JOURNAL OF BACTERIOLOGY, May 1986, p. 533–540
0021-9193/86/050533-08$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 166, No. 2

Cloning and Molecular Characterization of *csm* Mutations Allowing Expression of Catabolite-Repressible Operons in the Absence of Exogenous Cyclic AMP†

SUSAN E. GEORGE‡ AND THOYD MELTON*
Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615

Received 19 August 1985/Accepted 3 February 1986

The cyclic AMP (cAMP) suppressor mutation (*csm*) of *Escherichia coli* has been cloned from strain NCR30 in the *Hin*dIII-*Eco*RI site of pBR322. This mutation has been mapped in or near the *crp* gene. Wild-type *crp* DNA hybridized to recombinant plasmids pGM5 and pGM25 containing the cloned *csm* mutation. These recombinant plasmids encoded a protein product of identical molecular weight and charge as that of the wild-type cAMP receptor protein. Transformants of *cya crp* deletion strains harboring pBM5 or pGM25 exhibited phenotypic characteristics common to strain NCR30. These included the expression of catabolite-repressible enzymes, such as arabinose isomerase, tryptophanase, β-galactosidase, and threonine deaminase; the expression of chemotactic and motility genes; cAMP sensitivity; and the accumulation of toxic levels of methylglyoxal. DNA sequence analysis indicated that the Csm suppressor phenotype was attributable to the insertion of a guanosine residue 17 base pairs downstream from the termination codon of the *crp* structural gene. The guanosine insertion is located in the stem region of the presumed transcriptional termination loop. This stem region contained a unique *Bss*HII restriction site which was used to construct an in vitro deletion in the wild-type *crp* insert in plasmid pHA7. The resulting plasmid, pGM459, renders transformants having a phenotype common to that conferred by the chromosomal or cloned *csm* mutation. Our results indicate a novel role for the 3′ flanking region of the *crp* structural gene in the expression of the cAMP receptor protein.

The cyclic AMP (cAMP) receptor protein (CRP) of *Escherichia coli* is composed of two identical monomers of 22,500 daltons and, upon binding cAMP, becomes a positive effector for many catabolite-repressible operons (1). The cAMP-CRP complex binds to the promoter and activates transcription of the lactose (15, 24, 40), arabinose (32, 47), galactose (43), and maltose (11) operons and also stimulates the *tnaA* (16) and the pBR322 P4 promoter (53). Ebright (22) has compared the DNA sequence of several promoters of operons under CRP control and found a consensus sequence which reads 5′ AA-TGTGA--T---TCA-AT (A or T) 3′. If a single-site mutation is induced in the TGTGA portion of the sequence, such as in the case of the *lacL8* and *lacL29* mutations, CRP binding affinity is significantly decreased (5, 18, 31, 34). Other studies describe the CRP as a negative effector for glutamine metabolism (52), *ompA* transcription (42), adenylate cyclase activity (10, 33), and transcription termination (28, 62). Aiba (2) has reported that the CRP structural gene is autoregulated and that the *cya* gene is repressed by the cAMP-CRP complex.

The wild-type *crp* gene from *E. coli* K-12 has been cloned, and its DNA sequence has been determined (3, 12). The gene encoding the CRP contains 627 base pairs, which translate into 209 amino acids.

Recently cAMP-suppressor mutants of *E. coli* (*csm*) have been isolated by Melton et al. (35) and shown to be spontaneous pseudorevertants of adenylate cyclase-negative strains. The suppressor mutation is cotransducible with the *aroB* gene locus, indicating that it maps within or near the *crp* gene region. Characteristics of *csm* mutants include (i) the absence of a functional *cya* gene, (ii) expression of catabolite-repressible operons in the absence of cAMP, and (iii) hypersensitivity to cAMP, which coincides with a detrimental increase in methylglyoxal concentration (35). Other investigators have reported similar mutants having dysfunctional adenylate cyclase activity but still being capable of expressing catabolite-repressible operons (4, 10, 17, 56, 61). However, none of these mutants is sensitive to cAMP.

In this study we describe (i) the cloning of the *csm* mutation from *E. coli* NCR30, (ii) the physiological and biochemical characterization of recombinant strains containing plasmids carrying the cloned *csm*, (iii) molecular characterization of the cloned *csm* fragment, and (iv) in vitro construction of deletions in the *Bss*HII restriction site in the nontranslated 3′ flanking sequence of the *crp* gene which mimic the chromosomal *csm* mutation.

MATERIALS AND METHODS

Chemicals and enzymes. cAMP was obtained from United Biochemical Corp., Cleveland, Ohio. Isopropyl-β-D-thiogalactopyranoside, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), RNase T_1, RNase A, proteinase K, and DNase I were purchased from Sigma Chemical Co., St. Louis, Mo. Ampholytes (pH ranges, 5–8 and 3–10) were obtained from Bio-Rad Laboratories, Richmond, Calif., and DNA polymerase, Klenow fragment DNA polymerase, T4 ligase, and all restriction enzymes were supplied by Bethesda Research Laboratories, Inc., Gaithersburg, Md., except the restriction enzymes *Eco*RV, *Bst*EII, and *Bss*HII, which were obtained from New England BioLabs, Inc., Beverly, Mass. The sequencing nucleotides were also purchased from New England BioLabs. Bacteriophage M13mp8, M13mp9, and the M13 17-base primer and S1 nuclease were from P-L

* Corresponding author.
† Paper number 10062 in the journal series of the North Carolina Agricultural Research Service at Raleigh.
‡ Present address: Genetic Toxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.

TABLE 1. *E. coli* bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
Strain		
K-12	Wild type	63
CA8445	*Δcya-45 Δ crp-45*	55
NCR30	*cya-57 csm-57*	35
NCR31	*cya-57*	25
NCR430	*Δcya-45 Δ crp-45*/pBR322	J. G. Harmon and W. J. Dobrogosz[a]
NCR431	*Δcya-45 Δ crp-45*/pHA7	J. G. Harmon and W. J. Dobrogosz
NCR434	*Δcya-45 Δ crp-45*/pGM5	This study
NCR435	*Δcya-45 Δ crp-45*/pGM25	This study
NCR437	*cya-57 csm-57*/pHA7	This study
NCR459	*Δcya-45 Δ crp-45*/pGM459	This study
JM103	*(lac pro) this rpsL supE endA sbcB15 hsdR4* F' *traD36 proAB lacI^{q}ZΔM15*	36
HB101	*hsdR hsdM recA13 supE44 lacZ4 leuB6 proA2 thi-1* SmΔ^r	14
Plasmid		
pBR322	*amp$^+$ tet$^+$*	9
pHA7	*crp$^+$ amp$^+$ tet*	3
pBM5	*csm-57 amp$^+$ tet*	This study
pBM25	*csm-57 amp$^+$ tet*	This study
pGM17	*csm-57 amp$^+$ tet*	This study
pGM459	*crp amp$^+$ tet*	This study

[a] J. G. Harman and W. J. Dobrogosz, Abstr. Annu. Meet. Am. Soc. Microbiol., 1983, K41, p. 183.

