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Nitrogen Regulation of the *codBA* (Cytosine Deaminase) Operon from *Escherichia coli* by the Nitrogen Assimilation Control Protein, NAC

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Transcription of the cytosine deaminase (*codBA*) operon of *Escherichia coli* is regulated by nitrogen, with about three times more *codBA* expression in cells grown in nitrogen-limiting medium than in nitrogen-excess medium. β-Galactosidase expression from *codBp-lacZ* operon fusions showed that the nitrogen assimilation control protein NAC was necessary for this regulation. In vitro transcription from the *codBA* promoter with purified RNA polymerase was stimulated by the addition of purified NAC, confirming that no other factors are required. Gel mobility shifts and DNase I footprints showed that NAC binds to a site centered at position −59 relative to the start site of transcription and that mutants that cannot bind NAC there cannot activate transcription. When a longer promoter region (positions −120 to +67) was used, a double footprint was seen with a second 26-bp footprint separated from the first by a hypersensitive site. When a shorter fragment was used (positions −83 to +67), only the primary footprint was seen. Nevertheless, both the shorter and longer fragments showed NAC-mediated regulation in vivo. Cytosine deaminase expression in *Klebsiella pneumoniae* was also regulated by nitrogen in a NAC-dependent manner. *K. pneumoniae* differs from *E. coli* in having two cytosine deaminase genes, an intervening open reading frame between the *codB* and *codA* orthologs, and a different response to hypoxanthine which increased *cod* expression in *K. pneumoniae* but decreased it in *E. coli*.

Escherichia coli can utilize cytosine as its sole source of nitrogen by using cytosine deaminase to cleave cytosine to ammonia (a source of nitrogen) and uracil (a source of pyrimidines). Cytosine deaminase formation is subject to a complex regulation that responds to purines, pyrimidines, and the nitrogen supply of the medium (1). A mutant that expressed a constitutively active Ntr system constitutively expressed cytosine deaminase. This was taken as evidence that the Ntr system of *E. coli* is involved in the regulation of cytosine deaminase formation (1).

The Ntr system is a complex regulatory cascade that senses the quality of the available nitrogen supply and ultimately results in the activation of a transcriptional activator, NTRC, when the nitrogen source is growth rate limiting (20). Many of the genes in the Ntr regulon are directly regulated by the NTRC-mediated activation of RNA polymerase bearing the unusual sigma factor σ54. But some genes in the Ntr regulon are regulated indirectly, and their transcription is activated by the nitrogen assimilation control protein NAC, which activates RNA polymerase bearing the more common sigma factor σ70 (4).

When NAC activates transcription, it binds to a consensus sequence often abbreviated ATA-N_9-TAT (26). A search of available DNA sequence data for sequences resembling this site uncovered many such sequences within the *E. coli* genome (12), one of which was in the promoter region of *codBA*, the operon that encodes cytosine deaminase (*codA*) and a cytosine permease (*codB*). When a *nac* mutant of *E. coli* was tested for growth with cytosine as the sole nitrogen source, the *nac* mutant grew more slowly than the wild type (23). The presence of a NAC site in the *codBA* promoter region and the slow growth phenotype of a *nac* mutant when cytosine was the sole nitrogen course suggested that NAC was involved in the nitrogen regulation of *codBA*.

An analysis using microarrays to measure steady-state mRNA levels in response to physiological and genetic signals that affect nitrogen regulation has been recently reported (28). These data show that *codBA* expression is indeed regulated by nitrogen, that this response requires the Ntr system, and that the response also requires NAC. The experiments reported here were designed to determine whether NAC directly regulates the *codBA* operon or whether NAC might act indirectly.

MATERIALS AND METHODS

Bacterial strains and plasmids. Descriptions and genotypes of the bacterial strains used here are listed in Table 1. All *E. coli* strains were derived from *E. coli* strain K-12. All *Klebsiella pneumoniae* strains were derived from strain W70. This strain was formerly known as *Klebsiella aerogenes*; however, the species *K. aerogenes* has been subsumed into the species *K. pneumoniae*.

Enzyme assays. β-Galactosidase was measured as described previously (27) except that the assay was performed at 30°C. For cytosine deaminase assays, cells were grown to mid-log phase, collected by centrifugation, and washed once with cold assay buffer (50 mM HEPES, pH 7.8). The cells were resuspended in 1/10 of their original volume of assay buffer and disrupted by sonic oscillation. Cellular debris was removed by centrifugation. The resulting cell extract contained about 1 mg of total protein/ml, determined by the method of Lowry (19). The reaction mixture contained 0.4 ml of assay buffer containing 50 mM cytosine equilibrated to 37°C. The reaction was initiated by the addition of 50 μl of the cell extract, samples were removed at 0.5, 10, 20, and 30 min, and the ammonia released from cytosine was measured as described previously (11). Specific activities are reported in nanomoles of ammonia released per minute per milligram of total protein.

