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Transcriptional regulation of the *cytR* repressor gene of *Escherichia coli*: autoregulation and positive control by the cAMP/CAP complex

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Summary

The *Escherichia coli cytR*-encoded repressor protein (CytR) controls the expression of several genes involved in nucleoside and deoxynucleoside uptake and metabolism. The *cytR* promoter was identified by determining the transcriptional initiation site of the *cytR* gene. A chromosomal *cytR-lacZ*$^+$ operon fusion was isolated and used to study the regulation of *cytR*. We show that *cytR* expression is negatively controlled by the CytR protein and positively affected by the cAMP/CAP complex. Footprinting studies with purified CAP protein revealed two CAP binding sites upstream of the *cytR* promoter. A previously described mutation (*cytR) in the cloned *cytR* gene, which results in the phenotypic suppression of a CytR operator mutation in the *tsx* P2 promoter, was analysed. DNA sequence analysis of the *cytR** mutation revealed a G-C to an A-T base pair transition at position −34 bp relative to the translational initiation site of *cytR*. This point mutation activates a cryptic promoter that is stronger than the wild-type *cytR* promoter and leads to overproduction of the CytR repressor.**

Introduction

Escherichia coli can use nucleosides and deoxynucleosides as precursors in nucleic acid synthesis and as carbon and nitrogen sources (Munch-Petersen and Mygind, 1983; Neuhard and Nygaard, 1987). The uptake and metabolism of these compounds involve the products of at least eleven genes, nine of which share common regulatory features. The outer membrane protein Tsx and the inner membrane proteins NupC and NupG mediate the transport of the various nucleosides across the *E. coli* cell envelope, while the Cdd, Udp and DeoCABD proteins catalyse their metabolism. Their structural genes form a regulon controlled by the DeoR and CytR repressor proteins. The transcription initiated from all CytR-controlled promoters is activated by the cAMP/CAP complex (Hammer-Jespersen, 1983).

The *deoCABD* operon, whose expression is mediated by two promoters, P1 and P2, has been used as a model to study the mechanism of DeoR-mediated repression of transcription (Valentin-Hansen *et al.*, 1982; 1986b; Dandanell *et al.*, 1987). Mutant analysis and binding studies with the purified DeoR repressor have shown that DeoR recognizes a 16-bp palindromic sequence (Dandanell and Hammer 1985; Mortensen *et al.*, 1989). These DeoR-binding sites overlap the −10 regions and the transcription initiation sites in the *deo* P1 and P2 promoter regions; therefore, the bound repressor most likely prevents open-complex formation between these promoters and RNA polymerase. Transcription initiating from the *deo* P2 promoter is further regulated by the CytR repressor and positively controlled by the cAMP/CAP complex. Tandem binding sites for the activator complex have been mapped in the *deo* P2 promoter region (Valentin-Hansen, 1982), but the operator site for CytR has not yet been determined by footprint analysis. It has been suggested that CytR and the cAMP/CAP complex recognize similar DNA sequences (Valentin-Hansen, 1982). This proposal is supported by mutant and *in vitro* analysis of the *deo* P2, *tsx* P2 and *cdd* promoters, all of which are affected by CytR and cAMP/CAP and contain tandem cAMP/CAP binding sites (Valentin-Hansen, 1982; Martinussen *et al.*, 1988; Bremer *et al.*, 1988b; Valentin-Hansen *et al.*, 1989; unpublished data). These analyses showed that alterations in the cAMP/CAP targets in these promoters affected both cAMP/CAP and CytR binding and that most of the operator-constitutive mutants for CytR carried mutations in one of the two cAMP/CAP targets. Based on these results, a regulatory model has been proposed which suggests that the repressor and the activator complex compete for their DNA targets and that, consequently, the bound CytR repressor prevents the productive interaction of the activator complex with the various promoter regions (Valentin-Hansen, 1984; Martinussen *et al.*, 1988).

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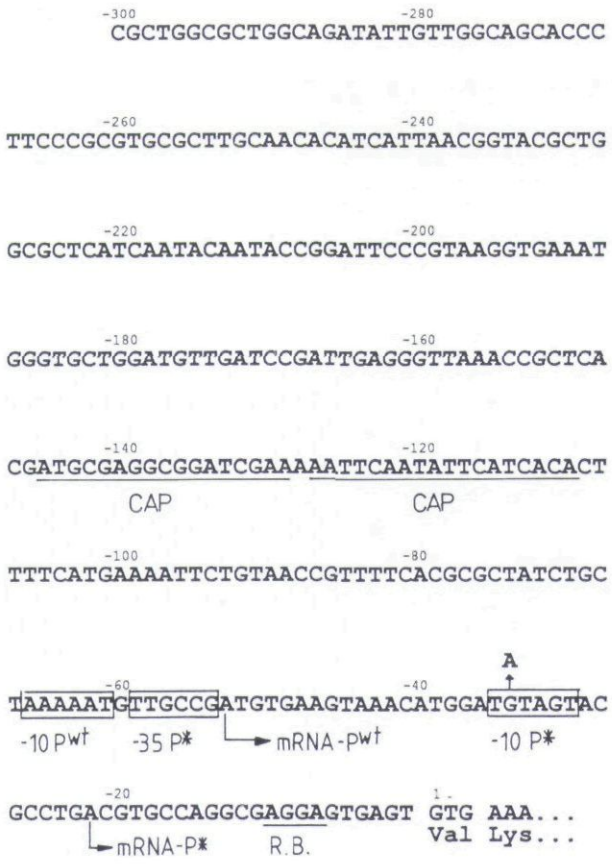

Fig. 1. Regulatory region of the *cytR* gene. The sequence and numbering shown is according to Valentin-Hansen *et al.* (1986a). The beginning of the *cytR* coding region and the presumed ribosome-binding site (R.B.) are indicated. The −10 region of the wild-type *cytR* gene (P^{wt}) and the −10 and −35 regions (P*) of the promoter created by the *cytR** mutation are boxed. The G to A exchange in the *cytR** allele is shown. The transcription initiation sites for both the *cytR* and *cytR** genes are marked by arrows. The binding sites for the cAMP/CAP complex are underlined. It should be noted that the 'A' at position −121 was incorrectly printed as a 'G' in the original publication (Valentin-Hansen *et al.*, 1986a). This correction has been submitted to the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries.

