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Analysis of CRP-CytR Interactions at the *Escherichia coli udp* Promoter

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Multiprotein complexes regulate the transcription of certain bacterial genes in a sensitive, physiologically responsive manner. In particular, the transcription of genes needed for utilization of nucleosides in *Escherichia coli* is regulated by a repressor protein, CytR, in concert with the cyclic AMP (cAMP)-activated form of cAMP receptor protein (CRP). We studied this regulation by selecting and characterizing spontaneous constitutive mutations in the promoter of the *udp* (uridine phosphorylase) gene, one of the genes most strongly regulated by CytR. We found deletions, duplications, and point mutations that affect key regulatory sites in the *udp* promoter, insertion sequence element insertions that activated cryptic internal promoters or provided new promoters, and large duplications that may have increased expression by *udp* gene amplification. Unusual duplications and deletions that resulted in constitutive *udp* expression that depended on the presence of CytR were also found. Our results support the model in which repression normally involves the binding of CytR to cAMP-CRP to form a complex which binds to specific sites in the *udp* promoter, without direct interaction between CytR protein and a specific operator DNA sequence, and in which induction by specific inducer cytidine involves dissociation of CytR from cAMP-CRP and then RNA polymerase interaction with cAMP-CRP bound to a site upstream of the transcription start point. The stimulation of *udp* expression by CytR in certain mutants may reflect its stabilization of cAMP-CRP binding to target DNA and illustrates that only modest evolutionary changes could allow particular multiprotein complexes to serve as either repressors or transcriptional activators.

Multiprotein complexes, long recognized as regulators of expression of many eukaryotic genes, can also be important in the regulation of prokaryotic gene expression (1). This is exemplified by the genes encoding the array of membrane proteins and intracellular enzymes that take up and catabolize deoxy- and ribonucleosides in *Escherichia coli* (19). Much of the expression of nucleoside utilization genes is coordinated by a repressor protein, CytR, acting in concert with the cyclic AMP (cAMP)-activated form of the cAMP receptor protein (CRP) (2, 9, 27). The three promoters that have been analyzed most extensively (*deoP2*, *cddP*, and *tsxP*) each contain two binding sites for CRP (CRP1 and CRP2) but are otherwise dissimilar in sequence (Fig. 1). The current view is that transcription from these promoters involves CRP binding to CRP1 and that repression involves the binding of CytR protein to CRPs that are, in turn, bound to both CRP1 and CRP2 in the promoter region. It had been speculated that CytR binding to specific DNA sequences would also be important in repression, since CytR exhibits considerable homology to other repressors (21, 32). An imperfect inverted-repeat motif (5′-TGCAAN$_{2-3}$TTGCA) is present in each of these promoters and is considered a candidate CytR recognition sequence (24). In this model, induction of expression by cytidine was shown to involve dissociation of CytR from CRP complexes, which results in transcription activation, possibly through effects on DNA topology or direct interaction with RNA polymerase.

Here we focus on the promoter for the *udp* (uridine phosphorylase) gene (*udpP*), one of the most strongly regulated genes in the CytR regulon. The *udpP* structure is similar to that of *cddP* in the spacing between CRP1 and CRP2 (52 bp) and in the spacing between CRP1 and the −10 site (29 bp) (Fig. 1) (31), and it contains the putative CytR operator (5′-TGCAA and TTGCG inverted-repeat motif) but with 5 bp between the half-sites (in contrast to 2 bp in *deoP2* and *tsxP2* and 3 bp in *cddP* and *cytRP*) (16, 24, 34). We study the interactions between DNA, CytR, and CRP proteins in the *udpP* region in vitro.

We further define the complex *udpP* mutationally, and in terms of mRNA start site and CRP and CytR binding sites, using gel retardation assays and DNase footprinting. Our results support and extend a model in which transcription is repressed when CRP moieties are bound to two appropriately positioned sites in the *udpP* and are themselves bound and stabilized by CytR.

MATERIALS AND METHODS

Strains. Bacterial strains used in this work are listed in Table 1. Phage P1*vir* was used to transduce alleles from one strain to another as described previously (15).

Growth of bacteria. Bacteria were grown in L broth (26) or Vogel-Bonner (33) minimal medium with 0.2% glucose or glycerol as a carbon source. To select *udp* constitutive mutants, strain AM271, which requires high levels (20 μg/ml) of thymine, was grown overnight in L broth, washed with 0.9% NaCl, spread on glucose or glycerol minimal agar plates with 5 μg of thymine per ml (10^8 cells per plate), and incubated for 2 days at 37°C.

Mutant detection and characterization. Some *udp* mutations were identified by a change in size of a PCR product generated with primers A and C that is 282 bp in the wild type (WT) (Table 2). PCR amplification was carried out with boiled extracts of mutant strains and 1 U of AmpliTaq thermostable polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in 25 μl of reaction mixture in a model TC480 Thermal Cycler (Perkin-Elmer Cetus) during 30 cycles of 30 s at 95°C, 40 s at 58°C, and 1 min at 72°C, using buffers provided by the supplier. PCR

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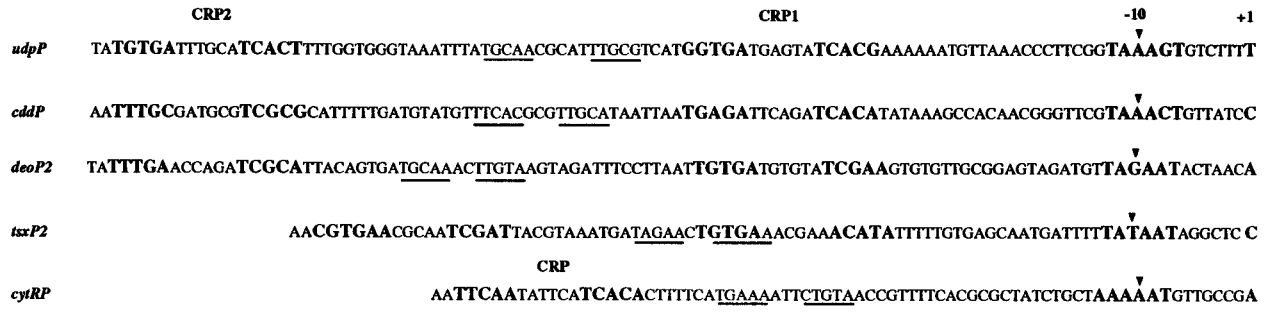

FIG. 1. Structures of CytR-regulated promoters. Putative CytR operators are underlined.

products were purified by using a Geneclean kit (Bio 101, Inc., La Jolla, Calif.) when needed.