In vitro transcription. In vitro transcription from the *codBA* promoter in plasmid pCB592 was carried out essentially as described by Maquat and Reznikoff (21) by using RNA polymerase with σ70 (purchased from Epicentre Technologies) and NAC (purified essentially as described by Goss and Bender [12]).

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TABLE 1. List of strains

Strain	Relevant genotype	Source or reference
E. coli[a]		
DH5α	Δ(*cod-lac*)	16
W3110	Wild type	R. Matthews
YMC10	Δ(*cod-lac*)*U169*	2
YMC15	Δ(*cod-lac*)*U169 glnL2302*	6
EB3264	DH5α/pCB630	This work
EB3364	W3110 *nac-28*	22
EB3846	W3110/pPC36	17
EB3870	YMC10 *nac-28*/pPC36/pCB911	This work
EB3872	YMC10 *nac-28*/pPC36/pCB910	This work
EB3874	YMC15/pCB910	This work
EB3875	YMC15 *nac-28*/pCB910	This work
EB3876	YMC10/pCB910	This work
EB3877	YMC10 *nac-28*/pCB910	This work
EB3878	YMC15/pCB911	This work
EB3879	YMC15 *nac-28*/pCB911	This work
EB3880	YMC10/pCB911	This work
EB3881	YMC10 *nac-28*/pCB911	This work
EB5550	YMC10/pCB1375	This work
EB5551	YMC10 *nac-28*/pCB1375	This work
K. pneumoniae[b]		
KC2668	Wild type	17
KC2725	KC2668 *nac-203*::Tn*5-131*	17

[a] All *E. coli* strains are derived from *E. coli* strain K-12.
[b] All *K. pneumoniae* strains are derived from *K. aerogenes* (now called *K. pneumoniae*) strain W70.

[α-^{32}P]UTP was included in the reaction mixture, and the resulting radioactive transcripts were separated by polyacrylamide gel electrophoresis and visualized by autoradiography.

Gel mobility shift assays. DNA fragments were isolated and labeled with [α-^{32}P]dATP by using the Klenow fragment (Roche) according to the manufacturer's instructions. Labeled DNA fragments were incubated with increasing concentrations of purified NAC protein with 50 mM Tris, pH 7.5, and 20 nM poly(d · IC) in a total volume of 5 μl. The binding mixtures were incubated for 20 min at 30°C, and after this incubation period, 1 μl of DNA loading buffer (2 mg of bromophenol blue, 2 mg of xylene cyanol, 40 mM Tris [pH 8.4], 4 mM EDTA [pH 8.0], 25% glycerol) was added. Each reaction was loaded onto a Tris-borate-EDTA 5% polyacrylamide gel, and the gel was run, treated, and exposed to film as previously described (26).

DNase I footprinting. Plasmid DNA was linearized by digestion with *Eco*RI or *Hin*dIII, and the resulting 3′ ends were labeled with the Klenow fragment of DNA polymerase I. The labeled DNA was digested with a second restriction enzyme to generate two end-labeled fragments, one less than 20 bp and the other carrying the promoter of interest. A 7-μl sample containing about 0.2 pmol of DNA (about 500,000 cpm) was mixed with purified NAC and allowed to incubate for 30 min at room temperature. Digestion was initiated by the addition of 10^{-3} U of DNase I and terminated 2 min later by the addition of 7 μl of formamide, and samples were incubated at 80°C for 5 min. Samples were prepared for electrophoresis, and the digested fragments were separated by electrophoresis in polyacrylamide gels containing 7 M urea. The results were visualized by autoradiography. DNase I digestion patterns were aligned with the DNA sequence by comparison with the G and/or A+G sequencing ladders that were run on the same gels.

Construction of plasmids. The plasmid pRAC82 (a kind gift of Jan Neuhard) carries a *Hae*III fragment of *E. coli* DNA cloned into the *Sma*I site in pUC19. The full-length *codBA* promoter region, extending from positions −120 to +67 relative to the start site of transcription, was subcloned as an *Eco*RI-*Bam*HI fragment into the *lacZ* expression vector pRJ800 (3), resulting in plasmid pCB1375. Plasmid pCB910 contains a shorter fragment extending from positions −83 to +67 that was generated by PCR. Plasmid pCB911 was constructed exactly as was pCB910, except that the upstream primer contained an A-to-C change in the first nucleotide of the NAC-binding sequence. Thus, plasmid pCB911 is identical to pCB910 except that pCB910 carries the sequence ATA-N_9-TAT and pCB911 carries CTA-N_9-TAT. Plasmids pCB816 and pCB864 (carrying the longer and shorter promoter regions, respectively) were used for DNase I footprinting. Plasmid pCB816 carries an *Eco*RI-*Hin*dIII fragment from positions −120 to +67 cloned into pBC KS+. Plasmid pCB864 carries the region from positions −83 to +67 cloned as an *Eco*RI-*Bam*HI fragment in pBC KS+. Thus, pCB816 and pCB864 are in opposite orientations in pBC KS+. Plasmid pCB592 carries the full-length *codBA* promoter region (positions −120 to +67) cloned upstream from the strong transcription terminator in pTE103 (9).