Biochemicals, Inc., Milwaukee, Wis. [α-^{32}P]dCTP tetra triethylammonium salt (specific activity, 800 C/mmol) and [α-^{32}P]dATP tetra triethylammonium salt (specific activity, 800 C/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

Strains and media. Bacterial strains used in this study are shown in Table 1. Bacteria were cultured in yeast extract-tryptone (YT), Luria broth (LB) (39), or polypeptone (35) medium. When minimal medium was required, either ECB (19) or M9 (39) medium was utilized. Minimal medium was supplemented with 80 μg of thiamine per ml, 0.2% carbon source, 50 μg of ampicillin per ml, 20 g of tetracycline per ml, or 5 mM cAMP where indicated. Eosin-methylene blue (EMB) without lactose or MacConkey indicator plates were used when fermentation was monitored. Solid agar plates were prepared by adding 15 g of agar per liter to M9 or YT media. Isopropyl-β-D-thiogalactopyranoside and X-gal (40 μg/ml) were added to YT plates where indicated. All cultures were incubated at 37°C on a rotary shaker incubator unless otherwise indicated.

Isolation of chromosomal and plasmid DNA. Strain NCR30 chromosomal DNA was isolated by the procedure of Schleif and Wensink (58). Large-scale plasmid DNA isolation was done by the procedure of Norgard (44). Small-scale isolation of plasmid DNA was carried out by the procedure of Birnboim and Doly (7).

Restriction enzyme digestions and recovery of DNA fragments. Restriction enzyme digestion of chromosomal and plasmid DNA was by the procedure of Davis et al. (14), and the conditions were those recommended by the company from which the enzymes were obtained.

The *crp* fragment was isolated via band interception from an agarose gel after *Hin*dIII-*Eco*RI double digestion of pHA7. The DNA was transferred to DEAE-cellulose as described by Dretzen et al. (21).

Probing of chromosomal or plasmid DNA with *crp* [^{32}P]DNA. Radiolabeled [α-^{32}P]dCTP was used to label the isolated *crp* DNA fragment by a nick-translation procedure similar to that of Rigby et al. (54). The ^{32}P-labeled probe (6 × 10^6 cpm) was hybridized to DNA bound to BA85 nitrocellulose filter paper as described by Davis et al. (14). The hybridized DNA was observed by autoradiography.

Ligation reactions and transformation. A typical ligation reaction contained 50 μg of isolated *Hin*dIII-*Eco*RI *csm* fragment, 10 μg of linearized pBR322, 70 mM Tris hydrochloride (pH 7.5), 7 mM $MgCl_2$, 70 μM ATP, 10 mM dithiothreitol, and 0.2 U of T4 ligase in a total volume of 5 μl (6). The ligation was carried out at 15°C for 18 h and terminated by the addition of 2 μl of 0.2 M disodium EDTA and 23 μl of distilled H_2O.

The procedure of Enea et al. (23) was used to prepare competent cells for transformation. Transformation of plasmid DNA (1 μg) into *E. coli* was performed by the procedure of Dagert and Ehrlich (13). After incubation for phenotypic segregation, 1 ml of cell suspension was added to 3.0 ml of soft YT agar (0.6%) and overlaid on YT-ampicillin (50 μg/ml) medium.

Preparation of proteins and NEPHGE. Two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE) was performed as described by O'Farrell et al. (45, 46). Bacterial strains were cultured in 500 ml of ECB medium and grown to late log phase. The cells were harvested and suspended in 10 ml of ECB. After disruption by sonication, the cell extract was centrifuged at 4°C for 1 h at 100,000 × *g*. The supernatant was treated by the procedure of O'Farrell (45). The protein concentration was determined with commercially obtained Bio-Rad concentrate. NEPHGE gels were loaded with 70 to 100 μg of protein and electrophoresed at 100 V for 8 to 10 h. Purified wild-type or mutant CRP protein (5 μg) was used as a marker to spike protein gels. NEPHGE gels were silver stained by the method of Morrissey (41).

Enzyme assays. β-Galactosidase activity was determined by the method described by Miller (39). Threonine deaminase degradative activity was measured spectrophotometrically by the assay of Shizuta and Takushige (59). Arabinose isomerase activity was assayed by the cysteine-carbazole method for ribulose determination as described by Schleif and Wensink (58). Tryptophanase activity was assayed spectrophotometrically by the method of Meyer et al. (38).

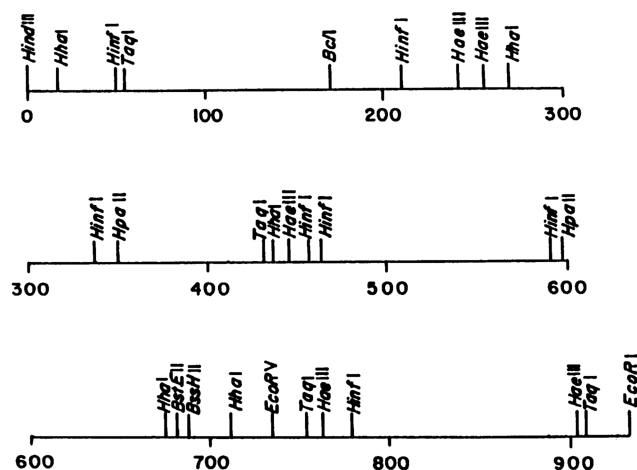

FIG. 1. Physical map of the *Hin*dIII-*Eco*RI *csm* insert in pGM25. The restriction map of the *Hin*dIII-*Eco*RI fragment from pGM25 was derived from restriction enzyme digestions and directly from DNA sequencing. Except for a unique *Bst*EII recognition sequence, the physical map is identical to that of the cloned *crp* (3, 12).

Methylglyoxal assay. The methylglyoxal concentration was determined colorimetrically by the method of Friedmann and Haugen (26) with modifications by Melton et al. (35).

