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Regulation of aspartate transcarbamoylase synthesis in *Escherichia coli*: Analysis of deletion mutations in the promoter region of the *pyrBI* operon

(attenuation/*galK* expression/pKO1/termination of transcription)

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ABSTRACT **The catalytic and regulatory polypeptide chains of *Escherichia coli* aspartate transcarbamoylase are encoded by the *pyrB* and *pyrI* genes, respectively, which constitute a single transcriptional unit in the *pyrBI* operon. The DNA sequence immediately preceding the first structural gene, *pyrB*, contains a short open reading frame that could encode a 44-amino acid leader peptide and a (G+C)-rich region of dyad symmetry followed by eight thymidine residues. Synthesis of the enzyme is negatively controlled at the level of transcription depending on the cellular level of UTP, and an attenuation mechanism has been proposed to account for the 70-fold increase in *pyrBI* expression on pyrimidine starvation. The potential role of the dyad and eight thymidines as an attenuator was tested with a plasmid containing the promoter region of the *pyrBI* operon upstream of the *galK* coding sequence. When cells containing this plasmid, pPYRB10, were grown in a medium low in uracil, there was an 83-fold increase in galactokinase activity compared with the same cells grown at high uracil levels. This regulation is similar to that for aspartate transcarbamoylase synthesis in cells depleted of pyrimidines. Deletions constructed in the promoter region of pPYRB10 from the 3′ side produced one plasmid that retained normal control of *galK* expression and five that exhibited greatly reduced regulation. Nucleotide sequence determination showed that the one deletion mutation that was functionally similar to the wild-type plasmid contained the entire region of dyad symmetry, including the eight thymidines. The plasmids with more extensive deletions lacked the region with dyad symmetry and the eight thymidines. One of the deletion mutants that exhibited very low levels of regulation lacks the entire sequence coding for the putative leader peptide up to the major promoter. The results demonstrating the crucial role of a 19-nucleotide sequence (from −33 to −15) support an attenuation model but indicate that other mechanisms also contribute to the regulation of the *pyrBI* operon.**

Regulation of pyrimidine biosynthesis in *Escherichia coli* and *Salmonella typhimurium* is achieved in part through the action of the allosteric enzyme aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase, carbamoylphosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2), which catalyzes the formation of carbamoylaspartate. The reaction between carbamoylphosphate and aspartate, which constitutes the first committed step in the pathway leading to pyrimidines, is controlled by different mechanisms. ATCase activity is inhibited by CTP, the end product of the pathway, and stimulated by ATP (1). In addition, the formation of carbamoylaspartate is regulated by ATCase through the sigmoidal dependence of enzyme activity on the concentration of both substrates (1, 2). An even greater level of control is achieved by changes in the biosynthesis of ATCase depending on the cellular concentration of uridine nucleotides (3). In bacterial strains containing mutations that affect enzymes in the pyrimidine pathway and lead to drastically reduced levels of uridine nucleotides, the synthesis of ATCase is increased several hundred-fold (3). Turnbough has shown that UTP is the principal regulatory effector responsible for much of the negative control (4). How UTP regulates gene expression leading to ATCase synthesis has been the subject of recent investigations, and in this paper we present results of studies aimed at testing an attenuation mechanism that has been proposed to account for this control (5–7).

ATCase is assembled *in vivo* (8) through a series of association reactions (9, 10) involving catalytic trimers and regulatory dimers that are formed independently from catalytic and regulatory polypeptide chains encoded, respectively, by the *pyrB* and *pyrI* genes (11, 12). These two structural genes are linked in the *pyrBI* operon in which the *pyrB* stop codon is 15 nucleotides upstream from the start codon of *pyrI* (12, 13). Recent determinations (5–7) of the nucleotide sequence upstream of the *pyrBI* operon revealed two regions showing considerable homology with the consensus sequence for *E. coli* promoters (14), a segment that could code for a 44-amino acid leader peptide and two sequences of dyad symmetry, each of which is followed by a pyrimidine-rich region. *In vitro* transcription experiments indicated that the first of these inverted repeats is implicated in transcriptional pausing at low UTP concentrations (6). The second sequence of dyad symmetry is similar to the *trp* attenuator of *E. coli* (15) and satisfies structural requirements for ρ-independent transcriptional termination sites (16).

