Examination of the internal promoter, P_E , in the *ilvGMEDA* operon of *E. coli* K-12

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ABSTRACT

The *ilvGMEDA* operon of *Escherichia coli* K-12 contains an internal promoter, P_E , in the distal portion of the *ilvM* gene immediately upstream from the *ilvE* gene. The location of this promoter was determined using S1 nuclease protection analyses of *in vivo* and *in vitro* transcripts. The transcriptional activity of the internal promoter was compared to the transcriptional activity of the operon-proximal promoter P1P2 using transcriptional fusion vectors and plasmid copy number determinations. These measurements showed that the P1P2 promoter is 52-fold stronger than the internal P_E promoter. Estimates of the transcriptional role of the internal promoter on *ilvE* gene expression during growth conditions in excess and limiting branch chain amino acids is presented.

INTRODUCTION

In addition to transcriptional promoters at the beginning of operons, internal promoters have been described for several operons in *Escherichia coli* (for examples see refs. 1-5). In each case these internal promoters are located either in an intercistronic region between genes or in the extreme distal end of a gene. This is probably because such locations of internal promoters ensure efficient transcription of downstream genes by minimizing the synthesis of non-translated mRNA which reduces transcriptional efficiency by rho-dependent polarity effects (6). Two functional roles have been described for internal promoters. The internal promoter can serve to regulate operon-distal genes to effect discoordinate expression of operon encoded products (3,4). Alternatively, the internal promoter is unregulated and its constitutive activity is assumed to function to maintain a basal level of expression of operon-distal genes (1,2).

There is evidence for an internal promoter, P_E , preceding the *ilvE* gene in the *ilvGMEDA* operons of *Escherichia coli* K-12 and *Salmonella typhimurium* LT2 (7-12). This operon encodes enzymes involved in the biosynthesis of the branched chain amino acids, isoleucine, valine and leucine. Expression of the *ilvGMEDA* operon is regulated by multivalent translational control of transcription termination at an attenuator site immediately preceding the first structural gene of the operon, *ilvG* (13,14,15). Transcription of this operon is initiated *in vivo* from the downstream promoter, P2, of tandem *in vitro* promoters, P1P2 (15). Early evidence for the internal promoter, P_E , came from

the observation that transposon insertions into the ilvG gene do not exert expected polarity effects on the expression of the downstream gene, ilvE (7,8,9). However, this conclusion must be prefaced by the observation that Tn10 insertions can provide a promoter for transcription of adjacent sequences (16,17). More recently, Calhoun et al. demonstrated that a 960 bp KpnI-HindIII restriction endonuclease fragment containing the ilv'GME' region of the operon contained promoter activity by translationally fusing this DNA fragment to the lacZ gene (12).

In this report we use the S1 nuclease protection method (18) to determine the 5'-end of in vivo and in vitro transcripts initiating from the P_E promoter. In the in vitro transcription of the P_E promoter, we use a transcriptional vector, pDD3, which is constructed so as to allow the use of supercoiled plasmids as a DNA template in the transcription reaction (D. Daniels and K. Bertrand, manuscript in preparation). This enhances transcriptional levels and is particularly applicable for internal promoters which are often relatively weak. Additionally, we quantitate transcription from the P_E promoter using multicopy galK transcriptional fusion plasmids (19) and plasmid copy number determinations (20). The relative transcriptional strength of the P_E promoter is compared to transcription from the P_{1P2} promoter. The transcriptional role of the internal promoter, P_E , in the overall expression of the ilvGMEDA operon is discussed.

MATERIALS AND METHODS

Materials

All enzymes were purchased from Boehringer Mannheim or New England Biolabs. Radiochemicals were obtained from Amersham.

