragment, yielded a population of plasmids in which we identified the desired plasmids by their ability to confer a Lac^+ Crp^- Ap^r phenotype to a Δ*crp* Δ*lac* host strain (plasmid pF1.31). By use of a *Bam*HI linker, we introduced a *Bam*HI site next to the *Sal*I site and recloned the *Bam*HI fragment carrying the *crp-lac* fusion in pBR322 (plasmid pF8-3). Then we replaced the deleted region upstream of *crp* by the wild-type sequences (pFF8). This was achieved by a digestion with *Eco*RI and a partial digestion with *Bcl*I. The region downstream of *crp* was introduced by replacing the *Bam*HI fragment containing the *crp* gene in

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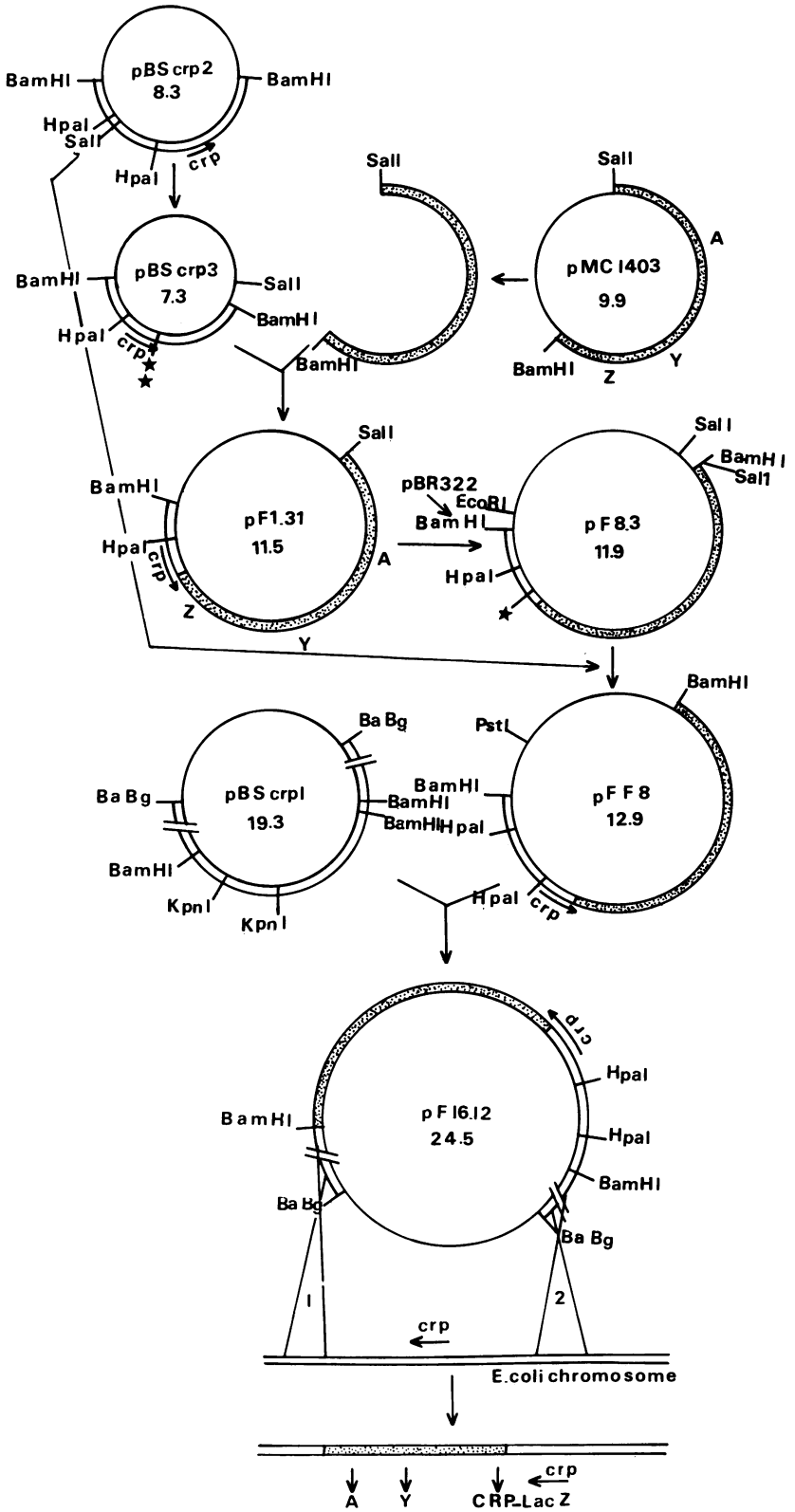

