JOURNAL OF BACTERIOLOGY, Feb. 1987, p. 654–659
0021-9193/87/020654-06$02.00/0

Vol. 169, No. 2

Generation of Deletions in the 3′-Flanking Sequences of the *Escherichia coli crp* Gene That Induce Cyclic AMP Suppressor Functions†

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Received 13 September 1985/Accepted 31 October 1986

The *crp* structural gene and its 3′-flanking sequences were subcloned into M13mp8, and in vitro deletions were constructed in both the 5′ and 3′ ends of the gene by using *Bal* 31 nuclease. Deletions ranged in size from 24 to 250 base pairs at the 5′ end of *crp*. Sixteen deletions generated at the 3′ end of the gene ranged in size from 133 to 675 base pairs. The majority of deletions extended into the *crp* structural gene. Another class of deletions, i.e., Δ*crp-4*, Δ*crp-17*, and Δ*crp-2*, had endpoints extending in the 3′-flanking sequences external to the *crp* structural gene. Deletions were subcloned into pBR322 and transformed into the *Escherichia coli cya crp* deletion strain NCR438. Transformants containing plasmid pBM4 with the Δ*crp4* mutation, a deletion of 133 base pairs, were cyclic AMP independent. Strain NCR440 harboring this plasmid expressed β-galactosidase and threonine dehydratase activities and fermented lactose, ribose, arabinose, and xylose in the absence of exogenous cyclic AMP. The Δ*crp-4* mutation also caused strain NCR440 to be hypersensitive to exogenous cyclic AMP. The cylic AMP receptor protein expressed in maxicells from pBM4 carrying the Δ*crp-4* mutation comigrated with the wild-type protein on eletrophoretic gels. The Δ*crp-4* mutation demonstrates that sequences distal to the *crp* structural gene can mediate cyclic AMP suppressor functions.

The cyclic AMP (cAMP) receptor protein (CRP) of *Escherichia coli* along with its effector molecule, cAMP, controls the transcription of some catabolite-repressible genes (1). Equilibrium studies have shown that CRP by itself is able to bind to nonspecific DNA sequences, but in the presence of cAMP the binding constant for specific recognition sites is increased by a factor of 10^5 (14). CRP is a dimer composed of two identical subunits consisting of 209 amino acids. The three-dimensional structure of the CRP-cAMP complex has been determined by X-ray crystallography (24). These data along with the study of fragments generated by proteolytic digests (4) have indicated two binding domains in the CRP monomer, one for the binding of cAMP and the other for the binding of specific DNA sequences. The gene coding the CRP has been cloned and sequenced (3, 10). The cAMP-CRP complex acts as a positive effector for the transcription of a number of genes including *lac* (11, 26) *ara* (21, 29), *malT* (9), and other operons (6, 30). The cAMP-CRP complex can also act as a negative effector for some genes such as the *ompA* gene (27), and it is an autogenous regulator of its own expression (2). Sequence data and DNase protection experiments reveal a consensus cAMP-CRP binding site (AATGTGA--T---TCA-AT A or T) that is important for the recognition of cAMP-CRP to its DNA target site in a number of cAMP-CRP-dependent promoters (13).

In our laboratory *E. coli* mutants have been isolated which have mutations that eliminate the requirement of cAMP for the expression of catabolite-sensitive operons (23). Because these mutations suppress the need for cAMP they are referred to as cAMP suppressor mutations (*csm*). Transductional analysis has shown these *csm* mutations map in or near the *crp* gene region of *E. coli* (23). Recently the *csm* mutation of *E. coli* NCR30 was cloned in our laboratory into the *Hin*dIII-*Eco*RI site of the plasmid vector pBR322 (16). Sequence analysis indicated that the *csm* suppressor phenotype was due to the insertion of a guanosine in the stem region of the potential transcriptional termination loop in the nontranslated 3′-flanking sequence of the *crp* gene. To investigate this phenomenon we generated sequential deletion mutations in the 3′-flanking sequence of the *crp* gene. In this paper we describe the construction and characterization of these in vitro deletions in the *crp* gene and its flanking sequences. Deletions were generated both from the 5′ and 3′ regions of the *crp* gene. One deletion, extending into the 3′-flanking region of the *crp* gene, allowed the expression of catabolite-repressible operons in the absence of exogenous cAMP and conferred hypersensitivity to exogenous cAMP, a character associated with the *csm* mutation (23). This deletion was located 164 base pairs (bp) distal to the translational termination codon. Our results suggest that sequences distal to the *crp* structural gene act to render the transcriptional function of CRP in *csm* mutants independent of cAMP.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are shown in Table 1.

