JOURNAL OF BACTERIOLOGY, July 1990, p. 3813–3820
0021-9193/90/073813-08$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 172, No. 7

Cloning and In Vivo and In Vitro Regulation of Cyclic AMP-Dependent Carbon Starvation Genes from *Escherichia coli*

PAUL H. BLUM,[1]* STEVAN B. JOVANOVICH,[2]† MICHAEL P. McCANN,[1] JOANNE E. SCHULTZ,[1] SCOTT A. LESLEY,[2] RICHARD R. BURGESS,[2] AND ABDUL MATIN[1]

Department of Microbiology and Immunology, Stanford University, Stanford, California 94305-5402,[1] and McArdle Laboratory, University of Wisconsin, Madison, Wisconsin 53706[2]

Received 7 December 1989/Accepted 17 April 1990

The regulation of three *Escherichia coli* carbon starvation (*cst*) genes fused to *lacZ* was examined. Expression of these genes is induced by starvation for a carbon source. The role of carbon and cyclic AMP (cAMP) availability and of an altered-function *crp* mutation were investigated for their effect on *cst* expression in vivo. The experiments indicated that cAMP concentrations controlled the absolute expression of one *cst* fusion, but the other two *cst* fusions were dependent upon some component not present in exponentially growing cells under conditions of glucose excess, even when cAMP was added. To examine the regulation of these genes in further detail, the three *cst*::*lacZ* fusions were cloned on multicopy plasmids. All three *cst*::*lacZ* fusions retained their inducible regulatory phenotype in the multicopy state. Analysis of the expression of the cloned *cst*::*lacZ* fusions in an in vitro-coupled transcription-translation cell-free system demonstrated that the predominant promoter(s) present on each cloned DNA was dependent on σ^{70} for expression. In vitro cAMP titration curves indicated that this molecule was necessary and sufficient for the expression of one fusion but not sufficient for the second fusion, while the third fusion exhibited constitutive levels of expression in vitro. The results are discussed in the context of the *E. coli* carbon starvation response.

Conditions in most natural environments require that bacteria be able to survive prolonged periods of starvation (26). It has been shown that cyclic AMP (cAMP) levels rise during carbon starvation (5, 10, 23), but only recently have studies focused on the molecular consequences of starvation on gene expression. Within the first 4 to 5 h of the onset of starvation for carbon substrates, *Escherichia coli* K-12 induces the synthesis of approximately 30 proteins, some of which confer a general-resistance phenotype, including resistance to starvation, oxidation, heat, and osmotic stress (14, 18, 19, 29–31). About two-thirds of the genes encoding these carbon starvation proteins (the *cst* genes) require the cAMP-cAMP recepter protein (cAMP-CRP) complex for induction during starvation (31), and it is likely that the increase in intracellular cAMP levels that occurs at the onset of carbon starvation (9, 24, 28) plays a role in this induction. It has remained unclear, however, whether variations in intracellular cAMP levels alone are sufficient to account for *cst* gene induction. A strict correlation did not exist between the expected cAMP levels and the level of induction of some *cst* genes during starvation for different carbon substrates. Furthermore, the Cst proteins encompassed all the temporal categories of starvation proteins (31), even though sequential fluctuations in cAMP levels do not occur in starving cells (9, 24, 28).

This investigation was undertaken to delineate further the role of cAMP in the regulation of selected *cst* genes. Both in vivo and in vitro approaches were used. The former consisted of investigating the pattern of β-galactosidase synthesis by selected *cst*::*lacZ* fusion strains in response to changes in carbon availability, cAMP availability, and an altered-function *crp* mutation (*crp***GQ141*). The carbon source used (glucose) was either depleted from the medium by cell catabolism or removed by centrifuging the culture. The effect of cAMP availability and the *crp***GQ141* mutation was also examined in strains deleted for *cya*. The CRP encoded by the *crp***GQ141* allele is relatively independent of cAMP in activating cAMP-CRP-dependent promoters (12; J. Kim, S. Garges, and S. Adhya, unpublished data), and the Δ*cya* mutation eliminates changes in cAMP concentration in response to starvation. The in vitro approach was a DNA-dependent cell-free coupled transcription-translation (S-30) system to characterize expression of the regulatory regions of cloned *cst*::*lacZ* fusions. This approach permitted exploration of the role of cAMP and the major RNA polymerase holoenzyme, the σ^{70} holoenzyme, in the transcription of *cst* genes.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The bacterial strains used are described in Table 1. Cells were grown at 37°C in the defined M9 medium (27), supplemented with glucose. A low glucose concentration (0.025% [wt/vol]) was used to establish carbon starvation conditions as described previously (14). For genetic manipulations, the defined medium was M9 salts supplemented with the specified carbon source at 0.4% (wt/vol). The rich media used were LB (27) and MacConkey base medium (Difco Laboratories). Ampicillin (50 μg/ml) was added to all media used for strains containing plasmids. The antibiotics used in strain construction were ampicillin (50 μg/ml), tetracycline (10 μg/ml), chloramphenicol (12 μg/ml), kanamycin (75 μg/ml), and fosfomycin (10 μg/ml). The sodium salt of cAMP was from Sigma Chemical Co.; cAMP solutions were prepared fresh for each experiment.

Genetic manipulations. All strains used in these experiments were constructed as isogenically as possible. Generalized transductions were done with phage P1 *vir* and were performed as described before (27). The *cst*::*lacZ* fusion strains were isolated by their ability to hydrolyze increased amounts of 5-bromo-4-chloro-3-indolyl-β-D-galactopyrano-

* Corresponding author.
† Present address: Abtox Inc., Fremont, CA 94566.