FIG. 1. Construction of the *crp-lacZ* fusion. The names of the plasmids involved in the construction (see the text) and their approximate molecular sizes in kilobase pairs are indicated inside the circular maps. The thick dotted line on the circular maps of the plasmids and on the chromosome represents the *lac* fragment, the thin line represents pBR322 sequences, and the double line corresponds to the bacterial *crp* region. Indicated restriction sites correspond to all sites present on the plasmids for *Bam*HI, *Hpa*I, *Sal*I, and *Kpn*I. In pBScrp3, the two stars correspond to the position of the *Sau*3A site which was ligated to the *lac* fragment. In pF8-3, the star corresponds to the unique cleavage site in a partial digest by *Bcl*I which led to the construction of pFF8. BaBg corresponds to the hybrid sites formed after inserting a *Bgl*II-*Bgl*II fragment in the *Bam*HI site of pBR322 (6). The symbol (//) indicates that the *Bam*HI-BaBg fragments are not represented in totality. The last step of the construction shows at positions 1 and 2 the double crossing of the *crp-lacZ* fusion with the homologous regions of chromosomal DNA. The arrows indicate the direction of transcription of gene *crp*.

pBScrp1 (a pBR322 derivative carrying a bacterial insert larger than that of pBScrp2) by the *Bam*HI fragment carrying the *crp-lacZ* gene fusion (plasmid pF16-12). The gene fusion was then transferred on the chromosome of the F^- strain MC1000 by in vivo recombination: we selected for *crp* homogenotes and eliminated the recombinant plasmid as previously described (6). The *crp-lacZ* fusion joint was verified to be at the expected location by crosses with several Hfr strains harboring different *crp* deletions (data not shown).

The chromosomal fusion was used to study the regulation of *crp* gene expression. To look for a possible autogenous regulation of *crp*, we transferred the fused *crp-lacZ* genes and the *cyaO6* allele by P1 cotransduction, using the *aroB* and *ilv* markers present, into a strain harboring the Δ*lacX74* deletion. This strain is referred to as Bof169. We then analyzed the effect of cAMP in cultures grown in rich medium, after introduction of the crp^+ allele on either a transducing phage (in a low copy number) or a pBR322 plasmid vector (in a multicopy number). We measured the differential rate of β-galactosidase synthesis during three to four generations times by assaying β-galactosidase activity at different time intervals. When gene *crp* was present in a low copy number or in a multicopy number, we observed a twofold decrease in *crp* expression when 5×10^{-3} M cAMP was added (Table 1). We failed to observe any effect of the crp^+ allele in the absence of cAMP (Table 1). In the absence of the crp^+ allele, cAMP had no effect on *crp* expression. When cultures were grown in minimal medium with glucose as carbon source, we obtained similar results. In a cya^+ background, we did not obtain a significant repression of β-galactosidase expression when the crp^+ allele was introduced. However, the addition of cAMP to the cultures led to a twofold repression, showing that high concentrations of cAMP-CAP complex are required to repress *crp*. These data demonstrate that the expression of the *crp* gene is repressed by the cAMP-CAP complex (Table 2).

Our results are consistent with the in vitro study reported by Aiba showing a negative autoregulation of *crp* at the transcriptional level and the presence of specific binding sites in the *crp*-controlling region (2). However, these results are at variance with those of a previous work in which the authors did not detect by radioimmunoassay any variation of CAP synthesis, in various media or in a cya^+ versus a *cya* background (11).

We calculated 1,500 dimers of CAP per cell based on the β-galactosidase activity. This value is of the same order as the 1,000 to 3,500 dimers assumed to be present in the cell (11, 20).

