

Positive and Negative Transcriptional Regulation of the *Escherichia coli* Gluconate Regulon Gene *gntT* by GntR and the Cyclic AMP (cAMP)-cAMP Receptor Protein Complex

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The *gntT* gene of *Escherichia coli* is specifically induced by gluconate and repressed via catabolite repression. Thus, gluconate is both an inducer and a repressor of *gntT* expression since gluconate is a catabolite-repressing sugar. In a *gntR* deletion mutant, the expression of a chromosomal *gntT::lacZ* fusion is both high and constitutive, confirming that GntR is the negative regulator of *gntT*. Indeed, GntR binds to two consensus *gnt* operator sites; one overlaps the -10 region of the *gntT* promoter, and the other is centered at $+120$ with respect to the transcriptional start site. The binding of GntR to these sites was proven in vitro by gel retardation assays and in vivo by site-directed mutagenesis of the binding sites. Binding of GntR to the operators is eliminated by gluconate and also by 6-phosphogluconate at a 10-fold-higher concentration. Interestingly, when *gntR* deletion strains are grown in the presence of gluconate, there is a twofold decrease in *gntT* expression which is independent of catabolite repression and binding of GntR to the operator sites. This novel response of *gntR* mutants to the inducer is termed ultrarepression. Transcription of *gntT* is activated by binding of the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex to a CRP binding site positioned at -71 upstream of the *gntT* transcription start site.

Escherichia coli metabolizes gluconate via the Entner-Doudoroff pathway (EDP) (6, 12, 13), which is, in addition to the gluconate transport and gluconate kinase activities, specifically induced by gluconate (15). Recent experiments suggested that some of the genes involved in gluconate metabolism are required for *E. coli* to colonize the mouse large intestine (43). It was shown that at least the *eda* gene, which encodes the 2-keto-3-deoxy-6-phosphogluconate aldolase of the EDP, and the *gntP* gene, which encodes one of the four gluconate transporters of *E. coli* (33), play a crucial role during the colonization (22, 43).

Two sets of genes are involved in transport and phosphorylation of gluconate (1, 20, 51). The main system, GntI, contains *gntT* and *gntU*, encoding high- and low-affinity gluconate transporters (approximate K_m s of 6 and 212 μ M), respectively, and *gntK*, a thermoresistant gluconokinase (8). The GntII system, which was discovered in a GntI deletion mutant, contains *gntW* and *gntV*, encoding another high-affinity gluconate transporter and a thermosensitive gluconokinase (20, 45). Thus, there are four known gluconate transporters, including GntP (22). Together with three other *E. coli* proteins of unknown function (Dsdx, ORF0454, and ORF1388), these seven proteins comprise a novel transporter family (33, 48). There is controversy as to why *E. coli* possesses so many gluconate transporters and which are expressed during growth on gluconate. Recent results show that *gntT* and *gntU* are expressed during growth on gluconate (34, 35, 45). In contrast to *gntU*, *gntT* shows a pronounced peak of expression very early in the logarithmic phase and is expressed at lower levels in late logarithmic phase (35).

It was also found that *gntT* is maximally induced by 0.5 mM gluconate, whereas *gntU* shows the highest expression in medium with 10 to 100 mM gluconate (unpublished data). Thus, it appears that GntT is important for growth on low gluconate concentrations, for entry into logarithmic phase, and for catabolism of gluconate and glucose (35).

Previous genetic studies indicated that the GntI system is, together with the EDP genes *edd* and *eda*, negatively regulated by the *gntR* gene product (10, 45, 11). GntR belongs to the GalR-LacI family of regulators and possesses an N-terminal helix-turn-helix DNA-binding motif, suggesting that GntR fulfills a regulatory role similar to that of LacI for the *lac* operon (45, 46, 47). Recently we postulated a consensus sequence for a *gnt* operator which was found in all gluconate-inducible genes (35). Furthermore, the genes of the GntI system are subject to catabolite repression. Interestingly, gluconate is itself a catabolite-repressing sugar (36). In this study, we show for the first time that the operator sequence is indeed the binding site for the negative regulator GntR and that gluconate is the true inducer of *gntT*, by inactivation of GntR binding to the operator. The results in this report also demonstrate that *gntT* is regulated by the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex, which was found to be essential for full induction.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains, plasmids, and phages used in this study are listed in Table 1. All chromosomal single *gntT::lacZ* fusion were constructed in P90C; DH5 α was used for constructing and propagating plasmids. *E. coli* strains were routinely grown at 37°C in Luria broth (LB) (28) with or without added carbohydrate (0.4%), and growth was monitored by measuring the turbidity (optical density [OD]) with a Spectronic 601 (Milton Roy Co.) spectrophotometer.

Construction of single-copy *gntT::lacZ* fusions. *gntT::lacZ* operon and protein fusions, present in the chromosome as lambda lysogens in single copy, were constructed by using the system of Simons et al. (41). DNA fragments of the

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TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Relevant genotype	Source or reference
<i>E. coli</i> strains		
DH5 α	<i>lacZ</i> Δ M15 <i>recA</i>	Bethesda Research Laboratories
P90C	<i>ara</i> Δ (<i>pro-lac</i>) <i>thi</i>	41
DPB271	<i>recD</i> ::Tn10 F ⁻ λ ⁻	B. Bender
HT28	W3110 Δ <i>cya</i> ::Kan ^r	19
RH74	Δ <i>cya-851 ilv</i> ::Tn10	26
RH77	Δ <i>cya-851 Δcrp zhd-731</i> ::Tn10	26
HT216	RH74 <i>gntR</i> ::Kan ^r	This study
M15	<i>recA</i> ⁺ <i>uvr</i> ⁺ F ⁻ <i>mtl gal ara lac</i> (pREP4)	50
NP100	P90C (ARS88 <i>gntT</i> :: <i>lacZ</i>)	35
PR201	NP100 <i>gntR</i> ::Kan ^r	This study
Plasmids		
pQE-30	Expression vector; His ₆ affinity tag	42
pRS551	<i>bla</i> ' <i>lacZ</i> ⁺ (operator fusion vector)	41
pRS552	<i>bla</i> ' <i>lacZ</i> ⁺ (protein fusion vector)	41
pTC229	pTC221 Kan ^r in <i>Stu</i> I	This study
pNP-41	<i>gntR</i> in pQE30 (<i>gntR</i> overexpression)	This study
pNP-52	<i>crp</i> in pQE30 (<i>crp</i> overexpression)	This study
Phage λ RS88 <i>bla</i> ' <i>lacZ imm</i> ⁴³⁴ <i>ind</i>		
		41

promoter region of *gntT* were amplified by PCR with primers carrying either an *Eco*RI or a *Bam*HI site, one at each end. These fragments were cloned into the vector pRS551 (operator fusion vector). Single-copy fusions were made by homologous recombination with the lambda phage λ RS88 and integration of the lysogenic phage into the chromosome (41). The copy number of the fusion in the chromosome was checked by the method of Powell et al. (37).