TABLE 1. Bacterial strains[a]

Strain	Genotype	Origin
Wild-type K-12 (F^- λ^-)		Stanford University
AMS2	*Δcya-854*	31
AMS6	*ΔlacU169*	31
AMS13	*cst-2*::λ*plac*Mu-9 (Kan^r); otherwise like MC4100	31
AMS28	*cst-2*::λ*plac*Mu-9 (Kan^r) *ΔlacU169*	31
AMS29	*Δcya-854*; otherwise like AMS28	31
AMS35	*cst-4*::Mu dX(*lac* Ap^r Tn*9*); otherwise like MC4100	14
AMS42	*cst-8*::λ*plac*Mu-9 (Kan^r) *ΔlacU169*	E. Auger and A. Matin
AMS66	*recA56 srl*::Tn*10* *ΔlacU169*	Transduction of AMS6 to Tet^r with NCM80 donor
AMS89	*crp*GQ141* Kan^r; otherwise like AMS2	
AMS93	*crp*GQ141*; otherwise like AMS29	
AMS94	*Δcya-854*; otherwise like AMS42	
AMS95	*crp*GQ141*; otherwise like AMS94	
AMS96	*cst-4*::Mu dX(*lac* Ap^r Tn*9*); otherwise like AMS6	
AMS97	*Δcya-854*; otherwise like AMS96	
AMS98	*crp*GQ141* Kan^r; otherwise like AMS97	
AMS100	pAMC3; otherwise like AMS66	
AMS101	pAMC6; otherwise like AMS66	
AMS106	pAMC5; otherwise like AMS66	
CSH26(pRZ5203)	*ara Δlac thi*	Munson, thesis
CSH44	*tonA Δlac* (λ cI857ts68*h*80) *thi* (λ cI857ts68*h*80d*lac*$^+$)	27
DH5α	F^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	
G839	*Δcya lac*$^+$ *gal*$^+$ HB101 r^- m^-	S. Garges
G976	G839 *crp*GQ141* Kan^r	S. Garges
G947	DH5α F′ *rec*$^+$ *srl*::Tn*10* *Δcrp*::*cat*	S. Garges
JV554	F^- *Δlac-74 galK phoA20 phoR trp*(Am) *strA relA nadA*::Tn*10*	
MC4100	F^- *araD139 rpsL150 ΔlacU169 relA1 ptsF25 flbB5301 deoC1*	
NCM80	*recA56 srl*::Tn*10*	
RK4349	*F*$^-$ *ilv metB proB entA Δlac strA his metE*::Tn*10*	R. Kadner

[a] All strains are *E. coli* K-12 derivatives. See Materials and Methods for strain constructions involving the *cst*, *crp*, and *cya* alleles. Unless otherwise indicated, all strains were constructed for this work or were from laboratory stocks. For *cst* gene identity of the plasmids, see Fig. 1.

side in glucose-depleted medium (14, 31). Fusion strains containing the *crp*GQ141* mutation and the *Δcya-854* mutation (Table 1) were constructed in one of two ways depending on the antibiotic resistance marker encoded by the *cst*::*lacZ* fusions. In strains containing λ*plac*Mu-9 insertions, which confer kanamycin resistance (7), the *crp*GQ141* mutation was introduced by generalized transduction before the *Δcya-854* mutation. An insertion-deletion of the *crp* gene containing a chloramphenicol resistance determinant was first introduced into the fusion strains by transduction with strain G947 as the donor, and then the *crp* insertion-deletion was replaced with the *crp*GQ141* mutation. The latter was done by selection for growth on cAMP-dependent carbon sources, ribose or maltose, after infection with phage grown on strain AMS89, which contains the *crp*GQ141* allele. AMS89 was constructed in a similar manner, but *crp*GQ141* was derived from strain G976. The *Δcya-854* mutation was introduced by transduction by selecting first for a *metE*::Tn*10* by using strain RK4349 as the donor and then introducing the genetically linked *Δcya-854* mutation with AMS2 as the donor, by selecting for methionine prototrophy and screening for cotransduction of the *Δcya-854* mutation. *cya* mutant cotransductants were identified as fosfomycin resistant on MacConkey-ribose plates as described before (1). This procedure resulted in construction of strains AMS93 (*cst-2*) and AMS95 (*cst-8*). The *cst-4*::*lacZ* fusion, which was constructed with the Mu dX(*lac* Ap^r Tn*9*) phage (3) in strain MC4100 (Table 1), was first transduced with phage P1 into a *Δlac* strain (AMS6), and then the *Δcya-854* mutation was introduced as described above, followed by the *crp*GQ141* mutation. In this case the *crp*GQ141* mutation was introduced by selecting for the linked Kan^r determinant in strain AMS89 (Table 1), and then the presence of the *crp*GQ141* mutation was confirmed by the ability of exogenous cyclic guanosine monophosphate (cGMP) to preferentially suppress the fermentation defect of a *Δcya-854* mutant in a *crp*GQ141* mutant background (12); the wild-type *crp* allele is not suppressed by cGMP. This procedure resulted in strain AMS98 (*cst-4*).

As stated above, the CRP protein encoded by the *crp*GQ141* allele is believed to be independent of cAMP in activating the cAMP-CRP-dependent promoters. To confirm this, the ability of the *crp*GQ141* mutation to restore induced β-galactosidase synthesis from *lacZ* in a *Δcya-854* mutant of *E. coli* was investigated. The strains were grown in M9 medium with 0.4% added glucose to mid-log phase, and then isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 1 mM and differential rates of β-galactosidase synthesis were determined. These rates were: wild-type *E. coli* K-12, 465 U; *Δcya-854* derivative (AMS2, Table 1), 32 U; and *crp*GQ141* derivative of AMS2 (AMS89, Table 1), 154 U. (Activity units are defined below.) Thus, the CRP protein encoded by the *crp*GQ141* allele activated the *lac* promoter up to ca. 30% of its full activity in the absence of cAMP. The results confirm that the *crp*GQ141* mutation does indeed make the expression of cAMP-CRP-activated genes partially independent of cAMP.

Cell cultivation and β-galactosidase assays. Cultivation of cells for glucose depletion experiments was performed by inoculating 50 ml of warm M9 medium containing 0.025% (wt/vol) glucose with 2.5 ml of cultures of the various fusion strains grown overnight in M9 medium with glucose 0.04%

TABLE 2. Regulatory responses of *cst*::*lacZ* fusions in vivo

Fusion	β-Galactosidase activity (U)					
	Glucose excess[a]	Glucose depletion[b]	Glucose removal[c]	Δ*cya*[d]	cAMP[e]	*crp***GQ141*[f]
cst-2::*lacZ*	27	158	101	3	332	7
cst-4::*lacZ*	4	40	5	4	5	5
cst-8::*lacZ*	6	10	10	6	6	10

[a] Values were determined from differential rate plots of β-galactosidase activity in exponentially growing cultures. (See text for definition of in vivo β-galactosidase activity units.)

[b] Values represent maximal levels of β-galactosidase activity produced in response to glucose depletion.

[c] Values represent maximal levels of β-galactosidase activity produced in response to glucose removal.

[d] Experiments were performed as for glucose depletion but were done with Δ*cya* mutant derivatives.

[e] Experiments were performed as for glucose excess but were done with cultures of Δ*cya* mutant derivatives, and cAMP was added at a final concentration of 30 mM.

