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Amino Acid Substitutions in the CytR Repressor Which Alter Its Capacity To Regulate Gene Expression

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In *Escherichia coli*, transport and catabolism of nucleosides require expression of the genes composing the CytR regulon. Transcription initiation of cistrons in this gene family is activated by cyclic AMP-catabolite activator protein (cAMP-CAP), repressed by the CytR protein, and induced by cytidine. A random proofreading mutagenesis procedure and a genetic screen using *udp-lac* fusions have allowed the identification of distinct regions of the 341-amino-acid CytR polypeptide that are critical for repression of gene expression and response to induction. Determination of the ability of various CytR mutants to control gene expression in vivo indicated that the intrinsic affinity of the CytR protein for operator DNA is gene specific and that efficient repression of transcription by wild-type CytR is dependent on the interaction of CytR with cAMP-CAP. CytR mutants that were cytidine induction defective (CID) were characterized; these mutant proteins had only Asp-281 replaced. Data obtained with *cytRΔM149*, a dominant negative allele, indicated that the native CytR repressor is an oligomeric protein. Representative *cytR* mutations were combined with *cytRΔM149*, and the resulting hybrid repressors were tested for transdominance in a CytR$^+$ *E. coli* strain. Amino acid substitutions A209E and C289Y suppressed the transdominance of CytRΔM149, suggesting that these replacements alter the normal protein contacts involved in repressor subunit-subunit association. In contrast, amino acid substitutions located in the N-terminal portion of the CytR protein had no effect on the transdominance of CytRΔM149. The results from this study suggest that the CytR repressor is an oligomeric, allosteric protein in which conformational changes are required for repression and derepression.

Escherichia coli has the ability to recycle nucleosides it encounters in its environment into nucleic acids or to utilize these molecules as a source of carbon and energy (15, 24). The transport proteins and catabolic enzymes required for these processes are, in *E. coli*, expressed from genes composing the CytR regulon. Members of this gene family include unlinked genes such as *cdd*, encoding cytidine deaminase (CDase); *udp*, encoding uridine phosphorylase (UDP); the *deo* operon that directs the synthesis of purine and pyrimidine phosphorylases and enzymes required for deoxyribose utilization; *tsx*, which codes for an outer membrane protein involved in nucleoside permeation; genes involved in nucleoside transport; and the *cytR* gene itself (12, 15, 22). Expression of these CytR-controlled cistrons is subject to both positive and negative control. Transcription initiation is activated by the cyclic AMP-catabolite activator protein (cAMP-CAP) complex. Where the gene regulatory regions have been examined in detail, each DNA region required for activation has been shown to contain tandem cAMP-CAP binding sites (19, 41–43). Negative regulation requires the CytR repressor (23), and derepression is dependent upon only cytidine in *E. coli* (23). The *cytR* structural gene has been cloned (2), its nucleotide sequence has been determined (44), and the *cytR* gene product has been identified in vivo as a polypeptide having a subunit M_r of ≈37,000 (37). Comparison of the amino acid sequence deduced for the CytR repressor with sequences of other DNA-binding proteins revealed the presence of a helix-turn-helix motif located at the N terminus which is proposed to function in repressor-DNA interaction (5, 30, 31, 44). The control of transcriptional initiations from the genes composing the CytR regulon is thought to involve cooperative protein-protein contacts formed between DNA-bound cAMP-CAP complex and the CytR repressor (39). Such a mechanism raises many interesting questions concerning the nature of the protein contacts formed between CytR and activated CAP and the role of helix-turn-helix domain in determining specific interactions with operator DNA.

To effectively regulate gene expression from each cistron of the CytR regulon, the CytR protein can be envisaged as having a number of distinct domains that are required for repressor function. These domains, which may function independently of, in concert with, or in response to other regions of the repressor, are (i) a region of the protein required for interaction with DNA, (ii) a repressor surface region that participates in CAP-CytR protein-protein contacts, (iii) a repressor region that is involved in subunit oligomerization, and (iv) a repressor domain that functions in inducer binding. The results derived from this mutational analysis of the CytR protein have identified domains that are critical for repressor function and have suggested that this repressor is an allosteric protein in which subunit conformational changes may be required both for repression and for derepression.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids, and phages used in this study are described in Table 1. SS6003 was constructed by moving a *lac* deletion linked to marker *proAB*::Tn*10* from strain SS5066 into strain SS100 by P1 transduction. The Tn*10* insertion was removed in a transduction by using a P1 lysate prepared on MC4100 (*pro*$^+$ Δ*lac*).

Indicator strains allowing the identification of mutations in

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or bacteriophage	Characteristics	Source or reference
E. coli strains		
SS100	F⁻ *thi leu rpsL*	This study
SS113	Hfr *thi upp udp ton* Δ*deo* Δ*lac* Φ(*tsx-lac*) [λp1(209)]	This laboratory
SS5066	*araD* Δ(*argF-lac*) *relA rpsL flbB proAB*::Tn*10* λ⁻	This laboratory
SS6003	SS100, Δ(*argF-lac*)*U169*	This study
SS6004	SS6003, Φ(*udp-lac*)*6*(Hyb) (λRS45)	This study
SS6005	SS6003, Φ(*udp-lac*)*8* (λRS45)	This study
SS6018	SS6005, *cytR*::Tn*10*dTet	This study
SS6083	SS6004, *recA56 srlC300*::Tn*10*	This study
JM103	Δ(*lac-pro*) *thi rpsL supE endA sbcB15* /F' *traD36 proAB lacI*^{q} *lacZ*ΔM15	J. Messing
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR*	M. Casadaban
KH1214	F⁻ *mutD5 zaf13*::Tn*10*	E. C. Cox
JC10240	Hfr(PO45) *srlC300*::Tn*10 recA56 thr-300 ilv-318 rpsE300*	A. J. Clark
Plasmids		
pCB002	Ap^r, pBR322 derivative carrying an inactivating, partial deletion of the *cytR* gene	2
pCB008	Ap^r, pBR322 derivative carrying the wild-type *cytR* gene	2
pCB030	Ap^r, pUC19 derivative carrying the *udp* gene isolated from pVMK27	This study
pCB031	Ap^r, *Eco*RI deletion derivative of pCB030 that carries the *udp* control region plus the first 49 codons of the *udp* gene	This study
pCB038	Kan^r, pACYC184 derivative carrying the *udp* gene	This study
pCB053	Ap^r, pRS591 derivative containing a *udp-lac* protein fusion	This study
pCB056	Ap^r, pRS528 derivative containing a *udp-lac* operon fusion	This study
pCB071	Ap^r, a pCB008 derivative. The *cytR* gene contains unique *Nde*I and *Cla*I sites.	
pCB072	Kan^r, The *kan* gene isolated from pNK862 was inserted into the blunt-ended *Pst*I site of pCB071.	This study
pCB093	Kan^r, a pCB071 derivative; contains a *Sma*I⁻, *Hin*dIII⁻, *Cla*I⁻ *kan* gene generated by site-directed mutagenesis. The *cytR* gene was mutagenized to contain unique *Hin*dIII and *Apa*I sites. All changes are silent.	This study
pCB094	Kan^r, a *cytR* deletion derivative of pCB093.	This study
pVMK27	Tet^r, a pBR325 derivative carrying the *udp* gene	S. Kushner
pRS528	Ap^r, a pBR322 derivative used to construct operon fusions to *lacZ*	R. W. Simons
pRS591	Ap^r, a pBR322 derivative used to construct protein fusions to *lacZ*	R. W. Simons
Bacteriophages		
λCB8	λRS45, Φ(*udp-lac*)*8*	This study
λCB6	λRS45, Φ(*udp-lac*)*6*(Hyb)	This study
λRS45	Lambda phage engineered to allow in vivo recombination with *lac* gene fusions constructed on pRS528 and pRS591. This phage integrates at *att* λ of the bacterial chromosome.	R. W. Simons