A decrease in the level of cAMP is observed in presence of glucose (12, 14). Therefore, it seemed interesting to test whether glucose also had an effect on CAP synthesis. If the cAMP-CAP complex represses *crp* expression, one could expect that glucose, which lowers concentrations of cAMP, would derepress *crp* expression. In fact, when cultures of the fusion strains containing the crp^+ allele on a transducing phage were grown with glycerol instead of glucose as carbon source, an increase in β-galactosidase synthesis was observed (Table 2). This result suggests that glucose exerts a slight repression of *crp* expression, which occurs independently of the effect of cAMP. Such a repression by glucose, which is independent of the presence of cAMP, has already been observed (8–10), suggesting that regulatory mechanisms other than the one directly involving cAMP and CAP could regulate the expression of catabolic operons.

Intracellular concentrations of cAMP (2 to 6 μM) and CAP (3 μM) are similar and close to the K_A of the cAMP-CAP complex (10 μM) (18). Therefore, slight variations in intracellular cAMP or CAP levels affect the concentration of the cAMP-CAP complex, leading to a modulation of expression of the catabolic operons. This expression is dependent on the formation of specific complexes. The cAMP concentration is known to vary from 2 to 6 μM in the cell. When higher levels of cAMP are present in the cell, one would expect that a higher proportion of nonspecific binding would occur, which could perturb the cell. Our results suggest that such a perturbation does not occur, due to a cAMP-dependent repression of *crp* gene expression, only detectable at high concentrations of cAMP. This repression factor is low, but it is sufficient to compensate for the increase in cAMP concentrations.

In summary, when cAMP concentration increases, transcription of the different catabolic operons gradually occurs (13), depending on the association constant of the respective ternary complexes (RNA polymerase, cAMP-CAP, DNA site). When cAMP levels are high, the CAP concentration levels off to prevent any increase of nonspecific binding. In addition, in the presence of glucose, concentrations of both cAMP and CAP are lowered. This might contribute to an explanation of why catabolic operons are, in those conditions, so strongly repressed.

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TABLE 1. Autogenous regulation of *crp*[a]

Strain[b]	Relevant genotype	β-Galactosidase synthesis	
		Without cAMP	With cAMP
Bof169	Δ*cya crp-lacZ*	2,300	2,150
Bof218	Δ*cya crp-lacZ*:λ *crp*	2,150 (1,600)	1,000 (830)
Bof219	Δ*cya crp-lacZ*:pBScrp2	2,350	850

[a] The different values correspond to experiments in which five to seven samples were withdrawn during exponential growth in LB rich medium at 30°C and assayed for β-galactosidase activities. Numbers in parentheses correspond to cultures in glucose M63 medium supplemented with 0.1% Casamino Acids. β-Galactosidase activity was assayed according to the method of Pardee et al. (15) in toluenized cell suspensions. The differential rates of enzyme synthesis are expressed as units of enzyme per milligram (dry weight) of bacteria deduced from the optical density at 600 nm (1 mg [dry weight] per ml = 3.7 optical density units at 600 nm).

[b] Bof169 is the *crp-lacZ cya* derivative (see the text) of GL246 [*leu* Δ(*proB-lac*) *thy ilv rpsL;* G. Lindhal] in which the Δ(*proB-lac*) deletion was replaced by Δ*lacX74* by using an $F^- \times$ Hfr cross with Hfr 3000X74 [collection de l'Institut Pasteur]). The *thy* marker was eliminated by transduction with P1 *vir* grown on 3000X74. *aroB* was introduced by isolating a spontaneous *malT* mutant and then eliminating this mutation by a cotransduction with P1 *vir* grown on Hfr G6 (*his aroB* [(H. H. Malamy]). Strain Bof218 corresponds to strain Bof169 lyzogenized with the phage λY1079 kindly given by Sankar Adhya. Strain Bof219 corresponds to strain Bof169 transformed with plasmid pBScrp2 (6).

TABLE 2. Regulation of *crp* in a cya^+ background[a]

Strain[b]	Relevant genotype	β-Galactosidase synthesis with:			
		Glucose		Glycerol	
		− cAMP	+ cAMP	− cAMP	+ cAMP
Bof129	*crp-lacZ*	940	ND	ND	ND
Bof143	*crp-lacZ*:λ *crp*	850	540	1,100	1,100

[a] Experimental conditions are described in Table 1, footnote *a*. ND, Not determined.

[b] Bof129 is a derivative of strain MC1000 (4) in which the *crp-lacZ* fusion was introduced by in vivo recombination (see the text and Fig. 1). Bof143 corresponds to Bof129 lysogenized with λ1079.

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