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Regulation of *Escherichia coli pyrC* by the Purine Regulon Repressor Protein†

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The purine regulon repressor, PurR, was identified as a component of the *Escherichia coli* regulatory system for *pyrC*, the gene that encodes dihydroorotase, an enzyme in de novo pyrimidine nucleotide synthesis. PurR binds to a *pyrC* control site that resembles a *pur* regulon operator and represses expression by twofold. Mutations that increase binding of PurR to the control site in vitro concomitantly increase in vivo regulation. There are completely independent mechanisms for regulation of *pyrC* by purine and pyrimidine nucleotides. Cross pathway regulation of *pyrC* by PurR may provide one mechanism to coordinate synthesis of purine and pyrimidine nucleotides.

In *Escherichia coli* and *Salmonella typhimurium*, a group of unlinked genes, *carAB*, *pyrBI*, *pyrC*, *pyrD*, *pyrE*, and *pyrF*, is required for the de novo synthesis of UMP (19). Previous studies have indicated that expression of these genes is noncoordinately regulated by the intracellular pools of pyrimidine nucleotides and arginine. Expression of *carAB*, which is involved in pyrimidine and arginine synthesis, is cumulatively repressed at tandem promoters by arginine and pyrimidine nucleotides (5, 9). Genes *pyrBI* (4, 14, 18, 24, 27, 34) and *pyrE* (1, 23) are regulated by a UTP-sensitive attenuation mechanism, as well as by a secondary attenuation-independent mechanism (13, 15). Expression of *pyrC* and *pyrD* appears to be repressed by a cytidine nucleotide, whereas *pyrF* is subject to repression by a uracil nucleotide other than UMP (21, 30). In addition to the regulation of *pyr* genes by pools of pyrimidine nucleotides, the results of growth experiments of *S. typhimurium guaB* mutants, in which the growth rate was limited by the rate of GMP synthesis, have suggested that these genes are also regulated by purine nucleotides (7).

Recent work on the regulation of *E. coli* genes involved in de novo purine nucleotide synthesis has led to the identification of a *purR*-encoded repressor protein which regulates transcription of unlinked *pur* regulon genes (10, 26). A binding site for PurR, the *pur* operator, has been identified or inferred in the 5′-flanking region of a number of *pur* genes (16, 29, 32, 33, 36). This site has the consensus sequence 5′-NCGCAAACGTTTNCNT. A similar sequence is present in the 5′-flanking region of *pyrC* (37) and *pyrD* (12) as well as *carAB* (9, 22) and *prsA* (2, 6), which encodes phosphoribosylpyrophosphate synthetase.

Here we report results of an investigation of the mechanism for purine regulation of *E. coli pyrC*. Purine repressor, PurR, was found to bind to the inferred control site in the *pyrC* promoter and repress transcription by twofold. Mutations which enhance binding of PurR to the control site were found to increase repression of *pyrC* in vivo. The PurR-mediated regulation of *pyrC* was independent of the approximately eightfold regulation by pyrimidines. Thus, the purine repressor is the first of the *trans*-acting factors involved in the regulation of *pyr* genes to be identified. This repressor provides one potential mechanism to coordinate the synthesis of pyrimidine and purine nucleotides.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. Strains, phage, and plasmids are described in Table 1.

Media and culture methods. Cells for β-galactosidase assays were grown in minimal medium containing salts (35), 0.4% glucose, 2 μg of thiamine per ml, 0.2% acid casein hydrolysate, and specific supplements as required. The pyrimidine source was either 0.24 mM UMP or 1 mM uracil. Adenine was used at a concentration of 100 μg/ml. LB (17) was used as the rich medium. Antibiotics were added at the following concentrations: ampicillin, 100 μg/ml for selection and maintenance of plasmids, 20 μg/ml for lysogen selection; kanamycin, 70 μg/ml for selection of P1 transductants.

