JOURNAL OF BACTERIOLOGY, Apr. 1993, p. 2363–2369
0021-9193/93/082363-07$02.00/0

Vol. 175, No. 8

Genetic Evidence that Promoter P_2 Is the Physiologically Significant Promoter for the *pyrBI* Operon of *Escherichia coli* K-12

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Received 16 December 1991/Accepted 13 February 1993

The *pyrBI* operon of *Escherichia coli* K-12 encodes the two nonidentical subunits of the pyrimidine biosynthetic enzyme aspartate transcarbamylase (ATCase). Expression of this operon is negatively regulated by pyrimidine availability primarily through UTP-sensitive transcriptional attenuation and, to a lesser extent, at the level of transcriptional initiation. Previous studies indicated that the *pyrBI* operon was transcribed from tandem σ^{70} promoters designated P_1 and P_2, with the large majority of transcription initiated at the more downstream promoter P_2. To more clearly define the roles of these promoters, mutations that severely impair or inactivate individual promoters were constructed in the chromosomal *pyrBI* operon, and their effects on ATCase synthesis were measured. In cells grown under conditions of either pyrimidine excess or pyrimidine limitation, more than 99% of all ATCase synthesis was directed by transcripts initiated at promoter P_2, indicating that it is the only physiologically significant *pyrBI* promoter. However, mutations that effectively inactivate promoter P_1 caused a 15% reduction in ATCase levels, apparently by inhibiting transcription from promoter P_2 by an unknown mechanism. Support for this explanation was provided by the demonstration that little, if any, transcriptional initiation occurred at promoter P_1 in a transcriptional fusion vector whereas a high level of transcription was initiated at promoter P_2 in an equivalent construction. Our results also provide evidence for pyrimidine-mediated regulation of transcriptional initiation at promoter P_2 over a severalfold range and show that cells can grow reasonably well with very low levels of ATCase, apparently because of changes in the concentration of allosteric effectors that increase the specific activity of the enzyme.

The *pyrBI* operon of *Escherichia coli* K-12 encodes the catalytic (*pyrB*) and regulatory (*pyrI*) subunits of the enzyme aspartate transcarbamylase (ATCase), which catalyzes the first committed step in the de novo synthesis of pyrimidine nucleotides. The product of the ATCase reaction, carbamyl aspartate, is toxic at high levels in the cell (22). ATCase activity is subject to allosteric regulation by the activator ATP and the inhibitors CTP and UTP, which bind to the regulatory subunit and alter substrate binding at the catalytic site (24). ATCase activity also is controlled by the level of *pyrBI* expression, which is negatively regulated over an approximately 300-fold range by pyrimidine availability. This regulation occurs primarily (i.e., 50-fold) through UTP-sensitive attenuation control, with additional pyrimidine-mediated regulation occurring independently at the level of transcriptional initiation (8, 9). Two putative *pyrBI* promoters designated P_1 and P_2, located approximately 350 and 160 bp upstream of the *pyrB* structural gene, respectively, were initially identified by in vitro transcription (23). Both promoters were recognized by σ^{70}-containing RNA polymerase holoenzyme. Subsequent quantitative S1 nuclease mapping experiments showed that a large percentage (ranging in three independent studies from approximately 95 to >99%) of cellular *pyrBI* transcripts were initiated at promoter P_2, with the remainder initiated at the more upstream promoter P_1 (4, 6, 13). Although these experiments strongly indicate that promoter P_2 is at least the major *pyrBI* promoter, a number of uncertainties associated with the quantitative S1 mapping procedure (e.g., internal cutting of probe DNA-mRNA heteroduplexes and different stabilities of P_1 and P_2 transcripts in vivo) preclude a precise assessment of the contributions of the two promoters to *pyrBI* expression. To more accurately determine the roles of these promoters, we have measured their contributions to operon expression by a different and more sensitive method. By site-directed mutagenesis or deletion, we constructed mutations that severely weaken or inactivate individual promoters and used strains carrying these mutations to measure their effects on ATCase synthesis. In addition, we examined transcription from each promoter after the promoters were cloned separately into a transcriptional fusion vector. The results indicate that essentially all ATCase synthesis is directed by transcripts initiated at promoter P_2 but that promoter P_1 does influence slightly the level of ATCase synthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used are *E. coli* K-12 and are described in Table 1. Bacteriophage P1-mediated transductions (11) and transformation of strain JC7623 with linearized plasmid DNA (9, 25) used in strain constructions were performed as previously described. Some plasmids used in this study and their constructions are described in Fig. 1. Plasmid pBHM74 is identical to plasmid pBHM42, except that the T in the sixth position of the −10 region of the *pyrBI* promoter P_2 has been changed to a G residue. The vector for generating individual *pyrBI* promoter::*lacZ* transcriptional fusions was plasmid pTL61T