[f] Experiments were performed as for glucose depletion but with *crp***GQ141* Δ*cya* mutant derivatives.

[g] Experiments involving *cst-8* were performed at least three times, and samples were assayed in duplicate. The standard error of the mean was no more than 10% of the values shown.

(wt/vol). Samples were removed at approximately three 1-h intervals before and at least four 1-h intervals after glucose depletion and immediately transferred into glass tubes prechilled on ice. The depletion of glucose was apparent when the culture optical density remained constant. Maximum induction of the *cst*::*lacZ* fusions was achieved within 2 h following the onset of starvation (Table 2).

For experiments involving the removal of glucose, cultures were inoculated as described for glucose depletion experiments, grown to mid-log phase in M9 medium containing 0.4% (wt/vol) glucose, and then harvested by low-speed centrifugation at room temperature. The cell pellet was washed once in M9 salts and then suspended in the original sample volume of fresh warm M9 salts medium lacking a carbon source. Samples were removed before and after centrifugation and assayed for β-galactosidase activity. The strains used for the glucose depletion and glucose removal experiments were AMS28 (*cst-2*), AMS42 (*cst-8*), and AMS96 (*cst-4*).

cAMP addition experiments were performed by growing cultures to mid-log phase in M9 medium containing 0.4% (wt/vol) glucose (as described for the glucose removal experiments) and diluting them 1:1 into fresh M9 medium containing 0.4% glucose with or without added cAMP. Samples were then removed every 10 min for up to 1 h and assayed for β-galactosidase activity. The *cya* mutant strains used for glucose deprivation experiments and cAMP addition experiments were AMS29 (*cst-2*), AMS94 (*cst-8*), and AMS97 (*cst-4*).

Assays for β-galactosidase activity in vivo and in vitro were performed as described previously (14, 20, 31). In vitro β-galactosidase specific activity units are expressed as 1,000 × [(A_{420} units in the no-DNA blank)/(assay time in min)]. In vivo β-galactosidase activity units are expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per A_{660} unit of cells as described before (14).

Cloning procedures. Cloning of *cst* regulatory regions from selected *cst*::*lacZ* fusions onto multicopy plasmids was performed essentially as described before (32, 36). The method relies on insertional activation of truncated plasmid marker genes by cloning the missing portion of the marker gene from genomic digests of the fusion strains. It assumes that a genomic restriction fragment that reconstitutes expression of the truncated plasmid marker gene would encompass promoter and promoter-proximal regulatory regions. The *cst-4*::*lacZ* fusion (strain AMS35, Table 1) is transcriptional and was cloned by using the cloning vector pBW2 (36). This vector has a truncated *bla* gene terminating at a *Pst*I restriction site. *Pst*I chromosomal DNA fragments from AMS35 were cloned into *Pst*I-cleaved pBW2. Ampicillin-resistant recombinant plasmids were selected, and all were found to have a starvation-inducible *lacZ* phenotype. One such plasmid, pAMC1, was selected for further analysis.

In initial attempts at cloning the *cst-4*::*lacZ* fusion, it became apparent that the pBW2 vector had appreciable constitutive promoter activity for β-galactosidase synthesis. Removal of cloned chromosomal sequences from pAMC1 by cleavage at a *Hin*dIII site within the Mu phage DNA adjacent to the fusion joint and the *Hin*dIII site in the pBW2 polylinker (36) yielded a plasmid which produced 200 U of β-galactosidase activity in exponentially growing cells in M9 glucose medium. A likely candidate for this promoter is the vector sequence which overlaps the pBR322 *tet* gene promoter but which directs transcription in the opposite orientation (anti-*tet* promoter) (8, 34). In subsequent fusion clones and vectors, including pAMC3, the anti-*tet* promoter sequences were removed.

The *cst-2*::*lacZ* and *cst-8*::*lacZ* fusions (strains AMS13 and AMS42, respectively, Table 1) are kanamycin-resistant translational fusions and do not have a *bla* gene, and therefore their cloning required a different vector. Vector pAMC4 (Fig. 1) was constructed from pAMC1 (see above) by removal of the *Eco*RI fragment, extending from the *Eco*RI site in *lacZ* (32) to the *Eco*RI site in the pBW2 polylinker (36). The anti-*tet* promoter sequences and part of the *tet* gene were then removed by cutting at the *Hin*dIII and *Sal*I sites of pBW2 (36), filling in the ends, and blunt-end ligating. pAMC4 contains a functional *bla* gene, the *lacYA* genes, and the C-terminus of *lacZ*. *Eco*RI chromosomal DNA fragments from AMS13 and AMS42 were ligated into *Eco*RI-cleaved pAMC4, and recombinant Lac$^+$ plasmids were recovered by selecting for *E. coli* DH5α transformants on M9 agar plates containing 0.4% (wt/vol) lactose and 2 μg of thiamine per ml. The use of *lacZ* reconstitution as a method for cloning chromosomal gene fusions has been described previously (32). Recombinant DNA procedures were essentially as described before (25).

Plasmid and phage DNA preparation. Plasmid DNA for use in the in vitro system and for cloning experiments was prepared from cultures grown in LB medium and treated with chloramphenicol to amplify plasmid copy number. The DNA was extracted by the alkaline lysis procedure (4), purified over two successive cesium chloride density gradients, and then dialyzed against two changes of 3 liters each of 10 mM Tris-acetate–0.1 mM EDTA (pH 7.8). The *lacZpUV5-lacZ* template was from strain CSH26(pRZ5203) (L. Munson, Ph.D. thesis, University of Wisconsin, Madison, 1983), and the phage λ DNA encoding the wild-type *lacZp-lacZ* template was from CSH44. Phage λ DNA for use in the in vitro system was prepared as described before (S. Jovanovich, Ph.D. thesis, University of California, Davis, 1983).