On the basis of their nucleotide sequence determinations, Roof *et al.* (5), Turnbough *et al.* (6), and Navre and Schachman (7) independently proposed an attenuation model for the regulation of the *pyrBI* operon. Fig. 1 illustrates the salient features of the model in which the coupling of transcription and translation can account for variations in expression of the *pyrBI* operon depending on the cellular UTP levels. If the UTP concentration within the cell is high, RNA polymerase is likely to terminate at the second dyad because transcription of a dyad region followed by a series of eight thymidine residues is thought to form a hairpin that blocks further RNA synthesis (16). However, if UTP is at low levels, RNA polymerase may pause for a sufficient time at the first dyad to allow translation of the leader peptide synchronously with transcription of the terminator structure. The presence of the ribosome directly behind RNA polymerase, according to the model, prevents formation of the termination hairpin and thereby permits RNA polymerase to transcribe both *pyrB* and *pyrI*. The basic premise of the model was tested by *in vivo* experiments using S1 nuclease mapping studies that demon-

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Abbreviations: ATCase, aspartate transcarbamoylase.
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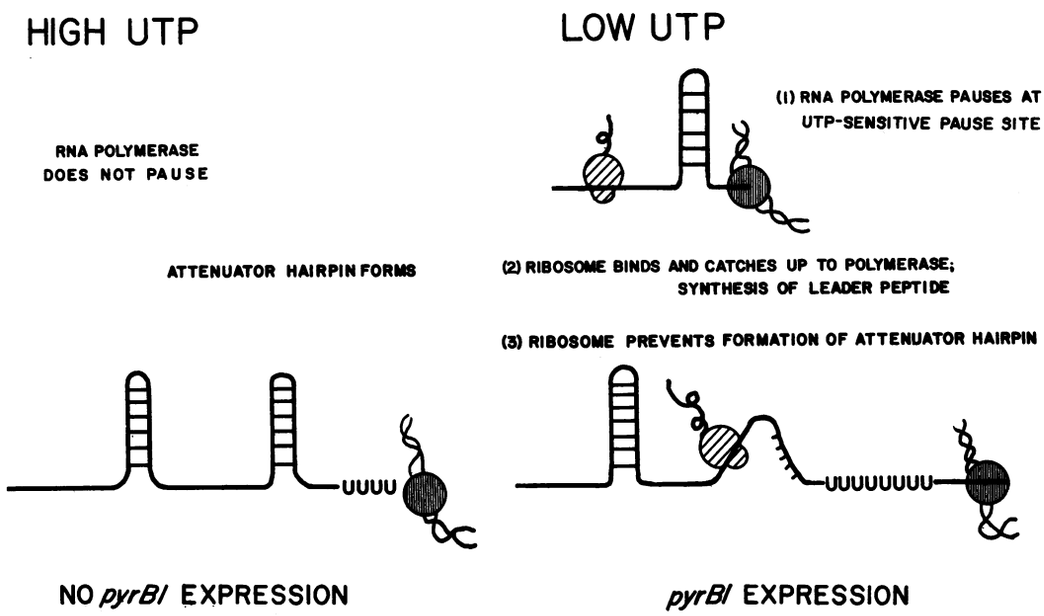

FIG. 1. Schematic representation of the attenuation model. The left side shows the basic features of the regulatory process for *pyrBI* expression in cells containing a high concentration of UTP. Under these circumstances, RNA polymerase (represented by the cross-hatched circle) does not pause and the transcript forms two hairpins corresponding to the dyads in the DNA sequence. Because the attenuator hairpins form, transcription terminates in the vicinity of the series of uridines. On the right is shown the antitermination mechanism that operates at low UTP levels. Because of the lack of UTP, RNA polymerase pauses after the first hairpin at nucleotide −80, thereby permitting the ribosome (represented by the widely cross-hatched object) to initiate translation of the leader peptide. The presence of the ribosome and the growing polypeptide chain are assumed to disrupt the terminating hairpin. In that way there is read through and RNA polymerase transcribes the operon.