Plasmid construction. The *pyrC* control region was isolated as a 153-base-pair (bp) *Bst*NI fragment from plasmid pBHM105 (37). After the ends were made blunt by filling in with dTTP and dATP, the fragment was cloned into the *Hinc*II polylinker site of pUC118 to yield pPyrC0. In this construction, sequence analysis indicated deletion of a guanosine residue at the 3′ *pyrC*-polylinker junction. This single-base deletion permitted the in-frame *pyrC-lacZ* fusion that was constructed. Single-stranded DNA from this phagemid was the template for oligonucleotide-directed mutagenesis, described below. For construction of *pyrC-lacZ* fusions, the *pyrC* control region was isolated from plasmid pPyrC0 and its mutant derivatives by digestion at the flanking *Hin*dIII and *Eco*RI polylinker sites. The *Hin*dIII site was made blunt by filling in with deoxynucleoside triphosphates, and the resulting 207-bp fragment was ligated into the *Eco*RI and *Sma*I sites of the *lacZ* fusion vector pMLB1034. The newly formed pKY plasmids were transformed into strain DH5αF′, and blue colonies were selected on LB plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and ampicillin. A *pyrC-lacZ* fusion is shown schematically in Fig. 6. The *pyrC-lacZ* fusions contained 100 bp of *pyrC* DNA upstream from the transcription start site, five *pyrC* codons, plus 30 bp of polylinker sequence ligated to codon 9 of *lacZ*. All constructions were verified by DNA sequence analysis (28).

Isolation of λ *pyrC-lacZ* lysogens. *pyrC-lacZ* fusions from pKY plasmids were crossed onto λRZ5 for single-copy chromosomal insertion (25).

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TABLE 1. *E. coli* K-12 strains, plasmids, and phage used

Strain, plasmid, or phage	Genotype or description	Comment or source
Strain		
DH5αF′	F′ φ80d *lacZΔM15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ$^-$ *thi-1*	Bethesda Research Laboratories, Inc., Gaithersburg, Md.
CLT75	Δ(*lacIPOZYA*)*U169 strA thi pyrB482*::*kan srl-300*::Tn*10 recA56*	C. L. Turnbough, Jr.
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 thiA1 relA1 deoC1 ptsF25 flbB5301*	26
KYC4100	MC4100 *pyrB482*::*kan*	P1(CLT75) × MC4100
KYC4101	KYC4100(λRC0)	Wild-type *pyrC-lacZ*
KYC4102	KYC4100(λRC1)	*pyrC* (−28C)[a]-*lacZ*
KYC4103	KYC4100(λRC2)	*pyrC* (−16T)-*lacZ*
KYC4104	KYC4100(λRC3)	*pyrC* (−28C and −16T)-*lacZ*
Plasmids		
pRRM127	15.5-kilobase *purR*$^+$ Km (26)	
pBHM105	6.0-kilobase *pyrC*$^+$ Ap^r (37)	
pMLB1034	*'lacZY'* fusion vector, Ap^r (31)	
pPyrC0	Wild-type *pyrC* control region in pUC118, Ap^r	
pPyrC1	pPyrC0 with −28C *pyrC* mutation	
pPyrC2	pPyrC0 with −16T *pyrC* mutation	
pPyrC3	pPyrC0 with −28C and −16T *pyrC* mutations	
pKY0	*pyrC-lacZ* wild type, Ap^r	
pKY1	pKY0 with −28C *pyrC* mutation	
pKY2	pKY0 with −16T *pyrC* mutation	
pKY3	pKY0 with −28C and −16T *pyrC* mutations	
Phage		
λRZ5	λ *'bla 'lacZY*$^+$ Lac$^-$ Ap^s	
λRC0	λ *pyrC-lacZ bla*$^+$ Lac$^+$ Ap^r, wild-type *pyrC* control region	
λRC1	λRC0 with −28C *pyrC* mutation	
λRC2	λRC0 with −16T *pyrC* mutation	
λRC3	λRC0 with −28C and −16T *pyrC* mutations	

[a] Designation for mutation is nucleotide position relative to the start of transcription, mutant base. Mutations are shown in Fig. 2.

Assay of β-galactosidase. *pyrC-lacZ* λ lysogens were grown to the log phase in media with either limiting pyrimidines (UMP) or excess pyrimidines (uracil). Adenine was added to signal excess purines. Cells were assayed for β-galactosidase activity as described previously (17).

