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Expression of *Escherichia coli pabA*

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Escherichia coli pabA **encodes the glutamine amidotransferase subunit of *p*-aminobenzoate synthase. *p*-Aminobenzoate synthase catalyzes the conversion of chorismate and glutamine to 4-amino-4-deoxychorismate, which is then converted to *p*-aminobenzoate by a 4-amino-4-deoxychorismate lyase. The 5′-terminal segment of *pabA* was previously shown to be transcribed from two different promoters, one near the *pabA* coding sequence (P_1) and one preceding *fic* (P_2). However, a *pabA-lacZ* translational fusion was expressed only from the mRNA originating at P_1. We have determined that expression of a *pabA-lacZ* chromosomal fusion is not changed by *p*-aminobenzoate limitation, growth rate, catabolite repression, overexpression of either *p*-aminobenzoate synthase subunit, or gene dosage of *pabA* and *pabB*. The lack of *pabA* expression from P_2 appears to be the result of a stable secondary structure in the intergenic space preceding *pabA* that sequesters the *pabA* ribosome binding site. Disruption of the secondary structure by mutation allowed expression of *pabA* from P_2, as did translation of ribosomes into the *fic-pabA* intergenic region.**

p-Aminobenzoate synthase (PABS) catalyzes the conversion of chorismate and glutamine to an intermediate compound, probably 4-amino-4-deoxychorismate, which is then converted to *p*-aminobenzoate (PABA) by a second enzyme, aminodeoxychorismate lyase (18, 25). The two subunits of PABS are encoded by two unlinked genes in *Escherichia coli*: *pabB*, at 40 min (10, 24), encodes the larger 54,000-molecular-weight subunit, i.e., component I, that catalyzes the synthesis of aminodeoxychorismate from chorismate and NH_3 (7, 21); and *pabA*, at 74 min (9, 10), encodes a 21,000-molecular-weight glutamine amidotransferase subunit, i.e., component II, that allows PABS to use glutamine as the source of NH_3 (12). *pabC*, which encodes aminodeoxychorismate lyase, is unlinked to either *pabA* or *pabB* (8).

PABA is essential for the biosynthesis of dihydrofolate, which in various forms participates in the synthesis of purines, thymidylate, *N*-formylmethionyl-tRNA, methionine, glycine, and pantothenate. As the folate moiety is not consumed during the reactions, de novo synthesis requirements for dihydrofolate and its precursors are small. Also, since exogenous folates cannot be transported and used by *E. coli*, dihydrofolate synthesis is essential, but only at a replenishment level as a cell grows and divides. Despite the fact that most of the enzymes involved in the biosynthesis of tetrahydrofolate have been characterized in *E. coli* (5), only two genes, besides the aforementioned *pabA* and *pabB* genes, have been mapped. These are *folA*, which encodes dihydrofolate reductase and maps at 1 min (4, 5), and *folC*, which encodes the bifunctional folylpoly-γ-glutamate synthetase–dihydrofolate synthetase and maps at 56 min (3).

Nucleotide sequence analysis of the *pabA* region from *E. coli* and *Salmonella typhimurium* revealed at least four closely linked genes near *pabA* (Fig. 1). The gene previously designated *utu* (22) has been identified and designated *rot* (15) and encodes peptidylprolyl *cis-trans* isomerase (rotamase). A short open reading frame (*orf1*) overlaps a Shine-Dalgarno sequence (19), an ATG triplet, and eight nucleotides (not including the termination codon) of *fic*. The *fic* coding region extends for 200 codons and encodes a product thought to be involved in cell division (13, 14). The *fic* coding region ends 31 nucleotides upstream of *pabA*. Eighty-five nucleotides downstream of *pabA* is an open reading frame that is conserved in *S. typhimurium* and *Klebsiella aerogenes*. The conserved open reading frame is designated *dtu* and is likely to encode *argD* (13, 14).

Transcriptional analysis of the *E. coli pabA* region has defined four promoter-containing regions (22). The relative strengths of these promoters, designated P_r, P_2, P_1, and P_d, are 10:1:1:3 (22). *rot* is preceded by a strong promoter and followed by a transcription terminator (22). P_2 lies between *rot* and *orf1*, and P_1 lies partially within *fic* and initiates transcription 22 bp upstream of *pabA*. We have shown previously by RNase protection and primer extension that the 5′-terminal segment of *pabA* is transcribed from both P_1 and P_2 (22). Deletion of P_2 or insertion of a polar kanamycin cassette between P_2 and P_1 blocked transcription of the *pabA* segment from P_2, as assayed by RNase protection and primer extension, but did not affect expression of a *pabA-lacZ* translational fusion. Deletion of five nucleotides from the −10 region of P_1 resulted in a 50-fold decrease in the expression of the *pabA-lacZ* fusion. This level of expression was not appreciably altered by the presence or absence of the polar insertion between P_2 and P_1. Further, strains containing the P_1 deletion and wild-type *pabA* demonstrated a requirement for PABA. These data indicated that mRNA derived from P_2 was not competent for *pabA-lacZ* translation and that the majority of *pabA* expression was derived from mRNA transcribed from P_1.