Motility studies. Bacterial strains were grown overnight on YT plates. Sterile applicator sticks were used to lift colonies off the plates and stab them into YT-soft agar swarm plates (containing 0.3% agar [Difco Laboratories, Detroit, Mich.]). After incubation at 37°C for 12 h, motility was monitored by measuring the size of the bacterial chemotactic zone extending from the initial stab.

Subcloning *csm* fragments into bacteriophage M13mp8 and M13mp9. The *Hin*dIII-*Eco*RI *csm* fragment was isolated from plasmid pGM25 and cleaved with *Taq*I or *Hae*III. The fragments were isolated by band interception (21) and purified. The isolated fragments were subcloned into the Messing and Viera (37) M13 phage vectors M13mp8 and M13mp9 cleaved with the appropriate enzyme. *E. coli* JM103 was made competent and transfected with the recombinant phage. Positive recombinant phage were identified as white plaques on YT-X-gal-isopropyl-β-D-thiogalactopyranoside plates.

Purification of template DNA for DNA sequencing. The method of Messing and Viera (37) was used to prepare DNA templates for dideoxy sequencing as described by Sanger et al. (57).

C test. To confirm the hybridization results and determine the orientation of inserts in M13 prior to sequencing, a C test was performed by the procedure of Poncz et al. (51).

In vitro construction of internal deletions. Plasmid pHA7 (5 μg) was digested with the restriction enzyme *Bss*HII for 2 h at 50°C. The single-stranded ends were then removed with S1 nuclease. The reaction mixture, which contained 30 μl (5 μg) of *Bss*HII-cleaved pHA7 DNA, 4 μl of S1 reaction buffer (500 mM sodium acetate [pH 4.5], 1.5 M NaCl, 5 mM $ZnSO_4$), 1 μl of S1 nuclease (1 U), and 5 μg of distilled H_2O, was incubated at 37°C for 30 min, and the reaction was terminated by the addition of 2 μl of 100 mM EDTA (pH 7.5) and 4 μl of 1 M Tris hydrochloride (pH 8.0). After phenol, chloroform, and ether extractions, the DNA was ethanol precipitated, dried, and suspended in 10 μl of dH_2O. The blunt ends were ligated for 18 h at 15°C with T4 ligase (2 U). Competent cells of strain CA8445 were transformed with the ligation mixture and spread plated on EMB-arabinose-ampicillin plates. After 24 to 48 h of incubation, positive colonies were cultured and tested for cAMP sensitivity. Plasmids were isolated by the method of Birnboim and Doly (7) and examined for failure to be digested by *Bss*HII.

RESULTS

Cloning of the chromosomal *csm* mutation from NCR30. The *Hin*dIII-*Eco*RI-generated fragment from pHA7 (*crp*$^+$) was isolated from a 1% agarose gel and labeled via nick translation with [α-^{32}P]dCTP. A Southern transfer of *Hin*dIII- and *Hin*dIII-*Eco*RI-cleaved NCR30 chromosomal DNA was hybridized to the ^{32}P-labeled *crp* probe, and an autoradiogram was produced. The probe hybridized to a 4.25-kilobase fragment in the *Hin*dIII digest of NCR30 chromosomal DNA. Subsequent *Eco*RI cleavage produced a smaller, hybridizable fragment of 0.930 kilobase. The 0.930-kilobase fragment was recovered from the gel via band interception and cloned into pBR322 double digested with *Hin*dIII and *Eco*RI.

Competent *E. coli* HB101 cells were transformed with the recombinant pBR322 vector containing the 930-base-pair fragment, and transformants were selected on YT-ampicillin plates. Thirty-two ampicillin-resistant colonies grew on the YT-ampicillin selection plates. Upon replica plating onto YT-tetracycline plates, 24 colonies were found to be tetracycline sensitive, indicating the presence of an insert in the *Hin*dIII-*Eco*RI site of pBR322.

Characterization of recombinant plasmids containing the cloned *csm* mutation. Plasmid DNA was isolated from the ampicillin-resistant, tetracycline-sensitive HB101 transformants by the procedure of Birnboim and Doly (7) and examined on a 1% agarose gel by ethidium bromide staining. Upon Southern transfer and subsequent ^{32}P-labeled *crp* hybridization, 13 positive clones were identified. Of these recombinant plasmids, pGM17 and pGM25 were isolated by CsCl gradient ultracentrifugation and restricted with *Pvu*II, *Pst*I, *Bam*HI, *Ava*I, *Hin*cII, and *Sal*I. The resulting cleavages were compared with pHA7 and found to be identical.

The *Hin*dIII-*Eco*RI fragments from plasmids pHA7, PGM17, and pGM25 were isolated and subjected to further restriction enzyme analysis. A physical map of pGM25 is shown in Fig. 1, and it can be seen that the cloned *csm* has a unique *Bst*EII (*Asp*AI) site (i.e., -GGTTACC-) that lies within the transcriptional termination loop of the *crp* gene.

Expression of gene product encoded by cloned sequences in pGM5 and pGM25. Harman and Dobrogosz (29) reported that the CRP isolated from the cAMP suppressor mutant strain NCR30 had the same molecular size (45,000 daltons) and charge as the wild-type CRP on NEPHGE gels. To examine the protein products of pGM5 and pGM25, these plasmids were isolated and transformed into strain CA8445 deleted for both *cya* and *crp* genes. Proteins from these strains (i.e., NCR434 and NCR435) as well as from strains NCR430 and NCR431 (harboring plasmids pBR322 and pHA7, respectively) were compared on NEPHGE gels. Recombinant plasmid pGM5 and pGM25 expressed a protein of identical molecular weight and charge as the wild-type CRP synthesized by plasmid pHA7 (Fig. 2a to d).