Preparation and use of the S-30 transcription-translation extract. The S-30 extract was prepared by a modification of the Zubay method (2, 20, 37; Jovanovich, thesis) with *E. coli* JV554. The essential modifications were: the preincubation step was performed at room temperature; the serine protease

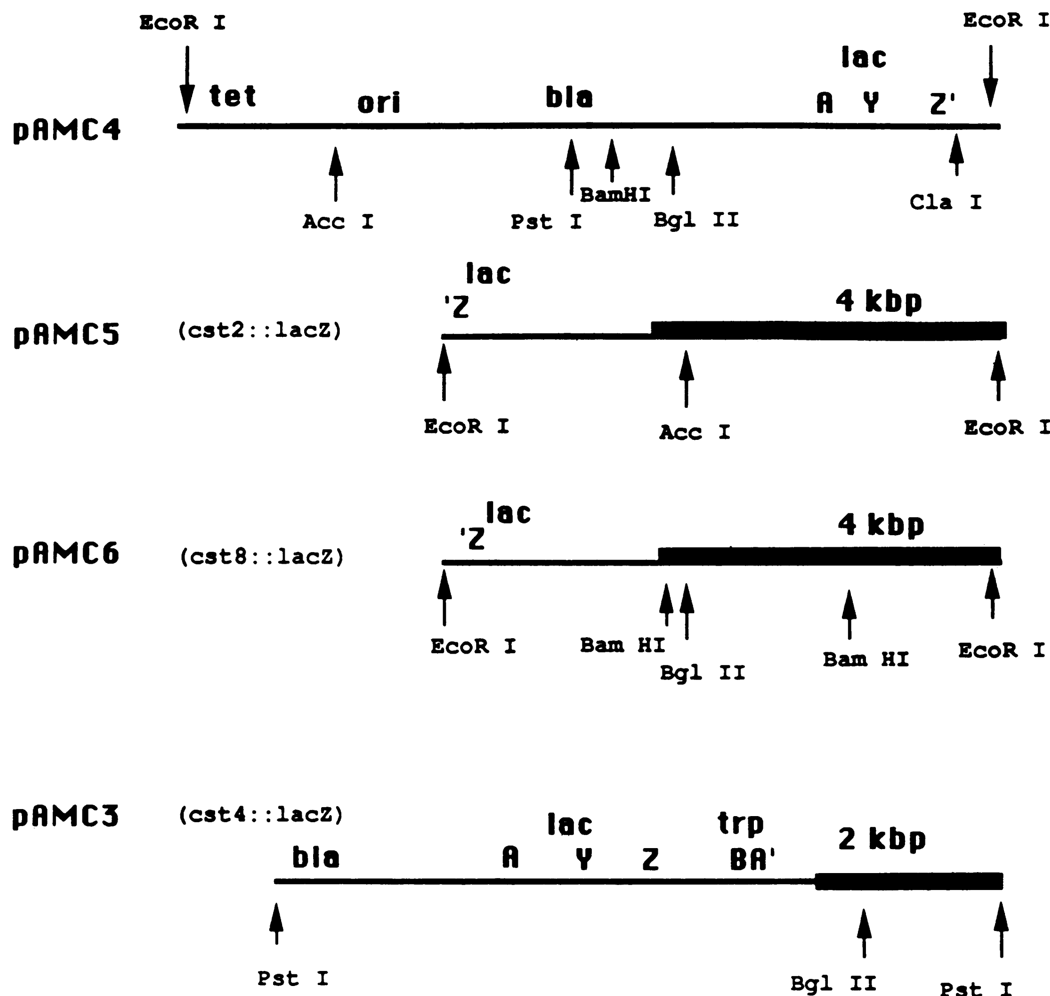

FIG. 1. Schematic representation of cloned *cst*::*lacZ* fusions. The plasmids consist of cloned *E. coli* K-12 chromosomal DNA fragments translationally fused to *lacZ* (pAMC5 and pAMC6) or transcriptionally fused to *lacZ* (pAMC3). The individual *cst*-containing fragments cloned into each plasmid are identified. The sizes of the cloned DNAs and locations of restriction sites are shown. pAMC4 is not drawn to scale. See Materials and Methods for details.

inhibitor *p*-toluene sulfonyl fluoride was included in the growth medium (50 μg/ml) and in all buffers (75 μg/ml); and four changes of dialysis buffer were made at 90-min intervals. The final protein concentration of the S-30 extract was 20.2 mg/ml, as determined by the method of Bradford (6).

The S-30 transcription-translation reactions were performed as described before (20). Briefly, the reaction mixes, in a final volume of 50 μl, contained 35 mM Tris-acetate, pH 8.0; 120 mM potassium glutamate; 27 mM ammonium acetate; 2 mM dithiothreitol; 12.2 mM magnesium acetate; 0.5 mM each of the 20 amino acids; 2 mM ATP; 0.5 mM each CTP, UTP, and GTP; 20 mM phosphoenolpyruvate; 1 μg of tRNA per ml; 35 μg of polyethylene glycol 8000 per ml; 20 μg of folinic acid per ml; and 319 μg of S-30 protein. The mixture was incubated on ice for 30 min. When specified, antibodies were added at the concentrations given in the text. The reaction was started by the addition of 5 μg of the appropriate DNA (10 μl) and incubation at 37°C for 70 min with rapid shaking. When anti-σ^{70} monoclonal antibodies or purified σ^{70} was added, 1 mM cAMP was included in the reaction mix.

Monoclonal antibodies were prepared as described before (22, 33) by the method of Fazekas de St. Goth and Scheidegger (11) and purified by ammonium sulfate precipitation as described before (20). σ^{70} was prepared from an overproducing strain as described elsewhere (13).

RESULTS

Effect of glucose availability, cAMP availability, and the *crp*GQ141* mutation on *lacZ* expression in chromosomal *cst*::*lacZ* fusion strains. Experiments involving glucose availability, cAMP availability, and the *crp*GQ141* mutation were performed with three *cst*::*lacZ* fusion strains to ascertain their regulatory characteristics. The results of these experiments are summarized in Table 2. Glucose depletion induced expression of all three *cst*::*lacZ* fusions (Table 2) approximately 6-fold, 10-fold, and 1.6-fold for the *cst-2*::*lacZ*, *cst-4*::*lacZ*, and *cst-8*::*lacZ* fusions, respectively. In contrast, when glucose was removed from the medium by centrifuging cells and transferring them to fresh medium lacking glucose, only the *cst-2*::*lacZ* and *cst-8*::*lacZ* fusions induced expression, by approximately 4-fold and 1.6-fold, respectively (Table 2). When the *cya* gene was deleted, rendering cells cAMP deficient, glucose deprivation no longer induced expression of any of the *cst*::*lacZ* fusions

(Table 2). In addition, the basal level of expression of the *cst-2*::*lacZ* fusion was reduced nearly 10-fold. Addition of cAMP to a final concentration of 30 mM to exponentially growing cultures of the three fusion strains greatly induced expression of the *cst-2*::*lacZ* fusion but had no effect on expression of the *cst-4*::*lacZ* or *cst-8*::*lacZ* fusions (Table 2).

