JOURNAL OF BACTERIOLOGY, July 1997, p. 4206–4212 Vol. 179, No. 13
0021-9193/97/$04.00+0

Promoters and Transcripts Associated with the *aroP* Gene of *Escherichia coli*

PEIXIANG WANG, JI YANG, AND A. J. PITTARD*

Department of Microbiology, University of Melbourne, Parkville, Victoria 3052, Australia

Received 29 January 1997/Accepted 22 April 1997

Analysis of in vitro transcriptional events initiating within the region immediately upstream of the *aroP* coding region has revealed the presence of three promoters, P1, P2, and P3. Both P1 and P2 give rise to mRNA encoding the AroP protein, whereas P3 initiates transcription in the opposite direction. Both P1 and P3 contain UP elements which contribute to promoter strength. Regulation of expression from these three promoters has been examined in vitro by using supercoiled DNA templates and in vivo by using *lacZ* transcriptional fusions and specific promoter mutants. Expression from P2 is partially repressed by TyrR alone both in vitro and in vivo. Addition of the aromatic amino acid tyrosine, phenylalanine, or tryptophan further increases this repression. P1 is not repressed by TyrR alone but is repressed in vivo in the presence of phenylalanine, tyrosine, or tryptophan. This also occurs in vitro but requires Ca^{2+} ions in the reaction mixture for its demonstration. Under these conditions, transcription from P3 is enhanced by TyrR protein with phenylalanine, tyrosine, or tryptophan. However, we were unable to demonstrate P3 expression in vivo. Under repressing conditions, there is no production of truncated RNA molecules (from P1), which would be expected if repression involved a roadblock mechanism.

In *Escherichia coli*, the gene *aroP* encodes an integral membrane protein that transports tyrosine, phenylalanine, and tryptophan into the cell (1). The *aroP* gene has been cloned and sequenced (2, 8), and primer extension analysis has identified its major promoter. The *aroP* gene is a member of the TyrR regulon (14), and its expression is repressed by the TyrR protein in conjunction with any one of the three aromatic amino acids tyrosine, phenylalanine, and tryptophan. However, because the binding site for the TyrR repressor is located downstream and outside the putative RNA polymerase binding site for the previously identified promoter, there is some uncertainty about the mechanism of TyrR-mediated repression of *aroP*. One possibility is that in this case, the binding of the TyrR repressor to the TyrR boxes interferes with RNA chain elongation rather than initiation. Such a model has been advanced to explain the repression of *purB* expression by PurR repressor (7). In the case of *aroP*, this kind of repression would result in the formation of truncated mRNA species of 30 to 50 bases. Another possibility is that the previously identified promoter is not the sole or principal promoter for the expression of *aroP* and additional promoters are located closer to the TyrR boxes. In order to investigate this situation in more detail, we have used in vitro transcription to look for short, truncated transcripts under conditions of repression and to examine the possibility of other promoters in the *aroP* regulatory region. In this paper, we describe the identification and analysis of various promoters involved in *aroP* expression and the use of site-directed mutagenesis in conjunction with *aroP-lacZ* fusions to analyze the contributions of these different promoters to *aroP* expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains used in this study were derivatives of *E. coli* K-12. The strains and plasmids used are listed in Table 1.

* Corresponding author. Mailing address: Department of Microbiology, University of Melbourne, Royal Parade, Parkville, Victoria 3052, Australia. Phone: 61 3 9344 5679. Fax: 61 3 9347 1540. E-mail: aj.pittard@microbiology.unimelb.edu.au.

Media and chemicals. The minimal medium used was prepared from half-strength buffer 56 described by Monod et al. (13) supplemented with 0.2% glucose and appropriate growth factors. To study regulation, we added tyrosine, phenylalanine, or tryptophan to the minimal medium at a final concentration of 1 mM each. Trimethoprim was used in nutrient and minimal media at final concentrations of 40 and 10 μg/ml, respectively. Ampicillin and kanamycin were used at final concentrations of 25 and 10 μg/ml, respectively. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a final concentration of 25 μg/ml. α-^{35}S-dATP (1,200 Ci/mmol; 10 mCi/ml) was obtained from Amersham Corporation. [γ-^{32}P]ATP (3,000 Ci/mmol; 10 mCi/ml) and [α-^{32}P]UTP (3,000 Ci/mmol; 10 mCi/ml) were obtained from NEN-DuPont.

Recombinant DNA techniques. Standard recombinant DNA procedures were performed essentially as described by Sambrook et al. (17). DNA sequences were determined by the chain termination method described by Sanger et al. (18) with T7 DNA polymerase (Pharmacia).

Site-directed mutagenesis. Oligonucleotides were synthesized on a Pharmacia Gene Assembler Plus apparatus. In vitro mutagenesis with synthesized oligonucleotides was performed on M13tg131 derivatives containing the *aroP* promoter-operator region from pMU1631 by using commercially available kits (Amersham Corporation and U.S. Biochemical Corporation). Mutations were confirmed by DNA sequencing analysis.

β-Galactosidase assay. β-Galactosidase activity was assayed as described by Miller (12). Specific activity is expressed in units described in reference 12. The data are the results of at least three independent assays.

