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Regulation of *Escherichia coli purA* by Purine Repressor, One Component of a Dual Control Mechanism†

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***Escherichia coli purA* encodes adenylosuccinate synthetase, one of two enzymes required for synthesis of AMP from IMP. *purA* is subject to two- to threefold regulation by *purR* and about twofold regulation by a *purR*-independent mechanism. The 5′-flanking region of *purA* confers *purR*-dependent transcriptional regulation of *purA* but not the *purR*-independent regulation. Two operator sites in the 5′-flanking region which bind purine repressor in vitro and are required for in vivo regulation were identified. The *purR*-independent regulation may be posttranscriptional. It is now established that all transcription units involved in de novo synthesis of purine nucleotides, nine *pur* operons, as well as *purR* itself and *guaBA*, are subject to *purR* control.**

Adenylosuccinate synthetase (EC 6.3.2.6), the product of the *purA* gene in *Escherichia coli*, catalyzes the first of two reactions from the IMP branch point to AMP in the de novo purine nucleotide biosynthetic pathway (21). The second reaction to AMP is catalyzed by adenylosuccinate lyase (EC 4.2.2.2), the product of *purB*. Genetically, *purA* is located at min 95 on the *E. coli* chromosome and is unlinked to other *pur* regulon genes (1). The *purA* gene has been cloned and sequenced (32), and its expression is modulated by the levels of purines in the growth medium (31). On the basis of assays of adenylosuccinate synthetase used to monitor *purA* expression and β-galactosidase for *purB-lacZ* expression, it was concluded that *purA* and *purB* are regulated separately (31). More recent information indicates that *purB* expression is coregulated with those of other *pur* regulon genes by the purine repressor (PurR) (9, 15). PurR requires binding of hypoxanthine or guanine corepressors for interaction with a conserved 16-bp operator sequence (16, 23). The position of the operator relative to the promoter varies among the *pur* regulon genes and in part determines the extent and mechanism of transcriptional regulation. In *purB*, the operator is far downstream from the promoter in the protein coding region (9). *purB* is regulated only about two- to threefold, whereas genes dedicated to the de novo synthesis of IMP are more highly regulated (8, 9, 15).

Here, we report the results of experiments to determine the regulatory mechanism for *purA*. The *purA* gene is subject to dual regulation by levels of purines in the medium. One mechanism, described here, involves PurR-operator control. In addition, there is *purR*-independent regulation of *purA*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this work are listed in Table 1. LB and 2× YT media (18) were used as rich media. The minimal growth medium has been described previously (8).

Plasmid construction. To clone the *purA* control region, a segment of *purA* from nucleotides −478 to +103, numbered relative to the start of transcription (Fig. 1), was amplified by PCR (20) from chromosomal DNA of *E. coli* W3110. The amplified fragment, containing *Sma*I and *Bam*HI adapters, was ligated into the *Sma*I and *Bam*HI sites of pRS415 to yield a *purA′-lacZ* transcriptional fusion in plasmid pBH599. Plasmid pRS415 contains an intact *lacZ* coding sequence and ribosome-binding site but lacks a promoter. Plasmids pBH140 and pBH170 were constructed as a source of the *purA* control region for the gel retardation assay. First, a 140-bp fragment of *purA* from nucleotides −193 to −54 was amplified by PCR. Second, this fragment, containing *Eco*RI and *Bam*HI adapters, was subcloned into the *Eco*RI-*Bam*HI site of pUC118, resulting in plasmid pBH140. In a similar way, a 178-bp fragment from nucleotides −72 to +103 was constructed and was ligated into the *Eco*RI and *Bam*HI sites of pUC118 to give plasmid pBH170.

Site-directed mutagenesis. Mutations were introduced at three invariant positions (7) in the *purA* operators. A 581-bp *purA* fragment from pBH599 was cloned into the *Hinc*II and *Bam*HI sites of pUC118 for mutagenesis by the procedure of Kunkel et al. (11). The primers which were used for operator mutations (with the mismatches underlined) were

5′ CTACATGTTGAGGTCAATGATTGGCTGAAC ($purA_{O_1}$) and
5′ GAATCCATTTTTAAGTGCACGGTGATTTTG ($purA_{O_2}$).