the *cytR* structural gene were constructed as follows. SS6003 (Δ*lac*) was transformed with pCB053 and pCB056, and the appropriate transformants were lytically infected with λRS45 (36). Recombinant λRS45 derivatives carrying each *udp-lac* fusion were identified, isolated, and plaque purified. λCB8 carries the *udp-lac* transcriptional fusion of pCB056, and λCB6 carries the *udp-lac* translational fusion of pCB053. SS6003 was infected with either λCB6 or λCB8, yielding the Lac$^+$, single lysogens SS6004 [(*udp-lac*)*6*(Hyb)] and SS6005 [(*udp-lac*)*8*]. Next, the chromosomal copy of the *cytR* gene was inactivated to allow identification of mutations carried by a plasmid-borne *cytR* gene. SS6005 was transformed with pNK861 (47), and bacteria having *cytR*::Tn*10*dTet insertions were selected as fast-growing colonies on minimal agar medium containing 1% lactose, leucine (50 μg/ml), vitamin B_1, and tetracycline (15 μg/ml). Several primary mini-Tet insertions were then moved by P1 transduction into SS113 (*tsx-lac metB*), and Lac$^+$ Met$^+$ Tet^r transductants were identified, thereby confirming insertion of the minitransposon into the *cytR* gene. One secondary *cytR*::Tn*10*dTet insertion was moved, by P1 transduction, back into SS6005, yielding SS6018.

Media and growth conditions. The *E. coli* strains were grown at 37°C in (i) a minimal medium containing Vogel and Bonner salts (45) supplemented with vitamin B_1 at 5 μg/ml, individual amino acids at 50 μg/ml or 0.2% Casamino Acids, and either 0.2% glucose or 0.4% glycerol as the carbon source; (ii) 1% Bacto Tryptone containing 0.27% NaCl (TN medium); (iii) TN containing either 0.4% glycerol, 1% lactose, or glucose plus gluconate, 0.5% each, as the carbon source; (iv) L broth (20); or 2× YT (20). The Lac$^{+/-}$ phenotypes of the various strains were determined on a solid medium (TTC-Lac) containing 1% Bacto Tryptone, 0.27% NaCl plates containing 50 μg of tetrazolium chloride (Eastman) per ml, and 1% lactose. On this indicator medium, colonies lacking β-galactosidase activity are red whereas colonies synthesizing this enzyme are white. Where required, antibiotics were added to the media at the following final concentrations: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; and kanamycin, 25 μg/ml (minimal media) or 50 μg/ml (rich media). Solid media contained 1.5% agar.

General bacterial and molecular genetic techniques. Lysate preparations and transductions involving lambda or P1 phages were carried out as described by Silhavy et al. (35) and by Miller (20). Competent *E. coli* cells were prepared and transformed as described by Maniatis et al. (18). Plasmid DNA and DNA extracted from M13mp19 phages were isolated and digested, and their restriction digests were analyzed as described previously (18, 34). Specific DNA fragments used in *cytR* and *udp* subcloning experiments were isolated from preparative agarose gels by electroelution or by binding to GeneClean glassmilk (Bio 101, La Jolla,

Calif.). DNA ligations using T4 DNA ligase (New England BioLabs, Inc., Beverly, Mass.) were carried out in TA buffer prepared as described by O'Farrell et al. (25). New restriction sites in the *cytR* and *kan* coding sequences were introduced by using M13mp19 constructs and a site-directed mutagenesis kit (Amersham Life Sciences, Arlington Heights, Il.) according to the manufacturer's instructions.

Double-stranded DNA template for sequencing was prepared by the rapid lysis procedure described by Weickert and Chambliss (48). Dideoxy-chain termination sequencing (33) was performed by using [α-^{35}S]dATP and Sequenase version 2.0 (United States Biochemical Corp., Cleveland, Ohio).

Cloning of the *udp* gene regulatory region. Plasmid pVMK27, a pBR325 derivative containing the *udp* gene, was obtained from S. Kushner (1). The *udp* gene was excised from pVMK27 as a *Pst*I- and *Mlu*I-ended DNA fragment and subcloned into a pACYC184 derivative, yielding pCB038, and into pUC19, yielding pCB030. pCB031 is an *Eco*RI deletion derivative of pCB030 and contains the regulatory region and the first 49 codons of the *udp* gene. Indicator strain SS113 (*tsx-lac*) (2) was transformed with each plasmid, and the presence of a CytR-regulated *udp* gene and/or the titration of chromosomally expressed CytR repressor was demonstrated by enzyme assays.

Construction of *udp-lac* fusions on multicopy plasmids. A 310-bp fragment containing the *udp* regulatory region bounded by *Pst*I and *Eco*RI restrictions sites on pCB031 was subcloned into vectors pRS591 and pRS528, obtained from R. W. Simons (36). The resulting plasmids, pCB053 and pCB056, have *udp-lac* translational and transcriptional gene fusions, respectively. Both plasmids conferred a Lac$^+$ phenotype to SS6003 and were sequenced across the fusion joints to verify each construction.