In vitro transcription. In vitro transcription reactions were carried out by a method based on the standard single-round conditions described by Igarashi et al. (9, 10). A mixture of template DNA (0.15 pmol) and RNA polymerase (0.5 pmol) was incubated at 37°C in transcription buffer (45 mM Tris · acetate, 150 mM KCl, 4.5 mM magnesium acetate, 0.1 mM EDTA, and 0.1 mM dithiothreitol) for 25 min. This pretranscription mixture had a total volume of 35 μl and included, when required, TyrR protein (200 nM), ATP (0.2 mM), and tyrosine (1 mM), phenylalanine (1 mM), or tryptophan (1 mM). When added, the final concentration of $CaCl_2$ was 6 mM. Following incubation, 15 μl of start solution (consisting of 1× transcription buffer with 0.67 mg of heparin per ml; 0.53 mM [each] ATP, CTP, and GTP; 0.053 mM UTP; and 2 to 3 μCi of [α-^{32}P]UTP) was added to initiate RNA synthesis. Transcription was allowed to proceed for 5 min before the reaction was terminated by addition of 50 μl of stop solution (40 mM EDTA and 300 μg of *E. coli* tRNA per ml). Protein was removed from the reaction tubes by phenol extraction, and the synthesized RNA was precipitated with 0.5 volumes of ammonium acetate (7.5 M) and 2.5 volumes of ethanol. Samples were pelleted, resuspended in sequencing dye mix, and loaded onto a 6% sequencing gel. Following electrophoresis, the gels were exposed to Kodak XA-R film at −70°C for 12 to 24 h.

In vitro primer extension. The RNA for in vitro primer extension was prepared by using 1.5 pmol of *aroP* template DNA and 5 pmol of RNA polymerase. The resulting transcripts were isolated on a 6% polyacrylamide gel. A ^{32}P-labelled oligonucleotide probe (0.05 pmol), complementary to the bottom or top strand of *aroP* fragment, was hybridized to half of each purified RNA sample by mixing in hybridization buffer [80% formamide, 20 mM piperazine-*N*,*N*′-bis(2-

TABLE 1. *E. coli* K-12 strains, plasmids, and phages used in this work

Strain, plasmid, or phage	Relevant characteristics	Source or reference
Strains		
JP7740	Δ*lacU169 recA56*	21
JP8042	Δ*lacU169 recA56 tyrR366*	21
Plasmids		
pDD3	Ap^r; pBR322 derivative	20
pMU1065	Km^r; 1.77-kb *Eco*RV-*Pvu*I fragment containing *tyrR* gene in the *Hin*dII site of pACYC177	4
pMU1631	Ap^r; 0.3-kb *Sma*I-*Bam*HI fragment of *aroP* regulatory region in pKK232.8	3
pMU2385	*galK′-lac′Z* Tp^r IncW; low-copy-number transcriptional fusion vector	15
pMU6257	Tp^r; pMU6263 derivative; P1(−35) mutation: GTGCAT→G**CA**CAT	This work
pMU6258	Tp^r; pMU6263 derivative; P1(−10) mutation: TATTCT→**AG**TTCT	This work
pMU6260	Tp^r; pMU6263 derivative; P2(−35) mutation: TTGATC→T**CA**ATC	This work
pMU6262	Tp^r; pMU6263 derivative; P2(−10) mutation: AACAAT→AAC**CGG**	This work
pMU6263	Tp^r; 0.31-kb *Sal*I-*Hin*dIII fragment containing wild-type *aroP* regulatory region in pMU2385; *aroP-lacZ* transcriptional fusion	This work
pMU6265	Ap^r; pMU6270 derivative; P1(−35) mutation: GTGCAT→G**CA**CAT	This work
pMU6266	Ap^r; pMU6270 derivative; P1(−10) mutation: TATTCT→**AG**TTCT	This work
pMU6268	Ap^r; pMU6270 derivative; P2(−35) mutation: TTGATC→T**CA**ATC	This work
pMU6269	Ap^r; pMU6270 derivative; P2(−10) mutation: AACAAT→AAC**CGG**	This work
pMU6270	Ap^r; 0.30-kb *Bgl*II-*Bam*HI fragment containing wild-type *aroP* regulatory region in pDD3	This work
pMU6310	Tp^r; pMU6263 derivative; 37-bp deletion of P1 upstream sequence from positions −46 to −82	This work
pMU6314	Tp^r; pMU6310 derivative; P2(−35) mutation: TTGATC→T**CA**ATC	This work
pMU6320	Ap^r; pMU6270 derivative; P3(−35) mutation: AAGACT→AA**CCA**T	This work
pMU6321	Ap^r; pMU6270 derivative; P3(−10) mutation: TGGTAT→**G**GGTAT	This work
pMU6322	Ap^r; pMU6270 derivative; 37-bp deletion of P1 upstream sequence from positions −46 to −82	This work
Phages		
M13tg131	*lacPOZ′*	11
pMU6271	Wild-type *aroP* cloned in the polylinker region of M13tg131	This work

ethanesulfonic acid) (PIPES)-NaOH (pH 6.5), 0.4 M NaCl], and incubation at 85°C for 10 min. The samples were then incubated for a further 4 h at 25°C, pelleted, and resuspended in 20 μl of reaction buffer (50 mM Tris-HCl [pH 8.3], 10 mM $MgCl_2$, 10 mM dithiothreitol, 60 mM NaCl) containing deoxynucleoside triphosphates at a final concentration of 1 mM. The extension reaction was commenced with the addition of 10 U of avian myeloblastosis virus reverse transcriptase and was allowed to proceed for 1 h at 42°C prior to termination with 150 μl of stop solution (0.15 M NaCl, 5 mM EDTA). Extension products were separated on a 6% denaturing polyacrylamide gel next to the sequence tracks which were generated by using an oligonucleotide probe (A or B) as a primer and an M13tg130 derivative (or an M13tg131 derivative) which contains the *aroP* regulatory region as a template.