A cytosine deaminase gene from *K. pneumoniae* was initially cloned by using the in vivo method based on phage Mu and described by Groisman and Casadaban (15). The resulting plasmid, pCB619, contained about 5 kb of *K. pneumoniae* DNA inserted into pEG5005. Plasmid pCB619 allowed strain YMC10 (Δ*cod-lac*) to grow with cytosine as the sole nitrogen source. A 7.4-kb *Hin*dIII fragment from pCB619 (containing the kanamycin resistance element from pEG5005 as well as the *codA* gene from *K. pneumoniae*) was subcloned into pGB2 (7), resulting in pCB628. A 6.6kb *Sac*I-*Eco*RI fragment from pCB628 was then subcloned into pBluescript (pBS KS−), resulting in pCB630.

RESULTS

NAC-induced sensitivity to fluorocytosine. Wild-type strains of *E. coli* are sensitive to the cytosine analog fluorocytosine because this compound is metabolized to the toxic 5-fluorouracil by cytosine deaminase (24). However, *codBA* mutants are resistant to this compound. As an initial estimate of NAC participation in *codBA* regulation, disks containing fluorocytosine were placed on lawns of wild-type and *nac* strains growing with various nitrogen sources. As expected, nitrogen-limiting medium resulted in larger zones of growth inhibition for the wild-type strain than nitrogen-excess medium (Table 2). In contrast, strains carrying *nac-28*, a null mutation of the *nac* gene, showed the same small zone of inhibition whether growing on nitrogen-limiting or nitrogen-excess medium.

The NAC protein from *K. pneumoniae* is very similar to NAC from *E. coli* and can functionally replace *E. coli* NAC in all assays tested (23). When strain EB3846 (W3110 with an isopropyl-β-D-thiogalactopyranoside [IPTG]-inducible *nac* gene from *K. pneumoniae*) was induced with IPTG, the strain was sensitive to fluorocytosine even when grown on nitrogen-excess medium. Thus, NAC was necessary for *codBA*-mediated sensitivity to fluorocytosine. Neither NTRC nor nitrogen limitation was required if NAC was provided.

TABLE 2. Sensitivity to 5-fluorocytosine

Strain	Relevant genotype	Medium[a]	Zone of clearing[b] (mm)
W3110	Wild type (*E. coli*)	GNArg	21
W3110	Wild type (*E. coli*)	GArg	71
EB3366	*nac-28*	GNArg	21
EB3366	*nac-28*	GArg	23
EB3846[c]	Wild type/pPC36 (inducible NAC_K)	GN	37
EB3846	Wild type/pPC36 (inducible NAC_K)	GN IPTG	>75
KC2668	Wild type (*K. pneumoniae*)	GN	12
KC2668	Wild type (*K. pneumoniae*)	GGlt	61
KC2725	*nac-203*::Tn*5-131*	GGlt	14
W3110	Wild type (*E. coli*)	GNArg+Hpxt	6
W3110	Wild type (*E. coli*)	GArg+Hpxt	10
KC2668	Wild type (*K. pneumoniae*)	GN+Hpxt	25
KC2668	Wild type (*K. pneumoniae*)	GGlt+Hpxt	75

[a] Growth medium contained the following: G, 0.4% (wt/vol) glucose; N, 0.2% (wt/vol) ammonium sulfate; Arg, 0.2% (wt/vol) L-arginine hydrochloride; IPTG, 0.5 mM IPTG; Glt, 0.2% (wt/vol) monosodium glutamate; Hpxt, 50 μg of hypoxanthine/ml.
[b] Small disks were saturated with a solution of 5-fluorocytosine at 50 μg/ml for *E. coli* strains and 600 μg/ml for *K. pneumoniae* strains.
[c] Plasmid pPC36 carries an IPTG-inducible gene that encodes NAC from *K. pneumoniae* (NAC_K).

TABLE 3. Cytosine deaminase activity and β-galactosidase expression from *codB-lacZ* fusions

Strain	Genotype or description	Growth medium[a]	Cytosine deaminase[b]	β-Galactosidase[b]
W3110	Wild type	GNArg	50	
W3110	Wild type	GArg	165	
EB3366	*nac-28*	GNArg	43	
EB3366	*nac-28*	GArg	52	
EB3846[c]	*nac*$^+$/pPC36 (NAC_K inducible)	GN	78	
EB3846	*nac*$^+$/pPC36 (NAC_K inducible)	GN IPTG	211	
EB5550	Δ(*cod-lac*)/pCB1375	GNArg		1360
EB5550	Δ(*cod-lac*)/pCB1375	GArg		7470
EB5551	Δ(*cod-lac*) *nac-28*/pCB1375	GNArg		1530
EB5551	Δ(*cod-lac*) *nac-28*/pCB1375	GArg		1420
EB3876	Δ(*cod-lac*)/pCB910	GArg		4830
EB3877	Δ(*cod-lac*) *nac-28*/pCB910	GArg		1932
EB3874[d]	Δ(*cod-lac*) *glnL2302*/pCB910	GN		4291
EB3875	Δ(*cod-lac*) *glnL2302 nac-28*/pCB910	GN		1430
EB3872	Δ(*cod-lac*) *nac-28*/pPC36/pCB910	GN		1432
EB3872	Δ(*cod-lac*) *nac-28*/pPC36/pCB910	GN IPTG		4152
EB3880	Δ(*cod-lac*)/pCB911	GArg		1110
EB3881	Δ(*cod-lac*) *nac-28*/pCB911	GArg		1233
EB3878	Δ(*cod-lac*) *glnL2302*/pCB911	GN		1267
EB3879	Δ(*cod-lac*) *glnL2302 nac-28*/pCB911	GN		1176
EB3870	Δ(*cod-lac*) *nac-28*/pPC36/pCB911	GN		939
EB3870	Δ(*cod-lac*) *nac-28*/pPC36/pCB911	GN IPTG		1241
EB3264[e]	Δ(*cod-lac*)/pCB630 ($codA_K$)	GNArg	311	
EB3264	Δ(*cod-lac*)/pCB630 ($codA_K$)	GArg	1545	