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Source
JC7623	*recB21 recC22 sbcB15 arg ara his leu pro thr*	G. Walker (25)
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 thiA1 relA1 deoC1 ptsF25 flbB5301 rbsR*	M. Casadaban (2)
CLT35	MC4100 *araD*⁺ *car-94 pyrB477 srl-300*::Tn*10 recA56*	This laboratory (15)
CLT42	MC4100 *araD*⁺ *car-94*	This laboratory (15)
CLT43	CLT42 *srl-300*::Tn*10 recA56*	This laboratory (15)
CLT96	MC4100 *zjg-2086*::*kan*	P1 (CLT150) × MC4100
CLT97	MC4100 *zjg-2086*::*kan pyrBp(2)485*[a]	P1 (CLT152) × MC4100
CLT100	CLT42 *zjg-2086*::*kan*	P1 (CLT150) × CLT42
CLT101	CLT42 *zjg-2086*::*kan pyrBp(1)484*	P1 (CLT151) × CLT42
CLT102	CLT42 *zjg-2086*::*kan pyrBp(2)485*	P1 (CLT152) × CLT42
CLT103	CLT42 *zjg-2086*::*kan* Δ*pyrBp(1)486*	P1 (CLT153) × CLT42
CLT150	JC7623 *zjg-2086*::*kan*	JC7623 × pBHM203[b]
CLT151	JC7623 *zjg-2086*::*kan pyrBp(1)484*	JC7623 × pBHM211
CLT152	JC7623 *zjg-2086*::*kan pyrBp(2)485*	JC7623 × pBHM210
CLT153	JC7623 *zjg-2086*::*kan* Δ*pyrBp(1)486*	JC7623 × pBHM204

[a] The number in parentheses following *pyrBp* indicates which *pyrBI* promoter is mutated.
[b] This designation indicates that the strain was constructed by transforming recipient cells with linearized plasmid DNA and selecting for kanamycin resistance.

(7), which was obtained from Tom Elliott (University of Alabama at Birmingham). Fusion plasmids were constructed by inserting a restriction fragment carrying either a wild-type version or a mutant version of one of the *pyrBI* promoters into the unique *Sma*I site upstream of the *lacZ* gene in the vector. The restriction fragments used were the 316-bp *Ssp*I fragment carrying promoter P_1 and the adjacent 345-bp *Ssp*I-*Pvu*II fragment that includes promoter P_2 (Fig. 2). All plasmid constructions were confirmed by restriction enzyme mapping, and when necessary, ligation junctions were confirmed by DNA sequence analysis (18).

DNA preparations, restriction digests, ligations, transformations, and in vitro oligonucleotide-directed mutagenesis. These procedures were performed as previously described (9, 15). The sequences of the oligodeoxynucleotides used for the in vitro mutagenesis of the −10 regions of *pyrBI* promoters P_1 and P_2 were 5′-GGAATAAAAGGCATATCTG and 5′-TTGTCCGGCCTTATACTCA, respectively (note that the oligonucleotides are complementary to different DNA strands and that the mutagenic nucleotide is underlined). To verify the oligonucleotide-directed mutations and to show that no other changes were introduced by the mutagenesis procedure, the sequence of the *pyrBI* promoter region and flanking DNA carried by the recombinant M13 phage genome in which the mutations were initially introduced was determined (18).

Introduction of promoter mutations into the chromosomal *pyrBI* operon. Promoter P_1 and P_2 mutations were introduced individually into the chromosomal *pyrBI* operon of the pyrimidine-auxotrophic strain CLT42 (*car-94*) in two steps. Initially, strain JC7623 was transformed with *Eco*RI-digested plasmid pBHM204 [*kan* Δ*pyrBp(1)486*], pBHM210 [*kan pyrBp(2)485*], or pBHM211 [*kan pyrBp(1)484*] with selection for kanamycin resistance. A kanamycin-resistant, wild-type-promoter control strain also was constructed by transforming strain JC7623 with *Eco*RI-digested plasmid pBHM203 (*kan pyrBp*⁺). Strain JC7623 was used as the recipient in this first step, because it can be readily transformed with linearized DNA (25). The resulting transformants were designated CLT150 (*zjg-2086*::*kan*), CLT151 [*zjg-2086*::*kan pyrBp(1)484*], CLT152 [*zjg-2086*::*kan pyrBp(2)485*], and CLT153 [*zjg-2086*::*kan* Δ*pyrBp(1)486*]. In the second step, the *pyrBI* regions of strains CLT150, CLT151, CLT152, and CLT153 were recombined into strain CLT42 by P1-mediated transduction with selection for kanamycin resistance to generate strains CLT100 (*zjg-2086*::*kan*), CLT101 [*zjg-2086*::*kan pyrBp(1)484*], CLT102 [*zjg-2086*::*kan pyrBp(2)485*], and CLT103 [*zjg-2086*::*kan* Δ*pyrBp(1)486*]. The *pyrBI* regions of strains CLT150 and CLT152 also were introduced into the pyrimidine-prototrophic strain MC4100 by P1-mediated transduction with selection for kanamycin resistance to obtain strains CLT96 (*zjg-2086*::*kan*) and CLT97 [*zjg-2086*::*kan pyrBp(2)485*].

Southern hybridization. Chromosomal DNA was subjected to restriction enzyme digestion and then prepared for hybridization and probed as described by Sambrook et al. (17) with modifications as indicated. DNA restriction fragments were separated by electrophoresis in a 1% agarose gel and transferred to nitrocellulose filter paper by vacuum blotting. The probes were 5′-^{32}P-labeled synthetic oligodeoxynucleotides, labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham Corp.), and ^{32}P-labeled DNA fragments complementary to the 758-bp *Pvu*II fragment of plasmid pBHM42, which were synthesized by random priming. The sizes of DNA fragments were determined by comparison with appropriate DNA standards.

