Attenuation of the ilvB operon by amino acids reflecting substrates or products of the ilvB gene product

(Escherichia coli K-12/in vivo attenuation/message degradation/acetoxyhydroxy acid synthase I)

CRAIG A. HAUSER AND G. WESLEY HATFIELD

Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, CA 92717

Communicated by Charles Yanofsky, September 12, 1983

ABSTRACT Transmission termination at the ilvB attenuator of Escherichia coli K-12 has been quantitated by measuring the in vivo rate of synthesis and degradation of mRNA segments proximal and distal to the attenuator. This analysis demonstrates that a 4-fold deattenuation results in vivo when the growth of cells is limited by the availability of valine or leucine. The result suggests that attenuation is the major mechanism by which this operon is regulated by these endproduct amino acids. On the basis of possible secondary structures of ilvB leader RNA, we predicted that attenuation of this operon should also be affected by growth of cells in limiting amounts of alanine or threonine. We report here that the ilvB operon is deattenuated when cells are starved for either of these amino acids. A rationale for the regulation of this operon by these four amino acids, which represent both the substrates and the products of the ilvB gene product, and by catabolite repression is presented.

The ilvB operon of Escherichia coli K-12 encodes the structural gene for one of the three acetoxyhydroxy acid synthase isoenzymes that catalyze the first common step in the biosynthesis of isoleucine, valine, and leucine (1). Expression of the ilvB gene increases about 5-10-fold in cells growing in media containing limiting concentrations of valine or leucine (2-4). This biosynthetic operon is also regulated by catabolite repression (5).

DNA sequence analysis of the ilvB promoter-regulatory region and in vitro transcription studies have suggested that the amino acid-mediated regulation of this operon is effected by an attenuator mechanism (4, 6). In vitro transcription of the ilvB regulatory region produces a leader RNA that encodes a 32-amino-acid leader polypeptide containing tandem valine and leucine codons. This leader RNA is capable of forming mutually exclusive secondary structures similar to those described for other amino acid biosynthetic operons regulated by attenuation (4, 6, 7).

The position of tandem codons for alanine and threonine in the polypeptide coding region of the leader RNA led us to propose that the ilvB operon should also deattenuate in response to growth-limiting concentrations of either of these amino acids (4). In this report we measure in vivo transcription through the attenuator region of the ilvB operon. This analysis confirms that the ilvB operon is regulated in vivo by attenuation and that deattenuation results when the growth of cells is limited by the availability of valine, leucine, alanine, or threonine. It is interesting that the expression of the ilvB gene product, acetoxyhydroxy acid synthase I, is regulated by alanine and threonine because both of these amino acids are only one metabolic step removed from the substrates (pyruvate and α-ketobutyrate) of acetoxyhydroxy acid synthase I. A rationale for the physiological basis of the regular-

MATERIALS AND METHODS

Materials. [5,6-3H]Uridine (40-50 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. Restriction endonucleases and other enzymes were from Bethesda Research Laboratories or New England BioLabs or were prepared in this laboratory. Aurintricarboxylic acid (practical grade) was from Eastman Kodak.

Bacterial Strains and Plasmids. The relevant genotypes of the E. coli K-12 strains used are: strain T31-4-505, Δilv-GEADYC, trpE9829, trpF89, trpA89, 9761 (8); strain AB4132, ala54, metC56 (9); and strain JA200, thr-1, leuB6, ΔtrpE7 (10). Plasmid pCH5 was constructed by cloning a 1.4-kilobase-pair (kb) HindIII restriction fragment of E. coli DNA containing the entire promoter-regulatory region and the proximal portion of the presumed coding region of the ilvB gene into the HindIII site of plasmid pBR322 (4). Plasmid pCH5 contains the identical ilvB regulatory region to pCH2, which was shown to be regulated in the same manner as the chromosomal ilvB gene (4).