Other mutant alleles were identified by electrophoresis of heteroduplexes between the WT and mutant PCR fragments in mutation detection enhancement gels, which are composed of a special formulation that allows even single-base differences to be detected (AT Biochem, Inc., Malvern, Pa.).

DNA sequences were determined by linear amplification (cycle) sequencing of PCR products or purified chromosomal DNA (13), using deoxy- and dideoxynucleoside triphosphates from Boehringer Mannheim (Indianapolis, Ind.). Chromosomal DNA used as a template for cycle sequencing was purified as described previously (3).

Isolation of RNA, primer extension, and S1 mapping. Cells were grown in 30 ml of liquid minimal medium to late log phase, and total RNA was purified from these cultures by the guanidinium thiocyanate method (26). Primer extensions with reverse transcriptase and S1 mapping were carried out by using ^{32}P-end-labeled primers D and E with 60 to 70 μg of total RNA as described previously (26).

Udp assays. Bacteria growing exponentially in 5 ml of minimal glucose or glycerol medium were harvested by centrifugation at a density of 5×10^7 cells per ml, resuspended in 3 ml of 50 mM sodium phosphate buffer (pH 7.0), and sonicated in an MSE disintegrator, and cell debris were removed by centrifugation. Udp was assayed as described previously (25). One enzyme unit catalyzes the conversion of 1 nmol of uridine to uracil per min per mg of protein at 37°C. The protein concentration of cell extracts was measured by the method of Lowry et al. (14), using bovine serum albumin (BSA) as a standard. The values listed represent the averages of at least three independent determinations.

Proteins. Purified CytR repressor protein was a generous gift of S. A. Short, Department of Microbiology, Wellcome Research Laboratories, Research Triangle Park, N.C. (2). CRP was purified by using CRP overproducer strain SA500/pHA5, kindly provided by S. Adhya, using his recommended procedure (1a). CRP was purified as follows. Cells were grown in 8 liters of 2× YT medium in the presence of 1 mM isopropylthiogalactopyranoside (IPTG) overnight, harvested, and washed in Tris-EDTA buffer. Fifty grams of frozen cells was lysed in 140 ml of lysis buffer (50 mM Tris [pH 8.0], 50 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% sucrose, 0.3 mg of lysozyme per ml) for 30 min on ice with occasional stirring. Lysis was completed by incubation of the solution for 10 min at 32°C followed by 10 min on ice to chill the lysate. $MgCl_2$ was added to a concentration of 2 mM, and DNA was removed from the lysate by treatment with pancreatic DNase. KCl was added to bring the final concentration to 0.3 to 0.4 M, and then the lysate was clarified by centrifugation at 40,000 rpm in a type 45 rotor for 3 h. The pH of the clarified supernatant was adjusted to 7.2. The lysate was subjected to ammonium sulfate fractionation at 0 to 30% $(NH_4)_2SO_4$, the supernatant was collected after centrifugation at 12,000 rpm, and its pH was adjusted to 8.5. CRP was precipitated by a second fractionation of the supernatant at 30 to 60% $(NH_4)_2SO_4$. After 30 min of incubation on ice and centrifugation at 12,000 rpm, the pellet was collected and resuspended in 10 ml of buffer A (20 mM potassium phosphate [pH 7.0], 0.2 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol), dialyzed against the same buffer overnight, and centrifuged at 12,000 rpm for 15 min. CRP was purified from the dialyzed lysate by fast protein liquid chromatography on a Mono-S column, using a linear gradient of 10 to 30% of KCl with A buffer containing 1 M KCl.

The activity of purified CRP was determined by a cAMP binding assay as follows. Twenty-microliter aliquots of CRP fractions were incubated for 5 min on ice in a reaction mixture containing 35 μl of 10 mM potassium phosphate (pH 7.0), 10 μl of 10 mM cAMP, 10 μl of 0.1 M AMP, 20 μl of casein (10 mg/ml), and 5 μl of a 1/10 dilution of [^{3}H]cAMP (0.1 μCi/μl) (Amersham). Then the protein was precipitated with 400 μl of saturated $(NH_4)_2SO_4$ and counted by using Aquasol.

Gel retardation and DNase I footprinting assays. For gel retardation assays, DNA fragments containing WT and mutant *udpP*s were PCR amplified with primers A and C (Table 2) and ^{32}P labeled by using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.). ^{32}P-labeled fragments and proteins were incubated in binding buffer (10 mM Tris-HCl [pH 7.8], 50 mM KCl, 1 mM EDTA, 50 μg of BSA per ml, 1 mM dithiothreitol, 0.05% Nonidet P-40, 50 μM cAMP) with 20 μg of competitor DNA per ml for 30 min at 37°C in a total volume of 10 μl (22), 2 μl of loading buffer (50% glycerol in binding buffer plus 0.1 mg of bromphenol blue per ml) was added, and the samples were loaded immediately on a vertical 6% polyacrylamide gel with the current on. The electrophoresis buffer consisted of 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 50 μM cAMP. Following electrophoresis, the gel was dried and autoradiographed.

For DNase I footprinting experiments, promoter-containing DNAs were amplified with primers B and C, one of which had been ^{32}P labeled by using T4 polynucleotide kinase as described above. Two nanomolar DNA was used for binding with CRP and CytR proteins in concentrations shown in the figure legends, using the same as for gel retardation binding buffer. DNase I footprinting reactions were carried out in a 100-μl volume with 0.5 U of DNase I (Promega) at room temperature essentially as described previously (7). Footprinting patterns were analyzed on 8% polyacrylamide sequencing gels.