Media. All strains were grown in YT medium (25) or M9 minimal medium (25). When necessary, ampicillin was added to a final concentration of 50 μg/ml. Strains tested for biodegradative threonine dehydratase activity were grown anaerobically in POPEP medium (16). Strains tested for β-galactosidase activity were grown in minimal M9 medium containing 10 mM isopropyl-β-D-thiogalactopyranoside with 0.2% glycerol as the sole carbon source. Fermentation of sugars was tested with eosin-methylene blue (EMB) agar containing 1% of the appropriate carbon sources.

Chemicals, enzymes, and DNA. Restriction enzymes, T4 DNA ligase, DNA polymerase 1, Klenow fragment, *Bal* 31 nuclease, and DNA size markers were purchased from

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† Paper no. 10087 of the Journal Series of the North Carolina State University, Agricultural Experiment Station, Raleigh, NC 27650.

TABLE 1. Bacterial strains

Strain	Genotype	Reference or source
CA8445	HfrH Δ*crp-45* Δ*cya-45 thi* Str^r	D. Sabourin (33)
NK530	Hfr *srl-1300*::Tn*10 recA5-recA6 ilv-318 thi thr-300 rel-1 spc-300*	N. Kleckner (22)
JM103	Δ(*lac pro*) *supE thi*; *upsL endA sbcB15 hsdR4* F$^-$ *traD36 proAB*	J. Messing (28)
NCR438	HfrH, Δ*crp-45* Δ*cya-45 thi* Str^r *srl*::Tn*10 recA5-recA6*	This paper
NCR439	NCR438(pHA7) (*crp*$^+$)	This paper
NCR440	NCR438(pBM4) (Δ*crp-4*)	This paper
NCR441	NCR438(pBM17) (Δ*crp-17*)	This paper
NCR442	NCR438(pBM2) (Δ*crp-2*)	This paper
NCR443	NCR438(pBM3) (Δ*crp-3*)	This paper
NCR444	NCR438(pBM16) (Δ*crp-16*)	This paper
NCR445	NCR438(pBM8) (Δ*crp-8*)	This paper
NCR446	NCR438(pBM9) (Δ*crp-9*)	This paper
NCR447	NCR438(pBM7) (Δ*crp-7*)	This paper
NCR448	NCR438(pBM21) (Δ*crp-21*)	This paper
NCR449	NCR438(pBM12) (Δ*crp-12*)	This paper
NCR450	NCR438(pBM13) (Δ*crp-13*)	This paper
NCR451	NCR438(pBM23) (Δ*crp-23*)	This paper
NCR452	NCR438(pBM5) (Δ*crp-5*)	This paper
NCR453	NCR438(pBM19) (Δ*crp-19*)	This paper
NCR454	NCR438(pBM10) (Δ*crp-10*)	This paper
NCR458	NCR438(pBM6) (Δ*crp-6*)	This paper
NCR456	NCR438(pBM61) (Δ*crp-61*)	This paper
NCR457	NCR438(pBM24) (Δ*crp-24*)	This paper
NCR458	NCR430(pBM68) (Δ*crp-68*)	This paper
SS5062	*lacI22 lacZ pro48 met-90 trpA trpR his rpsI gyrA* P1^s *recA56 src*::Tn*10*	Singer (37)

Bethesda Research Laboratory and used according to the manufacturer's specifications. S1 nuclease, dideoxy sequencing reagents, M13mp8 RF, and M13mp9 RF were purchased from P-L Biochemicals, Inc. Isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were purchased from Sigma Chemical Co. Adenosine 5',3' cyclic monophosphoric acid was obtained from United States Biochemicals Corp. ^{32}dATP (800 Ci/mmol) and L-[^{32}S]methionine (>1,000 Ci/mmol) were purchased from New England Nuclear Corp.

Construction of *E. coli* NCR438. A *recA* mutation was constructed in the *cya crp* deletion strain CA8445 via conjugative transfer of this marker from strain NK530 by the procedure of Kleckner et al. (20). One selected transconjugate, strain NCR438, was used in marker rescue experiments.