[a] W4 minimal medium (14) was supplemented with the following: G, 0.4% (wt/vol) glucose; N, 0.2% (wt/vol) ammonium sulfate; Arg, 0.2% L-arginine hydrochloride; IPTG, 0.5 mM IPTG.
[b] β-Galactosidase and cytosine deaminase assays are reported as the specific activity and are the averages from three or more experiments.
[c] Plasmid pPC36 carries an IPTG-inducible gene which encodes NAC from *K. pneumoniae.*
[d] Plasmid pCB910 carries a short fragment of the *codBA* promoter region; pCB911 carries the same region but with a mutation in the NAC-binding site. The *glnL2302* mutation causes high-level expression of the Ntr system, even in the presence of ammonia.
[e] Plasmid pCB630 carries the *K. pneumoniae codA* gene.

Cytosine deaminase in *K. pneumoniae*. Our laboratory has a longstanding interest in nitrogen regulation in *K. pneumoniae* where nitrogen-regulated genes are more numerous and often more tightly regulated than those of *E. coli*. Therefore, we examined the regulation of cytosine deaminase expression in *K. pneumoniae.* Wild-type *K. pneumoniae* strain KC2668 was much more sensitive to fluorocytosine under nitrogen-limiting conditions than under nitrogen excess (Table 2). Moreover, a *nac* mutation abolished the increased sensitivity (Table 2, strain KC2725), suggesting that *K. pneumoniae* has a NAC-regulated cytosine deaminase.

Surprisingly, *E. coli* and *K. pneumoniae* differed in their response to the presence of hypoxanthine. In *E. coli*, the presence of hypoxanthine in the medium reduced *codBA* expression because *codBA* is under the control of the *purR* repressor protein in addition to control by NAC (1). As a result, hypoxanthine reduced the sensitivity of *E. coli* to fluorocytosine (Table 2, strain W3110). In contrast, hypoxanthine increased the sensitivity of *K. pneumoniae* to fluorocytosine (Table 2, strain KC2668).

NAC-dependent activation of *codBA* expression in vivo. The data in Table 3 show that cytosine deaminase formation is derepressed about threefold by nitrogen-limited growth in *E. coli* (strain W3110). This confirms the earlier data of Anderson et al. (1) by using a different assay. This derepression was abolished in the *nac* mutant, strain EB3366. In strain EB3846, which carries the IPTG-inducible *nac* gene on pPC36, the addition of IPTG led to a threefold induction of cytosine deaminase even in nitrogen-excess medium. Thus, NAC was necessary for the derepression of cytosine deaminase formation.

To confirm that this derepression reflected a transcriptional control of *codBA* expression, we took advantage of three plasmids. Plasmid pCB1375 contains *codBA* promoter DNA from positions −120 to +67 relative to the start of transcription. (The key features of the *codBA* promoter region are illustrated in Fig. 1). Plasmid pCB910 contained the region from positions −83 to +67, and plasmid pCB911 was identical to pCB910 except that the conserved ATA of the recognized NAC consensus was changed to CTA (an A-to-C change at position −66). This change should abolish NAC binding (26).

The longer *cod-lac* fusion in pCB1375 showed a fivefold derepression in response to nitrogen limitation (Table 3, strain EB5550), and this derepression was not seen in the congenic strain carrying the *nac-28* mutation (Table 3, strain EB5551). The shorter *cod-lac* fusion in pCB910 showed a threefold response to NAC (the same as seen for chromosomally encoded cytosine deaminase) in three separate comparisons. First, a wild-type strain carrying pCB910 had high-level expression of the *cod-lac* fusion in response to nitrogen limitation (Table 3, strain EB3876). A congenic strain carrying the *nac-28* mutation had almost threefold less (strain EB3877). Second, a strain that had a constitutively active Ntr system caused by the *glnL2303* mutation had high-level expression of the *cod-lac* fusion even in nitrogen-rich medium (Table 3, strain EB3874); again, a congenic strain carrying the *nac-28* mutation had about threefold less (strain EB3875). Finally, a strain carrying the *nac-28* mutation and an IPTG-inducible *nac* gene (on plasmid pPC36) had high-level expression of the *cod-lac* fusion when IPTG was added, even though the cells were grown in nitrogen-rich medium (Table 3, strain EB3872). The same strain grown without