Marker rescue and expression of catabolite-sensitive operons. In vivo gene expression of catabolite-sensitive operons by the recombinant plasmids containing the cloned *csm* mutation was examined. The ability of the mutant CRP product encoded for by the recombinant plasmids pGM5 and pGM25 to stimulate growth of strains NCR434 and NCR435 on the catabolite-repressible substrates arabinose, lactose,

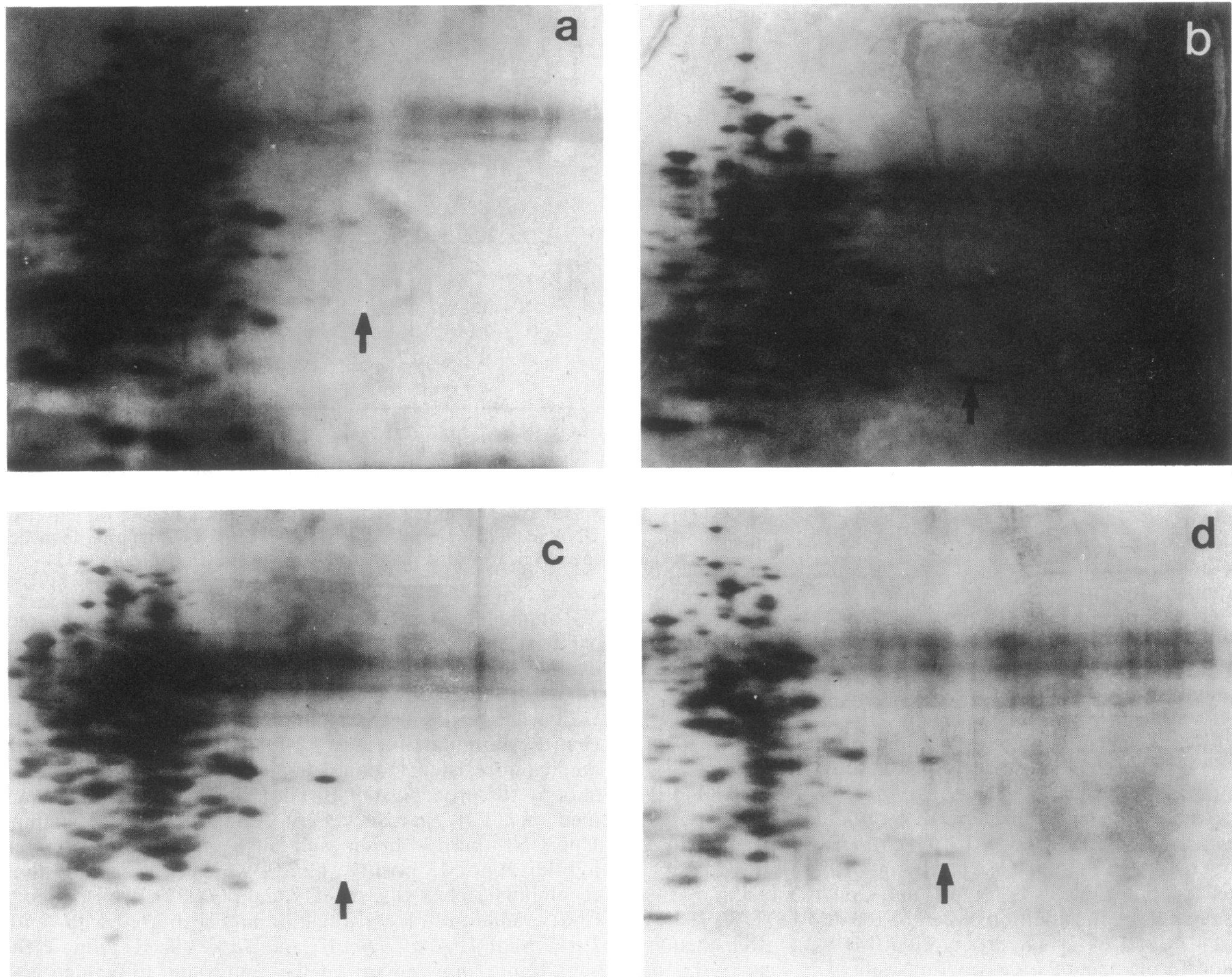

FIG. 2. NEPHGE analysis of proteins isolated from *E. coli* NCR430 (a), NCR431 (b), NCR434 (c), and NCR435 (d). Crude extracts of proteins were isolated from the above strains, prepared, and electrophoresed as described in Materials and Methods. The arrows depict the basic protein profile of NCR430 (a), NCR431 (b), NCR434 (c), and NCR435 (d).

maltose, xylose, and galactose and the noncatabolite-repressible substrate glucose was investigated. These strains were capable of wild-type growth on both arabinose and glucose media. However, limited growth was observed when these strains were grown on lactose, maltose, xylose, and galactose media.

The ability of the recombinant plasmids pGM5 and pGM25 to facilitate expression of a number of catabolite-repressible enzymes in the *cya crp* deletion strain CA8445 was also examined. Arabinose isomerase (32, 47), threonine deaminase (48) (degradative), and tryptophanase (16) and bacterial motility (20) systems whose expression is normally under cAMP-CRP control in the wild-type strain were measured. A comparison of the relative activities of the above enzymes for the wild-type *E. coli* K-12, the chromosomal *csm* mutant strain NCR30, and NCR434 and NCR435 carrying the *csm* recombinant plasmids pGM5 and pGM25, respectively, is shown in Table 2. The recombinant plasmids of strains NCR434 and NCR435 were capable of directing expression of the lactose operon. In vivo β-galactosidase activity was observed when strains NCR434 and NCR435 were grown on

TABLE 2. Relative enzymatic activities and motility response[a]

Strain	β-Galactosidase	Arabinose isomerase	Threonine deaminase	Tryptophanase	Motility[f]
K-12	1.30	1.00	1.00	2.25	+
NCR30	1.00[b]	1.00[c]	1.00[d]	1.00[e]	+
NCR430	0.00	0.40	0.00	0.03	−
NCR434	1.31	1.15	1.06	0.31	+
NCR435	1.21	1.20	1.01	0.22	+

[a] All reported numberical values are relative to the activity of strain NCR30.

[b] Measured activity was 1.88 mmol of *o*-nitrophenyl-β-D-galactopyranoside per min per mg (dry weight) of cells.

[c] Measured activity was 1.40 mol of ribulose per min per mg (dry weight) of cells.

[d] Measured activity was 2.32 mmol of α-ketobutyrate per min per mg (dry weight) of cells.

[e] Measured activity was 2.32 mmol of indole per min per mg (dry weight) of cells.

[f] A positive (+) response was defined as a chemotactic ring around the initial colony stab. A negative (−) response was no bacterial migration.

YT plates containing X-gal and isopropyl-β-D-thiogalactopyranoside. Measurement of in vitro β-galactosidase activity was negligible after a 30-min induction in strains NCR434 and NCR435. However, after a 90-min induction, wild-type enzyme levels were observed as shown in Table 2. Recombinant strains NCR434 and NCR435 were also capable of expressing activity for both arabinose isomerase and threonine deaminase comparable to those of strain NCR30 and the wild-type K-12. It can also be seen that these strains were positive for motility as well. Strain NCR30, the *cya crp* deletion strain, harboring only PBR322 and therefore lacking a functional cAMP-CRP transcriptional complex was unable to express motility. Strain NCR430 also lacked tryptophanase activity. Even though the recombinant strains exhibited a significant increase in tryptophanase activity compared with that of strain NCR430, the activity was lower than that observed for strains K-12 and NCR30.