The CytR repressor contains, near its amino terminus, the helix-turn-helix motif found in many regulatory proteins (Valentin-Hansen *et al.*, 1986a; Brennan and Matthews, 1989). Its primary sequence is highly homologous to GalR, DeoR, LacI, PurR, and MalI repressor proteins (Valentin-Hansen *et al.*, 1986a; Rolfes and Zalkin, 1988; Reidl *et al.*, 1989). Inspection of the regulatory region of the *cytR* gene has revealed several putative promoters and three regions that show homology to the DNA consensus sequences of cAMP/CAP binding sites (Valentin-Hansen *et al.*, 1986a). In view of the model for the CytR-mediated repression of gene expression, it has been speculated that transcription of *cytR* is subjected to regulation by CytR and the cAMP/CAP complex.

Using a chromosomal *cytR-lacZ*$^+$ operon fusion, we have investigated the expression of *cytR* and show that the transcription of *cytR* is autoregulated and positively affected by the cAMP/CAP complex. Binding of purified CAP protein to two sites in the *cytR* regulatory region was demonstrated. The *cytR* promoter was identified by mapping of the 5′-end of the *cytR* mRNA. We have also characterized a mutation in the cloned *cytR* gene, *cytR**, that results in the phenotypic suppression of a CytR operator-constitutive mutation in the *tsx* P2 promoter (Bremer *et al.*, 1988b).

Results

Mapping of the transcriptional initiation site of cytR

Several putative promoters have been noted in the *cytR* regulatory region (see Fig. 1), but the actual position of the *cytR* promoter is unknown (Valentin-Hansen *et al.*, 1986a). To identify this promoter we mapped the transcription initiation site of the *cytR* mRNA. Total RNA was isolated from the *cytR* strain BRE2050 carrying the *cytR*$^+$ plasmid pCB008 and hybridized to a radiolabelled *cytR*-specific primer. The primer sequence was extended *in vitro* using reverse transcriptase, and the start site for the *cytR*-directed mRNA was mapped by running appropriate sequencing ladders along with the primer extension product on a DNA sequencing gel (Fig. 2). We found a unique transcription initiation site that corresponds to the A residue at position −53 bp relative to the translational start of *cytR* (Fig. 1). Seven bp upstream of the *cytR* mRNA start site there is a region (AAAAAT) that shows homology to the consensus (TATAAT) Pribnow box. However, a −35 region with an appropriate spacing of 17 ± 1 bp to the −10 region (Harley and Reynolds, 1987) is not apparent. We show below that *cytR* expression is regulated by the cAMP/CAP activator complex. It is well established that many cAMP/CAP-dependent promoters do not possess a readily recognizable −35 region (Raibaud and Schwartz, 1984).

Isolation of a chromosomal cytR-lacZ$^+$ *operon fusion*

A bacterial strain containing a chromosomal *cytR-lacZ*$^+$ operon fusion permits quantitative studies of *cytR* expression. To construct such a strain, we isolated insertions of the transposable λp*lac*Mu55 phage (Bremer *et al.*, 1988a) in the chromosome of the *deoR*$^+$ *cytR*$^+$ strain, BRE2047. Strains containing the wild-type *cytR* gene cannot grow efficiently on inosine or uridine as a sole carbon source, but mutations in *cytR* permit efficient growth on both of these nucleosides (Hammer-Jespersen, 1983). We were therefore able to isolate a strain, GP100, containing a *cytR-lacZ*$^+$ operon fusion on the basis of its Lac$^+$ phenotype and its ability to grow efficiently on inosine (Ino$^+$) and uridine (Uri$^+$) (see *Experimental procedures*).

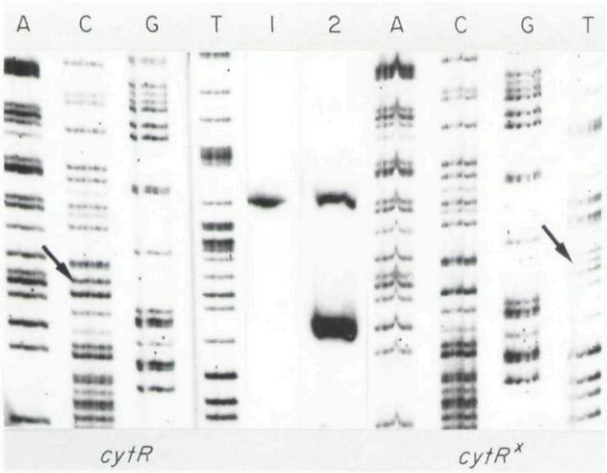

Fig. 2. Determination of the 5′ ends of the *cytR*- and *cytR**-directed mRNA. A single-stranded radiolabelled primer from the *cytR* coding region was annealed to total cellular RNA isolated from the *cytR* strain BRE2050 carrying either pCB008 (*cytR*$^+$) or pGP1 (*cytR**) and extended with reverse transcriptase. The extension products were analysed on a 4% sequencing gel. To calibrate the gel, we sequenced and electrophoresed in parallel M13 clones carrying the regulatory region from either the wild-type *cytR* gene (left side of the gel) or the *cytR** gene (right side of the gel). Lane 1, BRE2050(pCB008); lane 2, BRE2050(pGP1). The position of the G-C to A-T transition in the *cytR** mutation is indicated by arrows.