Plasmid Constructions

A 332 bp NarI-AluI restriction endonuclease fragment (Fig. 1A) was end-filled at the NarI 3'-recessed end using the Klenow fragment of E. coli DNA polymerase I (22), and ligated into the BamHI site of pKO9 (20), which was similarly blunt-ended. The NarI and AluI restriction endonuclease sites are GG $\downarrow$ CGCC and AG $\downarrow$ CT, respectively, and the ligation of these sites into the blunt-ended BamHI, G $\downarrow$ GATCC, generates BamHI restriction endonuclease sites at both fragment ends. A resulting plasmid, pRW37, contains the inserted fragment, flanked by BamHI sites, with the ilv'GME' region of the ilvGMEDA operon orientated in the same direction as the galK gene (Fig. 2).

The BamHI insert in pRW37, which contains the ilv'GME' region, was isolated and ligated into the unique BamHI site of the vector plasmid pDD3. The resulting plasmid pRW54 is illustrated in Figure 2, and the plasmid pRW52 has the identical BamHI insert in the opposite orientation. Plasmid pDD3 is a transcriptional vector constructed by D. Daniels and K. Bertrand (manuscript in preparation). This plasmid has a SalI to EcoRI (nucleotides 651 to 4361) pBR322 backbone which includes the ColEI origin of plasmid

replication and the selectable β -lactamase gene. A 470 bp EcoRI-HindIII restriction endonuclease fragment, which contains a unique BamHI site equidistant from the EcoRI and HindIII restriction endonuclease sites, is ligated between the EcoRI and Sall site of the pBR322. T1 transcriptional terminators from the E. coli K-12 rrnB ribosomal operon are located 150 bp on either side of the BamHI site. Therefore a promoter containing restriction endonuclease fragment inserted in either orientation in the unique BamHI site of this plasmid will produce in vitro transcripts of identical lengths.

Bacterial Strains and Growth Conditions

Strain IH-2 (proA2, his4, argE3, thi-1, lacY1, galK2, xyl-5, mlt-1, λ^- , supE44, ilvAGMEDA, rib::Tn5, KanR) was constructed in the laboratory (20). C600 galK is a derivative of C600 (F^- , thi-1, thr1, leuB6, lacY1, tonA21, supE44, λ^-). Strains were grown in minimal medium M63 (23) supplemented with 0.5% glucose, 1 mM $MgSO_4$ and 5 μ g/ml thiamine. All amino acid auxotrophies were satisfied by the addition of 50 μ g/ml of the required amino acid. Growth in minimal media of plasmid-containing strains resistant to ampicillin was in the presence of 50 μ g/ml of ampicillin. Growth of the plasmid-containing strains in LB broth (23) was in the presence of 100 μ g/ml of ampicillin.

In Vitro Transcription

Plasmids pRW52 and pRW54 were used as DNA templates in an in vitro transcription reaction essentially as described by Majors (24). Plasmid DNA (1 μ g) and RNA polymerase (0.5 units, New England Biolabs) were prebound in a 9 μ l solution of 100 mM KCl, 40 mM Tris-HOAc (pH 7.9), 4 mM $MgOAc$, 0.1 mM EDTA, 0.1 mM α -thiothreitol, 5% glycerol, 200 μ M ATP, and 20 μ M GTP including 10 μ Ci of (α - 32 P)GTP for 10 min at 37°C. At the end of this incubation, 1 μ l of a 2.0 mg/ml heparin solution was added and the reaction mixture was incubated at 37°C for an additional minute. The transcription reaction was initiated by the addition of a 1 μ l solution of 200 μ M CTP and 200 μ M UTP and incubated at 37°C for 10 min. To terminate the transcription reaction 50 μ g E. coli tRNA was added and a phenol/chloroform extraction was performed. The transcription sample was ethanol precipitated, washed with 70% ethanol, desiccated, and resuspended in a 15 μ l solution of 8 M urea, 0.1% sodium dodecyl sulfate, 0.25% xylene cyanol, and 0.25% bromphenol blue. Transcription products were analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea buffered with 50 mM Tris-borate (pH 8.3) and 1 mM EDTA. Plasmid pBR322 digested with restriction endonuclease Sau3AI was end-filled at the recessed 3'-ends with (α - 32 P)dGTP using the Klenow fragment of E. coli DNA polymerase I. The 32 P-labeled DNA fragments were used as markers on the polyacrylamide/urea gels. These markers were calibrated compared to other RNA transcripts to account for the electrophoretic differences between RNA and DNA molecules. The 255 nucleotide transcription product initiated from the P_E promoter was sliced from the polyacrylamide/urea gel and incubated in a 250 μ l solution of 10 mM Tris-HCl