Similar replacements in invariant positions in other *pur* gene operators are known to abolish binding of the repressor (10, 22, 24). All mutations were verified by nucleotide sequencing (25). The mutations were finally incorporated into the transcriptional fusion vector pRS415 by fragment exchange.

In vitro insertional mutagenesis. To mutagenize *purB*, a 1.2-kb fragment containing a kanamycin resistance (Kan^r) gene was isolated from pUC4KAC by *Bam*HI digestion, and the ends were made blunt with Klenow fragment. This fragment was inserted into the *Hpa*I site of the *purB* coding region in plasmid pBH105 to give plasmid pBH105B. pBH105B was linearized with *Acc*I and transformed into strain JC7623. A Kan^r Pur$^-$ colony was picked, and the disrupted *purB* gene was transferred into *purR*$^+$ and *purR* strains by P1 transduction (27) to give *purB* mutant strains BH804 (*purA′-lacZ purB*::Kan) and BH805 (*purA′-lacZ purB*::Kan *purR*), respectively.

Repressor-operator interaction. Gel retardation assays were performed as described previously with buffer system II for corepressor-dependent binding (23). Purified PurR was provided by K. Y. Choi of this laboratory. *purA* DNA fragments, nucleotides −478 to −193 and −193 to +103, were cloned by

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
MC4100	Δ(*argF-lac*)*169*	28
R320	MC4100 *purR300*	28
W3110	Wild type	Laboratory stock
CJ236	*dut-1 ung-1 thi-1 relA1* pJC105 (Cm^r)	11
MV1190	Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*)*306*::Tn*10* (Tet^r) F'::*traD36 proAB lacI*^{q}ZΔM15	17
JC7623	*recB21 recC22 sbcB15*	12
BH801	MC4100 (λpBH599) *purA'-lacZ* transcriptional fusion, Lac^+ Ap^r	This work
BH901	R320 (λpBH599) *purA'-lacZ* transcriptional fusion, Lac^+ Ap^r	This work
BH802	MC4100 (λpBH601) *purA'-lacZ* transcriptional fusion, $purA_{O_1^- O_2^+}$	This work
BH803	MC4100 (λpBH602) *purA'-lacZ* transcriptional fusion, $purA_{O_1^+ O_2^-}$	This work
BH804	BH801 *purB*::Kan	This work
BH805	BH901 *purB*::Kan	This work
pUC118	Phagemid cloning vector, Ap^r	30
pRS415	*lacZ* transcriptional fusion vector, Lac^+ Ap^r	28
pUC4KSAC	1.2-kb fragment containing Kan^r gene in pUC4	Pharmacia
pBH105	2.7-kb *Sau*3A fragment containing *purB* gene ligated into *Bam*HI site of pUC118	This work
pBH105B	*purB* gene in pBH105 disrupted by a Kan^r cassette	This work
pBH599	Transcriptional fusion of *purA* nucleotides −478 to +103 to *lacZ* in *Sma*I-*Bam*HI sites of pRS415	This work
pBH601	pBH599 $purA_{O_1^- O_2^+}$	This work
pBH602	pBH599 $purA_{O_1^+ O_2^-}$	This work
pBH140	140-bp *Eco*RI-*Bam*HI *purA* fragment from nucleotides −193 to −54 in pUC118	This work
pBH170	178-bp *Eco*RI-*Bam*HI *purA* fragment from nucleotides −72 to +103 in pUC118	This work

PCR from strain W3110. Subfragments of 140 bp, containing operator O_1, and 178 bp, containing operator O_2, were obtained from plasmids pBH140 and pBH170, respectively. Similar subfragments with operator mutations in O_1 and O_2 were amplified by PCR from plasmids pBH601 and pBH602, respectively. These fragments were end labeled at either *Bam*HI or *Eco*RI sites with [γ-^{32}P]ATP and T4 polynucleotide kinase. Incubations contained 10 fmol of ^{32}P-labeled DNA fragment, homogeneous PurR, 50 μM guanine corepressor, and buffer II (23) in 20 μl. For DNase I footprinting, PurR was bound to a 296-bp *purA* fragment from nucleotides −193 to +103 in buffer system I (23). The fragment was end labeled at either the *Eco*RI or *Bam*HI ends. Buffer I gives high-affinity PurR binding to the operator without the corepressor. Buffer II is used for corepressor-dependent binding of PurR to operator DNA.