We confirmed that the λp*lac*Mu55 insertion in GP100 was located in *cytR* by the following tests. (i) A P1*vir* lysate was prepared on this strain and used to transduce the λp*lac*Mu55 prophage into the parental strain BRE2047 by selecting for Km^R in the presence of XG. All transductants were LacZ$^+$ (more than 200 colonies were inspected) and all colonies tested (20/20) showed the typical CytR$^-$ phenotypes (Uri$^+$ Ino$^+$). Thus, the LacZ$^+$ Uri$^+$ Ino$^+$ phenotypes are genetically linked to the λp*lac*Mu55 prophage. (ii) The *cytR* gene is located at 88.8 min on the *E. coli* linkage map (Bachmann, 1983). We used a P1*vir* lysate grown on strain GD1 carrying the *zih-730*::Tn*10* insertion linked to the *glpK* gene at 88.4 min. When this Tn*10* insertion was transduced into strain GP100 (Φ(*cytR-lacZ*$^+$)1), the fusion was crossed out in 56% of the Tc^R colonies (i.e. they were Lac$^-$ Km^s), demonstrating that the operon fusion mapped in the expected chromosomal region. Furthermore, when several of these transductants were tested they showed a CytR$^+$ (Uri$^-$ Ino$^-$) phenotype. Conversely, Tc^R transductants which still carried the *cytR-lacZ*$^+$ operon fusion retained their CytR$^-$ (Uri$^+$ Ino$^+$) phenotype. These data provide strong genetic evidence that a *cytR-lacZ*$^+$ operon fusion had been obtained.

Positive control of cytR *expression by the cAMP/CAP complex*

We used the chromosomal Φ(*cytR-lacZ*$^+$) 1 operon fusion carried by GP100 to test whether *cytR* expression is positively controlled by the cAMP/CAP complex. The amount of cAMP/CAP complex present in the cell is influenced by the available carbon source (de Crombrugghe *et al.*, 1984). Strain GP100 was grown in minimal medium with glycerol or glucose as carbon source, and the β-galactosidase activity was measured to determine the level of *cytR-lacZ*$^+$ expression. Cells grown on glucose showed a two-fold reduced level of β-galactosidase activity relative to cells grown on glycerol as the carbon source (Table 1). The involvement of the cAMP/CAP activator complex in *cytR* expression was further investigated by transducing, with phage P1*vir*, the Φ(*cytR-lacZ*$^+$)1 fusion into strain BRE2055 (Table 1) carrying the Δ*crp*96 mutation. Expression of the fusion in the resulting strain, GP104, was two-fold lower than in the isogenic *crp*$^+$ strain GP100 in glucose-grown cultures (Table 1), strongly suggesting that the cAMP/CAP complex stimulates *cytR* expression.

Several putative cAMP/CAP binding sites have been noted in the *cytR* regulatory region (Valentin-Hansen *et al.*, 1986a). We used the DNaseI footprinting method (Galas and Schmitz, 1978) to test whether any of these DNA sequences are actually recognized by the cAMP/CAP complex. A 530 bp *Eco*RI-*Ava*I fragment that spans the *cytR* regulatory region (Valentin-Hansen *et al.*, 1986a) was labelled at the 3′- or 5′-end of the *Ava*I site with α-[^{32}P] and γ-[^{32}P], respectively, incubated with a fixed amount of purifed CAP protein in the presence of various amounts of cAMP, and digested with DNaseI. The reaction products were then electrophoretically separated on a DNA sequencing gel. Figure 3A/B shows the 5′-γ-[^{32}P]- and 3′-α-[^{32}P]-labelled *Eco*RI-*Ava*I restriction fragments, respectively. With both fragments, we detected that the DNA region around position −120 bp (see Fig. 1) was protected from DNaseI digestion by CAP in the presence of cAMP. A second and weaker protected region spanning the −140 bp position could be detected with the

Table 1. Effect of the cAMP/CAP complex on Φ(*cytR-lacZ*$^+$)1 expression.

Strain	Description[a]	β-Galactosidase Activity[b] in Cells Grown in MMA with glycerol	glucose
GP100	*deoR*$^+$, *crp*$^+$, Φ(*cytR-lacZ*$^+$)1	88.9 ± 16.8	44.4 ± 0.6
GP104	*deoR*$^+$, *crp*$^-$, Φ(*cytR-lacZ*$^+$)1	–[c]	17.2 ± 2.3
GP103	*deoR*$^-$, *crp*$^+$, Φ(*cytR-lacZ*$^+$)1	97.9 ± 14.7	53.3 ± 9.3

a. The Φ(*cytR-lacZ*$^+$)1 operon fusion is present as a single copy in the chromosome.

b. The strains were grown overnight in MMA with the indicated carbon source and the specific β-galactosidase activity was assayed as described by Miller (1972). The data shown are the mean values of at least three experiments and the error ratio in the β-galactosidase assays is given. A Δ*lac* strain was always included as a control.

c. Strains with a mutation in *crp* do not grow on glycerol as the sole carbon source.