Confirmation of chromosomal wild-type and mutant *pyrBI* promoters. The presence of the correct *pyrBI* promoter sequence in strains CLT100 (*zjg-2086*::*kan pyrBp*⁺), CLT101 [*zjg-2086*::*kan pyrBp(1)484*], and CLT102 [*zjg-2086*::*kan pyrBp(2)485*] was confirmed by Southern hybridization. A sample of chromosomal DNA from each strain was digested to completion with *Pst*I and *Pvu*II, a procedure which should generate a 348-bp *Pst*I fragment and a 418-bp *Pst*I-*Pvu*II fragment containing promoters P_1 and P_2, respectively (Fig. 1 and 2). Two identical nitrocellulose filter blots were prepared, each containing one lane of digested DNA (15 μg) from each of strains CLT100, CLT101, and CLT102. The blots were probed with either the oligonucleotide used to construct the point mutation in promoter P_1 (mutant P_1 oligo) or that used to construct the point mutation in promoter P_2 (mutant P_2 oligo). Hybridizations were done overnight at 40°C with the mutant P_1 oligo and at 47°C with the mutant P_2 oligo. The washes were performed for 15 min at 44°C with the mutant P_1 oligo and at 50°C with the mutant P_2 oligo. The mutant P_1 oligo hybridized only to the 348-bp *Pst*I fragment from strain CLT101 [*pyrBp(1)484*], and the mutant P_2 oligo hybridized only to the 418-bp *Pst*I-*Pvu*II fragment from

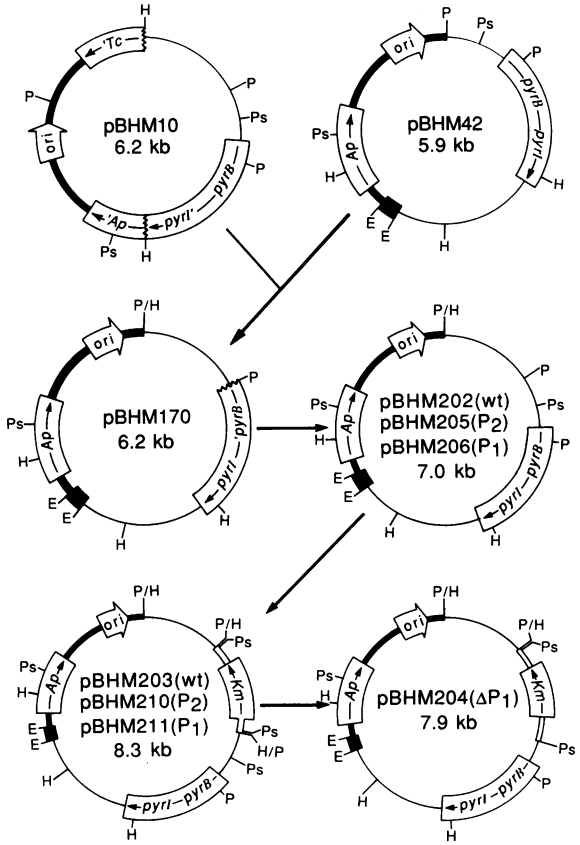

FIG. 1. Plasmid constructions. Plasmids pBHM10 (23) and pBHM42 (9) were constructed previously. DNA derived from plasmid pBR322 is shown as a thick line (with antibiotic resistance genes and the origin of replication [ori] indicated), and all other DNA is from the *E. coli* K-12 chromosome. The filled boxes in plasmid pBHM42 and plasmid derivatives represent a 177-bp *Eco*RI restriction fragment containing the T_1 transcriptional terminator of the *E. coli rrnB* operon. Restriction enzyme recognition sites: E, *Eco*RI; H, *Hin*cII; P, *Pvu*II; Ps, *Pst*I. Plasmid pBHM170 was constructed by ligation of the 5.1-kb *Pvu*II fragment of plasmid pBHM42 to the 1.1-kb *Hin*cII-*Pvu*II fragment of plasmid pBHM10 that is located upstream of the *pyrBI* operon. The 758-bp *Pvu*II fragment containing the wild-type *pyrBI* promoter-regulatory region from plasmid pBHM42 or the same fragment containing an oligonucleotide-directed point mutation in *pyrBI* promoter P_1 or P_2 was inserted into the *Pvu*II site of plasmid pBHM170 to generate plasmid pBHM202 (*pyrBp*$^+$), pBHM205 [*pyrBp(2)485*], or pBHM206 [*pyrBp(1)484*]. Plasmids pBHM203, pBHM210, and pBHM211 were constructed by inserting the 1.3-kb *Hin*cII fragment from plasmid pUC4K (Pharmacia) that carries the aminoglycoside 3′-phosphotransferase gene conferring kanamycin resistance into the *Pvu*II site located upstream of the *pyrBI* operon in plasmids pBHM202, pBHM205, and pBHM206, respectively. The *pyrBI* promoter P_1 deletion plasmid pBHM204 [*ΔpyrBp(1)486*] was obtained by ligation of a partial *Pst*I digest of plasmid pBHM203. wt, wild type.

