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Repression of *Escherichia coli purB* Is by a Transcriptional Roadblock Mechanism†

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***Escherichia coli purB* is regulated by a repressor-operator interaction. The *purB* operator is 242 bp downstream from the transcription start site and overlaps codons 62 to 67 in the protein-coding sequence (B. He, J. M. Smith, and H. Zalkin, J. Bacteriol. 174:130–136, 1992). The mechanism by which the repressor-operator interaction functions to repress transcription was investigated by a combination of promoter replacement experiments and RNA analyses. By using a *trp* promoter replacement that deleted 5′ flanking DNA to position −986, *purB* expression was increased sevenfold, yet normal two- to threefold regulation was maintained. This indicates that repressor-operator control is independent of the *purB* promoter and other 5′ flanking sequences. Transcriptional regulation was likewise independent of coupled translation. An approximately 260-nucleotide truncated in vivo *purB* mRNA was identified which was dependent upon repressor-operator interaction. Thus, binding of purine repressor to the *purB* operator inhibits transcription elongation by a roadblock mechanism. The roadblock was not influenced by a sevenfold increase in promoter strength or by an operator mutation resulting in a 2.5-fold increase in repressor-operator affinity.**

In *Escherichia coli* seven operons encode the 10 genes required for de novo synthesis of IMP. Gene *purB* encoding adenylosuccinate lyase is required in step 8 of the pathway to IMP and also for the second and final step from IMP to AMP. These seven operons are coregulated by a *purR*-encoded repressor (8, 9, 14). Upon binding of the hypoxanthine and/or guanine corepressor, the holorepressor can bind to a conserved 16-bp operator sequence in each of the coregulated genes and repress transcription (8, 14). In six of the operons, excluding *purB*, the operator is located between positions −46 and +10 relative to the start of transcription and overlaps the promoter region. For these genes binding of the repressor probably inhibits transcription initiation. On the other hand, the *purB* operator is located 224 bp downstream of the transcription start site in the protein-coding region (9). Previous experiments have demonstrated that PurR binds to this site in vitro, and deletion of this internal operator abolished repression, thus providing direct evidence for in vivo function. The mechanism by which the *purB* operator functions to repress transcription is not known. Two possibilities are (i) binding of the repressor to a single internal operator may block transcription elongation and (ii) binding of the repressor to the internal operator and an undetected secondary operator with DNA looping may inhibit transcription initiation or elongation. In this paper we report the results of experiments which demonstrated that *purB* is regulated by the internal single operator in the protein-coding sequence. Binding of the repressor to the *purB* operator blocks transcription elongation. This repression is independent of the *purB* promoter and coupled translation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids which we used are listed in Table 1.

Media. Luria-Bertani and 2XYT (17) were used as rich media. The minimal growth medium which we used has been described previously (8).

Plasmid construction. All of the plasmids used in this work are listed in Table 1. Translational P_{trp}-*purB′-′lacZ* fusions were constructed in two steps. A segment of *purB* from nucleotides (nt) −20 to 316 (9) containing the operator was amplified by the polymerase chain reaction (18) from plasmid pBH112 and was ligated into *Sma*I and *Bam*HI sites of pMLB1034 to give a *purB′-′lacZ* fusion. The junction between *purB* codon 93 and *lacZ* codon 8 is identical to the junction in pBLG2 used previously (9). Next, a 105-bp *Eco*RI-*Bam*HI fragment containing the *Serratia marcescens trp* operon promoter was isolated from plasmid pRK9, and the 3′ *Bam*HI site was made blunt with the Klenow fragment of polymerase I. The *trp* promoter was inserted to yield P_{trp}-*purB′-′lacZ* plasmid pBLG3 (Fig. 1). Plasmid pBLG3 is identical to *purB′-′lacZ* plasmid pBLG2 except that all of the *purB* DNA upstream of nt −20 has been replaced by the *trp* promoter. The *trp* promoter does not contain a *pur* operator-like sequence. In plasmid pBLG3 the *purB* operator is 270 bp from the transcription start site. A derivative of pBLG3 containing the λt_0 terminator inserted at the *purB-lacZ* junction was constructed. The λt_0 terminator was obtained on a 120-bp *Eco*RI-*Bgl*II fragment from plasmid pSS9.

To construct a P_{trp}-*purB′-lacZ* transcriptional fusion, a 430-bp *Eco*RI-*Bam*HI fragment containing the *trp* promoter and *purB* operator was isolated from pBLG3 and ligated into the *Eco*RI-*Bam*HI sites of pRS415, resulting in plasmid pBLG8. The parental P_{trp}-*purB′-lacZ* transcriptional fusion and subsequent derivatives were recombined into λRZ5 for insertion into the chromosome (20).