strated that the *pyrBI* operon is regulated at the transcriptional level (7). Further support for the attenuation model was obtained by Turnbough *et al.* (6), who found that RNA polymerase pauses at the first hairpin in *in vitro* transcription experiments and that the length of the pause depends on the UTP concentration. They also showed that the bulk of the transcripts terminated at the attenuator (6).

An *in vivo* test of the attenuation model, based on an analysis of expression from plasmids with deletions in the regulatory sequence, is presented here. These deletions were constructed to localize regions of sequence critical to the function of the regulatory mechanism. The investigation was facilitated by subcloning a 985-base fragment containing both promoters, the two dyads, and a nucleotide sequence coding for the first 163 amino acids of the catalytic chain into pKO1, a plasmid specially constructed to test promoter efficiency of DNA inserts (17). Removal of the terminating hairpin resulted in increased expression of the *galK* gene in pKO1, as expected for the attenuation model.

MATERIALS AND METHODS

Bacterial Strains and Growth Media. *E. coli* K-12 was the progenitor of all strains. Plasmids were screened for the Gal^+ phenotype by using MacConkey galactose indicator plates (17) and strain SA1943 obtained from Sankar Adhya. All galactokinase assays were performed on cultures of strain CLT53 [*araD139*, Δ(*lacI, P, O, Z, Y, A*)*u169, rpsL, thi, car-403*] obtained from Charles Turnbough. E minimal medium (18) was supplemented with arginine at 100 μg/ml, 0.25% glucose, thiamine at 5 μg/ml, and ampicillin at 50 μg/ml. Uracil was also added to the cultures in order to control expression of the *galK* gene under the influence of the promoter of the *pyrBI* operon; for high expression, the concentration of uracil was 4 μg/ml and 40 μg/ml was used for low expression.

Plasmid Construction. Plasmid pPYRB10 was constructed by inserting the *Bst*EII–*Eco*RI fragment from pPYRB3 (7) into the *Eco*RI and *Sma* I sites of pKO1 (17) after the 3′ recessed end produced by digestion with *Bst*EII had been filled by incubation with deoxynucleoside triphosphates and the large fragment obtained from DNA polymerase. The deletions pDel36, pDel25, pDel58, and pDel31 were produced by controlled BAL-31 digestion of pPYRB10 that had been cleaved at the unique *Pvu* II site. Solutions used for the BAL-31 digestions contained 0.02 enzyme unit and 2 μg of plasmid cleaved by *Pvu* II in 30 μl of buffer (12 mM $CaCl_2$/12 mM $MgCl_2$/20 mM Tris·HCl, pH 8.0/1 mM EDTA). The solution was incubated at 37°C for 30 min, and digestion was stopped by the addition of EDTA to a final concentration of 50 mM. All 3′ recessed DNA ends resulting from BAL-31 digestion were filled to yield double strands by incubating 2 μg of DNA at 20°C for 30 min in 50 μl of buffer (40 mM KPO_4 at pH 7.5/6.6 mM $MgCl_2$/1.0 mM 2-mercaptoethanol/1 mM dATP/1 mM dCTP/1 mM dGTP/1 mM dTTP/5 units of DNA polymerase large fragment). Blunt-ended fragments were circularized by incubating the solution for 2 hr at room temperature with 2 units of T4 DNA ligase; the reaction mixture contained 50 mM Tris·HCl at pH 7.8, 10 mM $MgCl_2$, 20 mM dithiothreitol, and 1 mM ATP. The resulting ligated DNA was used to transform competent SA1943 cells, which were then spread on MacConkey galactose/ampicillin plates. Colonies were isolated that exhibited the red, slow-growing phenotype characteristic of cells overproducing galactokinase; assays for *galK* expression were performed as described below. pDel1 was constructed by using the *Aha* II site located 15 nucleotides upstream from the ATG in *pyrB*. The *Eco*RI–*Aha* II fragment from pPYRB3 was inserted into the *Eco*RI and *Sma* I sites of pKO1 after the *Aha* II 3′ recessed end was filled in by treatment with deoxyribonucleoside triphosphates and DNA polymerase large fragment. pDel17 contains the *Eco*RI–*Nci* I pPYRB3 fragment ligated into pKO1 at the *Eco*RI and *Sma* I sites.

