Mutations That Affect Transcription and Cyclic AMP-CRP Regulation of the Adenylate Cyclase Gene (cya) of Salmonella typhimurium

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Manuscript received September 28, 1989
Accepted for publication May 2, 1990

ABSTRACT

We studied the expression of the cya promoter(s) in cya-lac fusion strains of Salmonella typhimurium and demonstrated cAMP receptor protein (CRP)-dependent repression by cAMP. Expression of cya was reduced about fourfold in cultures grown in acetate minimal medium as compared to cultures grown in glucose-6-phosphate minimal medium. Expression of cya was also reduced about fourfold by addition of 5 mM cAMP to cultures grown in glucose minimal medium. We constructed in vitro deletion and insertion mutations altering a major cya promoter (P2) and a putative CRP binding site overlapping P2. These mutations were recombined into the chromosome by allele replacement with M13mp::cya recombinant phages and the regulation of the mutant promoters was analyzed. A 4-bp deletion of the CRP binding site and a 4-bp insertion in this site nearly eliminated repression by cAMP. A mutant with the P2 promoter and the CRP binding site both deleted exhibited an 80% reduction in cya expression; the 20% residual expression was insensitive to cAMP repression. This mutant retained a Cya+ phenotype. Taken together, the results establish that the cya gene is transcribed from multiple promoters one of which, P2, is negatively regulated by the cAMP-CRP complex. Correction for the contribution to transcription by the cAMP-CRP nonregulated cya promoters indicates that the P2 promoter is repressed at least eightfold by cAMP-CRP.

The observation that crp mutants overproduce adenosine 3',5'-cyclic monophosphate (cAMP) in both Salmonella typhimurium (Raphaeli and Saier 1976) and Escherichia coli (Potter, Chalmers-Larson and Yamazaki 1974; Wayne and Rosen 1974) suggested that the synthesis of cAMP is inhibited by cAMP-CRP receptor protein (CRP). Either cya expression or adenylate cyclase activity might be negatively regulated by the cAMP-CRP complex. Although the positive control function of CAMP-CRP in regulating gene expression has been studied in greatest detail, it is important to note that the synthesis of an approximately equal number of proteins is repressed as is stimulated by cAMP (Mallick and Herrlich 1979).

Studies of the regulation of cya expression in enteric bacteria have led to confusing results and contradictory conclusions. Majerfeld et al. (1981) and Botsford and Drexler (1978) reported approximately 4-fold and 20-fold CRP-dependent inhibition of cAMP synthesis in Escherichia coli, respectively, but it was not determined whether the synthesis of adenylate cyclase was directly affected. Bankaitis and Basset (1982) isolated chromosomal cya-lac fusions in E. coli and observed a twofold or less repression of cya expression by addition of 5 mM cAMP to the growth medium, which they concluded was not physiologically significant. Kawamukai et al. (1985) reported a similar extent of repression of cya expression by cAMP in E. coli using cya-lac fusions carried on multicopy plasmids, and concluded the repression was physiologically significant. The findings of Kawamukai et al. (1985) disagree with those of Roy, Haziza and Danchin (1983) who also studied the regulation of E. coli cya-lac fusions carried on multicopy plasmids and reported that cAMP-CRP did not repress cya expression. In fact, they observed CRP-dependent stimulation of cya expression by cAMP. Aiba (1985) and Mori and Aiba (1985) observed binding of cAMP-CRP to the E. coli cya promoter region in vitro and demonstrated CRP-dependent inhibition of cya mRNA synthesis by cAMP in vivo. In work from this laboratory, strong CRP-dependent repression (about ninefold) was observed for S. typhimurium chromosomal cya-lac fusions when 25 mM cAMP was added to the growth medium (Jovanovich 1985).

The nucleotide sequence of the regulatory region of cya of S. typhimurium was reported in a companion study (Thorner, Fandl and Artz 1990) and shows regions of strong homology to the cya sequence reported for E. coli (Aiba et al. 1984) as well as regions of weak homology. The sequences are most homologous in regions proposed to be important for cya expression in E. coli (Aiba 1985). The regulatory
region appears to contain a major promoter (P2), two minor promoters (P1, P1') located approximately 200 bp upstream of P2, and a putative CRP binding site that straddles the −10 hexamer of P2.