Finally, the *crp*GQ141* mutation, which renders the *crp* gene product CRP partially independent of cAMP for stimulation of cAMP-CRP-dependent promoters due to a glycine (G) to glutamine (Q) change at position 141 (12; S. Garges, personal communication), was examined for its ability to suppress the cAMP requirement for induction of the *cst*::*lacZ* fusions (Table 2). These experiments were performed with *crp*GQ141* and *Δcya-854* derivatives of the fusion strains. The *crp*GQ141* mutation suppressed the effect of cAMP deficiency on the small but reproducible induction of the *cst-8*::*lacZ* fusion in response to glucose deprivation. No suppressive effect of the *crp*GQ141* mutation was seen on the cAMP requirement for induction of the *cst-2*::*lacZ* or *cst-4*::*lacZ* fusion. These experiments indicate that the three *cst*::*lacZ* fusions can undergo differential regulation in vivo. The *cst-2*::*lacZ* fusion appeared to be especially sensitive to cAMP, while both the *cst-4*::*lacZ* and *cst-8*::*lacZ* fusions may require some other factor or condition (besides cAMP) which is not present during exponential growth under conditions of glucose excess.

Cloning of selected *cst* regulatory regions. The procedure described in the Materials and Methods section enabled us to clone the regulatory regions of the three *cst* genes onto multicopy plasmids. The sizes of the cloned fragments of the three *cst* fusions (*cst-2*, pAMC5; *cst-4*, pAMC3; and *cst-8*, pAMC6]) are shown in Fig. 1, along with known information on the restriction sites within these fragments. For reference, the *lacZ* reconstitution vector, pAMC4 (see Materials and Methods), is also shown. It should be noted that the *cst-4*::*lacZ* fusion cloned in pAMC3 is a *lacZ* transcriptional fusion and has, in addition to the vector sequences shown, the N-terminus of *trpA* and probably all of *trpB*. The plasmids pAMC5 and pAMC6 contain *cst*::*lacZ* gene fusions and lack all *trp* sequences.

In vivo expression of the cloned promoters. All of the plasmid-bearing clones exhibited induction of β-galactosidase synthesis upon starvation for glucose (Fig. 2); furthermore, the induction ratio shown by the plasmid-bearing strains was very similar to that exhibited by the corresponding chromosomal fusion: 7-fold, 10-fold, and 1.5-fold in the *cst-2*::*lacZ*, *cst-4*::*lacZ*, and *cst-8*::*lacZ* fusions, respectively (Fig. 2 and Table 2). Thus, the *cst* promoters on multicopy plasmids remained as responsive as their chromosomal single-copy counterparts to the regulatory factor(s) that triggers their enhanced expression during glucose depletion.

In vitro analysis of the regulation of the *cst*::*lacZ* fusions. Several minor σ factors have been implicated in the control of different regulons in *E. coli*, e.g., for nitrogen starvation (σ^{54} [16, 17, 21]) and heat shock (σ^{32} [15]). The factor used during the cAMP-dependent stimulation of expression of the *cst* genes at the onset of carbon deprivation could be a minor σ factor. We therefore examined the expression of cloned *cst* regulatory regions in an in vitro coupled transcription-translation (S-30) system (37). Use of this system also allowed us to examine the dependence of *cst* expression on added cAMP.

Addition of the anti-σ^{70} monoclonal antibody 3D3 (20, 33) to the in vitro system at a final concentration of 0.47 mg/ml eliminated expression of β-galactosidase from plasmids encoding the three *cst* regulatory regions (pAMC5, *cst-2*::*lacZ*; pAMC3, *cst-4*::*lacZ*; pAMC6, *cst-8*::*lacZ*) and the control *lacZpUV5* promoter (Fig. 3). Previous studies have shown the inhibition of *lacZpUV5* by 3D3 to be specific and to be reversed by the addition of purified σ^{70} (20). The addition of a lower concentration of 3D3 (0.047 mg/ml) resulted in partial inhibition of expression of the promoters present in plasmids pAMC3, pAMC5, and pAMC6 and the *lacZpUV5* promoter.

To provide further confirmation that the inhibition by 3D3 was due to specific inhibition of σ^{70}-containing RNA polymerase, we added purified σ^{70} to the S-30 extract (Fig. 4). Since our S-30 extract is not saturated with functional σ^{70}, supplementation with σ^{70} will lead to an increase in expres-

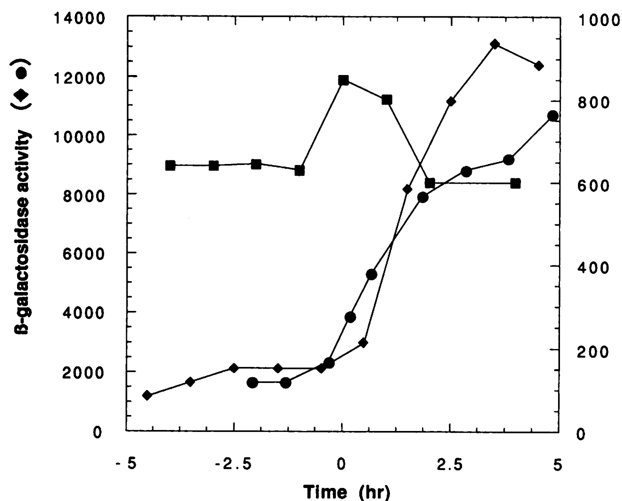

FIG. 2. Expression of cloned *cst*::*lacZ* fusions in vivo on multicopy plasmid vectors when subjected to carbon depletion. Symbols: ◆, *cst-4*::*lacZ* [AMS100(pAMC3)]; ●, *cst-2*::*lacZ* [AMS106 (pAMC5)]; ■, *cst-8*::*lacZ* [AMS101(pAMC6)]. Values shown for *cst-4* and *cst-2* correspond to the left vertical axis, and those for *cst-8* correspond to the right vertical axis.