All enzymes used in the plasmid constructions were obtained from New England Biolabs except for BAL-31, which was obtained from Bethesda Research Laboratories. The nucleotide sequences at the junctions of the deletion

plasmids were determined by the method of Maxam and Gilbert (19).

Galactokinase Assay. The procedure of McKenney *et al.* (17) was used for assays of galactokinase activity on 1 ml of CLT53 cultures grown to midlogarithmic phase. Cells were permeabilized by adding 2 drops of toluene and 40 μl of lysis buffer (100 mM EDTA and 100 mM dithiothreitol in 50 mM Tris·HCl at pH 8.0) and then mixing the suspensions in a Vortex for 1 min. After the toluene was removed in a Speed Vac evaporator (Savant), the suspension of permeabilized cells was assayed at 30°C for the amount of galactose converted to galactose 1-phosphate in a 15-min period. The reaction mixture included 10 μl of the permeabilized culture; 20 μl of a solution containing 5 mM dithiothreitol and 16 mM NaF; 50 μl of a solution containing 8 mM $MgCl_2$, 200 mM Tris·HCl at pH 7.9, and 3.2 mM ATP; and 10 μl of [^{14}C]galactose solution [Amersham D-[1-^{14}C]galactose at 50 mCi/mmol (1 Ci = 37 GBq) filtered through Whatman DE81 and diluted to a specific activity of 1.0×10^6 dpm/μmol]. After 15-min incubations, the solutions were filtered on 2.4-cm DE81 discs, which were then washed with H_2O and dried. Measuring the radioactivity on discs in a scintillation counter provided relative values of the amounts of phosphorylated galactose.

β-Lactamase Assay. The procedure of Samuni (20) was used to measure the concentration of β-lactamase in extracts of the permeabilized cells and the amounts determined by the spectrophometric assay provided relative values of the plasmid copy number in the cell cultures (21). Assays were performed by measuring the decrease in absorbance at 240 nm in 2-ml reaction mixtures containing 50 μl of the permeabilized cells and 1.4 mM benzylpenicillin in 0.2 M KPO_4 buffer at pH 7.0. The results of the β-lactamase assays were used to normalize each galactokinase activity in the same culture to correct for plasmid copy numbers.

RESULTS

Control of *galK* Expression by the Regulatory Region of *pyrBI*. To facilitate the construction and analysis of the deletions in the control region of the *pyrBI* operon, we subcloned a restriction fragment containing both promoters and 489 nucleotides of the *pyrB* gene into the plasmid pKO1. Fig. 2 shows relevant regions of the recombinant plasmid pPYRB10, which contains the *E. coli galK* gene preceded by 168 nucleotides of leader sequence. Because pKO1, as designed by McKenney *et al.* (17), is devoid of both galactose operon promoters, the expression of *galK* depends entirely on the promoter function of the DNA inserted into restriction sites upstream from the gene. Thus, pyrimidine starvation should cause pPYRB10 to express *galK* in the same way that reduced UTP pools cause overexpression of *pyrB* and *pyrI* in wild-type cells containing the intact *pyrBI* operon.