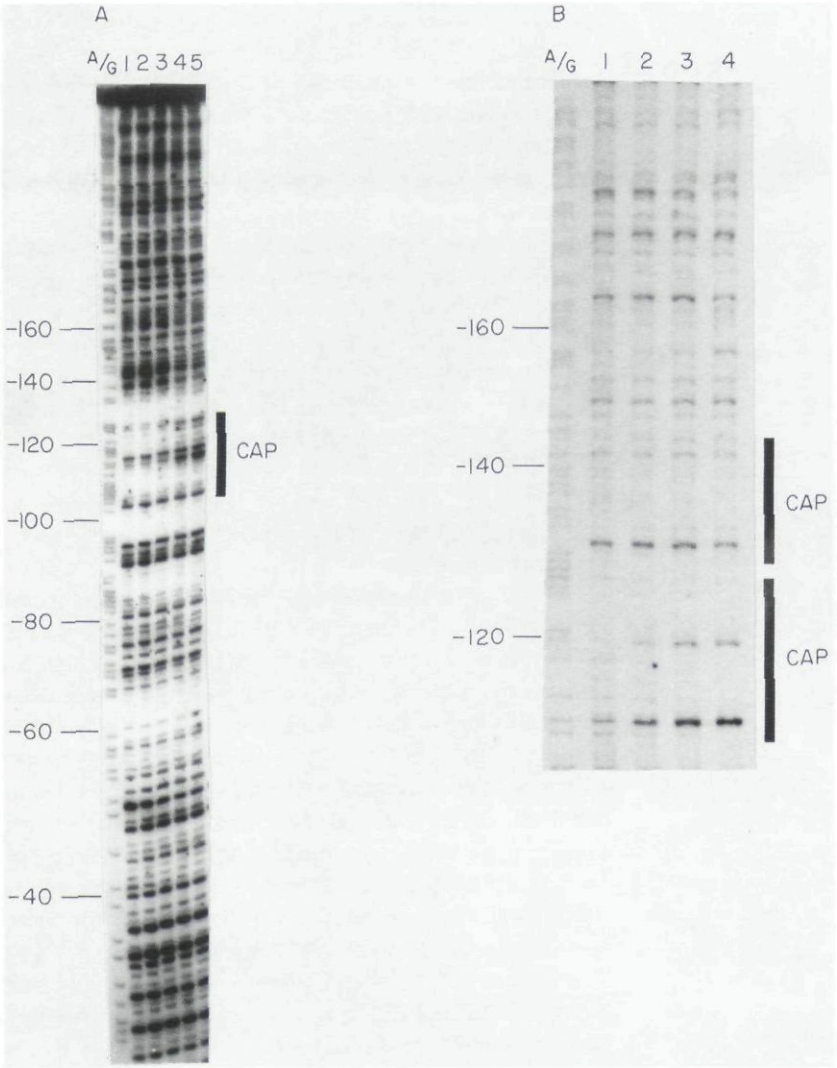

Fig. 3. Protection of the *cytR* regulatory region by CAP from DNaseI digestion. The autoradiograph shows the effect of CAP (A: 8 μg ml^{-1}; B: 10 μg ml^{-1}) and increasing concentrations of cAMP upon digestion with DNaseI (A: 0.5 μg ml^{-1}; B: 0.2 μg ml^{-1}) of the *Eco*RI-*Ava*I restriction fragment (approximately 0.2 μg ml^{-1}) spanning the *cytR* regulatory region. Treatment with DNaseI was carried out for 20 s (A) and 10 s (B), respectively. The restriction fragment used for the experiment shown in A was 5′-γ-[^{32}P]-labelled at the *Ava*I site; in (B) the restriction fragment was 3′-α-[^{32}P]-labelled at the same site.
A. The radiolabelled fragment was incubated with CAP and 2 μM cAMP (lane 1), CAP and 0.5 μM cAMP (lane 2), CAP and 0.1 μM cAMP (lane 3), CAP in the absence of cAMP (lane 4) and without CAP (lane 5).
B. The radiolabelled fragment was incubated with CAP (lane 1), with CAP and 0.2 μM cAMP (lane 2), with CAP and 1 μM cAMP (lane 3) and CAP with 5 μM cAMP (lane 4). In A and B the purine-specific cleavage of the radiolabelled *Eco*RI-*Ava*I fragment is shown on the left-hand side. The indicated base-pair numbering corresponds to that shown in Fig. 1.

3′-α-[^{32}P]-labelled restriction fragment (Fig. 3B). Within the protected regions are fragments whose rates of protection are enhanced. This is a common phenomenon in the footprinting procedure and is probably the result of protein-induced changes in the DNA structure (Valentin-Hansen 1982). Binding of the CAP protein to the *cytR* regulatory region was dependent on the presence and amount of cAMP in the reaction mixture (Fig. 3). Since expression of the *cytR-lacZ*$^+$ operon fusion is stimulated by the cAMP/CAP complex *in vivo* (Table 1) and since cAMP/CAP can bind *in vitro* to sequences upstream of the *cytR* promoter (Figs 1 and 3), we conclude that this activator complex plays a functional role in the expression of *cytR*.

Expression of cytR *is autoregulated*

Insertion of the transposable λp*lac*Mu55 bacteriophage (Bremer *et al.* 1988a) into *cytR*$^+$ disrupts the integrity of this gene and consequently no CytR protein is synthesized in Φ(*cytR-lacZ*$^+$)1 operon fusion strains. To test whether the CytR protein influences the expression of its own structural gene, we transformed the multicopy plasmid pCB008(*cytR*$^+$) and the vector pBR322 into strain GP103 carrying the chromosomal Φ(*cytR-lacZ*$^+$)1 operon fusion. Strain GP103 forms Lac$^+$ (red) colonies when plated on lactose-McConkey medium. The presence of pCB008 resulted in a Lac$^-$ (white) phenotype of the fusion strain, whereas pBR322 had no influence on the Lac$^+$ phenotype of GP103. When the expression of the Φ(*cytR-lacZ*$^+$)1 fusion in these strains was quantitated by β-galactosidase assays (Table 2), we found that the presence of the *cytR*$^+$ plasmid pCB008 reduced *cytR-lacZ*$^+$ expression approximately four-fold both in glycerol- and glucose-grown cells relative to the fusion strain carrying pBR322. The cloned *cytR*$^+$ gene specifically affected *cytR-lacZ*$^+$ transcription

Table 2. Repression of *cytR-lacZ* expression by the cloned *cytR* gene.

Strain	Description[a]	Plasmid	β-Galactosidase Activity[b] in Cells Grown in MMA With glycerol	β-Galactosidase Activity[b] in Cells Grown in MMA With glucose
GP103	Φ(*cytR-lacZ*$^+$)1	pBR322	115.6 ± 37.7	56.3 ± 15.1
GP103	Φ(*cytR-lacZ*$^+$)1	pCB008 (*cytR*$^+$)	26.7 ± 7.3	11.9 ± 2.1
GP105	Φ(*ompF-lacZ*$^+$)16–13	pBR322	829.6 ± 29.6	ND
GP105	Φ(*ompF-lacZ*$^+$)16–13	pCB008 (*cytR*$^+$)	844.4 ± 29.7	ND
GP100	Φ(*cytR-lacZ*$^+$)1	pHSG576	83.0 ± 3.4	44.5 ± 0.6
GP100	Φ(*cytR-lacZ*$^+$)1	pGP18 (*cytR*$^+$)	47.4 ± 8.2	23.4 ± 0.4
GP100	Φ(*cytR-lacZ*$^+$)1	pGP19 (*cytR**)	9.2 ± 0.2	8.6 ± 0.1
GP2	Φ(*tsx-lacZ*)hyb1	pHSG576	ND	533.3 ± 11.1
GP2	Φ(*tsx-lacZ*)hyb1	pGP18 (*cytR*$^+$)	ND	59.3 ± 0.4
GP2	Φ(*tsx-lacZ*)hyb1	pGP19 (*cytR**)	ND	23.1 ± 4.2

a. The various *lac* fusions are present as a single copy in the chromosome.