Plasmid constructions. To allow the isolation of the *cytR* coding sequence following in vivo mutagenesis, an *Nde*I restriction site was introduced by site-directed mutagenesis at the start codon of the *cytR* gene. This mutation transforms the GTG start codon of the wild-type *cytR* gene into an ATG codon, a change that has no effect on the activity of the CytR repressor. The modified *cytR* coding sequence was subcloned into a derivative of pCB008 (2), altered to remove the vector *Nde*I and *Cla*I sites. The resulting plasmid, pCB071, now has unique *Nde*I and *Cla*I endonuclease cleavage sites in the *cytR* coding sequence. Plasmid pCB072 is a pCB071 derivative in which the kanamycin resistance gene (*kan*) of Tn*903* was inserted into the *bla* gene at the *Pst*I site. Plasmid pCB093 was constructed to increase the number of unique restriction sites within the *cytR* coding sequence. The *kan* gene was modified as indicated in Table 1 and replaced DNA bounded by the unique *Pst*I and *Eco*RI sites of pCB071. Plasmid pCB094 is a pCB093 derivative obtained by deletion of the entire *cytR* gene.

Generation, identification, and characterization of *cytR* mutations. The *mutD* strain KH1214 (7, 8), obtained from E. Cox, was grown in minimal glucose medium and transformed with target plasmid pCB071. Transformants were selected, purified, and maintained on minimal glucose-ampicillin medium. To induce mutagenesis, an inoculum of ~10^5 cells was grown in 5 ml of rich medium for 24 h. The plasmid DNA was purified; indicator strain SS6018 was transformed with mutagenized pCB071 and plated on TTC-Lac-ampicillin medium. Pink, CytR$^-$ colonies were purified, and their plasmids were isolated. The *cytR* coding sequence from each clone was transferred into nonmutagenized pCB071, and the selection was repeated. All resulting transformants were pink, indicating that the mutations were located within the *cytR* coding sequence. Mutations within *cytR* that altered the ability of this protein to respond to induction were identified by using TTC-Lac-antibiotic plates containing 2 mM cytidine.

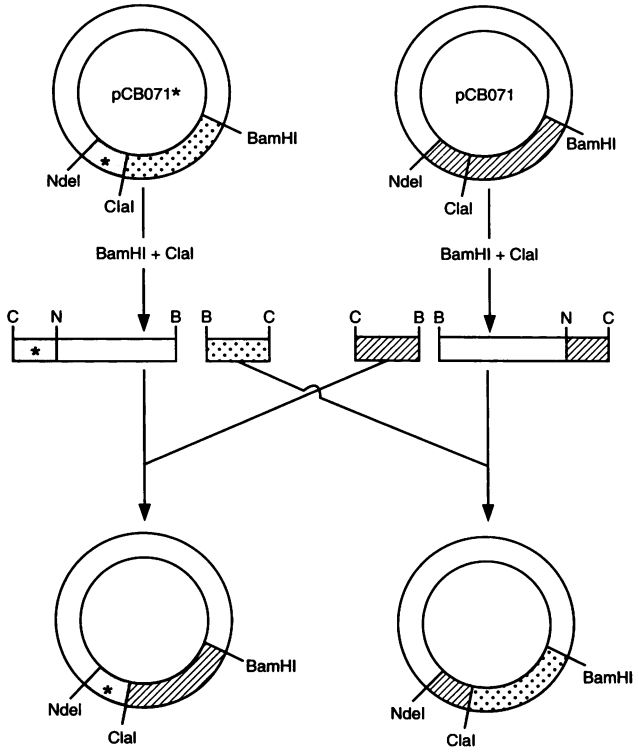

FIG. 1. Mapping of *cytR* mutations by fragment exchange. The hatched area of each plasmid represents the wild-type *cytR* gene, and the stippled and dotted areas represent 5′ and 3′ regions, respectively, of the mutagenized *cytR* gene. The open portion of each plasmid representation (both circular and linear) represents vector sequence and DNA 5′ to the *cytR* initiation codon. The endonuclease cleavage sites for *Nde*I, *Cla*I, and *Bam*HI are unique.

To determine whether the mutant genes directed the synthesis of full-length CytR protein in amounts comparable to that synthesized from the wild-type *cytR* gene, each CytR$^-$ strain was grown for ~18 h in 5 ml of 2× YT-ampicillin medium, and cells collected from 2 ml of culture were used for Western immunoblots. Plasmid DNA isolated from cells in the remaining 3 ml of culture was used to map the *cytR* mutations either upstream or downstream of the unique *Cla*I restriction site by fragment exchange (Fig. 1). In every case, only one *cytR* DNA fragment was found to determine the mutant phenotype, allowing the localization of the mutation and identification by DNA sequencing.

In other experiments, the *cytR* coding sequence from mutagenized pCB071 (Amp^r) was recloned directly into pCB072 (Kan^r), eliminating the need to purify the DNA fragments. When each *cytR* allele was transferred to pCB093, mutations located 5′ to the unique *Cla*I site were transferred on an *Nde*I- and *Cla*I-bounded fragment and those located 3′ to the *Cla*I site were transferred on a *Cla*I- and *Bam*HI-bounded fragment.

In this study, the *cytR* allele having an ATG start codon is referred to as the wild-type *cytR* gene. The designations for *cytR* mutations, and for the protein encoded by each mutant

TABLE 2. Expression from and regulation of the plasmid-borne *udp* gene

	E. coli strain	Induction with 2 mM cytidine	Sp act in cell extracts (U/mg of protein)	
			CDase	UDP
1a.	SS113(pVMK37)	−	94	6,500
1b.		+	202	13,957
2a.	SS113(pUC19)	−	8	0
2b.		+	252	0
3a.	SS113(pCB038)	−	32	800
3b.		+	197	13,082
4.	SS113(pCB030)[a]	−	64	67,000
5.	SS113(pCB031)	−	72	0

[a] Upon addition of cytidine, the growth rate of this strain markedly decreases, making a meaningful comparison of enzyme specific activities for induced and noninduced cells impossible.

allele, consisted of single-letter amino acid abbreviations indicating the substitution separated by the position in the CytR protein where the change occurred. Hybrid *cytR* genes and proteins have the appropriate designations separated by a hyphen.

Production of anti-CytR antibody. CytR protein was purified from an overproducing *E. coli* strain (3), resuspended in Freund's complete adjuvant, and used to immunize New Zealand rabbits. The rabbits were bled prior to the onset of the immunization regimen (preimmune serum) and 7, 14, and 21 days following injection of antigen.