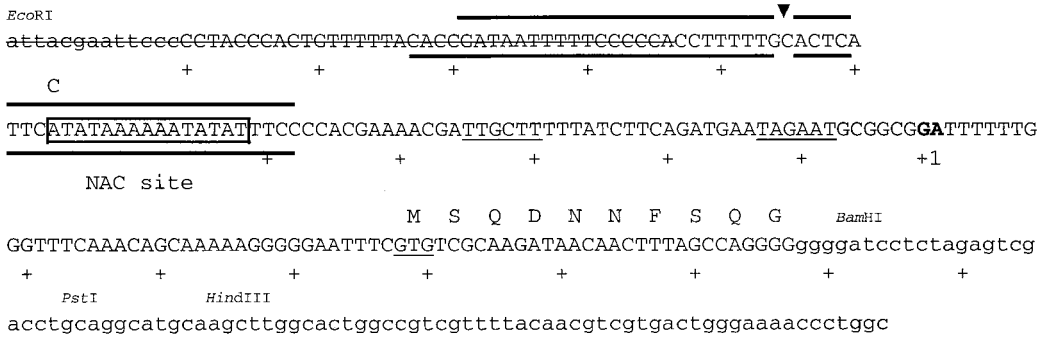

FIG. 1. DNA sequence of the *codBA* promoter region showing key features. The DNA sequence is as found in pRAC82. Small letters represent vector DNA; capital letters represent *E. coli* DNA. Boxed nucleotides are the NAC consensus sequence. Over- and underscoring show the extent of the DNase I footprint of NAC on the top and bottom strands of this fragment (Fig. 3 and data not shown). The arrowhead indicates the strong hypersensitive site. The letters with a line through them represent the segment that was deleted (replaced) in the shorter promoter in pCB910 and pCB911. The bold letters at position +1 are the start sites of transcription (8). Underlined sequences are putative −10 and −35 regions, and GTG is the start site of the *codB*-coding sequence. The C above the A at position −66 indicates the nucleotide changed in the construction of pCB911 from pCB910.

IPTG induction had about threefold less. Thus, NAC was necessary for the derepression of a *cod-lac* transcriptional fusion in vivo and the NAC-mediated control of cytosine deaminase formation functioned at the level of transcription.

Plasmid pCB911 with its defective NAC site was used in the same three tests as pCB910. Under nitrogen-limiting conditions, a wild-type strain showed no more *cod-lac* expression than a strain carrying the *nac-28* mutation (Table 3, strains EB3880 and EB3881). The Ntr constitutive strain showed no more *cod-lac* expression than the *nac-28* strain (Table 3, strains EB3878 and EB3879). Finally, even IPTG induction of the *nac* gene on pPC36 resulted in only a slight (about 30%) increase in *cod-lac* expression. Thus, the ATA-N_9-TAT sequence thought to be important for NAC binding was necessary for the NAC-mediated activation of *codBA* transcription in vivo.

NAC binds to the *codBA* promoter. The importance of the ATA-N_9-TAT sequence for NAC binding was confirmed by gel mobility shift analysis and by DNase I footprinting. Figure 2 shows that a DNA fragment containing the mutant *codBA* promoter from pCB911 was defective in NAC binding, whereas comparable wild-type fragments from pRAC82 and pCB910 did bind NAC. However, the fragments from pRAC82 and pCB910 showed different patterns of mobility shift by NAC. In both cases, two specific shifts were detected. When the longer fragment (positions −120 to +67) was used, the slower-migrating band predominated. When the shorter fragment was used, the faster-migrating band predominated. Curiously, the only shift detected with the mutant fragment was the slower-migrating one, which, though weak, was about as strong as the comparable band in the fragment from pCB910 (Fig. 2, lanes 10 and 15).

The DNase I footprints of NAC bound to the longer promoter (positions −120 to +67) showed strong protection of a 26- to 27-bp region from positions −48 to −75 with the NAC consensus ATA-N_9-TAT at its center (Fig. 3, lanes 1 through 3). On the promoter-distal side of this protected region was a site with increased DNase I sensitivity followed by an extended footprint of about 25 to 28 bp. When the shorter promoter (with DNA upstream from position −83 replaced by vector DNA) was analyzed, the core footprint from positions −48 to −75 centered on ATA-N_9-TAT was again observed (Fig. 3, lanes 4 through 8). The locations of the DNase I footprints and the DNA replacement are illustrated in Fig. 1. Neither the hypersensitive site nor the extended footprint were seen with the short promoter. Thus, the short promoter (from positions −83 to +67) bound NAC at the consensus site, protected a 26-bp region as seen elsewhere (12), and caused a modest gel mobility shift with a small fraction shifted to the more slowly migrating species. The long promoter (positions −120 to +67) gave two adjacent footprints of similar size (roughly 26 bp) separated by a hypersensitive site. In mobility shift assays, the longer promoter showed only a trace amount of the modest shift, with the majority of the shifted target belonging to the more slowly migrating species.