Accordingly, the effect of varying uracil concentration on the expression of *galK* in pPYRB10 was measured in the Pyr$^-$ strain CLT53. Midlogarithmic-phase cultures were permeabilized and the extracts were assayed for galactokinase activity, which was then normalized for plasmid copy number evaluated from the companion assay for β-lactamase. Cells grown in the presence of uracil at 4 μg/ml contained 83 times more galactokinase than those grown at 40 μg/ml. Thus, the regulatory region of the *pyrBI* operon inserted upstream of pKO1 did control *galK* expression and the dependence of galactokinase synthesis on uracil levels was similar to that observed with cells synthesizing ATCase from the intact *pyrBI* operon.

In control experiments with CLT53 cells containing pKO1, only trace amounts of galactokinase activity were detected. Moreover, there was no change in the amount of galactokinase resulting from variation in the amount of uracil in the growth medium. The low background level (less than 0.5%) of galactokinase activity was used to correct all results obtained from strains containing various deletions in pPYRB10.

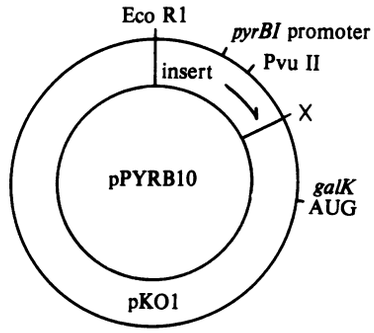

FIG. 2. Map of the plasmid pPYRB10. The arc labeled pKO1 shows the pBR322-derived vector containing the *galK* gene with the first codon indicated by AUG. The segment marked insert is the *pyrBI* fragment that was inserted into the *Eco*RI and *Sma* I sites of pKO1. The *Sma* I junction is designated by X because the ligation at this position destroyed the *Sma* I recognition site. The position of P_2, the promoter nearest the *pryB* gene is indicated along with an arrow to show the direction of transcription. Only one of the relevant restriction sites, *Pvu* II, used for the construction of the deletion mutations is shown. This site is located in the *pyrB* structural gene at position 140.

Nucleotide Sequences of the Various Deletion Mutations in pPYRB10. Fig. 3 summarizes the sequence determinations of the mutants containing the different deletions. pDel17 had the most extensive deletion, starting just 3′ to P_2 at nucleotide −148 and extending in the 3′ direction to the pKO1 junction at nucleotide 484. As indicated by the linear map of pPYRB10 in Fig. 3, the deletion in pDel17 includes the first dyad from −108 to −86, which is thought to be a transcriptional pause site, the second inverted repeat from −50 to −31, which appears to be the transcriptional termination site, and the nucleotide sequence represented by part of *pyrB*. The deletion in pDel36 started at nucleotide −53, just 5′ to the terminator, and continued for 331 nucleotides (to position 278), leaving intact the final 210 nucleotides of the insert. pDel25, pDel58, and pDel31 all contained deletions with 5′ junctions lacking portions of the terminator hairpin and the eight thymidines. The 3′ junctions in these three deletion mutants all occurred within 2 nucleotides of one another, leaving about 234 nucleotides of the insert. In pDel1, the deletion started downstream of the terminator at nucleotide −14 and continued in the 3′ direction to the pKO1 junction.

***galK* Expression in the Various Deletion Mutants.** The effect of each deletion on *galK* expression is summarized in Fig. 4, which presents a fine-structure map of the regulatory region and the results of galactokinase assays on extracts from cell cultures grown to midlogarithmic phase in the presence of uracil at either 40 μg/ml (high) or 4 μg/ml (low). As seen for the wild-type strain, containing the intact regulatory region of *pyrBI*, the galactokinase activity of cells grown at a high uracil concentration was only 1.2% that of the same cells when grown at low levels of uracil. All values listed in Fig. 4 are relative to that obtained for pPYRB10 in CLT53 cells at low uracil levels. The large deletion in pDel17, which eliminated both dyads, caused the loss of most of the decrease in expression at high uracil levels. In these cells, galactokinase synthesis at a high uracil concentration was 39% that of the fully expressed level in pPYRB10, a value 32 times that of the cells containing the intact regulatory region of *pyrBI*. Plasmids pDel36, pDel25, pDel58, and pDel31, which also lacked all or part of the terminator hairpin, produced 30, 19, 10, and 11%, respectively, of the galactokinase activity in cells grown