***Bal* 31 deletion pool construction.** *Bal* 31 deletion pool construction was done by the procedure of Poncz et al. (32). M13mp8 crp$^+$ RF DNA was digested with either *Eco*RI at the 3' terminus of the *crp* gene or with *Hin*dIII at the 5' terminus of the gene depending on which end of the *crp* gene was to be deleted. The linearized DNA (15 μg) contained in 100 μl of reaction buffer (consisting of 20 mM Tris [pH 8.1], 12 mM $MgCl_2$, 600 mM NaCl, and 1 mM disodium EDTA) was digested with 2 U of *Bal* 31 nuclease at 37°C. Samples of 10 μl each were removed every minute, and the reaction was terminated by extraction with equal volume of Tris-EDTA-saturated phenol. S1 nuclease was used to determine the approximate size of the *Bal* 31-generated deletions (32).

Subcloning of *crp* deletions into pBR322. The in vitro-generated *crp* deletions contained in M13 were liberated after digestion of the vector with either *Hin*dIII or *Eco*RI. DNA fragments were separated by electrophoresis and removed from gels (i.e., 1% low-melting-point agarose) via the procedure of Weislander (39) and concentrated by chromatography with Elut-Tip (Schleicher & Schuell Co.). The *crp* deletion fragments contained one cohesive end and one heterologous blunt end. A restriction enzyme site was added to the heterologous termini to enhance manipulations. The above *crp* deletion fragments were subcloned in M13mp9 RF DNA. M13mp9 RF DNA digested with *Hin*dIII and *Hin*cII or with *Eco*RI and *Hin*cII was ligated to *crp* DNA deletion fragments with 1 U of T4 DNA ligase. This ligation mix was used to transfect strain JM103. Recombinant plaques containing *crp* deletions were identified by dot hybridization with a ^{32}P-labeled *crp* probe. The deletion fragments were removed from the M13 RF DNA by double digestion with *Eco*RI and *Hin*dIII and were subcloned into pBR322 between its unique *Hin*dIII and *Eco*RI sites. The ligation mixture was used to transform strain NCR438, and transformants were selected as ampicillin-resistant colonies on YT-ampicillin plates. These colonies were screened for the presence of the cloned *crp* deletions by colony hybridization (17).

DNA sequence analysis. DNA sequence analysis was done by the method of Sanger et al. (35). The exact endpoints of *crp* deletions were determined by using M13 single-stranded DNA containing the appropriate deletion fragments as templates. Internal sequence analysis was acheived by using the 333-bp *Hae*III *crp* restriction fragment and the 186-bp *Hae*III fragment.

cAMP hypersensitivity. Strains NCR439, NCR440, NCR441, and NCR442, which carry the wild-type *crp* and the Δ*crp-4*, Δ*crp-17*, and Δ*crp-2* deletion inserts, respectively, were tested for hypersensitivity to exogenous cAMP. Strain NCR30 and other strains carrying the cloned *csm* mutation exhibit hypersensitivity to exogenous cAMP (16, 23). Hypersensitivity of strains to exogenous cAMP was determined by monitoring growth of strains of EMB indicator plates containing different carbon sources at a final concentration of 1% with and without 0.25 μM cAMP.

Enzyme assays. Biodegradative threonine dehydratase (TDA) activity was assayed by a modification of the procedure of Shizuta et al. (36).

β-Galactosidase activity was assayed by the procedure of Miller (25).

Labeling of plasmid-encoded proteins. Plasmid gene expression was monitored by the Maxicell procedure of Singer et al. (37). The presence of the desired plasmids and transformants was determined by small-scale plasmid isolation (7). *E. coli* SS5062 served as the maxicell strain and was transformed with plasmids pBR322, pHA7, pBM4, and pBM68. Plasmid-encoded proteins were labeled with [^{35}S]methionine (specific activity, >1,000 Ci/mmol).

RESULTS

***Bal* 31-generated deletions in the crp gene and its 3'-flanking sequence.** Previous sequence analysis of *csm* clones in our laboratory indicated that the suppressor phenotype of these mutants was due to the insertion of a guanosine 17 bp downstream from the termination codon of the *crp* structural gene (16). Within the stem region of the potential transcriptional termination loop is a unique *Bss*HII restriction site, which was used in vitro to generate a deletion in this sequence. Transformants which harbor plasmids carrying this in vitro-generated deletion were found to exhibit *csm* characteristics. These results suggested a more thorough examination of the 3'-flanking, nonstructural sequence of the *crp* gene was warranted. To accomplish this we generated