In vitro transcription of *codBA*. To confirm that NAC activation of *codBA* expression was direct and required no other factors, we analyzed the ability of NAC to activate transcription from the *codBA* promoter in vitro by using *E. coli* RNA polymerase in a purified system. The *codBA* promoter was

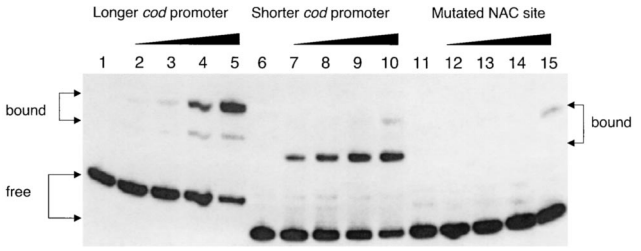

FIG. 2. Gel mobility shift assay of the interaction of NAC with the *codBA* promoter region. Each lane contains about 100,000 cpm of ^{32}P-end-labeled DNA. Lanes 1 through 5 (longer promoter), the *Eco*RI-*Hin*dIII fragment from pRAC82 containing promoter DNA from positions −120 to +67; lanes 6 through 10 (shorter promoter), an *Eco*RI-*Bam*HI fragment from pCB910 containing promoter DNA from positions −83 to +67; lanes 11 through 15 (mutant promoter), an *Eco*RI-*Bam*HI fragment from pCB911 containing the same promoter DNA (positions −83 to +67) but with an A-to-C mutation in the NAC-binding site. In each set of five, NAC was absent from the first lane and the remaining four samples were incubated with 7, 26, 66, or 132 ng of NAC before electrophoresis.

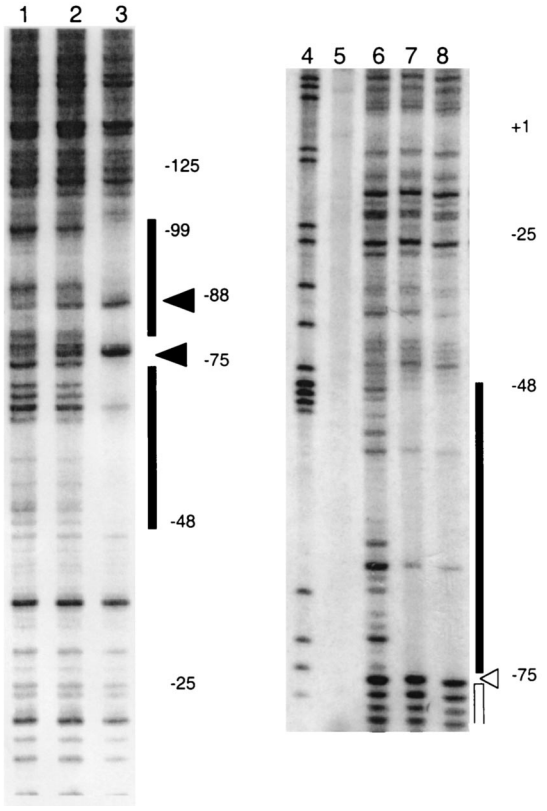

FIG. 3. DNase I protection assay of the *codBA* promoter. Lanes 1 through 3 show the top strand of pCB816 (labeled at the *Hin*dIII site), which contained the longer promoter fragment. Lanes 6 through 8 show the DNase I footprint of NAC on pCB864 (labeled at the *Hin*dIII site) which contained the shorter promoter fragment. Lane 4 contained the G ladder, and lane 5 contained the no-DNase I control. Lanes 1, 2, and 3 contained 0, 0.1, and 0.5 μg of NAC, respectively. Lanes 6, 7, and 8 contained 0, 0.4, and 0.8 μg of NAC, respectively. Protected regions are indicated with the black bar, and hypersensitive sites are marked with black arrows. The open bar and arrowhead indicate the protected region and hypersensitive site seen in the longer *codBA* promoter construct, respectively. For comparison, the protected sequences are indicated in Fig. 1.

cloned upstream of a strong transcriptional terminator such that transcription from *codBp* would yield a discrete RNA molecule. Figure 4 shows that RNA polymerase alone can generate a transcript of the expected size and that the addition of purified NAC increased the amount of this transcript at least threefold. This shows clearly that NAC alone is sufficient to activate *codBA* transcription by binding to a site centered at position −59.