Site-directed mutagenesis. PCR was used to generate mutations in the operator sequences of the *gntT* gene. Mutations within the internal operator sequence were produced by PCR using a set of mismatched 3' primers carrying substitutions and insertions within the internal operator site (P102 to P106) and a 5' primer with the wild-type sequence (P231) (16). These DNA fragments were cloned into the operon fusion vector pRS551. Mutations within the external *gnt* operator sequence and the CRP binding site were generated by a three-step recombinant PCR method first described by Higuchi et al. (16). In a separate reaction, two overlapping primary PCR products were synthesized by using outside primers (P230 and P231) of the *gntT* fragment and overlapping, mismatched inside primers (P206B to P221B) containing the same mutation. The two PCR products were purified by electroelution from an agarose gel and subjected to a second PCR to form a heteroduplex product. This secondary PCR product, containing the mutation in both strands, was amplified with the two outside primers. Double mutations were constructed by the same method, by using DNA containing the first mutation as a template for the three-step recombinant PCR method. The PCR products were cloned into the operator fusion vector pRS551 and then recombined into λ RS88 for construction of single-copy fusions as described above. The following primers were used. Wild-type primers P230 (5'-GCGGATCCCCGATAGCAACAATGACTAATG-3') and P231 (5'-CGGAATTCTGAAAGGTGTGCGGATCTCAC-3') were used to amplify the entire promoter region of *gntT*; wild-type primer P101 (5'-GCGGATCCCCA TTTGTTATGGGTAAACGTCATTT-3') and primers P102 (5'-GCGGATCCCCA TTTGTTATGCAAGGTAACGTCATTT-3'), P103 (5'-GCGGATCCCCA TTTGTTATGCAAGGTAACGTCATTT-3'), P104 (5'-GCGGATCCCCA TTTGTTAT GGCAGCAGTCAATTT-3'), P105 (5'-CATGCTTTTCATTTGCGTGG GTAACGTCAA-3'), and P106 (5'-GCGGATCCATTGTTCTGGGTAACGT CAATTT-3') were used to create mutations within the internal *gnt* operator site; primers P206 (5'-TGAATGATACGGTTCGACATCTGGCGTTT-3'), P206B (5'-AAACGCCAGATGTCGACCGTATCATTC-3'), P208 (5'-ATGATACGGG TACCCATGGCGTTTGGAGAA-3'), P208B (5'-TTCTCAAACGCCATGGG GTACCCGATCAT-3'), P211 (5'-CATGTAATGACCCGGTAAACATCT GGC-3'), P211B (5'-GCCAGATGTTACCCGGGTCATTCACATG-3'), P212 (5'-CCAGATGTTACCATGGTATCATTACA-3'), and P212B (5'-TGTGAA TGATACCATGGTAAACATCTGG-3') were used to create mutations within the external *gnt* operator site; primer P220s (5'-TGAGAGGTTGTCGACTT ATCGCGGGA-3'), P220B (5'-TCCC CGGATAAGTTCGACCAACCTCTC A-3'), P221 (5'-TTTAAATTCGATGGTTGGTTCATAT-3'), and P221B (5'-ATATGACCAACCATCGATAATTTAA-3') were used to create mutations within the cAMP-CRP binding site. All DNA fragments carrying PCR-generated mutations were verified by DNA sequence analysis.

DNA band migration retardation. DNA band migration retardation analyses were performed as described by Carey (5). DNA fragments used in the assays

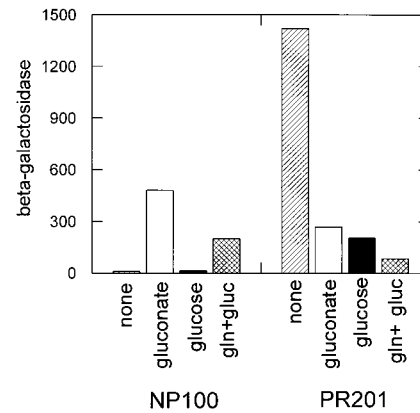


FIG. 1. Expression of the *gntT*::*lacZ* operon fusion in the wild type (NP100) and in a *gntR* mutant strain (PR201). The strains were growing in LB plus 0.4% of the indicated carbon sources. β -Galactosidase activities were measured in late log growth phase.

were labeled with [α -³²P]dATP during PCR. The binding reactions contained, in a 20- μ l final volume, the labeled DNA fragment, 2 μ g of sonicated herring sperm DNA, 4 mM Tris-HCl (pH 7.0), 5 mM sodium chloride, 2 mM magnesium chloride, 7.5% glycerol, 2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. For some experiments, the labeled DNA was first digested with a restriction enzyme and then purified by Sephadex-G50 chromatography. Protein, sugars, and cAMP were added as indicated. The samples were incubated for 10 min at room temperature and loaded onto a 5 or 6% polyacrylamide gel (20 cm by 16 cm by 1 mm thick; 29:1 acrylamide/bisacrylamide). The gel was equilibrated overnight at 4°C in 1 \times Tris-borate-EDTA buffer (pH 8.3) and prerun for 2 h at 200 V prior to sample loading. For the loading of the samples, the voltage was increased to 300 V. As soon as the last sample had entered the gel, the voltage was reduced to 200 V. After electrophoresis for 1 to 2 h, the gel was dried on paper at 80°C and autoradiographed on Kodak BIOMAX MS film.