b. The strains were grown overnight in MMA with the indicated carbon source and the specific β-galactosidase activity was assayed as described by Miller (1972). The data shown are the mean values of at least three experiments and the error ratio in the β-galactosidase assays is given. A Δ*lac* strain was always included as a control. Plasmids pBR322 and pCB008 are multicopy plasmids; plasmid pHSG576 and its derivatives pGP18 and pGP19 are low-copy-number plasmids. ND = not determined.

since the expression of an *ompF-lacZ*$^+$ operon fusion was not influenced by CytR (Table 2).

To analyse the effect of CytR on *cytR-lacZ*$^+$ expression under more physiological conditions, we constructed a plasmid, pGP18, that carried the *cytR*$^+$ gene in the pSC101-derived, low- copy-number vector, pHSG576, which is present in the cell in approximately six copies per chromosome (Takeshita *et al.*, 1987). When pGP18 and pHSG576 were transformed into strain GP100, the expression of the chromosomal *cytR-lacZ*$^+$ operon fusion was reduced approximately two-fold in the presence of pGP18 both in glycerol- and glucose-grown cultures, whereas vector pHSG576 did not influence the expression of the fusion (Table 2). As a control, we introduced the same two plasmids into strain GP2 carrying the Φ(*tsx-lacZ*) hyb1 protein fusion, which is known to be regulated by CytR (Bremer *et al.*, 1988b). As expected, the expression of the *tsx-lacZ* fusion was reduced (approximately nine-fold) when pGP18 was present in the cells relative to the same fusion strain carrying pHSG576 (Table 2). As a further test for the specific effect of CytR on the transcription of its own structural gene, we transformed plasmid pGP19 into strains GP100(Φ(*cytR-lacZ*$^+$) and GP2(Φ(*tsx-lacZ*)hyb1) and determined the specific β-galactosidase activities. Plasmid pGP19 is analogous to the *cytR*$^+$ plasmid pGP18 but carries a mutationally altered *cytR* gene, *cytR**, which results in a four- to five-fold overproduction of the CytR regulatory protein (see below). In both strains, the expression of the fusions was further reduced relative to the strains carrying pGP18 (Table 2). Taken together, the data shown in Table 2 demonstrate that the expression of the chromosomal Φ(*cytR-lacZ*$^+$)1 operon fusion is specifically reduced by the CytR repressor, strongly suggesting that *cytR* transcription is autoregulated.

The *deoR*-encoded repressor plays an important role in the regulation of several genes whose products are involved in nucleoside uptake and metabolism (Hammer-Jespersen, 1983). We tested whether DeoR influences *cytR* expression by introducing, with phage P1*vir*, the Φ(*cytR-lacZ*$^+$)1 operon fusion into the *deoR* strain BRE2050. β-galactosidase activity in the resulting strain, GP103, did not differ from that measured in the *deoR*$^+$ strain, GP100 (Table 1), demonstrating that DeoR does not control the expression of the *cytR* gene.

The CytR* *mutation results in increased CytR synthesis*

The *cytR** mutation we have previously described (Bremer *et al.*, 1988b) is a non-allele-specific suppressor mutation in the cloned *cytR*$^+$ gene. The *cytR**-encoded gene product exerts negative control on the transcription initiating from both the wild-type *tsx* P2 promoter and the CytR operator mutant, O^c-1, located in this promoter. The suppressor phenotype conferred by *cytR** could result either from an altered repressor with different operator specificity or from increased synthesis of CytR, since the O^c-1 mutation in *tsx* still allows the binding of CytR to some extent (Bremer *et al.*, 1988b). To test whether synthesis of CytR is increased, we expressed the parent *cytR*$^+$ plasmid pCB008 and its *cytR** derivative, pGP1, in minicells. The *cytR** mutation led to synthesis of a polypeptide whose electrophoretic mobility is identical to that of the wild-type CytR protein (M_r = 37 800; Valentin-Hansen *et al.*, 1986a; Singer *et al.*, 1985) but which is produced in greater amounts. Densitomer scanning of the

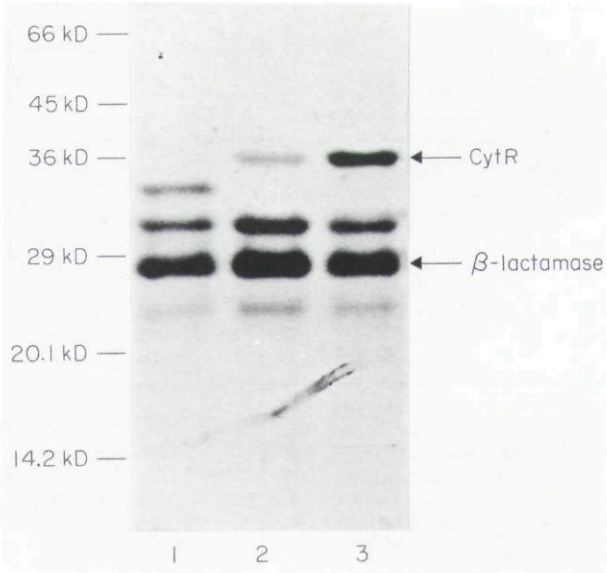

Fig. 4. Synthesis of CytR in minicells. Minicells from strain HB290 carrying the pBR322 (lane 1), pCB008(*cytR*$^+$) (lane 2), and pGP1 (*cytR**) (lane 3) plasmids, respectively, were radiolabelled with [^{35}S]-methionine, the proteins were electrophoretically separated on a 12% SDS-polyacrylamide gel, and newly synthesized proteins were visualized by autoradiography. The positions of CytR and β-lactamase are indicated by arrows, and the molecular weight standards are given in kD.

autoradiogram shown in Fig. 4 demonstrated that synthesis is increased approximately four- to five-fold (data not shown). These results suggest that the CytR* suppressor phenotype results from overproduction of the repressor protein and indicates that the *cytR** mutation affects either transcription or translation of the *cytR* regulatory gene.