The *fic-pabA* intergenic region contains a sequence capable of forming a stable secondary structure ($\Delta G^\circ = -8.5$) which, if formed in mRNA, would include the *pabA* ribosome binding site. P_1 transcription initiates within the putative structure-forming sequence, and no mRNA structure occluding the *pabA* ribosome binding site could be formed. The formation of such a structure within the *fic-pabA* intergenic region was hypothesized to inhibit *pabA* expression from P_2 mRNA at the level of translation initiation (11). In this work, we have investigated growth conditions that might affect *pabA* expression and have investigated the role

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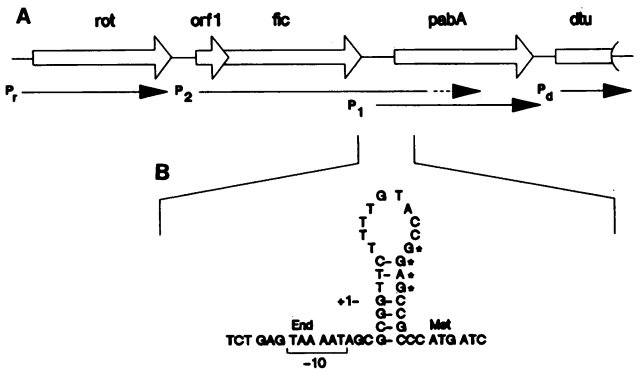

FIG. 1. (A) Chromosomal context of *pabA*. Open arrows represent the genes that have been identified near *pabA*. *orf1* and *dtu* have not been identified, but *dtu* is likely to be *argD* (13). Locations of promoters and their transcripts that have been identified in the region are shown below the genes. (B) Detail of the nucleotide sequence in the *fic-pabA* intergenic region, illustrating the secondary structure that may form in mRNA, a portion of promoter P_1 (−10), the site of initiation from P_1 (+1), and the putative *pabA* ribosome binding sequence (*).

of the putative secondary structure in the expression of *pabA* from promoter P_2.

MATERIALS AND METHODS

Strains, media, and genetic techniques. All bacterial strains, plasmids, and phages are described in Table 1. *E. coli* cultures were grown at 37°C with aeration either in NZY broth (16) or in Vogel-Bonner minimal salt medium (23). Amino acids and thiamine, when needed, were supplemented at the concentrations recommended by Davis et al. (6); PABA was used at 10 ng/ml unless otherwise indicated. Ampicillin and kanamycin were used at 50 μg/ml.

Transformations were carried out by the $CaCl_2$ method (16). P1 transduction and in vivo mutagenesis using ethyl methanesulfonate were done as described by Miller (17).

Chemicals and enzymes. Amino acids, PABA, antibiotics, isopropyl-β-D-thiogalactoside (IPTG), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was purchased from Research Organics. Oligonucleotides were synthesized by the Laboratory for Molecular Biology DNA Synthesis Facility at the University of Illinois at Chicago. Other oligomers used in DNA sequencing were synthesized or purchased from New England BioLabs. All enzymes used in standard DNA techniques and RNA analysis were purchased from Boehringer Mannheim Biochemicals, New England BioLabs, Promega Biotec, or United States Biochemical Corp. The T4 DNA ligase was prepared in this laboratory according to published procedures. All radioactive nucleotides were purchased from Amersham.

DNA manipulations. Standard recombinant DNA techniques such as plasmid DNA isolation, restriction enzyme analysis, agarose gel electrophoresis, and polyacrylamide gel electrophoresis were performed according to Maniatis et al. (16). Bacterial chromosomal DNA was prepared by the lysozyme-sodium dodecyl sulfate-phenol extraction method (16). Nick-translated DNA prepared by using [α-^{32}P]dCTP was used for DNA hybridization.

Genomic DNA amplification by PCR and genomic DNA sequence analysis. Asymmetrical amplification of chromosomal DNA for nucleotide sequencing was carried out by using the polymerase chain reaction (PCR) kit purchased from Perkin Elmer Cetus. The reaction was set up according the manufacturer's suggestions except that 1 μg of chromosomal DNA was used and the ratio of the primers was 50:1. The amplification reaction was carried out for 40 cycles in the DNA Thermal Cycler (Perkin Elmer Cetus). The parameters are as follows: time delay, 72°C for 10 min; thermal cycle (40 cycles), 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. Following 40 cycles of amplification, the PCR prod-