RESULTS

Definition of *udpP*. The site of initiation of *udp* transcription from the wild-type promoter was identified by primer extension and S1 nuclease mapping. It mapped to a site downstream of a −10 consensus sequence and a likely binding site for cAMP-activated CRP (CRP1) in accordance with predictions based on DNA sequence (Fig. 1). The same initiation site was seen in CytR$^+$ and CytR$^-$ strains (data not shown) and also under growth conditions that should lead to high and low levels of cAMP and thus of CRP (Fig. 2, first two lanes). The relative intensities of the extended chains from the glucose- and glycerol-grown cells support a greater expression of the *udp* gene

TABLE 1. *E. coli* K-12 strains used in this work

Strain	Genotype	Source
AM271	*thi thyA deoA metB*::Tn*5 lacZ43*	Collection of A. Mironov
AM276	*thi thyA deoA lacZ43* Δ(*metE-udp*) *zif-9*::Tn*10*	Collection of A. Mironov
AM277	*thi thyA deoA lacZ43 crp zhb*::Tn*10*	Collection of A. Mironov
SA500/pHA5	*rpsL his su*$^-$[pHA5][a]	S. Adhya

[a] pHA5 contains a *crp* gene.

TABLE 2. Primers used in this work

Primer	Sequence	Position[a]
A	5′-CCATGGTATTCTCCGTACCC	−226 to −206
B	5′-ATCTTTTATGCAGTCCTGCAG	−138 to −118
C	5′-ACATCAGACTTGGACATATACAAC	+55 to +34
D	5′-GCCAGGGACGATGGCAAGCG	+117 to +98
E	5′-AGTGAATTCGCGGTGAGATGCCA	+192 to +170

[a] Relative to the start point of *udp* transcription.

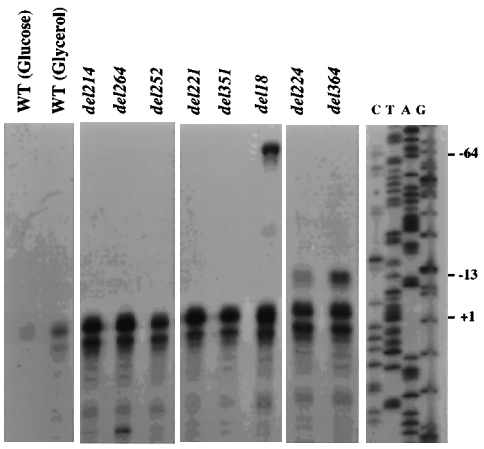

FIG. 2. S1 nuclease mapping of *udp*-specific RNAs from WT and mutant *E. coli*, using a fragment containing the WT *udpP* as a probe. This probe was PCR amplified with primers A and ^{32}P-labeled E. The first two lanes contained mRNA from WT cells grown in minimal medium with glucose and glycerol, respectively, as carbon sources. All mutant strains were grown with glucose as a carbon source. Lanes C, T, A, and G represent cycle sequencing reactions of WT *udpP*, using ^{32}P-end-labeled primer E. Nucleotide positions are indicated on the right.

in cells grown with glycerol as the sole energy source. The higher levels of mRNA correlate with higher levels of activities of the Udp enzyme in the cells grown in glycerol (Table 3).

The interaction of CRP and CytR with DNA regulatory sites in the WT *udpP* region was studied in vitro, using a PCR-amplified segment containing *udpP* and gel retardation and DNase footprinting techniques. Figure 3A shows that, given constant cAMP levels (50 μM), just one band was retarded when the CRP concentration was very low (0.25 ng/μl) and that an additional, more slowly migrating band appeared when higher CRP levels were used. These two bands were interpreted to reflect the binding of CRP first to one and then to the second CRP site. Even at a high CRP concentration (5 ng/μl), only a subset of the *udpP* DNA fragments contained protein bound to both CRP sites (Fig. 3A).

Gel retardation assays using CytR protein alone revealed only very weak binding of CytR to the *udpP* region, even at high CytR concentrations (>1 ng/μl; data not shown). In contrast, the additional presence of CRP allowed formation of a band which migrated more slowly than the DNA-CRP band in the gel retardation assay. This band was interpreted to contain a bound complex of CytR and CRP bound to one of the two CRP sites at a low CRP concentration (0.25 ng/μl); no band attributable to the binding of only CRP was seen. A second, larger complex was also observed. This complex is believed to contain two CRP molecules in addition to the DNA and CytR. At a higher CRP concentration (1 ng/μl), all *udpP* DNA migrated slowly at the position predicted of a CRP-CytR complex with CRP bound to both CRP sites (Fig. 3A). These results indicate that strong CytR-*udpP* interaction depends on binding by the cAMP-CRP complex and, conversely, that CytR itself enhances CRP binding to the *udpP* region.

To determine the promoter sites protected by CRP binding, DNase I footprinting experiments were undertaken. Low levels of CRP (0.2 ng/μl) protected only CRP2; a 10-fold-higher CRP concentration (2 ng/μl) partially protected CRP1 and more strongly protected CRP2. Inclusion of CytR repressor along with CRP (0.5 ng/μl) resulted in strong protection of both CRP sites and also the 52-bp segment between them (Fig. 4A). Thus, these experiments showed that CRP2 far from the transcriptional start has the higher affinity for CRP and demonstrated strong cooperativity in CytR-CRP-*udpP* interactions.