***purB* mutations.** A 430-bp P_{trp}-*purB* fragment from pBLG3 was cloned into the *Eco*RI and *Bam*HI sites of pUC118 for mutagenesis by the procedure of Kunkel et al. (11). The primers which we used for operator mutations (with the mismatches underlined) were 5′-GCATCAAGGTAACCAA TCGC GTCGGCAGC (*purB1*) and 5′-TGCATCAAGGTA AACGTTTGCGTCGGC (*purB2*). For mutations to abolish translation we used primers 5′-GTGATCTATCGAATTAT

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Source or reference
E. coli strains		
MC4100	Δ(*argF-lac*)*169* Lac$^-$	1
R320	MC4100 *purR320*	20
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}Z*ΔM15)	16
BH103	MC4100 (λLT3) Lac$^+$ Ap^r; *purB′-lacZ* transcriptional fusion in pRS415	9
BH301	MC4100 (λpBLG8) Lac$^+$ Ap^r; P_{trp}-*purB′-lacZ* transcriptional fusion	This study
BH302	MC4100 (λpBLG9) Lac$^+$ Ap^r; BH301 with ATG-to-ATC mutation in codon 1	This study
BH303	MC4100 (λpBLG10) Lac$^+$ Ap^r; BH301 with AAA-to-TAA mutation in codon 47	This study
Plasmids		
pRS415	*lacZ* transcriptional fusion vector; Ap^r	28
pMLB1034	*′lacZ* translational fusion vector; Ap^r	27
pUC118	Phagemid cloning vector	29
pBH112	1.3-kb *Kpn*I-*Eco*RI *purB* fragment (nt −986 to +316) in pUC118	9
pRK9	105-bp *Eco*RI-*Bam*HI fragment containing *S. marcescens trp* operon promoter in plasmid pBR322	19
pSS9	88-bp *Sau*3AI fragment containing λt_0 terminator	24
pBLG3	430-bp *Eco*RI-*Bam*HI P_{trp}-*purB′* fragment in which *purB* codon 93 is joined to codon 8 of *′lacZ* in pMLB1034	This study
pBLG4	pBLG3 with A-to-G and C-to-T changes in *purB* operator (*purB1*)	This study
pBLG5	pBLG3 with T-to-A and G-to-T changes in *purB* operator (*purB2*)	This study
pBLG6	pBLG3 with insertion of λt_0 terminator between the *purB* and *lacZ* fusion junction	This study
pBLG7	pBLG4 with insertion of λt_0 terminator between the *purB* and *lacZ* fusion junction	This study
pBLG8	430-bp *Eco*RI-*Bam*HI P_{trp}-*purB′* fragment containing wild-type *purB* operator in pRS415, transcriptional fusion to *lacZ*	This study
pBLG9	pBLG8 with ATG-to-ATC change at *purB* initiation codon	This study
pBLG10	pBLG8 with AAA-to-UAA change at codon 47 in *purB*	This study
pF_0	220-bp *Stu*I-*Nde*I *purF* fragment (nt −183 to +37) in pUC118	8
pCM2	2.2-kb upstream *purF* DNA in pMC1403	13
pRR1	T-26A, T27C *purF* operator-constitutive mutation in pCM2	20

CC and 5′-TGGCTGCAATAACTGGCCG (the mismatches are underlined). All mutations were verified by nucleotide sequencing (23). The mutations were finally incorporated into the parental P_{trp}-*purB-lacZ* translational and transcriptional fusions by fragment exchange.

Repressor-operator binding. Gel retardation assays were carried out as previously described by using buffer system II for corepressor-dependent binding (22). The incubation mixtures contained 10 fmol of ^{32}P-labeled 430-bp *Eco*RI-*Bam*HI P_{trp}-*purB* DNA from plasmid pBLG3, pBLG4, or pBLG5 or 220-bp *Eco*RI-*Hin*dIII *purF* fragment from plasmid pFo, homogeneous PurR, and 50 μM hypoxanthine corepressor in 20 μl of buffer system II. The purine repressor was a gift from Kang Yell Choi of our laboratory.

Enzyme assay. Plasmids were transformed into strains MC4100 (*purR*$^+$) and R320 (*purR*). Cells were grown to mid-log phase in minimal medium with or without adenine. β-Galactosidase activity was determined in permeabilized cells by the Miller assay (17).