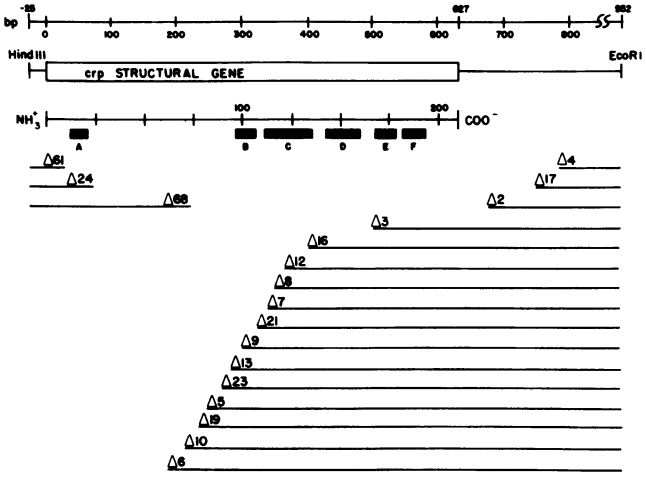

FIG. 1. Deletion map of *Bal* 31-generated deletions. Shown is a diagram of the *Hin*dIII-*Eco*RI fragment containing the *crp* structural gene and its 3′-flanking sequence. Above this fragment is a scale showing the size of the *crp* structural gene and the length of the 3′-flanking sequence in base pairs. Below the *Hin*dIII-*Eco*RI fragment is a map of the CRP. The vertical lines represent the number of amino acid residues. The shaded boxes correspond to the α-helices found in the CRP monomer. The individual deletions are represented as horizontal lines with specific allele numbers.

deletions in the *crp* gene and its 3′-flanking sequence by using *Bal* 31 nuclease. In vitro-generated deletions were constructed from both the 3′ and 5′ ends of the *crp* gene. The M13 RF vector containing the generated *crp* deletion fragments was doubly digested with *Hin*dIII and *Eco*RI, and the resulting fragments were sized on a 1% agarose gel. The deletions ranged in size from 24 to 250 bp from the 5′ terminus of the *crp* gene. Some 16 different in vitro-generated deletions were also generated in the 3′ region of the *crp* gene. These ranged in size from 133 to 675 bp and constitute two classes (Fig. 1). One class of deletions (i.e., Δ*crp-4*, Δ*crp-17*, and Δ*crp-2*) was external to the structural sequences of the *crp* gene. The other class of deletions extended into the *crp* structural gene. These deletion mutations lie in sequences coding for the various folded protein structure β-helices (i.e., A, B, C, D, E, and F) of the CRP (Fig. 1).

To determine the exact endpoint for each of the constructed deletions, M13mp8 or M13mp9 single-stranded vector DNAs containing the various *crp* deletions were used as

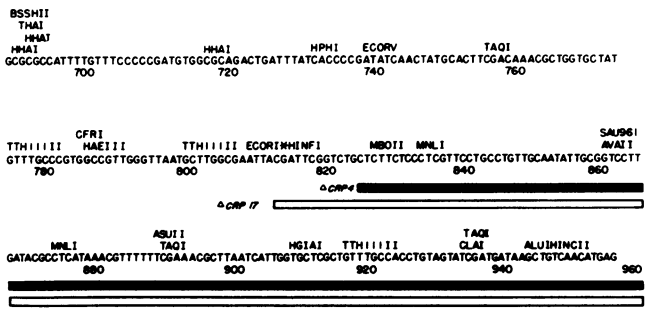

FIG. 2. Sequence of the *crp* 3′-flanking region from the *Bss*HII restriction site to the unique *Alu*I site. The endpoint for Δ*crp-4* and Δ*crp-17* in this adjacent 3′-flanking sequence is indicated. Also shown are endonuclease restriction sites that cut in this flanking sequence.

TABLE 2. Fermentation patterns and cAMP hypersensitivity of recombinant strains

Strain	Plasmid	cAMP[a]	cAMP hypersensitivity[b]	Fermentation of sugars[c]					
				Lac	Xyl	Rib	Ara	Mtl	Glu
NCR438	None	+	−	−	−	−	−	−	+
		−		−	−	−	−	−	+
NCR439	pHA7	+	−	+	+	+	+	+	+
		−		−	−	−	−	−	+
NCR440	pBM4	+	+	+	+	+	+	+	+
		−		+	+	+	+	+	+
NCR441	pBM17	+	−	+	+	+	+	+	+
		−		−	−	−	−	−	+
NCR442	pBM2	+	−	+	+	+	+	+	+
		−		−	−	−	−	−	+