Selection of *udp* constitutive mutants. Mutants with constitutive *udp* expression were isolated and characterized in order to better understand regulatory protein interactions at *udpP*. Constitutive mutants were selected on the basis of the ability of Udp to convert thymine to thymidine, albeit inefficiently, which is then phosphorylated and incorporated into DNA. This action of Udp allows strains deficient in thymidylate synthetase (*thyA*) and thymidine phosphorylase (*deoA*) to grow on mini-

TABLE 3. Udp activity in *udp* constitutive mutants

Allele	Size (bp) (position)	Udp activity[a]			Regulation[b]	
		cytR$^+$ *crp*$^+$	*cytR crp*$^+$	*crp cytR*$^+$	CytR	cAMP-CRP
WT		100 (200)	680 (1,200)	70	0.1	17
dup1	41 (−52 to −12)	2,700 (3,600)	890 (3,100)	100	3.0	31
dup361	8 (−90 to −83)	1,086 (2,774)	648 (3,603)	138	1.7	26
del224	11 (−42 to −32)	2,400 (2,300)	1,400 (2,500)	1,500	1.8	1.7
del364	11 (−38 to −28)	2,600 (4,500)	950 (5,600)	1,640	2.7	3.4
dup41	528 (−60 to +468)	1,200	1,300	980	0.9	2.5
dup262	206 (−198 to +8)	3,200	2,950	1,580	1.0	1.8
del214	159 (−188 to −30)	2,286	2,900	2,400	0.8	1.2
del264	52 (−90 to −39)	2,560	2,600	1,600	1.0	1.5
del252	23 (−40 to −18)	2,000	2,000	810	1.0	3.2
del221	22 (−47 to −26)	2,564	3,120	600	0.8	5.3
del351	3 (−82 to −80)	430 (2,300)	870	370	0.5	7.3
del18	1 (−79)	2,500	2,650	2,480	0.9	1.4
del232	1 (−49)	2,245	3,100	420	0.7	6.1
del305	1 (−80)	980	1,200	460	0.8	4.8
del320	1 (−83)	880	910	580	1.0	7.5
P304	(−97)	330 (1,390)	1,178	112	0.3	18.3

[a] For cells grown on minimal medium with glucose (0.4%). Values in parentheses indicate Udp activities on the glycerol (0.4%) media for WT, CytR-dependent mutants, and two mutants (*del351* and base substitution *P304*) selected on glycerol medium. Enzyme levels were measured during exponential growth at 37°C in Vogel-Bonner minimal medium, using glucose or glycerol as the carbon source. Samples were taken for enzyme assays between optical densities at 450 nm of 0.2 and 0.6. Each value is the average of at least three independent experiments (the variations in activity did not exceed 15%).

[b] Extent of regulation by CytR protein was estimated as the ratio of activities in *cytR*$^+$ versus *cytR* strains; regulation by cAMP-CRP was estimated as the ratio of activities in *crp*$^+$ *cytR* cells grown in minimal medium with glycerol (fully derepressed *udp* expression) versus *crp* cells grown in the same medium.

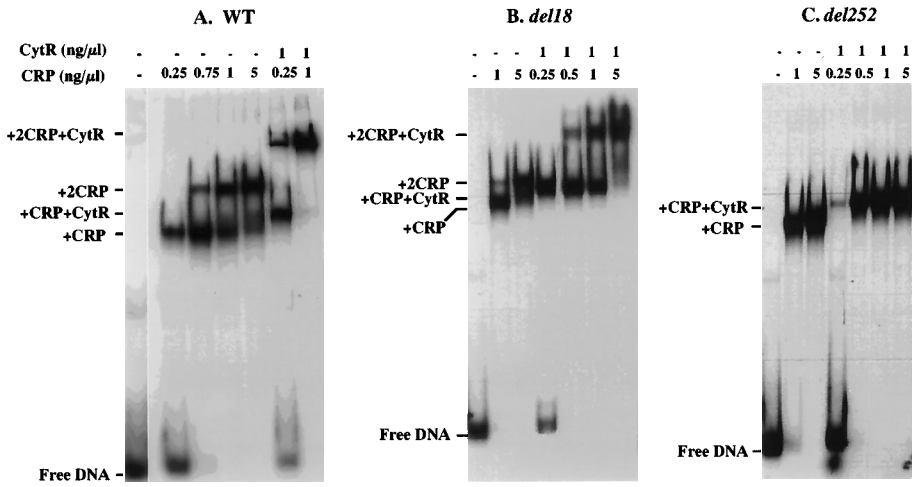

FIG. 3. Gel retardation of ^{32}P-end-labeled *udpP*-containing DNA fragments, using purified CytR and CRP in the presence of 50 μM cAMP. DNA fragments were PCR amplified by using primers A and C and ^{32}P end labeled with T4 polynucleotide kinase.

mal glucose medium containing thymine. At least 20 μg of thymine per ml is needed for growth if *udp* is expressed at basal levels. Use of one-fourth of this amount of thymine (5 μg/ml) generally selects for constitutive mutations in *udpP* (reference 17 and this work).

To isolate *udp* constitutive mutants, fresh overnight cultures of the *thyA deoA* strain AM271 were plated on minimal glucose medium with 5 μg of thymine per ml and incubated for 2 days at 37°C. Mutant colonies were obtained at frequencies of about 10^{-7}, and 354 mutants (two from each of 177 separate cultures) were characterized. Most (95%) mutants selected on 5 μg of thymine per ml could grow on 1 μg/ml, but none had completely lost their thymine requirement.

Nature of *udp* constitutive mutations. PCR amplification was used to help identify mutant *udpP* alleles, and a considerable variety was detected by simple agarose gel electrophoresis (Fig. 5). Many mutant alleles were defined by sequencing and further characterized by assays of Udp activity, primer extension, gel shift, and/or DNase I footprinting.