Isolation of GntR and CRP. GntR and CRP were isolated by using the His tag modification system from Qiagen (Hilden, Germany) (17, 42). A 1,130-bp DNA fragment containing the coding region of the *gntR* gene was synthesized by PCR using primers P701 (5'-GCGGATCCATGAAAAGAAAAGACCCCGTAC-3') and P702 (5'-GGGGTACCGTGCCCCACAATACAAGAA-3'), and a 634-bp DNA fragment containing the coding region of the *crp* gene was synthesized by using primers PCR1 (5'-GCGGATCCATGGTGTGGCAAACCGCAAA-3') and PCR3 (5'-GGGGTACCACGGGATTAACGAGTGCCGTA-3'). After being digested with the endonucleases *Bam*HI and *Kpn*I, the fragments were cloned into the vector pQE30, which contains an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible T5 promoter (4). The resulting plasmids, pNT70 and pNT82, containing the *gntR* and *crp* genes, respectively, fused to six histidine codons at the 5' end, were transformed into *E. coli* M15, which overexpresses the *lacI* gene from pREP4 (50). For the isolation of the recombinant proteins, the resulting *E. coli* strains M15(pNT70) and M15(pNT82) were grown in LB medium to an OD at 600 nm (OD₆₀₀) of 0.8 or 1.5, respectively. Expression of the proteins was induced by the addition of 1 to 2 mM IPTG for 2 to 4 h. The cells were then suspended in cold buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 10 mM imidazole) and disrupted by sonication. Cell debris was removed by centrifugation, and the supernatant was mixed with nickel-nitrilotriacetate matrix and incubated for 2 h on ice. The matrix was washed three times with buffer (50 mM sodium phosphate [pH 6.0], 300 mM NaCl, 10% glycerol, 20 mM imidazole). The proteins were eluted with the latter buffer, containing 250 mM imidazole. The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), carried out as described by Laemmli (25); the gel was stained with Coomassie brilliant blue as described by Sambrook et al. (40).

β -Galactosidase assays. β -Galactosidase activity was determined in permeabilized cells as described by Miller (30) and is expressed in Miller units. Each value is the average of at least three independent measurements.

Genetic methods. Transduction was performed as described by Lennox (27), using bacteriophage P1. A *gntR* (kanamycin-resistant [Kan^r]) insertion mutant was constructed as follows. A 1,020-bp Kan^r gene block (Pharmacia, Piscataway, N.J.) was ligated into the *Stu*I site of plasmid pTC221, carrying the *gntR* gene on a 3.4-kb DNA fragment. The resulting plasmid, pTC29, was digested with *Pvu*II, purified by electroelution from an agarose gel, and transformed by electroporation into *E. coli* DPB271. Kan^r transformants were analyzed by PCR.

Chemicals and enzymes. [α -³²P]dATP was purchased from Amersham International (Buckinghamshire, United Kingdom). Biochemicals and endonucleases were from Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany), or Sigma (St. Louis, Mo.).

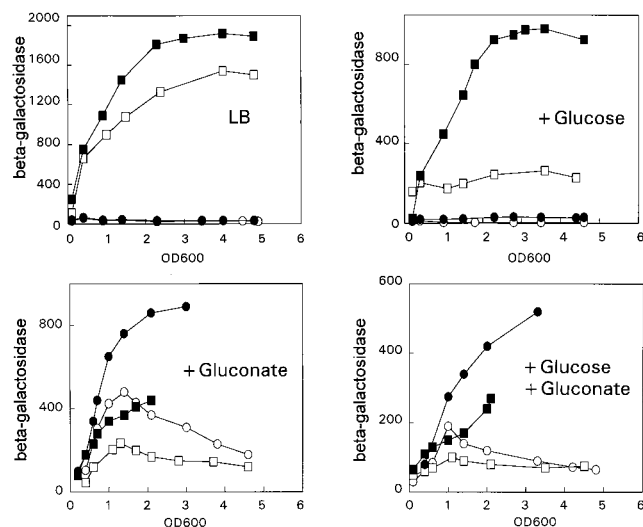


FIG. 2. Expression of the *gntT::lacZ* fusion in wild-type and *gntR* mutant strains in the presence and absence of cAMP. The cells were grown in LB medium with carbon sources as indicated. Symbols: circles, wild type (NP100); squares, *gntR* mutant (PR201); open and filled symbols, in the absence and presence, respectively, of 5 mM cAMP. β -Galactosidase was measured in the cultures at the indicated OD₆₀₀ points.

RESULTS

Effect of the *gntR* mutation on *gntT* expression. To investigate the role of GntR in repression of the *gntT* gene, the expression of a single-copy chromosomal *gntT::lacZ* fusion was measured in a *gntR* mutant. This *gntR* null mutant was constructed by insertion of a Kan^r cassette into the *StuI* site of the *gntR* gene, followed by linear transformation and specific recombination in a *recD* strain and then transduction of the *gntR* mutation into the *gntT::lacZ* fusion strain *E. coli* NP100 to create *E. coli* PR201. The *gntT::lacZ* fusion was fully derepressed in *E. coli* PR201 and showed a threefold higher β -galactosidase activity in the absence of gluconate compared to the fully induced level in the wild type grown on gluconate (Fig. 1). This result confirms that GntR is the negative regulator of *gntT*.

Catabolite repression of *gntT*. Expression of the *gntT::lacZ* fusion in the *gntR* mutant (PR201) remained subject to catabolite repression. Growth of *E. coli* PR201 in the presence of gluconate, glucose, or the mixture of glucose plus gluconate resulted in 5-, 7-, or 18-fold repression, respectively (Fig. 1). As expected, this catabolite repression order follows the relative cAMP concentrations measured for cells grown on these sugars (14). Thus, gluconate serves not only as an inducer of *gntT*

but also as a repressor, most likely via cAMP-dependent catabolite repression.

Next, cAMP-dependent catabolite repression was investigated in detail. The effect of exogenous cAMP on expression of the *gntT::lacZ* fusion was measured in the wild-type and *gntR* mutant strains during growth on LB and on LB containing glucose, gluconate, or a mixture of both sugars at different time points in batch cultures (Fig. 2). In the wild-type strain, there was no induction of β -galactosidase activity in the absence of gluconate, regardless of the presence of cAMP, which again indicates that the repression by GntR is cAMP independent. In wild-type cells grown in the presence of gluconate, the addition of cAMP caused a significant increase in the β -galactosidase activity, confirming that catabolite repression of the *gntT* gene is cAMP dependent (Fig. 2). Addition of cAMP to the wild type growing under catabolite-repressing conditions was in no case able to increase expression of the *gntT::lacZ* fusion to the fully derepressed level of the *gntR* mutant. Also, cAMP addition to the *gntR* mutant strain growing on glucose caused a fivefold increase in β -galactosidase activity, but again to a level lower than that of *E. coli* PR201 grown on LB without added sugar (Fig. 2). Thus, addition of cAMP was not sufficient to fully relieve catabolite repression. The recent finding that expression of the *crp* gene is modulated by catabolite-repressing sugars, including gluconate, may at least partially explain why addition of cAMP does not fully overcome catabolite repression (18).