The cytR* *mutation creates a new promoter*

To characterize the *cytR** mutation at the DNA level, we cloned a *Pvu*II-*Eco*RV fragment that carries the *cytR* regulatory region and part of the *cytR* coding sequences from pCB008(*cytR*$^+$) and pGP1 (*cytR**) into the *Sma*I site of vector M13mp18. We determined the *cytR* and *cytR** DNA sequences in parallel from position 230 bp in the coding region up to position −180 bp in the regulatory region (Valentin-Hansen *et al.*, 1986a). The *cytR** sequence differed from the wild-type *cytR* DNA sequence at only one position: a G-C base pair at position −34 bp relative to the start of translation was changed to an A-T base pair (Figs 1 and 2). Since this mutation is located downstream of the transcription initiation site of the *cytR*$^+$ gene (Fig. 1), we considered the possibility that the *cytR** mutation has created a new promoter. Inspection of the *cytR* DNA sequence around the mutation revealed the presence of putative −35 and −10 sequences (Harley and Reynolds, 1987) that were separated by 18 base pairs (Fig. 1). According to the algorithm of Mulligan *et al.* (1984), which allows estimation of promoter strength, this putative promoter gives a homology score of 49%. The *cytR** mutation, which lies within the −10 region of this putative promoter, increases the homology score to 60%, predicting a relatively efficient promoter (Mulligan *et al.*, 1984).

To test whether this putative promoter was functional in the *cytR** gene, we isolated the total RNA from strain BRE2050 carrying the *cytR** plasmid, pGP1, and determined the *cytR** transcriptional initiation site(s) using reverse transcriptase (Fig. 2, lane 2). In contrast to the results obtained with the *cytR*$^+$ gene, we found two mRNA species for the *cytR** gene. The longer one corresponds to the wild-type mRNA (Fig. 2, lane 1), while the second mRNA initiates at an 'A' residue at position −22 bp relative to the translational start site. This position is 31 bp downstream of the wild-type transcription initiation site and 8 bp downstream of the putative −10 region that is altered by the G-C to A-T transition in the *cytR** mutation (Fig. 1). The mRNA initiated at this new site is made in greater amounts than the wild-type transcript (Fig. 2, compare lanes 1 and 2).

Discussion

The number of CytR repressor molecules has been estimated to be approximately 100 per cell (Valentin-Hansen *et al.*, 1978) and there are nine genes whose expression is known to be under the control of this repressor (Hammer-Jespersen, 1983). We have studied the functional organization of the *cytR* regulatory region and the transcriptional control of *cytR* expression. The transcriptional initiation site of the *cytR* mRNA was mapped 53 bp upstream of the presumed GTG translational start codon for CytR (Fig. 1). Eight bp upstream of this site there is a hexamer (AAAAAT) that shows homology to the consensus Pribnow box of *E. coli* promoters (Harley and Reynolds, 1987); however, an appropriately spaced −35 region is not readily apparent. Promoters which deviate in such a way from the typical *E. coli* promoter sequences are often controlled by activator proteins (Raibaud and Schwartz, 1984). We found that the cAMP/CAP activator complex can bind *in vitro* to two regions upstream of the *cytR* promoter (Fig. 1) that show homology to the consensus cAMP/CAP binding site (de Crombrugghe *et al.*, 1984; Ebright *et al.*, 1984). This cAMP/CAP binding to the *cytR* regulatory region is of functional importance since *in vivo* the absence of the CAP protein results in a two-fold decrease in the expression of a chromosomal *cytR-lacZ*$^+$ operon fusion (Table 1). This cAMP/CAP-dependent stimulation of *cytR* expression is weak but indicates that the synthesis of the CytR repressor is linked to the metabolic status of the cell.

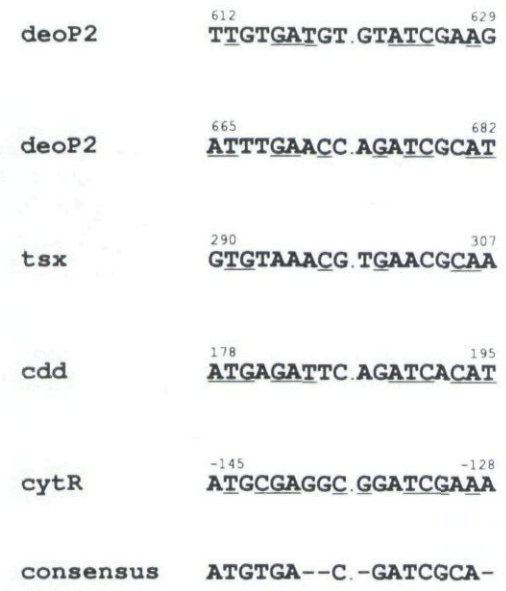

Fig. 5. Comparison of putative CytR operator sites. The putative CytR-binding sites from the *deo* P2 (Valentin-Hansen *et al.*, 1982), *cdd* (Valentin-Hansen *et al.*, 1989), *tsx* P2 (P. Valentin-Hansen, unpublished *results) and the cytR (Valentin-Hansen et al., 1986a) genes are* compared. Dyad symmetries are underlined and the centre of the symmetry is marked by a dot. Position numbers are listed above the alignment.