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-478
ATTCATCCGTAGCCTGCGTGCTTATGAGAACAGCTTCTCTGGCAATCAGG
-428
ACGTGATGGTCATGAGCCCGGATAGATTTCTTCCGCTACATGAAGACGCC
-378
GACTTCCGCAACGCGTTAATATAACGACTGCGGTACAGGTCAATAAAGCC
-328
ACCGCATCCTCAGGGATGTCGGTGGTTTTCTTTTTCTATAAGGATAATGA
-278
ATGAATTCGACAATCTGGCTGGCGCTTGCCCTGGTTTTGGTACTGGAAGG
-228
TTTAGGGCCGATGCTTTACCCGAAGGCATGGAAGAAGATGATCTCTGCGA
-178
TGACCAATTTGCCCGATAATATTTTACGTCGTTTTGGCGGTGGACTTGTGG
-127
TTGCGGGCGTTGTGGTCTACTACATGTTG[AGGAAAACGATTGGCT]GAAC
                                     O1
-78
AAAAAACAGACTGATCGAGGTCATTTTTGAGTGCAAAAAGTGCTGTAACT
                                          -35
-28
CTGAAAAAGCGATGGTAGAATCCATTTTT[AAGCAAACGGTGATTT]TGAAA
                 -10                  O2   SD
+23
AATGGGTAACAACGTCGTCGTACTGGGCACCCAATGGGGTGACGAAGGT
+72
AAAGGTAAGATCGTCGATCTTCTGACTGAACG
```

FIG. 1. Nucleotide sequence of *purA* 5'-flanking region. The sequence is numbered from the transcriptional start (arrow) (31). Operator sites O_1 and O_2 are boxed. The position −35 and −10 promoter elements and the ATG codon are underlined. SD, Shine-Dalgarno sequence.

Enzyme assays. Wild-type cells were grown to mid-log phase in minimal medium with 100 μg of adenine per ml or without adenine. Growth conditions for purine auxotrophs were essentially the same, except that a limiting concentration of adenine (5 μg/ml) was used to derepress *purA* expression. For repression, the purine concentration was 100 μg/ml. β-Galactosidase activity in permeabilized cells was determined by the procedure of Miller (18). For adenylosuccinate synthetase assays, cells were disrupted (22) in 50 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 1 mM dithiothreitol, and cell extracts were dialyzed against the same buffer. The activity of the enzyme was measured as described by Dong and Fromm (5). One unit of enzyme activity is defined as the amount of enzyme required to produce 1 nmol of product per min at 25°C with a molar absorption coefficient of 11.7×10^6 cm^2/mol for adenylosuccinate (5). A *purB* mutant was required for assaying in extracts to avoid conversion of adenylosuccinate into AMP. The protein concentration was determined by the method of Bradford (2).

RESULTS

Regulation of *purA* expression. Regulation of the intact chromosomal *purA* gene was determined by assay of adenylosuccinate synthetase activity from cells grown with limiting or excess purines. In a $purR^+$ background (strain BH804), there was sixfold repression by adenine and there was two- to threefold repression by hypoxanthine or guanine (Table 2). Regulation by hypoxanthine and guanine was not observed in the *purR* mutant (strain BH805), indicating that this repression in strain BH804 was mediated by *purR*. The data in Table 2 also show that part of the sixfold repression by adenine was dependent upon *purR*, and about one-half was *purR* independent. Therefore, the threefold *purR*-dependent repression by adenine was likely due to its conversion to hypoxanthine and guanine (21), which are corepressors for PurR (16, 23). Previously, Wolfe and Smith (31) reported repression of *purA*

TABLE 2. Adenylosuccinate synthetase assay of *purA* regulation

Growth medium	Sp act[a] (nmol/min/mg of protein) of:	
	BH804 ($purR^+$)	BH805 (*purR*)
Limiting adenine	24	21
Excess adenine	4	10
Limiting adenine, excess guanine	9	23
Limiting adenine, excess hypoxanthine	11	25

[a] Values are the average of two independent experiments with variation of 12% or less.