RESULTS

In vitro transcription of *aroP*. The sequence of the *aroP* regulatory region, including the various features that are referred to in this paper, is shown in Fig. 1. In order to produce a small supercoiled template for in vitro studies, the 299-bp *Bgl*II-*Bam*HI fragment shown in Fig. 1 was cloned into the *Bam*HI site of plasmid pDD3 to form plasmid pMU6270. The pDD3 plasmid has been designed specifically for in vitro transcription experiments and contains two T1 transcription terminators from the *E. coli rrnB* operon situated 150 bp on each side of the unique *Bam*HI site (20). The supercoiled molecules of pMU6270 were purified and used as templates in the in vitro transcription experiments. The results of one such experiment are shown in Fig. 2. Contrary to expectation, three transcripts, two strong and one weak, were found to be generated from the *aroP* regulatory region (Fig. 2). One of these, a transcript of 313 bases, corresponded to the one predicted to be formed from the previously identified promoter (designated P1) (3). The other two transcripts, whose sizes were about 290 and 250 bases, have not previously been reported and must either be generated from previously unidentified promoters, which we have designated P2 and P3, respectively, or be the breakdown products of the transcript from P1.

By adding TyrR protein alone or TyrR protein and each of the three aromatic amino acids to the reaction mixture, it was possible to measure the effects of these additions on the production of each of the three transcripts. As can be seen in Fig. 2, formation of the transcript from P2 was partially inhibited by TyrR protein alone and totally inhibited if one of the aromatic amino acids was also added to the reaction mixture. In these experiments, formation of the transcript from P1 was not affected by the presence of TyrR protein, with or without the aromatic amino acids. We also observed that the transcript from P3 was sometimes increased in the presence of TyrR and one of the aromatic amino acids (data not shown). However, the enhancement of this transcript was not reproducible from experiment to experiment. We were nevertheless able to obtain enough of this transcript and the other two from a number of gels to carry out primer extension analysis to determine the 5′ ends of these transcripts.

Primer extension of transcripts obtained in vitro. Each of the three transcripts described above was harvested from sequencing gels and hybridized with two ^{32}P-labelled oligonucleotides. Oligonucleotide A is complementary to the top strand of the *aroP* regulatory sequence, and oligonucleotide B is complementary to the bottom strand (Fig. 1). After reverse transcription, the extension products were analyzed against a sequence ladder generated from oligonucleotide A or B as a primer. This enabled the 5′ ends of the transcripts to be identified, as shown in Fig. 3. The transcripts from P1 and P2 were both extended when oligonucleotide A was used as a primer, and the transcript from P3 was extended only by oligonucleotide B. The transcription start site of P1 was found to be the same as that of the transcript previously identified by Chye and

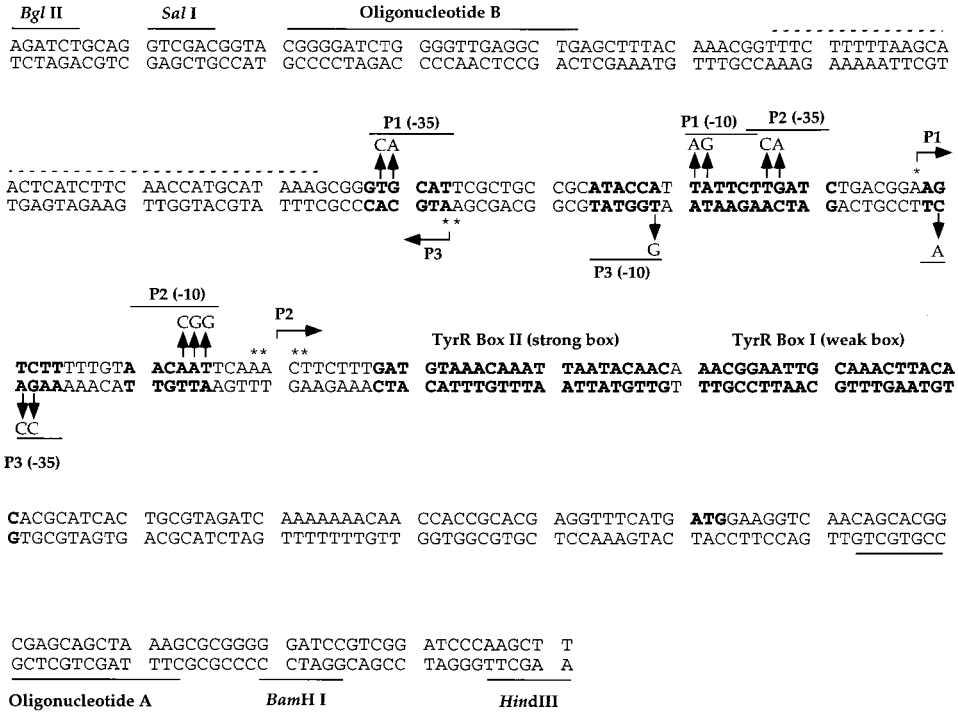

FIG. 1. Nucleotide sequence of 320-bp *Bgl*II-*Hin*dIII fragment carrying the *aroP* promoter-operator region. The two TyrR boxes (boldface), the −35 and −10 regions of the P1 and P2 promoters (overlined), the −35 and −10 regions of the P3 promoter (underlined), the transcription start points for the three promoters (asterisks), and the two oligonucleotides (A and B) used for primer extension analysis are indicated. The deleted region of the *aroP* fragments carried by plasmids pMU6310 and pMU6322 (dotted line) is also shown.

Pittard (3) (data not shown), and that of P2 was located 21 bases further downstream (Fig. 3A). On the other hand, the transcription start site of P3 is on the opposite strand from P1 and P2 and overlaps the P1 −35 sequence (Fig. 3B).

The putative −35 and −10 regions of P2 and P3 were identified by applying the rules of Hawley and McClure (5), and the proposed positions for these promoters and P1 within the upstream region of *aroP* are shown in Fig. 1. Both P1 and P3 show poor agreement with the −35 consensus hexamer, and the spacing between the putative −35 and −10 regions of P3 is not optimal. Inspection of the sequence in Fig. 1 shows that there are AT-rich sequences in the upstream regions of both P1 and P3 which may serve as UP elements for enhancement of transcription of these two promoters (16).