In this paper, we report experiments with chromosomal cya-lac fusions that support unequivocally a model of negative regulation of cya by CAMP-CRP in S. typhimurium. We present genetic evidence for this model based on analysis of site-directed mutations in the putative CRP binding site. We also report genetic evidence confirming the existence and physiological importance of multiple cya promoters in S. typhimurium.

MATERIALS AND METHODS

Bacterial strains, bacteriophages and media: S. typhimurium strains are described in Table 1. The E. coli strains JM103 and JM110 have been described (MESSING 1983; YANISCH-PERRON, VIEIRA and MESSING 1985). M13mp recombinant phages are derivatives of either M13mp8 or M13mp9 (MESSING and VIEIRA 1982) and are described in Table 2. Transductions were done using phage P22.

TABLE 1

Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2Z</td>
<td>Wild type</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>JV114</td>
<td>cya403 rpsL201</td>
<td>JOVANOVICH (1985)</td>
</tr>
<tr>
<td>AZ105</td>
<td>proA13 [F'128 lac' pros*]</td>
<td>PALMER, BLUM and ARTZ (1983)</td>
</tr>
<tr>
<td>AZ403</td>
<td>pspF146 trp43 leu500 recA1 srl::Tn10</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>AZ1156</td>
<td>trp::Tn5 [F'123 trp' finP301]</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>AZ2504*</td>
<td>[cya-lac] 696ins</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2541</td>
<td>DUPS1 [cya-lac] 696ins cya*</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2562</td>
<td>DUPS2 [cya-lac] 696ins cya* recA1 srl::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2612</td>
<td>trp::Tn5 cya706 [F'123 trp' finP301]</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2614</td>
<td>trp::Tn5 cya706 [F'123 trp' finP301]</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2616</td>
<td>trp::Tn5 [cya701-lac] 696ins [F'123 trp' finP301]</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2618</td>
<td>trp::Tn5 cya703 [F'123 trp' finP301]</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2620</td>
<td>trp::Tn5 cya702 [F'123 trp' finP301]</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2621</td>
<td>trp::Tn5 cya704 [F'123 trp' finP301]</td>
<td>This study</td>
</tr>
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<td>AZ2634</td>
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<td>This study</td>
</tr>
<tr>
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<td>This study</td>
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<td>AZ2638</td>
<td>trp::Tn5 [cya702] 696ins [F'123 trp' finP301]</td>
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<td>AZ2639</td>
<td>trp::Tn5 [cya702-lac] 696ins [F'123 trp' finP301]</td>
<td>This study</td>
</tr>
<tr>
<td>AZ2640</td>
<td>trp::Tn5 [cya704-lac] 696ins [F'123 trp' finP301]</td>
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<tr>
<td>AZ2641</td>
<td>trp::Tn5 [cya704-lac] 696ins [F'123 trp' finP301]</td>
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<tr>
<td>AZ2642</td>
<td>trp::Tn5 [cya702] 696ins [F'123 trp' finP301]</td>
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<tr>
<td>AZ2643</td>
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</tr>
<tr>
<td>AZ4344*</td>
<td>[cya-lac] 696ins trpD10 cyaB12 ara-1</td>
<td>Same as JV344 (JOVANOVICH 1985)</td>
</tr>
</tbody>
</table>

* This strain was isolated as one of a class of heat-resistant derivatives of strain AZ4344 using the procedure for immobilization (IMB designation) of Mu d1 fusions (see MATERIALS AND METHODS).

50 µg/ml ampicillin, 10 µg/ml tetracycline and 200 µg/ml streptomycin. Cyclic AMP was added at 1 mM unless otherwise specified.