by adenine, but the effects of hypoxanthine and guanine were not seen, and the role of *purR* was not investigated.

Identification of *cis* control sites for PurR. Given the *purR*-dependent regulation shown by the data in Table 2, we searched the *purA* 5′-flanking region for operator sites. A 581-bp fragment of the 5′-flanking region, nucleotides −478 to +103 (Fig. 1), was isolated from plasmid pBH599, labeled with ^{32}P, and assayed for interaction with PurR by gel retardation. Corepressor-dependent binding of PurR to the 581-bp DNA fragment was detected (data not shown). To further localize the PurR binding site, the 5′-flanking DNA was dissected into two subfragments containing nucleotides −478 to −193 and −193 to +103. PurR bound to the −193 to +103 subfragment (Fig. 2A) but not to the upstream distal DNA (not shown). At repressor concentrations greater than 33 nM, the mobility of

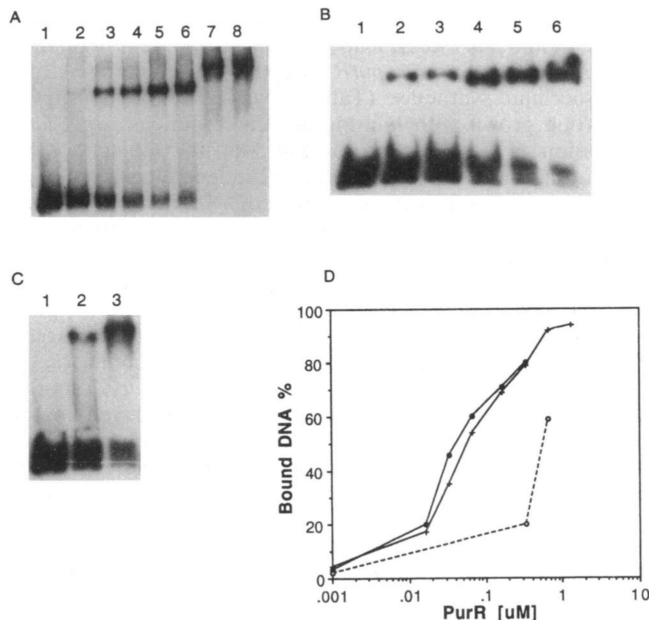

FIG. 2. Binding of PurR to the *purA* operators. Gel retardation assays were carried out as described in Materials and Methods. (A) Binding of PurR to O_1 and O_2. Concentrations of PurR (micromolar) are: lane 1, none; lanes 2 to 8, 0.017, 0.033, 0.066, 0.17, 0.33, 0.66, and 1.32, respectively. (B) Binding of PurR to O_1. Concentrations of PurR (micromolar) are: lane 1, none; lanes 2 to 6, 0.017, 0.033, 0.066, 0.17, and 0.33, respectively. (C) Binding of PurR to O_2. PurR concentrations (micromolar) are: lane 1, none; lanes 2 and 3, 0.33 and 0.66, respectively. (D) Quantitation of binding data. Bound DNA and unbound DNA were counted for radioactivity, and the percentage of bound DNA was plotted as a function of PurR concentration. ●, O_1; ○ (dashed line), O_2; +, O_1 plus O_2.

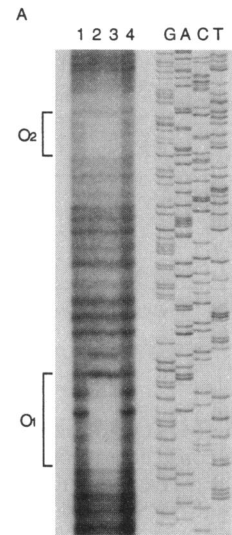

FIG. 3. DNase I footprinting for interaction of PurR with *purA* control sites. (A) A 296-bp *Eco*RI-*Bam*HI fragment (nucleotides −193 to +103) was labeled at the *Eco*RI site. Lanes 1 and 4, DNA alone; lanes 2 and 3, DNA plus G, A, C, and T (sequencing ladder for size standard). (B) Regions protected by PurR.

the repressor-DNA complex was further retarded, consistent with the possibility that PurR had bound to multiple operator sites.

To define the sites of repressor-DNA interaction, the 5′ end of the −193 to +103 *purA* fragment was labeled at either the *Eco*RI end (top strand) or *Bam*HI end (bottom strand), and the complex with PurR was footprinted with DNase I. The results of a typical experiment are shown in Fig. 3. PurR bound to two regions which are identified in the sequence as O_1 and O_2. Similar results were obtained for the footprint of the bottom strand (not shown). The protected regions O_1 and O_2 are each related to the 16-bp palindromic *pur* regulon operator 5′-ACGCAAACGTTTGCGT (8, 15, 22). There are 11 of 16 matches for O_1 and 10 of 16 matches for O_2 with the perfect palindromic sequence. These operator sites are identified in Fig. 1.

Gel retardation assays were conducted with flanking regions of the *purA* gene containing either O_1 or O_2 for comparison with PurR binding to O_1 plus O_2, which is shown in Fig. 2A. O_1 was in a 140-bp fragment (nucleotides −193 to −54), and O_2 was in a 178-bp fragment extending from nucleotides −72 to +103. Repressor-operator binding is shown in Fig. 2B for O_1 and in Fig. 2C for O_2. Summarized data are given in Fig. 2D. The results show binding of PurR to O_1 and to DNA containing O_1 plus O_2 with similar affinity. We therefore conclude that PurR binds initially to O_1 and then binds at higher PurR concentrations to O_1 plus O_2. Interactions between O_1 and O_2 appear unlikely. Mutations in conserved positions of O_1 and O_2 abolished binding of PurR to DNA fragments containing each of the mutant operators (not shown), as observed previously for other *purR* genes (10, 22, 24).