Western blots. A single colony was grown at 37°C for ~18 h in 2× YT medium supplemented with the required antibiotic. Cells from 2 ml of culture were harvested, and the pellets were resuspended in 0.4 ml of a lysis solution previously described (37). The proteins in the cell lysate were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (37) and electrotransferred to an Immobilon-P membrane (Millipore Corp., Bedford, Mass.) according to the manufacturer's instructions. Western immunoblots were performed as described previously (32). The CytR-antibody complex was visualized by using biotinylated goat anti-rabbit antibody, avidin, biotinylated alkaline phosphatase (Vectastain kit from Vector Laboratories Inc., Burlingame, Calif.), and alkaline phosphatase substrate (Promega Corp., Madison, Wis.) according to the manufacturers' instructions.

Enzyme assays and analytical procedures. Bacteria used for enzyme assays were grown for ~18 h at 37°C in 5 ml of complete glycerol minimal medium supplemented with the required antibiotic. Experiments involving induction of CytR-regulated gene expression in exponentially growing cultures were carried out as described previously (37). The procedure to obtain cell extracts and the spectrophotometric assays for CDase (EC 3.5.4.5) and β-galactosidase (EC 3.2.1.23) have been described elsewhere (37). Uridine phosphorylase (EC 2.4.2.3) was assayed as described previously (34). One enzyme unit catalyzes the conversion of 1 nmol of nucleoside substrate to product per min per mg of protein at 30°C. The protein concentration of cell extracts was measured by the method of either Lowry et al. (17) or Bradford (4), using bovine serum albumin as the standard.

RESULTS

Construction of plasmids and *E. coli* strains that permit detection of mutant CytR repressors. On the basis of the nucleotide sequence reported for the *udp* gene (46), DNA encoding this nucleoside phosphorylase was cloned from pVMK27 into pUC19 and a pACYC184 derivative, yielding plasmids pCB030 and pCB038, respectively (Table 1). The data given in Table 2 demonstrate that both plasmids encode functional UDP. While *udp* expression from low-copy-number plasmid pCB038 responded to cytidine induction (Table 2, lines 3a and 3b), UDP synthesis from pCB030 was not regulated by chromosomally encoded CytR, as expected from the *udp* gene dosage (Table 2). Moreover, high-copy-number plasmid pCB030 and its UDP^- derivative pCB031 increased synthesis of CDase due to titration of the CytR repressor (Table 2, lines 4 and 5). These results demonstrate that both the regulatory and structural regions of the *udp* gene have been cloned and allowed the localization of the CytR interactive region to a 310-bp segment of the *udp* DNA carried by pCB030.

Mutations within the *cytR* gene were identified by using *E. coli* indicator strains having either a transcriptional or a translational *udp-lac* gene fusion. Each gene fusion was transferred to a λ phage and then introduced, in single copy, into a $CytR^+$ strain lacking the *lac* operon but which contained a wild-type *udp* gene, thus providing an internal monitor for CytR-regulated β-galactosidase expression. Enzyme assays demonstrated that β-galactosidase expression from both the operon and protein *udp-lac* fusions was

TABLE 3. Comparison of β-galactosidase synthesis from *udp-lac* gene fusions with enzyme synthesis from the wild-type *udp* gene

E. coli strain	Gene fusion type	*cytR* genotype[a]	Induction with 2 mM cytidine	Sp act (U/mg of protein) in cell extracts[b]	
				UDP	β-Galactosidase
SS6003(pCB002)	None	+/−	−	126	0
			+	565	0
SS6004(pCB002)	Protein	+/−	−	96	575
			+	671	4,620
SS6005(pCB002)	Operon	+/−	−	83	841
			+	584	5,168
SS6018(pCB002)	Operon	−/−	−	1,557	17,951
			+	1,564	17,794
SS6018(pCB071)	Operon	−/+	−	66	134
			+	405	2,429

[a] The symbol to the left of the slash indicates a functional (+) or defective (−), chromosomal *cytR* gene, and the symbol to the right of the slash designates the status of the plasmid-borne *cytR* gene.

[b] Determined for cell extracts prepared and assayed as described in Materials and Methods.

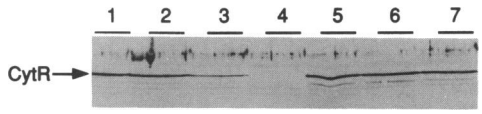

FIG. 2. Western blot analysis of wild-type and mutant CytR proteins. Equal amounts of cell extract protein prepared from cultures of SS6018 harboring plasmids expressing wild-type CytR, mutant CytR, or no CytR protein were prepared as described in Materials and Methods. The extracts were subjected to SDS-polyacrylamide gel electrophoresis, the proteins were transferred to an Immobilon-P membrane, and the CytR proteins were detected immunologically as described in Materials and Methods. All background bands were accounted for by probing the protein blots with preimmune rabbit serum. Cell extracts of SS6018 expressing mutant repressors from pCB071 and pCB072 produced results identical to those shown. Extracts analyzed were prepared from SS6018 harboring pCB093 derivatives expressing CytRS27F (lane 1), CytRA24V (lane 2), wild-type CytR (lane 3), no CytR protein (i.e., Δ*cytR*) (lane 4), CytRΔM149 (lane 5), CytRM151I (lane 6), and CytRM151V (lane 7).

regulated by the CytR repressor identically to the expression from the wild-type *udp* gene (Table 3). Insertional inactivation of the chromosomal *cytR* gene, as in SS6018 (*cytR*::Tn*10*dTet), resulted in constitutive expression of both UDP and β-galactosidase, and regulated expression from both genes was simultaneously restored by introduction of CytR$^+$ plasmid pCB071 (Table 3).

Selection and characterization of mutant CytR repressor proteins. In order to locate domains of the CytR protein that mediate the repression of gene expression, defective regulatory proteins encoded by a plasmid-borne gene were identified in SS6018. To increase the chances of finding stable repressor proteins having a functional rather than a structural defect, mutant SS6018(pCB071) transformants expressing partially active repressors, i.e., those producing pink colonies on TTC-Lac medium, were selected. After showing that their colony phenotype was due to mutations mapping to the *cytR* coding sequence, we subjected all potential CytR mutants to a Western blot screen to verify that the subunit size and the steady-state levels of mutant and wild-type repressors were equivalent.