TABLE 1. Bacterial strains, plasmids and phages

Strain, plasmid, or phage	Relevant properties	Reference or source
Strains		
CJ236	*dut ung*	Amersham
JM103	Δ(*lac-pro*)(F'*traD proAB lacI*^{q} *lacZM15*)	16
MC1000	Δ(*lacIPOZYA*)*X74*	20
BN151	MC1000 Φ(*pabA*::*lacZ*)*17 cam*	22
BN185	MC1000 *Bgl*II::*kan* Δ(*rot*[*Hpa*I]-*fic*[*Pvu*II$_b$]) Φ(*pabA*::*lacZ*)*34*(Hyb) *cam*	22
BN193	MC1000 *fic*[*Pst*I]::*kan* ΔP$_1$ Φ(*pabA*::*lacZ*)*34*(Hyb) *cam*	22
BN198	MC1000 *Bgl*II::*kan* ΔP$_1$ Φ(*pabA*::*lacZ*)*34*(Hyb) *cam*	22
BN501	MC1000 *Bgl*II::*kan*	22
BN502	MC1000 *Bgl*II::*kan* ΔP$_1$	22
BN503	MC1000 *fic*[*Pvu*II$_b$]::*kan*	22
BN73	*trpR lacZU118 cysB*	C. Yanofsky
BN75	*trpR lacZU118 cysB rho-103*	C. Yanofsky
BN502-6	BN502 *pabA*$^+$	This work
BN502-A2	BN502 *pabA*$^+$	This work
Plasmids		
pBS(+)	Riboprobe vector	Stratagene
pT40*Bal*213	Δ(*pabA-lacZ*)	22
pSZD51	*pabA* overproduction	18
pSZD52	*pabB* overproduction	18
Phage		
mpT4	M13mp9 containing *pabA-lacZ* fusion	22
P1*kc*	Generalized transducing phage	Laboratory collection

ucts were ethanol precipitated and eluted from 1.2% low-melting-point agarose gel (16). The eluted DNA was sequenced by using the primer BPN003 and the Sequenase kit purchased from United States Biochemical Corp.

Transcription analysis. RNA was isolated from mid-log cultures grown with aeration in NZY broth by the hot phenol method as modified by Arps and Winkler (1) and was followed by DNase treatment (RQ1 DNase; Promega Biotec) to remove residual contaminating DNA. RNase protection mapping was performed as described elsewhere (2). The uniformly labeled antisense RNA probe was transcribed from linearized templates [pBS(+) derivatives] containing the 159-bp *Sau*3AI fragment spanning the *pabA-dtu* intergenic region (bp 1845 to 2004 [22]) in the presence of [α-^{32}P]CTP, using T3 RNA polymerase. Approximately 10^5 cpm of probe was hybridized to 25 μg of *E. coli* RNA or yeast tRNA (as a control) in 30 μl of hybridization buffer (80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 0.4 M NaCl, 1 mM EDTA (pH 8.0)] at 45°C for approximately 12 h. Subsequent RNase A and RNase T_1 digestion was performed at 30°C for 90 min. Digestion products were electrophoresed on 5% polyacrylamide–7 M urea sequencing gels and analyzed by autoradiography. Sequencing reactions of M13mp18 were loaded on the same gel to approximate the size of protected fragments.

Oligonucleotide-directed mutagenesis. Uracil-containing DNA template of phage mpt4 was prepared from *E. coli* CJ236 (*dut ung*) and mutagenized (2) by using synthetic primers. Mutations were verified by DNA sequence analysis. The mutated inserts were transferred onto plasmid pT40*Bal*213 by in vivo homologous recombination as follows: (i) *E. coli* JM103 was infected with phage containing the mutated insert; (ii) strains harboring the phage were then transformed with pT40*Bal*213, and Ap^r colonies were selected; (iii) the Ap^r transformants were grown up in NZY broth containing ampicillin for about 5 h, after which time they were plated onto NZY agar plates containing ampicillin and kanamycin; and (iv) plasmid DNA from the Ap^r and Kan^r transformants was prepared for DNA restriction analysis, which showed that the DNA preparation contained both recombinant and nonrecombinant plasmids and phage DNA. To separate recombinant plasmids from nonrecombinant plasmids and phage DNA, the DNA preparation was retransformed into *E. coli* MC1000 *recA*, and Ap^r Kn^r transformants were selected. Restriction analysis of plasmid DNA prepared from these transformants indicated the presence of only the recombinant plasmids.

Enzyme assays. β-Galactosidase activity was measured as described by Miller (17). Mid-log cultures (25 ml) were harvested, washed with 0.85% NaCl, and resuspended in 1 ml of Z buffer. The cell suspension was sonicated for 30 s, cooled on ice for 30 s, and sonicated for an additional 30 s. Cell debris was removed from the lysate by centrifugation. The lysate was immediately assayed for protein by the Bio-Rad method with BSA as a standard and for β-galactosidase activity with ONPG as the substrate. One unit of β-galactosidase is defined as 1 nmol of ONPG hydrolyzed per min per mg of protein. An extinction coefficient for ONPG of 4500 was used to calculate moles of ONPG (20).

RESULTS

Alterations of PABA levels, growth rate, and *pabA* and *pabB* gene dosage do not affect *pabA* expression. To determine the minimal level of exogenous PABA supplementation required to allow full growth of a PABA auxotroph, *E. coli* BN151 (*pabA-lacZ*) was streaked on minimal medium agar plates supplemented with various PABA concentrations. After 2 days of growth at 37°C, colonies supplemented with PABA at 5 ng/ml or higher grew at about the same rate. Smaller colonies formed on plates containing lower PABA concentrations, indicating that PABA concentrations of less than 5 ng/ml were limiting for growth.