TABLE 3. Regulation of *purA'-lacZ* by *purR*

Strain	*purR*	β-Galactosidase activity (Miller units)[a]		Repression (fold)
		With adenine	Without adenine	
BH801 (O_1,O_2)	Wild type	204	483	2.4
BH901 (O_1,O_2)	Mutant	462	490	1.1
BH802 (O_2)	Wild type	292	426	1.5
BH803 (O_1)	Wild type	271	442	1.6

[a] β-Galactosidase values are the average of two or three independent experiments with a variation of 10%.

Regulation by the PurR-O_1-O_2 interaction. Transcriptional *purA'-lacZ* fusions were constructed to investigate the regulatory function of O_1 and O_2. The −478 to +103 *purA* fragment was ligated into plasmid pRS415 to give the *purA'-lacZ* transcriptional fusion plasmid pBH599. The transcriptional fusion was recombined into the chromosome to give *purR*$^+$ strain BH801 and *purR* strain BH901, each containing the wild-type *purA* control region. In a similar way, we constructed *purR*$^+$ strains BH802 and BH803 with an O_1 or O_2 mutation, respectively. Data in Table 3 show approximately 2.4-fold regulation by PurR and O_1 plus O_2 (compare strain BH801 grown with and without adenine and strain BH801 with BH901 grown with adenine). A mutation in either O_1 or O_2 led to a similar reduction to about 1.5- to 1.6-fold repression. Thus, both O_1 and O_2 are required for maximal regulation, and the interaction of PurR with each operator appears to contribute to a similar extent to the overall regulation.

DISCUSSION

The data reported here show that adenine exerted a sixfold regulation of *purA*-encoded adenylosuccinate synthetase activity. Part of this regulation resulted from the interaction of PurR with two operators in the *purA* 5'-flanking region, an interaction that is expected to repress transcription initiation. This regulation by PurR requires conversion of adenine into hypoxanthine and guanine, the corepressors needed for high-affinity repressor-operator binding (15, 22). The mechanism for the PurR-independent regulation of *purA* by adenine was not addressed.

The somewhat greater than 2.5-fold PurR-mediated regulation of *purA* is comparable to that for other genes in the IMP-to-AMP and -GMP branches of the purine nucleotide biosynthetic pathway; *purB* (9, 15) and *guaBA* (15) are regulated two- to threefold and about fivefold, respectively. This limited repression should permit the salvage of purine bases with phosphoribosyltransferases for adenine, guanine, and hypoxanthine (21) and conversion of IMP to AMP and GMP with enzymes encoded by *purA*, *purB*, and *guaBA* while expression of genes for de novo synthesis of IMP is shut down by PurR (8). It is now apparent that all of the transcription units for synthesis of purine nucleotides in *E. coli*, nine *pur* operons plus *purR* and *guaBA*, respond to the pools of purine bases via PurR, with genes dedicated to de novo synthesis of IMP exhibiting a greater response than those involved in the synthesis of AMP and GMP from IMP (8, 9, 15).