Construction of cya promoter mutations: The construction of mutations that disrupt the putative CRP binding site (Figure 1) was facilitated by the presence of a unique BclI recognition site adjacent to the P2 −10 hexamer. Phage M13mp9::cyaΔ53R (Table 2) replicative form (RF) DNA, isolated from strain JM10 (lacking the dam methylase), was digested with BclI then treated with either nuclease S1 to remove the 4-base single-strand ends, Klenow fragment of DNA polymerase I plus all four deoxyribonucleotides to fill in the single-strand ends, or BAL-S1 exonuclease to generate larger deletions (MANNITIS, FRITSCHE and SAMBROOK 1982). Ligated DNAs were transformed into strain JM103 as described (HANAHAN 1983). The nucleotide sequence of an isolate from each class of mutations (Figure 1) was determined as previously described (THORNER, FANDL and ARTZ 1990). Deletions 703 resulted in the loss of 4 bp immediately downstream of the P2 −10 hexamer and within the downstream half of the consensus CRP binding site palindromic. Insertion 704 resulted in the addition of 4 bp immediately downstream of the P2 −10 hexamer and increases the spacing between the two highly conserved halves of the CRP binding site. Deletions 705 and insertion 704 altered the putative CRP binding site without changing the adjacent P2 −10 hexamer. Deletions 702 removed 32 bp including all of the −10 hexamer and all but 1 bp of the −35 hexamer of P2. The nucleotide sequence of the entire cya regulatory region of each mutant revealed no additional changes.

Mutations removing the beginning of the cya gene (Δ700 and Δ701') were constructed for use in recombining the promoter mutations described above into the chromosome and for fusing the mutant promoter to lac. Deletions 700 and 701 were made by combining existing deletions of the...
**TABLE 2**

<table>
<thead>
<tr>
<th>Phage</th>
<th>Characteristics</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13mp8 and M13mp9</td>
<td>Cloning/sequencing vectors; amber mutations in genes I and II</td>
<td>MESSING and VIEIRA (1982)</td>
</tr>
<tr>
<td>M13mp8::cya</td>
<td>Contains 4.2-kb Smal-EcoRI fragment encoding the regulatory region and most of the cya structural gene</td>
<td>THORNER, FANDL and ARTZ (1990)</td>
</tr>
<tr>
<td>M13mp9::cyaΔ33R</td>
<td>Contains 507-bp PstI-AvaI fragment encoding the cya regulatory region and N terminus of the structural gene</td>
<td>THORNER, FANDL and ARTZ (1990)</td>
</tr>
<tr>
<td>M13mp8::cyaΔ23</td>
<td>BAL-3I generated deletion of M13mp8::cya extending from the upstream Smal site to 35 amino acids into the cya structural gene</td>
<td>This study</td>
</tr>
<tr>
<td>M13mp8::cyaΔ121</td>
<td>BAL-3I generated deletion of M13mp8::cya extending from the upstream Smal site to 13 amino acids into the cya structural gene</td>
<td>This study</td>
</tr>
<tr>
<td>M13mp8::cyaΔ700</td>
<td>HindIII-BclI fragment from M13mp8::cyaΔ33R inserted into the HindIII-BamHI site of M13mp8::cyaΔ121 (Figure 2); this cya mutation in M13mp8::cyaΔ700 corresponds to cyaΔ700 when in the chromosome</td>
<td>This study</td>
</tr>
<tr>
<td>M13mp8::cyaΔ701</td>
<td>HindIII-BclI fragment from M13mp8::cyaΔ33R inserted into the HindIII-BamHI site of M13mp8::cyaΔ23; this cya mutation in M13mp8::cyaΔ701 corresponds to cyaΔ701 when in the chromosome</td>
<td>This study</td>
</tr>
<tr>
<td>M13mp9::cyaΔ702</td>
<td>M13mp9::cyaΔ33R derivative with a 32 bp deletion of cya P2 from -3 to -34; this cya mutation in M13mp9::cyaΔ702 corresponds to cyaΔ702 when in the chromosome</td>
<td>This study</td>
</tr>
<tr>
<td>M13mp9::cyaΔ703</td>
<td>M13mp9::cyaΔ33R derivative with a 4-bp deletion of cya P2 from -3 to -6; this cya mutation in M13mp9::cyaΔ703 corresponds to cyaΔ703 when in the chromosome</td>
<td>This study</td>
</tr>
<tr>
<td>M13mp9::cyaINS704</td>
<td>M13mp9::cyaΔ33R derivative with a 4-bp insertion (GATC) between -3 and -4; this cya mutation in M13mp9::cyaINS704 corresponds to cyaΔ704 when in the chromosome</td>
<td>This study</td>
</tr>
</tbody>
</table>