Bacterial Growth and Starvation Conditions. Cells containing plasmid pCH5 to increase the amount of ilvB message synthesis were grown in glucose M63 minimal salts media (11) as described (4). Auxotrophic strains were supplemented with 50 μg of the appropriate amino acids per ml. Auxotrophs were starved for each amino acid by filtering 30 ml of a mid-logarithmic culture (0.5 OD₅₅₀) through 0.45-μm nitrocellulose filters and washing with 20 ml of a prewarmed M63 minimal salts solution. The washed cells were resuspended in 8 ml of prewarmed media containing all required amino acids (unstarved) or lacking one amino acid (starved) as indicated in Results. The resuspended cells were incubated at 37°C for 10 min and then pulse labeled with [3H]Uridine. Alanine starvation was accomplished in a media containing 0.75 M NaCl/0.075 M sodium citrate/0.2% NaDodSO₄/100 mM potassium phosphate, pH 7.0. A similar protocol was followed for the cells grown in the presence of 6 mM nitrocellulose filters, including 14 hr at 65°C in the presence of 3 mM aurintricarboxylic acid, a ribonuclease inhibitor (13).

RNA Hybridization. Amino acid-starved or unstarved cells containing the plasmid pCH5 were pulse labeled for 1.5 min with 40 μCi of [3H]Uridine per ml. Incorporation of [3H]Uridine was terminated by pouring the cells into 20 ml of −70°C ethanol. RNA was extracted by the method of Gegenheimer and Apirion (12), in which the cells are lysed in the presence of 3 mM aurintricarboxylic acid, a ribonuclease inhibitor (13).

Quantitative RNA hybridization was accomplished by incubating the labeled RNA from 5 ml of a midlogarithmic culture with 6 μg of an appropriate M13 DNA probe fixed to a 6-mm nitrocellulose filter, for 14 hr at 65°C in the presence of 0.75 M NaCl/0.075 M sodium citrate/0.2% NaDodSO₄/100 mM potassium phosphate, pH 7.0. The filter was washed and exposed to film for autoradiography.

Abbreviations: kb, kilobase pair(s); bp, base pair(s).
\[ \text{Rate of in Vivo RNA Degradation.} \] The half-life of the preattenuator and postattenuator RNA segments detectable by hybridization, incorporation of \([\text{H}]\)uridine was stopped at the end of the 1.5-min pulse by the addition of 0.4 mg of rifampicin and 2 mg of unlabeled uridine per ml. Aliquots were taken every 30 sec for 2 min and the amount of radiolabeled RNA that specifically hybridized to the M13pre or M13post probes was measured by liquid scintillation spectroscopy.

**RESULTS**

**Construction of Message Hybridization Probes.** DNA probes to hybridize \(ilv\)-specific mRNA transcribed from DNA sequences before or after the attenuator of the \(ilv\) operon were constructed. The preattenuator probe was constructed by inserting the Sau3A–HinfI restriction endonuclease fragment that contains DNA sequences before the attenuator site (Fig. 1) into the blunt-end SaMa1 site of phage M13mp8 DNA (14). This was accomplished by creating blunt ends on the Sau3A–HinfI fragment by filling in these sites with the Klenow fragment of DNA polymerase I. The DNA insert of this recombinant phage, M13pre, is shown schematically in Fig. 1. The postattenuator probe was constructed by isolating a 300-base-pair (bp) TaqI restriction endonuclease fragment (Fig. 1) that contains \(ilv\) DNA beginning 75 bp downstream of the \(ilv\) attenuator site. As before, the TaqI sites of this restriction endonuclease fragment were filled in prior to ligation of the fragment into the SaMa1 site of the replicative form of phage M13mp11 DNA (14). The DNA insert of this recombinant phage, M13post, is shown schematically in Fig. 1. The identity and orientation of the \(ilv\) DNA inserted in each of these recombinant phages was confirmed by DNA sequence analysis (data not shown). Single-stranded DNA isolated from phage in the culture media of cells infected with either of these recombinant phage or a control phage, M13mp8, was fixed to nitrocellulose and used for message hybridizations as described in Materials and Methods.

**Determining the Rate of Degradation of \(ilv\) RNA.** To accurately quantify in vivo attenuation of the \(ilv\) operon it is necessary to measure the amount of transcription termination at the attenuator under various cell growth conditions that affect this regulation. In vivo transcription termination can be measured by determining the amount of \(ilv\) RNA synthesized before and after the attenuator site. However, because degradation of newly synthesized radiolabeled RNA occurs throughout the pulse-labeling period, the apparent amount of transcription through the attenuator will be altered if the segments of pre- and postattenuator message measured by hybridization have different rates of degradation. The in vivo rate of degradation of these \(ilv\) RNA segments was measured and the results in Fig. 2 show that preattenuator message is degraded more rapidly than postattenuator message and that, under certain growth conditions (Fig. 2E), this difference can be as great as 4-fold. It is surprising that in most cases decreases in growth temperature resulted in increased rates of RNA degradation (Fig. 2).