The NAC used in this experiment was purified from *K. pneumoniae*. The *E. coli* NAC protein is insoluble, and we have not yet succeeded in purifying it in quantity (22). To show that the *E. coli* NAC can activate *codBA* transcription, we used a fusion of the *E. coli* maltose-binding protein (MBP) to the N terminus of NAC. The two proteins were joined by a linker that is readily cleaved by factor Xa protease. Although incubation of this fusion protein with factor Xa liberated intact MBP and NAC, the NAC that is released was rapidly degraded by factor Xa. Therefore, we incubated the MBP-NAC fusion protein (and a comparable fusion of MBP to NAC from *K. pneumoniae*) with factor Xa for a brief period and immediately added the mixture to the RNA polymerase and *codBA* DNA reaction. As can be seen in Fig. 4 (lanes 5 and 6), the *E. coli* NAC was as effective in activating *codBA* transcription as the *K. pneumoniae* NAC was.

Two cytosine deaminase genes in *K. pneumoniae*. We sought a clone of the gene for cytosine deaminase from *K. pneumoniae* for comparative study. The in vivo cloning procedure of Groisman and Casadaban (15) yielded plasmid pCB619 (moderate copy number). The *E. coli* Δ*cod-lac* strain, YMC10, carrying this plasmid (strain EB3246) was able to grow with cytosine as the sole nitrogen source and was sensitive to fluorocytosine. A DNA fragment from pCB619 was subcloned into a low-copy-number plasmid (pCB628) and a high-copy-number plasmid (pCB630) (see Materials and Methods). Plasmid pCB628 allowed the *E. coli* Δ*cod-lac* strain DH5α to grow with cytosine as sole nitrogen source. Plasmid pCB630 conferred cytosine growth and fluorocytosine sensitivity on strain EB3264 (DH5α/pCB630), but it also made this strain unable to grow on minimal medium unless supplemented with cytosine. Enzyme assays confirmed that strain EB3264 produced a cytosine deaminating activity and that nitrogen limitation led to a fivefold derepression of this cytosine deaminase (Table 3). Thus the cloned fragment contained all the information to produce cytosine deaminase whose formation was regulated by nitrogen.

The DNA sequence of two contiguous *Bam*HI fragments from pCB630 was determined, representing about 5.8 kb of DNA. This sequence was compared to the genomic sequence of *K. pneumoniae* strain MGH78578 that was determined by the Genome Sequencing Center at Washington University at St. Louis (http://www.genome.wustl.edu) and was identical except for 1 nucleotide. This 5.8-kb region contained a number of open reading frames (ORFs), of which three were of particular

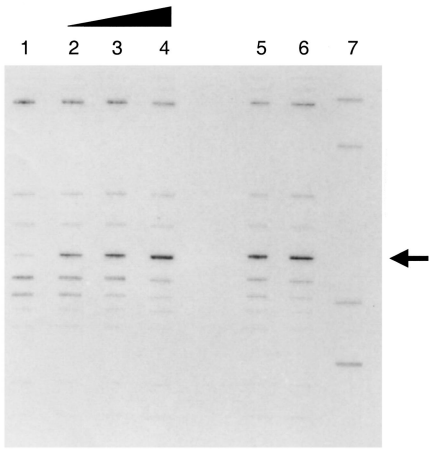

FIG. 4. In vitro transcription from the *codBA* promoter. The DNA template was supercoiled plasmid pCB592. The reactions in lanes 1 through 6 contained purified RNA polymerase from *E. coli.* Lane 1 contained no NAC. Lanes 2 through 4 contained 0.1, 0.2, and 1.0 μg of NAC derived from *K. pneumoniae*, respectively. Lanes 5 and 6 contained 1 μg of NAC (from *K. pneumoniae* and *E. coli*, respectively) prepared as an MBP-NAC fusion protein and cleaved with factor Xa to release active NAC immediately before addition to the reaction mixture. Lane 7 contained labeled DNA size standards of 210, 245, 400, and 510 bp. The arrow indicates the runoff transcript from the *codB* promoter.

interest. These three ORFs span a continuous sequence of 3,657 bp such that the termination codon of each ORF (TGA in each case) overlapped the initiation codon of the next ORF. When the deduced amino acid sequences of these three ORFs were compared to the sequences of deduced *E. coli* proteins, the first ORF showed similarity to the *codB* (cytosine permease) protein and the third ORF showed strong similarity to the *codA* (cytosine deaminase) protein. The deduced amino acid sequence of the intervening ORF (predicted to be 411 amino acids long) showed no strong similarity to any *E. coli* protein. Thus, the organization of this putative operon differs from the *codBA* operon of *E. coli.*

Surprisingly, when the *codA* gene from *E. coli* was compared to the genomic sequence of *K. pneumoniae* strain MGH78578, a second unlinked gene that was even more similar to *E. coli codA* than was the ortholog on pCB630 was found. Perhaps this may explain another peculiar difference between *E. coli* and *K. pneumoniae.*

DISCUSSION

The data presented here confirm previous observations that cytosine deaminase formation is regulated by the Ntr system acting through the NAC protein (1, 28). The present study also shows that the activation of *codBA* transcription by NAC is direct. However, the activation of *codBA* transcription by NAC is unusual in several respects.