Assays for protein-DNA binding. A 207-bp fragment containing the *pyrC* control region was isolated from pPyrC plasmids by digestion at *Eco*RI and *Hin*dIII polylinker sites. The *Hin*dIII end was labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase before digestion with *Eco*RI. The labeled fragment was purified by electrophoresis on a 5% polyacrylamide gel and isolated by electroelution. Gel retardation assays were conducted as previously described (26). The 20-μl binding reaction contained 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0), 50 mM KCl, 1 mM EDTA, 1 μg of poly(dI-dC)-poly(dI-dC), 10% glycerol, 10 fmol of labeled DNA fragment, and varied amounts of cell extract. Cell extract was prepared from *purR*$^+$ strain R303(pRRM127) and *purR* strain R303 (26). After electrophoresis to separate protein-DNA complexes from free DNA (26), the gels were fixed, dried, and exposed to X-ray film at −70°C. After exposure, bands containing protein-DNA complexes and free DNA were excised and counted for radioactivity. DNase I footprinting was performed as described previously (26), using the same DNA fragments as for gel retardation assays. Free DNA and protein-DNA complexes were separated as for gel retardation experiments and were then isolated by electroelution. Samples were resolved on 6% polyacrylamide sequencing gels.

Site-directed mutagenesis. The procedure of Kunkel et al. (11) was used for mutagenesis. Phagemid DNA prepared from pPyrC0 was the source of single-stranded template DNA. The mutagenic oligonucleotides were 5′-GAAACGTT TGCCTTTGCAC and 5′-CAAAGGATAAACGGAAACG (mismatched bases are underlined). A double mutation was constructed with single-stranded DNA from mutant plasmid pPyrC2 and the appropriate oligonucleotide. Each of the mutations was verified by DNA sequencing (28).

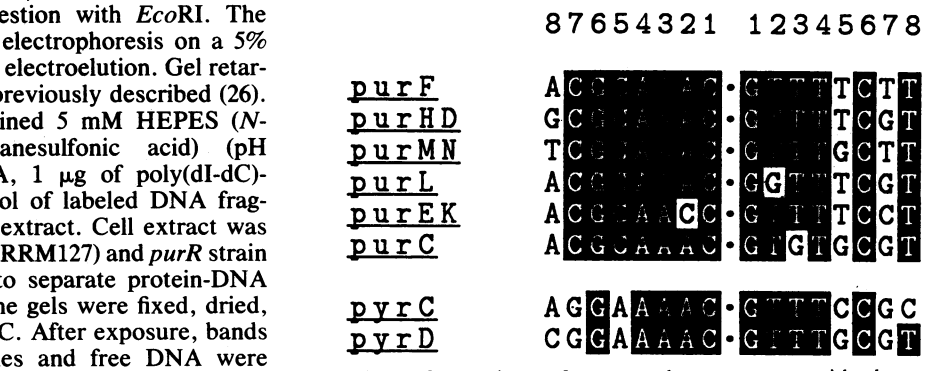

FIG. 1. Comparison of *pur* regulon operators with the *pyrC* purine regulatory sequence and with the putative *pyrD* regulatory sequence. Sequences are from *purF* (16), *purHD* (K. A. Flannigan, S. H. Hennigan, H. H. Vogelbacker, J. S. Gots, and J. M. Smith, Mol. Microbiol., in press), *purMN* (32), *purL* (29), *purEK* (33, 36), *purC* (A. A. Tiedeman, D. J. DeMarini, J. Parker, and J. M. Smith, submitted for publication), *pyrC* (37), and *pyrD* (12). Conserved positions are highlighted. The center of symmetry is marked by a dot.

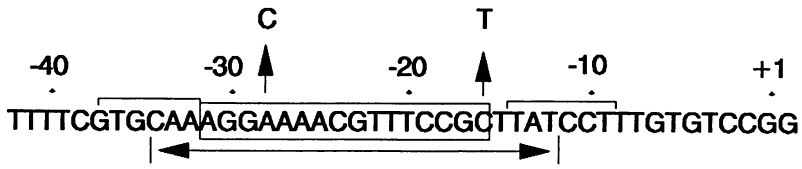

FIG. 2. Nucleotide sequence of the *pyrC* promoter region. The sequence is taken from Wilson et al. (37) and is numbered from one of four possible mRNA 5′ ends. Brackets identify −35 and −10 promoter elements. The *pur* regulon control site is boxed. Single-nucleotide replacements at positions −28 and −16 are marked by arrows. The region protected from DNase I by repressor is marked by an extended arrow.