**Calculation of the Rate of Synthesis of \(ilv\) RNA.** Because the rates of degradation of preattenuator and postattenuator \(ilv\) RNA are rapid, the amount of RNA measured by hybridization is an underestimate of the actual amount of this RNA synthesized during the 90-sec pulse-labeling period. The shorter the half-life of the RNA, relative to the labeling period, the greater this disparity becomes. However, it is possible to calculate the ratio of RNA synthesized to RNA measured for any labeling period (T) if the half-life (\(t_\text{1/2}\)) of the hybridizable RNA segment is known. This ratio can be calculated by using the following equation, which relates linear synthesis to simultaneous logarithmic degradation (15):

\[
\frac{\text{RNA synthesized}}{\text{RNA measured}} = \frac{\ln 2}{1 - e^{-\ln 2/T/t_\text{1/2}}}.
\]

**Quantitation of in Vivo Attenuation.** The amount of radiolabeled \(ilv\)-specific RNA hybridized to nitrocellulose filters containing preattenuator DNA (M13pre) or postattenuator DNA (M13post) was determined by measuring the cpm of \([\text{H}]\)RNA hybridized to these filters and subtracting the cpm of \([\text{H}]\)RNA hybridized to filters containing control DNA (M13mp8). The nonspecific radioactive background in each of these experiments varied between 75 and 150 cpm. The radioactivity (cpm) of \(ilv\)-specific RNA hybridized from cultures grown under various conditions is shown in Table 1. The \(ilv\) DNA sequence in the postattenuator probe hybridizes to a 300-nucleotide region of \(ilv\) RNA that contains 66 uridine residues (unpublished data). The preattenuator probe hybridizes to the first 148 nucleotides of \(ilv\) leader RNA that contains 28 uridine residues (4). Multiplying the cpm of the hybridized preattenuator RNA by 2.3 (the ratio of the uridine content of the post- to preattenuator RNA segments) normalizes the cpm measured to molar amounts of RNA on either side of the attenuator. It is assumed for this calculation that \([\text{H}]\)uridine residues are equally distributed throughout the message. Finally, the effects of differential RNA degradation during the \([\text{H}]\)uridine pulse-labeling period were corrected for using the equation shown above. For example, in the most extreme case (Fig. 2E) the measured ratio of postattenuator to preattenuator RNA (Table 1), corrected for differential uridine content, was reduced by a factor of 2.1 when actual RNA synthesis was calculated. These corrected data were used to calculate the molar ratios of postattenuator to preattenuator RNA synthesis under the various growth conditions described in Table 1. These molar ratios are proportional to the fraction of RNA polymerase molecules that transcribe through the \(ilv\) attenuator. This fraction is expressed in Table 1 as percent read-through at the attenuator. The data in Table 1 show that starvation of appropriate auxotrophic strains of \(E.\ coli\) for leucine, valine, alanine, or threonine causes deattenuation of the \(ilv\) operon, whereas

---

**Biochemistry: Hauser and Hatfield**

\(\mu g\) of yeast tRNA per ml. All hybridizations were performed in duplicate. Each vial contained only one filter to eliminate competition between the DNA probes for attenuator-terminated and read-through \(ilv\) transcripts. Under these conditions the amount of RNA hybridized was proportional to the amount of RNA added. After hybridization the nitrocellulose filters were washed twice in 0.3 M sodium chloride/0.03 M sodium citrate containing 10 \(\mu g\) of RNase A per ml for 30 min at room temperature and two more times in 10 mM potassium phosphate (pH 7.0). The filters were subsequently dried, in vacuo, and the filter-bound radioactivity was measured by scintillation spectroscopy.