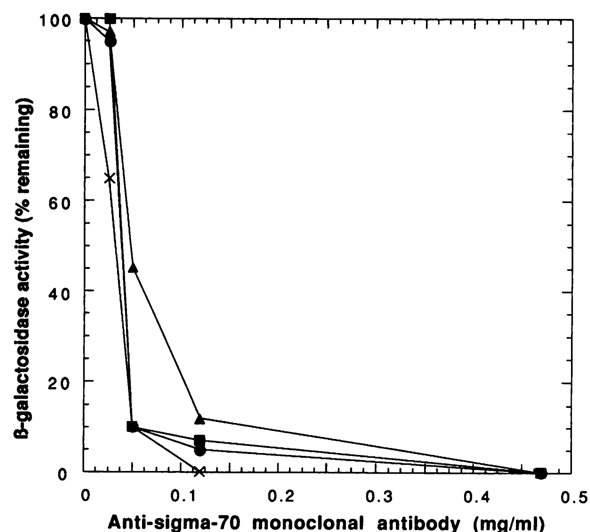

FIG. 3. Anti-σ^{70} monoclonal antibody (3D3) inhibition of *cst* expression in vitro. 3D3 was added to the in vitro reaction mixes at the concentrations indicated, and the relative expression of the *cst* and *lacZpUV5* templates was determined. Symbols: ●, *cst-2*::*lacZ* (pAMC5); ×, *cst-4*::*lacZ* (pAMC3); ▲, *cst-8*::*lacZ* (pAMC6); ■, *lacZpUV5*.

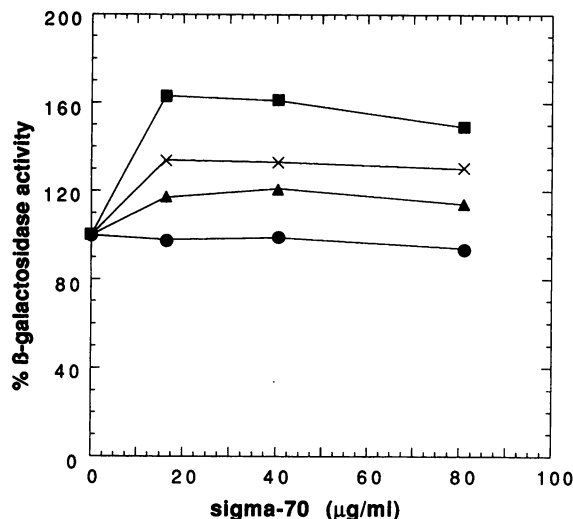

FIG. 4. Effect of σ^{70} addition on *cst* expression in vitro. Purified σ^{70} was added at the concentrations indicated to the S-30 extracts containing the various DNA templates. The symbols are the same as in Fig. 3. The values represent the amount of β-galactosidase produced in the presence of added σ^{70} versus the amount produced in the absence of added σ^{70}.

sion of σ^{70}-dependent promoters and an inhibition of noncognate promoters such as those which are recognized by σ^{54} or σ^{32}, as well as presumably any other non-σ^{70}-dependent promoters. The addition of σ^{70} stimulated the *lacZpUV5* promoter and the promoters in plasmids pAMC3 and pAMC6, whereas no stimulation was seen with the promoter in plasmid pAMC5. Significantly, no inhibition was seen when purified σ^{70} was added. These data, the inhibition by anti-σ^{70} monoclonal antibody and the stimulation by purified σ^{70}, suggest that in this S-30 extract, the strongest promoter(s) fused to *lacZ* is recognized by σ^{70}-containing RNA polymerase. The existence of other minor promoters present on the cloned DNAs may go undetected in these experiments.

We also investigated the effect of cAMP addition on the expression of the cloned *cst* regulatory regions and the control *lacZpUV5* (cAMP independent) and *lacZp* (cAMP dependent) promoters; both *lacZpUV5* and *lacZp* require σ^{70} RNA polymerase holoenzyme for transcription. As expected, strong *lacZpUV5* expression occurred without the addition of exogenous cAMP to the reaction mixture, but expression of the *lacZp* promoter was completely dependent on cAMP addition, with maximum expression occurring at about 0.5 mM cAMP (Fig. 5A). Expression of β-galactosidase from the cloned *cst* regulatory regions in pAMC5 (*cst-2*::*lacZ*) was very similar to that of *lacZp*, i.e., a total dependence on exogenous cAMP addition for expression, with maximal expression occurring at about 0.5 mM cAMP (Fig. 5B). The response of *cst-2* to added cAMP in vitro also paralleled the response seen in vivo (Table 2). In contrast, the *cst-4* regulatory region in pAMC3 exhibited only a low level of expression even at relatively high concentrations of added cAMP. Again, the in vitro (Fig. 5B) and in vivo (Table 2) responses of *cst-4* to added cAMP were similar. The *cst-8* regulatory region in pAMC6 resembled the *lacZpUV5* promoter in being highly expressed without the addition of exogenous cAMP (Fig. 5B).

DISCUSSION

Induction of expression of the *cst-4*::*lacZ* fusion in vivo depended on the manner in which glucose was removed from the growth medium of cultures of the *cst*::*lacZ* fusion strains, suggesting that some component present in the cells or the medium of cultures which have been allowed to catabolize glucose to exhaustion is not present in cultures of cells growing under conditions of glucose excess. In contrast, induction of expression of the *cst-2*::*lacZ* and *cst-8*::*lacZ* fusions in vivo was independent of the manner in which carbon became unavailable. Expression of the *cst-2*::*lacZ* fusion under all conditions depended only on the concentration of cAMP. This dependence on cAMP by the *cst-2*::*lacZ* fusion also extended to the basal levels of expression seen during growth under conditions of glucose excess. Use of the Δ*cya* mutation, which prevents endogenous cAMP synthesis, severely reduced expression of the *cst-2*::*lacZ* fusion but did not further reduce the low basal levels of expression of the *cst-4*::*lacZ* and *cst-8*::*lacZ* fusions. These results suggest that the maintenance of basal levels of expression of some *cst* genes reflects parameters other than the cAMP concentration. Since cAMP addition strongly stimulated expression of the *cst-2*::*lacZ* fusion but not the *cst-4*::*lacZ* and *cst-8*::*lacZ* fusions during growth under conditions of glucose excess, the *cst-2*::*lacZ* fusion appears not to require components present in glucose-starved cells or medium.

The glucose depletion experiment conditions should result in the accumulation of mixed acid fermentation products in the medium. Therefore, all three fusion-bearing strains were examined for the ability to metabolize these excretion products as an indication of whether the fusion vector insertion sequences might lie in genes involved in the catabolism of these substances. All three fusion-bearing strains were proficient in the metabolism of acetate and could oxidize lactate and formate, indicating that the insertion mutations were not in genes involved in these processes (E. Auger, M. McCann, J. Schultz, and A. Matin, unpublished). The identity and function of the *cst* genes discussed in this work are unknown, but they appear to be previously undescribed genes, as determined by genetic map analysis (J. Schultz and A. Matin, unpublished; M. McCann and A. Matin, unpublished).