RESULTS

Identification of PurR control site. A *pur* regulon operator, inferred from sequence alignments (16, 29, 32, 33, 36; K. A. Flannigan, S. H. Hennigan, H. H. Vogelbacker, J. S. Gots, and J. M. Smith, Mol. Microbiol., in press; A. A. Tiedeman, D. J. DeMarini, J. Parker, and J. M. Smith, submitted for publication), has been identified by mutational analysis (25) and by footprinting of PurR-binding sites (26; B. He, A. Shiau, K. Y. Choi, H. Zalkin, and J. M. Smith, submitted for publication). Figure 1 shows an alignment of these operators. Wilson et al. (37) identified a sequence with hyphenated dyad symmetry in the 5′-flanking region of *pyrC* between the −35 and −10 promoter elements. This sequence, shown in Fig. 2, resembles that for the *pur* operator (Fig. 1). Results of

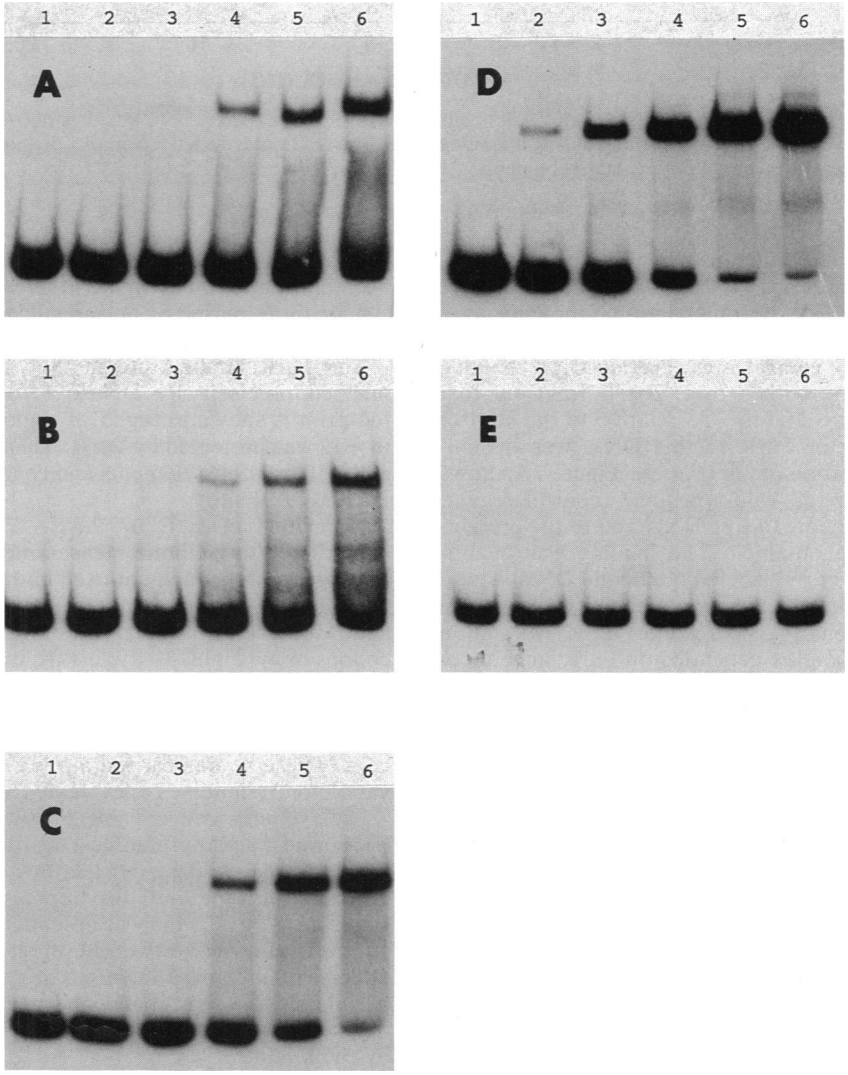

FIG. 3. Gel retardation assay for binding of PurR to the *pyrC* purine control site. The 207-bp *Eco*RI-*Hin*dIII DNA fragment contains 153 bp of *pyrC* DNA. (A) Fragment from pPyrC0 (wild type); (B) pPyrC1 (−28C mutation); (C) pPyrC2 (−16T mutation); (D) pPyrC3 (−28C and −16 T mutations); (E) pPyrC0 with *purR* extract. The slower-migrating band is the protein-DNA complex, and the faster-migrating band is free DNA. Extract added (micrograms): lane 1, none; lane 2, 1.25; lane 3, 2.5; lane 4, 5.0; lane 5, 10; lane 6, 20.