(pH 7.5), 1 mM EDTA, 0.3 M NaCl and 1% phenol at 37°C with shaking at 200 rpm overnight. The acrylamide debris was removed from the eluted RNA sample by centrifugation in an Eppendorf microfuge (15,000 X g) for 5 min. This transcript was precipitated in ethanol in the presence of 25 µg *E. coli* tRNA.

S1 Nuclease Analysis

The P_E promoter in vivo and in vitro transcriptional initiation site was determined by the following modification of the Berk and Sharp S1 nuclease protection method (19). Total cellular RNA was isolated from *E. coli* K-12 strain C600 *galK* transformed with plasmid pRW37 grown in 500 ml of LB medium containing 100 µg/ml ampicillin to mid log phase (100 Klett units) as described previously (25). A 240 bp *MluI*-*Bam*HI restriction endonuclease fragment (Fig. 1A) labeled with (γ-³²P)ATP at the *Bam*HI 5'-terminus using T4 polynucleotide kinase (22) was used as the DNA hybridization fragment. This ³²P-labeled *MluI*-*Bam*HI double-stranded DNA fragment was added to 100 µg of total cellular RNA isolated from the plasmid pRW37 transformed strain in a 20 µl solution of 80% formamide, 400 mM NaCl, 40 mM pipes (pH 6.4), and 1 mM EDTA. This solution was heated at 80°C for 5 min and incubated at 49°C for 3 h. The hybridization mixture was diluted with 180 µl solution of 60 mM NaOAc (pH 6.4), 100 mM NaCl and 2 mM ZnCl₂ and incubated at room temperature for 30 min and at 4°C for 15 min in the presence of 1500 U of S1 nuclease (Boehringer Mannheim). The digested sample was precipitated with 2.5 volumes of ethanol, washed with 70% ethanol, dessicated, and resuspended in a 15 µl solution of 80% (v/v) formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (w/v) xylene cyanol and 0.1% (w/v) bromophenol blue. After heating at 90°C for 2 min, this sample was analyzed by electrophoresis on a 6% polyacrylamide/urea gel.

For the S1 nuclease analysis of in vitro transcripts, a 255 nucleotide ³²P-labeled RNA band from the plasmid pRW54 in vitro transcription reaction was eluted from the polyacrylamide/urea gel, hybridized to the ³²P-labeled *MluI*-*Bam*HI restriction endonuclease DNA fragment and treated with S1 nuclease as described. The digested sample was ethanol precipitated, resuspended in 100 µl 0.1 N NaOH, and heated to 100°C for 20 min to hydrolyze the ³²P-labeled RNA. The S1 resistant DNA was ethanol precipitated, resuspended in loading buffer, and analyzed by gel electrophoresis. The *MluI*-*Bam*HI restriction endonuclease DNA fragment labeled at the *Bam*HI 5'-terminus was chemically cleaved using the sequence method of Maxam and Gilbert (26), and the sample was used as a sequence marker alongside of the S1 resistant DNA fragments.

Galactokinase Assays and Plasmid-specific Number Determinations

E. coli K-12 strain IH-2 transformed with plasmid pKO9 or a derivative, was grown in minimal media with amino acid supplements in the presence of 50 µg/ml ampicillin to saturation overnight. Saturated cultures were diluted 1:40 in 12.5 ml of minimal media, containing amino acid supplements and ampicillin, grown to 100 Klett units, chilled on