PCR products from 24 mutants (of 354) were at least 1 kb larger than those from the WT strain. DNA sequencing revealed insertions of the transposable insertion sequence (IS)

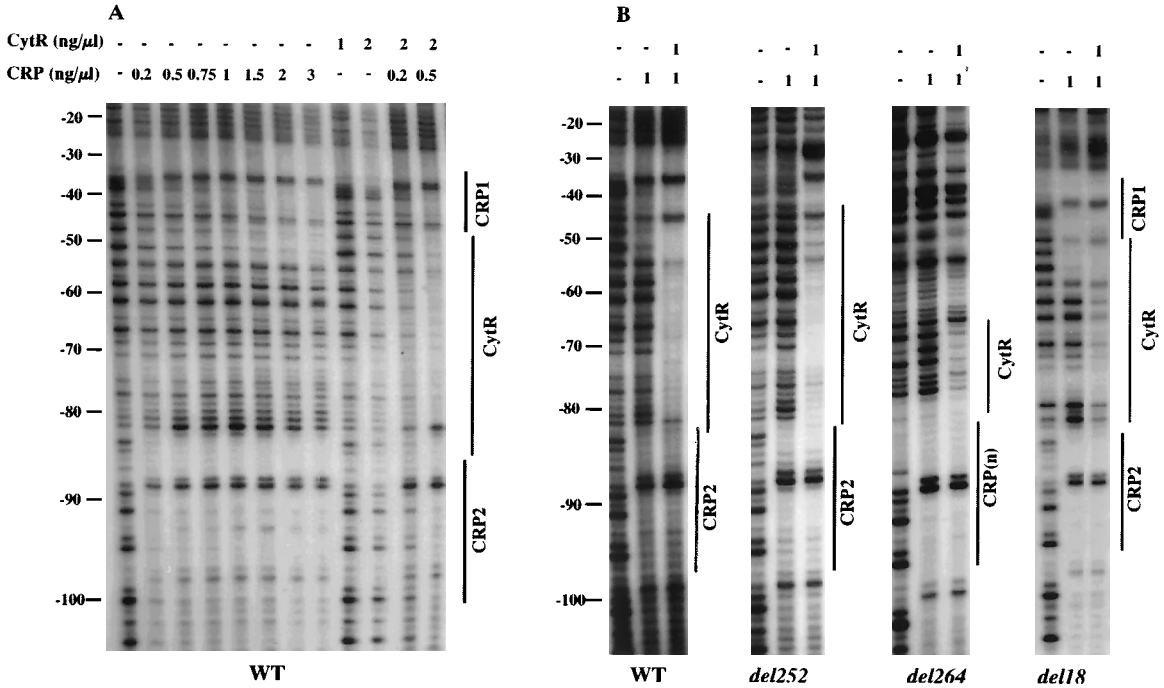

FIG. 4. DNase I footprinting of complexes between WT, *del252*, *del264*, and *del18 udpP*s, CRP, and CytR. *udpP* fragments were PCR amplified with primer C and ^{32}P-labeled primer B and purified by using a Geneclean kit (Bio 101). Two nanomolar DNA was incubated with CytR and CRP in concentrations shown above the gel in the conditions described in Materials and Methods in the presence of 50 μM cAMP, and the mixtures were treated with 0.5 U of DNase I (Promega). (A) Titration of CytR and CRP on WT *udpP*; (B) CytR-CRP-DNA interactions in WT, *del252*, *del264*, and *del18 udpP*s. Numbers on the left indicate nucleotides upstream of the start site of transcription (+1) and correspond to the coordinates shown in Fig. 3.

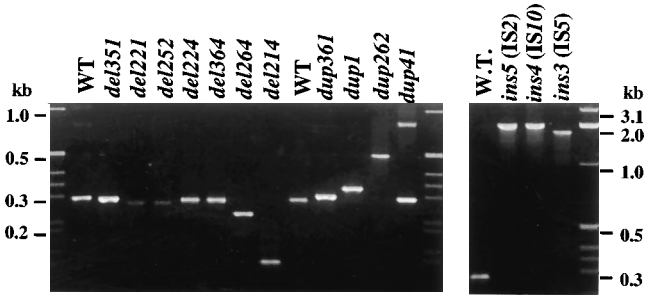

FIG. 5. Identification of mutant *udpP*s by PCR. Fragments corresponding to the *udpP* region were amplified by using primers A and C (Table 2) and analyzed by electrophoresis in a 2% agarose gel. A 1-kb DNA ladder (Gibco BRL) was used as a size standard.

elements IS*2* (*ins1* and *ins5*), IS*5* (*ins3*), and IS*10* (*ins2* and *ins4*) at different sites (Fig. 5 and 6C). Primer extension experiments indicated that the constitutivity of *ins3* (IS*5*), *ins1* (IS*2*, orientation I), and *ins5* (IS*2*, orientation II) alleles resulted from juxtaposition of an IS element-borne −35 region either to the original −10 region of *udpP* (*ins3* and *ins5*) or to a newly created −10 region (as in *ins1*) (Fig. 6C) and that the constitutivity of *ins2* and *ins4* resulted from transcription from the P_{out} promoter of IS*10* (data not shown). (We note that IS*10* is not normally resident in *E. coli* K-12 and infer that in these strains, it must derive from a Tn*10* element introduced into one of the ancestors of the AM271 strain used here.) These insertion mutations illustrated the well-known principle that IS elements can supply active promoters, or help create new promoters for gene expression (6), but they do not give other insights into the regulation of *udpP* expression.

Four other alleles also yielded PCR products larger than those from the WT strain, and sequencing showed that they consisted of tandem duplications (Fig. 6B). The *dup1* allele contained a 41-bp duplication of a segment containing CRP1 but not the −10 site. The *dup41* isolate was unstable and resulted in the PCR amplification of three bands whose relative yields varied among single-colony isolates: a band that comigrated with that from the WT strain, a 0.5-kb-larger band, and a 1-kb-larger band (Fig. 5). DNA sequencing of the 0.8-kb (middle) band showed that this allele consisted of a 528-bp tandem duplication extending from upstream of CRP1 through part of the *udp* open reading frame. Accordingly, the first and third bands were inferred to consist of the resolved (nonmutant) sequence and of a tandem triplication of the same 528-bp segment, respectively, each generated by homologous recombination or strand slippage during replication. A third allele (*dup262*) consisted of a duplication of the entire *udpP* region, and a fourth (*dup361*) contained a partial duplication of CRP2 that also increased the spacing between the CRP sites by 8 bp.

Eleven constitutive alleles were deletions that ranged from 1 to 159 bp in size (Fig. 6A). In particular, we observed by sequencing (i) small deletions that removed part of CRP1 (*del252* [23 bp], *del221* [22 bp], *del224* [11 bp], and *del364* [11 bp]) or that changed the spacing or angular presentation of CRP sites (*del351*); (ii) a deletion that fused CRP1 and CRP2 (*del264*); and (iii) a deletion of 159 bp that removed the *udpP* region and may have fused *udp* to a new −35 region (*del214*). Four additional 1-bp deletions representing nine different isolates are discussed below.