To investigate the role of the CRP and adenylate cyclase in catabolite repression of the *gntT* gene by glucose and gluconate, *crp* and *cya* null mutations were transduced into the strain carrying the chromosomal *gntT::lacZ* fusion. In the presence of gluconate, both the *crp* and the *cya* mutants showed fourfold-lower β -galactosidase activity (Table 2). However, this low level of expression was still gluconate inducible. As expected, addition of cAMP to the *cya* mutant, but not to the *crp* mutant, relieved the catabolite repression to a level equivalent to that of the wild type. In summary, these results demonstrate that *gntT* is positively regulated by CRP.

Site-directed mutagenesis of the putative operator sites of the *gntT* promoter. Two putative *gnt* operators were identified on the basis of similarity to sequences found in all other gluconate-inducible promoters (35). To check whether the postulated *gnt* operator sites within the *gntT* promoter region are in fact binding sites for the GntR protein, the effects of mutations within these sequences were measured. Mutations of the internal operator of the *gntT::lacZ* fusion were constructed by a recombinant PCR method using mismatched primers (Fig. 3). Mutations of the external operator site were generated by using a three-step PCR (Fig. 3). The activities of these fusions were determined from single-copy fusions generated by homol-

TABLE 2. Effects of the *cya* and *crp* mutations on *gntT::lacZ* expression

<i>E. coli</i> strain	Relevant genotype	β -Galactosidase activity (U) with indicated carbon source					
		None		Gluconate		Glucose	
		-cAMP	+cAMP ^a	-cAMP	+cAMP	-cAMP	+cAMP
NP100	Wild type	10	10	460	680	0	0
RH112	<i>crp</i>	20	15	105	70	0	0
HT110	<i>cya</i>	30	25	115	620	0	0
RH114	<i>crp cya</i>	0	10	90	60	0	0
RH216	<i>crp gntR</i>	50	60	45	35	50	55
HT216	<i>cya gntR</i>	75	585	55	380	60	290

^a Added to a final concentration of 5 mM.

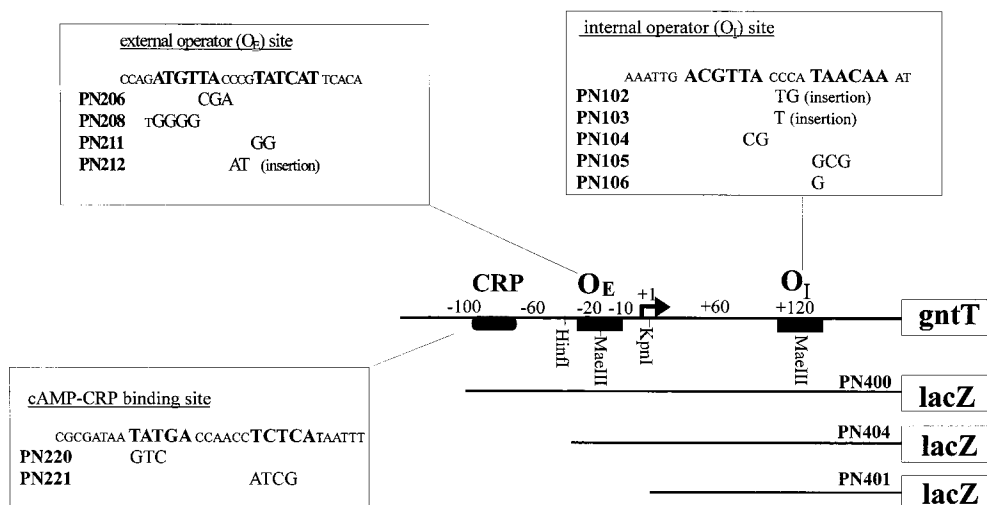


FIG. 3. Schematic representation of the *gntT* promoter region showing the site-directed mutations within the two operator sites and the cAMP-CRP binding site. The binding sites are shown in boldface; the specific mutations are indicated below the wild-type binding sequences. The deletion subclones (PN400, PN401, and PN404) of the *gntT* promoter region are shown under the line map.

ogous recombination with λ RS88 followed by integration into the chromosome.

Replacement of highly conserved bases within the internal operator site in strains PN104 and PN105 caused constitutive expression of β -galactosidase in cells grown in medium without gluconate (Table 3). Even the replacement of a single conserved base (PN106) caused a significant derepression of *gntT*::*lacZ* fusion activity. Insertion of two base pairs (TG) into the spacer sequence of the operator site (strain PN102) resulted in a similar constitutive expression of the *gntT*::*lacZ* fusion (Table 3), suggesting that the length of the spacer is crucial for binding of GntR. Even a single-base-pair insertion in mutant NP103 had a significant effect on the regulation of the *gntT*::*lacZ* fusion. None of the internal operator mutations were affected with respect to catabolite repression by glucose or gluconate. These results demonstrate that the internal operator site is indeed an important regulatory sequence. Thus, it appears that

any change to the highly conserved operator site is sufficient to affect GntR binding.

Strains NP206, NP208, and NP212 carry different substitutions or insertions within the external operator site (Fig. 3), which overlaps the -10 region of the promoter. Each of these mutations showed a level of β -galactosidase activity in the absence of gluconate which was twice as high as that of the internal operator mutants and similar to the level measured in the *gntR* mutant grown in the absence of sugar. External operator site mutants grown in medium with gluconate or the mixture of gluconate and glucose showed a level of catabolite repression equivalent to that of the wild type. Cells grown with glucose exhibited a derepressed level of β -galactosidase which was almost twofold higher than that of the internal operator mutants. The mutation in strain PN211, with a two-base substitution in the external operator which also replaces the most highly conserved region of the -10 hexamer, TATCAT, with GGTCAT, resulted in only a low constitutive synthesis of β -galactosidase under all growth conditions. The double mutant NP1256, which contains mutations in both operator sites, was constructed by using one of the mutant *gntT*::*lacZ* fusion as the template for a second site-directed mutagenesis. This strain showed a level of β -galactosidase equivalent to the fully derepressed level of the *gntR* mutant. In summary, the results clearly demonstrate that both operators are required for repression of *gntT* by GntR. Of the two operators, it appears that the external operator is perhaps the more important, since these mutants are derepressed to a greater extent than the internal operator mutants.