[a] Presence (+) or absence (−) of cAMP at a final concentration of 0.1 mM.
[b] cAMP hypersensitivity was determined with EMB agar containing glucose, arabinose, xylose, ribose, or mannitol at 1% and cAMP at a final concentration of 0.25 mM: (−) Normal growth and no cAMP hypersensitivity; (+) no growth and cAMP hypersensitivity.
[c] All sugars in EMB agar were at 1%. Scoring of fermentation: (+) fermenting colonies; (−) nonfermenting colonies. Sugars: Lac, lactose; Xyl, xylose; Rib, ribose; Ara, arabinose; Mtl, mannitol; Glu, glucose.

templates for dideoxynucleotide DNA sequencing. Several of the deletion endpoints were found to reside in a number of restriction sites, e.g., the Δ*crp-2* endpoint lies within one of the *Taq*I restriction sites. A cluster of deletion endpoints was found to be located between 180 and 400 bp, i.e., Δ*crp-19*, Δ*crp-5*, Δ*crp-23*, Δ*crp-13*, Δ*crp-6*, Δ*crp-10*, Δ*crp-9*, Δ*crp-21*, Δ*crp-7*, Δ*crp8*, Δ*crp-12*, and Δ*crp*-16. The endpoints of the shortest deletions, Δ*crp-2*, Δ*crp-17* and Δ*crp-4*, were all located within the 3′-flanking sequence of the *crp* structural gene. The exact endpoints of Δ*crp-17* and Δ*crp*-4 are shown in Fig. 2. These deletions were of interest since they were the shortest deletions located in the 3′-flanking sequence of the *crp* gene.

Expression of catabolite-sensitive operons in strains carrying the in vitro-generated *crp* deletions. We wanted to determine the effect of various *crp* deletions on the expression of catabolite sensitive operons. We subcloned the individual fragments containing deletions from the M13 vector between the unique *Hin*dIII and *Eco*RI sites of pBR322. Strain NCR438 containing chromosomal deletions in both the *cya* and *crp* genes is deficient for the synthesis of both the CRP and adenylate cyclase. Therefore, this strain does not ferment substrates whose utilization depends on the expression of catabolite-sensitive operons (i.e., lactose, arabinose, xylose, ribose, etc.). Plasmid pHA7, which carries the wild-type *crp* insert, was transformed into strain NCR438 as a positive control. The resulting transformant, strain NCR439, fermented lactose, ribose, xylose, arabinose, and mannitol only in the presence of exogenous cAMP (Table 2). All of the transformants harboring plasmids carrying *crp* deletions extending into the structural gene for CRP, e.g., strains NCR443 through NCR458 (Table 1), were negative for the fermentation of the above substrates in the presence or absence of exogenous cAMP. Strains NCR441 and NCR442 containing the *crp* deletions Δ*crp-17* and Δ*crp-2*, which are external to the structural gene, were negative for the fermentation of these substrates in the absence of cAMP and positive in the presence of cAMP (Table 2). Strain NCR440 was of particular interest, since it fermented all of the tested substrates in the presence or absence of exogenous cAMP (Table 2). This strain harbors plasmid pBM4, which carries Δ*crp-4*, the smallest of the deletions extending into the 3′-flanking sequences of the *crp* gene.

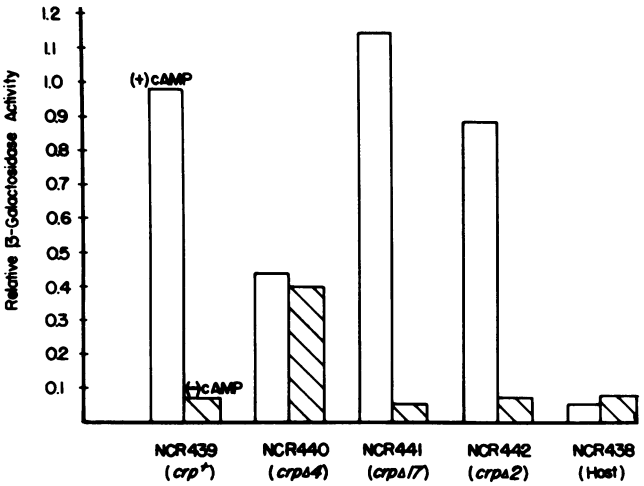

FIG. 3. Expression of β-galactosidase activity in strains harboring plasmids containing deletions in the 3'-flanking sequence of the *crp* gene. Strains were grown overnight in minimal glycerol media as described in Materials and Methods. Cells were induced for β-galactosidase for 30 min in the presence of 1 mM isoprophyl-β-D-thiogalactopyranoside. Strains were grown in the presence (□) or absence of (▨) of 0.1 mM cAMP. β-Galactosidase activity is reported relative to that of strain NCR439, which carries plasmid pHA7 containing the wild-type *crp* gene insert. Strain NCR439 expressed a specific activity of 10 μmol of *o*-nitrophenol per min per μg of dry cell weight.