By following this strategy, 11 partially active CytR mutants were identified from 16 independent mutagenesis experiments. In addition, a spontaneous totally inactive mutant, designated CytRΔM149, was isolated during the construction of pCB071. When the mutants were examined by Western immunoblots, a unique anti-CytR reactive band ($M_r \simeq 37{,}000$) was obtained for all partially active mutant repressor proteins (Fig. 2, lanes 1, 2, 6, and 7) and for the totally inactive mutant repressor encoded by *cytRΔM149* (lane 5), demonstrating that the subunit size for each CytR mutant was identical to that of the wild-type CytR protein (lane 3). In addition, since equal amounts of cells were analyzed in the Western blots, the data suggest that the mutant repressors were all synthesized in amounts that were equal to or greater than that observed for wild-type CytR. The enhanced intensity for the CytR band obtained with the cell extracts having mutant repressors is consistent with the known *cytR* autoregulation (12).

The mutations were mapped in relation to the unique *Cla*I site located within the *cytR* gene by fragment exchange (Fig. 1) and identified by DNA sequencing. Table 4 shows that the mutations were not evenly distributed throughout the protein but were predominantly confined to two clusters. The first cluster consists of four mutations (alleles *cytRV15A* through *cytRL30S*) that map to the proposed helix-turn-helix domain of the CytR protein (44). The second grouping contained three mutations (alleles *cytRΔM149*, *cytRM151V*, and *cytRM151I*), located in the central portion of the CytR primary amino acid sequence.

The ability of each CytR mutant to regulate gene expression was examined by measuring its influence on enzyme synthesis from the CytR-regulated genes *cdd* and *udp* and on the *udp-lac* gene fusion. The data given in Table 4 show that there is a clear correlation between CytR repressor function and colony phenotype on TTC-Lac medium for all CytR mutants except for the repressor encoded by allele *cytRM151V*. This data also indicate that not all mutant CytR repressors were capable of regulating transcription from the

TABLE 4. Summary of *cytR* mutations and their effects on CytR repressor efficiency[a]

cytR allele	*cytR* mutation		Increase in sp act		
	bp[b]	Nucleotide change	CDase	UDP	β-Galactosidase
cytRV15A	44	T→C	2.3	1.1	2.2
cytRA24V	71	C→T	2	1.9	2
cytRS27F	80	C→T	2.3	1.5	2.5
cytRL30S	89	T→C	1.9	1.4	1.7
cytRT40P	118	A→C	5	2.4	2.4
cytRY53C	158	A→G	4.3	3.1	3.8
cytRΔM149	445–447	Deletion	12	10	12
cytRM151V	451	A→G	0.3	0.7	0.6
cytRM151I[c]	453	G→A	1.6	2.1	2.5
cytRA209E	626	C→A	2	2.8	3.5
cytRC289R	865	T→C	4.5	4.4	5.1
Δ*cytR*[d]			17.7	10	12

[a] The indicator strain, SS6018 (*cytR*) harboring plasmids expressing each CytR mutant, was grown for ~18 h in minimum glycerol medium. CDase, UDP, and β-galactosidase activities were assayed as described in Materials and Methods. The results are presented as the ratio obtained by dividing the enzyme specific activity determined for the CytR mutant cell extract by the specific activity determined for that enzyme in a cell extract prepared from SS6018(pCB093). Plasmid pCB093 encodes wild-type CytR. SS6018(pCB093) had specific activity values for CDase, UDP, and β-galactosidase of 60, 214, and 1,515 U/mg of protein, respectively.

[b] Base pair numbering refers to the *cytR* coding sequence such that the A of the ATG initiation codon is assigned bp 1.

[c] Isolated twice independently.

[d] Control plasmid pCB094 in which the entire *cytR* gene is deleted.

TABLE 5. Effect of carbon source on the regulated expression of β-galactosidase from a *udp-lac* fusion[a]

cytR allele	β-Galactosidase sp act (U/mg of protein) in tryptone medium supplemented with:		
	Control	Lac	Glu/Gluc
Δ*cytR*	61,117	4,891	672
Wild type	1,134	560	200
cytRM151V	1,262	2,594	473
cytRM151I	2,940	2,270	557

[a] Strain SS6018 expressing wild-type CytR and each CytR mutant from a pCB093 derivative plasmid was grown for ~18 h in unsupplemented TN-kanamycin medium and in this tryptone medium supplemented with either 1% lactose (Lac) or with 0.5% glucose plus 0.5% gluconate (Glu/Gluc). Cells from each culture were collected, cell extracts were prepared, and the β-galactosidase specific activities were determined as described in Materials and Methods.

TABLE 6. Effect of coexpression of wild-type and mutant *cytR* genes on enzyme synthesis from *cdd*, *udp*, and *udp-lac*[a]

cytR gene	Sp act (U/mg of protein)		
	CDase	UDP	β-Galactosidase
Δ*cytR*	37	179	1,161
Wild type	13	102	313
cytRΔM149	133	653	5,429
cytRA209E	29	400	2,461

[a] *E. coli* SS6005 expressing each CytR mutant repressor from a pCB093 derivative plasmid was grown for ~18 h at 37°C in glycerol minimal medium. CDase, UDP, and β-Galactosidase activities were assayed as described in Materials and Methods.

reporter genes in an identical fashion. Specifically, synthesis of CDase and UDP (and β-galactosidase from *udp-lac*) responded uniquely to mutant repressors encoded by alleles *cytRT40P* and *cytRM151V*. For *cytRT40P*, the derepression of CDase synthesis relative to UDP synthesis was reproducibly increased twofold, whereas with *cytRM151V*, the level of UDP synthesis was reproducibly increased twofold compared with the level of CDase synthesis.