Sensitivity to cAMP and methylglyoxal production in recombinant strains. Strains containing the *csm* mutation are sensitive to exogenous cAMP (35). Strains NCR434 and NCR435 along with strain NCR30 were examined for cAMP sensitivity (Table 3). Strain NCR437, which has the chromosomal *csm* mutation and carries plasmid pHA7, was also tested to determine whether *csm* or *crp* was dominant. Strain NCR31 carries the *cya-57* chromosomal mutation and fermented arabinose only at high exogenous cAMP levels. Strain NCR431, which is deleted both for *cya* and *crp* on the chromosome, carries the wild-type *crp* allele on pHA7. This strain, unlike strain NCR31, fails to ferment arabinose at concentrations above 0.5 mM cAMP. Strain NCR30 has the *csm-57* mutation and exhibits sensitivity to exogenous cAMP. When the wild-type crp^+ allele is introduced into this mutant, as is the case with strain NCR437, it can be seen that the *csm* allele is dominant to the crp^+ allele since this strain remains sensitive to exogenous cAMP. Both strains NCR434 and NCR435 carrying the cloned *csm-57* mutation exhibit sensitivity to exogenous cAMP. The cAMP hypersensitivity of strain NCR30 has been correlated with the accumulation of toxic levels of methylglyoxal (35). The recombinant strains NCR434 and NCR435 both produced levels of methylglyoxal comparable to that of strain NCR30 in the presence of 5 mM exogenous cAMP (Table 4). These strains were also inhibited for growth at this concentration of cAMP (Table 3). The fact that methylglyoxal accumulation is dependent on a functional cAMP-CRP complex is indicated by the significant decrease in methylglyoxal production for the *cya crp* deletion strain NCR430 which harbors pBR322 (Table 4).

TABLE 3. Response of strains to exogenous cAMP[a]

Strain	Fermentation response to indicated concn of cAMP (mM)[b]				
	5.0	1.0	0.5	0.1	0.05
NCR434	I	I	I	+	+
NCR435	I	I	I	+	+
NCR431	–	–	–	+	+
NCR430	–	–	–	–	–
NCR437	NG	NG	NG	I	I
NCR30	NG	NG	NG	NG	+
NCR31	+	+	–	–	–
K-12	+	+	+	+	+
CA8445	–	–	–	–	–

[a] Fermentation patterns of organisms were examined on EMB–1% arabinose in the presence of various cAMP concentrations and 50 μg of ampicillin per ml where resistance was established. Plates were incubated for 24 to 36 h at 37°C.

[b] NG, No growth; I, inhibited growth (arabinose fermentation was undetectable). Where dense growth was observed, the arabinose fermentation response is indicated as positive (+) or (–).

TABLE 4. Methyglyoxal production of strains grown on minimal arabinose medium with and without cAMP

Strain	Relative methylglyoxal production of strains grown as follows[a]:	
	Without cAMP	With cAMP (5 mM)
K-12	0.23	0.43
NCR30	0.12[b]	1.00[c]
NCR430	0.06	0.08
NCR434	0.24	0.90
NCR435	0.24	0.89

[a] All values are relative to the activity of strain NCR30 grown in the presence of exogenous cAMP.

[b] The measured activity was 90 μmol of methylglyoxal per reaction.

[c] The measured actitity was 747 μmol of methylglyoxal per reaction.

Sequence analysis of the cloned *csm* mutation. The 930-base-pair fragment carrying the altered *crp* gene was sequenced by the dideoxy chain termination method of Sanger et al. (57). The coding sequence for the wild-type *crp* gene (3) was compared and found to be identical.

Since no base change was detected in the structural gene sequence of the cloned *csm* mutation, or interest turned to comparing the nontranslated 3′ distal sequence from the translation stop codon of the cloned *crp* genes. The ribosomal terminator loop is located 10 base pairs from the 5′-TAA-3′ codon at sequence position 598 (12) (Fig. 3). The presumed terminator loop of the wild-type *crp* gene consists of 23 bases and contains a unique *Bss*HII restriction site (i.e., 5′-GCGCGC-3′) in the stem of the terminator loop (Fig. 3a). Examination of the cloned *csm* sequence revealed an insertion of a guanosine residue 17 base pairs downstream from the translational termination codon (Fig. 3b). The insertion of this guanosine residue generated a unique *Bst*EII (*Asp*AI) recognition site (5′-GGTTACC-3′) within the presumed transcriptional loop-stem structure. Even though the recognition sequence was present, *Bst*EII did not cleave at this site under the conditions tested.

Generation of the in vitro deletions in the *Bss*HII terminator sequence. To determine whether the Csm phenotype was the result of a structural change in the presumed transcription terminator for *crp*, we generated a four-base-pair deletion in the unique *Bss*HII restriction sequence located in the terminator loop (Fig. 3). Plasmid pHA7 was digested with the *Bss*HII restriction enzyme. The single-stranded termini generated during *Bss*HII cleavage were removed with S1 nuclease. Blunt termini generated by S1 nuclease treatment were ligated to form in vitro deletion plasmid pGM459. Deletion plasmid pGM459 was isolated and restricted with the enzyme *Bss*HII along with the recombinant plasmids pHA7 and pGM25. Plasmids pHA7 and pGM25 were linearized with *Bss*HII; however, in vitro-constructed deletion plasmid pGM459 was not cleaved by this restriction enzyme, as predicted. Transformation of strain CA8445 with plasmid pGM459 gave rise to strain NCR459. A comparison of some of the properties of strains NCR431, NCR435, and NCR459 is presented in Table 5. It can be seen that strain NCR459 carrying the in vitro deletion is capable of fermenting arabinose in the absence of cAMP and exhibits cAMP sensitivity just as strain NCR435 which harbors the cloned chromosomal *csm* mutation (i.e., pGM25) does.