strain CLT102 [*pyrBp(2)485*]. Hybridized probe on each of the two blots was removed by washing at 85°C for 30 min in a solution containing 0.3 M NaCl, 0.03 M sodium citrate, and 0.5% sodium dodecyl sulfate. The blots were then probed with either dCAGATATGCATTTTATTCC (wild-type P_1 oligo) or dTGAGTATAATGCCGGACAA (wild-type P_2 oligo), which includes the sequence of the wild-type −10 region of promoter P_1 or P_2, respectively. Hybridizations

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          .          20         .          40         .
CTTTCACACTCCGCCCTATAAGTCGGATGAATGGAATAAAATGCATATCT
                          P1         -10        ***

         60          .          80         .         100
GATTGCGTGAAAGTGAAAAAGGAAAAAGCAGGGAATGTCTGCAATTATTG
*

          .         120         .         140         .
ATACCGAAGGACAGTTCCCCTGCAGAATCACATCAAATAAAAATGCATAT
                     PstI

        160          .         180         .         200
ACCTTGACTTTTAATTCAAATAAACCGTTTGCGCTGACAAAATATTGCAT
                                                SspI

          .         220         .         240         .
CAAATGCTTGCGCCGCTTCTGACGATGAGTATAATGCCGGACAATTTGCC
        -35          P2         -10        ***