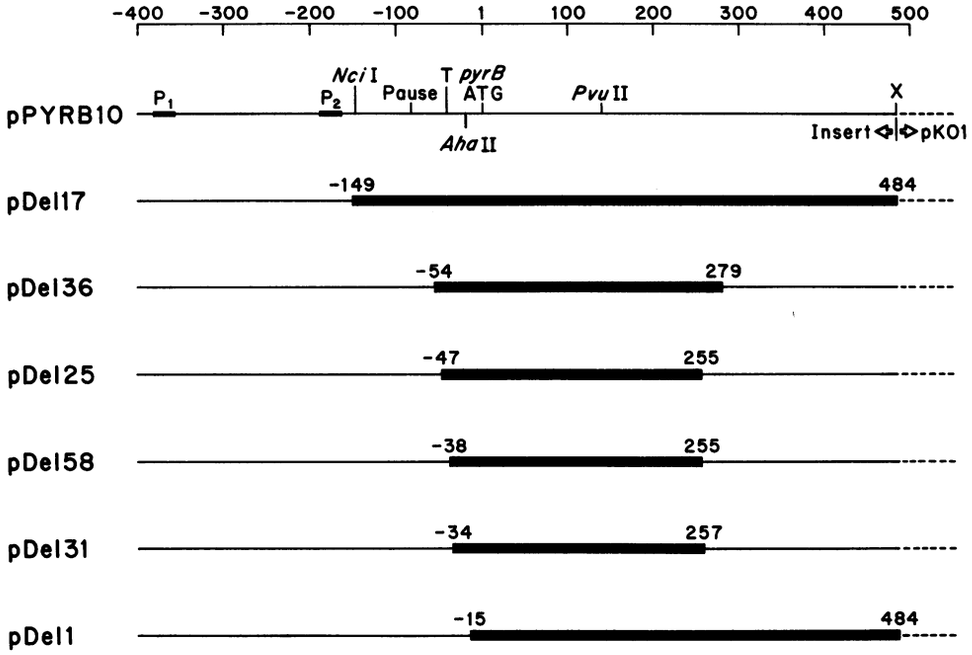

FIG. 3. Linear maps of various deletion mutations in pPYRB10. At the top is a scale indicating distances in nucleotides from the start (labeled 1) of the *pyrB* gene. The map of pPYRB10 shows the two promoter sites, P_1 and P_2, the pause site at the first region of dyad symmetry (nucleotides −108 to −86), and the transcriptional termination site (designated by T) at the second region of dyad symmetry (−50 to −31). The locations of the *Nci* I, *Aha* II, and *Pvu* II restriction sites are also shown; these sites were used for the construction of the various deletion mutations shown. The deleted regions are designated by the heavy line and the junctions are designated by the numbers. Although the numbering begins at position −400, the insert actually extends further upstream. X indicates the right-hand junction between the insert and pKO1.

at high uracil levels compared with the wild-type strain at low uracil concentrations. All of these deletion mutants lost most of the decrease in expression caused by high uracil levels as shown by the observation that they synthesized much more galactokinase than the wild-type strain when the cells were grown at high uracil levels. In contrast, when pDel1, which contained the bulk of the regulatory region of the wild-type plasmid pPYRB10, was grown in a high-uracil medium, the cells produced only 1.9% the galactokinase activity of pyrB10 grown at a high uracil level. This value is close to that (1.2%) of the wild-type strain grown under comparable conditions. These results for the various deletion mutants grown at a high uracil concentration demonstrate the role of the second dyad (that from −50 to −31) and the sequence of eight thymidines in controlling expression.