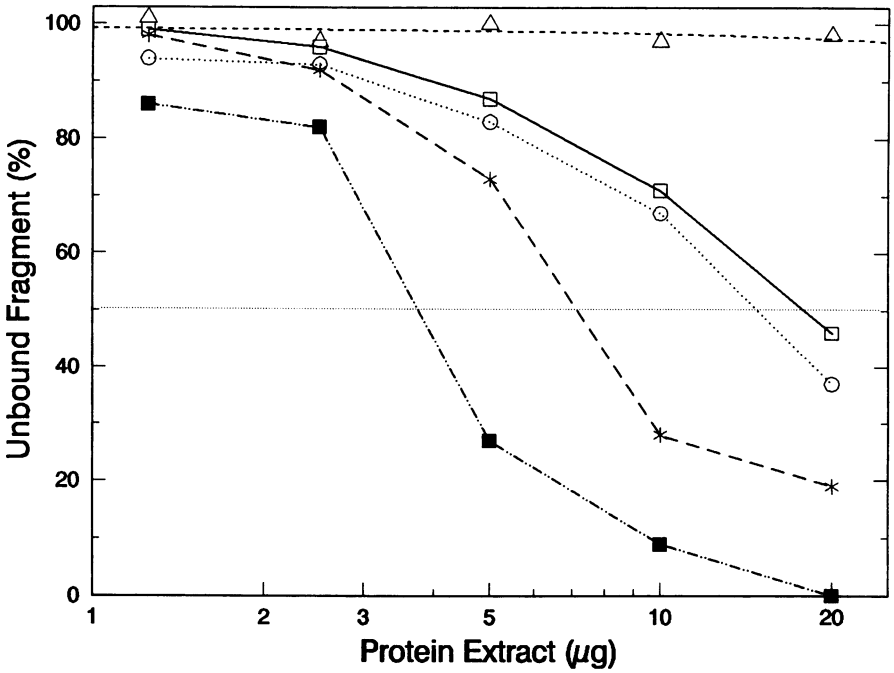

FIG. 4. Binding curve for *pyrC*-PurR. Bands containing nonbound DNA from the experiment in Fig. 3 were counted for radioactivity, and relative values are plotted against the protein added, using a semilog scale. The horizontal dotted line marks the position for 50% binding. The binding curve for PurR$^-$ is with wild-type *pyrC* DNA. Symbols: △, PurR$^-$; □, wild-type *pyrC*; ○, −28C *pyrC*; *, −16T *pyrC*; ■, −28C and −16T *pyrC*.

PurR binding and mutational analysis, described below, established that the sequence between nucleotides −31 and −16 relative to the start of transcription functions as a control site for regulation by PurR.

Binding of PurR to control site. Protein-DNA binding experiments were done with a *pyrC Bst*NI fragment that extends from positions −100 to +53 relative to the start of transcription. Binding of PurR to this DNA was demonstrated by a gel retardation assay (Fig. 3). Figure 3A shows a titration of PurR to the wild-type *pyrC* control region. Single bands corresponding to free DNA and to the protein-DNA complexes were obtained. The binding was quantitated by measurement of ^{32}P-labeled nonbound DNA. These results are plotted in Fig. 4. Approximately 18 μg of protein was required for 50% binding. As seen in Fig. 3E, formation of the protein-DNA complex depended on PurR since there was no functional binding protein in an extract from a *purR* mutant.