RNA analyses. The assay mixtures for in vitro transcription contained, in a volume of 25 μl, 40 mM Tris-HCl (pH 7.9), 1 mM dithiothreitol, 0.1 mM EDTA, 100 μM KCl, 4.0 mM $MgCl_2$, 50 μM hypoxanthine, 5% glycerol, different amounts of PurR, and 10 fmol of DNA template. After

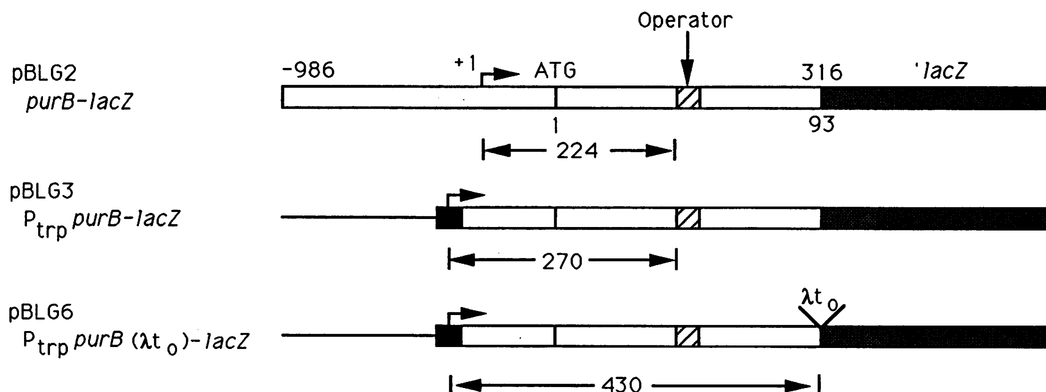

FIG. 1. Schematic representation of *purB* 5′ flanking DNA used for promoter replacement in translational *lacZ* fusion. The numbers above the diagrams refer to the nucleotide sequence, and the numbers below the diagrams refer to the amino acid sequence. The bent arrows indicate transcriptional start sites. Solid box, *S. marcescens trp* operon promoter; open box, *E. coli purB* and flanking DNA from positions −986 to 316; cross-hatched box, *purB* operator; line, vector DNA.

	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
Consensus	N	C	G	C	A	A	A	C	G	T	T	T	N	C	N	T
purF	A	C	G	C	A	A	A	C	G	T	T	T	T	C	T	T
purB	A	C	G	C	A	A	T	C	G	G	T	T	A	C	C	T
purB1	A	C	G	C	**G**	A	T	**T**	G	G	T	T	A	C	C	T
purB2	A	C	G	C	A	A	**A**	C	G	**T**	T	T	A	C	C	T

FIG. 2. Alignment of the wild-type and mutant *purB* operators with the *purF* operator and the consensus sequence for *pur* regulon genes. The nucleotides in the black boxes are nucleotides that are changed in the *purB* operator.

incubation at room temperature for 20 min, ATP, GTP, and CTP (each at a concentration of 0.15 mM) and *E. coli* RNA polymerase (Boehringer Mannheim Biochemicals) were added, and the mixtures were incubated at 37°C for 10 min. Multiround transcription was started by adding 25 μM [α-^{32}P]UTP and was allowed to continue for 20 min at 37°C. Reactions were terminated by adding phenol. After 50 μg of carrier tRNA was added, the RNA was precipitated with ethanol, and the ^{32}P-labeled transcripts were analyzed by electrophoresis on a 6% polyacrylamide–8 M urea sequencing gel. After autoradiography, bands were scanned with an LKB laser densitometer.

For analysis of *E. coli* RNA, strains MC4100 (*purR*$^+$) and R320 (*purR*) bearing appropriate plasmids were grown in 50 ml of minimal medium supplemented with 100 μg of adenine per ml. When the cell density corresponded to a Klett reading of 70 (determined by using a type 66 filter), each culture was poured onto ice chilled to −20°C, and the cells were isolated by centrifugation. The cells were suspended in cold 10 mM Tris-HCl (pH 7.6)–0.1 mM EDTA, and the RNA was isolated by hot phenol extraction (26). The RNA was dissolved in 10 mM Tris-HCl (pH 7.6)–0.1 mM EDTA and stored at −70°C.

For Northern blot analysis, RNA was fractionated by electrophoresis on a 6% polyacrylamide–8 M urea sequencing gel, soaked in transfer buffer (12 mM Tris, 6 mM sodium acetate, 0.3 mM EDTA) for 20 min, and electroblotted onto a Nytran nylon membrane for 10 h at 10 V. The membrane was prehybridized and hybridized (4) with a random primed (5) *purB* probe corresponding to nt −20–+316. In some cases RNA bands were quantitated by densitometric scanning.