promoter region (THORNER, FANDL and ARTZ 1990) (Table 2). The HindIII-BclI fragment of M13mp9::cyaΔ33R was subcloned into the HindIII-BamHI site of either M13mp8::cyaΔ121, resulting in M13mp8::cyaΔ700, or M13mp8::cyaΔ23 resulting in M13mp8::cyaΔ701. The strategy is shown in Figure 2 for construction of M13mp8::cyaΔ700. These deletions extend from the P2 promoter to 13 codons (Δ700) or 35 codons (Δ701) into the cya structural gene relative to the TTG initiation codon (THORNER, FANDL and ARTZ 1990).

**Allele replacement:** The allele replacement theory and procedures have been described in detail (ARTZ et al. 1983, BLUM et al. 1989). Briefly, the method is based on the ability to select for M13 lysogens and segregants of lysogens. The amber mutation in gene II of M13mp8 and 9 prevents phage replication in nonsuppressing host cells, but the phage can integrate into the chromosome if homology exists between the chromosome and the insert DNA in the recombinant phage. M13 lysogens are resistant to killing by male-specific phages (e.g., phage R17) because F-pili function is repressed. This provides a positive selection for integrants of the recombinant M13mp phage. M13 lysogens are sensitive to deoxycholate. This provides a selection for segregants of lysogens. Allele replacement using M13mp recombinant phages can be accomplished by selecting for either the M13 lysogen phenotype or the phenotype conferred by an inserted DNA fragment.

Phages M13mp8::cyaΔ700 and M13mp8::cyaΔ701 (Table 2) were recombined into the chromosome by selecting for the M13 lysogen phenotype (Figure 3, left). A fresh overnight culture of strain AZ1516 grown in VBG was diluted to 5 × 10⁷ cells/ml and 100 µl was mixed with 10 µl (10⁶ plaque forming units (pfu)); multiplicity of infection (MOI) approximately 200) of M13mp8::cyaΔ700 or M13mp9::cyaΔ701 and incubated without shaking at 37°C for 90 min. The mixture was then spread on green indicator plates supplemented with 5 mM CaCl₂ together with 150 µl (10⁴ pfu) R17 phage and incubated overnight at 37°C. Lysogens were picked and purified by single colony isolation three times successively on MacConkey glycerol agar containing an additional 0.15% (w/v) sodium deoxycholate to select segregants. Segregant colonies that were white on MacConkey glycerol and that were unable to grow on carbon sources other than glucose (Cya⁻ phenotype) were saved. Segregants containing Δ700 and Δ701 were designated strains AZ2612 (cyap700) and AZ2614 (cyap701) (Table 1).

Phages M13mp9::cyaΔ702, M13mp9::cyaΔ703 and M13mp9::cya INS704 (Table 2) were recombined into the chromosome of the Cya⁻ recipient strain AZ2612 by selecting for Cya⁺ on C⁻ citrate agar (Figure 3, right). The phages containing the promoter mutations were concentrated by polyethylene glycol precipitation (YAMAMOTO et al. 1979) to approximately 10¹⁰ pfu/ml and mixed with 200 µl of a fresh overnight culture of strain AZ2612 grown in VBG to give an MOI of about 1200. This mixture was incubated at 37°C without shaking for 90 min, spread on C⁻ citrate agar, and the plates were incubated at 37°C for 48 hr. Cya⁻ transductants were picked and purified on MacConkey glycerol agar containing an additional 0.15% (w/v) sodium deoxycholate.
and the segregant colonies appearing red (Δ703) or pink (Δ702) and INS704 were saved. Segregants containing Δ702, Δ703 and INS704 and an intact cya structural gene were designated strains AZ2620 (cyaP702), AZ2618 (cyaP703) and AZ2621 (cyaP704) (Table 1).

In all cases, segregation of M13 prophages was confirmed by testing segregants for sensitivity to killing by R17 phage. Cells were patched on NB agar supplemented with 5 mM CaCl₂ and 2 µl of an R17 lysate (10⁹ pfu) was immediately spotted in the center of each patch. After incubation at 37°, about 5 hours a clearing appeared if the host cells had segreaged the prophage.