Of the original 354 mutants, 142 were not distinguished from the WT strain in our agarose gels. Tests of these 142 mutants showed that each could be transduced by linkage to *zif9*::Tn*10* (a Tet^r marker close to *udp*) to another strain, indicating that their phenotypes were due to changes in the *udp* region, not in *cytR*. Fifty of these mutants were analyzed by using mutation detection enhancement heteroduplex gels and DNA sequencing. One A/T-to-G/C transition in CRP2 was identified, and nine 1-bp deletions were found (Fig. 6A). Among the 1-bp deletions, six were at the same site (deletion of one of three G's at −77 to −79; hereafter designated −79), and three others were at nearby sites (−80, −83, and −49) (Fig. 6A).

The other 40 mutants (of 50 tested) had no changes in the 282-bp *udpP* segment, and their constitutive phenotypes were quite unstable. For example, about 15% of the colonies obtained by plating cultures grown overnight in rich medium containing thymidine (i.e., without selection for constitutivity) were unable to grow on medium containing a low concentration of thymine (5 μg/ml) (like their AM271 ancestor). Deletion Δ(*met-udp*) was introduced by P1 transduction into the chromosomes of four of these strains. The frequency of cotransduction of *udp* constitutive alleles and Δ(*met-udp*) was found to be 15 to 22%. This result indicated that the second mutation was positioned at about 50 kb from the *udp* location, according to reference 15. A likely explanation would be a large duplication involving the *udp* gene. This was shown to be the case by direct sequencing of chromosomal DNA isolated from the four *udp* constitutive strains containing Δ(*met-udp*), using primers from the *udpP* region. The results showed that the *udp* gene was duplicated and that these rearrangements were mediated by IS*10*, which was now 1,334 bp upstream of *udp* and positioned such that *udp* transcription could be initiated from the IS*10* P_{out} promoter (data not shown).

Udp levels. Most constitutive mutants growing on glucose as the carbon source exhibited 9- to 27-fold-higher *udp* activity than did their udp^+ parents when the *cytR* and *crp* genes were functional. As exceptions, the activities of *P304* and *del351* were only three- and fourfold higher than WT activity. This result correlated with a requirement for higher levels of thymine in strains carrying these alleles relative to other mutants (5 μg/ml versus 1 to 2 μg/ml). Further tests showed that for most alleles, the levels of *udp* expression did not depend strongly on CRP or CytR function (Table 3). However, two deletions (*del224* and *del364*) and two duplications (*dup1* and *dup361*) were remarkable: the Udp activities of strains carrying these alleles were slightly but reproducibly higher in the $cytR^+$ than in the *cytR* background (Table 3). This CytR-dependent stimulation was seen only with cells from glucose-containing medium, in which the intracellular cAMP concentration is very low. The stimulation by CytR also required CRP in the cases of *dup1* and *dup361* but apparently not in the cases of *del224* and *del364.*

Other *udpP* alleles that lacked CRP1 (*del264*, *del252*, and *del221*) and small deletions (*del232*, *del351*, *del305*, and *del320*) exhibited near-complete CytR independence and significantly reduced levels of CRP dependence (1.5- to 7.5-fold, compared with 17-fold in WT *udpP*), and each allowed 4- to 12-fold-higher *udp* expression than did the WT promoter in a $cytR^+$ crp^+ background (Table 3). Mutant *del18*, containing a deletion of one G/C pair at the −79 position, was remarkable. Udp activity in this strain was as high as in constitutive mutants with larger deletions and was completely CytR and CRP independent.

In vitro characterization of mutated *udpP*s. With all mutants, S1 nuclease mapping and primer extension analysis revealed the significantly higher levels of mRNA synthesis expected with constitutive alleles (Fig. 2). In most mutants, transcription started from the same site as in the WT promoter, indicating a strengthening of the original promoter or loss of negative control. New transcriptional starts superim-