---

**Fig. 1.** Schematic representation of the \(ilv\) DNA fragments used for the construction of the preattenuator and postattenuator and probes, M13pre and M13post (see Results for details). Heavy lines represent regions of DNA segments that hybridize \(ilv\) RNA transcripts.
starvation for isoleucine, tryptophan, or methionine does not cause deattenuation of this operon. To effect starvation for each of seven amino acids, three different strains were used; consequently, the fold deattenuation of each starved culture shown in Table 1 is expressed relative to the amount of transcription read-through at the attenuator of the same strain unstarved. To decrease the in vivo level of aminoacylated alanyl-tRNA it was necessary to use a temperature-sensitive alanyl-tRNA synthetase mutant strain. To test for possible nonspecific effects of elevated temperatures during amino acid starvation, in vivo attenuation was measured in strain pCH5/JA200 at 42°C during starvation for tryptophan. These conditions did not affect attenuation of this operon (Table 1).

**DISCUSSION**

Evidence has accumulated during the last several years suggesting that the ilvB operon of *E. coli* K-12 is regulated by an attenuator mechanism. Briefly, this evidence is: (i) ilvB is regulated at the level of transcription (3, 4); (ii) inhibition of in vivo aminoacylation of tRNA^Ala^ or undermodification of tRNA by the hisT mutation causes elevated expression of the ilvB operon (2, 4); (iii) the DNA sequence of the regulatory region of the ilvB operon includes a promoter, a region that encodes a 32-amino acid polypeptide containing multiple valine and leucine codons, and a transcription termination site (4, 6); and (iv) in vitro transcription of this region produces a terminated leader transcript capable of forming secondary structures similar to those formed by leader RNAs of other attenuated operons (4, 6, 7).

We have now demonstrated that the ilvB operon is regulated in vivo by attenuation. The data in Table 1 show that this operon is deattenuated in response to growth-limiting concentrations of valine or leucine. These results were obtained by measuring the amount of transcription termination at the ilvB attenuator under various growth conditions. Direct examination of the molar ratio of ilvB RNA synthesized before the attenuator to RNA synthesized after the attenuator during each pulse-labeling period allows an accurate quantitation of in vivo attenuation. Because this method is based on the ratios of RNA synthesis before and after the attenuator for each growth condition, it precludes problems associated with variations in cellular uptake and internal pool sizes of uridine or fluctuations in in vivo plasmid copy number. We have found that it is not possible to accurately quantitate in

---

**Table 1. Quantitation of ilvB attenuation in vivo**

<table>
<thead>
<tr>
<th>Plasmid/strain and relevant phenotype</th>
<th>Growth condition</th>
<th>Radioactivity, cpm</th>
<th>% read-through at attenuator^†</th>
<th>Deattenuation, fold^‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCH5/T31-4-505 and ilv^−, Trp^−</td>
<td>Unstarved</td>
<td>2,145</td>
<td>4</td>
<td>(1.0)</td>
</tr>
<tr>
<td></td>
<td>−Leu</td>
<td>5,673</td>
<td>17</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>−Val</td>
<td>1,530</td>
<td>16</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>−Ile</td>
<td>788</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>−Trp</td>
<td>1,268</td>
<td>6</td>
<td>1.5</td>
</tr>
<tr>
<td>pCH5/AB4132 and Met^−, Ala^^− (ts)</td>
<td>30°C (unstarved)</td>
<td>1,321</td>
<td>4</td>
<td>(1.0)</td>
</tr>
<tr>
<td></td>
<td>30°C −Met</td>
<td>1,483</td>
<td>3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>42°C −Ala</td>
<td>867</td>
<td>12</td>
<td>3.0</td>
</tr>
<tr>
<td>pCH5/JA200 and Thr^−, Leu^−, Trp^−</td>
<td>Unstarved</td>
<td>744</td>
<td>5</td>
<td>(1.0)</td>
</tr>
<tr>
<td></td>
<td>−Thr</td>
<td>338</td>
<td>21</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>−Leu</td>
<td>291</td>
<td>16</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>−Trp</td>
<td>740</td>
<td>3</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>42°C −Trp</td>
<td>847</td>
<td>3</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Cultures were grown at 37°C unless indicated.

*Radioactivity of ilvB-specific RNA hybridizing to the preattenuator probe.

†Adjusted for the different uridine content and rate of degradation of preattenuator and postattenuator RNA. The half-life used for these calculations was that of the message segment in cells grown at the same temperature and either starved or unstarved as appropriate. (These data are shown in Fig. 2.)