        260          .         280         .
GGGAGGATGT ATG GTT CAG TGT GTT CGA CAT TTT GTC TTA
           Met Val Gln Cys Val Arg His Phe Val Leu
```

FIG. 2. Nucleotide sequence of the *pyrBI* promoter region. The sequence of the nontemplate strand is shown; numbering is from the 5′ end. The −10 and −35 regions of the *pyrBI* promoters P_1 and P_2 are underlined and labeled (note that there is no consensus-like −35 region for promoter P_1). Major and minor transcriptional start sites detected in vivo and in vitro are indicated (asterisks) (4). The coding region for the *pyrBI* leader polypeptide begins at nucleotide 261. Also shown is the *Pst*I site which marks the downstream end of the *ΔpyrBp(1)486* mutation and the *Ssp*I site used in the construction of individual *pyrBI* promoter::*lacZ* transcriptional-fusion plasmids.

and washes with the wild-type P_1 and P_2 oligos were performed as described for the mutant P_1 and P_2 oligos, respectively. The wild-type P_1 oligo hybridized only to the 348-bp *Pst*I fragments from strains CLT100 (*pyrBp*$^+$) and CLT102 [*pyrBp(2)485*], and the wild-type P_2 oligo hybridized only to the 418-bp *Pst*I-*Pvu*II fragments from strains CLT100 (*pyrBp*$^+$) and CLT101 [*pyrBp(1)484*].

Southern hybridization also was used to show that the introduction of the *pyrBI* promoter region in the construction of strains CLT100, CLT101, and CLT102 was not accompanied by large insertions, deletions, or rearrangements and to confirm the deletion of the promoter P_1 region in strain CLT103 [*zjg-2086::kan ΔpyrBp(1)486*]. Two separate hybridization experiments were performed. In the first, chromosomal DNAs (10 μg each) from strains CLT100, CLT101, CLT102, and CLT103 were digested to completion with *Pst*I and *Bgl*II and used to prepare a nitrocellulose filter blot. Under the conditions described by Sambrook et al. (17), this blot was probed with DNA fragments complementary to the 758-bp *Pvu*II fragment of plasmid pBHM42, which contains the entire *pyrBI* promoter region. On the basis of the sequence of the *pyrBI* operon (19, 23), digestion with *Pst*I and *Bgl*II should generate a 348-bp *Pst*I fragment containing promoter P_1 and a 1,398-bp *Pst*I-*Bgl*II fragment containing promoter P_2. The results showed that the probe hybridized to these two fragments from strains CLT100, CLT101, and CLT102 and hybridized only to the 1,398-bp fragment from strain CLT103, as expected. In the second experiment, another blot, containing *Bst*EII-digested chromosomal DNAs (10 μg per lane) from strains CLT42 (Km^s *pyrBp*$^+$), CLT100, CLT101, CLT102, and CLT103, was prepared. Digestion with *Bst*EII should generate a large *pyrBI* promoter-containing fragment with one end far upstream of the operon and the other within the *pyrB* structural gene. The blot was hybridized as described above. The results showed that the probe hybridized to a 5.4-kb fragment from strain CLT42, to 6.7-kb fragments from strains CLT100, CLT101, and CLT102, and to a 6.4-kb fragment from strain CLT103.

These sizes are as predicted for the correct *pyrBI* promoter regions in these strains. The increases in sizes of the hybridizing fragments from strains CLT100, CLT101, and CLT102 compared with the size of the hybridizing fragment from strain CLT42 correspond to the size of the kanamycin resistance cassette (1.3 kb) which should be present just upstream of the *pyrBI* promoters in strains CLT100, CLT101, and CLT102 but absent in strain CLT42. The hybridizing fragment from strain CLT103 also contains the kanamycin resistance cassette, but it is 0.3 kb shorter than the fragments from strains CLT100, CLT101, and CLT102 because of the 348-bp deletion which eliminates promoter P_1.

In vitro transcription. Purified RNA polymerase holoenzyme containing σ^{70} (23) and restriction fragments used as DNA templates (9) were prepared as previously described. Transcription reaction mixtures (50 μl each) contained 20 mM Tris hydrochloride (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM disodium EDTA, 0.1 mM dithiothreitol, 0.2 mM GTP, 0.2 mM CTP, 0.2 mM UTP, 0.2 mM [α-^{32}P]ATP (1 Ci/mmol; ICN Pharmaceuticals Inc.), 10 nM DNA template, and 100 nM RNA polymerase (active enzyme, 20%). Reaction mixtures lacking ribonucleoside triphosphates were preincubated for 5 min at 37°C. For multiple-round assays, reactions were initiated by the addition of the nucleoside triphosphates and reaction mixtures were incubated for 5 min at 37°C. Heparin (Sigma Chemical Co.) was then added to a final concentration of 0.1 mg/ml, and the mixtures were incubated for an additional 10 min. For single-round assays, reactions were initiated by the addition of the nucleoside triphosphates containing heparin (final concentration, 0.1 mg/ml) and reaction mixtures were incubated for 10 min at 37°C. All reactions were terminated and RNA samples were prepared and analyzed as previously described (9), except that the electrophoretic separation of transcripts was done in a 1.5-mm-thick 10% polyacrylamide–7 M urea gel.

Media and culture methods. Cells used for enzyme assays or growth rate determinations were grown in N^-C^- medium (1) supplemented with 10 mM NH_4Cl, 0.4% glucose, 0.015 mM thiamine, and 1 mM arginine for strains carrying the *car-94* mutation, 100 μg of ampicillin per ml for plasmid-containing strains, and 1 mM uracil or 0.25 mM UMP as a pyrimidine source, as indicated. Cultures were grown with shaking at either 30 or 37°C, as indicated. Culture densities were measured with a Gilford model 260 spectrophotometer, and doubling times were determined between optical densities at 650 nm of 0.1 and 0.2. The solid media used for strain constructions were LB (11) with ampicillin (25 μg/ml) or kanamycin sulfate (50 μg/ml) added when required. Growth on solid media was at 37°C.

Enzyme assays. Cells from exponential-phase cultures were harvested at an optical density at 650 nm of 0.5 or 0.25 for plasmid-containing strains. Cell extracts were prepared and assays for ATCase (3), β-galactosidase (14), and β-lactamase (14) activities were performed as previously described.

RESULTS AND DISCUSSION