Mutations were constructed in the PurR control site to further verify its function. Two mutations, −28C and −16T, shown in Fig. 2, increased the similarity of this site to the *pur* gene operator consensus. Results of protein binding are shown in Fig. 3 B to D and are quantitated in Fig. 4. The amount of extract protein needed for half-maximal binding was 16 μg for the −28C mutation, 7 μg for −16T, and 4 μg for DNA having the double mutation. For comparison, approximately 2.5 μg of extract protein was required for half-maximal binding to *purF* under these conditions of assay (data not shown). These results demonstrate the importance of two of the conserved bases in the control site for interaction with PurR. In these and in previous (26) experiments with PurR, binding to DNA was obtained without the addition of a purine or purine nucleotide corepressor. There are at least three possibilities to account for lack of a requirement for corepressor. (i) Crude repressor may contain tightly bound corepressor; (ii) conditions have been chosen that may bypass the requirement for corepressor; or (iii) a purine or purine nucleotide corepressor is not required for binding of PurR to the control site.

The PurR binding site in the *pyrC* control region was mapped precisely by DNase I footprinting. A DNase I footprint is shown in Fig. 5. A region from nucleotides −34 to −12 was protected by PurR. This region overlaps the −35 and −10 promoter elements and covers the intervening 16-bp PurR control site.

Regulation of *pyrC* by PurR. A *pyrC-lacZ* fusion was constructed to facilitate measurements of gene expression. The *pyrC Bst*NI fragment used above for binding to protein was ligated to a *lacZ* reporter gene as described in Materials and Methods. In this translational fusion, the first five codons of *pyrC* plus 10 triplets from a polylinker were joined to codon 9 of *lacZ*. This construction is outlined schematically in Fig. 6. Plasmid-borne *pyrC-lacZ* was crossed onto λ phage RZ5 and then lysogenized into *purR* and *purR*$^+$ host cells. These strains carried a *pyrB* mutation to permit starvation for pyrimidines to evaluate regulation by pyrimidines.

Data summarized in Table 2 shows the regulation of *pyrC* by purines and pyrimidines, as determined by assay of β-galactosidase from *pyrC-lacZ* fusions. In the *purR*$^+$ background, wild-type *pyrC* was repressed twofold by adenine. Repression by adenine was essentially abolished in the *purR* strain. In contrast to twofold repression by adenine, pyrimidine regulation was eightfold. Regulation by purines and pyrimidines was independent since similar twofold purine regulation was obtained in cells in which *pyrC* was repressed by pyrimidines.

The effect of mutations that enhance binding of PurR to the *pyrC* control region is also given in Table 2. Mutation −28C, which was without significant effect on binding of PurR, had no significant effect on repression by adenine.