ice and harvested by centrifugation at 3,000 X g for 10 min. The bacterial pellet was resuspended in a 1 ml solution of 20 mM Tris-HCl (pH 7.5), 8 mM MgCl₂ and 2.2 mM dithiothreitol, and disrupted by sonication by four 5 sec bursts at a microtip setting of 4 (Tekmar Sonic Disruptor Model TM250B). The extracts were transferred to 1.5 ml microfuge tubes and cell debris was removed by centrifugation for 15 min in an Eppendorf microfuge (15,000 X g). Supernatant solutions were divided into two aliquots. One aliquot was stored frozen at -20°C for plasmid-specific number determination. The other aliquot was used immediately to assay for galactokinase activity (nanomoles of galactose 1-phosphate/min/mg of protein) as described previously (20). The assay was linear within a range of 1,000 to 30,000 cpm/30 µl sample/min. Sonicated extracts were diluted up to 250-fold in order to achieve this linear range. Total protein in the sonicated extract was measured by the method of Bradford (27) using the Bio-Rad protein assay kit.

The moles of plasmid/mg of protein in the sonicated cell extract was determined by hybridization with nick-translated pKO9 plasmid as described by Adams and Hatfield (20). Samples of the sonicated protein cell extract containing 0.1, 2, 6, 10, 30 µg protein were added to 1.3 ml solution of basic 15X SSPE (100 ml of 20X SSPE, pH 7.0, and 30 ml 2 N NaOH) and incubated at 80°C for 10 min. The solution was placed in an ice water bath and neutralized by the addition of 0.4 ml acidic Tris-HCl (100 ml of 2 N 2-amino-2-hydroxymethyl-1,3-propanediol base and 21 ml of concentrated HCl). One-tenth of this sample was filtered onto nitrocellulose paper placed in a dot blot apparatus (Schleicher and Schuell) and each sample was washed three times with 0.5 ml of 10X SSPE. Cesium chloride purified plasmid samples (22) sheared by sonication were used as standards in this assay. Samples of plasmid pKO9 at a range of concentrations between 2.5 nanograms and 400 nanograms were incubated in the basic 15X SSPE at 80°C for 10 min, neutralized in the acidic Tris-HCl and one-tenth of the sample was blotted onto the nitrocellulose paper. After filtration, the nitrocellulose paper was baked under vacuum at 80°C for 2 h. The nitrocellulose paper was prehybridized, hybridized to nick-translated pKO9 plasmid and washed under stringent conditions as previously described (22). After washing, the nitrocellulose filter was dried and exposed to X-ray film to determine the quality of hybridization. The individual nitrocellulose dots for each sample were then punched out with a cork borer and quantitated using scintillation counting. The moles of plasmid/mg protein in the sonicated cell extracts were determined by a comparison to the pKO9 plasmid standard.

RESULTS

A 332 bp NarI-AluI restriction endonuclease fragment (Fig. 1A), which contains the ilv'GME' region of the ilvGMEDA operon, was examined for promoter activity using the galK transcriptional fusion system (19). This restriction fragment was ligated into the

Figure 1. (A) Restriction endonuclease map of the *ilvGMEDA* operon of *E. coli* K-12. The open lines indicate the extent of the coding region for each of the designated genes. Preceding the first gene of the operon, *ilvG*, the tandem *in vitro* promoters (P_1P_2), leader (*e*), and attenuator (*a*), are similarly designated. The restriction endonuclease map is shown on the solid line. Below the restriction endonuclease map of the operon is an enlargement of a 332 bp *NarI*-*AluI* restriction endonuclease fragment containing the *ilv'GME'* region. The internal promoter, P_E , is located within the fragment. The restriction endonuclease map and the location of the genes of this operon is based on previous data (11, 21, 25). The letter designations for restriction endonucleases are as follows: A, *AluI*; B, *BglII*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; M, *MluI*; N, *NarI*; P, *PvuII*; S, *SalI*; X, *XhoI*. (B) The nucleotide sequence of the internal promoter, P_E . The -10 and -35 hexamer regions of the P_E promoter, and the *SalI* restriction endonuclease site which flanks the -10 region are designated. The nucleotide positions are relative to the *ilvGMEDA in vivo* transcriptional initiation site (11, 15).

unique *Bam*HI site of plasmid pKO9 in such a way as to generate *Bam*HI restriction sites at both ends of the fragment (see methods and materials). A resulting plasmid, pRW37 (Fig. 2), contains the *ilv'GME'* region in the same orientation as the *galK* gene. *E. coli* K-12 strains C600 *galK* and IH-2 were transformed with plasmid pRW37 and plated onto MacConkey agar plates containing 10 g/l galactose and 100 μ g/ml ampicillin. Colonies of both transformed strains were red in color, indicating promoter activity.