TABLE 2. Effects of PABA supplementation and growth rate on β-galactosidase expression

Strain	Genotype	Carbon source or supplement	Generation time (min)	β-Galactosidase activity (U)
BN151	P_1^+ P_2^+	PABA (5 ng/ml)	ND[a]	33
BN151	P_1^+ P_2^+	None	ND	31
BN151	P_1^+ P_2^+	PABA (10 ng/ml)	ND	27
BN185	P_1^+ ΔP_2	Glucose,[b,c] CAA[d]	40	22
BN185	P_1^+ ΔP_2	Glucose[c]	60	20
BN185	P_1^+ ΔP_2	Glycerol[c]	75	22
BN185	P_1^+ ΔP_2	Succinate[c]	120	30
BN198	ΔP_1 P_2^+	Glucose, CAA[c]	40	0.4
BN198	ΔP_1 P_2^+	Glucose[c]	60	0.5
BN198	ΔP_1 P_2^+	Glycerol[c]	75	0.6
BN198	ΔP_1 P_2^+	Succinate[c]	120	0.5

[a] ND, Not determined.
[b] All carbon sources were supplied at 0.2%.
[c] Also supplemented with PABA (10 ng/ml).
[d] CAA, Casamino Acids, 0.5%.

To test the effect of PABA limitation on the expression of *pabA*, *E. coli* BN151 was grown in minimal medium supplemented with PABA (5 ng/ml) to 20 Klett units. The culture was centrifuged, and the cells were resuspended in 0.85% NaCl. A portion of the culture was saved for β-galactosidase assay, and two other portions were used to inoculate fresh medium either without PABA or with PABA at 10 ng/ml. The growth of each culture was monitored turbidimetrically. After 4 h of growth (70 Klett units), the PABA-supplemented culture was harvested for β-galactosidase assay. After 6 h of incubation, growth of the unsupplemented culture reached a plateau; after 9 h (60 Klett units), it was harvested. The data presented in Table 2 show that there were no significant differences in β-galactosidase produced by each of the three cultures, indicating that the *pabA-lacZ* fusion was not induced by PABA limitation.

To test whether *pabA* expression from either P_1 or P_2 was affected by growth in media with different carbon sources or was affected by different growth rates, *E. coli* BN185 (P_1^+ ΔP_2) and *E. coli* BN198 (ΔP_1 P_2^+) were grown in the presence of either glucose plus Casamino Acids (Difco), glucose alone, glycerol, or succinate, and β-galactosidase production was determined. Table 2 shows that β-galactosidase expression was not significantly affected, suggesting that neither growth rate control nor catabolite repression affected *pabA* expression from either P_1 or P_2.

Since *pabA* encodes one of the subunits of PABS, we tested the effects of overproduction of each subunit separately on the expression of the *pabA-lacZ* fusion. pSZD51 and pSZD52 overproduce wild-type products of *pabA* and *pabB*, respectively, but the *pab* promoters and ribosome binding sites have been replaced by sequences that function at a high level (18). pSZD51 overproduces PabA activity over 500-fold, while pSZD52 overproduces PabB activity (PABS component I) approximately 275-fold (18). β-galactosidase assay of *E. coli* BN151 harboring either pSZD51 or pSZD52 did not show any changes in the level of β-galactosidase expression (data not shown). In addition, plasmids

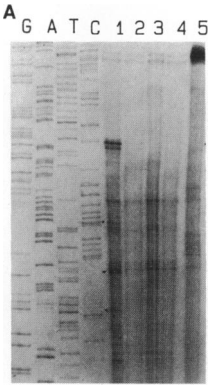

FIG. 2. Identification of 3′ termini of *pabA* transcripts. (A) RNase protection of the 193-bp probe containing the 159-bp *Sau*3AI fragment spanning the *pabA-dtu* intergenic region. Lanes: G, A, T, and C, sequencing ladder of M13mp18 used for size standards; 1, RNA from *E. coli* BN503 (P_1^+ *fic*::*kan*); 2, RNA from *E. coli* BN502 (ΔP_1 P_2^+); 3, RNA from *E. coli* BN193 (ΔP_1 *fic*::*kan pabA*::*lacZ*); 4, yeast tRNA; 5, undigested probe. Dots adjacent to lane 1 indicate protected fragments unique to *E. coli* BN503. (B) Nucleotide sequence of the *pabA-dtu* intergenic region. Lowercase letters refer to the positions of the 3′ termini of *pabA* transcripts.

containing *pabA* and *pabB* genes with wild-type expression signals did not affect β-galactosidase expression when present in BN151. These data suggest that the expression of *pabA* is not coordinated by an excess of one of the enzyme subunits and also indicate that the wild-type genes do not titrate a *trans*-acting factor involved in *pabA* expression.