Mutations of the cAMP-CRP binding site. Two potential binding sites for the cAMP-CRP complex within the promoter region of *gntT* were identified on the basis of similarity to the consensus binding site, positioned at -71 (TATGAccaacTC TCA) and -13 (TGTTAccgtaTCATT) with respect to the transcriptional start site (Fig. 3). The latter CRP binding site overlaps the external operator site and could be a hybrid site for both proteins. However, the effects of base pair substitutions in the left half of this site (NP206 and NP208) that should have eliminated cAMP-CRP binding did not affect the positive regulation of *gntT*. On the other hand, replacement of three or

TABLE 3. Effects of mutations in the regulatory region of *gntT*

<i>E. coli</i> strain	β -Galactosidase activity (U) with indicated carbon source(s)			
	None	Gluconate	Glucose	Gluconate + glucose
NP100 (wild type)	10	510	0	280
NP102	780	520	100	320
NP103	280	490	60	260
NP104	840	490	100	330
NP105	920	500	90	310
NP106	110	610	40	300
PN206	1,320	560	190	190
PN211	60	80	40	40
PN208	1,260	460	170	210
PN212	1,180	480	160	190
PN220	0	130	60	60
PN221	0	110	50	70
NP1256	1,460	640	190	280
PN400	0	590	0	290
PN401	10	10	0	0
PN404	0	140	40	60

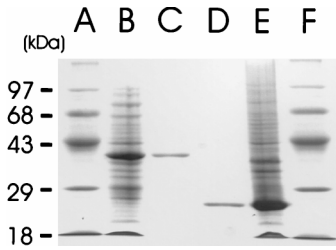


FIG. 4. Overexpression and purification of GntR and CRP, demonstrated by SDS-PAGE of the purified His-tagged proteins. Lanes A and F, molecular weight standards; lanes B and E, cell extracts of strains M15(pNP-41) and M15(pNP-52) after IPTG induction; lanes C and D, purified GntR and CRP, respectively.

four base pairs within the left or right half of the putative cAMP-CRP binding site at -71 bp upstream of the transcriptional start site resulted in a strong decrease in β -galactosidase activity in strains PN220 and PN221 (Table 3), respectively, a phenotype similar to that observed in the *crp* and *cya* mutants. These results clearly demonstrate that the cAMP-CRP binding site at -71 is indeed involved in the catabolite repression and the positive regulation of *gntT*. PN220 and PN221 remained gluconate inducible, again indicating that induction and catabolite repression are independent.

Deletion mutants. To check whether additional regulatory elements are present within the *gntT* promoter region, deletions of the *gntT::lacZ* fusion were constructed. Deletion of the entire region upstream of the cAMP-CRP binding site beyond position -91, in mutant PN400 (Fig. 3), resulted in no significant difference in regulation by comparison to the wild type (Table 3). Mutant strain PN404, which was constructed by removing the distal *EcoRI-HinI* fragment containing the cAMP-CRP binding site, showed very low but still gluconate-inducible β -galactosidase activity, confirming results described above. During the course of previous work, primer extension analysis had revealed two ends for the *gntT* mRNA (35), but the existence of a second promoter is not confirmed by these data. Since no expression was measured in the mutant carrying a deletion of the entire promoter region (PN401), it appears that there is only a single promoter for *gntT*, and it is suggested that the second primer extension signal is the result of transcript processing.

Additional regulatory effect of GntR. During the course of this work, it became apparent that GntR might affect *gntT* expression by a mechanism that is independent of negative control by operator binding as well as activation by cAMP-CRP. Unexpectedly, expression of the *gntT::lacZ* fusion in the *gntR* mutant grown in the presence of gluconate was approximately one-half of that of the wild type grown under the same conditions (a finding called the 50% effect) (Fig. 1 and 2). This result was duplicated in the presence of cAMP (Fig. 2). Furthermore, the *crp gntR* double mutant expressed the *gntT::lacZ* fusion in the presence of gluconate at one-half of the level of the *crp* mutant in a *gntR*⁺ background, regardless of whether cAMP was added (Table 2). Similarly, the *cya gntR* double mutant expressed the *gntT::lacZ* fusion in the presence of gluconate at one-half of the level of the *cya* mutant in a *gntR*⁺ background. In this case, addition of cAMP increased expression, but the *gntR* mutant retained the 50% effect. Thus, it appears that the twofold decrease in expression of the *gntT::lacZ* fusion in the *gntR* mutant when grown in the presence of gluconate is independent of catabolite repression. The fully derepressed double-operator-site mutant, PN1256, expressed the *gntT::lacZ* fusion when grown on gluconate at a level sim-

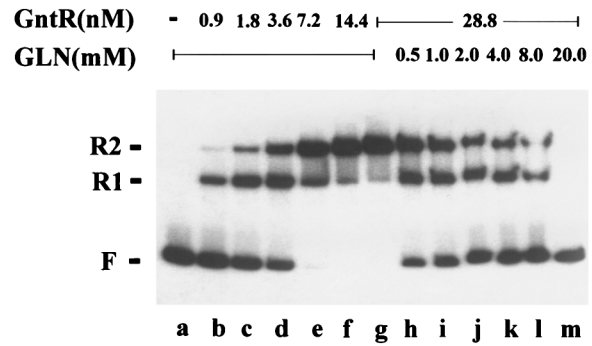


FIG. 5. Electrophoretic mobility of the GntR-operator complexes. Increasing amounts of purified GntR were added to a 456-bp *gntT* fragment containing the putative regulatory region of the *gntT* gene in the absence or presence of different concentrations of gluconate. In the presence of GntR, two GntR-operator complexes (R1 and R2) were formed. F, free DNA. All samples contained 8.1 nM *gntT* DNA and the designated concentrations of GntR and gluconate.

ilar to that of the induced level of the wild type grown under the same conditions (Table 3), indicating that the 50% effect is independent of GntR binding and, hence, the negative regulation of the *gntT* gene; that is, that the *gntR* mutation is required for the observed 50% effect during growth on gluconate.