RESULTS

In vitro analysis of *purB* operator mutations. Mutations were constructed in the 16-bp *purB* operator to aid in the analysis of function. The consensus sequence derived from nine *pur* regulon operator sites (8, 9, 14) is shown in Fig. 2 along with sequences for operators from wild-type *purB* and *purF*, as well as two *purB* mutants. Mutations in *purB1* (Fig. 2, black boxes) were chosen to decrease the affinity for PurR, whereas replacements in operator *purB2* were designed to increase similarity to the consensus sequence and thereby increase the affinity for PurR. These nucleotide changes were chosen to minimize amino acid replacements in the *purB* coding region. The wild-type adenylosuccinate lyase amino acid sequence is maintained in the mutant *purB1* operator, while the mutations in *purB2* result in Ile-to-Asn and Gly-to-Val changes at amino acid positions 64 and 65 (9).

Corepressor-dependent binding of PurR to the wild-type and mutant *purB* operators is shown in Fig. 3. Figure 3 shows that binding of PurR was abolished by the mutations in *purB1*, whereas the *purB2* mutations resulted in an approximately 2.5-fold increase in binding affinity compared

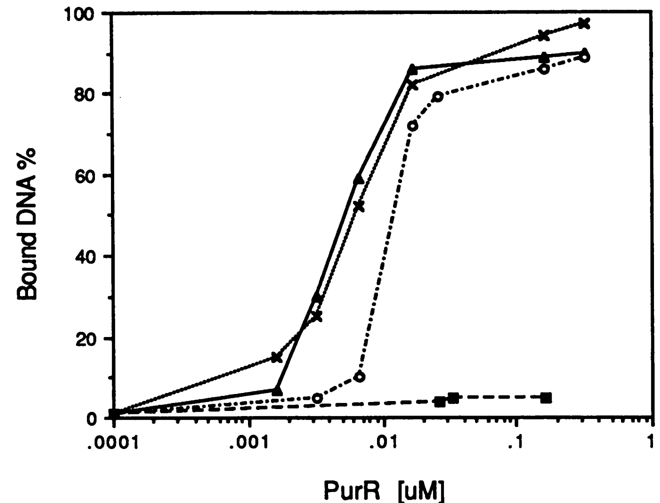

FIG. 3. Binding of *pur* repressor to *purF* and *purB* operators. Binding was determined by a gel retardation assay. Incubation mixtures contained 10 fmol of ^{32}P-labeled DNA fragment, homogenous PurR, and 50 μM hypoxanthine corepressor in 20 μl of buffer system II. The *purB* operator was on a 430-bp *Eco*RI-*Bam*HI fragment in which the *trp* promoter was joined to *purB* DNA containing nt −20 to +316. For *purF* an *Eco*RI-*Hin*dIII fragment (nt −183 to +37) was used. Bound and unbound DNA fragments were counted for radioactivity, and the percentage of bound DNA was plotted as a function of PurR. Symbols: ○, *purB*; ×, *purF*; ■, *purB1*; ▲, *purB2*.

with the wild type. The *purB2* operator sequence is identical in 14 of 16 positions to the *purF* operator, and repressor binding to these two operators was similar.

Promoter replacement and regulation by *purR*. Regulation of *purB* expression was previously shown to be dependent upon the operator at nucleotide positions 224 to 239 in the coding sequence (9); a secondary *purB* operator was not detected by DNase I footprinting, by a gel retardation assay, or by a computer search of the sequence between nt −986 and +382 (9, 10). Nevertheless, to eliminate the possibility of an additional *cis* element in the *purB* promoter or 5′ flanking sequence, this entire region was replaced by a *trp* operon promoter (Fig. 1). This replacement resulted in the deletion of all of the *purB* DNA from positions −986 to −20 relative to the start of transcription and abolished *purB* promoter function. Expression of *purB* from the *trp* promoter was monitored by using a translational fusion to a *lacZ* reporter. Schematic representations of the P_{trp}-*purB′*-′*lacZ* fusion in plasmid pBLG3 and the parent pBLG2 plasmid are shown in Fig. 1. Since previous work demonstrated comparable two- to threefold purine-dependent regulation from multicopy *purB′*-′*lacZ* plasmids or chromosomal integrants, expression of *purB′*-′*lacZ* from P_{trp} was determined by using plasmid pBLG3. Table 2 shows an approximately twofold regulation of *purB* expression from the *trp* promoter by *purR*. Regulation was dependent upon *purR*$^+$ and a *purB* operator that binds repressor. Expression was not regulated by operator *purB1*. The twofold regulation in this experiment is comparable to that previously obtained for expression from the *purB* promoter (9). Thus, repressor-operator control is independent of the *purB* promoter and 5′ flanking DNA. Repression of *purB* appears to result from a blockade in transcription elongation.