Isolation of mutants defective in either P1 or P2. In order to determine the relative contributions that P1 and P2 make to the transcription of the *aroP* message, site-directed mutagenesis was used to disable one or the other of these promoters. The −35 sequence of P1 was changed from GTGCAT to G**CA**CAT. The −10 sequence of P1 was changed from TAT TCT to **AG**TTCT. The −35 sequence of P2 was changed from TTGATC to T**CA**ATC, and a −10 mutant was made with the sequence AAC**CGG** instead of AACAAT. After the mutations had been verified by sequencing, these mutant templates were each cloned into pMU2385 to construct transcriptional fusions for in vivo studies and into plasmid pDD3 for in vitro studies.

In vitro studies with promoter mutants. Using different pDD3 derivatives (Table 1) as supercoiled templates, in vitro transcription experiments were carried out as described above for the wild-type template, and the results are shown in Fig. 4. Both mutant templates with alterations to the P1 promoter (pMU6265 and pMU6266) yield one major transcript (from P2), and the two templates with mutations inactivating the P2 promoter (pUM6268 and pMU6269) yield only one strong transcript from P1. The template pMU6268 also yields a stronger-than-usual transcript from P3.

In vivo studies of transcription from P1 and P2. Along with the wild-type *aroP-lacZ* fusion plasmid pMU6263, plasmids

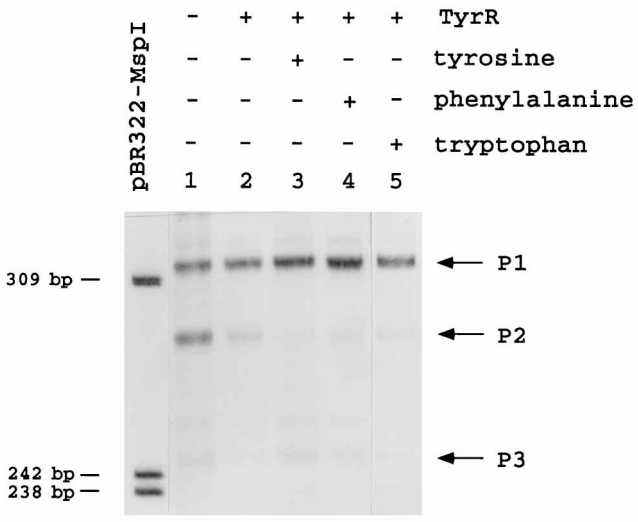

FIG. 2. In vitro transcription of pMU6270 (wild-type *aroP* template). Single-round in vitro transcription was performed as described by Igarashi et al. (9, 10). The reaction mixtures contained 0.5 pmol of RNA polymerase and 0.15 pmol of plasmid pMU6270. When added (+), the final concentration of the TyrR protein was 200 nM, and the final concentrations of tyrosine, phenylalanine, and tryptophan were 1 mM.

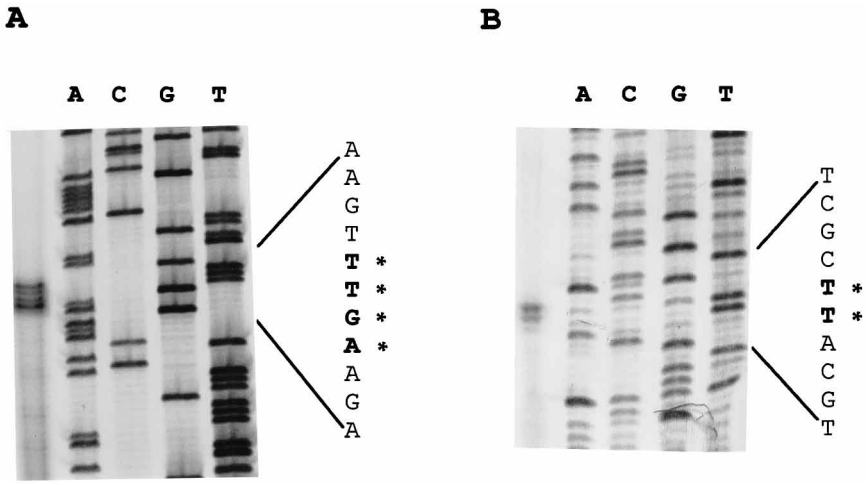

FIG. 3. Primer extension analysis to determine the transcription start sites of the RNA products generated from plasmid pMU6270 (wild-type *aroP* template). The sequence ladders are shown, and the final extension points are indicated (asterisks). (A) Extension generated from the P2 transcript when oligonucleotide A (Fig. 1) was used as a primer. (B) Extension generated from the P3 transcript when oligonucleotide B (Fig. 1) was used as a primer.

pMU6257, pMU6258, pMU6260, and pMU6262 with the mutant promoters (for a detailed description of these plasmids, see Table 1) were each transformed into a haploid *tyrR*$^+$ strain (JP7740), into a *tyrR366* strain with a TyrR null phenotype (JP8042), and into strain JP8042 containing a multicopy *tyrR*$^+$ plasmid (pMU1065). These strains were grown in minimal medium alone and in minimal medium supplemented with each of the three aromatic amino acids and assayed for β-galactosidase activity. The results are shown in Table 2.