DISCUSSION

We earlier reported the isolation of spontaneous and chemically induced pseudorevertants of *cya* mutants of *E.*

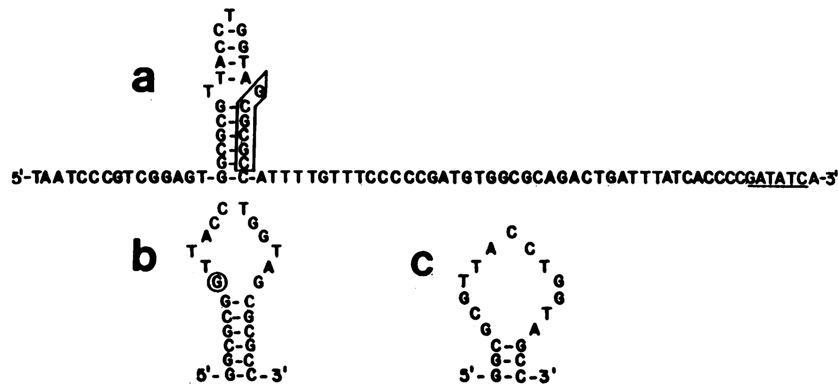

FIG. 3. Proposed structure of transcription termination loops in pHA7, pGM25, and pGM459. (b) The guanosine insertion in the mutant *csm* gene (G) is within the transcription termination loop. If a loop forms, its structure, which is quite different from that of the wild-type *crp* (a), will be as shown (b). The *Bss*HII recognition sequence is shown in the boxed region (a). The possible loop structure for the constructed deletion plasmid, pGM459, has little sequence that can generate a loop (c). It also may not form. The sequence on the 5′ end of the wild-type *crp* (a) commences at the termination codon (-TAA-). The adjacent 3′ region is shown up to the *Eco*RV site (underlined).

coli (35). One class of such mutants was designated cAMP suppression mutants (*csm*) and phenotypically resembled wild-type cells but retained the original *cya* mutation. The *csm* mutation mapped in or near the *crp* gene on the *E. coli* chromosome. The *csm* mutant strains grew on catabolite-repressible substrates in the absence of exogenous cAMP and were sensitive to catabolite repression mediated by α-methylglucoside (35).

To define the molecular basis for the *csm* mutation, we cloned the *csm* from strain NCR30 into the *Hin*dIII-*Eco*RI site of the plasmid vector pBR322. Recombinant plasmids pGM5 and pGM25 carry the cloned *csm*. Transformants carrying deletions in the chromosomal *cya* and *crp* genes that harbored plasmids pGM5 or pGM25 expressed enzymes encoded by catabolite-repressible operons (i.e., arabinose isomerase, threonine deaminase, tryptophanase, and β-galactosidase). Mutants defective for the synthesis of cAMP have been shown to be also defective for motility (20). However, transformants harboring pGM5 or pGM25 were motile. Like the original pseudorevertant strain NCR30, all transformants harboring the cloned *csm* were also sensitive to exogenous cAMP. Both recombinant strains NCR434 and NCR435 exhibited inhibition of growth in the presence of exogenous cAMP. We showed earlier (35) that this growth inhibition in strain NCR30 corresponded with a concurrent overproduction of intracellular methylglyoxal. The present investigation demonstrated the accumulation of toxic concentrations of methylglyoxal in both strains NCR434 and NCR435 when grown in the presence of exogenous cAMP (Table 4). Our results indicate the cloned *csm* manifests the same phenotypic characteristics as the chromosomal *csm*. It would appear that the *csm* mutation has the same effect on the cells whether it is in a multicopy state (i.e., on plasmid pBR322) or exists as a single chromosomal copy.

The cloned *csm* encoded a CRP which was identical in molecular weight and charge to the purified wild-type CRP. Harmon and Dobrogosz (29) reported that the CRP encoded by the chromosomal *csm* of strain NCR30 has a basic isoelectric point and a molecular size of 45,000 daltons.

Our results indicate that the suppressor phenotype of *csm* strains is due to a single base insertion in the 3′ flanking region of the *crp* structural gene. DNA sequencing data for the cloned *csm* indicated an insertion of a guanosine residue in the presumed termination loop sequence of the *crp* gene (Fig. 3). The fact that a mutation in this region could be responsible for the observed phenotype of *csm* mutants was substantiated by the generation of an in vitro deletion with the unique *Bss*HII restriction site. The *Bss*HII restriction site is located within the stem of the presumed termination loop of the *crp*. The *crp cya* deletion strains harboring plasmids containing the in vitro *Bss*HII-generated deletion exhibited a phenotype similar to those of the *csm* mutants (Table 5). Thus it appears that mutations within this 3′ flanking sequence of the *crp* structural gene are capable of rendering a CRP product that is able to promote the transcription of catabolite-repressible operons in the absence of exogenous cAMP. Recently we have performed in vitro, BAL 31-generated deletions in the 3′ flanking sequence of the *crp* structural gene which also elicit a cAMP suppressor phenotype like that observed with the chromosomal and *Bss*HII-generated *csm* mutations (J. W. Barton and T. Melton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, H2, p. 91).

Results reported in this article describe a novel function for the 3′ flanking sequences of the *crp* structural gene. Our results clearly implicate a role for these 3′ flanking sequences in the expression of CRP in cAMP-independent mutants. However, the molecular nature of other cAMP-independent mutations isolated in other laboratories appears to be different. Several laboratories have reported the isolation of *crp* mutations capable of extragenic suppression of *cra* mutations (4, 10, 17, 56, 61). Recently, sequence data for several of the cAMP-independent *crp* mutants have been

TABLE 5. Comparison of plasmids harboring wild-type and mutant *crp* genes

Strain and plasmid	Arabinose fermentation[a]	cAMP sensitivity[b]	Presence of *Bss*HII recognition sequence[c]
NCR431(pHA7)	−	−	+
NCR435(pGM25)	+	+	+
NCR459(pGM459)	+	+	−

[a] Strains harboring plsmids were cultured on EMB-arabinose-ampicillin plates at 37°C for 24 to 48 h.

[b] Strains harboring plasmids were examined for growth on EMB-arabinose-ampicillin plates containing 5 mM cAMP after culturing at 37°C for 24 to 48 h.

[c] Plasmids were isolated and restricted with *Bss*HII as described in Materials and Methods.

obtained. These results indicated that the cAMP-independent *crp* mutants result from mutations at different sites within the *crp* structural gene. Harman and co-workers have examined the DNA sequence of the *crp* from three cAMP-independent *crp* mutants (personal communication). The suppressor mutations of these mutants were attributed to a C-to-T transition, a G-to-A transition, a C-to-A transversion, and a T-to-G transversion occurring at positions 127, 144, 170, and 195 of the *crp* structural gene.