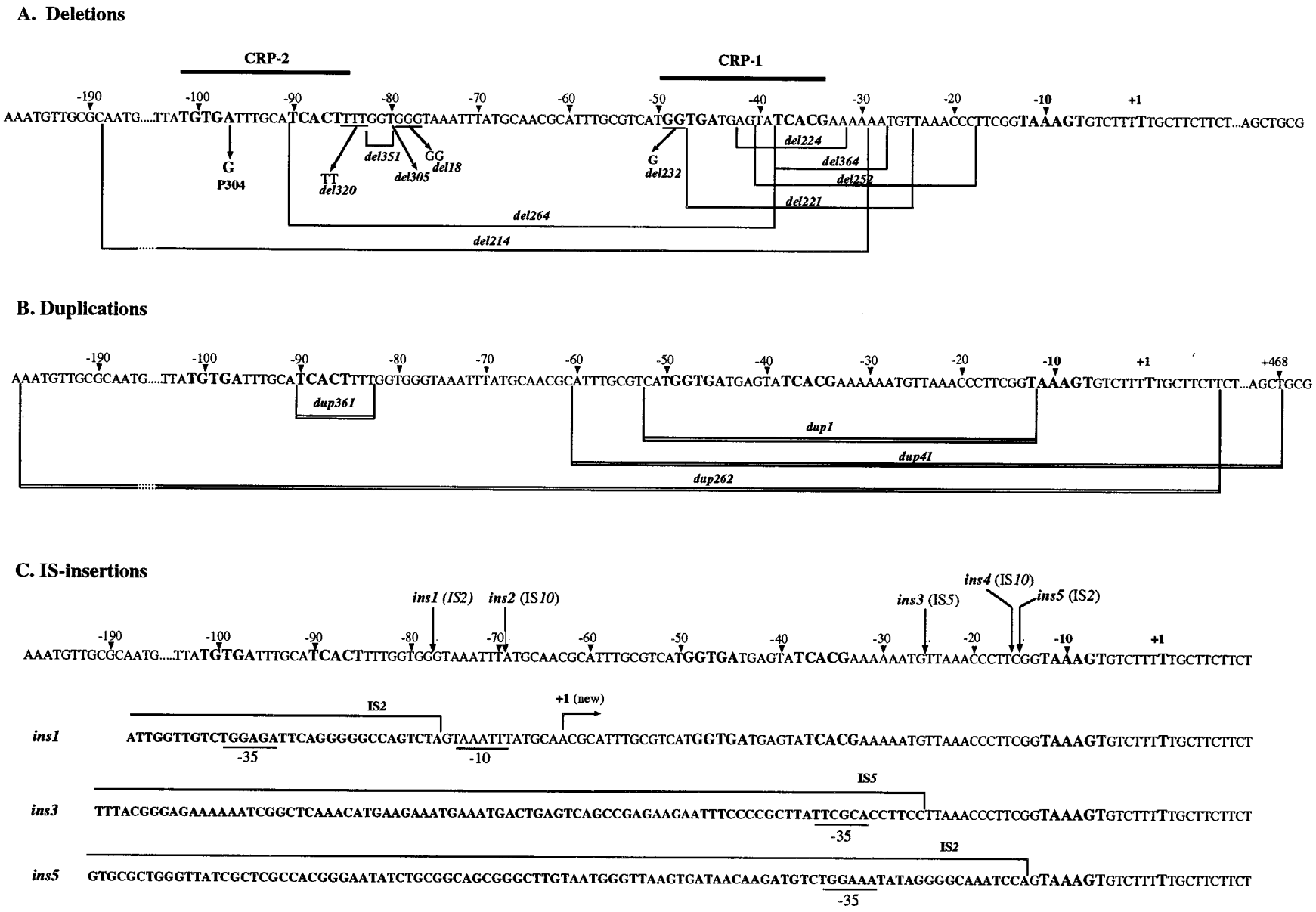

FIG. 6. Structures of WT and mutant *udpP*s. Arrows in panel A indicate the 1-bp deletions and A/T-to-G/C transition resulting in *udp* constitutivity. Arrows in panel C indicate sites of IS insertions and the new transcriptional start in *ins1* (position −64, the same as in *del18*); newly created −35 and −10 regions are underlined.

posed on the original low-level transcription were seen only with one duplication allele (*dup262*) and with the IS*10* insertions (*ins2* and *ins4*) (data not shown).

Two weak new promoters were also found (Fig. 2). Transcription started 13 bp upstream of the original +1 site in *del224* and *del364* and 64 bp upstream of the original +1 site in *del18* and *ins1*. The level of transcription from these promoters was significantly lower than that from the WT promoter (Fig. 2) and thus should not contribute directly to the observed constitutivity of *udp* expression.

Gel retardation and DNase I footprinting analysis were carried out with DNA fragments from mutant *udpP*s with concentrations of cAMP, CRP, and CytR matching those used to study WT *udpP* (see above). CRP bound only to intact CRP2 in gel shift assays of DNAs with a mutant CRP1 (*del224*, *del364*, *del252*, and *del221*) (Fig. 3C). A second, more retarded band was seen with these alleles when CytR was present, indicating formation of a DNA-CRP-CytR complex. CRP-DNA interaction at this site and protection of the interstitial region by CytR were both evident in DNase I footprints (Fig. 4B). The region of the relatively weak protection extended into the mutated CRP1 in *del224*, *del364*, and *del252* alleles, which retain only the left pentamer, GGTGA, of the CRP recognition palindrome. This could be the result of weak, CytR-mediated CRP-DNA interactions in this region that were not detected in gel retardation experiments. Alternatively, the interaction of the CRP-CytR complex with the deleted DNA made the DNA in this region partially inaccessible to DNase I. Moreover, even in *del264*, which lacks the putative CytR operator region, there was some CRP-dependent protection by CytR a few nucleotides immediately downstream of the chimeric CRP site created by the deletion (Fig. 4B). These results suggest strong interactions between CytR and CRP rather than between CytR and any particular DNA sequence within *udpP*.

Two bands corresponding to CRP bound to one or two sites were found in gel retardation experiments with 3-bp (*del351*) and 1-bp (*del320*, *del305*, and *del18*) deletions with CRP alone at concentrations of 1 and 5 ng/μl (Fig. 3B). It is interesting that with these mutants, the *udpP* DNA fragment complexed with two CRP molecules migrated faster than did the corresponding WT fragment (Fig. 3C), perhaps reflecting a changed angular conformation of the mutant DNAs. In contrast, the mobility of the complex CRP-CytR-DNA was not changed. In DNase footprinting assays with these mutants, CytR protein (in the presence of CRP) only slightly protected the corresponding promoter region (Fig. 4B).

DNase footprinting analysis was done with the two duplication mutants that showed CytR-dependent constitutivity (*dup1* and *dup361*). In *dup1*, CRP was bound to CRP2 and to each CRP1. Inclusion of CytR repressor resulted in strong protection of the region between CRP2 and the original CRP1 but not the duplicate CRP1. CRP bound to this duplicate site seemed not to interact with CytR. Inspection of the protected areas in the promoter of the *dup361* allele (8-bp duplication) revealed only weak protection by CytR of the segment between CRP2 and CRP1 (data not shown).

DISCUSSION

We used biochemical and mutational tests to better understand how CRP and CytR repressor interact with each other and target DNA to result in the regulation of transcription of the *udp* gene. We found that constitutive CRP-independent *udp* expression in many of the isolated mutants was caused by loss of part or all of CRP1, indicating that it is important in CRP activation and CytR repression of *udp* transcription. This site at −41.5 bp is at the same position as CRP sites for activation of transcription of several other promoters (e.g., *galP1* [8], *deoP2*, *cddP*, and *tsxP* [31]). CRP2 at −93.5, a location from which CRP cannot by itself activate transcription (29), is important along with CRP1 for repression. In *udpP*, as well as in *cddP* and *deoP2*, the positions of these two CRP binding sites are similar. There are nevertheless significant regulatory differences between *deoP2* and *cddP*. CRP2 is essential for repression by CytR of *deoP2* (27) but not of *cddP* (11) and is the higher-affinity site for CRP in *deoP2* (30) but not in *cddP* (11).