Analysis of 3′ termini of *pabA* mRNA. Previous results (22) indicated that the 5′ segment of *pabA* is transcribed from two different promoters P_1 (initiating near *pabA*) and P_2 (initiating upstream of *fic*). However, a *pabA-lacZ* fusion protein was effectively expressed only from mRNA transcribed from P_1 (22; Table 2). To determine the extent of transcription from each promoter through *pabA*, we analyzed endpoints of transcripts within the *pabA-dtu* intergenic region, using RNA prepared from strains in which transcription from P_1 or P_2 was blocked. A 159-nucleotide *Sau*3AI fragment spanning most of the *pabA-dtu* intergenic region was cloned into the riboprobe plasmid pBS(+) and used to generate a ^{32}P-labeled antisense RNA probe. RNA from *E. coli* BN503 (P_1^+ *fic*::*kan*) and BN502 (ΔP_1 P_2^+) was used to analyze the P_1 and P_2 transcripts, respectively. RNA from BN193 (ΔP_1 *fic*::*kan pabA-lacZ*), containing a *pabA-lacZ* fusion which is not expressed because of disruption of transcription from both promoters, and yeast tRNA were used as negative controls. Figure 2A shows the presence of several minor protected RNA species present in the control lanes (lanes 3 and 4) that represent background in the experiment. Comparison of lanes 3 and 4 also shows that no RNA 5′ termini arose from within the *pabA-dtu* intergenic region when that region was removed from the 5′ segment of *pabA* by the insertion of *lacZ* and a chloramphenicol resistance gene (compare BN193 [lane 3] with yeast tRNA [lane 4]). This comparison suggests that under the growth conditions used, the *dtu* promoter was inactive, and no 5′ termini of RNA complicated the analysis. Full-length protection and three partially protected signals were generated by using RNA from *E. coli* BN503 (containing mRNA transcribed from P_1) (Fig. 2A, lane 1), but these signals were absent or greatly reduced when RNA from BN503 (containing mRNA transcribed from P_2) was used. The protected fragments must represent the 3′ termini of the P_1 mRNA, and the deduced endpoints are indicated in Fig. 2B. The *pabA-dtu* intergenic region does not contain any obvious secondary structures that may serve as *rho*-independent transcription terminators but is rather rich in thymidine, suggesting that transcription termination of the P_1 transcript is probably mediated by Rho factor. That no 3′ ends of the P_2 mRNA were detected in this experiment supported the earlier suggestion that transcription from the P_2 promoter does not proceed to the 3′ end of *pabA*, possibly because of polarity as the result of poor translation of *pabA*. Since *pabA* is more efficiently translated from the P_1 mRNA, the posttranscriptional events that led to the termination or degradation of the P_2 mRNA are not exerted on the P_1 mRNA.

Expression of a *pabA-lacZ* fusion in *rho*$^+$ and *rho* backgrounds was examined to determine whether polarity per se was responsible for lack of *pabA* expression when transcribed from the P_2 promoter. The *pabA-lacZ* fusion from *E. coli* BN198 (ΔP_1 *Bgl*II::*kan*) was transduced into *E. coli* BN75 (*rho-103 trpR lacZU118 cysB*) and the isogenic *rho*$^+$ strain (BN73) by generalized P1 transduction. β-Galactosidase assays of extracts derived from the two strains did not reveal a significant difference in the level of *pabA-lacZ* expression (data not shown), suggesting that *rho*-mediated polarity is not primarily responsible for the lack of expression from the P_2 promoter.

Genetic selection for *pabA* expression from mRNA derived from promoter P_2. *E. coli* strains lacking P_1 promoter activity grew very slowly in the absence of exogenous PABA supplementation. The generation time for *E. coli* BN502 (ΔP_1 P_2^+) was approximately 180 min, compared with approximately 60 min for BN501 (P_1^+ P_2^+). We took advantage of this phenotype to select mutants that had wild-type growth rates on minimal media without PABA supplementation. *E. coli* BN502 was mutagenized with ethyl methanesulfonate, and colonies with wild-type growth characteristics on minimal media without PABA supplementation were selected. Mutations resulting in increased growth were identified as *cis* to the *pabA* region by P1 cotransduction of the fast-growth phenotype with the Kn^r marker located at the *Bgl*II site upstream of *pabA*. The recipient strain was *E. coli* BN151, which contained a *pabA-lacZ* fusion and was therefore *pabA*. The *fic-pabA* intergenic region and the flanking coding regions of the *cis* mutants were amplified by PCR, and the PCR products were subjected to nucleotide sequence analysis.

Ten fast-growth mutant strains were obtained. Four of the mutations were not linked to the Kn^r marker by P1 transduction, and these mutants were not further analyzed. The remaining six mutations were cotransducible with Kn^r, indicating a *cis* configuration. Chromosomal sequence analysis of the mutants is shown in Fig. 3. Five of the mutations were identical, containing a nucleotide change of cytidine (nucleotide −4 in Fig. 3C) to thymidine (this mutation is designated BN502-A2), and one showed a nucleotide change of guanine (nucleotide −1 in Fig. 3C) to adenine (designated BN502-6).