Regulation of gene expression by mutant repressor CytRM151V. As noted above, when tested for its ability to regulate gene expression in cells that were grown in glycerol minimal medium, the mutant CytRM151V repressor was found to be equivalent to, if not more efficient than, wild-type protein in controlling enzyme synthesis from the *cdd* and *udp* genes (Table 4). However, this CytR mutant was isolated on the basis of a colony phenotype indicative of partial repressor activity. When β-galactosidase activity (CDase or UDP activity [3]), was measured in bacteria expressing either CytRM151V or CytRM151I following growth in a liquid medium identical in composition to the solid indicator medium, CytRM151V was found to be defective only in bacteria grown in lactose-containing medium, whereas repression by CytRM151I was equally impaired in bacteria grown in the absence or presence of lactose (Table 5). This surprising observation was confirmed by strain reconstruction and shown to be due exclusively to the presence of the plasmid-borne *cytRM151V* allele. Consideration of the facts that *udp* (and thus *udp-lac*) expression is activated by cAMP-CAP (15) and that CytR repression of transcription from *deoP2* and *tsxP2* requires cAMP-CAP (11, 38) plus the observation that β-galactosidase synthesis decreased in both $CytR^-$ and $CytR^+$ control strains grown in lactose-containing medium (Table 5) suggested that repression of enzyme synthesis from *udp-lac* is also sensitive to catabolite repression. To test this possibility, the experiment was repeated by using a medium containing glucose plus gluconate which would produce a marked decrease in the cellular cAMP-CAP concentration (10). In the presence of these sugars, β-galactosidase synthesis measured for all strains was reduced as expected, but in bacteria expressing either wild-type or mutant CytR, the β-galactosidase activity was lower than that found for the control $CytR^-$ strain. The data in Table 5 shows that *udp-lac* repression produced by wild-type CytR was at least two- to fourfold stronger than that produced by CytRM151V under conditions producing catabolite repression. These results, which were obtained from the study of β-galactosidase synthesis as a function of repressor type and the carbohydrate added to the medium, suggest that (i) even under growth conditions producing severe catabolite repression, when synthesized from multicopy plasmids, both wild-type and mutant proteins retain some capacity to repress gene expression and (ii) repression of *udp-lac* expression by CytRM151V is more dependent on the cellular cAMP-CAP concentration than is repression produced by wild-type CytR.

Studies with a dominant negative CytR mutant. The transdominance of the protein encoded by the most defective *cytR* allele, *cytRΔM149*, expressed from a plasmid was tested in a genetic background having a chromosomal, wild-type *cytR* gene. Comparison of enzyme synthesis from the CytR-regulated reporter genes in SS6005 ($cytR^+$, but otherwise isogenic with SS6018) showed that CytR synthesis from the additional copies of the plasmid-borne, wild-type gene increased repression two- to fourfold (Table 6). In contrast, the presence of subunits encoded by *cytRΔM149* resulted in a 7- to 17-fold derepression of enzyme synthesis relative to the same strain expressing the wild-type repressor from pCB093 (Table 6). This data demonstrates that *cytRΔM149* is a dominant negative allele. The dominant phenotype of CytRΔM149 may be explained in two ways. First, as a result of the Met-149 deletion, the mutant repressor could be in a conformation that would activate gene expression and compete with wild-type repressor for operator sites. However, the genetic and biochemical data presently available demonstrate that CytR is strictly a negative regulator of transcription initiation (11, 23, 39). Accordingly, deletion of a single codon would not be expected to transform the CytR repressor into an activator of gene expression, although this possibility will be tested in DNA footprinting studies. The second explanation for the CytRΔM149 transdominance data proposes that the active form of the CytR repressor is an oligomer. Overexpression of mutant subunits that are defective in repression of gene expression but still able to oligomerize would titrate the wild-type subunits, thereby increasing gene expression by preventing formation of active, wild-type repressor oligomers. Data supporting the second proposal have been obtained from experiments that examined the size of wild-type CytR contained in cell extracts prepared from cells grown in glycerol minimal medium (3). When the cell extracts were fractionated by centrifugation through sucrose density gradients or by gel filtration chromatography, the immunoreactive CytR fractions were found between the CDase (dimer, subunit M_r = 32,540) and purine nucleoside phosphorylase (hexamer, subunit M_r = 25,950) standards, suggesting that native CytR repressor is at least a dimer. This information plus our CytRΔM149 transdominance data are consistent with the interpretation that derepression resulted from titration of

TABLE 7. Influence of secondary mutations within the *cytRΔM149* gene on CytR repressor subunit association[a]

cytR gene	β-Galactosidase sp act (U/mg of protein)	Ratio of β-galactosidase sp act,[a] CytR hybrid/ CytRΔM149
cytRΔM149	2,187	1.0
cytRA24V-ΔM149	1,955	0.9
cytRT40P-ΔM149	1,890	0.9
cytRY53C-ΔM149	2,376	1.1
cytRC289Y-ΔM149	1,309	0.6
cytRA209E-ΔM149	963	0.4

[a] *E. coli* SS6083 (*cytR*$^+$ *udp-lac recA56*) harboring a pCB093 derivative expressing each of the *cytR* alleles indicated was grown for ~18 h at 37°C in TN medium containing 50 μg of kanamycin per ml and 0.4% glycerol. β-Galactosidase activity was assayed as described in Materials and Methods.
[b] A ratio of 1.0 indicates that the second mutation introduced into the *cytRΔM149* gene did not affect the interaction of mutant, hybrid repressor subunits with the subunits encoded by the wild-type *cytR* gene. A ratio less than 1.0 indicates that the second mutation introduced into the *cytRΔM149* gene reduced formation of hybrid/wild-type heteroligomeric repressors.

wild-type CytR subunits by oligomerization-competent, repression-defective CytRΔM149 mutant subunits.

The transdominance of the other *cytR* alleles was also investigated. Except for *cytRA209E*, none were found to have a measurable effect on the expression of the reporter genes in SS6005 (3). Expression of *cytRA209E* in SS6005 produced a two- to eightfold increase in enzyme activity compared with SS6005 expressing the wild-type protein from both the chromosome and plasmid (Table 6).

Examination of wild-type and mutant CytR subunit association. If the transdominance of CytRΔM149 results from the titration of wild-type subunits, then subtle changes in repressor subunit association produced by other *cytR* mutations might be detected by constructing gene hybrids with the *cytRΔM149* allele. Any mutation which affects the efficiency of subunit oligomerization should suppress the transdominance of repressor subunits having the Met-149 deletion. For this approach, it is assumed that each mutation of the hybrid gene will affect CytR function independently. CytR repressor double mutants were constructed in vitro by combining the mutations encoded by alleles *cytRA24V*, *cytRL30S*, *cytRT40P*, *cytRY53C*, *cytRA209E*, and *cytRC289R* with the *cytRΔM149* mutation. These constructions were verified by DNA sequencing, the repressor encoded by each hybrid gene was shown to be inactive when tested in SS6018 (*cytR*), and the amount of each hybrid CytR subunit synthesized in SS6018 was shown by Western blots to be equivalent to that for subunits having a single amino acid substitution (3). Each hybrid *cytR* gene was expressed in SS6083 (*cytR*$^+$ *recA56 udp-lac*). The influence of each secondary mutation on the transdominance of CytRΔM149 is shown by the data given in Table 7. Clearly, the mutations found in alleles *cytRL30S*, *cytRT40P*, and *cytRC53Y* do not affect repressor subunit oligomerization, since *udp-lac* expression was equally derepressed in bacteria synthesizing mutant CytR repressor either from the *cytRΔM149* allele or from the hybrid *cytR* genes. The data obtained for the *cytRL30S* hybrid were identical to the data for the *cytRA24V* double mutant (3). However, hybrid repressor subunits encoded by genes having either the *cytRC289Y* or the *cytRA209E* mutation combined with the *cytRΔM149* mutation were impaired in their interaction with wild-type CytR subunits. That is, repression of transcription from *udp-lac* increased (β-galactosidase specific activities decreased) in bacteria expressing these CytR double mutants because of an increase in the cytoplasmic concentration of wild-type CytR repressor (Table 7). These data also suggest that the loss of repressor function produced by the C289Y substitution results from changes in the normal pattern of protein-protein contacts formed during assembly of wild-type subunits into an oligomeric repressor. The results obtained with the CytRA209E-ΔM149 hybrid protein were unexpected, since repressor subunits encoded by each single mutant allele were either strongly (CytRΔM149) or weakly (CytRA209E) transdominant (Table 6). The data given in Table 7 show that CytRA209E-ΔM149 hybrid subunits do not efficiently interact with wild-type repressor subunits despite the fact that subunits of both single and double mutants were synthesized in equivalent amounts (3).