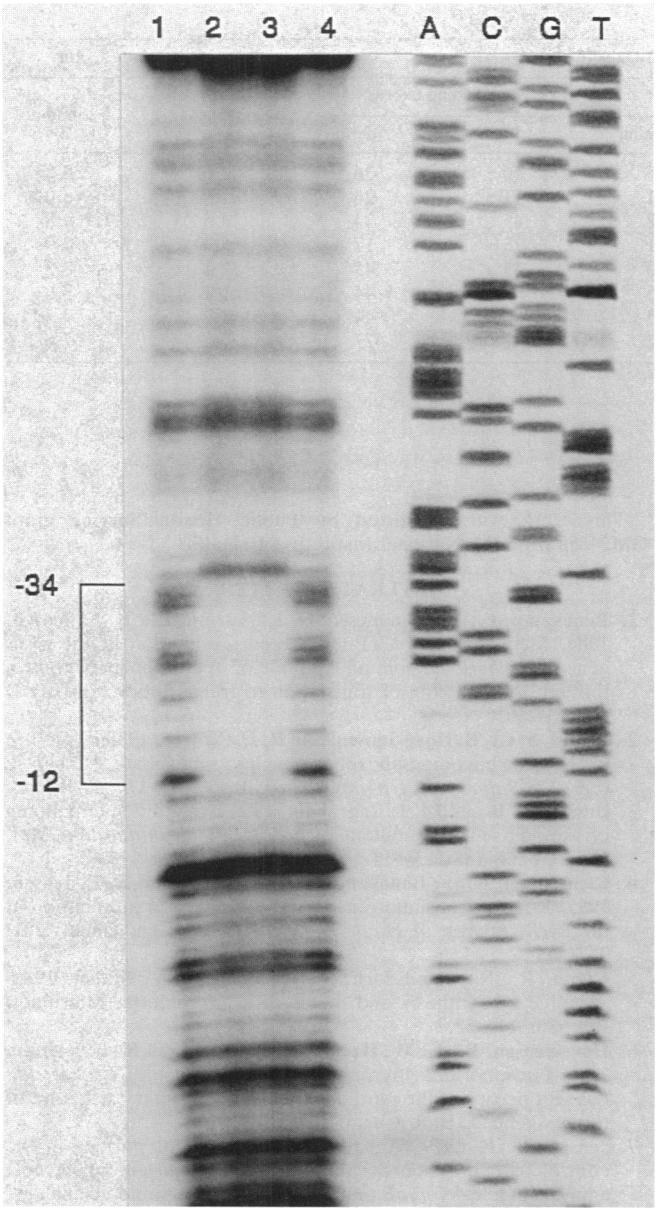

FIG. 5. DNase I footprint of purine repressor interaction at the control site in the *pyrC* promoter. The binding reaction and DNA fragment are described in Materials and Methods. Lanes 1 and 4, No extract; lanes 2 and 3, 20 μg of extract from *purR*$^+$ strain R303(pRRM127). A sequencing ladder (lanes A, C, G, T) of the coding strand of the same fragment provides the size standard. The region protected from DNase I digestion is indicated as a bracket.

However, repression by adenine was increased to 4.5-fold in the −16T mutant and to 6.4-fold in the double mutant. Adenine regulation was similar when expression of *pyrC* was repressed by pyrimidines. Thus, in vivo regulation of *pyrC* correlates with the increased in vitro affinity of PurR to the control site. In all mutants, regulation by adenine was invariably abolished in the *purR* background, indicative of the requirement for purine repressor. However, a constant 7- to 10-fold regulation by pyrimidines was maintained, independent of *cis*- or *trans*-acting mutations in the system for control by purines.

The data in Table 2 show increased *pyrC* expression in the −28C/−16T double mutant. This may reflect enhanced interaction of RNA polymerase to the mutant promoter. The increased expression of *pyrC* was maximal in the *purR* strain where PurR was not available to compete for the promoter with RNA polymerase.

DISCUSSION

UTP-dependent attenuation in *pyrBI* and *pyrE* is the only well-characterized mechanism for pyrimidine control of genes involved in de novo synthesis of UMP (4, 14, 18, 24, 27, 34). Examination of the nucleotide sequences of the control regions of other *pyr* genes indicates that attenuation control similar to that for *pyrBI* and *pyrE* is not involved (37). Mutations that influence expression of *pyr* genes in *S. typhimurium* (3) and *E. coli* (20) have been described, but there is presently no information on other mechanisms for pyrimidine-dependent regulation of *pyr* gene expression. *trans*-acting factors involved in pyrimidine regulation of *pyr* genes have yet to be characterized.

We identified the purine regulon repressor, PurR, as a component of the regulatory system for *pyrC*. PurR was found to bind in vitro to a *pur* operatorlike control site at nucleotides −31 to −16 and to repress expression by approximately twofold. Two lines of evidence indicate that there are independent mechanisms for regulation by purines and pyrimidines. (i) Similar repression by adenine was obtained in pyrimidine-starved cells having derepressed *pyrC* and in cells grown with excess pyrimidines having repressed *pyrC* expression. (ii) Mutations in the *pur* operator sequence resulted in enhanced repression of *pyrC* by purines without altering pyrimidine regulation.

In Fig. 1, six operator sequences for *E. coli pur* regulon genes are listed, along with the purine control site in *E. coli pyrC* and the putative site in *pyrD*. Most of the highly conserved positions in the *pur* regulon operator are also conserved in *pyrC* and in *pyrD*. Our results demonstrate that position 8 in the right-hand symmetry is important for repressor interaction. Replacement of C by T at this position significantly increased in vitro repressor binding and in vivo