Characterization of deletions in the 3'-flanking sequence of the *crp* gene. Strains NCR440, NCR441, and NCR442 were further characterized for their ability to express catabolite-sensitive operons. These strains harbor plasmids pBM4, pBM17, and pBM2, respectively. The expression of the lactose and TDA (biodegradative threonine dehydratase) operons, which are known to require the cAMP-CRP complex for efficient transcription, was examined. The expression of the lactose operon was examined by assaying for β-galactosidase activity (Fig. 3). Strain NCR438 contains chromosomal deletions in both the *cya* and *crp* genes. β-Galactosidase was not expressed in this strain in the presence or absence of exogenous cAMP. On the other hand, strain NCR439, a transformant which harbors plasmid pHA7 containing the wild-type *crp* insert, exhibited expression of β-galactosidase activity only in the presence of exogenous cAMP. The same result was observed for strains NCR441 and NCR442, which contain plasmids harboring the external 3'-flanking mutations Δ*crp-17*, and Δ*crp-2*. Strain NCR440, which carries the Δ*crp-4* external deletion on plasmid pBM4, exhibited β-galactosidase activity in the absence or presence of exogenous cAMP. Levels of β-galactosidase activity in this strain were about 50% of the activity observed in strain NCR439 (when cAMP was present). Similar results were observed for TDA expression in the above strains (Fig. 4). Strains NCR439, NCR441, and NCR442 exhibited expression of TDA only in the presence of exogenous cAMP. Strain NCR438 showed no TDA activity in the presence or absence of exogenous cAMP. However, TDA activity was expressed in strain NCR440 in the absence or presence of exogenous cAMP. The level of TDA produced by strain NCR440 was lower than that produced by strain NCR439, which harbors plasmid pHA7. Therefore the expression of the TDA and lactose operons in strain NCR440 is independent of cAMP. The ability of this strain to ferment and express catabolite-sensitive operons in the absence of exogenous cAMP suggests that the Δ*crp-4* mutation mimics the spontaneous cAMP suppressor mutations isolated previously in the laboratory (23).

cAMP hypersensitivity in strain NCR440. Chromosomal cAMP suppressor mutations previously isolated by Melton et al. have been characterized and shown to express a hypersensitivity to exogenous cAMP (23). Strain NCR440, which harbors pBM4 carrying the shortest external deletion, exhibited cAMP hypersensitivity on EMB agar containing either glucose, arabinose, xylose, ribose, or mannitol (Table 2). Strains NCR441 and NCR442, which contain plasmids pBM17 and pBM2, respectively, did not exhibit cAMP hypersensitivity. Revertants were isolated from strain NCR440 that were not sensitive to exogenous cAMP. These revertants were negative for the fermentation of substrates tested in the presence of cAMP and therefore exhibited a Crp^- phenotype. Such revertants were also isolated by Melton et al. for the chromosomal *csm* mutant strain NCR30 (23).

Expression of CRP in maxicells. DNA sequencing of the chromosomal cloned *csm* from strain NCR30 showed the *csm* mutation to be located outside of the *crp* structural gene in the 3'-flanking region (16). We sequenced all of the *crp* inserts containing *Bal* 31 in vitro-generated deletions isolated in this study. We were particularly interested in determining whether the *crp* structural gene of pBM4 might have other mutations which could account for the observed suppressor phenotype. DNA sequencing analysis indicated that there were no point mutations or other structural alterations in the *crp* structural gene adjacent to the 3'-flanking Δ*crp-4* mutation. To substantiate this we used the Maxicell technique to examine the CRP expressed from pBM4, which carries the Δ*crp-4* mutation (Fig. 5). The maxicell strain SS5062 was transformed with plasmids pBR322, pHA7, pBM4, and pBM68. Plasmids pHA7, pBM4, and pBM68 carry inserts of the wild-type *crp* gene, the 3'-flanking Δ*crp-4* mutation, and