Construction and characterization of *pyrBI* promoter P_1 and P_2 mutations that effectively block transcriptional initiation. As the first step in measuring the contributions of the two *pyrBI* promoters to operon expression, we constructed three mutations designed to severely inhibit or eliminate transcriptional initiation from either promoter P_1 or promoter P_2. The first two mutations were constructed by oligonucleotide-directed mutagenesis in separate recombinant M13 phage carrying the *pyrBI* promoter-regulatory region. The mutations changed the highly conserved T in the sixth position of the −10 region of either promoter P_1 or promoter P_2 to a G residue. On the basis of previous studies, this change was predicted to cause a large reduction in promoter activity (12). The two mutations, designated *pyrBp(1)484* and *pyrBp(2)485*, were transferred to *pyrBI*-containing plasmids as shown in Fig. 1. The third mutation, designated Δ*pyrBp(1)486*, was generated by deleting the 348-bp *Pst*I fragment from the *pyrBI*-containing plasmid pBHM203 (Fig. 1). This deletion eliminates promoter P_1 but leaves wild-type promoter P_2 and the 87 bp upstream of the −35 region of promoter P_2 intact (Fig. 2).

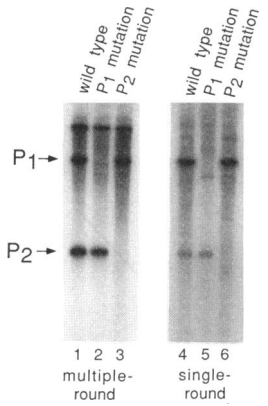

FIG. 3. Effects on in vitro transcription of the T-to-G substitutions in the sixth position of the −10 regions of *pyrBI* promoters P_1 and P_2. The autoradiogram is of a 10% polyacrylamide gel used to separate ^{32}P-labeled transcripts synthesized from the *pyrBI* promoter-containing 758-bp *Pvu*II fragments of plasmids pBHM42 (*pyrBp*$^+$), pBHM205 [*pyrBp(2)485*], and pBHM206 [*pyrBp(1)484*] (Fig. 1). The bands corresponding to transcripts initiated at promoter P_1 (approximately 290 nucleotides) and transcripts initiated at promoter P_2 (approximately 135 nucleotides) are indicated. The identities of these bands were established previously (4, 23). The third major band in lanes 1 through 3, which migrates more slowly than P_1 transcripts, is apparently the result of nonspecific transcriptional initiation near the end of the template (23).

To measure the effects of the two point mutations on transcription, the 758-bp *Pvu*II fragments from plasmids pBHM42 (*pyrBp*$^+$), pBHM205 [*pyrBp(2)485*], and pBHM206 [*pyrBp(1)484*] containing the entire *pyrBI* promoter region were transcribed in vitro (Fig. 3). Transcription was examined with multiple-round and single-round assays, in which different relative levels of transcription from the two wild-type *pyrBI* promoters occur (4). The results show that the two mutations eliminate virtually all activity from the mutant promoters under both assay conditions. To accurately determine the level of residual in vitro transcription from the mutant promoters, a series of dilutions of the reaction mixtures also were analyzed by gel electrophoresis and autoradiography (data not shown). A comparison of the lanes of the autoradiogram showed that the mutant promoter P_1 was less than 1% and the mutant promoter P_2 was between 1 and 2% as active as the corresponding wild-type promoters.

Effect of the promoter P_1 and P_2 mutations on *pyrBI* expression. To examine the effects of the three promoter mutations on *pyrBI* expression in vivo, each mutation was introduced individually into the chromosomal *pyrBI* operon

TABLE 2. Effects of the *pyrBI* promoter mutations on ATCase synthesis[a]

Strain	ATCase activity (nmol/min/mg)[b]		
	Repressed[c]	Derepressed[d]	Fold derepression
CLT42 (*pyrBp*$^+$)	20.5	7,300	356
CLT100 (*zjg-2086::kan pyrBp*$^+$)	22.6	7,200	319
CLT101 [*zjg-2086::kan pyrBp(1)484*]	19.2	6,330	330
CLT102 [*zjg-2086::kan pyrBp(2)485*]	ND[e]	3.62	
CLT103 [*zjg-2086::kan ΔpyrBp(1)486*]	19.1	5,990	314

[a] Cultures were grown at 30°C. Doubling times were 68 min and 110 ± 2 min for strains grown on uracil and UMP, respectively.
[b] Values are averages for two experiments; variation was less than or equal to ± 5%.
[c] Cells grown in uracil-supplemented medium.
[d] Cells grown in UMP-supplemented medium.
[e] ND, not detectable.

of strain CLT42 (*car-94*), as described in Materials and Methods. Strain CLT42 is a pyrimidine auxotroph, because the *car-94* mutation prevents the synthesis of carbamyl phosphate, a substrate for the ATCase reaction. A *kan* gene located just upstream of the *pyrBI* promoter region (and transcribed in the opposite direction) was used as the selectable marker in these constructions. The derivative strains were designated CLT101 [*zjg-2086::kan pyrBp(1)484*], CLT102 [*zjg-2086::kan pyrBp(2)485*], and CLT103 [*zjg-2086::kan ΔpyrBp(1)486*]. For use as a control, an additional derivative of strain CLT42 carrying the *zjg-2086::kan* insertion and the wild-type *pyrBI* operon was constructed and designated CLT100. The effects of the mutations were then determined by measuring the levels of ATCase in strains CLT42, CLT100, CLT101, CLT102, and CLT103 grown in a glucose-minimal salts medium containing either uracil or UMP as the pyrimidine source. Growth on uracil causes repressed *pyr* gene expression, and growth on UMP, which is only slowly used by the cells, results in pyrimidine limitation and derepressed *pyr* gene expression.

The results show that the point mutation in promoter P_2 of strain CLT102 caused a large reduction in ATCase levels (Table 2). The level in uracil-grown cells was too low to be detected, and the level in cells grown on UMP was reduced by a factor of 2,000, indicating that essentially all ATCase synthesis was directed by transcripts initiated at promoter P_2. However, both the point mutation in promoter P_1 of strain CLT101 and the complete deletion of this promoter in strain CLT103 caused an approximately 15% reduction in the level of ATCase in cells grown on either uracil or UMP (Table 2). This small reduction was detected reproducibly even with uracil-grown cells, in which ATCase levels were relatively low. Taken together, these results suggest that the wild-type promoter P_1 sequence provides a small, and indirect, stimulatory effect on transcription of the *pyrBI* operon, which is initiated almost exclusively at promoter P_2.