Since a pronounced peak of *gntT* expression was observed during early log phase (35), a pattern of expression similar to that of the Fis protein (3), it was possible that Fis is the regulator of *gntT*. Although a sequence (GTTTGAGAATCA

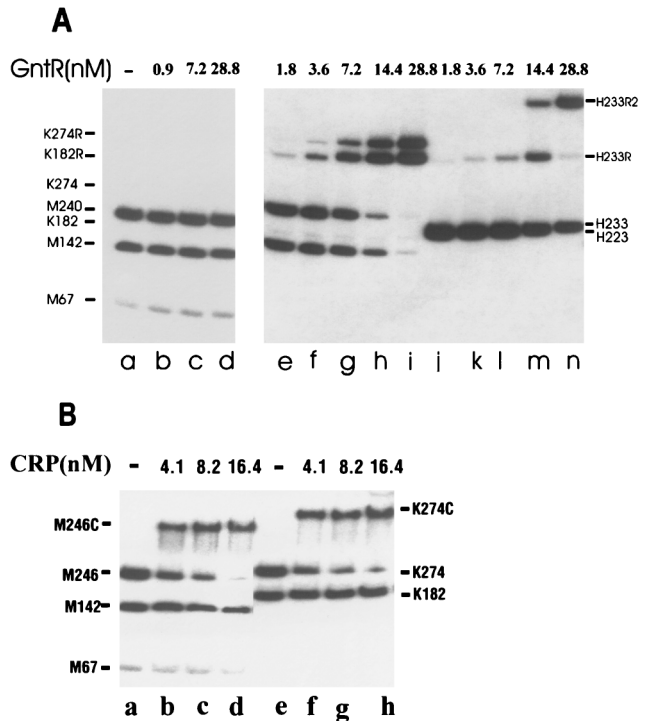


FIG. 6. Formation of GntR- and CRP-DNA complexes with cleaved *gntT* DNA fragment. Digestion of the *gntT* fragment with different restriction enzymes resulted in following fragments: *MaeIII* (67, 142, and 240 bp), *KpnI* (182 and 274 bp), and *HinI* (223 and 233 bp). The formation of complexes was tested with GntR (A) and CRP (B). Complexes of the DNA fragment with one GntR molecule, two GntR molecules, and Crp were given the suffixes R, R2, and C, respectively.

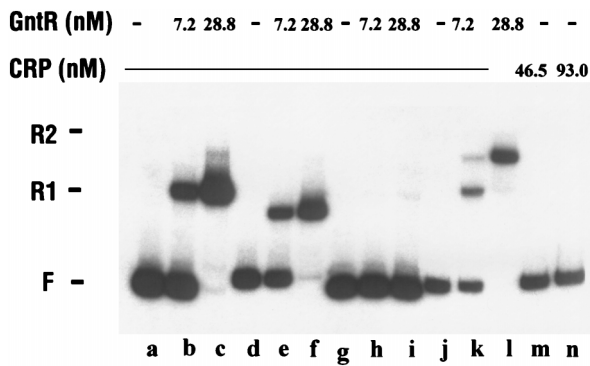


FIG. 7. Effects of mutations in the internal and external operator sites on the formation of the repressor-operator complex. Addition of different concentrations of purified GntR to the 456-bp *gntT* fragment gave a mixture containing free DNA (F) and 1:1 (R1) and 2:1 (R2) repressor complexes, respectively. Samples a to i and j to n contained 6 and 2.2 nM *gntT* DNA, respectively. Samples a to c, d to f, g to i, and j to n contained *gntT* fragments from strains NP105, NP206, NP1256, and NP220, respectively.

CCA, at -42) with similarity to the consensus binding sequence of Fis (GNN[C/T][A/G]NN[T/A]NN[T/C][G/A]NNC) (3) could be identified within the *gntT* promoter region, a *fis* null mutation had no effect on expression of the *gntT::lacZ* fusion.

Purification of GntR and CRP. GntR and CRP were isolated by using a His tag modification system as described in Materials and Methods. The coding regions of both genes, including the original start and stop codons, were amplified by PCR and cloned into the expression vector pQE30. The resulting fusion genes carried six additional His codons at their 5' ends. After overexpression of the His-tagged GntR and CRP in *E. coli* M15(pNP-41) and M15(pNP-52), respectively, the proteins were isolated in native form by high-affinity chromatography on nitrilotriacetate resin. The purified GntR and CRP had apparent molecular masses of 35 to 37 and 23 to 25 kDa, respectively, as determined by SDS-PAGE (Fig. 4), in agreement with the predicted values of 36.8 and 24.3 kDa, respectively. The procedure yielded proteins of greater than 95% purity.

Binding of GntR to the *gntT* operator sites. To test the binding of GntR to the operators of *gntT* and the effects of

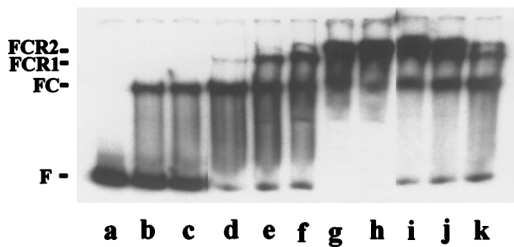
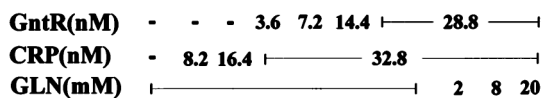


FIG. 8. Formation of a ternary and a tertiary complex of GntR, CRP, and *gntT* operator shown by electrophoretic mobility assay. The *gntT* promoter fragment was incubated with different amounts of CRP and GntR in the presence of 5 mM cAMP. Besides the free DNA (F), three DNA bands with reduced mobility, FC, FCR1, and FCR2, corresponding to DNA bound to one CRP, one CRP and one GntR, and one CRP and two GntR molecules, respectively, were formed.