When the cells containing the same deletions were grown at low uracil levels, there were only slight changes in *galK* expression. As shown in Fig. 4, the galactokinase activities

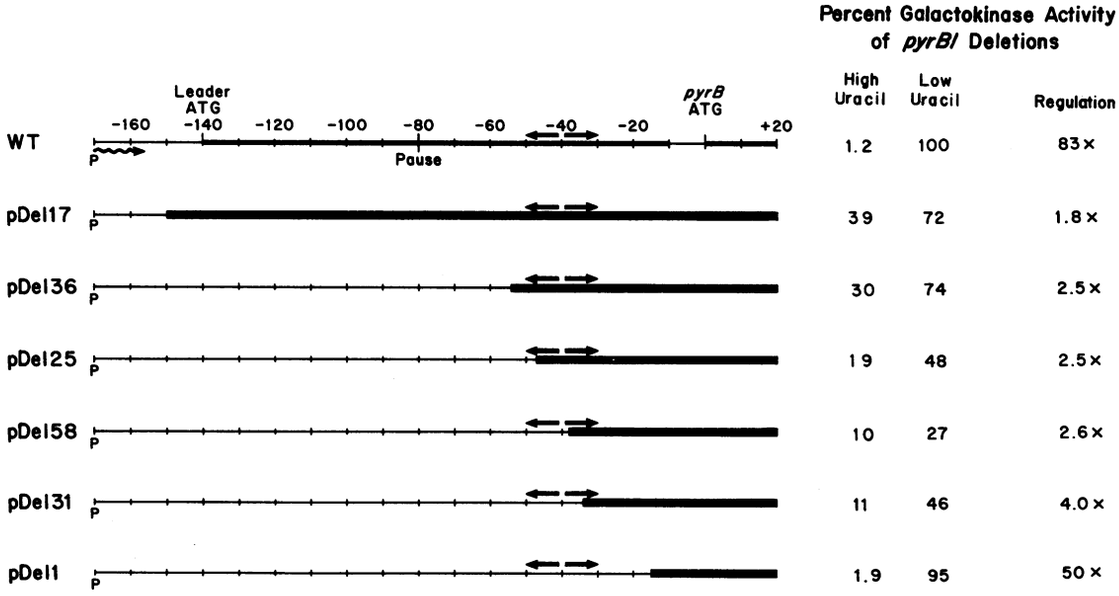

FIG. 4. Fine-structure map of deletion mutations and *galK* expression of each mutant grown at high and low concentrations of uracil. The wild-type (WT) map is shown at the top with P designating the position of P_2, the wavy line and arrow showing the direction of transcription, leader ATG designating the start codon for the leader peptide and Pause indicating the location of the dyad symmetry centered at −97. Double arrows between −50 and −31 designate the location of the second dyad, which leads to the termination hairpin in the transcript. The start of the structural gene is represented by *pyrB* ATG. Heavy lines on the WT map show sequences that yield a transcript that is translated. Deleted regions in the various mutations are indicated by the heavy lines in pDel17, pDel36, pDel25, pDel58, pDel31, and pDel1. Results of galactokinase assays of extracts of CLT53 cells transformed with each of the deletion plasmids are shown in the table. The columns marked high uracil and low uracil correspond to cells grown in media containing uracil at 40 and 4 μg/ml, respectively, and the values are presented as percentage of enzyme activity produced by the wild-type plasmid grown in a low-uracil medium. All values are corrected for background and for copy number. The column designated Regulation gives the ratios of galactokinase activity measured for cells grown in low-uracil medium relative to those for cells in high-uracil medium.

for the different mutants varied from 27 to 95% that observed for pPYRB10. Deletion of the entire terminator region as in pDel36 yielded 74% of the galactokinase activity of the wild-type strain, whereas pDel1, which contains the entire terminator, produced virtually the same amount (95%) of galactokinase as observed for intact pPYRB10.

DISCUSSION

In a Pyr$^-$ strain of *E. coli* containing a single copy of the *pyrBI* operon (on the chromosome) and a mutation in *pyrC*, the level of ATCase activity was shown by Gerhart (22) to be 70-fold higher for cells grown in low-uracil medium than for the same cells in medium containing a high uracil concentration. It is of interest that multiple copies (about 20) of pPYRB10, a plasmid with the control region of *pyrBI* placed 5′ to the *galK* coding sequence, produced an 83-fold increase in galactokinase activity in cells on growth in uracil-poor medium. This result indicates that the *cis*-acting *pyrBI* regulatory sequence in pPYRB10 is sufficient for normal levels of control. Although these observations do not rule out the possibility that *trans*-acting products contribute to the large enhancement caused by uracil depletion, it is evident that increasing the copy number of the control region had little impact on the observed regulation.