Support for this interpretation was provided by examining transcriptional fusions in which individual *pyrBI* promoters were inserted in the correct orientation into the cloning site preceding the *lacZ* gene of plasmid pTL61T. This plasmid is a multicopy vector specifically designed to measure promoter strength (7). It contains an RNase III processing site between the cloning site and the *lacZ* gene, which leads to the production of *lacZ* transcripts with the same 5′ end regardless of the promoter region insert. The vector and recombinant plasmids were introduced by transformation into strain CLT43 (*car-94 recA56*), and β-galactosidase activities in cells grown under pyrimidine-limiting conditions were measured (Table 3). The results show that the enzyme level in cells carrying the wild-type promoter P_1::*lacZ* fusion plasmid (pBHM501) was only approximately 40% higher than the basal enzyme level in cells carrying the promoterless vector. A slightly higher, but still low, level of enzyme activity was found in cells carrying an equivalent recombinant plasmid (pBHM503) in which the promoter P_1 insert contained the *pyrBp(1)484* point mutation. The reason for the higher enzyme level with the mutant promoter is not clear. However, these results indicate that very little, if any, transcriptional initiation occurred at promoter P_1. In contrast, the β-galactosidase activity in cells carrying the wild-type promoter P_2::*lacZ* fusion plasmid (pBHM498) was nearly 100 times higher than the activity in vector-containing cells. This high level of activity corresponds to a protein level that is approximately 20% of the total soluble protein of the cell (15) and clearly indicates that P_2 is a strong promoter. It should be noted that the promoter P_2 insert in plasmid pBHM498 includes the *pyrBI* attenuator; however, the effects of attenuation were eliminated by pyrimidine-limited growth.

To further examine the effect of the promoter P_2 point mutation *pyrBp(2)485* on *pyrBI* expression, particularly in uracil-grown cells, derivatives of strain CLT35 (*car-94 pyrB477 recA56*) carrying either plasmid pBHM42 or plasmid pBHM74 were grown on uracil or UMP. Plasmid pBHM42 contains the wild-type *pyrBI* operon; plasmid pBHM74 is identical to plasmid pBHM42, except that it carries the *pyrBp(2)485* mutation. The ATCase levels in both strains carrying the multicopy plasmids were measured. The results show that the promoter P_2 mutation again caused

TABLE 3. Expression of *pyrBI* promoter::*lacZ* transcriptional fusions derived from vector pTL61T[a]

Plasmid	*pyrBI* promoter	β-Galactosidase activity (nmol/min/mg)[b]
pTL61T	None	1,630 (1.00)
pBHM501	Wild-type P_1	2,260 (1.39)
pBHM503	Mutant P_1	3,390 (2.08)
pBHM498	Wild-type P_2	157,000 (96.3)

[a] Transformants of strain CLT43 carrying the indicated plasmids were grown at 30°C with UMP as the pyrimidine source. Doubling times were 116 ± 2 min for strains carrying plasmids pTL61T, pBHM501, and pBHM503 and 151 ± 1 min for the strain carrying plasmid pBHM498.
[b] Relative activities are given in parentheses. Values are averages for two experiments; variation was less than or equal to ± 10%. Plasmid copy numbers in all strains were the same, as judged by measurement of quantitatively extracted plasmid DNA (15).

TABLE 4. Effect of the *pyrBp(2)485* mutation on plasmid-encoded ATCase synthesis[a]

Strain[b]	ATCase activity (nmol/min/mg)[c]		
	Repressed[d]	Derepressed[e]	Fold derepression
CLT35/pBHM42 (*pyrBp*$^+$)	706	48,000	68
CLT35/pBHM74 [*pyrBp(2)485*]	3.56	80.6	23

[a] Cultures were grown at 30°C. Doubling times were 79 ± 1 min for both strains when grown on uracil; doubling times were 171 ± 9 min for strain CLT35/pBHM42 and 114 ± 2 min for strain CLT35/pBHM74 when the strains were grown on UMP.

[b] With the indicated plasmid genotype.

[c] Activities were normalized to plasmid copy number by using relative levels of plasmid-encoded β-lactamase activity, which were 1.00 and 0.98 for strain CLT35/pBHM42 grown on uracil or UMP, respectively, and 0.88 and 0.80 for strain CLT35/pBHM74 grown on uracil or UMP, respectively. Values are averages for two experiments; variation was less than or equal to ±4%.

[d] Cells grown in uracil-supplemented medium.

[e] Cells grown in UMP-supplemented medium.

large reductions in ATCase levels. In uracil-grown cells, the enzyme level was reduced by a factor of 200, and in UMP-grown cells it was reduced by a factor of 600 (Table 4). This effect is essentially the same as that caused by a 6-bp deletion that removes the −10 region of promoter P_2 (5), and this similarity indicates that the apparently low level of *pyrBI* transcription on plasmid pBHM74 is initiated at promoter P_1 or some other uncharacterized upstream promoter. Because ATCase activity could be detected in uracil-grown cells in this experiment, it was possible to measure the effect of the promoter P_2 mutation on regulation. Compared with regulation observed with the wild-type operon (68-fold), the level of regulation with the mutant operon (23-fold) was reduced by a factor of 3 (Table 4). This result indicates that some aspect of pyrimidine-mediated regulation is defective with the mutant promoter P_2. We presume that this regulation requiring promoter P_2 corresponds to the previously described attenuation-independent control of *pyrBI* expression (9).

The severalfold-lower level of regulation observed when the wild-type *pyrBI* operon is carried on a multicopy plasmid compared with that observed when it is present in single copy on the chromosome (68- and 356-fold, respectively) is due to a lower-than-expected level, on the basis of plasmid copy number, of ATCase in plasmid-containing UMP-grown cells. The synthesis of ATCase in these cells, which represents nearly 30% of total soluble protein synthesis (21), inhibits growth (Table 4, footnote *a*). Apparently, the cells respond to this inhibitory effect by capping ATCase synthesis in a presently unknown way. Because ATCase synthesis in strain CLT35/pBHM74 is low, even in UMP-grown cells, it should not be subject to this capping effect. Consequently, the threefold reduction in regulation determined by comparing ATCase levels in strains CLT35/pBHM42 and CLT35/pBHM74 is likely an underestimate of the effect of the mutant promoter P_2.