**Im mobilization of Φ (cya-lac)** 696 and duplication of the cya locus: To facilitate genetic manipulation and growth at higher temperatures of strains containing the cya-lac fusion, it was necessary to immobilize the fusion (BLUM, BLAHA and ARTZ 1986) in strain AZ4344. Heat-resistant derivatives of strain AZ4344 were obtained by spreading 0.1 ml of an overnight NB culture on NB agar plates supplemented with cysteine and CAMP, followed by incubation at 42° overnight. Colonies were pooled in 1 ml NB, diluted 1:10 into NB + cysteine + CAMP, and incubated at 37° with shaking for 7 hr. A phage P22 lysate was made on the pooled culture and the phage were pelleted (DAVIS, BOTSTEIN and ROTH 1980) and resuspended in an equal volume of T2 buffer (1 mM MgSO₄, 0.1 mM CaCl₂, 20 mM Na,HPO₄, 8.6 mM KH₂PO₄, 68 mM NaCl, 28.7 mM K₂SO₄, and 0.001% gelatin, pH 7.0) to remove extracellular β-lactamase. This lysate was then used to transduce strain LT22Z to ampicillin resistance again scoring for Cya⁺. This procedure was repeated until a lysate was obtained that yielded a high frequency (99%) of Cya⁺ transductants.

Transduction of the immobilized fusion from strain AZ2590 into a Cya⁺ recipient should yield 100% Cya⁺ transductants. However, we repeatedly observed that about 1% of the transductants were Cya⁺, Lac⁺, and Ap⁺. Transduction of the immobilized fusion into one cya locus of a recipient containing a preexisting duplication of cya would yield this phenotype. The duplications were confirmed as such by the following tests: (1) The duplication phenotype was unstable (Cya⁺, Lac⁺, Ap⁺ and Cya⁺, Lac⁺, Ap⁺ segregants were observed) but was stabilized by introduction of a recA mutation into the strain. For example, the duplication in strain AZ2590 was stabilized by transducing the recA mutation from strain AZ403 to construct strain AZ2599 (Table 1). (2) Lysates grown on duplication strain AZ2599 were shown to transduce Cya⁻ recipients to Cya⁺, Lac⁺, Ap⁺ or Cya⁻, Lac⁺, Ap⁺. (3) Genes proximal to cya (e.g., metE and ilv) were frequently included in the duplications.

Genetic complementation experiments in S. typhimurium often are done with heterologous E. coli F' episomes since there are few S. typhimurium F' episomes available. The method of isolating and stabilizing duplications with immobilized Mu d1 insertions (similar to the method of ANDERSON and ROTH (1981) for isolation of tandem duplications with Tn10) should be generally useful for studying complemented gene expression of homologous genes.

**Construction of cya promoter mutations fused to lac**

To facilitate study of the regulation of mutant cya promoters we placed the cya-lac fusion downstream of the mutations. A lysate grown on the Lac⁻ strain AZ2617 (θ(cyaP701-lac)696;MIN) was used to transduce the cya promoter mutants AZ2618, AZ2620 and AZ2621 to ampicillin resistance on MacConkey lactose agar. Lac⁺ transductants (red or pink colonies) contained the Cya⁺ mutant promoters of the recipient strains fused to lac. Isogenic crp mutants were constructed by transducing the (cyap-lac) fusion strains to streptomycin resistance with a P22 lysate made on strain JV114 (crp404 rpsL201).

**β-Galactosidase assay:** Cultures were grown overnight in
endonucleases were from Bethesda Research Laboratories, with five samples taken at ODtis0's in the range of 0.2-0.6. Acrylamide, and ammonium persulfate were from Bio- and were the average of two experiments.

DNA polymerase I (Klenow fragment), and all restriction chemicals, Milwaukee, Wisconsin. Isopropyl-β-D-thiogalactopyranoside (IPTG), O-nitrophenyl-β-D-galactopyranoside (ONPG), cAMP, low gelling temperature agarose (type VII), and sodium deoxycholate were from Sigma Chemical Co., St. Louis, Missouri.