To address the possibility that twofold *purB* regulation is a

TABLE 2. Promoter replacement and regulation by *purR*

Plasmid (operator)	*purR*	β-Galactosidase activity[a]		Repression (fold)[b]
		With adenine	Without adenine	
pBLG3 (*purB*$^+$)	+	669	1,338	1.8–2.0
	–	1,236	1,338	
pBLG4 (*purB1*)	+	1,249	1,208	0.91–0.97
	–	1,134	1,177	
pBLG5 (*purB2*)	+	555	1,233	2.0–2.2
	–	1,125	1,171	

[a] β-Galactosidase activity is given in Miller units. Values are the averages from two or three independent experiments.

[b] Repression was calculated in two ways and was expressed as a range, as follows: (i) (enzyme activity in *purR*$^+$ without adenine)/(enzyme activity in *purR*$^+$ with adenine) and (ii) (enzyme activity with adenine in *purR*)/(enzyme activity with adenine in *purR*$^+$).

result of weak repressor-operator affinity, the wild-type operator was replaced by the *purB2* operator, which binds repressor with a higher affinity that is similar to the *purF* operator affinity (Fig. 3). Expression of *purF* is regulated 11- to 18-fold by purines (8, 20). Table 2 shows comparable twofold regulation by operator *purB2* and by wild-type operator. Thus, twofold regulation is not simply a consequence of repressor-operator affinity.

Effect of RNA polymerase and coupled translation on repression of *purB*. Since a single repressor-operator interaction in the *purB* coding region appears to act as a roadblock to transcription, it was important to determine whether coupled translation has any effect on repression. To determine the possible contribution of coupled translation on repression of *purB*, a series of transcriptional fusions to *lacZ* were constructed and integrated into the chromosome. Table 3 shows that there was a 2.9-fold repression of transcription from the *purB* promoter. This is similar to the regulation that was obtained previously in this strain (9). In strain BH103 the *purB* coding sequence preceding *lacZ* is translated. Table 3 shows that replacement of the *purB* promoter with the *trp* promoter in strain BH301 increased transcription approximately sevenfold, yet repression by *purR* was not affected. In strains BH302 and BH303 *purB* translation was abolished. Strain BH302 has an ATG-to-ATC mutation which prevents translation initiation. Strain BH303 has an AAA-to-TAA mutation that terminates translation at codon 47 prior to the operator which overlaps codons 62 to 67. Neither of the

TABLE 3. Effect of translation on repression of *purB*

Strain	Translation	β-Galactosidase activity[a]		Repression (fold)
		With adenine	Without adenine	
BH103 (P_{purB})	+	81	233	2.9
BH301 (P_{trp})	+	590	1,600	2.7
BH302 (P_{trp})	–	529	1,520	2.9
BH303 (P_{trp})	–	573	1,570	2.7

[a] β-Galactosidase activity is given in Miller units. Values are the averages from two experiments.

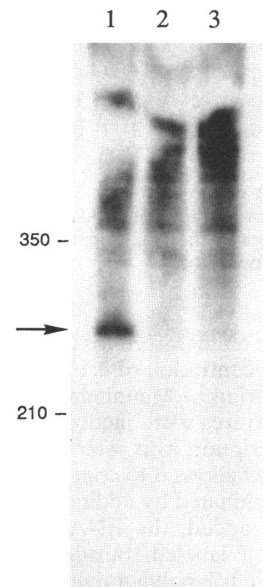

FIG. 4. Northern blot of *E. coli* RNA. RNAs from strain MC4100 (*purR*$^+$) carrying either pBLG3 (*purB*$_O^+$) or pBLG4 (*purB1*) and from strain R320 (*purR*) carrying pBLG3 were electrophoresed on a 6% polyacrylamide gel containing 8 M urea, blotted onto a nylon membrane, and hybridized with a radioactive probe specific for *purB*. Lane 1, RNA from strain MC4100 (*purR*$^+$) carrying pBLG3 (*purB*$_O^+$); lane 2, RNA from strain R320 (*purR*) carrying pBLG3 (*purB*$_O^+$); lane 3, RNA from strain MC4100 (*purR*$^+$) carrying pBLG4 (*purB1*). A total of 50 μg of RNA was loaded onto each lane. The arrow indicates the position of the truncated mRNA species. DNA fragments of 210 and 350 bp were used as size standards.

mutations which interfered with translation had any effect on *purB* regulation.