Six deletion mutations, spanning the region thought to control *pyrBI* expression, were constructed to determine which sequences are critical for regulation of *galK* expression. In cells grown in excess uracil, all but one of the six deletions caused an overproduction of galactokinase compared with the plasmid containing no deletion. pDel17 and pDel36 produced 32 and 25 times more *galK* expression than the undeleted plasmid. Cells containing pDel25, pDel58, and pDel31 yielded 16-, 8.3-, and 9.2-fold increases in galactokinase activity relative to pPYRB10. In contrast to these five deletion mutants, pDel1 caused only a 1.6-fold overproduction, which is almost a normal level of *galK* expression. The increased levels of galactokinase encoded by pDel17, pDel36, pDel25, pDel58, and pDel31 indicate that these five deletion mutants lack sequences that usually serve to reduce *galK* expression. The only sequence in pDel1 not present in pDel31 is between nucleotides −33 and −15. Thus the location of at least part of the nucleotide sequence responsible for reduced *galK* expression is between −33 and −15. According to the attenuation model in Fig. 1, there is a transcriptional termination site located in precisely this region of the *pyrBI* operon. Further evidence supporting the model that the dyad from −50 to −31 and the eight thymidines serve as a transcriptional termination site was obtained from *in vitro* experiments. As shown by Turnbough *et al.* (6), ρ-independent transcriptional termination occurs in a region near the attenuator. However, fragments from pDel17 or pDel25 plasmids that lack that hairpin cause no termination when used as templates for RNA polymerase (data not shown).

Interpretations of the extent of *galK* overproduction in the various deletion mutants must include possible effects of polarity because each deletion produced a frameshift that may cause a reduction in *galK* expression. As a result, the degree of overproduction of galactokinase activity may be artificially low and the differences among the various deletion mutants may be attributable in part to variations in the effects of polarity.

Each of the plasmids was tested to determine the effect of the deletions on the extent of overproduction of galactokinase in cells grown in low levels of uracil. As shown in Fig. 4, cells with plasmids pDel17, pDel36, pDel25, pDel58, and pDel31 showed only a 2- to 4-fold increase in *galK* expression when grown in a low-uracil medium as compared with the growth of the same cells in excess uracil. These ratios are markedly lower than the 83-fold overproduction observed with the plasmid containing no deletion and with pDel1, which showed a 50-fold increase in galactokinase synthesis. On the basis of the comparison between pDel31 and pDel1, we conclude that the 19 nucleotides present in the former and absent from the latter define a region required for normal function of the *pyrBI* regulatory mechanism. The results indicate that nucleotides −33 to −15 not only function to decrease *galK* expression in cells grown in high-uracil medium but also participate in the regulation leading to overproduction of galactokinase when uracil is little present during growth of the cells. According to the attenuation model illustrated in Fig. 1, expression of the operon is reduced by termination of transcription between nucleotides −33 to −15 when the UTP concentration is high, and overexpression results when cells lack UTP and the effectiveness of the terminator is reduced. The results presented here are consistent with both the termination and the antitermination aspects of the model.

Although the attenuation mechanism accounts satisfactorily for many of the experimental observations, there still appears to be a 2- to 4-fold residual regulation exhibited by cells containing plasmids that lack the terminator. The slight increase in galactokinase activity in these cells grown in a low-uracil medium indicates that another regulatory mechanism independent of attenuation may be operating. This second mechanism may function at the promoter itself since pDel17, which lacked all regulatory regions of the nucleotide sequences up to P_2, still exhibited a 2-fold increase in *galK* expression when the cells were grown at low uracil levels.

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