Garges and Adhya have also sequenced the *crp* genes from several cAMP-independent *crp* mutants (27). A total of six base substitutions were identified of which five were transitions from G-C to A-T and one was a transversion from A-T to C-G. These mutational changes occurred at positions 72, 141, 142, and 144 in the *crp* sequence of the various cAMP-independent mutants. Most of the above mutational changes occurred in the C, D, E, and F α-helices of the carboxy domain of the CRP. It would therefore seem that mutations rendering cAMP independence can be located within the *crp* structural gene or within sequences located in the 3′ flanking region of the *crp* gene.

Interestingly, a number of investigators have studied the molecular nature of the 3′ flanking region of a number of other genes. These genes have in common a consensus sequence found in a large dyad of symmetry. This dyad region has been identified in the 3′ flanking region of the *trpR* (8), *fol* (60), *glnS* (64), *glyA* (50), *malk*, *nisJ*, and *hisG* (30) genes. The sequences are palindromic and are never found within a coding sequence. The exact function of these repetitive extragenic palindromic sequences is not known presently. Some evidence suggests that these sequences might have some effect upon transcription. Plamann and Stauffer (49) have reported a *cis*-acting regulation mutation that maps between the end of the *glyA* structural gene and its proposed transcription termination site. The Mu *cts*-generated *glyA* mutant exhibits only 30% of the level of serine hydroxymethyltransferase activity. The same results were found when the 3′ nontranslated region was deleted. These authors postulate the lower level of enzyme activity might be due to the decreased stability of the upstream *glyA* mRNA. We presently are investigating the possibility of homology of the 3′ flank sequences of the *crp* with the consensus repetitive extragenic palindromic sequences and also the stability of CRP mRNA in *csm* mutant strains.

ACKNOWLEDGMENT

We thank J. G. Harman for his generous gift of the purified wild-type and mutant CRP.

LITERATURE CITED

1. **Adhya, S., and S. Garges.** 1982. How cyclic AMP and its receptor protein act in *Escherichia coli*. Cell **29**:287–289.
2. **Aiba, H.** 1983. Autoregulation of the *Escherichia coli crp* gene: CRP is a transcriptional repressor for its own gene. Cell **32**:141–149.
3. **Aiba, H., S. Fujimoto, and N. Ozaki.** 1982. Molecular cloning and nucleotide sequencing of the gene for *E. coli* cAMP receptor protein. Nucleic Acids Res. **10**:1345–1361.
4. **Alexander, J. K.** 1980. Suppression of defects in cyclic adenosine 3′,5′-monophosphate metabolism in *Escherichia coli*. J. Bacteriol. **144**:205–209.
5. **Beckwith, J., T. Grodzicker, and R. Arditti.** 1972. Evidence for two sites in the *lac* promoter region. J. Mol. Biol. **69**:155–160.
6. **Bethesda Research Laboratories.** 1980. M13 cloning/"dideoxy" sequencing manual. Bethesda Research Laboratories, Inc., Gaithersburg, Md.
7. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **1**:1513–1523.
8. **Bogosian, G., R. L. Sommerville, K. Nishi, Y. Kano, and F. Imamoto.** 1984. Transcription of the *trpR* gene of *Escherichia coli*: an autogeneously regulated system studied by direct measurements of mRNA levels in vivo. Mol. Gen. Genet. **193**:244–250.
9. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlack, H. L. Heynecker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
10. **Botsford, J. L., and M. Drexler.** 1978. The cyclic 3′,5′-adenosine monophosphate receptor protein and regulation of cyclic 3′,5′-adenosine monophosphate synthesis in *Escherichia coli*. Mol. Gen. Genet. **165**:47–56.
11. **Chapon, C.** 1982. Role of the catabolite activator protein in the maltose regulon of *Escherichia coli*. J. Bacteriol. **150**:722–729.
12. **Cossart, P., and B. Gicquel-Sanzey.** 1982. Cloning and sequence of the *crp* gene of *Escherichia coli* K12. Nucleic Acids Res. **10**:1363–1378.
13. **Dagert, M., and D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23–28.
14. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics, p. 159–180. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **deCrombrugghe, B., B. Chen, W. Anderson, R. Nissley, M. Gottesman, and I. Pastan.** 1971. *lac* DNA, RNA polymerase and cyclic AMP receptor protein, cyclic AMP, *lac* repressor and inducer are all essential elements for controlled lac transcription. Nature (London) New Biol. **231**:139–142.
16. **Deeley, M. C., and C. Yanofsky.** 1982. Transcription initiation at the tryptophanase promoter of *Escherichia coli* K-12. J. Bacteriol. **151**:942–951.
17. **Dessein, A., M. Schwartz, and A. Ullmann.** 1978. Catabolite repression in *Escherichia coli* mutants lacking cyclic AMP. Mol. Gen. Genet. **162**:83–87.
18. **Dickson, R., J. Abelson, R. Johnson, W. Reznikoff, and W. Barnes.** 1977. Nucleotide sequence changes produced by mutation in the *lac* promoter of *Escherichia coli*. J. Mol. Biol. **111**:65–76.
19. **Dobrogosz, W. J.** 1966. Altered end-product patterns and catabolite repression in *Escherichia coli*. J. Bacteriol. **91**:2263–2269.
20. **Dobrogosz, W. J., and P. B. Hamilton.** 1971. The role of cyclic AMP in chemotaxis in *Escherichia coli*. Biochem. Biophys. Res. Commun. **42**:202–207.
21. **Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. **112**:295–298.
22. **Ebright, R.** 1982. Sequence homologies in DNA in six sites known to bind the catabolite activator protein of *Escherichia coli*, p. 91–100. *In* J. Griffen and W. Duax (ed.), Molecular structure and biological activity. Elsevier/North-Holland Publishing Co., New York.
23. **Enea, V., G. F. Vovis, and N. D. Zinder.** 1975. Genetic studies with heteroduplex DNA of bacteriophage F1. Asymmetric segregation, base correction and implications for the mechanism of genetic recombination. J. Mol. Biol. **96**:495–509.
24. **Eron, L., and R. Block.** 1971. Mechanism of initiation and repression of *in vitro* transcription of the *lac* operon of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **68**:1828–1832.
25. **Ezzell, J. W., and W. J. Dobrogosz.** 1975. Altered hexose transport and salt sensitivity in cyclic adenosine 3′-5′-monophosphate-deficient *Escherichia coli*. J. Bacteriol. **124**:815–824.
26. **Friedmann, T. E., and G. E. Haugen.** 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. **147**:415–442.
27. **Garges, S., and S. Adhya.** 1985. Sites of allosteric shift in the structure of the cyclic AMP receptor protein. Cell **41**:745–751.
28. **Guidi-Rontani, C., A. Danchin, and A. Ullmann.** 1980. Catabolite repression in *Escherichia coli* mutants lacking cAMP