Many of the previous examples of promoters strongly activated by NAC have a NAC-binding site centered at −64 relative to the start site of transcription (4). An analysis of NAC-mediated activation by using the *lacZ* promoter region confirmed that the strongest activation occurred when the NAC site was centered at position −64 (25). However, significant activation (about threefold) was observed when the NAC site was centered at position −42, −52, or −54. Consistent with these artificial constructs, we found that the alanine utilization operon (*dadAB*) from *K. pneumoniae* is activated about threefold and has a NAC site centered at position −44 (17). However, no activation of the *lacZ* promoter was seen when the NAC-binding site of the artificial constructs was moved 5 bp from these sites to position −47, −49, or −59 (25). Yet NAC can activate *codBA* transcription from a site centered at position −59. Such a location should put the NAC binding on the opposite side of the DNA helix from all other known NAC activation sites.

The location of the NAC-binding site at *cod* is not its only unusual feature; the architecture of the site is also unusual. The consensus sequence for NAC-activated promoters is ATA-N_9-TAT. At these sites, NAC generates a simple footprint of about 26 bp with the consensus sequence located at the center. Occasionally, a slight increase in DNase I sensitivity is seen just at the promoter-proximal edge of the footprint but it is always very slight at best. The consensus-binding site for NAC-repressible promoters (*gdhA* and *nac*) includes ATA-N_9-GAT (13). At these sites, NAC has a complex footprint consisting of a core footprint of 26-bp that includes the ATA-N_9-GAT sequence, a region of strong DNase I hypersensitivity, and an extended footprint of similar size covering a nucleotide sequence that bears no recognizable similarity to the known consensus sequences for NAC binding. At *nac*, the extended footprint is strong and has distinct endpoints (10). At *gdhA*, the extended footprint is weaker and its endpoint is less distinct (13). In addition, gel mobility shift assays at *gdhA* show two retarded species in contrast to the single species seen at activatable promoters (18). The extended footprints seen in DNase I footprints and the slower-migrating bands seen in gel shifts at *gdhA* and *codBA* suggest the presence of a second NAC dimer (possibly NAC tetramers) bound to the DNA. Thus, the footprints and gel mobility shift pattern of NAC at *codBA* resemble those of NAC-repressible promoters more closely than other NAC-activatable promoters. The significance of this observation is unclear, especially since replacement of part of the extended footprint with vector DNA sequences abolished the complex footprint and gel mobility shift patterns without abolishing the NAC-mediated activation.

One other feature of the NAC site at *codBA* is its extreme AT richness. The *codBA* promoter region (positions −120 to +67) includes five runs of T_5 or T_6 as well as a run of A_6 that lies in the center of the NAC-binding site. In fact, the 15 bp consensus region for NAC binding at *codBA* contains only A's and T's.

The identification of two putative cytosine deaminase genes in *K. pneumoniae* was unexpected. The gene that was cloned in pCB630 certainly encodes a cytosine deaminase. First, it allows a Δ*cod-lac* deletion of *E. coli* to use cytosine as its sole nitrogen source. Second, it confers sensitivity to fluorocytosine on strains that carry it. And third, cell extracts of strains that carry this gene contain an activity that can release ammonia from cytosine. We have no direct evidence that the other *codA* ortholog is an authentic cytosine deaminase gene, but its strong similarity to the *E. coli codA* gene makes it likely.

The *codBA* operon of *E. coli* is part of the "insertion bubble" that distinguishes the *lacZYA* region of *E. coli* from other enteric bacteria (5). Thus, one may ask where the *codA* genes of *K. pneumoniae* lie. The ORFs on pCB630 give no clue to this question. However, a comparison of the genes on the same DNA segment of the *K. pneumoniae* genomic sequence strongly suggests that the *codA* ortholog on pCB630 lies at a position analogous to min 13 of the *E. coli* genetic map and that the other *codA* ortholog lies at min 75. About 15 kb from the *codA* ortholog found on pCB630 is a sequence of ORFs that are orthologous to *E. coli fepB*, *entC*, *entE*, *entA*, *cstA*, and *ybdH* (in that order and transcribed convergently toward the 3′ end of *codA*). That places this ortholog near min 13. The other *codA* ortholog appears to be neatly inserted between the *nirB* gene (part of the nitrite reductase operon) and an unidentified ORF (*orf383*). In *E. coli*, *orf393* and *nirB* are separated by 262 bp and are transcribed in the same direction. In *K. pneumoniae*, the *codA* ortholog is 39 bp downstream from *orf393*, 239 bp upstream from *nirB*, and transcribed in the direction opposite both of them. This places the other *codA* ortholog at min 75, near the *crp* gene.

The regulation of cytosine deaminase formation in *E. coli* is quite complex. This is expected because the deamination of cytosine can lead to an imbalance in pyrimidine pools. The threefold increase in cytosine deaminase formation mediated by NAC has a profound effect on fluorocytosine sensitivity. The 10-fold increase seen when the high-copy-number clone pCB630 was present seems to have led to such rapid degradation of endogenous cytosine that the cells were unable

to synthesize enough cytosine to allow growth and could grow only when an exogenous source was provided. In other words, even small changes in expression can have serious consequences and therefore, tight regulation is necessary.

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