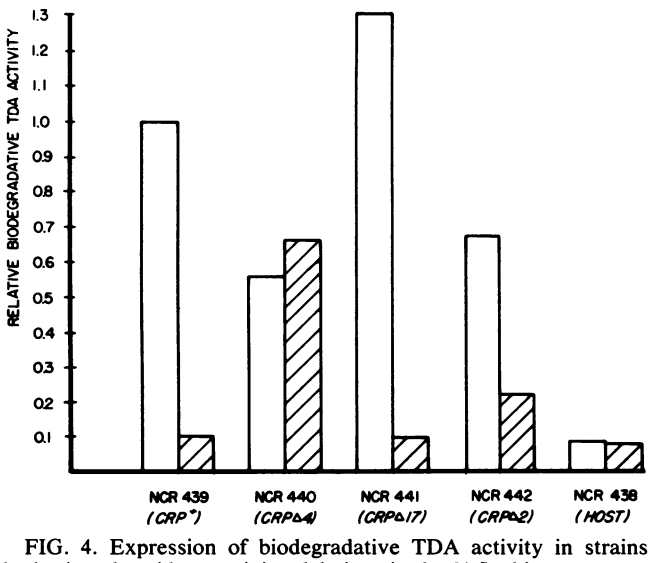

FIG. 4. Expression of biodegradative TDA activity in strains harboring plasmids containing deletions in the 3'-flanking sequence of the *crp* gene. Strains were grown anaerobically for 16 h in POPEP medium in the presence (□) or absence of (▨) of 0.1 mM cAMP. Strain NCR439, carrying the plasmid pHA7 with the wild-type *crp* gene insert, exhibited a specific activity for biodegradative threonine dehydratase of 0.1 μmol of α-ketobutyrate per min per μg dry cell weight. All other activities are reported as relative to that of strain NCR439.

the Δ*crp-68* mutation located in the 5′ region of the *crp* gene, respectively (Fig. 1). No CRP was synthesized by strains harboring plasmid pBR322 or pBM68, as expected. Both plasmids pHA7 and pBM4 expressed CRP having identical molecular weights (Fig. 5). Therefore, the *crp* structural gene of pBM4 is intact, and the observed cAMP suppression is a consequence of the Δ*crp-4* mutation external to the *crp* structural gene.

DISCUSSION

Previously we described the isolation and characterization of *csm* mutations in *E. coli* K-12 which promoted the expression of catabolite-sensitive operons in *cya* mutant strains (23). Workers in other laboratories have also reported the isolation of cAMP-independent mutations (5, 8, 12, 34, 38). Spontaneous and in vitro-generated *csm* mutations isolated in this laboratory share the following characteristics: (i) they are each mutation is located outside of the structural gene for the CRP, i.e., reside in the 3′-flanking sequence of the *crp* gene; (ii) they confer cAMP hypersensitivity; (iii) they manifest the expression of catabolite-sensitive operons in the absence of exogenous cAMP; and (iv) revertants of these mutations selected as resistant to exogenous cAMP fall into several similar classes (23). The cAMP-independent mutations isolated in our laboratory and by other investigators fall into two major classes. Class I mutations include suppressor mutations which are intercistronic, i.e., those located in the 3′-flanking sequence of the *crp* structure gene. Class II suppressors include mutations located in the *crp* structural gene itself. Garges and Adhya (15) have identified the alterations in the amino acid sequence of the CRP encoded by *crp** mutants by determining the nucleotide sequence of the mutant genes. These cAMP-independent missense mutations are class II cAMP suppressors. They caused substitution of amino acids with bulky side chains in the D-α-helix of the carboxy domain of the CRP, near the hinge region which connects the carboxy domain to the amino domain. Harman and co-workers have sequenced the *crp* genes from three cAMP-independent mutants (personal communication). Sequence data show that these mutations also are located within the *crp* structural gene and result in amino acid substitutions in the CRP.