Mutation BN502-A2 mapped outside of the sequence involved in the putative secondary structure formed by the P_2 mRNA and lay within the sequence corresponding to the position of the P_1 −10 sequence. Since the nucleotide change

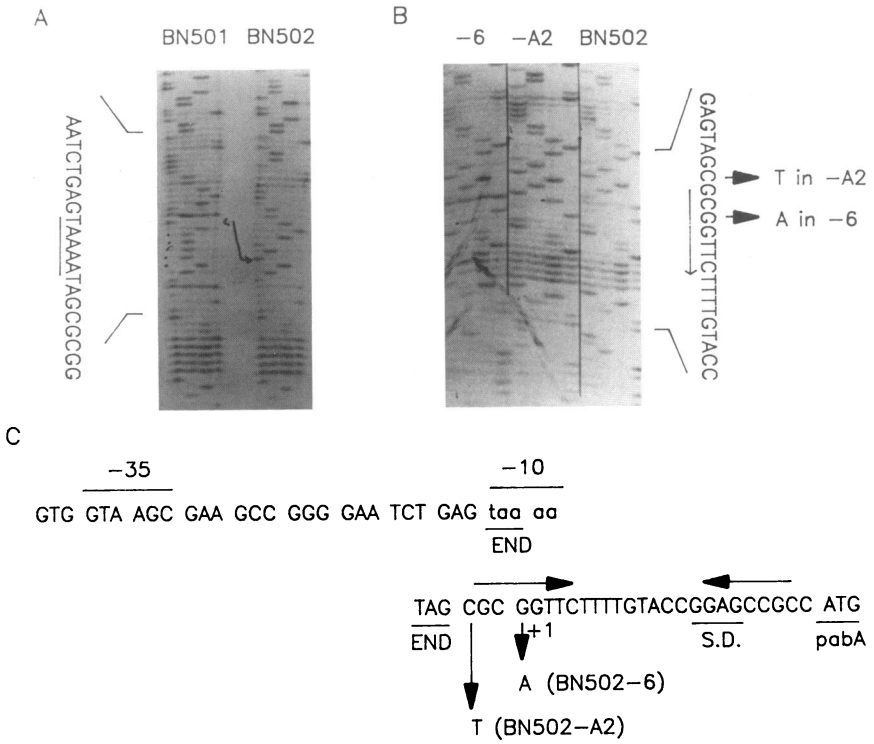

FIG. 3. Nucleotide sequences of wild-type and mutant P_1 promoters. Templates were prepared by PCR amplification of chromosomal DNA as described in Materials and Methods. (A) Comparison of the wild-type (BN501) and deleted (BN502) P_1 promoters; (B) sequences of mutations in *E. coli* BN502-6 and BN502-A2 that restore *pabA* expression to the P_1 deletion strain (BN502); (C) nucleotide sequence of the *fic-pabA* intergenic region illustrating the positions of promoter P_1 (−35, −10, and +1), the P_1 deletion (lowercase letters), mutations that restore *pabA* expression in the P_1 deletion strain (vertical arrows), secondary structure (horizontal arrows), and the *pabA* ribosome binding site (S.D.).

would bring the new −10 sequence closer to the *E. coli* consensus promoter sequence, the nucleotide change probably restored promoter P_1 activity, although this interpretation was not verified experimentally. (A mutation in the adjacent base also partially restored P_1 function; see below.) The mutation in BN502-6 mapped within the 5′ end of the putative secondary structure stem and disrupted the base pairing of the putative stem loop structure (Fig. 3C), and this mutation was chosen for further analysis.

To measure the levels of *pabA* transcription and translation resulting from the BN502-6 mutation, the mutation was reconstituted on a plasmid harboring a *pabA-lacZ* fusion, in which β-galactosidase is expressed only from P_2. M13mpt4 is an M13mp19 derivative that contains portions of the Kn^r determinant, the *fic-pabA* intergenic region, and a portion of a *pabA-lacZ* fusion gene (22). The mutations present in *E. coli* BN502-6 were recreated by oligonucleotide-directed mutagenesis of M13mpt4 (22). Mutant phage were identified by DNA sequence analysis, and the mutation was transferred to a plasmid by homologous recombination. The recipient plasmid, pT40*Bal*213, contained portions of the Kn^r determinant and *lacZ* but was deleted for the *fic-pabA* intergenic segment (22). The resulting plasmid was designated pT93. The Kn^r cassette blocking transcription from P_2 was removed by digestion with *Pst*I and religation, and the plasmid was designated pT94. As shown in Table 3, mutation BN502-6 in pT63 (ΔP_1 *fic*::*kan*) did not restore P_1 promoter activity. Removing the polar Kn^r block, thereby allowing transcription from P_2 into *pabA* (pT94), resulted in a 6.5-fold increase of β-galactosidase specific activity relative to the activity of a plasmid containing the wild-type intergenic region (pT55).