potential inducers on such binding, we studied GntR-operator complex formation with the purified GntR protein by gel electrophoretic mobility assays. A DNA fragment containing the proposed regulatory region of *gntT* including both operator sites was amplified and radioisotopically labeled by using PCR. The DNA was incubated with different amounts of purified GntR before electrophoresis. Two DNA bands with reduced mobility, corresponding to DNA with one or two GntR molecules bound, were formed (Fig. 5 and 7). The DNA-protein interaction was found not to be cooperative, since the formation of the DNA-protein complex was linearly proportional to the concentration of DNA and GntR protein (data not shown). If the binding of GntR to the operator sites of *gntT* is specific, unlabeled DNA carrying these binding sites should compete for binding, whereas nonspecific DNA should not. Addition of a 10-fold excess of unlabeled operator DNA completely abolished the formation of a labeled GntR-DNA complex, whereas a 100-fold molar excess of unlabeled sonicated herring sperm DNA was insufficient to compete for the binding with the labeled fragment (data not shown). These results indicated that GntR binding is specific for the operator. To test the effect of gluconate on the formation of the GntR-operator complex, the binding assay was carried out in the presence of different gluconate concentrations. As shown in Fig. 5, gluconate reduced the formation of the binary complex in proportion to its concentration, probably by reducing the binding affinity of GntR to the operator sites. Several other sugars and sugar acids were tested for this effect on formation of the repressor-DNA complex, and only 6-phosphogluconate was found to inhibit the protein-DNA interaction, but a concentration 10-fold higher than that of gluconate was required, suggesting that the phosphorylated product of gluconate can act as a weak inducer of the *gntT* gene (data not shown). The sugars glucuronate, 5-ketogluconate, glucose-6-phosphate, fructose, glycerol, and L-idonate had no effect on binding (data not shown).

The presence of several restriction sites within the *gntT* regulatory region afforded the opportunity to test whether cleavage of the consensus sequence of the operators would eliminate binding and to confirm the relative locations of the operator sites. The restriction enzyme *MaeIII* specifically cleaves the *gntT* fragment within the left half-site of each operator. None of the three labeled *MaeIII* fragments showed binding to GntR in the gel retardation assay (Fig. 6A), indicating that the GntR binding sites were destroyed by digestion with *MaeIII*. Both the 182- and 274-bp *KpnI* fragments, each carrying one of the operator sites, were shifted in the presence of GntR, while only one of the two *HinfI* fragments produced DNA bands with lower mobility during electrophoresis (Fig. 6A). These data indicated that the two operator sites are separated by the *KpnI* site and are downstream of the *HinfI* site.

To prove that GntR binds to the postulated operator sequences, we constructed mutants carrying base pair substitutions and insertions which were predicted to affect the binding of GntR to these sites (Fig. 7 and unpublished data). The substitution of two, three, or four base pairs within the left or right half of the internal or the external operator site and the insertion of two base pairs into the spacer sequences prevented the binding of GntR in gel retardation assays. On the other hand, the mutants carrying a substitution of one base pair in the right half site and an insertion of a single base pair into the spacer sequence of the internal operator site (PN106 and PN103, respectively) could form complexes only in the presence of eight- and fivefold-higher GntR concentrations (data not shown), suggesting that these mutations caused a lower affinity of GntR to these sites. Likewise, the mutant carrying a two-base-pair substitution in the right half-site of the internal operator

(PN211) could form the binary complex, but only with a 12-fold-higher GntR concentration (data not shown). Mutations of just one of the two operators eliminated formation of the binary complex, while no complex was observed with a mutation which eliminated both operators (Fig. 7).

Binding of CRP to the *gntT* promoter. The purified CRP protein was tested for binding to the *gntT* promoter region by gel electrophoretic mobility assay (Fig. 6B). When the labeled *gntT* fragment was incubated with CRP, only one band with reduced mobility was formed, and as expected, this occurred only in the presence of cAMP. Since it could be argued that the His-tagged CRP might have different binding properties, the native enzyme was tested and found to give the same results (data not shown). A *gntT* DNA fragment carrying a mutation within the CRP binding site showed no formation of a complex with cAMP-CRP. Also, assays with restriction endonuclease cleaved *gntT* DNA showed that only the fragments carrying the CRP site at -71 were able to bind CRP. When the *gntT* promoter fragment was incubated with both GntR and CRP-cAMP, a ternary complex consisting of one GntR molecule and one cAMP-CRP complex bound or two GntR molecules and one cAMP-CRP complex bound was formed (Fig. 8). These results indicated that cAMP-CRP binds specifically to the site centered at -71 but not to the hypothetical site at -13 . The binding of the cAMP-CRP complex is essential for full induction of *gntT*, and this binding is neither cooperative nor inhibited by GntR.

DISCUSSION

In *E. coli*, *gntT* is specifically induced in the presence of gluconate, fully repressed in the absence of gluconate, and partially induced on a mixture of glucose and gluconate. A strain in which the *gntR* gene is knocked out by the insertion of a Kan^r cassette showed constitutive expression of a single-copy chromosomal *gntT::lacZ* operon fusion, even in the absence of an inducer (Fig. 1). Since this constitutive expression was threefold higher than the gluconate-induced expression in wild-type cells, it appears that gluconate can serve as both an inducer and a repressor for *gntT* expression.

It has long been known that gluconate is a very effective substrate for catabolite repression and acts by decreasing the cellular cAMP concentration to nearly the same extent as glucose. In fact, a mixture of glucose and gluconate caused one of the most catabolite repressing conditions measured (14, 31, 32). The results presented here demonstrate that a higher cellular cAMP concentration relieves catabolite repression of *gntT* by gluconate and also by glucose. The fact that *gntT* expression is very low in the absence of adenylate cyclase (*cya*) or catabolite receptor protein (*crp*), even in the presence of gluconate, shows that the positive regulation by cAMP-CRP is essential for full induction of *gntT*. However, addition of cAMP does not fully relieve the repressing effect of glucose and gluconate, which indicates that besides cAMP, another regulatory factor is involved in the catabolite repression. In the general model, the variable cAMP concentration alone is believed to be the mediator of catabolite repression. The intracellular cAMP pool is regulated by phosphorylated EIIA^{Glc}, which stimulates adenylate cyclase to produce cAMP (36, 23, 39). More recently, Hogema et al. (18) showed that catabolite-repressing sugars lower not only the intracellular cAMP pool but also the intracellular concentration of CRP. Furthermore, EII^{Glc} seemed not to be involved in the regulation of the CRP concentration by the non-phosphoenolpyruvate:sugar phosphotransferase system (PTS) substrate gluconate, since a *crr* mutation did not affect their results (18, 49). The reason why

exogenous cAMP does not fully relieve the catabolite repression of *gntT* by gluconate could be the simultaneous, cAMP-independent reduction of the CRP concentration by gluconate (18). It should be pointed out that it is still not known how gluconate, a non-PTS carbon source, regulates the intracellular concentrations of cAMP and CRP. One possibility is that gluconate by itself, or a regulatory component of the gluconate regulon, interacts directly or indirectly with adenylate cyclase and the *crp* gene.