In *tyrR366* cells, in which no regulation of *aroP* occurs, inactivation of the P2 promoter by changing the −10 sequence (pMU6262) only reduces the β-galactosidase level to about 80% of that of wild-type cells. Inactivation of the P2 promoter by changing the −35 region, however, results in an approximately 50% reduction in *aroP* expression. We hypothesize that the change to the −35 sequence of P2, because of its proximity to the −10 sequence of P1, may also be affecting P1 activity. Inactivation of promoter P1 reduces overall *aroP* expression to less than 20% of that of the wild type, confirming previous observations that in vivo, transcription from P1 is the predominant event (3). With regard to regulation, it can be seen that expression from both P1 and P2 is strongly repressed in vivo by TyrR protein with either tyrosine or phenylalanine. In the presence of tyrosine, for example, the repressibility of P1 and P2 combined (wild type) is 14-fold for haploid *tyrR*$^+$ and 33-fold for multicopy *tyrR*$^+$, and the repression of the P1 promoter (pMU6260) shows similar values of 13- and 26-fold. In the case of pMU6262, repression of P1 by either tyrosine or phenylalanine is significantly reduced (this mutant is discussed in more detail in the accompanying paper [19]).

While there is practically no repression of P1 in minimal medium, under the same conditions, repression of the P2 promoter (for both pMU6257 and pMU6258) is 5.4- to 6-fold in the haploid *tyrR*$^+$ strain and 11- to 14-fold in the multicopy *tyrR*$^+$ strain. In the presence of tyrosine or phenylalanine, the TyrR-mediated repression of P2 is increased to about 20- to 40-fold in the haploid *tyrR*$^+$ strain and about 35- to 50-fold in the strain carrying a multicopy *tyrR*$^+$ plasmid.

If the amino acid tryptophan is substituted for phenylalanine or tyrosine in the growth medium, repression of expression from both P1 and P2 can also be observed. In this case, however, the level of repression is significantly less than that observed with tyrosine or phenylalanine. The repression of P1 is about 2- to 4-fold in either the haploid or the multicopy *tyrR*$^+$ background, and the repression of P2 is about 10- to 20-fold and 20- to 30-fold, respectively, in the haploid and multicopy *tyrR*$^+$ backgrounds (Table 2).

Is there an UP sequence associated with promoter P1? Ross et al. (16) have identified an AT-rich sequence in the −40 to −60 region of the *rrnB* P1 promoter which acts as a separable promoter module to significantly stimulate transcription. Such sequences have been referred to as UP elements. It has been shown that stimulation of a promoter by an UP element is prevented by mutations in the carboxyl-terminal region of the α subunit of RNA polymerase (16).

When RNA polymerase with a truncated α subunit (α-235) (9, 10) was used in an in vitro assay (performed with the wild-type template pMU6270 in the absence of TyrR protein), transcription from P1 but not P2 was dramatically reduced

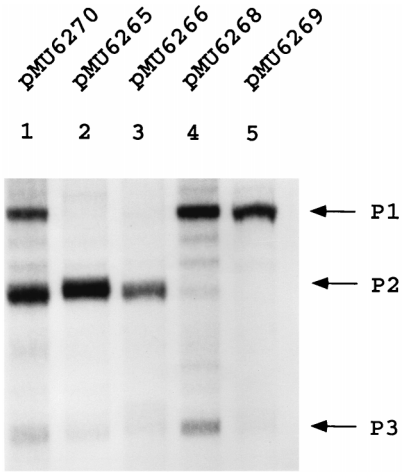

FIG. 4. In vitro transcription of various *aroP* templates by wild-type RNA polymerase. Single-round in vitro transcription was performed as described in the legend to Fig. 2. pMU6270, wild-type *aroP* template; pMU6265, *aroP* P1 −35 mutant template; pMU6266, *aroP* P1 −10 mutant template; pMU6268, *aroP* P2 −35 mutant template; and pMU6269, *aroP* P2 −10 mutant template.

TABLE 2. β-Galactosidase activities of various *aroP-lacZ* transcriptional fusions

Plasmid	Active promoter(s)	β-Galactosidase sp act (U)[a]							
		tyrR366	Haploid *tyrR*$^+$				Multicopy *tyrR*$^+$			
			MM	Tyr	Phe	Trp	MM	Tyr	Phe	Trp
pMU6263 (wild type)	P1 + P2	642	450 (1.4)	46 (14)	64 (10)	146 (4.4)	326 (1.9)	19 (33)	26 (25)	139 (4.6)
pMU6260 (P2 −35 mutant)	P1	294	184 (1.6)	24 (13)	27 (11)	72 (4.1)	231 (1.3)	11 (26)	14 (21)	65 (4.5)
pMU6262 (P2 −10 mutant)	P1	525	484 (1.0)	101 (5.4)	170 (3.2)	262 (2)	408 (1.3)	84 (6.5)	103 (5.3)	273 (1.9)
pMU6257 (P1 −35 mutant)	P2	90	16.6 (5.4)	4.6 (20)	3.5 (26)	9.4 (9.6)	8.4 (11)	2.6 (35)	1.8 (50)	4.2 (22)
pMU6258 (P1 −10 mutant)	P2	79	13 (6)	2.5 (32)	2.1 (38)	3.8 (21)	5.6 (14)	1.8 (44)	1.5 (53)	2.8 (28)

[a] Units are those defined by Miller (12). The following strains were used: *tyrR366*, JP8042; haploid *tyrR*$^+$, JP7740; and multicopy *tyrR*$^+$, JP8042/pMU1065. MM, minimal medium; Tyr, minimal medium containing 1 mM tyrosine; Phe, minimal medium containing 1 mM phenylalanine; and Trp, minimal medium containing 1 mM tryptophan. Values in parentheses are fold repression, i.e., the ratio of β-galactosidase activity in *tyrR366* strain JP8042 to that in *tyrR*$^+$ strains.