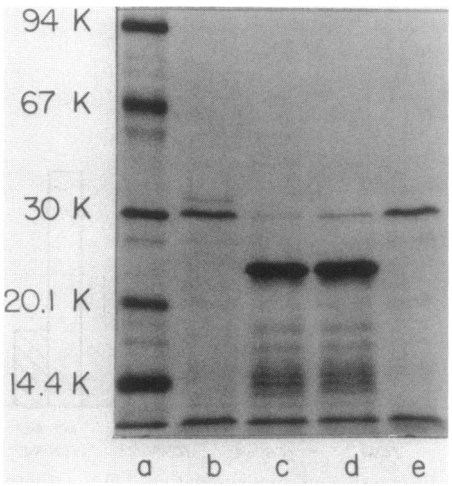

FIG. 5. Maxicell expression of cloned Δ*crp-4* and the wild-type *crp* genes. The maxicell experiment involved the use of cells containing plasmid pBR322 (lane b) and derivates of pBR322 with the cloned wild-type *crp* (lane c), the Δ*crp-4* mutation (lane d), and the Δ*crp-68* mutation (lane e). Protein molecular mass markers (lane a) included α-lactalbumin (14.4 kilodaltons); soybean trypsin inhibitor (20.1 kilodaltons); carbonic anhydrase (30 kilodaltons); bovine serum albumin (67 kilodaltons); phosphorylase *b* (94 kilodaltons).

Recently, we cloned and sequenced the *csm* mutant allele of the *crp* gene from *E. coli* NCR30 (16). Sequence analysis of the *csm*-57 mutant allele revealed the insertion of a guanosine residue in the stem region of the presumed transcriptional termination loop of the *crp* structural gene. We also constructed an in vitro deletion in the unique *Bss*HII site of the stem-loop, which generated a cAMP-independent phenotype.

We have described in this article in vitro-generated deletions in the cloned crp^+ gene and its 3′-flanking sequences. One deletion mutation (Δ*crp-4*), which extended only into the 3′-flanking sequences of the *crp* gene, was shown to be involved in the expression of the cAMP suppressor function.

Our laboratory is engaged in studies to delineate further the molecular role of the *crp* 3′-flanking sequences in the suppression of *cya* mutations. Below we discuss some possible mechanisms for the involvement of the *crp* 3′-flanking sequences in suppression of *cya* mutations. One might envision the presence of an additional gene coded by the 3′-flanking region of the *crp* gene. The peptide or protein encoded by such a gene in the *csm* mutants could bind and modify the CRP conformation such that it activates transcription of catabolite-sensitive operons in the absence of cAMP. An analysis of the sequences in the 3′-flanking region of the *crp* gene reveals the presence of possible open reading frames (manuscript in preparation).

Also, deletions or point mutations within the 3′-flanking region of the *crp* gene could abolish transcription termination, causing readthrough into distal genes whose products regulate CRP. Guarante et al. (18) have shown that deletions ranging in size from 200 to 1,000 bp, which lie past the end of the *trp* operon, can cause readthrough into distal genes. Plamann et al. have found a long region of symmetry of the *glyA* gene located between the site of translation termination and the proposed transcription termination region (31). They proposed that this region may play a role in the expression of *glyA* and other procaryotic operons with which it shares homology. It is also possible that the 3′-flanking sequences of the *crp* gene affect the stability or secondary structure of *crp* mRNA in *csm* mutants.

We are currently investigating the effect of mutations in this region on the biochemical properties of the CRP. Harman and Dobrogosz (19) showed that the CRP isolated from the *csm* strain NCR30 differed from the protein isolated from wild-type *E. coli*. The mutant protein activated the in vitro synthesis of β-galactasidase in the absence of cAMP. Unlike the wild-type CRP, the mutant CRP in its native form was sensitive to digestion by chymotrypsin, subtilisin, and trypsin in the absence of the effector cAMP. These results were interpreted as providing biochemical evidence that the mutant *crp* allele encoded a CRP that existed in a cAMP-CRP complex-like conformation in the absence of cAMP. In this study we have shown the CRP protein expressed in maxicells from pBM4 carrying the in vitro-generated *csm* mutation Δ*crp-4* is identical in molecular weight to the wild-type protein. Our laboratory has recently initiated an investigation of the proteolytic sensitivity of maxicell-expressed CRPs for the wild-type and exogenic mutated *crp* genes. Our preliminary results agree with the findings of Harmon and Dobrogosz (19) in that the mutant CRP expressed from pBM4 is sensitive to digestion by certain

proteases in the absence of the effector cAMP (manuscript in preparation).

Our results strongly indicate that the 3'-flanking sequences of the *crp* gene is involved in the induction of cAMP suppressor functions of CRP, i.e., the ability of CRP to promote expression of catabolite-repressible operons in the absence of cAMP. The elucidation of the precise mechanism of this suppression is forthcoming.

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