**METHODS.**

Optical density (OD) at 650 nm with a Gilford model 250 spectrophotometer. The cultures were sampled (1 ml) into iced test tubes and assayed for β-galactosidase activity and its correlation with cAMP levels:

\[ \text{specific activity} = \frac{\text{activity} (\text{min})}{\text{OD}_{600} \times 1000} \]

Values are the average of two experiments.

**RESULTS**

Carbon source regulation of (cyu-lac)696 and its correlation with cAMP levels: Table 3 shows the results of experiments in which β-galactosidase activity was assayed as a measure of cyu-lac expression in strain AZ2599 grown on different carbon sources. Strain AZ2599 carries an immobilized cyu-lac fusion (Mu d1 insertion) and a cyu" gene as the result of a duplication. The strain, therefore, is Cya+ owing to complementation by the homologous S. typhimurium cyu" gene. The differential rate of β-galactosidase synthesis in strain AZ2599 was 3.8-fold higher in cultures that were grown in glucose-6-phosphate minimal medium than in cultures grown in acetate minimal medium. Growth in glucose, gluconate, glycerol and glucosamine gave intermediate levels of cyu-lac expression. The differential rates of the homologously complemented cyu-lac fusion were similar to those previously observed for the nonimmobilized fusion (E. coli F'-cyu"--data not shown). In addition, the results for the immobilized fusion were similar to those previously observed for the nonimmobilized fusion (JOVANOVICH 1985). These data indicate that cyu expression and regulation were not affected by the immobilization procedure, and that carbon source regulation of cyu-lac expression is the same whether the complementing cyu" gene is derived from S. typhimurium or E. coli.

The carbon sources shown in Table 3 are arranged in the order of the intracellular level of cAMP that they elicit in E. coli when acting as the sole source of carbon in the medium. For example, in the order reported by EPSTEIN, ROTHMAN-DENES and HESSE.
(1975), growth on glucose-6-phosphate resulted in the lowest relative intracellular cAMP concentration in E. coli whereas growth on glucosamine elicited the highest concentration. It is clear that E. coli F' lac expression in S. typhimurium strain AZ105 is derepressed during growth on carbon sources known to elicit high intracellular cAMP levels (Table 3). Therefore, F' lac expression in S. typhimurium parallels lac expression in E. coli during growth on different carbon sources. Since lac expression varies directly with intracellular cAMP concentrations in E. coli (Epstein, Rothman-Denes and Hesse 1975), we conclude that F' lac expression in S. typhimurium can be used as a measure of relative cAMP levels. The intracellular concentration of cAMP resulting from growth on acetate was inversely related during growth on different carbon sources, we conclude that cya expression is negatively regulated by cAMP.

Effects of cya P2 mutations on promoter activity: Table 4 shows the effects of cya P2 mutations (Figure 1) on transcriptionally fused lac expression. β-galactosidase activities were determined in a constitutively derepressed crp mutant background in order to quantify promoter activities independent of regulation by cAMP-CRP (the results were unaffected by addition of cAMP—data not shown). The wild-type strain (AZ2642) grown in minimal glucose medium gave a β-galactosidase specific activity of about 700. The 32-bp deletion mutation (cyap702) in strain AZ2638, which removes the −10 and −55 hexamer sequences of cya P2, reduced total cya expression about fivefold.

<table>
<thead>
<tr>
<th>cya-lac strain</th>
<th>Promoter genotype</th>
<th>β-Galactosidase specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ2642</td>
<td>cyap+</td>
<td>706 (559)</td>
</tr>
<tr>
<td>AZ2638</td>
<td>cyap702</td>
<td>147</td>
</tr>
<tr>
<td>AZ2634</td>
<td>cyap703</td>
<td>681 (554)</td>
</tr>
<tr>
<td>AZ2640</td>
<td>cyap704</td>
<td>235 (88)</td>
</tr>
</tbody>
</table>

Cultures were grown at 37° in M56 minimal medium with 40 mM glucose as described in MATERIALS AND METHODS.

Values shown are specific activities determined from differential rate plots and the units are A405/OD600 × 1000. Values are the average of at least two experiments.

Values in parentheses are the specific activities attributed to the P2 promoter and were calculated by subtracting the specific activity attributed to the upstream promoters (P1, P1') in strain AZ2638. For example, the activity attributed to P1, P1' in strain AZ2638 (147) was subtracted from the activity in strain AZ2642 (706) to get the activity attributed to P2 in strain AZ2642 (559).