below levels observed with the wild-type enzyme (Fig. 5A, lane 2). In the same experiment, we also observed a significant reduction in transcription from P3 (Fig. 5A, lane 2). These results suggested that either or both of these promoters may have associated UP elements. An examination of the −40 to −60 regions of both promoters reveals relatively AT-rich sequences that could function as UP sequences. We have tested this in promoter P1 by deleting a 37-bp sequence from positions −46 to −82 (relative to the P1 promoter) and measuring the overall effect on promoter activity, using a *lacZ* transcriptional fusion. The results in Table 3 show that in pMU6310, the overall transcription levels (P1 and P2) were reduced to one-quarter of the wild-type levels (in the *tyrR366* background), while repression was left largely unchanged (in both haploid and multicopy *tyrR*$^+$ backgrounds). By introducing an additional mutation which disabled the P2 promoter in this construct with a deleted P1 UP element (pMU6314), we were able to show that the transcription activity of P1 was decreased by about 10-fold. These results were also confirmed in an in vitro transcription assay in which a mutant *aroP* template, with the 37-base sequence containing the putative P1 UP element (pMU6322) deleted, was used (Fig. 5B). The expression from P1 but not P2 is greatly reduced with this template.

Analysis of the P3 promoter. As described above, some in vitro transcription experiments appeared to show activation of P3 expression by TyrR protein and phenylalanine or tyrosine. However, the effects were weak and not reproducible. Furthermore, in these early experiments, we were unable to demonstrate repression of transcription from P1. In an attempt to solve these problems, we first altered our in vitro assay conditions by either utilizing linear DNA templates or including histone-like host proteins such as HU and IHF in the transcription reaction mixture, but none of these had any impact on these experiments (data not shown). This situation was changed, however, when Ca^{2+} (6 mM) was added to the reaction mixture (Ca^{2+} has been shown to facilitate in vitro binding of RNA polymerase to promoters [10a]). As can be seen in Fig. 6, under these conditions, TyrR protein and phenylalanine caused a marked activation of P3 expression and a significant repression of expression of P1. Similar results were also obtained with tyrosine and tryptophan.

In order to confirm the identity of the putative P3 promoter, we made two mutant derivatives of *aroP* with changes in either the −35 or the −10 region of P3 (Table 1). The DNA fragments containing these two P3 mutations were each cloned into plasmid pDD3. The two resulting plasmids (pMU6320 and pMU6321) were then used to carry out in vitro transcription experiments. As shown in Fig. 7, under conditions in which P3 is strongly expressed, each of these putative P3 promoter mutations severely inhibits in vitro transcription from P3, suggesting that the putative P3 promoter is the binding site for RNA polymerase.

To analyze P3 transcription in vivo, DNA fragments carrying the putative P3 promoter and the two P3 mutations described above were each introduced into the transcriptional fusion plasmid pMU2385. The resulting plasmids were then each transformed into strain JP8042 (*tyrR366*) or JP7740 (haploid *tyrR*$^+$), and the specific activity of β-galactosidase in these strains was measured under conditions shown to repress P1. The wild-type P3 produced very low levels of β-galactosidase (approximately 10 Miller units) in either the *tyrR366* or the haploid *tyrR*$^+$ background, and the rate of β-galactosidase synthesis was not affected by either of the P3 mutations (data not shown). These results suggest that P3 does not function as a productive promoter in vivo.

DISCUSSION

In this study, we have carried out in vitro transcription experiments with a supercoiled *aroP* template and analyzed the

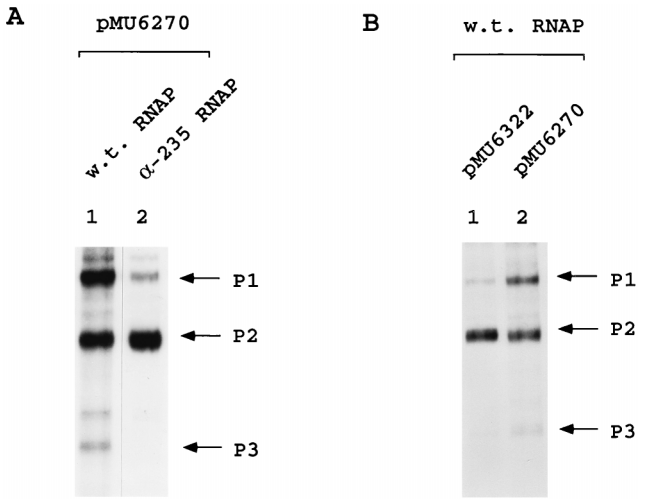

FIG. 5. (A) In vitro transcription of pMU6270 (wild-type *aroP* template) by wild-type RNA polymerase (w.t. RNAP) and mutant polymerase containing the C-terminally truncated α subunit (α-235 RNAP). Single-round in vitro transcription was performed as described by Igarashi et al. (9, 10). The reaction mixtures contained 0.5 pmol of either wild-type RNA polymerase or mutant RNA polymerase (α-235) and 0.15 pmol of plasmid pMU6270. (B) In vitro transcription of pMU6270 (wild-type *aroP* template) and pMU6322 (*aroP* P1 UP element deletion) by wild-type RNA polymerase. Single-round in vitro transcription was performed as described for panel A. The reaction mixtures contained 0.5 pmol of wild-type RNA polymerase and 0.15 pmol of either pMU6270 or pMU6322.