To test whether the increased *pabA-lacZ* expression was due to alteration of the DNA sequence or alteration of the putative secondary structure, a second mutation was introduced to restore the base pairing potential of the secondary structure. The compensating mutation changed a C to T at position 19 to allow formation of a stable structure (ΔG° = −6.7). The plasmid containing the Kn^r cassette blocking P_2 transcription was designated pT95, and a derivative with the Kn^r cassette removed was designated pT96. β-Galactosidase assays of plasmids carrying the double mutation indicated that the level of *pabA-lacZ* expression was reduced to about the same level as that of the plasmid containing neither

TABLE 3. Effects of putative secondary structure mutations on *pabA-lacZ* expression from P_2

Plasmid	Mutation	Genotype	β-Galactosidase activity (U)	Relative activity[a]
pT55	None	ΔP_1	4.8	1.0
pT53	None	ΔP_1 *fic*::*kan*	0.5	0.1
pT94	−1A	ΔP_1	31.1	6.5
pT93	−1A	ΔP_1 *fic*::*kan*	0.5	0.1
pT96	−1A, 19T	ΔP_1	4.8	1.0
pT95	−1A, 19T	ΔP_1 *fic*::*kan*	0.5	0.1

[a] β-Galactosidase activity relative to that of pT55.

TABLE 4. Effects of extension of *fic* translation on expression from P_2

Plasmid	Nucleotide sequence of intergenic region[a]	Genotype	β-Galactosidase activity (U)[b]	Deduced expression[c]		Expression relative to pT55	
				P_1	P_2	P_1	P_2
pT55	TCT GAG taaaa TAG CGC GGT TCT TTT GTA CCG GAG CCG CC ATG	ΔP_1	2.5				1.0
pT53		ΔP_1 *fic*::*kan*	0				0
pT84	C TAG	ΔP_1	10.9	0.9	10.0	0.4	4.0
pT83	C TAG	ΔP_1 *fic*::*kan*	0.9	0.9	0	0.4	0
pT86	C TAG	ΔP_1	39.1	6.7	32.4	2.7	13.0
pT85	C TAG	ΔP_1 *fic*::*kan*	6.7	6.7	0	2.7	0
pT88	C AG	ΔP_1	40.7	6.7	34.0	2.7	13.6
pT87	C AG	ΔP_1 *fic*::*kan*	6.7	6.7	0	2.7	0
pT92	C TA	ΔP_1	130.0	6.7	123.3	2.7	49.2
pT91	C TA	ΔP_1 *fic*::*kan*	6.7	6.7	0	2.7	0

[a] The sequence of pT55 is shown. Lowercase letters represent the P_1 deletion, and underlined nucleotides designate the putative secondary structure. Only differences from this sequence are illustrated for the remainder of plasmids.

[b] Corrected for background measured in MC1000(pT40*Bal*213) (deleted for *lacZ*).

[c] P_1 expression is derived from plasmids containing the P_1 deletion and the polar *kan* cassette in *fic* (blocking transcription from P_2). P_1 expression is assumed to be similar in plasmids lacking the *kan* cassette. P_2 expression is derived by subtracting deduced P_1 expression from plasmids lacking the polar *kan* cassette (e.g., pT84 − pT83).

mutation. These data indicated that the formation of a stable secondary structure in mRNA transcribed from P_2 was responsible for inhibiting translation of *pabA*.

Effects of ribosome translocation into the *fic-pabA* intergenic region on *pabA* expression. The previous transcriptional analysis (22) indicated that the 5′ region of *pabA* was transcribed to about the same steady-state level from P_1 and P_2. If expression from each mRNA were equivalent, then expression from mRNA originating at P_2 should be as high as that from mRNA originating from P_1. However, a *pabA-lacZ* fusion was expressed at 50-fold-higher levels from mRNA transcribed from P_1 (22; Table 2). Destabilizing the putative secondary structure increased expression by 6.5-fold (Table 3), which is still a factor of 7 to 8 less than the expression observed from mRNA transcribed from the P_1 promoter. In an attempt to achieve full expression of a *pabA-lacZ* fusion from mRNA transcribed from P_2, we designed mutations that would allow a ribosome to traverse the *fic-pabA* intercistronic region.

The *fic* stop codon (TAG) of a P_1 deletion plasmid was converted to the sense codon TAC, and a new stop codon was introduced two nucleotides upstream of the *pabA* initiation codon by using the oligonucleotide-directed mutagenesis system described above. Since one of the nucleotide changes (TAG to TAC) was located within the deleted P_1 region, we also measured the level of β-galactosidase expression in plasmids in which transcription from P_2 was blocked by a polar Kn^r cassette. The data are summarized in Table 4. In the absence of P_2 transcription, we observed a rather low level of β-galactosidase expression, 2.7-fold higher than in a plasmid deleted for P_1. The low level of expression from plasmids deleted for P_1 and in which transcription from P_2 was also blocked suggested that the nucleotide change of guanine to cytidine partially restored P_1 promoter activity (compare pT91 with pT55 in Table 4). The level of β-galactosidase expression from the P_2 mRNA harboring the nucleotide change was determined by subtracting the P_1-derived expression from the total activity. As shown in Table 4, allowing translation very close to the *pabA* initiation codon led to a 49-fold increase in β-galactosidase expression from mRNA transcribed from the P_2 promoter (compare pT92 with pT55 in Table 4). This value is about equal to the expression level of β-galactosidase expressed from the P_1 promoter (22) and probably represents maximum expression of β-galactosidase from the P_2 mRNA.