‡Relative to the same strain unstarved.
vivo attenuation by using transcription fusion vectors that rely upon the measurement of enzyme activity such as the pK0 gapK transcription fusion system of McKenney et al. (16). This is because even short periods of growth during amino acid limitation cause dramatic increases in plasmid copy number and decreases in the apparent specific activity of the assayable plasmid gene product (unpublished data).

Previous analysis of the sequence of the ilvB operon leader RNA predicted that the deattenuation of this operon should also occur upon limitation for alanine or threonine due to the location of tandem codons for these amino acids in the coding region of the leader polypeptide (4). The data in Table 1 demonstrate that deattenuation of the ilvB operon is effected by growth-limiting amounts of alanine or threonine. Thus, the ilvB operon of E. coli K-12 is deattenuated during growth limitation by the supply of any one of the four amino acids, valine, leucine, alanine, or threonine. The specificity of this effect is demonstrated by the fact that growth-limiting amounts of isoleucine, tryptophan, or methionine do not cause deattenuation of this operon (Table 1). Because threonine is involved in posttranscriptional modification of several rRNA families, it is possible that the deattenuation observed by cellular limitation for this amino acid is indirect (17). We feel this is unlikely, because a 10-min amino acid limitation in a rel′ strain would not be a sufficient period of time for significant accumulation of new rRNA species.

An interesting question raised by the results reported here is why should an operon that expresses a gene product involved in the biosynthesis of isoleucine, valine, and leucine be regulated by alanine and threonine? Furthermore, why is this operon also regulated by catabolite repression? In consideration of these questions it is interesting to note that: (i) both alanine and threonine are immediate metabolic precursors of pyruvate and α-ketobutyrate, respectively, the substrates of the ilvB gene product; (ii) the ilvB operon encodes only one enzyme; and (iii) this operon is regulated independently from the other genes of the ilv regulon. Thus, fluctuations in the expression of the ilvB gene product, an isozyme catalyzing the first common step in the branched-chain amino acid biosynthetic pathway, could affect the flow of carbon through this pathway. We propose that such a regulatory mechanism could be important for maintaining the biosynthesis of these aliphatic amino acids during periods when cells are shifted from one carbon source to another. Under some carbon shift conditions, the availability of pyruvate or α-ketobutyrate could be severely compromised. A carbon shift could also stimulate the synthesis of cyclic AMP (18). Both of these circumstances should cause elevated expression of the ilvB operon. The decreased levels of pyruvate or α-ketobutyrate would be reflected by decreased levels of alanine or threonine and cause deattenuation of the ilvB operon. Elevated cellular levels of cyclic AMP could also increase the expression of this gene due to relief of catabolite repression. Thus, the flow of carbon through the ilv biosynthetic pathway could be coupled to the supply of branched-chain amino acid anabolic substrates. If this is so, then it seems that it is very important for cells to maintain a constant supply of the aliphatic branched-chain amino acids during critical periods of adaptation to growth on a new carbon source, even at the expense of further depleting intracellular levels of alanine or threonine. This may be due to the relatively high abundance of isoleucine, leucine, and valine in proteins.

In summary, we have shown that the ilvB operon of E. coli K-12 is regulated by an attenuator and that this attenuator responds to intracellular limitations for not only the end-product amino acids valine and leucine (end-product attenuation) but also the amino acids reflecting the substrates of branched-chain amino acid biosynthesis, alanine and threonine (substrate attenuation). We propose that substrate attenuation is a unique form of gene regulation designed to maintain an adequate cellular supply of isoleucine, valine, and leucine for protein synthesis during conditions of severe metabolic perturbation. For example, during growth adaptation to a new carbon source the ilv pathway could respond to a transient depletion of substrates by increasing the cellular concentration of the first enzyme of the pathway, the ilvB gene product, by relief of catabolite repression (coarse control), and by substrate deattenuation (fine control). In addition, this same operon is responsive to the cellular demands for the branched-chain amino acids by end-product attenuation.

We thank Craig Adams for many helpful suggestions and discussions. This work was supported in part by a grant (GM 24330) from the National Institutes of Health. C.A.H. was the recipient of a National Institutes of Health predoctoral training grant (GM 07134).