The full level of *purA* repression by PurR requires O_1 and O_2. The mutational analysis indicates that each operator contributes about half of the PurR-mediated regulation. Thus, repressor function is dependent upon operator position as well as binding affinity. O_1 binds the repressor with about 10-fold greater affinity than O_2, but O_2, with closer proximity to the promoter and transcription start site than O_1, is more favorably positioned to modulate transcription initiation. The proximity of an operator site to the promoter is known to be an important factor in determining the efficiency of transcriptional regulation (13).

There are several examples of multiple operator sites in prokaryotic gene regulation that interact by DNA looping (14). Although the distance of 99 bp between the *purA* operators is potentially capable of placing the two sites for repressor binding on the same face of the helix, there was no evidence for cooperative binding of PurR to DNA containing O_1 and O_2, and there was no evidence for loop formation. Furthermore, interaction of PurR dimers, which would be needed for loop formation, has not been detected by protein cross-linking (4). Dual operators are used for autoregulation in *purR*, and also in this gene, cooperative PurR binding and DNA looping were not detected (24). Whether DNA supercoiling might be required to form repression loops in *purA* and *purR*, as occurs in the *lac* operon (3), is not known. In the absence of DNA looping, how could *purA*$_{O_1}$ function to repress *purA*? Although *purA*$_{O_1}$ is somewhat distant from the typical repressible σ^{70} promoter (6), it is possible that a DNA bend might position PurR bound to O_1 close to the promoter, as occurs in other cases (19, 26, 29).

What is the basis for the *purR*-independent regulation of *purA*? The following discussion leads to the suggestion that this regulation of *purA* does not rely solely on a *cis*-acting element upstream of the start of translation and may result from posttranscriptional events. We first consider whether different growth conditions for the assay of adenylosuccinate synthetase (Table 2) and *purA'-lacZ* lead to *purR*-independent regulation only in the former case. *purR*-independent regulation of adenylosuccinate synthetase (Table 2) was observed in a purine auxotroph grown with limiting adenine, whereas *purA'-lacZ* expression in a purine prototroph was not subject to this regulation (Table 3). However, *purR*-independent regulation of *purA'-lacZ* was likewise not detected in strains BH804 (*purB purR*$^+$) and BH805 (*purB purR*) limited for adenine (not shown). Therefore, the different growth conditions for the experiments in Tables 2 and 3 do not adequately account for our inability to detect *purR*-independent regulation of *purA* with the *lacZ* reporter strain BH901 (*purR purA'-lacZ*) (Fig. 3). There are at least two possibilities that could account for the difference in *purA* regulation monitored by the *lacZ* reporter and by adenylosuccinate synthetase activity. (i) More than the first 80 bp of *purA* that are included in the transcriptional fusion to *lacZ* could be required. Sequences downstream of the *purA'* fusion junction could have a role in transcriptional regulation, similar to that in *purB* (9, 10), or may interact with an undetected control site in the *purA* 5'-flanking region. (ii) The *purR*-independent regulation may be posttranscriptional.

Overall, our results indicate that regulation of *purA* expression is more complex than that for *purB*. While both genes are subject to a two- to threefold regulation that requires PurR, there is an additional twofold regulation of *purA* that is independent of PurR.

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