Three additional constructions were made to allow ribosomes to translate into the *fic-pabA* intergenic region, extending the *fic* reading frame one (pT83 and pT84), two (pT85 and pT86), and three (pT87 and pT88) additional codons. The nucleotide changes in each of the mutations altered the 5′ portion of the putative secondary structure and allowed ribosome movement beyond the normal *fic* termination site. As in the case of the pT91 construction, P_1 promoter activity was found to be partially restored at a low level, probably because of the nucleotide change in the P_1 promoter. The level of β-galactosidase expression from the P_2 promoter of these constructions was therefore determined as described above. Table 4 shows that allowing ribosomes to translocate for one additional codon beyond the normal *fic* termination site increased expression from P_2 4-fold (pT84), while translation two and three additional codons past the normal *fic* termination site increased the level of β-galactosidase expression by 13- to 14-fold (pT86 and pT88).

These data suggest that destabilization of the putative secondary structure resulted in a 7-fold increase in β-galactosidase expression (as represented by mutation BN502-6; see above), while a combination of putative secondary structure disruption and extension of *fic* translation resulted in up to a 13- to 14-fold increase in β-galactosidase expression. Allowing ribosomes to translate to within two nucleotides of the *pabA* initiation codon allowed for a 49-fold increase in β-galactosidase expression, suggesting that an additional mechanism, possibly translational coupling, affected the expression of β-galactosidase from mRNA originating from P_2 in this mutant.

DISCUSSION

E. coli pabA encodes PABS component II, the glutamine amidotransferase subunit of the enzyme that converts chorismate and glutamine to an intermediate in PABA biosynthesis. Previous transcriptional analysis had determined that although the 5′ portion of *pabA* was transcribed from two promoters, *pabA* was expressed only from mRNA transcribed from P_1. Analyses of the 5′ (22) and 3′ termini of *pabA* mRNA indicated that mRNA originating from P_1 was

monocistronic, initiating 22 nucleotides prior to the *pabA* initiation codon and terminating at several sites within the *pabA-dtu* intergenic region. mRNA transcribed from P_2 was polycistronic, encoding *orf1*, *fic*, and at least the first 100 nucleotides of *pabA*. However, transcription did not proceed to the *pabA-dtu* intergenic region, and PABS component II was not expressed from mRNA initiating at P_2. The lack of *pabA* expression from P_2 was not due to premature transcription termination within *pabA*, since suppression of polarity in a *rho* strain did not lead to increased expression.

Rather, the lack of *pabA* expression appeared to be the result of a stable secondary structure in the *fic-pabA* intergenic region masking the *pabA* ribosome binding sequence. Disruption of the putative secondary structure allowed a 6.5-fold increase in expression of *pabA* from mRNA arising from P_2, presumably by allowing ribosomes access to the binding sequence, while a double mutation that restored base pairing also restored the wild-type level of expression. The lack of *pabA* translation initiation in the wild-type condition probably resulted in either premature transcription termination within *pabA* or degradation of the *pabA* portion of the P_2 transcript.

Allowing ribosomes to translate various distances into the *fic-pabA* intergenic region increased *pabA* expression to various extents. Mutations that affected the stability of the putative stem-loop structure and allowed ribosomes to translate into the intergenic region increased expression of a *pabA-lacZ* fusion 4- to 14-fold. Only when ribosomes were allowed to move to within two nucleotides of the *pabA* initiation codon was high-level expression of *pabA* observed from mRNA originating at P_2. This may be the result of coupling translation between *fic* and *pabA* or may be the combined results of nucleotide changes that alter the stability of the putative secondary structure and the efficiency of the ribosome binding site. The role of the putative secondary structure in the *fic-pabA* intergenic region is likely to be twofold: to uncouple expression of *pabA* from *fic* on mRNA transcribed from promoter P_2 by allowing *rho*-dependent transcription termination or mRNA degradation initiation within *pabA*, and protection of *fic* mRNA from degradation by 3′-5′ exonucleases. In this sense, the putative secondary structure replaces a specific transcription terminator. The putative structure neutralizes translation of *pabA* and ensures the destruction of its mRNA.

The presence of two overlapping transcripts containing *pabA*, the presence of an apparently stable secondary structure in the *fic-pabA* intergenic region, and the lack of expression of *pabA* from P_2 mRNA led us to investigate the parameters that influence *pabA* expression. Since expression of a *pabA-lacZ* chromosomal fusion was not changed by PABA limitation, growth rate, catabolite repression, overexpression of PABS component I or II, or gene dosage of *pabA* and *pabB*, we conclude that *pabA* is expressed constitutively, and essentially all of the expression is from mRNA transcribed from promoter P_1.

So far, none of the folate biosynthetic enzymes have been shown to be regulated by physiological conditions (3, 26). Since folate derivatives are essential for cellular metabolism, and *E. coli* cannot utilize exogenous folates, it is possible that the folate biosynthetic enzymes have not evolved genetic regulatory responses to extra- or intracellular levels of folate derivatives. However, if PABA biosynthesis is regulated, this regulation may occur by genetic regulation of PABS component I or enzyme X, or it may occur only at the level of regulation of enzyme activity.

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