Given the similarity in position of CRP sites in *deoP2*, *cddP*, and *udpP*, it is interesting to consider how each site contributes to regulation of transcription of distal genes. Our DNase I footprinting tests showed that in *udpP*, CRP2 is the higher-affinity site (as in *deoP2*). CRP1 has a much lower affinity for CRP binding. Full protection of CRP1 was observed only when CytR was included along with CRP. The CytR protein stabilized binding of CRP to the CRP sites in *udpP*. No direct interaction between CytR protein and DNA was observed. However, one cannot rule out possible CytR interaction with the DNA after binding to CRP. The interaction of CytR with *udpP* DNA (full protection) required the presence of CRP and CRP2. The absence of the putative CytR operator (*del264*) did not significantly affect the ability of CytR to mediate full protection of *udpP*, in accord with the results of studies of *deoP2* (28). These data indicate strong cooperativity in CytR-CRP action and direct CRP binding to its recognition sites at *udpP*, as at other CytR-regulated promoters.

The constitutivity of each of our *udpP* mutants could be explained by their sequences. For example, in deletion mutation *del214*, constitutivity could reflect a repositioning of a cryptic −35 region (TTGCGC) to a proper 17-bp distance from the original −10 region (Fig. 6). In two other deletion mutations (*del252* and *del221*), constitutivity could similarly reflect repositioning of another cryptic −35 region (TTGCGT) 17 bp from the original −10 region. The three- to fivefold activation of these mutant promoters by CRP can be ascribed to a repositioning of CRP2 from 93 to 71 bp from the transcriptional start, a position from which CRP activates transcription from the WT *malT* promoter (4).

The fusion of CRP1 and CRP2 in *del264* created a site ($TGTGAN_6TCACG$) almost identical to the consensus CRP binding site ($TGTGAN_6TCACA$). Despite this, the constitutivity of this mutant was dependent only slightly on CRP (Table 3). We propose that this reflects the importance of both CRP sites and perhaps a special conformation (bending) of the DNA between them in the normal regulation of *udp* transcription.

The significance of the spacing between CRP sites for activation of *udp* transcription was illustrated by the phenotypes of small deletions, especially *del18*. This 1-bp deletion at the −79 position created a new promoter 64 bp upstream of the original +1 site and made *udp* transcription independent of CRP and CytR (Table 3; Fig. 2). The same cryptic promoter was activated by IS*2* insertion in *ins1* (Fig. 2 and 6) but not by other 1- or 3-bp deletions (*del305*, *del320*, and *del350*). Two cryptic promoters activated in *del18* and *ins1* and in *del224* and *del364* each contain T at position −15 (Table 4). Other studies (10, 12, 18, 23) indicate that T at position −15 and G at position −14 are needed for efficient transcription in promoters that lack clear −35 regions, or for the strengthening of already functional promoters (10), and suggest that T at position −15 is a key element in the activation of these two cryptic *udpP*s. We further propose that the several cryptic promoter-like se-

TABLE 4. Comparison of putative −35, −15, −14, and −10 regions in different *udpP* mutants

Promoter[a]	Activity of cryptic promoter	Sequence			
		−35	−15	−14	−10
WT	−	acGAaA	c	G	TAaAgT
del18[b]	+	gTGAtt	T	G	TAaAtT
del305, *del320*	−	gTGAtt	g	G	TAaAtT
del351	−	aTGtgA	g	G	TAaAtT
ins1[b]	+	TgGAgA	T	a	TAaAtT
del224[c]	+	TTGcgt	T	G	TAaAcc
del364[c]	+	TTGcgt	T	G	TAaAcc
Consensus	+	TTGACA	(T)	(G)	TATAAT

[a] In all mutant promoters, the size of the spacer between the putative −35 and −10 regions is 17 bp, while in WT *udpP*, the spacer is 18 bp.

[b] Cryptic promoter with transcriptional start at −64 relative to the transcriptional start site in WT *udpP*.

[c] Cryptic promoter with transcriptional start at −13.

quences in the *udpP* region may also contribute to regulation at WT *udpP*, perhaps by sequestering RNA polymerase or by facilitating DNA bending or melting.

Of particular interest were mutations that resulted in CytR-dependent constitutive *udp* expression when intracellular cAMP-CRP concentrations were limiting (glucose medium) (*del224*, *del364*, *dup1*, and *dup361*). We propose that CytR repressor binding to CRP2 and CRP1 (original positions) in the *dup1* promoter ensured that CRP at the second copy of CRP1 could stimulate RNA polymerase to transcribe *udp* (Fig. 6B). The mechanism by which CytR repressor stimulates the *udp* expression in *dup361*, *del224*, and *del364* mutants is more difficult to explain. Possibly changes in DNA topology allow CytR-CRP-DNA binding to create structures that are more favorable for RNA polymerase activity than those created by binding to the WT sequence. Alternatively, an unknown surface on CytR protein might interact directly with RNA polymerase and increase its activity. In either model, this class of mutants suggests a new regulatory potential of CytR protein, which can act as an activator as well as a repressor of transcription.

A family of pentamer motifs matching or nearly matching the sequence 5′-TGCAA has been found as inverted repeats in all CytR-regulated promoters studied to date, including *udpP* (Fig. 1), and had been postulated to constitute a CytR binding site (24). We note, however, that the spacing between these pentamers is 5 bp in *udpP*, versus 3 bp in *cddP* and 2 bp in *deoP2*, and that the pentamers are positioned more asymmetrically with respect to the two CRP sites in *udpP* than in *deoP2* and *cddP*. There are well-known precedents for variable spacing of DNA-binding motifs, in particular the 2-bp range of spacing of −10 and −35 regions of promoters (5, 20), and one can imagine CytR-CRP complexes being quite flexible relative to other well-studied DNA-binding proteins in prokaryotes. Further studies will be needed to determine whether these sites play any, albeit very subtle, role in CytR-CRP-mediated regulation of gene expression.

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