TABLE 3. Specific β-galactosidase activities of strains containing *aroP-lacZ* fusion plasmids with or without a P1-associated UP element

Plasmid	β-Galactosidase sp act (U)[a]						
	tyrR366	Haploid *tyrR*$^+$			Multicopy *tyrR*$^+$		
		MM	Tyr	Phe	MM	Tyr	Phe
pMU6263 (UP$^+$ P1$^+$ P2$^+$)	642	450 (1.4)	46 (14)	64 (10)	326 (1.9)	19 (33)	26 (25)
pMU6310 (UP$^-$ P1$^+$ P2$^+$)[b]	150	60 (2.5)	4.6 (20)	3.8 (22)	58 (2.6)	3.5 (47)	2.9 (49)
pMU6260 (UP$^+$ P1$^+$ P2$^-$)[c]	294	184 (1.6)	24 (13)	27 (11)	231 (1.3)	11 (26)	14 (21)
pMU6314 (UP$^-$ P1$^+$ P2$^-$)	34	27 (1.2)	4.1 (8)	3.7 (8)	25 (1.3)	2.9 (12)	2.7 (11)

[a] See Table 2, footnote *a*.
[b] UP$^-$, deletion mutation in the P1 upstream region.
[c] P2$^-$, mutation which changes TTGATC to TC**A**ATC in the −35 region of P2.

transcriptional expression initiated from the region immediately upstream of the *aroP* gene. We have shown that three transcripts can be produced from the *aroP* template. The transcriptional origin of each of these has been determined by primer extension. On the basis of these results, coupled with an analysis of the DNA sequence, three promoters (P1, P2, and P3) corresponding to these transcripts have been proposed. Promoter P1 corresponds to the previously identified *aroP* promoter (3), P2 is positioned 21 bases downstream of P1, and P3 is transcribed in the opposite direction. The identities of these promoters were confirmed by carrying out in vitro transcription experiments with *aroP* templates which contain various promoter knockout mutations.

To quantify the in vivo contributions of P1 and P2 to the expression of *aroP*, we have constructed various forms of *aroP-lacZ* fusions (e.g., P1-*lacZ* and P2-*lacZ*). In vivo results with these mutants showed that in the *tyrR366* background, approximately 80% of transcripts originate from P1 and only about 20% originate from P2. These results do not agree with the in vitro observation that promoters P1 and P2 are of about equal strength. However, we tend to favor the in vivo results, as we could detect only the transcript from the P1 promoter in a primer extension experiment using RNA isolated from cells grown under derepressing conditions (data not shown). Although promoter P1 has a relatively weak −35 sequence, an associated UP element appears to significantly increase promoter strength.

There was no difficulty in demonstrating TyrR-mediated repression of the P2 promoter in the in vitro system. However, in the case of the major promoter P1, TyrR-mediated repression could be shown only when Ca^{2+} ions were included in the transcription reaction mixture. The role played by the Ca^{2+} ions in the TyrR-mediated repression of P1 is unclear, but it is possible that Ca^{2+} can act as a cofactor for either RNA polymerase or TyrR or that it has an effect on the structure of the supercoiled DNA templates. The fact that the putative RNA polymerase binding site for the P2 promoter overlaps the TyrR boxes probably accounts for the significant repression of P2 in minimal medium, as binding of a TyrR dimer to the strong box (box II) alone could interfere with RNA polymerase binding to P2. This minimal-medium repression would also diminish the overall contribution of P2 to *aroP* transcription in *tyrR*$^+$ cells in vivo. The P3 promoter contains a weak −35 sequence and has an unusually high number of bases (19 bp) separating the −10 and −35 sequences. The data from the in vitro transcription experiment involving a mutant RNA polymerase (α-235) suggest the existence of a P3-associated UP element which makes a significant contribution to P3 expression in vitro. Although in the presence of the Ca^{2+} ion, TyrR protein, and phenylalanine, tyrosine, or tryptophan P3 can cause significant levels of divergent RNA to be synthesized in vitro, no such transcription can be detected in vivo. Nevertheless, as described in the accompanying paper (19), we see this promoter as playing a vital role in regulation of *aroP* expression. The positioning of promoters P1, P2, and P3 is such that it seems likely that RNA polymerase bound at any one of these sites would preclude a second RNA polymerase molecule binding to any one of the other sites. We are carrying out further experiments to test this hypothesis.

Work by Zalkin and colleagues has shown that the operator (PurR binding site) of the *purB* gene of *E. coli* is 242 bp downstream of the transcriptional start site and overlaps codons 62 to 67 in the structural gene (6). Further studies involving promoter-swapping experiments and Northern (RNA) blot analysis have indicated that the PurR-mediated repression of *purB* occurs by a transcriptional roadblock mechanism (7). In a Northern blot experiment, a truncated *purB* mRNA species was detected. It was hypothesized that this truncated RNA is formed when RNA polymerase meets the PurR repressor bound to the *purB* operator. Although the relative positions of the TyrR boxes of *aroP* are such that TyrR protein bound to its recognition site could theoretically interfere with elongation of the RNA chain initiated from principal promoter P1, no sign of the predicted small RNA product could be seen in any in vitro transcription experiments (data not shown). Furthermore, in the Northern blot analysis using RNA isolated from cells under repressing conditions, we failed to detect any truncated *aroP* mRNA molecules (data not shown). To investigate the mechanism of repression of the *aroP* P1 promoter, we also made a construct in which a 60-bp fragment was inserted between the

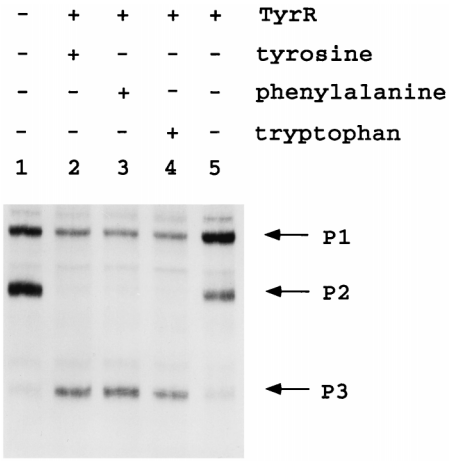

FIG. 6. In vitro transcription of pMU6270 (wild-type *aroP* template). Single-round in vitro transcription was performed as described in the legend to Fig. 2. The Ca^{2+} ion was added to a final concentration of 6 mM.