To quantitate the strength of this internal promoter and compare its strength to the *ilvGMEDA* P_1P_2 promoter, the galactokinase-specific activity was determined for the plasmid pKO9 derivatives containing these promoters. Previously, it was demonstrated *in vivo* that plasmid copy number fluctuations are inversely correlated to promoter strength in these *galK* transcriptional fusion vectors (20). Therefore, in order to correct for these fluctuations we measured, *in vivo*, the moles of galactokinase product

Table 1. Promoter activity of P₁P₂ and P_E promoters of the *ilvGMEDA* operon of *E. coli* K-12.

^a This number is derived from the plasmid-specific number and is based on the observations that sonication of 5×10^9 cells produces 1 mg of cellular protein, that the amount of protein/cell is proportional to the genome number/cell, and that cells grown in minimal medium contain 1.5 genomes/cell (28, 20).

Figure 3. *In vitro* transcription products of the plasmids pRW54 and pRW52. The 255 nucleotide transcript is initiated from the P_E promoter and terminates at the ribosomal operon *rrnB* T1 terminator. The 108 nucleotide transcript is derived from the *ColE1* origin of replication. Lanes designated M are 32 P-labeled pBR322 *Sau*3AI restriction endonuclease markers of the following size in base pairs: 1374, 655, 359, 341, 317, 272, 258, 207 and 105. The DNA markers were compared to other RNA transcripts to calibrate for electrophoretic differences between RNA and DNA molecules.

To determine the location of this promoter in vitro, plasmid pRW54 was constructed (Fig. 2). This plasmid contains the BamHI insert from plasmid pRW37 ligated into the unique BamHI restriction endonuclease site of the plasmid pDD3. In plasmid pDD3 a T1 transcriptional terminator of the rrnB ribosomal operon of E. coli K-12 has been placed 150 bp on either side of a unique BamHI restriction endonuclease site. Therefore, when plasmid pRW54 is used as a DNA template in an in vitro transcription reaction, RNA polymerase will initiate transcription at the internal promoter located in the BamHI insert and terminate transcription at a rrnB terminator. Plasmid pRW52 contains the identical BamHI fragment insert ligated into plasmid pDD3 in the opposite orientation. RNA polymerase was prebound to plasmid templates pRW54 or pRW52 in the presence of 20 μ M GTP including 10 μ Ci of (α - 32 P)GTP and 200 μ M ATP, incubated with heparin, and the transcription reaction was initiated with the addition 200 μ M CTP and UTP. The in vitro transcription samples were analyzed by electrophoresis on a 6% polyacrylamide/urea gel (Fig. 3). In this assay, both plasmids pRW52 and pRW54, produce transcripts of identical size, 255 nucleotides. This result shows that transcription from the internal promoter originates approximately 105 nucleotides upstream from the AluI site (Fig. 1A) and demonstrates a single promoter site in the ilv'GME' region of the ilvGMEDA operon.