Transcript analysis in vivo. To determine directly whether repression of *purB* in vivo can result from a block in transcription elongation, *E. coli* RNA was isolated from repressed cells bearing plasmid pBLG3 (P_{trp}-*purB'*-*'lacZ*). The RNA was blotted onto a nylon membrane and probed with a ^{32}P-labeled 5'-proximal *purB* DNA fragment. Figure 4 shows the results of a representative Northern blot analysis in which *purB* expression was repressed by adenine. A truncated RNA of approximately 260 nt was detected as a result of repression by *purR*$^+$ (Fig. 4, lane 1). This RNA was the size expected from a block in transcription elongation by PurR in plasmid pBLG3 (Fig. 1). The production of truncated RNA was dependent upon repression. The RNA was not found in *purR* cells (Fig. 4, lane 2) or in cells with the nonrepressible *purB1* operator mutation (lane 3).

To confirm that the truncated *purB* RNA resulted from a block of transcription elongation rather than from RNA degradation, RNA was isolated from plasmid-bearing cells in which a transcription terminator, t_0 from phage λ, was inserted 430 bp downstream from the *trp* promoter (Fig. 1). Figure 5 shows that an approximately 430-nt RNA resulted from termination by λt_0. In addition, repression by *purR*$^+$ led to the production of a 260-nt *purB* RNA (Fig. 5, lanes 1 and 2). The appearance of the 260-nt RNA was dependent upon *purR*$^+$ and a wild-type *purB* operator. The integrity of the 430-bp λt_0-terminated *purB* mRNA does not support the idea that the 260-nt truncated RNA results from a hypothetical degradative scheme. Rather, the experiments in Fig. 4

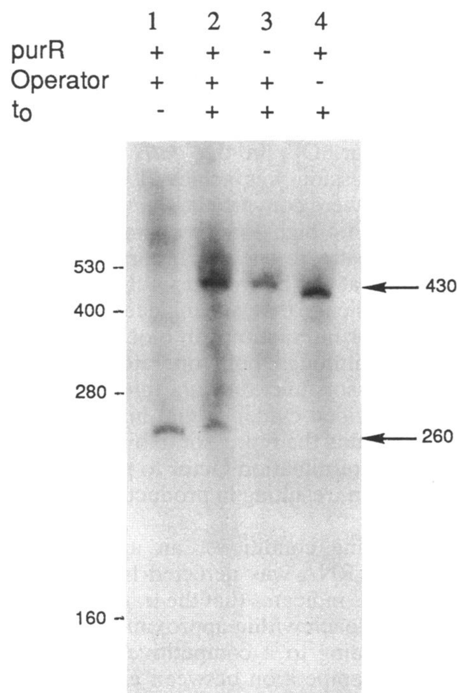

FIG. 5. Northern blot of *E. coli* RNA. Plasmids pBLG3 ($purB_O^+$), pBLG6 ($purB_O^+$ λt_0) and pBLG7 (*purB1* λt_0) were transformed into either strain MC4100 ($purR^+$) or strain R320 (*purR*). Cells were grown in the presence of adenine to mid-log phase, and then RNA was isolated. RNA was fractionated on a 6% polyacrylamide sequencing gel containing 8 M urea, blotted onto a nylon membrane, and hybridized with a radioactive probe specific for *purB*. A total of 50 μg of RNA was loaded onto each lane. Species of 430 and 260 nt reflect RNA that terminated at λt_0 and the *purB* operator, respectively. An RNA ladder (Bethesda Research Laboratories) was used to provide size standards.