Additionally, we found that the synthesis of the CytR protein directed by a low copy-number *cytR*$^+$ plasmid resulted in a moderate (two-fold), but specific, reduction in the expression of a chromosomal *cytR-lacZ*$^+$ operon fusion (Table 2). Our data therefore suggest that the transcription of the chromosomal *cytR*$^+$ gene is autoregulated. The regulation of *cytR* expression appears to be very similar to that of other *E. coli* regulatory genes (*araC, fur* and *uidR*) (Casadaban, 1976b; De Lorenzo *et al.*, 1988; Blanco *et al.*, 1985). A comparison of the *cytR* regulatory region with other proposed CytR targets (Fig. 5) revealed a region of homology in *cytR*, around position −136 bp, that overlaps one of the regions recognized by the cAMP/CAP complex (−145 bp to −129 bp; see Fig. 1). Such an overlap, and the presence of two closely spaced cAMP-CAP binding sites, appears to be typical for promoters regulated by both CytR and cAMP/CAP and seems to be essential for CytR-mediated repression (Martinussen *et al.*, 1988). However, DNA-binding studies with purified CytR are required to assess whether CytR actually recognizes this DNA sequence motif.

Finally, we have analysed a previously described mutation in the cloned *cytR* gene (*cytR**) that results in the phenotypic suppression of a CytR operator mutation in the *tsx* P2 promoter (Bremer *et al.*, 1988). To gain insight into the mechanism by which the *cytR** gene confers the suppressor phenotype, we have analysed the *cytR** regulatory region and found that it differed at a single position from that of the wild-type gene. This nucleotide exchange leads to the activation of a cryptic promoter (P*) positioned downstream of the wild-type *cytR* promoter (P^{wt}). The mutational activation of cryptic promoters has also been described for the *E. coli lac* operon (Karls *et al.*, 1989; Rothmel and LeClerc, 1989). Transcription initiating from the newly created promoter in *cytR** is much stronger than that directed by the wild-type promoter and consequently results in increased synthesis of CytR. Thus, it is this overproduction of the CytR repressor that confers the suppressor phenotype associated with the *cytR** gene.

Experimental procedures

Bacterial strains, bacteriophages and plasmids

The bacterial strains, phages and plasmids used in this study are listed in Table 3. All strains are derivatives of *E. coli* K12.

Growth conditions, media and chemicals

Bacteria were grown aerobically at 37 °C in LB medium or minimal medium A (MMA) supplemented with 0.4% glucose or 0.4% glycerol (Miller, 1972; Silhavy *et al.*, 1984). Solid media were prepared as described by Miller (1972). Minimal medium was supplemented with a solution of methionine, valine, and isoleucine to a final concentration of 0.02% as required. Agar plates spread with 0.1 ml of a 10 mg ml^{-1} solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) in dimethylformamide were used to distinguish between LacZ$^-$ and LacZ$^+$ phenotypes. Kanamycin (Km), tetracycline (Tc), ampicillin (Ap) and chloramphenicol (Cm) were added to media at 40 μg ml^{-1}, 5 μg ml^{-1}, 50 μg ml^{-1} and 25 μg ml^{-1}, respectively. Restriction enzymes, T4 ligase, *E. coli* DNA polymerase I (large fragment), bacterial alkaline phosphatase, T4 polynucleotide kinase, bovine pancreas deoxyribonuclease I (DNaseI), and reverse transcriptase were obtained from New England Biolabs and Boehringer Mannheim. The γ-[^{32}P]- and α-[^{32}P]-labelled nucleotide triphosphates were from I.C.N. Corp. The CAP protein was purified as described by Ghosaini *et al.* (1988).

Genetic procedures and construction of bacterial strains

Standard techniques were used for the growth of bacteriophages and for generalized transduction with phage P1*vir* (Miller, 1972; Silhavy *et al.*, 1984). The *ompF-lacZ*$^+$ operon fusion was introduced into strain BRE2050 using a P1*vir* lysate grown on strain MH513; Lac$^+$ transductants were selected on lactose-minimal plates. To construct a chromosomal *cytR-lacZ*$^+$ operon fusion, we used the λp*lac*Mu system (Bremer *et al.*, 1984; 1988a). Strain BRE2047(*deoR*$^+$ *cytR*$^+$) was infected with phage λp*lac*Mu55 in the presence of the Mu*A*$^+$*B*$^+$ λpMu507 helper phage, and Km^R colonies were selected. Approximately 8000 colonies were pooled and washed twice with 10 ml of a 10 mM $MgSO_4$ solution; the cells were then resuspended in 5 ml of a 10 mM $MgSO_4$ solution. Mutations in *deoR* allow utilization of inosine but not of

Table 3. Bacterial strains, bacteriophages and plasmids.

Strain	Description[a]	Reference/origin
Strains derived from *E. coli* K12		
BRE2047	F⁻, *metB, ilv, rpsL,* Δ(*argF-lac*)*U*169	Bremer *et al.* (1988b)
BRE2048	F⁻, *metB, ilv, rpsL,* Δ(*argF-lac*)*U*169, *cytR*9	Bremer *et al.* (1988b)
BRE2050	F⁻, *metB, ilv, rpsL,* Δ(*argF-lac*)*U*169, *cytR*9, *deoR*8	Bremer *et al.* (1988b)
BRE2055	BRE2047, Δ(*crp*)96, *zhd-732*::Tn*10*	Bremer *et al.* (1988b)
GP2	BRE2048, Φ(*tsx-lacZ*)hyb1, (λp*lac*Mu9)	Bremer *et al.* (1988b)
GP100	BRE2047, Φ(*cytR-lacZ*⁺)1, (λp*lac*Mu55)	This study
GP103	BRE2050, Φ(*cytR-lacZ*⁺)1, (λp*lac*Mu55)	This study
GP104	BRE2055, Φ(*cytR-lacZ*⁺)1, (λp*lac*Mu55)	This study
GP105	BRE2050, Φ(*ompF-lacZ*⁺)16–13, (λp1(209))	This study
MC4100	F⁻, Δ(*argF-lac*)*U*169, *araD*139, *rpsL*150, *deoC*1, *relA*1, *ptsF*25, *flbB*5501, *rbsR*	Casadaban (1976a)
GD1	MC4100, *glpR, zih-730*::Tn*10*	G. Sweet
MH513	MC4100, Φ(*ompF-lacZ*⁺)16–13, (λp1(209))	Hall and Silhavy (1981)
TG1	Δ(*lac-pro*), *supE, thi, hsd*5/F′, *traD*36 *proA*⁺*B*⁺, *lacI*ᵠ, *lacZ*ΔM15	Carter *et al.* (1985)
HB290	*minB, rpsL, mgl*	G. Hazelbauer through K. Heller
Plasmids		
pBR322	Cloning vector	Bolivar *et al.* (1977)
pVH002	*cytR*⁺	Valentin-Hansen *et al.* (1986a)
pCB008	*cytR*⁺	Barbier and Short (1985)
pHSG576	Cloning vector	Takeshita *et al.* (1987)
pGP1	pCB008 with *cytR** mutation	Bremer *et al.* (1988b)
pGP18	pHSG576 with *cytR*⁺	This study
pGP19	pHSG576 with *cytR**	This study
M13mp18	Cloning vector	Norrander *et al.* (1983)
Bacteriophages		
λp*lac*Mu55	Mucts62, *ner*⁺, *A*′am1093, ′*uvrD*′, Mu*S*′, ′*trp*′ *lacZ*⁺, *lacY*⁺, *lacA*′ *kan, imm*λ	Bremer *et al.* (1988a)
λpMu507	Mucts62, *A*⁺*B*⁺, λcIts857, *S*am7	Bremer *et al.* (1988a)