To determine more precisely the location of the internal promoter, the S1 nuclease protection method was used to analyze the 5'-end of in vitro and in vivo transcripts initiated from the P_E promoter. The 32 P-labeled RNA band of approximately 255 nucleotides from the plasmid pRW54 in vitro transcription reaction was sliced from the polyacrylamide/urea gel. The 32 P-labeled RNA was eluted from the gel slice and precipitated with ethanol in the presence of 25 μ g E. coli tRNA. For the determination of the internal promoter in vivo initiation site, total cellular RNA was isolated from E. coli K-12 strain C600 galK transformed with the plasmid pRW37, which was grown to mid-log phase in LB medium. The purified in vitro transcription product from the plasmid pRW54 or 100 μ g of total cellular RNA purified from the pRW37 transformed C600 strain was hybridized to a 240 bp MluI-BamHI restriction endonuclease fragment (Fig. 1A), which was labeled at the 5'-terminus with (γ - 32 P)ATP at the BamHI site. The BamHI site which was labeled is linked to the blunt-ended AluI site in the original plasmid pRW37 construction. The DNA:RNA hybrids were treated with S1 nuclease and the S1 nuclease resistant DNA was analyzed by gel electrophoresis (Fig. 4). Alongside of the S1 nuclease treated lanes are the corresponding Maxam and Gilbert sequence reactions of the same 32 P-labeled MluI-BamHI restriction endonuclease fragment. The DNA fragments resistant to S1 nuclease, indicated by arrows in Figure 4, are the C and T residues which correspond to G or A residues, respectively, in the noncoding strand. These are the probable nucleotides at which the internal promoter, P_E , initiates transcription. The -35

Figure 4. Determination of the P_E promoter *in vivo* and *in vitro* transcriptional initiation site by S1 nuclease protection analysis. A double-stranded MluI-BamHI DNA fragment (Fig.1A) was 5'-end labeled at the BamHI site. This BamHI restriction endonuclease site is linked to the AluI site in the plasmid pRW37 (see materials and methods). The labeled transcribed strand was hybridized to total cellular RNA isolated from an *E. coli* strain transformed with plasmid pRW37 or to a 255 nucleotide transcript purified from the *in vitro* transcription products of plasmid pRW54. After S1 nuclease treatment the protected DNA was analyzed by electrophoresis on a 6% polyacrylamide/urea gel in parallel with the Maxam and Gilbert sequence reactions of the same ^{32}P -labeled

MluI-BamHI restriction fragment. A single DNA fragment spanning several nucleotides was resistant to the S1 nuclease treatment. The arrows indicate C and T residues which correspond to G and A residues, respectively, in the nontranscribed strand. Either the G or A residue is the probable site of the P_E promoter transcription initiation. No S1 nuclease protected DNA fragments were observed when the labeled double-stranded MluI-BamHI fragment was incubated in the presence of 100 μ g of E. coli tRNA (data not shown).

and -10 hexamer regions of this promoter are illustrated in Figure 1B. E. coli promoter DNA sequence homologies show that the spacing between the -10 hexamer and the site of transcription initiation is usually 6 or 7 base pairs, with a range between 4 and 8 base pairs (29). Based on this statistical preference and the consensus promoter sequence of Hawley and McClure (29) we suggest that the transcription initiation site of the internal promoter, P_E , of the ilvGMEDA operon is the A residue, which is 7 nucleotides from the -10 hexamer region (Fig. 1B).

DISCUSSION

An internal promoter in the ilvGMEDA operon of E. coli K-12 was analyzed by multicopy galK transcriptional fusion plasmids and S1 nuclease protection analyses of in vitro and in vivo transcripts. This internal promoter, designated P_E , is estimated to initiate transcription at position 2101 in the distal portion of the ilvM gene of this operon (11,15). Transcription from the P_E promoter was compared to the P1P2 promoter at the beginning of the ilvGMEDA operon. The E. coli K-12 strain IH-2 transformed with plasmid pCAP1P2, a pKO9 derivative containing the P1P2 promoter, was determined to produce 52-fold more galactokinase activity per mole of plasmid than did the IH-2 strain transformed with plasmid pRW37, a pKO9 derivative containing the P_E promoter.

It is important to understand the structure of the ilvGMEDA operon in E. coli K-12, in order to perceive what transcriptional role the internal promoter, P_E , might have. The ilvGMEDA operon encodes enzymes involved in the biosynthesis of the branched chain amino acids, isoleucine, valine, and leucine (review 30). Transcription proceeding into this operon is regulated by an attenuation mechanism, which is subject to control by isoleucine, valine and leucine limitation. The first two genes of the operon, ilvG and ilvM, encode the large and small subunits, respectively, of the acetohydroxy acid synthase (AHAS) II isozyme (31,32). AHAS II is one of three isozymes which catalyze the first step of the parallel pathway for the biosynthesis of isoleucine and valine. In previous work analyzing the role of the ilvGMEDA attenuator, Adams et al. quantitated the in vivo transcription through the attenuator site preceding the ilvG gene during limiting and excess amino acid growth conditions (15). During growth in excess isoleucine, valine and leucine, 9% of the transcripts initiating from the P1P2 promoter readthrough the attenuator while in growth conditions limiting in isoleucine 28% of transcripts read-

Table 2. Percent total transcription of *ilvE* gene from P_E internal promoter.