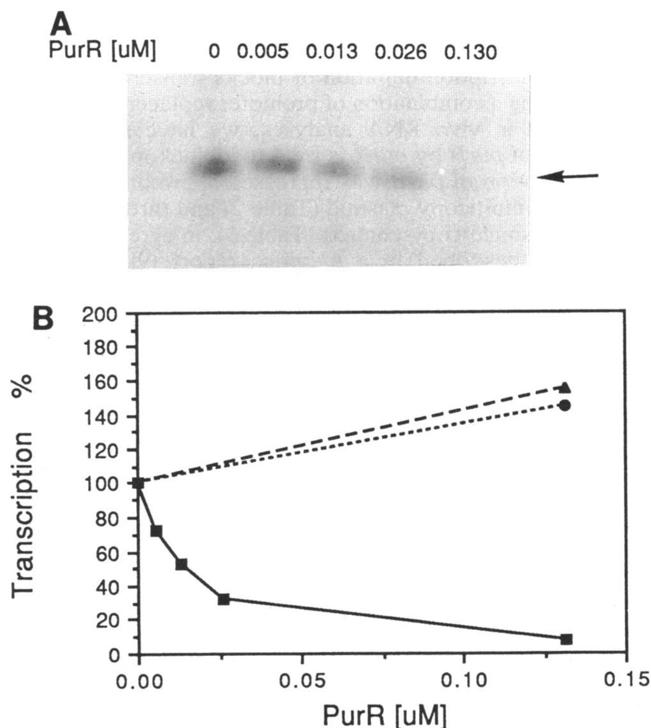

FIG. 6. Repression of *purF* transcription in vitro by PurR. (A) Transcription assay with different concentrations of PurR. The arrow indicates the position of the 290-nt run-off transcript. (B) Amount of transcript plotted as a function of PurR. Symbols: ■, transcription of wild-type *purF* with hypoxanthine corepressor; ●, wild-type *purF*, no corepressor; ▲, *purF* operator constitutive plus corepressor.

and 5 provided direct evidence for repression of *purB* by a blockade of transcription elongation and release of a truncated mRNA. From the estimated relative intensities of the 260- and 430-nt species (Fig. 5, lane 2), we estimated that the termination efficiency by $purR^+$ was about 18% (calculated by dividing the intensity of the 260-nt band by that for the 260-nt plus the 420-nt bands).

Transcription in vitro. An in vitro assay was used to determine whether PurR could repress *purB* transcription. Initially, however, it was necessary to establish conditions for in vitro repression by PurR. Therefore, we determined the conditions required for repression of *purF* transcription. Figure 6 shows that the purine holorepressor inhibited the synthesis of a 290-nt *purF* runoff transcript. On the basis of the results of densitometric scanning of the autoradiogram shown in Fig. 6A, approximately 1.5×10^{-2} μM repressor was required for 50% inhibition (Fig. 6B). This inhibition was dependent upon corepressor and upon a functional *purF* operator. Transcription from a *purF* template with an operator-constitutive mutation was not repressed by PurR in vitro. This mutation abolished binding of repressor in vitro (21) and repression in vivo (20). Thus, these data establish the functional integrity of purified PurR and its capacity to repress transcription of *purF* in vitro. Since the *purF* operator overlaps the promoter region, PurR probably represses transcription initiation. The apparent activation of transcription by purine repressor in the absence of corepressor or from a *purF* operator-constitutive template was not investigated further but probably results from a nonspecific effect of protein in the assay.

Next, the effect of PurR on transcription of *purB* initiated from the *trp* promoter was evaluated. A linear *purB* template was prepared from plasmid pBLG3. *purB* from this plasmid was regulated twofold by *purR* in vivo (Table 2). The 350-nt runoff RNA transcribed from this template was not repressed by PurR at concentrations up to 3.3 μM (data not shown). A number of conditions were varied, including nucleotide concentration (20 to 150 μM), temperature (25 to 37°C), single-round transcription, and salt concentration. We did not detect PurR-dependent inhibition of transcription or a prematurely truncated RNA. The results of control experiments verified that PurR bound to *purB* operator DNA under these assay conditions, although repressor-operator binding could not be evaluated by gel retardation in the presence of RNA polymerase. We do not know whether a missing factor, the physical state of the template DNA, or the ratio of polymerase to repressor prevented *purB* regulation in vitro.

DISCUSSION

Transcriptional regulation of *purB* requires a 16-bp operator site located 224 nt downstream from the transcriptional start site overlapping codons 62 to 67 in the protein-coding sequence (9). No other *pur* operator sites were detected between positions −986 and +382 by DNase footprinting, by a gel retardation assay, or by a computer search of the

sequence (9, 10). Previous experiments did not address the issue of whether binding of PurR to the *purB* operator inhibits transcription initiation or blocks transcription elongation. Using a combination of promoter replacement experiments and in vivo RNA analyses, we have shown that repression of *purB* by *purR* is by a roadblock mechanism.

Transcription of *purB* was repressed approximately twofold from a multicopy plasmid (Table 2) and threefold when it was in a single-copy context (Table 3), in agreement with the results described in a previous report (9). The data indicate that two- to threefold repression was maintained when the *purB* promoter and 5′ flanking DNA were replaced by a *trp* operon promoter. Therefore, transcriptional regulation of *purB* does not involve secondary operator sites in the 5′ flanking region, and repression of *purB* is independent of promoter strength. Transcriptional regulation was solely dependent upon the PurR interaction with the internal operator site (Table 2). This *purR*-dependent regulation is small compared with the 5- to 17-fold regulation of other *pur* regulon genes (8) and may reflect the dual adenylosuccinate lyase requirement for purine nucleotide synthesis. Not only is adenylosuccinate lyase required for de novo synthesis of IMP and AMP, but it is also involved in synthesis of AMP by the salvage pathway when the de novo pathway is shut down by repression.