The symbol Φ indicates the presence of a *lacZ* fusion, and the abbreviation 'hyb' indicates that the gene fusion encodes a hybrid protein. The symbols *lacZ*⁺ and *lacZ* denote *lacZ* genes with or without translational initiation signals, respectively. Strains carrying the λp*lac*Mu9 or λp*lac*Mu55 prophages are resistant to kanamycin. The genetic nomenclature is according to B. Bachmann (1983).

uridine, while strains carrying *cytR* mutations can grow efficiently on either nucleoside (Hammer-Jespersen, 1983). To enrich for λp*lac*Mu55 insertions in the *cytR* gene, we inoculated a 20 µl portion of these cells into 5 ml of MMA with 0.4% inosine as the carbon source and grew this culture overnight at 37 °C (Hammer-Jespersen, 1983). Serial dilutions of this culture were prepared, and 0.1 ml of each was spread onto inosine MMA plates containing 40 mM sodium citrate; the plates were incubated for two days at 37 °C. These colonies were then replica-plated onto inosine- and uridine-minimal plates with XG to identify LacZ⁺ (blue) CytR⁻(Ino⁺ Uri⁺) strains.

Methods used with nucleic acids and plasmid constructions

All standard DNA methods were performed as described (Maniatis *et al.*, 1982; Silhavy *et al.*, 1984). Plasmids pGP18 and pGP19 were constructed by ligating a 2.8 kb *Xho*II-*Xho*II restriction fragment from the *cytR*⁺ plasmid, pCB008 (Barbier and Short, 1985), and the *cytR** plasmid, pGP1 (Bremer *et al.*, 1988b), respectively, into the unique *Bam*HI site of the vector pHSG576 (Takeshita *et al.*, 1987). Restriction analysis showed that the orientation of the *cytR*⁺ and *cytR** genes in pGP18 and pGP19, respectively, is such that these genes are expressed from their own promoter and not from the *lacPO* regulatory region present in plasmid pHSG576. The construction and analysis of recombinant M13mp18 phage followed the procedures described by Messing (1983). DNA sequencing was done according to Sanger *et al.* (1977) with the modifications described by Biggin *et al.* (1984). Single-stranded ^{32}P-labelled hybridization probes were prepared from recombinant M13mp18 phage carrying the *Pvu*II-*Eco*RV restriction fragments isolated from pCB008 and pGP1 by *in vitro* primer extension of the universal *lac* primer. The radiolabelled double-stranded DNA was then cut with *Sma*I, and the primer fragment was isolated from a polyacrylamide gel. This *cytR*-specific primer was hybridized to total RNA isolated from LB-grown cultures (mid-log phase) of strain BRE2050, carrying either pCB008 or pGP1, by the hot acid-phenol technique. The probes were extended with reverse transcriptase as described by Honoré *et al.* (1986). The 530 bp *Eco*RI-*Ava*I restriction fragment which spans the *cytR* regulatory region was isolated from plasmid pVH002 (Valentin-Hansen *et al.*, 1986a) and was labelled with ^{32}P at the *Ava*I site at either the 5′-end using polynucleotide kinase or the 3′-end using the *E. coli* DNA polymerase I (large fragment).

The end-labelled fragments were incubated with a fixed amount of purified CAP protein in the presence of various concentrations of cAMP and treated with DNaseI. The reaction products were electrophoretically separated on a DNA sequencing gel as described (Galas and Schmitz, 1978; Valentin-Hansen, 1982). An A/G-specific sequencing reaction was performed with the radiolabelled *Eco*RI-*Ava*I fragment (Maxam and Gilbert, 1977), and the reaction products were electrophoresed along with those of the DNaseI protection assay to calibrate the sequencing gel.

Radiolabelling of proteins in minicells and SDS-polyacrylamide gel electrophoresis

Minicells were isolated from strain HB290 carrying plasmids pBR322, pCB008, and pGP1 (Table 3) as described by Reeve (1979). Labelling of minicells (0.5×10^9 cells) was performed in 500 μl of 0.4% glucose MMA using 12 μCi of [^{35}S]-methionine (1000 mCi mmol^{-1}, Amersham) for 1 h at 37°C. Minicells were then pelleted by centrifugation, washed once with 1 ml of TNE buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 1 mM EDTA), resuspended in 25 μl of sample buffer, and solubilized for 20 min at 50°C before loading onto a 12% SDS-polyacrylamide gel. The proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) and the separated proteins were visualized by staining with Coomassie Brilliant Blue. The radiolabelled proteins were detected by autoradiography using Kodak X-omatic 100 film. The autoradiogram was scanned using an LKB 2220 recording integrator.

β-galactosidase assay

Specific β-galactosidase activity was assayed as described by Miller (1972). The protein concentration was estimated from the optical density (at 600 nm) of the culture, assuming that an optical density at 600 nm of 1.4 corresponds to approximately 150 μg of protein per ml of culture (Miller, 1972).

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