Growth conditions	Relevant phenotype ^a	Percent of transcription from P1P2 through attenuator ^b	Percent total transcription of <i>ilvE</i> from P_E promoter ^c
Excess Ile, Val, Leu	<i>IlvG</i> ⁺	9%	18%
	<i>IlvG</i> ⁻	9%	41%
Limiting Ile	<i>IlvG</i> ⁺	28%	6%
	<i>IlvG</i> ⁻	28%	18%
Limiting Val	<i>IlvG</i> ⁺	23%	8%
	<i>IlvG</i> ⁻	23%	21%
Limiting Leu	<i>IlvG</i> ⁺	29%	6%
	<i>IlvG</i> ⁻	29%	17%

^a In wild type *E. coli* K-12 *ilvG* contains a frameshift mutation which renders the AHAS II isozyme nonfunctional (11).

^b Data from Adams et al. (15).

^c Calculation assumes the P_E promoter is in no way occluded by transcription from P1P2 promoter. Wild type *E. coli* K-12 contains a frameshift mutation in the *ilvG* gene (phenotype *IlvG*⁻), and a rho-dependent polarity factor of 3.2 was used in this calculation (34). The internal promoter is assumed to be constitutive with respect to limiting Ile, Val, or Leu as discussed in the text.

through the attenuator (Table 2). In cells grown in media limiting in valine or leucine, 23% or 29% of the transcripts initiating from the P1P2 promoter readthrough the attenuator, respectively. Although the role of the internal promoter, P_E , is not yet understood, there is evidence to suggest that this promoter is not subject to amino acid mediated regulation. Polar transposon insertions into the *ilvG* gene do not eliminate expression of the downstream *ilvEDA* genes due to expression from the internal promoter, P_E , and in these mutants the expression of the *ilvEDA* genes is not affected by branched chain amino acid limitations (8). In other examples of internal promoters in amino acid biosynthetic operons it appears that these promoters are also unaffected by operon specific amino acid limitation (1,2). For example, in the case of the internal promoter P2 in the distal portion of the *trpD* gene of the tryptophan operon (1), and the internal promoter P2 in the distal portion of the *hisC* gene of the histidine operon (2), there is evidence that these promoters are constitutive with respect to the availability of tryptophan or histidine, respectively. It is possible, however, that these internal promoters are subject to a more general form of regulation, such as growth rate dependent metabolic regulation.

The *in vivo* promoter analyses using the pKO9 derived plasmids indicate that transcription from the P1P2 promoter is 52-fold greater than that of the internal promoter, P_E . However, as previously noted, only 9% of the transcripts initiating from the P1P2 promoter readthrough the attenuator in cells grown in excess branched chain amino

acids. When this is the case, 18% of the total transcription into the *ilvE* gene is initiated from the P_E promoter (Table 2). This calculation neglects rho-dependent polarity effects on transcription in the *ilvG* and *ilvM* genes. Additionally, it assumes that initiation of transcription from the internal promoter, P_E , is in no way occluded by transcription from the proximal-end promoter, P1P2. In *E. coli* K-12 the *ilvG* gene contains a frameshift mutation which renders the AHAS II isozyme nonfunctional (11); therefore, wild type *E. coli* K-12 has an $IlvG^-$ phenotype. This frameshift mutation results in rho-dependent polarity effects upon the downstream genes of the operon (33,34). This polarity is illustrated by the effect a rho mutant has on the expression of the *ilvE* gene product, transaminase B. In *E. coli* K-12, grown in minimal media, the *rho221* mutant results in a 3.2-fold elevation in the expression of transaminase B activity (34). Therefore, using this estimate for the rho-dependent polarity effect upon transcripts initiated from the *E. coli* K-12 P1P2 promoter, 41% of the total transcription into the *ilvE* gene is initiated from the P_E promoter in cells grown in excess branched chain amino acids (Table 2).