The other important question is why gluconate acts on *gntT* expression both as an inducer and as a repressor. Derepression of the *gnt* regulon by addition of cAMP during growth on gluconate or by deletion of *gntR* caused a growth inhibition, probably as a result of the accumulation of the toxic metabolite methylglyoxal as has been reported previously (2). The growth inhibition caused by cAMP and gluconate was accentuated in the *gntR* mutant (Fig. 2), indicating that the growth inhibition is likely the result of overexpression of the gluconate catabolic pathway. The accumulation of methylglyoxal is probably the result of an unregulated flux of carbon from gluconate to glyceraldehyde-3-phosphate, which bypasses the key allosteric control points, phosphofructokinase and pyruvate kinase. The formation of methylglyoxal from dihydroxyacetone phosphate is catalyzed by the methylglyoxal synthetase. Obviously, there is an important balance between induction and repression of gluconate catabolism.

A comparison of negative control of the *gnt* regulon to that of the *gal* and *lac* regulons, the two paradigms of negative control (7), highlights an interesting difference. The *galR* mutant, when grown in the presence of the inducer galactose, shows a twofold-higher level of induction by comparison to the fully induced wild type (44). The mechanism of ultra-induction in the *gal* regulon is now understood to be due to the presence of an additional repressor, *galS* (47). The *lacI* mutant is derepressed for *lacZ* expression to exactly the same extent as the fully induced wild type, and the inducer IPTG has no effect on *lacZ* expression in the *lacI* mutant (21). Paradoxically, the *gntR* mutation causes a 50% lower expression of the *gntT::lacZ* fusion in the presence of the inducer gluconate by comparison to the wild type (Fig. 1). Furthermore, the *crp gntR* and *cya gntR* double mutants expressed the *gntT::lacZ* fusion in the presence of gluconate at one-half of the level of the *crp* or *cya* mutant in a *gntR*⁺ background. Finally, mutation of both operators in the fully derepressed mutant, PN1256, did not cause a 50% lowering of *gntT* expression when cells were grown on gluconate. Thus, it appears that the twofold decrease in expression of the *gntT::lacZ* fusion in the *gntR* mutant when grown in the presence of gluconate is independent of catabolite repression, as well as GntR binding to the operators. The simplest interpretation of this phenotype, which we can call ultrarepression, by analogy to the ultra-induction of the *gal* operon in a *galR* mutant (44), is that another regulatory factor may be involved in the regulation of *gntT*. One possibility is that GntR is essential for the expression of an activator, which acts positively on the transcription of *gntT* in the presence of gluconate, and that this activation is lost in the *gntR* mutant. The data presented here indicate that Fis (3) is not involved. However, it is possible that the closely related YjgS protein, which is 46% identical to GntR, is involved in the positive regulation of *gntT*. This possibility is particularly intriguing since the regulatory region of the GntII genes contains two *gnt* operator consensus sequences (35).

In the second part of this work, we used site-directed mutagenesis and DNA band migration retardation assays to prove that the *gnt* operators serve as binding sites for the negative regulator GntR and that the true inducers of the *gnt* regulon

are gluconate and, to a lesser extent, 6-phosphogluconate. The putative site for binding of the cAMP-CRP complex was also tested. One or two copies of the highly conserved consensus sequence postulated to be the *gnt* operator, consisting of two inverted hexamers separated by a GC-rich spacer sequence of 4 bp (ATGTTA[N₄; GC rich]TAACAT), are present in the regulatory regions of all gluconate-inducible genes (*edd*, *gntKU*, *gntT*, *gntV*, and *yjgV*) (35). Gel retardation assays showed that GntR binds to two different sites within the promoter region of *gntT*. Gluconate and also a 10-fold-higher concentration of 6-phosphogluconate were found to inhibit the formation of the GntR-DNA complexes (Fig. 5). These results confirm that regulation of the *gntT* gene by GntR is similar to other negatively regulated systems. In the absence of an inducer, the GntR repressor binds to the two operator sites, resulting in repression of the *gntT* gene. The inducer, gluconate (or 6-phosphogluconate), acts to eliminate binding by GntR, probably by reducing the DNA binding affinity of GntR to the operator. These results confirm earlier genetic studies indicating that gluconate, and 6-phosphogluconate in *pgi gnd* double mutants which can accumulate this intermediate, serves as an inducer of the *gnt* regulon (24, 38).

Proof that GntR binds to the postulated operator sequences was obtained by using mutants which were predicted to affect the binding of GntR to these sites. Substitutions within the left or right half of the operator sites and insertions in the spacer sequences resulted in a high, constitutive expression of the *gntT::lacZ* fusion in the absence of gluconate. Binding assays showed that these mutations specifically prevented the binding of GntR to these sites. Furthermore, when the operators were destroyed by digestion with *Mae*III, no binding of GntR was detected. In summary, these results demonstrate that GntR binds to two operator sites at *gntT* and that these protein-DNA interactions are highly specific.

CRP was found to bind, under *in vitro* conditions in the presence of cAMP, to the binding site at -71 (Fig. 6B and 8) but not to the putative site at -13, which was postulated to be a hybrid of GntR and CRP binding sites. Mutations within the CRP binding site at -71 prevented the binding of the cAMP-CRP complex. Furthermore, these mutations caused a significant decrease of *gntT::lacZ* fusion expression under induced conditions (Table 2), as did deletion of the distal region of the *gntT* promoter, including the CRP binding site at -71. Together these results demonstrated that the cAMP-CRP complex activates the transcription of *gntT* by binding to the -71 binding site and that full activity of the *gntT* promoter is dependent on this activation.

Since mutation of either of the operator sites caused a derepressed, constitutive expression of the *gntT* gene, it appears that there may be some kind of interaction between these sites. We favor the idea that the repression of *gntT* is the result of DNA looping through interaction between the two GntR molecules as shown for many other operons in *E. coli*, such as the *ara*, *gal*, *lac*, and *deo* operons (29). A greater extent of derepression results from mutation of the external operator site by comparison to the internal operator site mutants, suggesting that binding of GntR to the external site results in a stronger repression of the *gntT* gene. This can be explained by the fact that the external operator site overlaps the -10 region of the promoter, perhaps causing competition between RNA polymerase and GntR for binding to the promoter.

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