The *crp*GQ141* mutation made the expression of *lacZ* about 30% independent of cAMP in vivo. This degree of independence appeared to cause the induction of the *cst-8*::*lacZ* fusion at the onset of carbon starvation to become completely independent of cAMP in vivo, although the weak induction of this promoter precludes firm conclusions. It is, however, clear that the mutation had little or no effect on the cAMP dependence of the induction of the *cst-2*::*lacZ* and *cst-4*::*lacZ* fusions upon starvation. One explanation for the lack of suppression of the *crp*GQ141* mutation on expression of the *cst-2*::*lacZ* fusion could be that it possesses a degenerate CRP-binding site (35) to which the mutant CRP protein cannot bind effectively. *crp*GQ141*-mediated suppression of the cAMP requirement for induction of expression of the *cst-8*::*lacZ* fusion suggests the existence of a distinct regulatory mechanism which is activated at the onset of the stationary phase and requires cAMP as well as an additional signal which is specifically made (or activated) at the approach of the stationary phase. A carbon starvation regulatory mechanism in addition to the cAMP-CRP regulatory complex must also be proposed to explain the pattern of expression of the *cst-4*::*lacZ* fusion, since

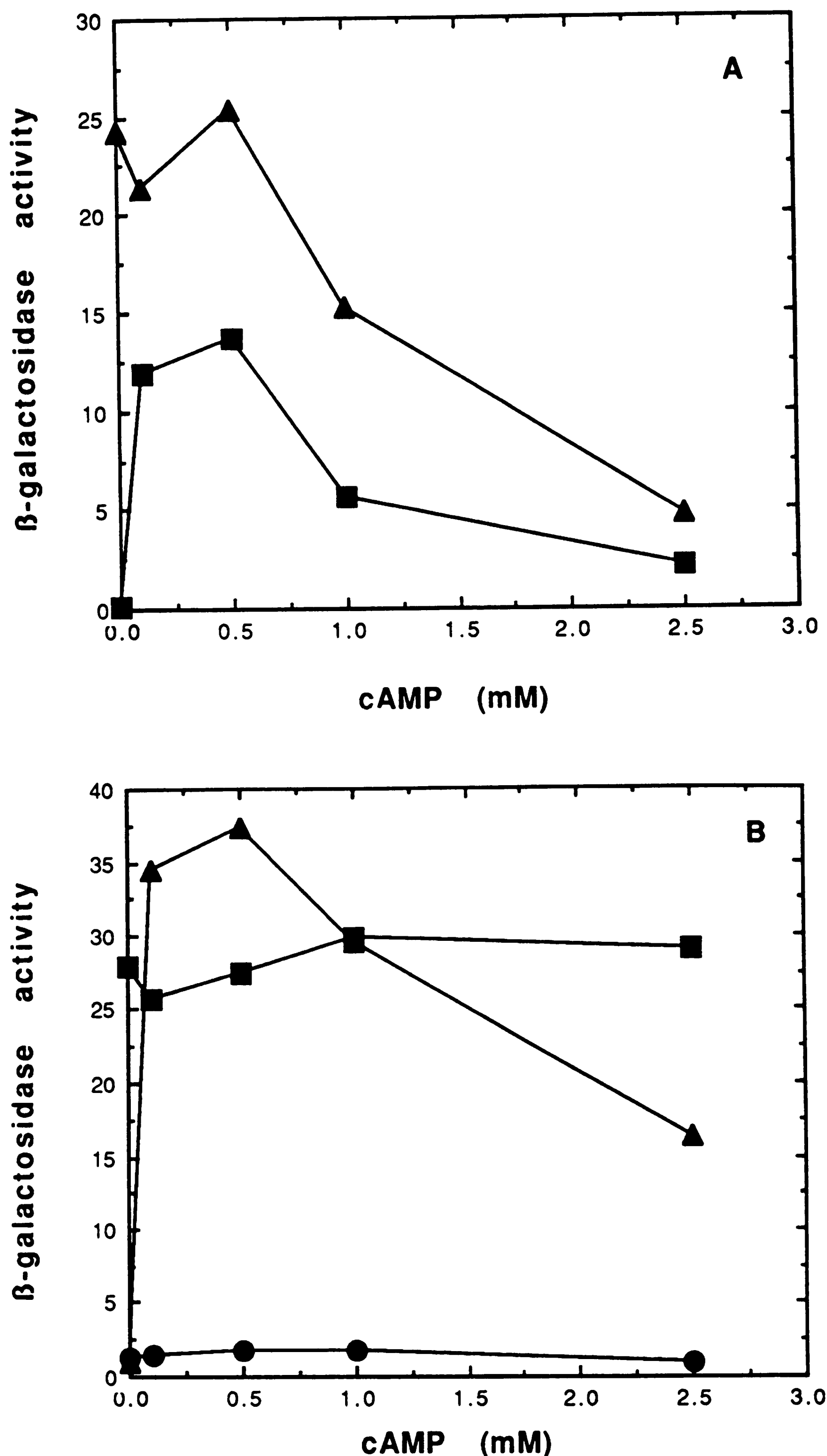

FIG. 5. Effect of exogenous cAMP addition on expression of cloned *cst*::*lacZ* promoters in vitro. See Materials and Methods and text for details. (A) Control promoters *lacZp* (■) and *lacZpUV5* (▲). (B) *cst-2*::*lacZ* (pAMC5, ▲); *cst-4*::*lacZ* (pAMC3, ●); *cst-8*::*lacZ* (pAMC6, ■).

cAMP addition in vivo and in vitro failed to stimulate expression.

That the different *cst*::*lacZ* fusions exhibit differences in their mechanism of regulation in vivo is consistent with the results obtained with the in vitro transcription-translation system. Thus, while σ^{70} RNA polymerase holoenzyme was necessary for expression of all three of the *cst*::*lacZ* fusions, the *cst-2*::*lacZ* fusion responded strongly to added cAMP, whereas the *cst-4*::*lacZ* fusion and *cst-8*::*lacZ* fusion did not respond to added cAMP.

Since the S-30 extracts used were prepared from exponentially growing cells, we postulate that the relatively low level of expression of the *cst-4*::*lacZ* fusion and its failure to respond to added cAMP are due to a requirement for factors,

in addition to cAMP, that are present only in the postexponential phase. Investigations are in progress to sequence the *cst* regulatory regions and determine the effect of nested deletions of upstream promoter-proximal *cst* regions on carbon starvation induction of gene expression, and the results may shed further light on the regulation of these genes.

ACKNOWLEDGMENTS

We thank Susan Garges for the generous gift of strains. We also thank E. Auger and S. Garges for critical reading of the manuscript.