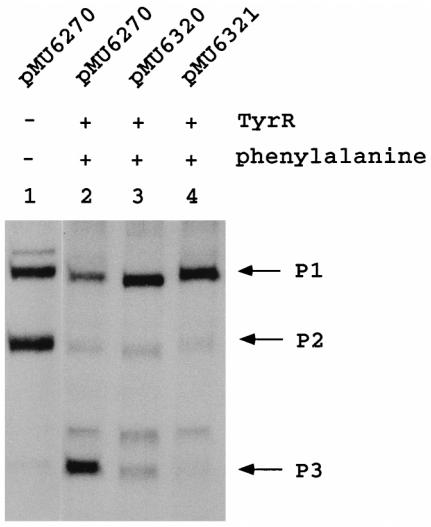

FIG. 7. In vitro transcription of pMU6270 (wild-type *aroP* template), pMU6320 (*aroP* P3 −35 mutant template), and pMU6321 (*aroP* P3 −10 mutant template). Single-round in vitro transcription was performed as described in the legend to Fig. 2. The Ca^{2+} ion was added to a final concentration of 6 mM.

transcriptional start site of P1 and the TyrR boxes (at positions +22 and +23 relative to the P1 transcriptional start site). In vivo assays showed that this insertion mutation caused complete loss of TyrR-mediated repression of P1 (data not shown). This result is consistent with the model for P1 repression proposed in the accompanying paper (19) but would not be expected if repression occurs by a roadblock mechanism.

ACKNOWLEDGMENTS

This work was funded by a grant from the Australian Research Council. P. Wang is the recipient of an Australian Agency for International Development scholarship.

We thank Wesley Hatfield for plasmid pDD3, Akira Ishihama for mutant RNA polymerase, and Barrie Davidson and colleagues for wild-type TyrR protein. We also thank Helen Camakaris for comments on the manuscript and Jing Hong An and Yan Jiang for excellent technical assistance.

REFERENCES

1. **Brown, K. D.** 1970. Formation of aromatic amino acid pools in *Escherichia coli* K-12. J. Bacteriol. **104:**177–188.
2. **Chye, M.-L., J. R. Guest, and J. Pittard.** 1986. Cloning of the *aroP* gene and identification of its product in *Escherichia coli* K-12. J. Bacteriol. **167:**749–753.
3. **Chye, M.-L., and J. Pittard.** 1987. Transcription control of the *aroP* gene in *Escherichia coli* K-12: analysis of operator mutants. J. Bacteriol. **169:**386–393.
4. **Cornish, E. C., V. P. Argyropoulos, J. Pittard, and B. E. Davidson.** 1986. Structure of the *Escherichia coli* K-12 regulatory gene *tyrR*: nucleotide sequence and sites of initiation of transcription and translation. J. Biol. Chem. **261:**403–412.
5. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
6. **He, B., J. M. Smith, and H. Zalkin.** 1992. *Escherichia coli purB* gene: cloning, nucleotide sequence, and regulation by *purR*. J. Bacteriol. **174:**130–136.
7. **He, B., and H. Zalkin.** 1992. Repression of *Escherichia coli purB* is by a transcriptional roadblock mechanism. J. Bacteriol. **174:**7121–7127.
8. **Honoré, N., and S. T. Cole.** 1990. Nucleotide sequence of the *aroP* gene encoding the general aromatic amino acid transport protein of *Escherichia coli* K-12: homology with yeast transport proteins. Nucleic Acids Res. **18:**653.
9. **Igarashi, K., A. Hanamura, K. Makino, H. Aiba, H. Aiba, T. Mizuno, A. Nakata, and A. Ishihama.** 1991. Functional map of the α subunit of *Escherichia coli* RNA polymerase: two modes of transcription activation by positive factors. Proc. Natl. Acad. Sci. USA **88:**8958–8962.
10. **Igarashi, K., and A. Ishihama.** 1991. Bipartite functional map of the *E. coli* RNA polymerase α subunit: involvement of the C-terminal region in transcription activation by cAMP-CRP. Cell **65:**1015–1022.

10a. **Ishihama, A.** Personal communication.

11. **Kieny, M. P., R. Lathe, and J. P. Lecocq.** 1983. New versatile cloning and sequencing vectors based on bacteriophage M13. Gene **26:**91–99.
12. **Miller, J. H.** 1974. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Monod, J., G. Cohen-Bazire, and M. Cohen.** 1951. Sur la biosynthese de la β-galactosidase (lactase) chez *Escherichia coli*. La specificite de l'induction. Biochim. Biophys. Acta **7:**585–599.
14. **Pittard, A. J., and B. E. Davidson.** 1991. TyrR protein of *Escherichia coli* and its role as repressor and activator. Mol. Microbiol. **5:**1585–1592.
15. **Praszkier, J., I. Wilson, and A. J. Pittard.** 1992. Mutations affecting translational coupling between the *rep* genes of an IncB miniplasmid. J. Bacteriol. **174:**2376–2383.
16. **Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse.** 1993. A third recognition element in bacterial promoters: DNA binding by the α subunit of RNA polymerase. Science **262:**1407–1413.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
19. **Wang, P., J. Yang, B. Lawley, and A. J. Pittard.** 1997. Repression of the *aroP* gene of *Escherichia coli* involves activation of a divergent promoter. J. Bacteriol. **179:**4213–4218.
20. **Wek, R. C., and G. W. Hatfield.** 1986. Examination of the internal promoter, PE, in the *ilvGMEDA* operon of *E. coli* K-12. Nucleic Acids Res. **14:**2763–2777.
21. **Yang, J., S. Ganesan, J. Sarsero, and A. J. Pittard.** 1993. A genetic analysis of various functions of the TyrR protein of *Escherichia coli*. J. Bacteriol. **175:**1767–1776.