Therefore, cya P2 accounts for approximately 80% of derepressed cya expression; the remaining 20% is the consequence of upstream promoter sequences (presumably the P1 and P1' promoters) (Thorner, Fandl and Artz 1990). In E. coli, the P1 and P1' promoters were identified by S1 mapping (Alba 1985) and exist in a region that is nearly identical with the analogous region in S. typhimurium (Thorner, Fandl and Artz 1990). The 4-bp deletion mutation (cyap703) in strain AZ2634, which removes bp −3 to −6, but leaves the cya P2 RNA polymerase-binding sequence intact, had essentially no effect on cya expression. In contrast, the 4-bp insertion mutation (cyap704) between bp −3 and −4 in strain AZ2640 resulted in a threefold decrease in cya expression even though the cya P2 RNA polymerase binding sequence remained intact. Subtraction of the contribution of the P1 and P1' promoters indicated that the reduction of P2 promoter activity caused by cyap704 is about sixfold (Table 4, values in parentheses).

Strains AZ2620 (cyap702) and AZ2621 (cyap704) have an intact cya structural gene and carry mutations that eliminate or greatly reduce P2 promoter activity (Table 4). These strains grew on the poor carbon source citrate indicating that, although it is the major carbon source, we conclude that strain AZ2639 as the reference for no P2 activity.

Effects of cya P2 mutations on regulation by cAMP-CRP: Table 5 shows the response of wild-type and mutant cya promoters (Figure 1) to exogenous cAMP in crp+ strains grown in minimal glucose medium. Addition of 5 mM cAMP to the growth medium repressed wild-type cya expression 3.7-fold in strain AZ2643. When the contribution of the upstream promoters (defined by strain AZ2639 which lacks cya P2) was subtracted from the total activity in the wild-type strain, we calculated that P2 expression in strain AZ2643 was repressed 7.8-fold by the addition of 5 mM cAMP. Repression by cAMP was eliminated in the isogenic crp mutant (data not shown). Deletion
mutation cya703 in strain AZ2635, which removed 4 bp of the putative CRP binding site, nearly eliminated cya repression by cAMP-CRP; cya P2 was repressed 1.5-fold by addition of 5 mM CAMP. Similarly, repression by CAMP-CRP was greatly reduced by the 4-bp insertion mutation cya704 in strain AZ2641 and by the P2 deletion mutation cya702 in strain AZ2639. These results indicate that cya P2 is the primary target of CAMP-CRP repression.

**DISCUSSION**

In this paper we demonstrated genetically that the CAMP-CRP complex represses cya expression in *S. typhimurium* by interacting at a site overlapping the major cya promoter, P2. Mutations altering the CAMP-CRP binding site were constructed and recombined into the chromosome allowing us to study the effects of the mutations under physiological conditions, in single copy and at the appropriate chromosomal locus. The mutant analysis supports the existence of multiple cya promoters consistent with the comparison of the cya DNA sequences of *S. typhimurium* and *E. coli* (Thorner, Fandl and Artz 1990) and nuclease S1 mapping experiments in *E. coli* (Aiba 1985).

The nucleotide sequence of the putative CRP binding site at cya P2 is

5' AG-TGTTA----TCACG-TT 3'
3' TC-ACAAT-----AGTGG-AA 5'

(Thorner, Fandl and Artz 1990) which closely resembles the consensus CRP binding site

5' AA-TGTTA----TCACA-TT 3'
3' TT-ACTCA-----AGTGT-AA 5'

(Ebright et al. 1987; Berg and von Hippel 1988). Important contacts between CRP and the lac operon CRP binding site were identified by chemical protection studies (Reznikoff and Abelson 1980), X-ray crystallography (McKay and Steitz 1981), and binding site mutations (Dickson et al. 1977; Ebright et al. 1987; Jansen, Gronenborn and Clore 1987). These studies indicated that the two TGTTA pentamers, which occur in the symmetric left- and right-half binding sites are most important in specifying CRP-DNA interactions. The cya CRP binding site deviates from the TGTTA consensus at two positions (shown in boldface): position 4 of the left-half binding site (T instead of G) and position 1 of the right-half binding site (C instead of T). The C at position 4 is critical for specific binding while the T at position 1 is relatively unimportant (Ebright et al. 1987; Jansen, Gronenborn and Clore 1987). Interestingly, Jansen, Gronenborn and Clore (1987) and Ebright et al. (1987) found that, of the possible substitutions at position 4, T had the least detrimental effect on specific CRP binding.