receptor protein. Proc. Natl. Acad. Sci. USA **77**:5799–5801.
29. **Harman, J. G., and W. J. Dobrogosz.** 1983. Mechanism of CRP-mediated *cya* suppression in *Escherichia coli*. J. Bacteriol. **153**:191–199.
30. **Higgins, C. F., G. F.-L. Ames, W. M. Barnes, J. M. Clement, and M. Hofnung.** 1982. A novel intercistronic regulatory element of prokaryotic operons. Nature (London) **298**:760–762.
31. **Kolb, A., S. Busby, M. Herbert, D. Kotlarz, and H. Buc.** 1983. Comparison of the binding site for the *Escherichia coli* cAMP receptor protein at the lactose and galactose promoters. EMBO J. **2**:217–222.
32. **Lee, N., G. Wilcox, W. Gielow, J. Arnold, P. Clery, and E. Englesburg.** 1974. *In vitro* activation of the transcription of *ara*BAD operon by *ara*C activator. Proc. Natl. Acad. Sci. USA **71**:634–638.
33. **Majerfeld, I. H., D. Miller, E. Spitz, and H. V. Rickenberg.** 1981. Regulation of the synthesis of adenylate cyclase in *Escherichia coli* by the cAMP-cAMP receptor protein complex. Mol. Gen. Genet. **181**:470–475.
34. **Majors, J.** 1975. Specific binding of CAP factor to *lac* promoter DNA. Nature (London) **256**:672–674.
35. **Melton, T., L. L. Snow, C. Freitag, and W. J. Dobrogosz.** 1981. Isolation and characterization of cAMP suppressor mutants of *Escherichia coli* K12. Mol. Gen. Genet. **182**:480–489.
36. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9**:309–321.
37. **Messing, J., and J. Viera.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene **19**:269–276.
38. **Meyer, R. G., J. Germershausen, and S. R. Susking.** 1970. Tryptophan synthetase. Methods Enzymol. **17A**:406–414.
39. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Mitra, S., G. Zubay, and A. Landy.** 1975. Evidence for the preferential binding of the catabolite gene activator protein (CAP) to DNA containing the *lac* promoter. Biochem. Biophys. Res. Commun. **67**:857–863.
41. **Morrissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
42. **Movva, R., R. Green, K. Nakamura, and M. Inouye.** 1981. Interaction of cAMP receptor protein with the *omp*A gene, a gene for a major outer membrane protein in *Escherichia coli*. FEBS Lett. **128**:186–190.
43. **Musso, R. E., R. DiLauro, S. Adhya, and B. deCrombrugghe.** 1977. Dual control for transcription of the galactose operon by cAMP and its receptor protein at two interspersed promoters. Cell **12**:847–854.
44. **Norgard, M. V.** 1981. Rapid and simple removal of contaminating RNA from plasmid DNA without the use of RNase. Anal. Biochem. **113**:34–42.
45. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
46. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acid proteins. Cell **12**:1133–1142.
47. **Ogden, S., D. Haggerty, C. M. Stoner, D. Kolodrubetz, and R. Schleif.** 1980. The *Escherichia coli* L-arabinose operon: binding sites of the regulatory proteins and a mechanism of positive and negative regulation. Proc. Natl. Acad. Sci. USA **77**:3346–3350.
48. **Phillips, A. T., R. M. Egan, and B. Lewis.** 1978. Control of biodegradative threonine dehyratase inducibility by cyclic AMP in energy-restricted *Escherichia coli*. J. Bacteriol. **135**:828–840.
49. **Plamann, M. D., and G. V. Stauffer.** 1985. Characterization of a *cis*-acting regulatory mutation that maps at the distal end of the *Escherichia coli gly*A gene. J. Bacteriol. **161**:650–654.
50. **Plamann, M. D., L. T. Stauffer, M. L. Urbanowski, and G. V. Stauffer.** 1983. Complete nucleotide sequence of the *E. coli glyA* gene. Nucleic Acids Res. **11**:2065–2075.
51. **Poncz, M., D. Solwiejczyk, M. Ballantine, E. Schwartz, and S. Surrey.** "Nonrandom" DNA sequence analysis in bacteriophage M13 by the dideoxy chain termination method. Proc. Natl. Acad. Sci. USA **79**:4298–4302.
52. **Prusiner, S., R. E. Miller, and R. C. Valentine.** 1972. Adenosine 3':5'-cyclic monophosphate control of the enzymes of glutamine metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **69**:2922–2926.
53. **Queen, C., and M. Rosenberg.** 1981. A promoter of pBR322 activated by cAMP receptor protein. Nucleic Acids Res. **9**:365–377.
54. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
55. **Sabourin, D., and J. Beckwith.** 1975. Deletion of the *Escherichia coli crp* gene. J. Bacteriol. **122**:338–340.
56. **Sanders, R., and D. McGoech.** 1973. A mutant transcription factor that is activated by 3':5'-cyclic guanosine monophosphate. Proc. Natl. Acad. Sci. USA **70**:1017–1021.
57. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
58. **Schleif, R. F., and P. C. Wensink.** 1981. Practical methods in molecular biology. Springer-Verlag, New York.
59. **Shizuta, Y., and M. Takushige.** 1971. Threonine deaminase (degradative) (*Escherichia coli*). Methods Enzymol. **17B**:575–586.
60. **Smith, D. R., and J. M. Calvo.** 1980. Nucleotide sequence of the *E. coli* gene coding for dihydrofolate reductase. Nucleic Acids Res. **8**:2255–2274.
61. **Takebe, Y., M. Shiguya, and Y. Kaziro.** 1978. A new extragenic suppressor of *cya* mutation. Mutant cyclic AMP receptor protein with an increased affinity for cyclic AMP. J. Biol. Chem. **83**:1615–1623.
62. **Ullmann, A., E. Joseph, and A. Danchin.** 1979. Cyclic AMP as a modulator of polarity in polycistronic transcriptional units. Proc. Natl. Acad. Sci. USA **76**:3194–3197.
63. **White, R. J.** 1968. Control of amino sugar metabolism in *Escherichia coli* and isolation of mutants unable to degrade amino sugars. Biochemistry **106**:847–858.
64. **Yamao, F., H. Inokuchi, A. Cheung, H. Oxeki, and D. Soll.** 1982. *Escherichia coli* glutaminyl-tRNA synthetase. I. Isolation and DNA sequence of the *glns* gene. J. Biol. Chem. **257**:11639–11643.