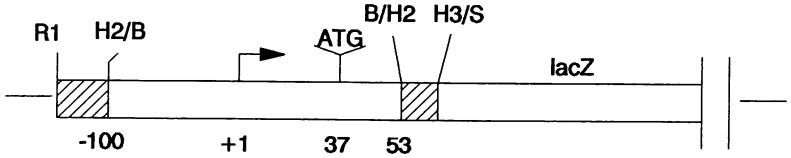

FIG. 6. Schematic diagram of *pyrC-lacZ* fusions. A 153-bp fragment containing the *pyrC* control region was cloned into the *Hin*cII site of a pUC118 polylinker. This fragment with flanking polylinker sequence was then ligated into the *Eco*RI and *Sma*I sites of *lacZ* fusion vector pMLB1034. Nucleotide positions in *pyrC* are given relative to the start of transcription. Hatched areas are polylinker sequences. The starts of transcription and translation are indicated. Abbreviations for restriction sites are R1, *Eco*RI; H2, *Hin*cII; B, *Bst*NI; H3, *Hin*dIII; S, *Sma*I. Sites connected by a slash are junctions in which both sites are destroyed. Only the 5′-proximal portion of *lacZ* is shown. Figure is not drawn to scale.

TABLE 2. Purine and pyrimidine regulation of *pyrC-lacZ*

Strain	*purR*	*pyrC-lacZ*	β-Galactosidase[a]				Repression factor	
			Pyrimidine starved[b]		Pyrimidine excess[c]		Purines[d]	Pyrimidines[e]
			−Adenine	+Adenine	−Adenine	+Adenine		
KYC4101	+	Wild type	400	205	50	26	2.0	8.0
KYC4102	+	−28C	407	231	51	26	1.8	8.0
KYC4103	+	−16T	439	98	45	14	4.5	9.8
KYC4104	+	−28C and −16T	522	81	75	17	6.4	7.0
KYC4105	−	Wild type	384	350	45	45	1.1	8.5
KYC4106	−	−28C	419	385	49	44	1.1	8.6
KYC4107	−	−16T	474	303	50	44	1.2	9.5
KYC4108	−	−28C and −16T	608	532	87	77	1.1	7.0

[a] Miller units.
[b] Grown with UMP.
[c] Grown with uracil.
[d] Calculated from pyrimidine-starved cells.
[e] Calculated from cells grown without adenine.

regulation. Replacement of A at position 5 with a C, in the left-hand symmetry, had little if any effect in vitro and in vivo. However, when combined with the C-to-T replacement, there was a modest increase in affinity of repressor and in vivo regulation. Based on the fact that the putative *pyrD* control site has a T at position 8 in the right-hand symmetry, PurR is expected to have a higher affinity for *pyrD* relative to *pyrC*. However, this putative purine control site in *pyrD* is somewhat upstream and does not overlap the −35 promoter element. Thus, repression by PurR, if it takes place, should be less than that observed for the *pyrC* −16T mutant.

Jensen (7, 8) has previously correlated expression of *pyr* genes with purine and pyrimidine nucleotide pools in mutant strains of *S. typhimurium*. Expression of *pyrC* was found to correlate best with the ratio of GTP to CTP pools in a manner suggesting coordinated stimulation by GTP and inhibition by CTP. Our experiments did not identify the purine or purine nucleotide corepressor, since binding of crude repressor occurred in the absence of added nucleotides. However, the interaction of purine repressor with the control site in *pyrC* was in all respects similar to its binding to the control site in *purF* (26) and other *pur* regulon genes (He et al., submitted), and thus the same corepressor is anticipated. In the simplest model, an adenine nucleotide would enhance binding of repressor to operator, a situation not in accord with the observations of Jensen (8). An alternative model, more in line with the observations of Jensen, would utilize GTP to prevent binding of purine repressor to the *pyrC* control site. We do not favor this latter possibility since it does not appear to be compatible with the mechanism for interaction of PurR with *pur* genes. Determination of the corepressor must await studies with the purified repressor protein.

It is interesting that all binding of repressor to the *pyrC* control site was abolished by using extract from a *purR* strain. This identifies the binding factor as a purine repressor and also indicates that a putative pyrimidine-specific repressor did not bind to *pyrC* under these conditions. The Pur repressor is thus the first *trans*-acting control element shown to regulate a pyrimidine gene. This cross pathway regulation should contribute to coordinating the rates of de novo synthesis of purine and pyrimidine nucleotides.

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