The Δ703 mutation destroyed the right-half binding site for CRP at cya P2 (Figure 1). When this deletion was recombined into the chromosome we observed nearly complete loss of repression of cya expression by CAMP-CRP (Table 5). In crp mutants, the activity of the Δ703 promoter was similar to that of the wild-type promoter (Table 4), indicating that Δ703 did not affect binding of RNA polymerase. In contrast, the 4-bp insertion mutation (INS704) which increased the spacing between the highly conserved CRP binding site pentamers reduced both cya P2 activity and CAMP-CRP regulation. Decreased promoter activity in this mutant was surprising because INS704 did not change the −10 hexamer of P2. We speculate that the presence of tandem GATC sequences (dam methylation sites) near the P2 −10 hexamer and transcription startpoint in this mutant may interfere with the interaction of RNA polymerase. The presence of dam methylation sites adjacent to certain promoters has been shown to reduce their activity (Hoopes and McClure 1987).

Our results and other studies (Botsford and Drexler 1978) indicate that under conditions where glucose is available and CAMP is less required, the cell derepresses synthesis of (and presumably accumulates) adenylate cyclase, while inhibiting the activity of the enzyme. When glucose in the medium is exhausted, the cell can rapidly react to the new conditions by activating its store of adenylate cyclase and accumulate CAMP. This allows activation of transcription of those genes required for the use of available alternative carbon sources. When the cell must use a carbon source with a lower energy yield than glucose it conserves energy by repressing the synthesis of adenylate cyclase as well as the synthesis of many other proteins (Mallick and Herrlich 1979) that presumably are less necessary under these growth conditions.

Although repression of cya expression by CAMP-CRP is now clearly established, this regulation cannot readily explain the 10- to 100-fold overproduction of CAMP by *crp* mutants growing in minimal glucose medium (Potter, Chalmers-Larson and Yamazaki 1974; Wayne and Rosen 1974; Raphaeli and Saier 1976). This is apparent from the observation that a *crp* mutant (strain AZ2642; Table 4) expresses cya less than twofold higher than *crp* strains (AZ2599 and AZ2643; Tables 3 and 5, respectively) during growth in minimal glucose medium. Therefore, overproduction of CAMP by *crp* mutants must be the direct or indirect consequence of a defect in CRP-mediated regulation of adenylate cyclase activity rather than cya expression, a conclusion realized by others as well (Raphaeli and Saier 1976; Botsford and Drexler 1978).
In addition to carbon source regulation involving cAMP-CRP, the cell may utilize other mechanisms to increase its store of adenylate cyclase under rapid growth conditions. The location of cya is near the origin of replication and therefore will be present in multicopy during rapid growth due to multiple replication forks. In addition, ANDERSON and ROTH (1981) observed that rapid growth rates increase the frequency of rRNA-mediated duplications. The finding that the cya locus experiences a high frequency of duplication (1% see MATERIALS AND METHODS) is consistent with its location between rRNA A and rRNA C and this may provide another method to maximize adenylate cyclase synthesis during rapid growth. Finally, the GATC sequence proximal to the cya P2-10 hexamer is a dam methylase recognition site and dam methylation near promoters is known to inhibit transcription except transiently during periods of hemimethylation (HOOPES and MCCLURE 1987). DNA is hemimethylated immediately after DNA replication. Therefore, during rapid growth, the GATC adjacent to the P2 promoter would be hemimethylated more often than during slow growth.

This work was supported by U.S. Public Health Service grant GM27307 from the National Institutes of Health. We thank LeWanna Archer for typing the manuscript.

LITERATURE CITED


Majerfeld et al., 1981; Joseph et al., 1982; Dobrogosz et al., 1983.
cya Promoter Mutations

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Communicating editor: J. R. Roth