Figure 3 shows that the *purB* operator has a somewhat lower affinity for PurR than the *purF* operator does. However, in vivo repression was not dictated solely by operator-repressor affinity. A 2-base mutation that increased operator-repressor affinity had no significant effect on in vivo repression. The important distinction between the *purF* operator and the high-affinity *purB2* operator is location relative to the promoter. The *purF* operator overlaps the promoter and exerts 10- to 18-fold regulation, whereas the high-affinity *purB2* operator is 224 bp downstream from the transcription start site and exerts 2- to 3-fold repression. Large differences in *lac* operon repression are known to result from placement of an operator in different positions relative to the promoter (12). Collado-Vides et al. (2) have noted the variable placement of an operator relative to the promoter in the different transcription units of a regulon and have suggested that this provides a mechanism which allows each promoter of the regulon to be regulated by the same repressor but in a different manner. In a compilation of 76 repressible promoters (2), there were no other occurrences, similar to *purB*, of a single operator that is remote from the promoter. However, there are several genes that are subject to regulation by duplicate operators, one of which is remote from the promoter and is in the coding sequence. In the *lac* operon, $lacO_2$ located in the *lacZ* coding sequence has two roles. The remote operator $lacO_2$ strengthens binding of repressor to $lacO_1$ by about threefold, and indirect evidence suggests that $lacO_2$ contributes to the overall repression by blocking transcription elongation (6). Evidence that $lacO_2$ can function to block transcription elongation was indirect, since truncated *lacZ* mRNA was not directly determined. To our knowledge, *purB* is the only native gene in which expression is regulated by a single repressor-operator roadblock that functions to inhibit transcription elongation.

However, it is known that engineered *lac* control elements can regulate gene expression by inhibiting transcription elongation. Deuschle et al. (3) have shown that high levels of *lac* repressor can block transcription elongation from a coliphage T5 promoter and repress synthesis of a downstream chloramphenicol acetyltransferase gene in vivo and in vitro. Analysis of in vivo and in vitro transcripts showed that bound repressor does not simply cause RNA polymerase to pause but rather acts as a transcription terminator. Termination in vivo was approximately 90%. In the *E. coli gal* operon, transcription initiation is inhibited by repressor bound to an operator upstream from the promoter, O_E^G, and a remote operator, O_I^G, in the *galE* structural gene (7). Comparable repression was achieved by the *lac* repressor when O_E^G and O_I^G were converted to *lac* operators O_E^L and O_I^L by using a synthetic high-affinity operator sequence. Upon removal of the upstream operator, repression by *lac* repressor was retained at O_I^L but not at O_I^G by the *gal* repressor. These results indicate that an engineered high-affinity *lac* repressor-operator interaction can block transcription elongation in *galE*, although both operators are required for normal *gal* repressor function. In a third example, Selitti et al. (25) have provided evidence that high-level synthesis of *lac* repressor permits the repressor to bind to *lac* operator O_1 and to serve as a termination factor to prevent read-through into the *lac* operon, resulting in production of monocistronic *lacI* mRNA.

Under repressing conditions, an approximately 260-nt truncated *purB* mRNA was detected from plasmid pBLG3 (Fig. 4 and 5). This indicates that the transcription elongation complex can approach within approximately 10 bp of bound repressor. According to a competitive kinetic model (30), there is kinetic competition between elongation and termination. A pause in elongation is expected to result from an encounter between the elongation complex and bound repressor. A pause in elongation lowers the activation free energy for the termination barrier relative to the elongation barrier, thus increasing the probability of transcription termination. On the basis of the results of previous in vitro experiments in which *lac* repressor blocked transcription elongation (3), accessory proteins are not required for transcript release.

In the simplest model in which the mRNA and enzyme levels are directly proportional, the PurR roadblock should be about 50 to 67% efficient to yield two- to threefold repression of *purB*. From the densitometric scan of truncated mRNAs in Fig. 5, we estimated a blockage efficiency of about 18%. Perhaps there was incomplete recovery of the 260-nt truncated mRNA. It is noteworthy that the blockade efficiency for repression was not affected by the sevenfold difference in transcription rates resulting from the *purB* and *trp* promoters or by a 2.5-fold increase in binding affinity of the repressor to a mutant *purB* operator.

Figure 6 shows the repression of *purF* transcription in vitro by purified PurR. This repression was dependent upon the corepressor and an intact operator capable of binding the holorepressor. For reasons that are not presently understood, repression of *purB* was not observed in vitro.

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