Effect of the promoter P_2 mutation on cell growth. Because the *pyrBp(2)485* mutation caused such a large reduction in the level of ATCase synthesis, we examined the possibility that this mutation could cause pyrimidine auxotrophy. The mutation was introduced into the chromosomal *pyrBI* operon of the pyrimidine-prototrophic strain MC4100 as described in Materials and Methods to generate strain CLT97. In this construction, the *zjg-2086::kan* insertion again was used as the selectable marker, with selection on an LB plate to avoid the requirement for pyrimidine biosynthesis. For use as a control, another derivative of strain MC4100 carrying the *zjg-2086::kan* insertion and the wild-type *pyrBI* operon was constructed. This strain was designated CLT96. Strains MC4100, CLT96, and CLT97 then were grown in glucose-minimal salts medium without or with a uracil supplement. Cell growth was examined at both 30 and 37°C (Table 5). The results show that the *pyrBp(2)485* mutation does not cause auxotrophy and reduces the growth rate only by a factor of 1.7 to 1.8 in the absence of uracil at both temperatures. Strains CLT96 and CLT97 grew almost identically at both temperatures when the medium contained uracil, indicating that pyrimidine limitation was the only cause of the slower growth of strain CLT97 without uracil. Strains MC4100 and CLT96 grew essentially the same under all conditions, indicating a lack of effect of the *zjg-2086::kan* insertion. Additionally, we examined a transformant of strain CLT97 carrying plasmid pBHM42 (*pyrBI*$^+$) and showed that it grew the same (within experimental error) as strain CLT96 with or without uracil (Table 5), confirming that the slow growth of strain CLT97 without uracil was due to low ATCase levels.

TABLE 5. Effect of the *pyrBp(2)485* mutation on cell growth

Strain	Doubling time (min)[a]			
	37°C		30°C	
	Without uracil	With uracil	Without uracil	With uracil
MC4100 (*pyrBI*$^+$)	48	50	73	79
CLT96 (*zjg-2086::kan pyrBI*$^+$)	50	49	74	76
CLT97 [*zjg-2086::kan pyrBp(2)485*]	91	50	127	76
CLT97/pBHM42 (*pyrBI*$^+$)	52	52	76	75

[a] In glucose-minimal salts medium. Values are averages for two experiments; variation was less than or equal to ±4%.

The growth of strain CLT97 in the absence of uracil indicates that even with a severely impaired promoter P_2, ATCase activity in the cell is sufficient to permit fairly rapid growth. To understand this response, we measured ATCase levels in strains CLT96 and CLT97 grown in the absence of uracil at 30°C. The levels were 71.7 and 3.75 nmol/min/mg in strains CLT96 and CLT97, respectively, representing a 19-fold difference. This difference is much less than the 2,000-fold difference in strength between the wild-type and mutant P_2 promoters indicated by the data in Table 2. The smaller difference is due to a high level of derepression of *pyrBI* expression in the pyrimidine-limited mutant strain CLT97, caused primarily by read-through transcription at the attenuator, coupled with only an approximately fourfold derepression of operon expression in wild-type strain CLT96 (data not shown). On the basis of growth rates, the level of derepression in strain CLT97 should be higher than the 23-fold derepression demonstrated with strain CLT35/pBHM74 (Table 4, footnote *a*, and Table 5). Derepression in strain CLT97 could not be determined directly, however, because the ATCase level in uracil-grown cells was too low to be measured reliably.

Even with the higher level of derepression, strain CLT97 still has only 5% of the level of ATCase found in strain CLT96 in the absence of uracil, a difference which is not reflected in their similar growth rates. Assuming that ATCase activity is the rate-limiting step in pyrimidine biosynthesis in wild-type *E. coli* (a situation which is highly likely because of the enzyme's position in the pathway, its

pattern of regulation, and the toxicity of its product), strain CLT97 must compensate for its low enzyme level. A likely explanation for this adjustment is that in strain CLT97 the specific activity of ATCase is increased by changes in the concentrations of allosteric effectors. In fact, it is known that the level of the positive effector ATP increases and the levels of the negative effectors CTP and UTP decrease in pyrimidine-limited cells (16). These changes, perhaps coupled with an increase in substrate levels, apparently provide ATCase total activity that is slightly more than half of that of strain CLT96.

The primary purpose of this study was to more clearly define the roles of *pyrBI* promoters P_1 and P_2. The results demonstrate that in cells grown under conditions of either pyrimidine limitation or pyrimidine excess more than 99% of all ATCase synthesis is directed by transcripts initiated at the more downstream promoter P_2, indicating that this promoter is the only physiologically significant promoter for the *pyrBI* operon. However, genetic inactivation of promoter P_1, by either a point mutation or deletion of the entire promoter region, causes a 15% reduction in ATCase level. The inability to detect significant transcription from promoter P_1 suggests that this reduction is due to the loss of a stimulatory effect of the wild-type promoter P_1 on transcription from promoter P_2. Perhaps the binding of RNA polymerase to promoter P_1 increases the local concentration of the enzyme or alters the local DNA structure so that transcription from promoter P_2 is facilitated. The mechanism and physiological significance of this activation remain to be determined. With respect to the proposed role for promoter P_2, it should be noted that in addition to pyrimidine-mediated regulation, *pyrBI* expression is subject to stringent control (20). Transcription from promoter P_2, but not from promoter P_1, is inhibited by physiological levels of guanosine tetraphosphate in vitro (4). Thus, transcription from promoter P_2 appears to be sufficient to account for all observed regulation of *pyrBI* expression. At present, we cannot eliminate the possibility that under some physiological conditions promoter P_1 or perhaps some other unidentified weak promoter is activated (or promoter P_2 is inactivated) so that a large fraction of the *pyrBI* transcripts are initiated at one of these sites. Such a mechanism involving promoter P_1 appears unlikely, however, because the DNA sequence of the promoter P_1 region in *E. coli* is not conserved in the closely related bacterium *Salmonella typhimurium* (10).

ACKNOWLEDGMENTS

We thank Don Court and Stuart Austin for helpful suggestions regarding the manuscript.

This work was supported by Public Health Service grant GM29466 from the National Institutes of Health.

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