This work was supported by National Science Foundation grant ECE86-13227 and by Public Health Service grant GM42159 from the National Institutes of Health (to A.M.).

LITERATURE CITED

1. **Alper, M. D., and B. N. Ames.** 1978. Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. **133:**149–157.
2. **Artz, S. W., and J. R. Broach.** 1975. Histidine regulation in *Salmonella typhimurium*: an activator-attenuator model of gene regulation. Proc. Natl. Acad. Sci. USA **72:**3453–3457.
3. **Baker, T. A., M. M. Howe, and C. A. Gross.** 1983. Mu dX, a derivative of Mu dl (*lac* Ap^r) which makes stable *lacZ* fusions at high temperature. J. Bacteriol. **156:**970–974.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Botsford, J. L.** 1981. Cyclic nucleotides in procaryotes. Microbiol. Rev. **45:**620–642.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Bremer, E., T. J. Silhavy, and G. M. Weinstock.** 1985. Transposable lambda p*lac* Mu bacteriophages for creating *lacZ* operon fusions and kanamycin resistance insertion in *Escherichia coli*. J. Bacteriol. **162:**1092–1099.
8. **Brosius, J., R. L. Cate, and A. P. Perlmutter.** 1982. Precise location of two promoters for the beta-lactamase gene of pBR322. J. Biol. Chem. **257:**9205–9210.
9. **Buettner, M. J., E. Spitz, and H. V. Rickenberg.** 1973. Cyclic adenosine 3',5'-monophosphate in *Escherichia coli*. J. Bacteriol. **114:**1068–1073.
10. **Busby, S., and H. Buc.** 1987. Positive regulation of gene expression by cyclic AMP and its receptor protein in *Escherichia coli*. Microbiol. Sci. **4:**371–375.
11. **Fazekas de St. Goth, S., and D. Sheidegger.** 1980. Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods **35:**1–21.
12. **Garges, S., and S. Adhya.** 1985. Sites of allosteric shift in the structure of the cyclic AMP receptor protein. Cell **41:**745–751.
13. **Gribskov, M., and R. R. Burgess.** 1983. Overexpression and purification of the sigma subunit of *Escherichia coli* RNA polymerase. Gene **26:**109–118.
14. **Groat, G. R., J. E. Schultz, E. Zychlinsky, A. Bockman, and A. Matin.** 1986. Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. J. Bacteriol. **168:**486–493.
15. **Grossman, A. D., J. W. Erickson, and C. A. Gross.** 1984. The *htpR* gene product of *E. coli* is a sigma factor for heat-shock promoters. Cell **38:**383–390.
16. **Hirschman, J., P. K. Wong, K. Sei, J. Keener, and S. Kustu.** 1985. Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription in vitro: evidence that the *ntrA* product is a sigma factor. Proc. Natl. Acad. Sci. USA **82:**7525–7529.
17. **Hunt, T. P., and B. Magasanik.** 1985. Transcription of *glnA* by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, *glnG*, and *glnL*. Proc. Natl. Acad. Sci. USA **82:**8453–8457.
18. **Jenkins, D. E., S. A. Chaisson, and A. Matin.** 1990. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J. Bacteriol. **172:**2779–2781.
19. **Jenkins, D. E., J. E. Schultz, and A. Matin.** 1988. Starvation-induced cross-protection against heat or H_2O_2 challenge in *Escherichia coli*. J. Bacteriol. **170:**3910–3914.
20. **Jovanovich, S., S. Lesley, and R. R. Burgess.** 1989. In vitro use of monoclonal antibodies in *Escherichia coli* S-30 extracts to determine the RNA polymerase sigma subunit required by a promoter. J. Biol. Chem. **264:**3794–3798.
21. **Keener, J., P. Wong, D. Popham, J. Wallis, and S. Kustu.** 1986. A sigma factor and auxilary proteins required for nitrogen regulated transcription in enteric bacteria, p. 159–175. *In* W. S. Reznikoff, R. R. Burgess, J. E. Dahlberg, C. A. Gross, M. T. Record, and M. P. Wickens (ed.), RNA polymerase and the regulation of transcription. Elsevier Science Publishing Co., Inc., New York.
22. **Lesley, S. A., N. E. Thompson, and R. R. Burgess.** 1987. Studies of the role of the *Escherichia coli* heat shock regulatory protein sigma-32 by the use of monoclonal antibodies. J. Biol. Chem. **262:**5404–5407.
23. **Magasanik, B., and F. C. Neidhardt.** 1987. Regulation of carbon and nitrogen utilization, p. 1318–1324. *In* J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, H. E. Umbarger, and F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
24. **Makman, R. S., and E. W. Sutherland.** 1965. Adenosine 3',5'-phosphate in *Escherichia coli*. J. Biol. Chem. **240:**1309–1314.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in "nondifferentiating" bacteria. Annu. Rev. Microbiol. **43:**293–316.
27. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Peterkofsky, A., and C. Gazdar.** 1971. Glucose and the metabolism of adenosine 3':5'-cyclic monophosphate in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **68:**2794–2798.
29. **Reeve, C. A., P. S. Amy, and A. Matin.** 1984. Role of protein degradation in the survival of carbon-starved *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **157:**758–763.
30. **Reeve, C. A., P. S. Amy, and A. Matin.** 1984. Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. J. Bacteriol. **160:**1041–1046.
31. **Schultz, J. E., G. I. Latter, and A. Matin.** 1988. Differential regulation by cyclic AMP of starvation protein synthesis in *Escherichia coli*. J. Bacteriol. **170:**3903–3909.
32. **Silhavy, T. J., M. C. Berman, and L. W. Endquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Strickland, M., N. E. Thompson, and R. R. Burgess.** 1988. Structure and function of the sigma-70 subunit of *Escherichia coli* RNA polymerase. Monoclonal antibodies: localization of epitopes by peptide mapping and effects on transcription. Biochemistry **27:**5755–5762.
34. **Stuber, D., and H. Bujard.** 1981. Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. USA **78:**167–171.
35. **Studnicka, G. M.** 1987. Nucleotide sequence homologies in control regions of prokaryotic genomes. Gene **58:**45–57.
36. **Wanner, B. L.** 1987. Molecular cloning of Mu d(*bla lacZ*) transcriptional and translational fusions. J. Bacteriol. **169:**2026–2030.
37. **Zubay, G.** 1973. In vitro synthesis of protein in microbial systems. Annu. Rev. Genet. **7:**267–287.