TABLE 8. Synthesis of CytR-controlled enzymes in *E. coli* expressing either wild-type CytR or mutant CID repressors[a]

cytR allele	Induction with 2 mM cytidine	Sp act (U/mg of protein) in cell extracts		
		CDase	UDP	β-Galactosidase
Wild type	−	4	79	373
	+	59	575	6,072
cytRD281N	−	3	66	260
	+	5	101	397
cytRD281Y	−	5	79	412
	+	7	105	509

[a] Strain SS6018 (*cytR*) harboring plasmids expressing each allele was grown in minimum glycerol medium as described previously (2). Cell extracts were prepared, and the enzyme activities were measured as described in Materials and Methods.

Isolation and characterization of CytR mutants that do not respond to cytidine induction. Cytidine induction-defective (CID) CytR mutants were isolated following in vivo proofreading mutagenesis of the *cytR* gene carried by pCB071. Two mutants were purified, the *cytR* mutation of each was mapped and sequenced, and the presence of the proper-size CytR polypeptide was verified by the Western blot assay. Both CID CytR repressors (which were independently derived) contained a substitution for Asp-281 of wild-type CytR. SS6018 expressing either CID CytR repressor produced only dark red colonies on TTC-Lac medium containing 10 mM cytidine, a concentration of inducer 10- to 20-fold higher than that required to produce an observable phenotypic change with SS6018 expressing wild-type repressor. The data given in Table 8 corroborate the phenotype noted for the CID CytR mutants on TTC-Lac medium. In contrast to the 7- to 16-fold increase in enzyme specific activities obtained upon addition of cytidine to exponentially growing CytR$^+$ SS6018(pCB093), addition of inducer to cultures of SS6018 expressing either CytRD281N or CytRD281Y resulted in less than a 50% increase in the specific activities for these CytR-regulated enzymes.

DISCUSSION

The use of random proofreading mutagenesis and a genetic screen employing *udp-lac* fusions has allowed the identification of distinct regions of the 341-amino-acid CytR polypeptide that are critical for repressor function. This survey identified two regions of the CytR repressor that are involved in repression of gene expression and one region that mediates the responsiveness of repressor to the inducer, cytidine. In addition, the fact that a dominant negative *cytR*

allele was obtained indicates that the active form of the CytR repressor is an oligomer.

Four of the *cytR* mutations reported here change amino acids in the proposed DNA-interactive, helix-turn-helix motif of the CytR protein (5, 44). All of these mutations reduced the ability of the CytR protein to efficiently repress gene expression. Two mutations encode conservative substitutions (V15A and A24V), whereas the two others encode a change in side chain polarity (L30S) and hydrodynamic volume (S27F). By analogy with the role of helix-turn-helix domains in other bacterial repressors (5, 6, 26), we propose that these mutations weaken the interaction of CytR with its DNA operator site. A more extensive analysis of the CytR helix-turn-helix domain using saturation mutagenesis has revealed that missense mutations, which did not alter the steady-state amount of CytR protein, all encoded repressors with partial activity similar to the four helix-turn-helix mutant repressors described in this study (9). Mutations *cytRV15A* and *cytRS27F*, which are equivalent to *lacIV9A* and *lacIS21F* mutations, respectively (13, 31), are of particular interest. Both *cytR* mutations are recessive and yield a repressor with partial activity, whereas the comparable *lacI* mutations are dominant and yield defective repressors (13). The partial defect in repressor function imparted by amino acid substitutions in the putative helix-turn-helix domain is perhaps attributable to the fact that these substitutions do not alter residues directly involved in CytR-DNA interaction. Alternatively, the overexpression of the mutant proteins from a plasmid-borne gene and/or the cooperative interaction between activated CAP and CytR protein (11, 27, 39) might compensate for the effects that single amino acid substitutions have on protein-DNA interaction. Perhaps studies using either an in vivo indicator system involving expression of mutant repressors from the bacterial chromosome or an in vitro biochemical system using purified proteins will provide insight into the effects that individual missense mutations have on the interaction of CytR with operator DNA.

One additional point should be mentioned concerning CytR-DNA interaction. The CytR protein appears to interact with the different operators composing the CytR regulon with unequal specificity and/or affinity. This suggestion derives from the observation that several *cytR* mutations isolated in this study differentially affect the ability of the mutant proteins to repress synthesis of CDase and UDP (and β-galactosidase from *udp-lac*; Table 4) and from an independent study of CAP mutants altered in their interaction with CytR (39). Since the same pattern of regulation was obtained regardless of whether the indicator bacteria expressed wild-type CAP and mutant CytR or mutant CAP (impaired in its interaction with CytR) and wild-type CytR, these data sets are consistent with the suggestion that the intrinsic affinity of the CytR repressor for operator DNA is gene specific.