During growth conditions limiting in branched chain amino acids, there is about a 3-fold elevation of transcription through the attenuator site (15). Under these conditions the percentage of total transcription into the *ilvE* gene initiating from the P_E promoter is decreased. If we assume as previously discussed that transcription from the P_E promoter is unaltered by branch chain amino acid availability, we can use the *galK* transcriptional fusion determination of P_E promoter strength to estimate the percentage of the total transcription into the *ilvE* gene which is initiated from the P_E promoter in growth conditions limiting for isoleucine, valine or leucine. In cells grown in limiting isoleucine, 28% of transcription initiating from the P1P2 promoters reads through the *ilvGMEDA* attenuator. This results in 6% of the total transcription into the *ilvE* gene initiating from the P_E promoter (Table 2). In *E. coli* K-12, which contains a frameshift mutation in the *ilvG* gene, 18% of the total transcription into the *ilvE* gene initiates from the internal promoter, P_E , in growth conditions limiting for isoleucine (Table 2).

Our estimates of the percentage of the total transcription into the *ilvE* gene initiating from the P_E promoter in cells grown in media containing excess branched chain amino acids can be compared to a previous analysis by Calhoun et al. (12). This analysis used *ilvE::lacZ* translational fusions to estimate the role of the internal promoter, P_E . A 4.8 kb *Hind*III restriction fragment (*ilvGME'* region, Fig. 1A), containing the P1P2 promoter and the P_E promoter, was translationally fused to the *lacZ* gene. A similar translational fusion was constructed using a 960 bp *Kpn*I-*Hind*III restriction fragment (*ilv'GME'* region, Fig. 1A) containing only the P_E promoter. In cells grown in minimal media nonlimiting for branched chain amino acids, β -galactosidase expression from the plasmid containing only the P_E promoter was 67% of the levels expressed from the fusion plasmid containing both the P1P2 and P_E promoters. A *lacZ* translational fusion also was

constructed using a 4.8 kb HindIII restriction fragment from an E. coli K-12 strain containing a functional ilvG gene. The KpnI-HindIII construct containing only the P_E promoter expressed 15% of the β -galactosidase levels determined for the plasmid containing both the P_{1P2} and P_E promoters from the E. coli K-12 strain containing a functional ilvG gene. These values approximate our estimates of the total transcription into the ilvE gene initiated from the P_E promoter (Table 2).

The internal promoter, P_E , is conserved in Salmonella typhimurium, as well as Escherichia coli (7,8,9, J. Lopes and R. Lawther, in press). It is possible that this promoter sequence is fortuitous and is conserved because it is incorporated in the coding region of the ilvM gene. However, due to silent nucleotide changes inherent in the codon dictionary, such conservation of the P_E promoter sequence would appear unlikely. Additionally, the Sall restriction endonuclease site, which flanks the -10 hexamer region of the P_E promoter (Fig. 1), is found in several other members of the Enterobacteriaceae family (35). It is improbable that the Sall restriction endonuclease site is maintained as a result of amino acid coding conservation since the first three nucleotides of this site, GTC, encode a valine residue, which is variable in its third position. Thus, the conservation of this Sall restriction endonuclease in many other enteric bacteria suggests that the internal promoter, P_E , is conserved, and therefore, that this promoter serves a physiological role in branched chain amino acid metabolism.

In conclusion, the internal promoter, P_E , appears to be a conserved element of the ilvGMEDA operon of E. coli K-12. The percentage of the total transcription into the ilvE gene initiated from the P_E promoter varies within a specific range depending on the cell growth conditions (Table 2). It appears that the contribution of the P_E promoter, for which there is evidence for constitutive expression with respect to branched chain amino acid availability (8), is to maintain a basal level of operon-distal gene expression of the ilvGMEDA operon.

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