The regulation of the genes composing the CytR regulon requires both CytR repressor and activated CAP protein (16, 21, 23, 42). Data from a biochemical examination of the *deoP2* and *tsxP2* promoters indicated that efficient interaction of CytR with operator DNA is dependent upon the interaction of repressor with DNA-bound cAMP-CAP complex (11, 38, 40). Thus, a defect in repressor function may result from impaired CytR-CAP interaction as well as from altered repressor-DNA interaction. The mutant protein encoded by allele *cytRM151V* is particularly interesting in this respect. In *E. coli* expressing this *cytR* allele, repression of *udp-lac* expression was dependent on the degree of catabolite repression (i.e., the cellular cAMP-CAP level) to which the bacteria were subjected (Table 5). These results, which were obtained in vivo with *udp-lac* fusions, are consistent with the in vitro studies of CytR-*deoP2* interaction (27, 40) and show that efficient control of gene expression by wild-type CytR repressor is dependent on its interaction with activated CAP. The fact that CytR repressors producing markedly different phenotypes were obtained by substitution of Met-151 with two hydrophobic residues that differ in side chain size plus the loss-of-function mutant produced by deletion of Met-149 signals the importance of this region of the CytR protein. Recently, we constructed a CytR protein lacking the last 22 C-terminal amino acids which was stable but inactive when expressed alone and transdominant when synthesized concomitantly with the wild-type repressor (3). In these studies, the transdominance of CytRΔ320-341 was not affected by either Met-151 substitution or by the Met-149 deletion which would be as expected if the C-terminal CytR domain was responsible for repressor-CAP interaction. Collectively, the CytRΔM149, CytRM151V, CytRM151I, and CytRΔ320-341 mutant repressors define two domains that are important for repression of gene expression. We propose that the CytR domain containing amino acids 149 through 151 functions in signal transduction between the N-terminal, DNA-interactive helix-turn-helix domain and the C-terminal, cAMP-CAP interactive domain of the CytR protein.

The transdominance of CytRΔM149 provides additional information relative to the active form of the wild-type repressor. The results obtained with this CytR mutant suggest that the dominant phenotype of CytRΔM149 results from the formation of inactive, mixed oligomers containing wild-type and CytRΔM149 subunits. Thus, the active, repression-competent form of the wild-type CytR repressor would exist, in the bacterial cell, as an oligomer.

Since the CytRΔM149 data suggest that mutant subunits form inactive heteroligomers with wild-type subunits, any secondary *cytR* mutation that alters repressor subunit association might also suppress the transdominance of mutant subunits having the Met-149 deletion. This assumption was tested by constructing hybrid repressor genes in which the *cytRΔM149* mutation was combined with mutations located in either the N-terminal (*cytRA24V*, *cytRL30S*, *cytRT40P*, and *cytRY53C*) or C-terminal (*cytRA209E* and *cytRC289Y*) region of the CytR protein. None of the N-terminal amino acid substitutions modified the transdominance of repressor subunits carrying the Met-149 deletion (Table 7). In contrast, chimeric repressor genes containing the *cytRΔM149* mutation in combination with either the *cytRA209E* or *cytRC289Y* mutation encoded subunits that were significantly impaired in their ability to interact with wild-type repressor subunits (Table 7). At present, a clear understanding of the structural alterations produced by the A209E and C289Y substitutions is not possible and must await solution of the CytR crystal structure. However, from the genetic approach described in this study, we tentatively conclude that the A209E-ΔM149 combination may nonspecifically alter the conformation of the protein surface involved in subunit-subunit contact, while mutant allele *cytRC289Y* encodes a repressor specifically impaired in its ability to oligomerize. This proposal is consistent with the weak transdominant phenotype of CytRA209E and with the facts that CytRC289Y is the weakest repressor having a missense substitution in our collection (Table 4) and is recessive when coexpressed with the wild-type protein. The influence of amino acids around residue 300 on subunit assembly has recently been examined by using oligonucleotide saturation mutagenesis. From the initial mutant screen, a number of mutations that alter CytR

residues 305 through 311 were found (3). These mutations, like *cytRC289Y*, encode inactive repressors, do not reduce synthesis of repressor protein, and when combined with the *cytRΔM149* mutation, suppress the dominant negative phenotype produced by repressor subunits carrying the Met-149 deletion. Thus, amino acids 290 through 310 may define the CytR domain responsible for subunit association.

The last region of the CytR polypeptide to be identified in this study is represented by the CID mutants. Like the mutations that influence subunit oligomerization, the mutations producing CID repressors map to the 3′ end of the *cytR* gene and encode substitutions of Asp-281. The formation of a bond between the Asp-281 carboxyl group and the cytidine 4-amino group could serve as the trigger for induction of CytR-controlled gene expression. This specific interaction between repressor and inducer could explain why uridine, which has the same conformation as cytidine (14, 28, 29), is not an inducer of CytR-regulated gene expression in *E. coli*. The question of whether CID mutants are defective in cytidine binding or in their response to bound cytidine is currently being examined. Induction could result from cytidine-dependent conformational changes that modify the cooperativity of CytR-CAP interaction or the efficiency of repressor-operator interaction. Given the proximity of the *cytR* mutations producing CID and subunit association-defective repressors, it is tempting to speculate that cytidine binding actually prevents oligomerization of CytR subunits.

The CytR repressor and the LacI, GalR, PurR, and MalI repressors have a high degree of amino acid similarity and have been proposed to belong to the same family of DNA-binding proteins (30, 31). Although our mutational analysis would not be expected to disclose all of the mutation-sensitive amino acids in the CytR protein, comparison of our results with the mutation-sensitive sites of the LacI repressor reveals some interesting features (13). Nine CytR mutation-sensitive amino acids align with mutation-sensitive sites of LacI and produce similar functional defects in both proteins. Strikingly, the *cytR* mutations proposed to affect CytR-CAP interaction align with LacI regions that are mutationally silent (13). Thus, comparison of the positions of amino acid substitutions in CytR and LacI with the phenotype for each mutation suggests that the functional domains of both repressors (and thus GalR, PurR, and MalI) might be organized in a similar fashion. This alignment of repressor functional domains also lends support to our positioning of the CytR domains proposed to interact with cAMP-CAP complex. As additional *cytR* mutations are collected, it will be informative to determine whether mutations producing similar functional defects continue to cluster in comparable repressor regions and whether mutations that uniquely affect each protein map to distinct repressor regions.

The results obtained from our genetic and biochemical studies of the *E. coli* CytR repressor are consistent with the interpretation that this *trans*-acting, negative regulator of gene expression is an oligomeric, allosteric protein that undergoes distinct conformational changes for both repression and induction. Our working model proposes that the protein-protein contacts resulting from interaction of CytR oligomer with DNA-bound CAP could elicit reorientation of the N-terminal helix-turn-helix domain, thereby permitting efficient interaction with operator DNA. The binding of cytidine to its C-terminal inducer binding site is thought to trigger subunit conformational changes that result in a loss of affinity for either DNA or CAP protein or possibly result in depolymerization of the repression-competent CytR oligomer. Genetic and biochemical studies currently in progress will test the validity of this model for the regulation of gene expression by the CytR repressor.

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